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Receptor Tyrosine Kinases: Family and Subfamilies

Humana Press

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Dr. Wheeler dedicates this work to the loving support of his wife Shawna and daughter Morgan.

Dr. Yarden would like to thank Rachel and his children for their support and patience.

Preface

As the Human Genome Project completed the sequencing of the human genome, analysis of this robust resource has identified and classified one of the largest gene families, the protein kinases. These proteins are characterized by their ability to transfer phosphate from adenosine triphosphate to specific amino acids on recipient proteins. Collectively, 518 protein kinases have been identified and are broken down into distinct families and subfamilies based on sequence similarity and ultimate function within the cell. Multicellular organisms have evolved complex cellular communication pathways, many of which are controlled by protein kinases, to enable individual cells to communicate in specific ways. A definitive example is provided by the diverse group of cell surface receptors, including a group called receptor tyrosine kinases (RTKs). Receptor tyrosine kinases are endowed with an intrinsic ability to phosphorylate tyrosine residues on substrate proteins, serving as important signals for the maintenance of cellular homeostasis or changes in cellular function. Accordingly, RTKs regulate a diverse array of cellular functions, including cellular proliferation, survival, differentiation, migration, and metabolism, and they perform these functions in response to activation by specific polypeptide ligands.

Early classification of the family identified 58 human RTKs that fall into 20 subfamilies, based on the ligands they bind, their sequence homology, and their structures. However, on the basis of in-depth functional analyses, it becomes increasingly accepted that the lemur group of three tyrosine kinases (LMTKs) phosphorylate serine/threonine residues, thus altering the enumeration of the RTK family to 19 subfamilies and 55 RTKs.

The architecture of all RTKs is highly conserved from the nematode *Caenorhabditis elegans*, with an extracellular ligand binding domain, a single transmembrane α helix, an intracellular tyrosine kinase domain, and a tyrosine-rich C-terminal tail. RTKs are activated upon binding to ligands present in the extracellular milieu, leading to receptor homo- or heterodimerization, kinase domain activation, and subsequent phosphorylation of tyrosine residues located within the cytoplasmic tail. Phosphorylated tyrosines serve as docking sites for a variety of intracellular adaptors and effector enzymes that transmit signals to the cytoplasm

and nucleus, resulting in changes in cell function or fate. Termination of RTK activation is tightly controlled through the activation of a variety of tyrosine phosphatases, receptor-mediated endocytosis, and subsequent receptor degradation. Indeed, the importance of many RTKs in mammalian development has been displayed through the study of genetically altered mice, resulting in either severe developmental abnormalities or embryonic lethality. Collectively, RTK activation of signaling networks provides an essential mechanism by which cells communicate to regulate a multitude of different cellular responses. Since RTKs regulate both developmental and regulatory cellular processes, it is not surprising that abnormalities in RTK structure or activity can result in human diseases. Several diseases result from RTK mutations, constitutive activation, and/or overexpression. Examples include cancer, diabetes, inflammation, arteriosclerosis, angiogenesis, autoimmune disorders, and skeletal diseases. Due to this causal relationship, therapeutic targeting of specific RTKs is clinically approved for treatment of several human diseases.

In this comprehensive book entitled *"The Receptor Tyrosine Kinases: Family and Subfamilies,"* we have attempted to create an all-inclusive text for graduate students, postdoctoral fellows, medical students, practicing doctors, and active researchers who are interested in the incredible field of RTK biology. In this attempt, we have made every effort to highlight the information in an organized fashion and show uniformity between each chapter. We have divided this book into 19 chapters, one chapter dedicated to each subfamily of RTK. Within each chapter, we have organized distinct information, including a brief introduction to the family and the role of this family of RTKs in development and physiology, as well as human disease. Note that each chapter ends with some information on the respective gene and protein structure, ligands, activation and signaling, attenuation of signaling, and unique features of the 55 RTKs.

We take this opportunity to thank the many contributors for their remarkably extensive work. It is our hope that this book will provide students, researchers, and clinicians with a better understanding of the incredibly important RTK family.

Madison, WI, USA — Deric L. Wheeler
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Born in Washington State, Deric Wheeler received his Bachelor of Science degrees in Botany and Zoology from the University of Idaho (Moscow, ID), followed by a Masters in Pathology from the University of Iowa (Iowa City, IA) and a Ph.D. in Cancer Biology from the University of Wisconsin (Madison, WI). After his graduate training, Deric Wheeler started his postdoctoral training at the Fred Hutchinson Cancer Research Center in Seattle, WA (c/o Larry R. Rohrschneider), and at the University of Wisconsin School of Medicine and Public Health in Madison (c/o Paul M. Harari). He completed his postdoctoral training and transitioned to faculty, at the level of Assistant Professor, at the University of Wisconsin School of Medicine and Public Health in the Department of Human Oncology. Dr. Wheeler's lab research centers on the role of subcellular localization of receptor tyrosine kinases and their roles in oncogenesis and therapeutic resistance. Currently, Dr. Wheeler's research is supported by the American Cancer Society, Mary Kay Foundation, the Department of Defense Lung Cancer Research Program, and the Clinical and Translational Science Award (CTSA) program through the NIH National Center for Advancing Translational Sciences (NCATS).

Born in Israel, Yosef Yarden received his B.Sc. in Biological and Geological Sciences from the Hebrew University of Jerusalem, and a Ph.D. in Molecular Biology from the Weizmann Institute of Science. His postdoctoral training was undertaken at Genentech, Inc. (c/o Axel Ullrich) in San Francisco and at the Massachusetts Institute of Technology (c/o Robert A. Weinberg). He returned to the Weizmann Institute of Science as an Assistant Professor, eventually becoming a Full Professor there. His past administrative responsibilities at the Weizmann Institute include Dean of the Faculty of Biology, Vice President for Academic Affairs, Director of the M.D. Moross Institute for Cancer Research, and Dean of the Feinberg Graduate School. Dr. Yarden's lab research centers on the roles played by growth factors and receptor tyrosine kinases in tumor progression. Currently, Yarden's research is supported by the US National Cancer Institute (MERIT Award), a grant from the European Research Council (ERCAdG), the German Research Foundation (DIP), and a professorship from the Israel Cancer Research Fund (ICRF).

Chapter 1
The ALK Receptor Family

Ruth H. Palmer and Bengt Hallberg

1.1 Introduction to the ALK/LTK RTK Kinase Family

The leukocyte tyrosine kinase (LTK) and anaplastic lymphoma kinase (ALK) receptor tyrosine kinases (RTKs) define a subgroup of the insulin receptor superfamily. Leukocyte tyrosine kinase (LTK) was initially discovered by screening a mouse pre-B-lymphocyte library with the avian *v-ros* gene [1]. Subsequent work revealed the presence of an LTK extracellular domain [2], but it required significant further cloning efforts to clearly define the structure of this RTK. Cloning of the full-length LTK receptor revealed an intracellular PTK domain accompanied by a 347-amino-acid extracellular domain, which is relatively small when compared with other members of the receptor kinase superfamily. The LTK cDNA was subsequently shown to encode a glycosylated receptor harbouring in vitro kinase activity (Fig. 1.1) [3, 4].

Several years later a novel kinase showing similarities to LTK was described as a novel tyrosine phosphoprotein in anaplastic large cell lymphoma (ALCL) cell lines [5, 6]. Subsequent cloning of the ALCL translocation breakpoint between chromosomes (2;5)(p23;q35) revealed the identity of this chimeric protein as a fusion between NPM (nucleophosmin) and the intracellular kinase domain containing portion of a novel receptor tyrosine kinase (RTK). This novel kinase was named anaplastic lymphoma kinase (ALK) after the disease from which the breakpoint was first cloned [5]. Three years later the cloning of full-length ALK was reported, identifying a novel RTK with a unique extracellular domain composition among the RTKs [7, 8]. The *ALK* locus encodes for a classical RTK containing an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain.

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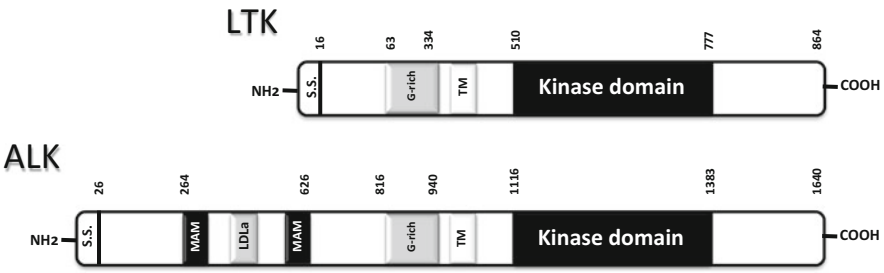

Fig. 1.1 Domain structure of ALK and LTK. ALK and LTK are classical receptor tyrosine kinases (RTK), which contain an N-terminal extracellular domain, transmembrane domain and a C-terminal intracellular domain. Both receptors contain a signal peptide (aa 1–26); ALK contains two MAM domains (amino acids 264–427 and 480–626) and one LDLa domain (amino acids 453–471). Both receptors contain a glycine-rich region (residues 816–940 in ALK and residues 63–334 in LTK). Both harbour a transmembrane segment which is followed by the intracellular kinase domain (ALK, amino acids 1116–1383 and LTK, amino acids 510–777, LTK). ALK and LTK proteins share 80 % amino acid identity in their kinase domain. Not drawn to scale

Both ALK and LTK make up a subfamily within the insulin receptor superfamily based upon kinase domain similarities. Their extracellular domains are unique among the RTKs, containing in the case of both: a glycine-rich region, and in the case of ALK also containing: an LDLa domain [9, 10] and MAM domains (named after meprins, A-5 protein and receptor protein-tyrosine phosphatase mu). The MAM domain of about 160 amino acids is found in transmembrane proteins such as the meprins and receptor protein-tyrosine phosphatases, where they have been suggested to function in cell/cell interactions (Fig. 1.1) [11–13]. In simpler organisms such as *Caenorhabditis elegans* (*C. elegans*) [14] and *Drosophila melanogaster* [15] one ALK/LTK family member exists; however, in zebrafish [16] and mammals duplication of a common ancestral gene appears to have resulted in the presence of both LTK and ALK. In this chapter we will focus on our present knowledge concerning the ALK/LTK family of RTKs.

1.2 The Role of the ALK/LTK Receptor Tyrosine Family in Embryonic Development and Adult Physiology

Despite intense efforts, the physiological role of ALK/LTK receptor family in humans remains rather enigmatic. From a human genetics standpoint one study has shown an association of polymorphisms in ALK with schizophrenia in a Japanese population [17]. Systemic lupus erythematosus patients, also from a Japanese population study, displayed LTK polymorphism with a significantly higher frequency compared with the healthy controls [18]. Transgenic LTK mice, in which the LTK gene was placed under the control of cytomegalovirus enhancer and the beta-actin

promoter, exhibited growth retardation and died within months after birth. In this work the authors noted a tissue specific activation of LTK in the heart, resulting in cardiac hypertrophy, cardiomyocyte degeneration and gene reprogramming [19]. Overall, the expression pattern of ALK in chicken, mice, rats and humans suggests key roles in development of the nervous system [7, 8, 20–24], and functional physiological roles have been observed in several model systems, such as *Drosophila*, *C. elegans*, zebrafish and mouse model systems.

1.2.1 Drosophila melanogaster *ALK/LTK*

The function of ALK in the context of an intact organism has probably been best described in *Drosophila melanogaster. Drosophila* ALK (dALK) was identified and characterised in vivo as a RTK able to stimulate ERK (extracellular-signal-regulated kinase) activation and play a vital role in the formation of the visceral musculature of the *Drosophila* gut [15, 25]. Subsequent identification of the dALK ligand—Jeb (Jelly Belly)—led to the definition of a ligand–receptor pair that functions in vivo to specify a specific founder cell type in the developing embryonic visceral mesoderm. The specification of founder cells, which then fuse with fusion competent myoblasts, is a critical step in the development of the multinucleated visceral musculature of the *Drosophila* gut [26–28]. In these studies, the kinase activity of the dALK RTK was shown to be essential for internalisation of the Jeb-dALK complex together with the downstream activation of ERK, and a number of downstream target genes were shown to be transcriptionally regulated by dALK signalling, including *dumbfounded/kirre* and *org-1* [26–28]. Since then additional targets of dALK signalling in the *Drosophila* visceral mesoderm have been reported, such as the bHLH transcription factor Hand and the Rap GEF C3G [29–31]. Moreover, the dALK/Jeb pathway in *Drosophila* plays an indirect role in endoderm development, since Dpp (a TGFβ homologue in the fly) transcription is dependent on Jeb/dALK signalling activity [29].

Besides development of the embryonic gut muscle, dALK and Jeb play a number of important roles in the *Drosophila* nervous system including acting as an anterograde signalling pathway mediating neuronal circuit assembly in the *Drosophila* visual system. Lack of either dALK or Jeb protein results in mistargeting of the photoreceptor axons during later maturation of the optic lobe neuropile [32]. Jeb-dALK signalling is also employed in the developing synapse at the *Drosophila* neuromuscular junction, where dALK on the postsynaptic membrane responds to secreted Jeb. Indeed, in *jeb* or *dALK* mutant animals, synaptic transmission frequency and strength together with larval locomotion are strongly impaired as a result of loss of ALK signalling [33].

dALK also functions in *Drosophila* as an upstream activator of dNf1-regulated Ras signalling. A reduction in dALK signalling—either genetically or pharmacologically—rescues the body-size and learning defects as well as the ERK overactivation of the *dNf1* mutant phenotype [34]. In a recent microarray based investigation dALK was

identified as target of the dLMO transcription factor. This work implicated dALK in adult responses to ethanol, with flies containing transposon insertions in dALK demonstrating increased resistance to the sedating effect of ethanol [34–36].

dALK also activates PI3K signalling in *Drosophila*, where ALK signalling in the CNS responds to Jeb expression from glial cells to protect neuroblasts during periods of nutrient restriction [35]. Interestingly, in the honeybee *Apis mellifera* a recent report suggests that royal jelly leads to epigenomic methylation modification of a number of genes, including *AmALK*, to determine development of a new queen instead of a short-lived worker bee [37].

1.2.2 Caenorhabditis elegans *ALK/LTK*

The ALK homologue in *Caenorhabditis elegans* (*C. elegans*) was originally described as a suppressor in a genetic screen of TGFβ pathway mutants that cause constitutive dauer formation [38]. Here the TGFβ pathway mediates transduction of environmental signals to regulate the dauer formation response through chemosensory neurons. This work led to the naming of the *C. elegans* ALK locus as *scd-2* (*suppressor of constitutive dauer 2*), and characterisation of these mutant *C. elegans scd-2* strains revealed that they were less efficient at dauer formation in response to pheromone or starvation as compared with wild-type animals [38]. The subsequent isolation of a wild *C. elegans* strain from a desert oasis which was unable to respond to dauer pheromone at 25 °C, but not at higher temperatures, led to the characterisation of *scd-2* as the T10H9.2 open reading frame encoding the *C. elegans* ALK homologue [39]. This work defined an ALK-mediated sensory pathway, consisting of the ligand HEN-1 (an LDL-containing protein with similarities to *Drosophila* Jeb) [40], the adaptor SOC-1 [41] and the MAP kinase SMA-5 [42], that regulates DAF-3 (SMAD3) activity in the TGFβ dauer pathway. The *C. elegans* ALK gene, *scd-2*, contains 22 exons and encodes a putative protein of 1421 amino acids displaying the conserved kinase domain, transmembrane domain, two MAM domains and the LDLa domain found in human ALK [39]. Reiner et al. also constructed a constitutively active ALK (*scd-2-neu**) in which the SCD-2/ALK transmembrane domain was replaced with the mutant transmembrane domain of the mammalian ErbB2/Her2/Neu RTK oncogene. This resulted in an early arrest phenotype which has been interpreted as a negative regulation of feeding behaviour, suggesting that SCD-2/ALK signalling may function to transduce a food signal as a chemosensory input in *C. elegans*.

Interestingly, genetic screening examining mechanisms modulating sensory integration initially identified HEN-1 as a regulator of sensory integration non-cell-autonomously in *C. elegans* [40]. Subsequent screening efforts led to the identification of mutants in SCD2/ALK, and characterisation of these mutant animals suggests that SCD2/ALK signalling functions to modulate activity in the AIA pair of interneurons in which conflicting sensory cues may converge [43, 44]. Thus, although neither HEN-1 nor SCD-2/ALK is required for *C. elegans* development, they appear to be required for integration of conflicting sensory inputs.

An additional role for ALK in the regulation of presynaptic differentiation at the *C. elegans* neuromuscular junction was reported when SCD-2/ALK was found to be a binding partner of the F-box protein FSN-1, binding via the SPRY domain of FSN-1. FSN-1 interacts with the RING finger protein RPM-1 in an SCF-like ubiquitin ligase complex in developing synaptic termini, targeting downstream effectors such as SCD-2/ALK to control the presynaptic differentiation process [14].

Finally, SCD-2/ALK has also been reported to be required in neurosecretory control of aging in *C. elegans*, with *scd-2* mutant animals displaying a decreased lifespan as measured by lysosomal deposits of lipofuscin [45].

1.2.3 Danio rerio *(Zebrafish) ALK/LTK*

Genetic mapping, together with confirmation by morpholino-mediated knockdown, identified the *shady* locus in the zebrafish genome as encoding the leukocyte tyrosine kinase (LTK) RTK. The zebrafish genome contains two ALK/LTK family members—both a LTK and an ALK gene. Interestingly, while all mammalian LTK proteins are lacking the MAM (meprin/A5/μ) domain, the zebrafish LTK contains a MAM domain and is in this respect—together with its expression pattern in the neural crest—more similar to ALK. In *Danio rerio* LTK signalling is required cell-autonomously for the specification of iridophores from the neural crest linage and *shady* mutants display pigmentation pattern defects [16]. ALK, on the other hand, is important for embryonic neurogenesis and is a critical player for the balance between neural progenitor growth, differentiation and survival [46]. Transgenic zebrafish have also been employed in examining the role of ALK in neuroblastoma development. Overexpression of both human MYCN and activated ALK^{F1174L} in the fish analogue of the adrenal medulla promoted neuroblastoma progression [47]; see section below). The zebrafish model system offers a number of advantages for screening small molecules in vivo, and this model may be useful in testing and identifying inhibitor strategies for ALK in neuroblastoma.

1.2.4 Mammalian ALK/LTK

A clear role for ALK in mammalians has been difficult to define. The reported ALK mRNA and protein expression patterns during mouse embryogenesis suggest a role for ALK in the nervous system [7, 8, 23]. It should be noted that the ALK transcript and protein appear to diminish after birth, reaching minimum levels at 3 weeks of age and is thereafter maintained at low levels in the adult animal [7]. Work defining ALK mRNA expression in the developing CNS of the chicken and rat is in keeping with mouse expression of ALK, with localisisation in a subset of spinal motor neurons, sympathetic ganglia and dorsal root ganglia [20, 21].

Several groups have generated models in which the ALK gene has been deleted (Bisland, Lasek, Weiss, Hallberg/Palmer). All of these animals, generated by different genetic deletion strategies, result in mice that are viable in the absence of wild-type ALK. The initial report, in which transmembrane and kinase domain of ALK were deleted, reported an increase in hippocampal performance of resulting adult mice, leading the authors to suggest ALK-based treatment strategies may be of use in the treatment of psychiatric illness [48].

A more recent study implicates ALK in the regulation of alcohol consumption in mice suggesting that the normal function of ALK may be to curb excessive alcohol intake [36]. This work was based on initial microarray results in *Drosophila*, identifying dALK as a target of the *Drosophila* LIM-domain only protein (dLMO) which is implicated in behavioural responses to alcohol. The authors employed ALK knockout mice in which exons encoding the juxtamembrane domain and amino-terminal portion of the kinase domain were targeted, resulting in both increased ethanol consumption and a delayed righting reflex under the influence of alcohol. Interestingly, they also reported polymorphisms in human ALK that appear to be associated with a decreased sensitivity to alcohol [49].

One legitimate concern associated with the lack of gross phenotype in ALK knockout mice has been the potential redundancy with LTK. This has recently been addressed experimentally, with analysis of single as well as double mutant ALK and LTK mice [50]. Expression of ALK and LTK mRNA was found to overlap extensively in the hippocampus, consistent with a possible functional interaction between the two receptors. In ALK single knockout mice reduced levels of adult neurogenesis, measured as doublecortin-positive cells, were observed. These reduced levels of adult neurogenesis in ALK knockout mice contrast with the earlier findings of Bisland et al [48]. Moreover, while no obvious effect was seen in LTK knockout mice, the authors found a more severe effect in the ALK/LTK double knockout mice [50]. In a battery of behavioural tests ALK knockout animals were found to have enhanced spatial memory and enhanced cognitive performance, whereas LTK knockout mice performed poorly in tests of sensimotor function on the rotorod. While an important first step in addressing ALK/LTK function in vivo, it is clear that further characterisation of the physiological function of both ALK and LTK is needed.

Indications of physiological roles of ALK in humans have arisen from clinical trials employing the crizotinib ALK/c-Met inhibitor. A number of enrolled patients treated with crizotinib for ALK-positive non-small cell lung cancer (NSCLC) developed asymptomatic sinus bradycardia (HR $\leq$45) [51]. Further, crizotinib therapy caused rapid suppression of testosterone levels in men, which suggest some kind of function in the hypothalamic or pituitary disturbance [52]. A significant portion of patients treated with crizotinib experience mild visual disturbance symptoms during treatment, an effect that is reversible upon discontinuation of treatment [53, 54]. However, in one case crizotinib treatment was associated with optic neuropathy and blindness [55]. Although no function has been described for human ALK in the visual system, there is robust expression of ALK within the lens and the neural and pigment layer of the mouse retina [23]. Interestingly, in *Drosophila* ALK signalling is involved in the maturation of the optic lobe in the fly brain [32]. In vitro studies

in mammalian cells also support a role for ALK in neuronal development. ALK activity has the capacity to induce neuronal differentiation of PC12 cells [56–60]. From a mouse model it was shown that activated ALK did not mediate tumour development but trigger a prolonged neurogenesis of the sympathetic ganglia, but it was noted that this mouse model show increased neonatal lethality [61]. In addition, employing antibodies which activate ALK results in neurite outgrowth, involving both the MAPK pathway and activation of the small G-protein Rap1 in both PC12 and neuroblastoma cell lines [58, 62, 63]. FRS2 (fibroblast growth factor receptor substrate 2)/SNT has also been reported to bind ALK and may be important in the induction of PC12 cell differentiation [62]. Finally, ALK can stimulate the ERK5 MAPK protein via PI3-K/Akt/PKB/MEKK3/MEK5 pathway, which promotes further downstream expression of the MYCN in neuroblastoma [64]. One outcome of activation of MYCN is that MYCN as a transcription factor has the ability to activate transcription of ALK as well [65].

1.3 The Role of the ALK Receptor Tyrosine Kinase in Human Disease

While knowledge of the physiological function of ALK is limited, we now understand significantly more about the role of ALK in pathogenic disease. Over the last decade a large body of data has accumulated concerning the role of ALK in the development and onset of a large number of cancer types. We now know that ALK is involved in the initiation and progression of multiple cancer types via several different mechanisms: (1) as a fusion protein, (2) as a result of ALK overexpression or (3) due to mutation of the ALK coding region. In this section we will look at each of these mechanisms in turn (Fig. 1.2).

1.3.1 ALK as a Fusion Protein

The original discovery of ALK as a fusion protein NPM-ALK [5, 6] turned out to be tip of the iceberg in terms of the many chromosomal translocations involving ALK as a partner. In addition to ALCL, these ALK fusion proteins are found in a number of cancers, such as in inflammatory myofibroblastic tumours (IMTs), non-small cell lung cancer (NSCLC), ALK-positive diffuse B-cell lymphoma (DBCL), squamous cell carcinoma of the esophagus (SCC), breast cancer, colon cancer, renal medullary carcinoma and renal cell carcinoma. All of the ALK fusion partners share a number of common features; firstly, the initiation of transcription of the new fusion protein is driven via the regulatory regions of the ALK partner protein. Secondly, the spatial localisation of the ALK fusion protein is determined by the ALK partner protein. Finally, the dimerisation process via the ALK partner protein induces trans-autophosphorylation and activation of the ALK kinase domain (Fig. 1.2).

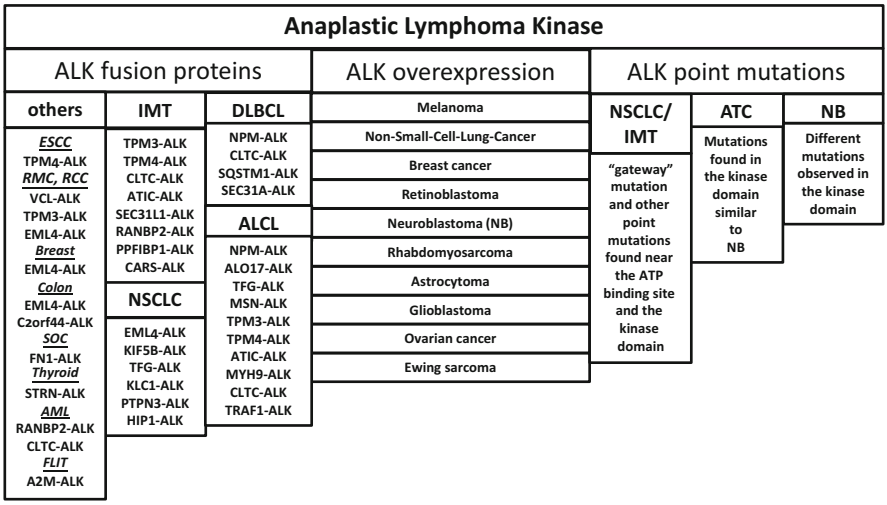

Fig. 1.2 Schematic overview of anaplastic lymphoma kinase (ALK) lesions in cancer. ALK fusion proteins, in which the kinase domain of ALK is fused to portion of various proteins, have been described in many cancers, such as ALCL (anaplastic large cell lymphoma), IMT (inflammatory myofibroblastic tumours), fetal lung interstitial tumour (FLIT), DLBCL (diffuse large B-cell lymphomas), NSCLC (non-small cell lung cancer), serous ovarian carcinoma (SOC), RMC (renal medulla carcinoma), RCC (renal cell carcinoma), breast and colon cancer, AML (acute myelomonocytic leukaemia), thyroid cancer and ESCC (esophageal squamous cell carcinoma). Moreover, secondary mutations in the context of ALK fusions have been described in NSCLC, IMT and ATC (anaplastic thyroid cancer); see Table "Receptor at a glance: ALK/LTK". ALK overexpression has been reported in a number of cancer types and cell lines, such as melanoma, NSCLC, breast cancer, retinoblastoma, neuroblastoma, ovarian cancer, thyroid carcinoma, astrocytoma, Ewings sarcoma and rhabdomyosarcoma. ALK point mutations have been found mainly in neuroblastoma, where most of the mutations are situated within the kinase domain of ALK, but also in ALK of NSCLC and ATC cancer origin. Abbreviations: *ALO17* ALK lymphoma oligomerisation partner on chromosome 17, *ATIC* 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase, *FN1* fibronectin 1, *STRN* striatin, *CLTC* Clathrin heavy chain like, *EML4* echinoderm microtubule-associated protein-like 4, *KIF5B* kinesin family member 5B, *MSN* moesin, *MYH9* non-muscle myosin heavy chain, *NPM* nucleophosmin, *PPFIBP1* F polypeptide-interacting protein-binding protein 1, *RANBP2* RAN binding protein 2, *SEC31L1* SEC31 homologue A (*S. cerevisiae*), *SQSTM1* sequestosome 1, *TFG* TRK-fused gene, *TPM3/4* tropomyosin 3 and 4, *VCL* vinculin, *HIP1* huntingtin interacting protein 1, *A2M* alpha-2-macroglobulin, *TRAF1* tumour necrosis factor receptor-associated factor and *C2orf44*, which contains a coiled-coil domain

1.3.1.1 Anaplastic Large Cell Lymphoma

Most of the studies concerning ALK as a fusion partner have been conducted in connection with ALCL. This disease, described for the first time in 1985, belongs to the group of non-Hodgkin's lymphoma arising from T cells, or less commonly B-cell origin [66], which are large cells that are different in size and shape and express a specific marker on the lymphoma cells called CD30 [67, 68]. Morris and

Yamamoto later described one determinant for the disease as the ALK fusion protein [7, 8]. ALK is expressed in 60–80 % of ALCL, a cancer mainly observed in children and young adults [69–71]. The fact that ALK-positive patients have a higher 5-year survival rate compared to ALK-negative patients makes ALK expression an important prognostic factor in ALCL [71–75]. Interestingly, alongside ALK, active caspase 3 expression is also used as a prognostic indicator for favourable outcome in ALCL; caspase 3 activity is strongly correlated with the expression of ALK [76]. Expression of STAT3 (signal transducer and activator of transcription 3) in both ALK-positive and ALK-negative ALCL has led to the suggestion that activated STAT3 may be a negative prognostic factor independent of ALK expression in ALCL; similarly survivin and MUC-1 indicate a poorer outcome in ALCL regardless of ALK status [77–79]. Clinical trials for ALK-positive neoplasia directly targeting ALK (http://clinicaltrials.gov/ct2/home) are currently ongoing. It should also be mentioned that there are reports of detectable NPM-ALK in the blood and lymphoid tissue of healthy individuals, raising the question of whether NPM-ALK alone is powerful enough to drive tumourigenesis [80, 81]. Besides NPM, numerous other fusion partners for ALK have been reported in ALCL, such as ALK lymphoma oligomerisation partner on chromosome 17 (ALO17) [82], 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC) [82–84], TRK-fused gene (TFG) [85, 86], moesin (MSN) [87], tropomyosin 3 and 4 (TPM3/4) [88–90], TRAF1-ALK [91], non-muscle myosin heavy chain (MYH9) [92] and Clathrin heavy chain like (CLTC) [93] (Fig. 1.2).

1.3.1.2 Inflammatory Myofibroblastic Tumour

IMTs belong to the category of ‘inflammatory pseudotumours’, with particular clinical, pathological and molecular characteristics. While they mostly occur in younger persons, they may also appear in patients of higher age [94]. Tumours can be located at any site in the body, mostly in soft tissues, although the most common sites are the head; neck, including upper respiratory tract; pelvis; abdomen; and retroperitoneum [95]. A recent review reported that around 33 % of cases were pulmonary and 67 % were extra-pulmonary [96]. Further, histologically, IMTs possess a variably cellular spindle cell proliferation in a myxoid to collagenous stroma with inflammatory infiltrate containing plasma cells and lymphocytes [94, 95]. The first connection between IMT and ALK was shown by Griffin et al., in 1999, who documented a 2p23 chromosomal rearrangement and ALK expression in IMT, suggesting a novel involvement of ALK in solid tumours for the first time [97]. Since then, a number of ALK fusion proteins have been described in IMT displaying a subcellular localisation that appears to be determined by the fusion partner. These ALK fusions include TPM3-ALK, TPM4-ALK [98], CARS-ALK [99], ATIC-ALK [100], SEC31L1-ALK [101] and PPFIBP1-ALK [102], all of which are localised in the cytoplasm of the cell. RANBP2-ALK [103] displays a nuclear localisation, in keeping with the role of RANBP2 as a nuclear pore protein. The fusion protein CLTC-ALK [104, 105] has a granular cytoplasmic staining, reflecting CLTCs

function as a structural protein of coated vesicles. Overall, approximately 50 % of all IMTs seem to have ALK rearrangements, which is similar to ALCL. It is also worth highlighting that the common ALCL NPM-ALK translocation has not been identified in IMT [97, 98, 106].

Currently, there is no clear relationship between ALK expression and prognosis in this rare tumour type. In a study addressing this, a small cohort of eight children, four of which were ALK positive, were examined [107] leading the authors to suggest that ALK expression might be associated with an improved prognosis. Clearly larger patient sets must be evaluated in order to validate this hypothesis.

So far one case report describes a partial response in a 44-year-old patient with recurrent RANBP2-ALK-positive IMT who was treated with the ALK inhibitor crizotinib [108]. This patient later acquired resistance to crizotinib in the form of a secondary mutation in the kinase domain of the RANBP2-ALK fusion. This acquired RANBP2-ALK^{F1174L} resistance mutation is similar to the ALK^{F1174L} mutation observed in neuroblastoma (see below) [108, 109].

1.3.1.3 Non-Small Cell Lung Cancer

In 2008, lung cancer was the leading cause of cancer deaths in males worldwide. In females, it accounts for the second leading cause of death after breast cancer. In total it was estimated to be responsible for 1.4 million deaths worldwide in 2008 and 1.6 million new cases every year [110]. Lung cancer can clinically be divided into two major subgroups: SCLC and NSCLC; patients are treated differently depending on the subgroup. NSCLC accounts for approximately 80 % of all lung cancers and include subtypes such as squamous cell lung carcinoma, large cell lung carcinoma and adenocarcinoma, all of which respond poorly to conventional cancer treatments [111]. It is estimated that smoking accounts for 80 % of all death in males and 50 % in females [112, 113]. ALK first entered the field of NSCLC in 2007, when two groups employing very different experimental approaches simultaneously reported the presence of ALK fusion proteins in lung tumours [114, 115]. Rikova et al. employed one of the first global phosphotyrosine proteomic analyses of NSCLC cell lines resulting in the identification of two aberrant fusion proteins including EML-4 and TRK-fused gene-ALK (TFG-ALK) [114]. In a more classical approach, Soda et al. employed a retroviral cDNA expression library from a lung adenocarcinoma specimen surgically resected from a 62-year-old man with history of smoking [115]. Since these original discoveries the group of NSCLC patients harbouring EML4-ALK or indeed other ALK fusion partnered translocation products is now recognised, as an increasing population of lung cancer patients, with adenocarcinoma histology, are younger and non-smokers [116–119].

Ongoing investigations carried out by the National Cancer Institute's Lung Cancer Mutations Consortium have reported results from more than 1,000 patient samples in an attempt to catalogue driver mutations and translocations in NSCLC. Their results suggest that NSCLC should be regarded as a number of molecularly different diseases, with 60 % of tumours exhibiting driver mutations.

These include KRAS (25 %) and EGFR (23 %) and others such as BRAF, HER2 and EML4-ALK. In this analysis ALK translocations were observed in 6 % of the cases [120–122].

From the start, ALK translocations and mutations in the other well-known NSCLC oncogenes such as EGFR and KRAS appear to be mutually exclusive in NSCLC patients [117, 123–126]. Although cases with multiple expression of drivers, such as ALK and/or EGFR/KRAS, have been observed [127–130]. A rather sobering extrapolation from the consortiums data is the fact in in 40–50 % of all NSCLC cases, we are currently unable to define a driver mutation. Of note, while to date 6 ALK fusion products have been described in NSCLC, including the kinesin family member 5 B (KIF5B-ALK), huntingtin interacting protein 1 (HIP1-ALK), protein-tyrosine phosphatase, nonreceptor Type 3 (PTPN3-ALK) and kinesin light chain 1 (KLC1-ALK), very few patient cases have been reported which do not involve the EML4-ALK fusion [5, 131–136].

Although ALK fusions in NSCLC are present in only ~5 % of patients, this extrapolates to ~40,000 patients/year worldwide. Clinical diagnostics for ALK-positive NSCLC are under active development [137]. Currently, fluorescence in situ hybridisation (FISH) is used for detecting ALK rearrangements, employing break-apart 5′ and 3′ ALK probes to identify tumour cells containing an ALK gene rearrangement [138]. Another clinical method with potential for use with ALK-positive NSCLC patients might be chromogenic in situ hybridisation (CISH) [139]. Development of PCR-based detection techniques is ongoing, and they remain attractive as they offer specificity and the products can be sequenced and verified [140]. However, this is complicated by the increasing number of different EML4-ALK fusion proteins that exist, providing real problems in the development of a robust RT-PCR diagnostic test which would be capable of identifying all ALK translocations. Obviously, since ALK should not be expressed in the lung of a grown up, immunohistochemistry is being actively tested and would offer a powerful tool to identify ALK-positive NSCLC. Hopefully, validated ALK antibodies will soon be included in the tool box of antibodies employed by the pathologist offering an alternative to current options.

1.3.1.4 ALK-Positive Diffuse Large B-cell Lymphoma

ALK-positive diffuse large B-cell lymphoma (ALK^+-DLBCL) is a rare subtype of lymphoma first described in 1997 [71, 141, 142]. It has an aggressive clinical course with a poor prognosis and has been documented in both paediatric and adult patient populations [143]. The most common ALK fusion partner is the *CLATHRIN (CLTC)* gene, producing a CLTC-ALK fusion protein with a characteristic granular pattern in the cytoplasm [73, 93, 142, 144–146]. Other less frequently occurring ALK-DLBCL fusions in the form of NPM-ALK, SEC31A-ALK and SQSTM1-ALK fusions have also been reported [147–150] together with an insertion of ALK sequence to chromosome 4q22-24 where the potential fusion partner is yet to be precisely molecularly defined [151].

1.3.1.5 Renal Carcinoma and Esophageal Squamous Cell Carcinoma and Other Tumours

A novel fusion partner for ALK, namely, vinculin (VCL-ALK), was recently identified in young individuals diagnosed with renal cell carcinoma (RCC) [152, 153]. On the basis of these reports, examination of a large cohort of renal tumours was carried out to assess ALK involvement. In this study, 2 ALK-positive RCC cases were detected, and the ALK fusions TPM3-ALK and EML4-ALK were identified [154]. Thus, while few cases have been reported so far, it will be of interest to investigate this patient population further for the presence of ALK fusions. In 2014 a few more novel ALK translocation products were found in different cancer types, such as striatin (STRN)-ALK in thyroid cancer [155, 156], alpha-2-macroglobulin (A2M)-ALK in fetal lung interstitial tumour (FLIT) [157], and RANBP2-ALK and CLTC-ALK in acute myelomonocytic leukaemia (AML) [158, 159].

In proteomics-based analyses of tumour tissue from squamous cell carcinoma of the esophagus, two groups have independently described the presence of TPM4-ALK fusion protein [160, 161]. This tumour type is more frequent in certain regions of the world, including Iran and China where these studies were carried out, and further studies are required to define the extent of ALK fusion involvement in esophageal squamous cell carcinoma in these and other populations.

Exon array profiling of 153 tumours, including 84 breast, 26 colorectal adenocarcinoma and 43 NSCLC samples, led to the identification of EML4-ALK fusions in breast, colorectal as well as in NSCLC tumours [162]. In a few cases of sarcomatiod carcinoma of head and neck that scored ALK FISH positive, the fusion partner to ALK has not been identified, although the patient benefited from ALK specific crizotinib treatment [163].

1.3.2 ALK Overexpression in Cancer

Amplification of the ALK locus and overexpression of ALK have been reported in a variety of different cancer cell lines and in cancers such as thyroid carcinoma, NSCLC, breast cancer, melanoma, neuroblastoma, glioblastoma, astrocytoma, retinoblastoma, Ewing's sarcoma leiomyosarcoma, peripheral nerve sheath tumours, malignant fibrous histocytoma and rhabdomyosarcoma and lately in colorectal carcinoma (Fig. 1.2) [164–170]. It has been suggested from neuroblastoma derived cell lines and from an 82-patient cohort with neuroblastoma that overexpression of either mutated or wild-type ALK defines poor prognosis patients [171]. This has been investigated further by Schulte and co-workers who examined genomic alterations of ALK in 263 neuroblastomas. Their data suggest that high levels of either mutated or wild-type ALK may contribute to initiation and progression of neuroblastoma [172]. ALK has also been reported to be differentially expressed in epithelial ovarian cancer as compared with normal ovarian tissue [173]. Furthermore, in mice pancreatic neuroendocrine tumours it has been noted that ALK is expressed at

significantly lower levels in invasion-resistant C3H mice as compared with invasion-susceptible B6 mice [174]. Taken together, these findings suggest that ALK overexpression in cancer may have consequences for tumour progression; however, further clarification/characterisation of the molecular mechanisms is required to understand the significance of this in a clinical setting.

1.3.3 Activating ALK Mutations in Cancer

1.3.3.1 Neuroblastoma

Neuroblastoma is a childhood cancer that arises from neural crest cells of the sympaticoadrenal lineage and can occur throughout the sympathetic nervous system [175, 176]. Genetically cases of neuroblastoma often display amplification of the *MYCN* gene on chromosome 2p (~24 % of all cases), deletions of parts of the chromosomes 1p and 11q, gain of parts of 17q and triploidy [177–180]. In 2008, ALK point mutations were reported by several groups as being in both familial and somatic neuroblastoma [181–186]. In fact ALK mutation accounts for the majority of familial neuroblastoma cases [187]. In some familial and somatic neuroblastoma patients, mutations in the transcription factor paired-like homeobox 2B (PHOX2B) are acquired. Interestingly, a recent study has reported that ALK is a PHOX2B target gene, suggesting that part of the molecular mechanism underlying the effects of PHOX2B mutations in mutations may involve ALK [188]. Recently, two cases of congenital neuroblastoma carrying somatic, heterozygous ALK mutations (F1174L and F1245V, respectively) were reported as showing severe encephalopathy and brainstem abnormalities, suggesting that inappropriate ALK activation is deleterious to the development of the central nervous system [189].

A recent meta-analysis of neuroblastoma has reported ALK gain-of-function mutations to be present at a frequency of 6.9 % of investigated neuroblastoma tumours. Further, a comparison of the ALK mutation frequency in relation to genomic subtype revealed that ALK mutations were most frequently observed in *MYCN* amplified tumours (8.9 %), correlating with a poor clinical outcome [177]. The most frequent mutations are identified at positions F1174 and R1275 [177]. A mutation in the kinase domain of ALK, F1174S, was observed in a relapse neuroblastoma patient, and this relapsed tumour was extremely aggressive in nature, and it was shown in various preclinical setting that the relapsed mutation transformed ALK to a gain-of-function mutation [185]. This initial finding was followed up by an investigation of paired tumours, i.e., primary tumours vs relapsed tumours, which verified information that ALK mutation can be observed in 5–8 % of primary tumours, but the mutation rate of ALK in relapsed tumours is 27–30 % [190]. Of notice in cases where ALK-positive tumours were detected at relapse, a small fraction of mutant cells could already be detected in the primary tumour by employing deep sequencing NGS technology. This indicates that a tiny fraction of the primary tumour can be selected and take over, which result in a ‘bad

to worse with poor prognosis' relapsed tumour. An unanswered question in the field is whether ALK mutation alone is able to initiate NB and the progression of the disease, acting as a so-called oncogenic driver. This issue is further complicated by recent data investigating the relationship between ALK and MYCN, for which amplification is well characterised as a negative prognostic factor in neuroblastoma [177]. In neuroblastoma cell culture-based studies, both wild-type and mutant ALK drive initiation of MYCN transcription, while co-expression of activated ALK and MYCN increased NIH3T3 cell transformation in a foci analysis [191]. Thus, if ALK is able to drive oncogenesis in neuroblastoma, with its ability to upregulate MYCN, another prominent oncogene in NB may in part explain the poor outcome for patients with driver ALK mutations. The hypothesis that ALK collaborates with MYCN in neuroblastoma pathogenesis is bolstered by elegant work in transgenic zebrafish, where co-expression of activated ALK accelerates the development of MYCN-induced neuroblastoma-like tumours 3-fold [47, 192]. Further, ALK F1174L mutation potentiates the oncogenic activity of MYCN in neuroblastoma [193]. Whether ALK can initiate and drive tumour formation in the mouse has not yet been reported. However, Schulte et al. show that double transgenic mice for ALK F1174L expression to the neural crest induces tumour formation employing the DBHiCre or TH-Ires-Cre mice [194]. We know that knockdown of ALK expression by siRNA resulted in decreased proliferation of neuroblastoma cell lines [186]. Furthermore, expression of the F1174L and K1062M ALK mutants in NIH3T3 cells and nude mice lead to the rapid formation of subcutaneous tumours, demonstrating the oncogenic and transforming potential of these ALK mutants [182].

The nature of the various ALK mutations found in neuroblastoma is currently a topic of active research. At present, most mutations reported are point mutations in the kinase domain (Table 1.1 and Fig. 1.3); however, deletions also occur and a number of mutations are observed outside the kinase domain [208, 209]. Out of all mutations reported to date a number have been experimentally confirmed as activating kinase activity, including the most commonly occurring mutations—F1174 and R1275Q, and the recently described ALK^{del2-3} deletion reported in the NB-1 cell line [209]. Further, intragenic ALK rearrangements, genomic rearrangements with unbalanced translocations and chromothripsis have been observed, which show activation of downstream targets, is a novel mechanism in neuroblastoma [208, 210].

Currently the reported ALK mutations fall into three classes: (1) gain-of-function (G-O-F) ligand independent mutations (such as F1174I/S/L) [182, 183, 185, 195], (2) kinase-dead ALK mutants [198] or (3) ALK mutations which are ligand dependent in nature, such as the human ALK mutations (T1087I, D1091N, A1099T, T1151M, M1166R, A1234T, R1464STOP), which are not constitutively active but which display ligand-dependent activation using agonist antibodies [195]. Importantly, to date, the activity of all ALK mutants tested can be abrogated by treatment with ALK small molecular inhibitors, albeit with differing sensitivity (see below [196, 202, 211]).

Table 1.1 Reported mutations in ALK

Amino acid mutation	ALK region	Phenotype (Ref.)	Tumour/cell line	Reference for the mutation
Neuroblastoma				
K1062M	Juxtamemb. dom.	G-O-F [182]	T/C	[182]
T1087I	Juxtamemb. dom.	Wild type [195]	T	[182]
D1091N	β1-strand	Wild type [195]	T/C	[186]
A1099T	β2-strand	Wild type [195]	T	
G1128A	P-loop	G-O-F [196]	T	[186]
T1151M	β3-strand	Wild type [195]	T	[183]
T1151R	β3-strand	?	T	[197]
M1166R	αC-helix	G-O-F or WT [195]	T	[186]
I1170T/S	αC-helix	?	T	[172]
I1171N	αC-helix	G-O-F [196]	T	[186]
F1174L/S	End of αC-helix	G-O-F [182, 183, 185]	T/C	[181–184, 186]
F1174I	End of αC-helix	G-O-F [195]	T	[186]
F1174C/V	End of αC-helix	?	T/C	[182–184, 186]
R1192Q	In between β4 and β5	G-O-F [196]	T	[186]
A1234Y	αE-helix	Wild type [195]	T	[183]
F1240V	αE-helix	?	S	[172]
F1245C	−2 to HRD	G-O-F [196]	T	[183, 186]
F1245I/L/V	−2 to HRD	?	T/C	[181–184, 186]
I1250T	+1 to HRD	KD [198]	T	[186]
R1275Q	+2 to DFG	G-O-F [183, 196]	T/C	[181–184, 186]
R1275L	+2 to DFG	?	T	[184]
Y1278S	A-loop	G-O-F[a]	T	[184]
1464STOP	C-term to kin. dom.	Wild type [195]	T	
Selected through prolonged drug treatment or mutagenesis of NPM-ALK and EML4-ALK expressing in Ba/F3 or SH-SY5Y cell lines				
F1174L+ L1198P	C-helix + In between β1 and β2	A.R. G-O-F [199]	SH-SY5Y	[199]
F1174L+ G1123S or D	C-helix + In between β1 and β2	A.R. G-O-F [199]	SH-SY5Y	[199]
L1198P	Between β4 and β5	A.R. G-O-R [199]	Ba/F3	[199]
G1269S	−1 to DFG	A.R. G-O-F [199]	Ba/F3	[199]
D1203N	Between β4 and β5	A.R. G-O-F [199]	Ba/F3	[199]
Y1278H	1278-YRASYY-1283	N,D [199]	SH-SY5Y	[199]
Mutations found in lung adenocarcinomas patient expressed in lung H1299 and NIH3T3cells				
S413N	First MAM domain	A.R. G-O-F [200] ??	T and H1299	[200]
V597A	Second MAM dom	A.R. G-O-F [200] ??	T and H1299	[200]
H694R	Between MAM and G-rich domain	A.R. G-O-F [200]	T, NIH3T3 H1299	[200]
G881D	G-rich domain	A.R. G-O-F [200] ??	T and H1299	[200]
Y1239H	Catalytic loop	I.R. G-O-F [200] ??	T and H1299	[200]
Y1278S	Catalytic loop	A.R. G-O-F [200]	T and H1299	[200]

(continued)

Table 1.1 (continued)

Amino acid mutation	ALK region	Phenotype (Ref.)	Tumour/cell line	Reference for the mutation
D1311A	Catalytic loop?	Not driver, wt or KD?		[201]
E1384K	C-term of Kin. Dom	A.R. G-O-F [200]	T, NIH3T3 H1299	[200]
K1518N	C-term of Kin. Dom	Not driver, wt or KD?	MEF's	[201]
K1525E	C-term of Kin. Dom	Not driver, wt or KD?	MEF's	[201]
Secondary mutations found in NSCLC and IMT with acquired resistance (A.R.) to crizotinib expressed and tested in cells				
I1151Tins	End of β3	A.R. G-O-F [202]	Ba/F3	[202]
L1152R	End of β3	A.R. G-O-F	Ba/F3, H3122	[130]
C1156Y	End of β3	A.R. G-O-F [203]	Ba/F3	[203]
I1171N/T/S	αC-helix	A.R. G-O-F		[204–206]
F1174L	End of αC-helix	A.R, G-O-F [109]	Ba/F3	[109]
V1180L	End of αC-helix	A.R. G-O-F		[204]
L1196M	Gateway	A.R. G-O-F [127, 202, 203]	Ba/F3	[203]
G1202R	Between β4 and β5	A.R. G-O-F [202]	Ba/F3	[202]
S1206Y	Between β4 and β5	A.R. G-O-F [202]	Ba/F3	[202]
G1269A	Catalytic loop	A.R. G-O-F	Ba/F3	[127]
Secondary mutations in ATC				
L1198F	Between β4 and β5	A.R, G-O-F	NIH3T3	[207]
G1201E	Between β4 and β5	A.R. G-O-F	NIH3T3	[207]
Burkitt's lymphoma				
R412C	First MAM domain	Not driver, wt or KD?	MEF's	[201]
Osteosarcoma				
C1021Y	Extra cellular, close to TM	Not driver, wt or KD?	MEF's	[201]
Carcinoma of the endometrium (CL)				
A1252V	Catalytic loop	Not driver, wt or KD?	MEF's	[201]
Uterine leiomyosarcoma				
R1192P	In between β4 and β5	Not driver, wt or KD?	MEF's	[201]

G/S germline/somatisk point mutations, *ND* not determinded, *wt* wild type, *KD* kinase dead, *kin. dom*. kinase domain, *?* not been investigated
[a]Chand, D et. al., manuscript submitted

1.3.3.2 ALK Point Mutations in Other Cancers

ALK mutations in other cancers are now also being described, perhaps as a result of increased awareness of the role of ALK as an oncogene (Table 1.1). In anaplastic thyroid cancer (ATC), two point mutations—L1198F and G1201E—have been described. These are located in the hinge region between the N- and C-terminal lobes of the ALK kinase domain, in close proximity to the ATP/inhibitor binding

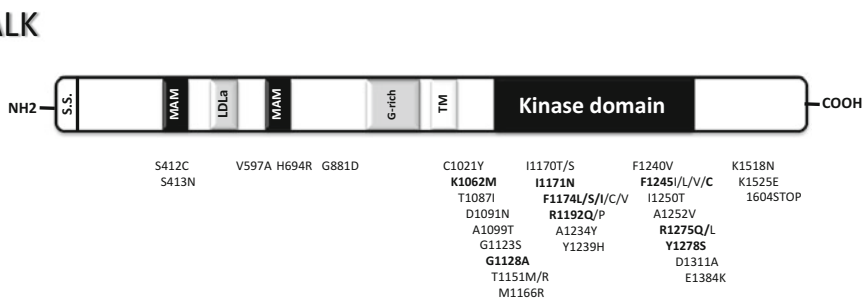

Fig. 1.3 ALK mutations identified in neuroblastoma. Point mutations found in neuroblastoma (for more information, see Table 1.1). *Bold* numbers indicate gain-of-function mutations. All other mutations show ligand-dependent activation abilities or mediate a kinase-dead ALK (mutation I1250T); see Table 1.1 for more information

site, and display increased kinase activity and ability to form foci and anchorage independent colonies [207].

Wang and co-workers investigated ALK in lung cancer, identifying six novel mutations—S413N, V597A, H694R and G881D in the extracellular domain for ALK as well as Y1239 and E1384K in the intracellular kinase domain (Table 1.1). Four of these mutations V597A, H694R, G881D and E1384K promote ligand independent phosphorylation of ALK as well as activation of downstream targets. Interestingly, in nude mice all six mutations identified were able to promote tumour growth, although H694R and E1384K produced a more robust response in xenograft assays that was sensitive to treatment with the WHI-P154 and the NVP-TAE684 ALK inhibitor [200].

Treatment of cancer patients with small molecular inhibitors has led to another challenging problem that of acquired drug resistance, in which secondary mutations appear that overcome drug inhibition at levels deliverable in patients. Such secondary mutations have been described in response to crizotinib (Xalkori) treatment regimes in NSCLC, IMT and ATC (discussed in more detail below, Table "Receptor at a glance: ALK/LTK").

1.4 The Role of the LTK Receptor Tyrosine Kinase in Human Disease

1.4.1 LTK in Human Cancer

LTK signalling drives both growth promoting and antiapoptotic pathways; thus, any dysregulation of LTK should result in consequences in human disease, particularly for neoplastic cell growth. It has been reported that high expression of LTK in non-small cell lung cancer (NSCLC) patients correlates with an increased risk of

Table 1.2 Reported mutations in LTK

Amino acid mutation	LTK region	Phenotype (Ref.)	Tumour/ cell line	Reference for the mutation
P116S	G-rich domain	?	SCC	[131]
G310E	G-rich domain	?	SCC	[131]
E398D	Between G-rich and kin. dom.	?	?	COSMIC domain
A432T	Between G-rich and kin. dom.	?	LAC	[213]
V480I	Between G-rich and kin. dom.	?	LAC	[213]
F586L	End of αC-helix	G-O-F [214]		[186]
R608*	Equivalent to ALK position R1275	?	LAC	[213]
Y616*	Third Y in 'YRASYY'	?	LAC	[213]
P625S	Equivalent to ALK position P1292	?	SCC	[131]
R669Q	+2 to DFG	G-O-F [214]		[186]
Q717K	Equivalent to ALK position 1384	?	LAC	[213]

SCC squamous cell carcinoma, *LAC* lung adenocarcinoma, *?* not been investigated

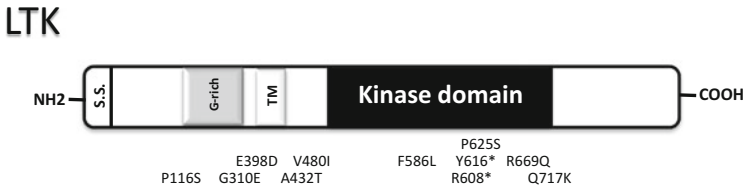

Fig. 1.4 LTK mutations identified in SCC and lung adenocarcinoma. Point mutations found in squamous cell carcinoma and in lung adenocarcinoma patients according to data deposited in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Table 1.2; http://www.sanger.ac.uk/genetics/CGP/cosmic/)

metastasis [212], suggesting LTK dysregulation may have consequences for cancer progression in various tumour types. In fact, according to data deposited in the Catalogue of Somatic Mutations in Cancer (COSMIC) database, a number of mutations in LTK have been found in human cancers (Table 1.2 and Fig. 1.4). To date, none of these mutations have been characterised; thus, we do not currently know whether they are activated versions of the LTK RTK or whether they represent passenger or drivers in the tumourigenic process. One study has been recently carried out, in which two LTK mutants, F586L and R669Q, were generated that correspond to two of the activating ALK mutations (F1174L and R1275Q) found in neuroblastoma. Similar to results in ALK, they observed that the F568L (F1174L in ALK) substitution transformed Ba/F3 cells to IL-3 independent growth, but not the R669Q mutations [214]. Moreover, the F586Q mutation was able to transform epithelial cells and induce PC12 cell neurite outgrowth, in keeping with the equivalent ALK mutations [196, 214]. Thus, as in ALK, mutations in LTK at these conserved residues result in activation of the kinase. However, these mutations have not been

described in LTK in any tumour samples to date (http://www.sanger.ac.uk/genetics/CGP/cosmic/) (Table 1.2 and Fig. 1.4). Controversely to ALK, and extracellular screen identified FAM150A and B as LTK ligand's with a binding affinity of 28 nM, which stimulate LTK phosphorylation [215]. Regarding downstream signalling the LTK mutants (F586L and R669Q above) induce the activation of various signalling pathways including Shc, Erk and the JAK/STAT pathways [214], suggesting that the signalling potential of LTK will share some similarities with ALK signalling.

1.4.2 LTK in Other Human Disease

Systemic lupus erythematosus patients also from a Japanese population study observed *LTK* polymorphism (*Ltk9464A-type* allele; Glu763Lys) with a significantly higher frequency compared with the healthy controls. This human polymorphism is close to that observed in the systemic lupus erythematosus (SLE)-prone NZB mouse which harbour a gain-of-function polymorphism (Gly746Glu) in the LTK kinase domain near the PI3K binding motif [18]. This polymorphism has been suggested to play a role in the aberrant activation of B cells observed in SLE patients. Only a limited number of studies have addressed the role of LTK in human disease, and it is clear that further investigation will be required to understand more fully the role of LTK in human pathology.

1.5 ALK (Synonyms: CD246)

1.5.1 ALK Gene, Transcripts and Protein Structure

The full-length human ALK RTK was first cloned in 1997 [7, 8]. The *ALK* locus, found on human chromosome 2, encodes a 1,620-amino-acid protein comprising an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. The ALK extracellular domains are unique among the RTKs, containing an LDLa domain [9, 10] and two MAM domains (named after meprins, A-5 protein and receptor protein-tyrosine phosphatase mu). MAM domains are comprised of ~160 amino acids and are present in transmembrane proteins such as the meprins and receptor protein-tyrosine phosphatases, in which they seem to function in cell–cell interactions [11–13]. In addition, a glycine-rich region is also found in the ALK extracellular domain.

ALK expression is observed in both the central and peripheral nervous systems among other tissues [7, 8, 20–24]. Although we know little about the transcriptional control of ALK, a recent report demonstrated that PHOX2B drives ALK gene transcription by directly binding its promoter. In keeping with a role in the regulation of ALK transcription, PHOX2B is involved in the specification of the noradrenergic phenotype during the development and differentiation of neural crest derivatives [188].

Furthermore, in human cells, the microRNA miR-96 has been identified as being able to bind the ALK 3′UTR. miR-96 appears to target alK through the 3′ UTR of ALK and may play a role in down regulation of ALK protein levels [216].

The ALK extracellular domain contains 16 highly conserved putative sites of N-linked glycosylation and is known to be glycosylated, resulting in a mature receptor with a molecular weight of approximately 220 kDa [217]. Interestingly, glycosylation has been shown to be important for correct activation of ALK and subsequent downstream signalling [217, 218].

At this point, there is no structural information concerning the ALK extracellular region. However, several groups have published the crystal structure of the ALK kinase domain in an inactive conformation providing important insight into the mechanisms underlying ALK activation [219, 220]. Like the InR and other members of the InR superfamily, ALK has a Y′XXX′YY autophosphorylation motif (Y′RAS′YY) in the activation loop (A-loop) [221–223]. In the case of the InR, the second tyrosine is proposed to be first residue phosphorylated. However, in ALK the first tyrosine, at position Y^{1278}, is initially phosphorylated. Moreover, some distinctive structural features are observed in the ALK structure, with a unique inhibitory position of the ALK A-loop packing a short proximal A-loop α-helix against the αC-helix of ALK, while a β-turn motif obstructs the substrate binding region (Fig. 1.5). In this arrangement Y^{1278} is inaccessible for phosphorylation since it is engaged in the interaction interface through bonding with Cys^{1097} in the N-terminal β-sheet [219, 220]. Interestingly, the tyrosine at position 1096 (Y^{1096}) in ALK, which is adjacent to Cys^{1097} in the N-terminal β-sheet, has also been identified as an ALK phosphorylation site

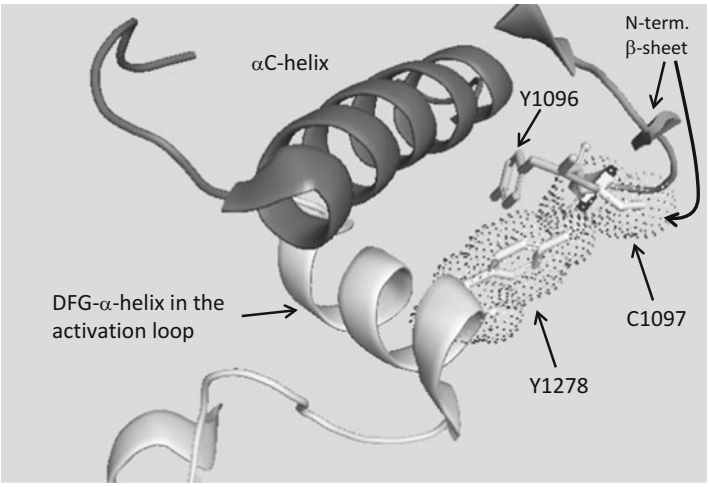

Fig. 1.5 A unique inhibitory position of the ALK activation loop. The αC helix is shown in cartoon and is *darker grey*. The short helical segment included in the activation loop following the DFG motif is in *white*. The N-terminal two-stranded antiparallel β-sheet is indicated and in *lighter grey*. Residues Y1278, C1097 and Y1096 are indicated as *sticks*. Y1278 is inaccessible for phosphorylation since it is engaged in the interaction interface through bonding with Cys1097 in the N-terminal β-sheet indicated with *sticks*/*dots*

[224, 225]. This proximity has led to the suggestion that the initial activation of ALK may be mediated by phosphorylation of Y^{1278} or by the nearby Y^{1096}, thus releasing ALK from inactive conformation restraints [219, 220]. While crystal structure(s) of activated ALK would facilitate future structure–function investigations, some deductions as to the nature of ALK activating mutations can be made. For example, R^{1275} in ALK—which is mutated in neuroblastoma—contributes interactions between the A-loop and the αC helix, stabilising the autoinhibited ALK kinase domain conformation. Mutation of R^{1275} to glutamine would be predicted to disrupt these autoinhibitory interactions and thus lead to ALK activation. Another hotspot for mutation of ALK in neuroblastoma is at position F^{1174}, which lies at the C terminus of the αC helix chain where it is a central residue in a small well-packed hydrophobic 'core' between the αC and the A-loop. It is thought that reducing the size of the F^{1174} side chain, as observed in neuroblastoma mutations (see Sect. 1.3.3.1), results in disruption of this packing, subsequently weakening autoinhibitory interactions and allowing the ALK kinase domain to more easily adopt its active configuration.

1.5.2 ALK Ligands

Currently no Jeb-like ligand for mammalian ALK has been described (Fig. 1.6). The ability of both mouse and human ALK to respond to *Drosophila* Jeb has also been analysed, and it is clear that they cannot respond to the *Drosophila* Jeb ligand [60, 185]. The expression of ALK in the neural crest and its involvement in

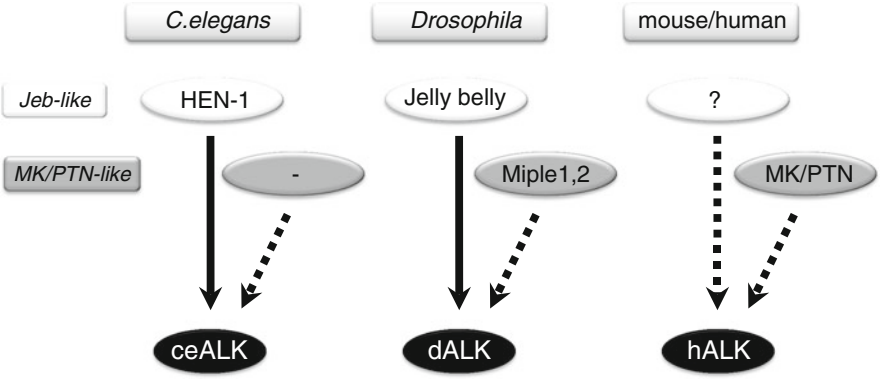

Fig. 1.6 Activation of ALK in *C. elegans*, *Drosophila* and mouse/human. Signalling by ALK is triggered known ligands in some model organisms. In *C. elegans* ALK (ceALK) and *Drosophila* ALK (dALK) is activated upon binding of the HEN-1 and Jelly Belly (Jeb) ligands, respectively. In human and mouse no Jeb/HEN-1-like ligand has been reported to date; however, the small heparin-binding growth factors pleiotrophin (PTN) and midkine (MK) have been suggested to be ALK ligands. There is no structural similarity between PTN/MK and the HEN-1/Jeb proteins. PTN/MK does not appear to be present in the *C. elegans* genome; however, the *Drosophila* genome contains two related genes: *Miple 1* and *Miple 2* [226]

neuroblastoma suggest that a potential ALK ligand may be presented to the developing neural crest cells at some point during development. While the issue of an ALK ligand may not be as important in the context of NSCLC or other cancer types where ALK exists as a fusion protein, this may be of critical importance in, e.g., neuroblastoma development.

The activation of ALK by the small heparin-binding growth factors midkine (MK) and pleiotrophin (PTN) has been reported [227, 228]. However, some questions remain regarding activation of ALK by MK/PTN, since no genetic evidence exists and several groups have reported contradictory findings [57, 58, 165, 167, 229, 230]. MK and PTN also signal via various other receptors such as receptor protein-tyrosine phosphatase β/ζ (RPTPβ/ζ), N-syndecan, LRP and integrins [231–236]. Thus, ALK could be activated intracellularly through the RPTPβ/ζ signalling pathway [237] or recruited and activated to the LRP/integrin/MK receptor complex, since the MK dimer binds to integrin and the chondroitin sulphate chain of RPTPβ/ζ, thereby inhibiting this phosphatase and resulting in an increase in the tyrosine phosphorylation of proteins included in the complex, such as ALK [234]. A genetic approach by Hugosson et al. employing *Drosophila* as a model system indicates that *Drosophila* ALK is not activated by *Drosophila* miple 1 and miple 1 which are the equivalent to human MK and PTN [238]. The authors also reported that neither human MK nor PTN can activate hALK in vivo when ectopically co-expressed in the fly. This information strongly indicates that ALK is still an orphan RTK.

1.5.3 Mechanism of Activation and Signalling via the Wild-Type ALK Receptor

The wild-type ALK receptor is considered to transmit signals from the external cellular environment to targets in the cytoplasm and the nucleus impacting upon cell growth, metabolism and differentiation. Compared with the extensive data accumulated from studies on ALK fusion proteins, in particular, NPM-ALK and EML4-ALK (see below), there are fewer published studies concerning signalling via the wild-type ALK. This is mainly due to the technical restrictions imposed by the lack of a clearly defined ligand for mammalian ALK. Work in the worm and fruit fly model organisms, where the ligands Jeb and HEN-1 activate ALK, has led to discovery of core components of the pathway within a physiological context (see above). In mammalian cell systems several approaches have been taken to investigate signalling by wild-type ALK (Fig. 1.7):

A. Activation by Artificial Chimeric Receptor Dimerisation Early efforts to study ALK signalling pathways relied on artificial dimerisation via replacement of the ALK extracellular domain to produce a chimeric receptor. These have included replacing the EC domain of ALK with (1) the mouse IgG 2b Fc domain to create an ALK.Fc chimera [59], (2) the extracellular domain of the EGFR [239] as well as (3) addition of a binding site in the intracellular domain of ALK for a small, synthetic dimeriser which induces dimerisation of the engineered ALK receptor [56].

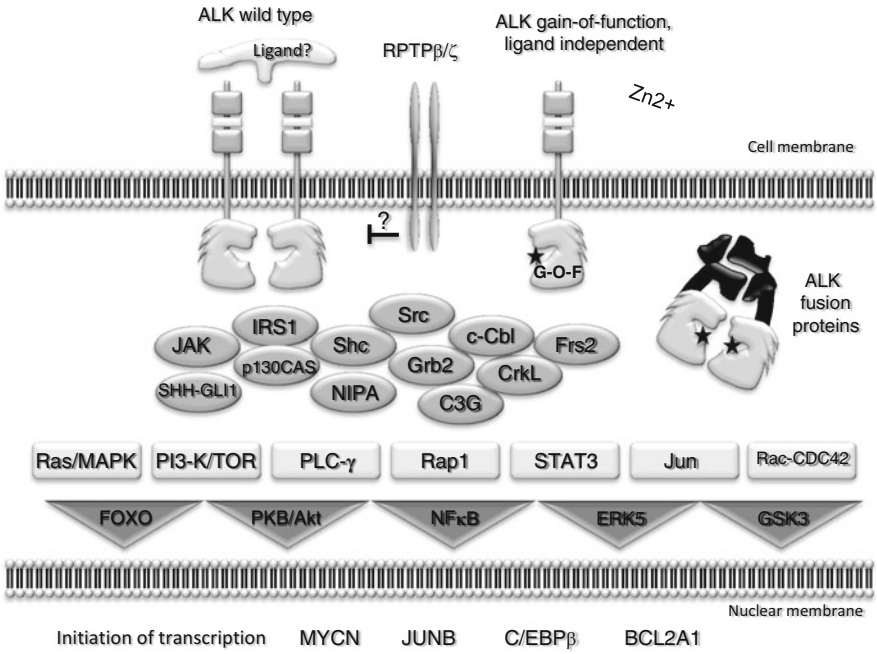

Fig. 1.7 Activation and signalling via wild-type and various ALK mutated RTK. Signalling via human ALK likely occurs through ligand mediated dimerisaton in response to ligand(s), activating antibodies or increased levels of Zink. ALK mediates signalling via the Ras/MAPK, PI3-kinase/TOR/PKB/Akt, ERK5, PLCγ, Rap1, JAK/STAT, Rac-CDC42 and the Jun pathways. Proteins such as SHH-GLI1, NIPS, Src, IRS1, p130-CAS, Shc, Grb2, C3G, c-Cbl, CrkL and Frs-2 interact and are phosphorylated by ALK upon activation. ALK regulates a number of genes at the transcriptional level, some which have been validated, such as MYCN, JunB, DEBPB and BCL2A1. Activation of ALK via RPTPβ/ζ, independently of direct ALK ligand interaction, has also been proposed

Employing chimeric ALKs such as the above has revealed that ALK dimerisation drives tyrosine autophosphorylation of ALK [56, 59, 239] with subsequent ERK activation and neurite outgrowth in PC12 cells [56, 59]. Furthermore, the neurite outgrowth observed is blocked by addition of the MEK-1 inhibitor PD98059, but not by PI3K or PLCγ inhibition, implying that the activation of MAPK is functionally important in the induction of neurite outgrowth in PC12 cells.

B. Activation Through the Use of Monoclonal Antibodies Investigation of endogenous ALK signalling has been aided by the identification of activating monoclonal antibodies generated by immunising mice with the entire extracellular domain of human ALK [57, 185]. Taken together, studies employing these reagents show that wild-type ALK activates a number of intracellular signalling pathways, such as MAPK, ERK5, PI3-K, PLC γ and C3G/Rap1 [56–60, 63, 64, 239]. Indeed, the prolonged ERK 1/2 activation seen upon ALK activation is associated with

differentiation of PC12 cells. Moreover, ALK is also able to regulate MYCN transcription and protein levels [191]. Phosphorylation of a number of key signalling components including IRS-1, STAT3, FRS2, Shc, and c-Cbl by ALK has also been reported [57, 58]. Interestingly, flotilin-1, a plasma membrane protein involved in internalisation of proteins into the cytosol by endocytosis, is a binding partner for ALK and regulates degradation of ALK in lysosomes through endocytosis [240]. ALK has also been reported to function as a dependence receptor harbouring an activation independent function [230].

C. Activation by Zinc Finally, it has been reported that ALK can by activated by zinc. The activation is dependent on ALK kinase activity and dimerisation of the ALK receptor but is independent of Src family kinase activity. Interestingly, zinc is involved in synaptic transmission in the mammalian brain, with zinc stored in glutamatergic and gabaergic vesicles being released into the extracellular space following high-frequency stimulation [241], leading the authors to suggest that in the central nervous system zinc could constitute a physiological ligand of ALK. The relevance of these results remains to be explored more fully [242].

1.5.4 Oncogenic ALK Signalling

Most information regarding ALK signalling comes from studies of the oncogenic ALK fusion proteins. This work has mainly examined the first described ALK fusion, namely, NPM-ALK. Further, since the discovery of EML4-ALK, a significant body of work has now begun to accumulate regarding signalling from this ALK fusion protein in the context of NSCLC.

When considering signalling mediated by the oncogenic ALK fusion proteins, it should be remembered that, firstly, the subcellular localisation of the ALK fusion proteins are out of context as compared with the membrane-bound wild-type ALK receptor, reflecting instead the cellular localisation determined by the ALK fusion partner. Secondly, the ALK fusion partner of ALK is likely to determine the level and tissue specific pattern of transcription. Thirdly, the fusion partner drives the dimerisation of the ALK kinase domain, thereby activating the kinase domain, leading to subsequent downstream signalling event. Substrates phosphorylated by the ALK fusion proteins most likely include both legal and 'illegal' targets and pathways, given that ALK has lost its normal spatial and temporal restraints, which are now determined by the fusion partner of ALK (Fig. 1.7).

NPM-ALK activates major signalling pathways such as PLCγ, JAK/STAT-, PI3-kinase/PKB/Akt, JunB and MAPK signalling pathways impacting on downstream target proteins, such as FOXO proteins, affecting cell growth, transformation and antiapoptotic signals (Fig. 1.7) [64, 243–245]. ALKs also directly regulate the abundance of HIF1α and HIF2α proteins in hypoxic conditions [246].

In cell culture, PLCγ has been shown to interact with NPM-ALK through the tyrosine equivalent to position 1604 in wild-type ALK and is also important for

transformation. Mutation of tyrosine 1604 abrogates the interaction between NPM-ALK and PLCγ and results in a loss of PLCγ phosphorylation and activation [247].

One important difference between wild-type ALK and fusion ALK proteins is the strong activation of the JAK/STAT cascade by NPM-ALK. While the mechanism of NPM-ALK-mediated STAT3 activation is unclear, a number of groups have reported STAT3 phosphorylation and activation [248–253]. JAK3 has been shown to associate with NPM-ALK, and upon inhibition of JAK3, STAT3 activation is reduced [252, 254, 255]. Alternatively, it has been suggested that ALK binds and activates STAT3 directly via the first tyrosine in the YxxxYY motif [222]. Adding to the complexity involved, Shp1 and protein phosphatase 2A, regulators of the JAK/STAT pathway, have been reported to be aberrantly expressed in ALK-positive ALCL [253, 256, 257]. The Shp1 tyrosine phosphatase associates with and dephosphorylates NPM-ALK leading to a reduction of NPM-ALK tumourigenicity [132, 256].

NPM-ALK has also been reported to activate STAT5B, leading to apoptosis and cell-cycle arrest, although a number of groups have been unable to detect STAT5 activation [252, 253, 258]. A recent interesting report investigated an observed correlation between ALK-positive ALCL lines and a lack of STAT5A expression. The resulting data suggests that STAT5A acts as a tumour suppressor in part by suppressing expression of NPM-ALK and that the NPM-ALK fusion itself acts via STAT3 to epigenetically silence STAT5A ALK-positive ALCL cell lines [259].

Another important NPM-ALK interaction partner is PI3K, leading to the activation of Akt and antiapoptotic signalling. This interaction has been reported to occur via NPM-ALK interaction with the p85 subunit of PI3K but may also be mediated via other adaptor molecules [260–263]. The PI3K downstream target PKB/Akt is also important for transformation since expression of a dominant negative PKB/Akt leads to delayed growth and tumour formation [263]. One NPM-ALK regulated component downstream of PI3K/Akt appears to be GSK3β, which is phosphorylated and inhibited by NPM-ALK activity, favouring growth and protection from apoptosis [264].

A later study, in which SNT-2/FRS2 was identified as an NPM-ALK binding partner by two-hybrid, examined a mutant version of NPM-ALK in which the SNT-2/FRS2 binding sites were mutated. This mutant, in which Y156 (equivalent to hALK Y1096), Y567 (equivalent to hALK Y1507) and a 19-amino-acid sequence (aa 631–649, equivalent to hALK L1571-G1589) were mutated, exhibited significantly reduced transforming activity still interacted with PI3K and PLCγ [224]. A more recent study has suggested a link between the Sonic Hedgehog (SHH) and the PI3K pathway downstream of NPM-ALK, where NPM-ALK-induced PI3K activation regulates SHH/GLI1 signalling, contributing to NPM-ALK-mediated oncogenicity [265].

NPM-ALK activation also phosphorylates the transcription factor FOXO3a (Forkhead Box O 3a) via the PI3K/PKB/Akt pathway in an inducible NPM-ALK Ba/F3 cell culture model. The modulation of PI3K/PKB/Akt/FOXO3a activity subsequently affects expression of FOXO3a target genes such as Bim-1, $p27^{Kip1}$ and cyclin D2 [266, 267]. Furthermore, NPM-ALK induces activation of the rapamycin-sensitive mTOR signalling pathway, although this activation may be supported by both RAS/

Erk and PI3K pathway [268, 269]. In the Ba/F3 cell system inhibition of mTOR with rapamycin results in enhanced G1 cell-cycle arrest and apoptosis [270].

In addition to activation of JAK/STAT and PI3K signalling, ALK fusion proteins such as NPM/ALK also activate the Ras/MAPK pathway, interacting with IRS1, Shc and Grb2, leading to subsequent downstream signalling [261, 268, 271, 272].

Additional work employing the two-hybrid approach to identify novel partners of NPM-ALK led to the identification of NIPA (nuclear interacting partner of ALK) [273]. Overexpression of NIPA in Ba/F3 cells protects from apoptosis induced by IL3 withdrawal, leading the authors to suggest an antiapoptotic role for NIPA in NPM-ALK signalling. While subsequent work has characterised NIPA as an F-box-containing protein that defines an SCF-type E3 ligase controlling mitotic entry [274], its role in NPM-ALK signalling requires further analysis.

Other downstream targets of NPM-ALK include the small GTPases Rac1 and Cdc42 [275, 276]. Moreover, p130 Crk-associated substrate (p130Cas) has been reported to mediate the transforming potential of NPM-ALK via Grb2 [277]. Additional potentially important NPM-ALK interactions are with the cytoplasmic tyrosine phosphatases Shp1 and Shp2 as well as Src kinases such as pp60Src, the significance of which is not well understood currently [256, 278, 279]. NPM-ALK has also been shown to interact the PIKfyve lipid kinase and has been reported to play a role in ability of NPM-ALK-positive cells to promote degradation of the extracellular matrix and invasiveness [280]. JNK activity has been shown to be important in the NPM-ALK-mediated development of lymphomas in mice and cell-cycle progression and oncogenesis in ALCL cell lines [281, 282]. A further role for JNK appears involve in inhibition of the p53 tumour suppressor pathway by NPM-ALK [283].

A number of groups have identified novel ALK targets by proteomics leading to a substantial list of potential players in ALK signalling including Dok2, IRS1, SHC, Crk, CrkL, STAT3, VASP, PSF, MSH2 and ATIC [225, 271, 279, 284–290]. Analysis of the transcriptomes of ALCL cell lines identified a number of ALK regulated genes such as the antiapoptotic protein BCL2A and the transcription factor C/EBPβ [291, 292].

Recently a number of genes regulated by NPM-ALK at the transcriptional level have also been reported, some also validated by siRNA analysis such as *JunB*, *DEBPB*, *BCL2A1*, *MMP-9*, *p16INK4a* and *HIF1α* [191, 293–295]. MicroRNAs (miRNAs) miR-135b, miR-29a and miR-16 have also been shown to be modulated downstream of NPM-ALK activity, raising an additional level of complexity in ALK-mediated oncogenic signalling [296–298].

Of the known ALK fusion proteins NPM-ALK has been studied most extensively. The other fusion proteins are assumed to signal in a similar fashion, although this has not been confirmed for all and in fact some differences have been observed between different fusion proteins. For example, it has been described that ATIC-ALK associates with Grb2 and Shc, while TFG-ALK interacts with Grb2, Shc and PLCγ [86, 299]. An additional ALK fusion protein, KIF5B-ALK, detected in non-small cell lung cancer, has been shown to activate STAT3 and Akt [136]. An investigation of NPM-ALK, TPM3-ALK, TFG-ALK, CLTC-ALK and ATIC-ALK revealed contrasting transforming and tumourigenic potential for these different fusion proteins [300]. The recent focus on the EML4-ALK fusion protein has increased our knowledge of this ALK fusion in particular [182, 301]. EML4-ALK exhibits

transforming potential [182, 301], and inhibition with the ALK specific inhibitor TAE684 induces apoptosis in lung cancer cells via the ERK/BIM and STAT3–survivin signalling pathways [302]. Clearly the signalling pathways utilised by the various ALK fusion proteins will share common components, but given the variable cellular contexts a number of differences can be expected, based upon tumour type and ALK fusion partner. While NPM-ALK signalling has been well studied, it is clear that many questions remain to be answered not only for this fusion protein but also for the numerous other ALK fusion proteins now identified.

1.6 LTK

There have been few studies published concerning leukocyte tyrosine kinase (LTK), and the function of this enigmatic RTK remains poorly understood. One important task for the future will be to define LTK signalling and its role in both development and disease.

1.6.1 LTK Gene, Transcripts and Protein

Leukocyte tyrosine kinase (LTK) was initially cloned from mouse [1] and subsequently from human [303]. Subsequent work revealed that both murine and human LTK possessed larger extracellular domains than initially reported [2–4, 304, 305]. In the mouse, CTG and ATG codons present several hundred base pairs upstream of the initially proposed ATG have been shown to serve as translation initiation codons [2, 3, 304]. In mammals LTK is expressed in pre-B and B lymphocytes and in the adult brain, as well as in the placenta [1, 2, 306].

The *LTK* locus is located on human chromosome 15 and produces a number of transcripts. The predominate cDNA isoform directs the synthesis of an 864-amino-acid protein, approximately 100 kDa, consisting of an extracellular domain, transmembrane domain, a tyrosine kinase domain and a short carboxy terminus [305]. While the extracellular domain of LTK contains a glycine-rich region, similar to that found in ALK, no other distinguishing features are obvious, and no ligand for LTK has been described in any species. The LTK kinase domain falls within the insulin receptor superfamily and is most similar to that of ALK, including conservation of the 'YRASYY' sequence found in the activation loop, which has been shown to be critical in the activation of ALK kinase activity (see Sect. 1.4.2 above).

1.6.2 LTK Mechanism of Activation and Signalling

Initial studies overexpressing LTK in Cos-1 cells results in tyrosine phosphorylation of the 100 kDa LTK RTK and the co-immunoprecipitation of a number of cellular phospho-proteins. LTK was found to associate with several Src homology (SH)

2-containing proteins, such as the p85 subunit of phosphatidylinositol 3 kinase (PI3 kinase), phospholipase C-γ (PLC-γ) and p21 ras GTPase activating protein (GAP) and the serine/threonine kinase, Raf-1. Furthermore, these interactions were dependent upon the kinase activity of LTK [306]. A subsequent study, in which a chimeric molecule, EGFR-LTK, was generated by replacing the extracellular region of LTK with that of the EFG receptor, suggested that LTK might function to transduce a signal, through its kinase activity, in response to binding of an as yet unidentified ligand. The EGFR-LTK chimeric receptor associated with Shc, via tyrosine 862 in the $NPXY^{862}$ motif, in a ligand-dependent manner recruiting Grb2 and Sos to the activated receptor [307]. In fact, LTK contains two NPXY motifs within the intracellular domain—$NPXY^{485}$ and $NPXY^{862}$—which are binding sites for Shc and IRS-1. While both NPXY motifs appear to contribute to activation of the Ras pathway and generation of mitogenic signals, only tyrosine 485 to which IRS1 binds transmits cell survival signals [308]. Inhibition of PI3K with wortmannin abolishes the survival effects of LTK. The p85 subunit of PI3 kinase binds directly to tyrosine 753 of LTK, within a YXXM motif, a consensus binding amino acid sequence for the SH2 domain of p85. Importance of the PI3 kinase pathway for the survival effects of LTK is illustrated by the fact that Ba/F3 cells stably expressing the EGFR-LTK chimeric receptor mutated at tyrosine 753 enter apoptosis even in the presence of EGF [309]. The creation of an additional chimeric receptor, colony-stimulating factor-1 (CSF1R)-LTK, in which the extracellular region of LTK was replaced by that of the receptor for CSF1, was used to examine the function of LTK in PC12 cells and to investigate effects on neurite outgrowth. Here, CSF1R-LTK activation resulted in activation of PKB/Akt and ERK together with robust neurite outgrowth [310].

In a recent study, the potential for activation of LTK was explored, with mutations in LTK analogous with the ALK^{F1174} and ALK^{R1275} mutational hotspots found in neuroblastoma patients generated in the LTK protein (LTK^{F568L} and LTK^{R669Q}) resulting in activated versions of the LTK RTK [214]. On expression in 293 T cells, these activated LTKs were able to induce phosphorylation of downstream signalling proteins, including Shc, ERK, Jak1, STAT3 and PKB/Akt.

Clearly much remains to be learned about LTK activation and signalling. Identification of a ligand (FAM150A and B) [215] recently for this RTK will be a significant step forward in unravelling the roles of LTK signalling processes, mechanisms of activation and its importance in development processes in vivo.

1.7 Treatment of ALK-Mediated Disease

Patients presenting with tumours in which ALK is activated represent a population for which individualised medicine regimes with ALK tyrosine kinase inhibitors (TKIs) show promise. Xalkori (formerly known as PF2340166 and crizotinib) was FDA approved in 2011 for use with non-small cell lung cancer (NSCLC) patients who express ALK gene fusions. The time encompassing the discovery of ALK fusion oncoproteins in NSCLC, development of Xalkori, preclinical and clinical

trials up to the point of an approval of Xalkori by FDA was less than 4 years. Initial reports of a novel ATP analogue inhibitor with specificity for ALK and c-Met (then named PF2341066) in experimental models of ALCL together with the appearance of ALK fusion proteins in NSCLC patients [114, 115, 311] were quickly acted upon and developed further. Early trial results treating patients with advanced ALK-positive NSCLC with Xalkori has shown benefit and improved survival [54, 312]. Among the 82 ALK-positive patients who were given crizotinib, median overall survival from initiation of crizotinib has not been reached yet; 1-year overall survival was 74 % (95 % CI 63–82), and 2-year overall survival was 54 % (40–60) [312]. However, the usefulness of long term treatment with drugs such as crizotinib and other TKIs, such as EFGR TKIs, is limited due to development of drug resistance in patients. These acquired inhibitor resistance mutations are a serious complication in the treatment of the cancer patient population, which may be overcome by the development of more effective clinical drugs and combination therapy strategies. Although, from a recent and open label trial comparing crizotinib with chemotherapy, crizotinib is superior in patients with previously treated, advanced ALK-positive NSCLC [313].

A number of studies have reported patients with acquired crizotinib resistance in ALK-positive NSCLC and IMT patients [116, 127, 130, 202, 203]; see Table 1.1. One of the first secondary mutations to be described was L1196M, which lies in the classical 'gatekeeper' residue in the ALK kinase domain [203, 314]. This mutation is similar to the T790M gefitinib-resistance mutation observed in the EGFR and the T315I mutation in ABL, suggesting the possible explanation for the drug resistance is an increased affinity for ATP, which is able to outcompete crizotinib [315]. The mechanism behind the other secondary mutations, C1156Y [203] and L1152R [130], which are not situated at the gatekeeper position but in the loop between β3 and the αC-helix, is yet to be clarified. Since no structure is available with ALK in an active mode, it is difficult to project the conformational disturbance by these mutations from leucine and cysteine to arginine and tyrosine, respectively. However, it is clear that stably expressed with above mutations in the lung cancer cells line H3122 mediate an increased resistance to crizotinib, i.e., the IC_{50} value increases threefold for crizotinib. The novel ALK mutation described by Doebele and co-workers at position G1269A was observed in two patients and is more resistant to crizotinib [127]. Amino acid G1269 is critically situated for ATP/crizotinib binding and may act by reducing crizotinib binding due to steric hindrance or by increasing the affinity for ATP over crizotinib similar to the L1196 mutation [315].

Katayama and co-workers investigated patients with ALK-positive NSCLC that had relapsed on crizotinib and identified four resistance mutations close to the ATP binding pocket, including the L1196M 'gateway' mutation and three novel mutations: G1202R, S1206Y and an insertion at position 1151 (T-ins) [202]. Mutation of G1202R is analogous to the BCR–ABL mutation G340W which has not yet been reported in a patient. Both G1202R and S1206Y are positioned in the kinase domain abutting the crizotinib binding site and are thought to decrease the affinity of crizotinib to the mutant ALK. The T insertion which lies further away in αC-helix at position 1151 is intriguing and has been suggested to disrupt a critical hydrogen

bond between T1151 and E1129 of the P-loop. This particular mutation displays a very high level of resistance to crizotinib [202]. Further to the secondary mutations in ALK kinase domain, Katayama and colleagues also observed in addition to ALK fusion proteins concomitant ALK gene gain amplifications, aberrant gain amplifications of Kit and increased autophosphorylation of EFG receptor in crizotinib-resistant tumours from patients [202]. This study also investigated the effect of several alternative ALK TKIs as well as the HSP90 inhibitor 17-AAG towards crizotinib-resistant forms of ALK in Ba/F3-based cell assays. Interestingly, they found that CH5424802 [316] and ASP-3026 [202] showed differing selectivity profiles, while the HSP90 inhibitor was active against all four EML-ALK crizotinib-resistant mutants [301, 317, 318]. In line with these findings, Doebele and co-workers also described crizotinib-resistant patients with copy number gain of the *ALK* gene, as well as one patient that demonstrated a novel EGFR L858R mutation and two patients with KRAS G12V mutations [127]. Taken together, these findings show that the mechanisms underlying crizotinib resistance may be complex, including ALK kinase domain mutations, copy number gain of the ALK gene fusion and separate driver oncogenes. The presence of EGFR and ALK or RAS and ALK co-mutations is not commonly reported in primary cases but has been observed in later stages and might arise due to treatment [121, 319, 320]. Further, EML4-ALK translocation has been observed in non-tumour material while absent in matching tumour samples from the same patient, and the explanation for this observation is not clear [321]. However, it instantly raises the question whether presence of multiple oncogenes in a tumour sample is the same tumour or whether they are different tumours. With next-generation DNA and RNA sequencing at our doorstep, we are moving into an era when it will be possible to harness these technologies to improve the therapeutic treatment of patients, especially to detect molecular mutation in heterogeneous tumour samples, specifically point mutations. A critical step will be to characterise point mutations and verify whether they are driver or passenger mutations for tumour initiation or progression.

An explosion has already occurred regarding the next generation of ALK inhibitors [322], some of which have been approved for patients with ALK-rearranged NSCLC, such as ceritinib [323] and alectinib (http://www.chugai-pharm.co.jp/english/news/detail/20140704150000.html). Both are regarded as generally well tolerated and indicate efficacy in crizotinib-refractory patients, i.e., show potency against cells expressing secondary EML4-ALK mutations and show activity against CNS metastases and selective inhibitor of ALK [324, 325].

Many others putative drugs have reached clinical trials already with preclinical verifications to overcome the resistance to the gateway mutation, like ASP3026 from Astrella [202] (NCT01401504), AP26113 from Ariad [317] (NCT02094573) and X-396 from Xcovery [326] (NCT01625234). It should be noted that all inhibitors show different abilities to block activity of different resistant mutations of ALK. An inhibitor that saw daylight in 2014 was PF06463922, which is a novel ALK inhibitor with good potency against crizotinib-resistant mutations and has the ability to cross the blood–brain barrier [327, 328], and currently this novel drug is in a phase I trail (NCT01970865).

Developments of TKIs for ALK are not the only strategy for ALK inhibition being pursued. Inhibitory antibodies are an interesting alternative to small molecules as exemplified by the use of trastuzumab (Herceptin). In patients with breast cancer trastuzumab (Herceptin), a monoclonal antibody significantly improves the median overall survival [329–331]. Vigny and colleagues have generated a panel of monoclonal antibodies recognising the extracellular domain of ALK [57]. One of these—mAb30—acted to block ALK activation, as measured by ALK receptor phosphorylation and activation of ERK and STAT3 proteins [57]. Interestingly, while preventing activation, mAb30 still appears to induce the internalisation of ALK [332]. The use of antibodies therapeutically is attractive, and a recent encouraging data would seem to back this approach. Using both mAb30 and mAb49 antagonistic antibodies [57], Carpenter et al. have shown that antagonistic ALK antibodies inhibit cell growth and induce cytotoxicity of neuroblastoma cells. Furthermore, they observed that the combination of the blocking antibody together with crizotinib induced a significantly higher level of cell death than either treatment alone and also reduced the IC50 for crizotinib treatment by half [333]. Results from a phase I crizotinib monotherapy against paediatric ALK-positive ALCL, NSCLC and IMT patients show very promising results; however, the same treatment for a group of ALK-positive neuroblastoma patients did not show similar positive results [334]. Could combination therapy be an option as a treatment procedure? Berry and colleagues reported that combined treatment with crizotinib together with an inhibitor against mTORC1/PI-3-kinase overcame tumour resistance in a ALK/TH-MYCN mouse model [193, 335]. A combinatorial treatment of crizotinib and a novel ERK5 inhibitor exhibited a synergistic effect superior to single-agent treatment of neuroblastoma in mice [64]. ERK5 also mediates ALK-induced transcription of MYCN [64], suggesting that targeting both ERK5 and ALK may be beneficial on neuroblastoma patients, since these patients with poor prognosis often have MYCN amplified. The real question of whether it will be possible to employ this therapeutic strategy in patients remains to be answered. However, these early results indicate that this path might be a relevant therapeutic strategy for neuroblastoma and might offer a real capacity to extend and improve patient survival.

Another approach for ALK inhibition that appears promising is inactivation of HSP90. A number of highly potent and pharmaceutically improved HSP90 inhibitors that avoid some of the drawbacks of the first-generation inhibitors are now in clinical trial [336]. ALK is a sensitive HSP90 client and treatment with the Hsp90 antagonist, 17-AAG, disrupts the NPM-ALK/HSP90 complex and leads to degradation of NPM-ALK and subsequent apoptosis in ALCL cell lines [337]. In a proteomics approach, ALK-positive ALCL cells were treated with the HSP90 inhibitor geldanamycin and the cellular effects examined. This treatment led to G2/M cell-cycle arrest and caspase-3-mediated apoptosis, while pathway analysis revealed changes in MAPK, WNT, NF-kappaB, TGF-beta, PPAR and integrin signalling components [338]. Retaspimycin hydrochloride (IPI-504), an analogue of 17-AAG, has been tested in in vitro and in vivo models [339, 340]. Studies in patients with NSCLC suggest that IPI-504 has clinical activity in particular with patients carrying ALK rearrangements [318]. In support of these observations,

IPI-504 rapidly lowers EML4-ALK levels and induces tumour regression in ALK-driven NSCLC xenograft mouse models [341]. Another interesting finding is the ability of the 17-AAG HSP90 inhibitor to block the activity of the recently described crizotinib-resistant ALK mutants, namely, L1196M, G1202R, S1206Y and T1151-ins [202], making these inhibitors particularly attractive for further investigation. Several clinical trials indicate HSP90 is well tolerated with promising clinical activity as single-agent therapy, as well as in combination with other drugs, although similar response as crizotinib or other ALK inhibitors has not been observed (https://clinicaltrials.gov/). A more challenging task is the development of RNAi-based therapeutics. siRNA has been reported to have antitumour activity and prolong survival in mouse models of neuroblastoma [342, 343] and ALCL [292]. If therapeutically deliverable, such strategies could prove clinically useful and should also act on TKI-resistant forms of ALK. While demanding, advances in siRNA delivery are being made and may become technically feasible in ALCL and NB patients in the coming years [344]. The combination of RNAi-based strategies with TKIs has also been explored, with a combination of ALK shRNA acting synergistically with the ERK1/2 inhibitor U0126 in ALCL cell lines and mouse xenografts [345]. Considering NB, a combination of ALK TKIs together with siRNA targeting MYCN may be an attractive choice for a subset of these young patients.

An interesting report investigating DNA vaccination suggests that this approach leads to protection from ALCL growth in mouse models of ALCL [244]. Whether this approach would offer therapeutic advantages or be employed together with ALK TKIs remains to be seen.

Finally, the issue of the vertebrate ALK ligand is therapeutically relevant. This is clearly more important in NB, where neural crest cells presumably express the ALK receptor, either as wild-type or as a mutant version. An understanding of the physiological ligand/activation mechanism(s) and the developmental biology underlying this will be critical to understand and exploit anti-ALK therapies.

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Appendix

Receptor at a glance: ALK/LTK

Chromosome location	ALK, 2p23; LTK, 15q15.1
Gene size (bp)	ALK, 728,793; LTK, 10,303
Intron/exon numbers	ALK, 29 exons; LTK, 20 exons
mRNA size	ALK, 6,267; LTK, several mRNA forms, predominant 3,046
Amino acid number	ALK, 1620 a.a; LTK, 864 a.a.
kDa	ALK, 176 kDa; LTK, 91 kDa
Post-translational modifications	ALK: glycosylated, phosphorylated LTK: glycosylated, phosphorylated

(continued)

Domains	ALK: MAM, LDLa, glycine-rich domain, kinase domain LTK: glycine-rich domain, kinase domain
Ligands	ALK: unclear (Jeb and HEN-1 in *Drosophila* and *C. elegans*; no Jeb-like ligand reported in mammalians as yet) LTK: FAM150A and B
Known dimerising partner	ALK: ? LTK: ?
Pathway activated	ALK: Ras/MAPK, Rap1, PI3-K/TOR, ERK5, PLCγ, STATs, Jun LTK: Ras/MAPK, PI3-K/TOR, PLCγ
Tissues expressed	ALK: central and peripheral nervous systems among other tissues LTK: pre-B and B lymphocytes and in the adult brain, as well as in placenta
Human diseases	ALK: different types of cancer
Knockout mouse phenotype	Both single and double knockouts are viable and fertile ALK: enhanced spatial memory and enhanced cognitive performance, effects on neurogenesis LTK: reduced sensimotor function ALK/LTK: reduced neurogenesis when compared with ALK KO

References

1. Ben-Neriah Y, Bauskin AR. Leukocytes express a novel gene encoding a putative transmembrane protein-kinase devoid of an extracellular domain. Nature. 1988;333:672–6.
2. Bernards A, de la Monte SM. The ltk receptor tyrosine kinase is expressed in pre-B lymphocytes and cerebral neurons and uses a non-AUG translational initiator. EMBO J. 1990;9:2279–87.
3. Haase VH, Snijders AJ, Cooke SM, Teng MN, Kaul D, Le Beau MM, et al. Alternatively spliced ltk mRNA in neurons predicts a receptor with a larger putative extracellular domain. Oncogene. 1991;6:2319–25.
4. Krolewski JJ, Dalla-Favera R. The ltk gene encodes a novel receptor-type protein tyrosine kinase. EMBO J. 1991;10:2911–9.
5. Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science. 1994;263:1281–4.
6. Shiota M, Fujimoto J, Semba T, Satoh H, Yamamoto T, Mori S. Hyperphosphorylation of a novel 80 kDa protein-tyrosine kinase similar to Ltk in a human Ki-1 lymphoma cell line, AMS3. Oncogene. 1994;9:1567–74.
7. Iwahara T, Fujimoto J, Wen D, Cupples R, Bucay N, Arakawa T, et al. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. Oncogene. 1997;14:439–49.
8. Morris SW, Naeve C, Mathew P, James PL, Kirstein MN, Cui X, et al. ALK, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). Oncogene. 1997;14:2175–88.
9. Daly NL, Scanlon MJ, Djordjevic JT, Kroon PA, Smith R. Three-dimensional structure of a cysteine-rich repeat from the low-density lipoprotein receptor. Proc Natl Acad Sci USA. 1995;92:6334–8.
10. Fass D, Blacklow S, Kim PS, Berger JM. Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. Nature. 1997;388:691–3.

11. Beckmann G, Bork P. An adhesive domain detected in functionally diverse receptors. Trends Biochem Sci. 1993;18:40–1.
12. Cismasiu VB, Denes SA, Reilander H, Michel H, Szedlacsek SE. The MAM (meprin/A5-protein/PTPmu) domain is a homophilic binding site promoting the lateral dimerization of receptor-like protein-tyrosine phosphatase mu. J Biol Chem. 2004;279:26922–31.
13. Jiang YP, Wang H, D'Eustachio P, Musacchio JM, Schlessinger J, Sap J. Cloning and characterization of R-PTP-kappa, a new member of the receptor protein tyrosine phosphatase family with a proteolytically cleaved cellular adhesion molecule-like extracellular region. Mol Cell Biol. 1993;13:2942–51.
14. Liao EH, Hung W, Abrams B, Zhen M. An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. Nature. 2004;430:345–50.
15. Loren CE, Scully A, Grabbe C, Edeen PT, Thomas J, McKeown M, et al. Identification and characterization of DAlk: a novel Drosophila melanogaster RTK which drives ERK activation in vivo. Genes Cells. 2001;6:531–44.
16. Lopes SS, Yang X, Muller J, Carney TJ, McAdow AR, Rauch GJ, et al. Leukocyte tyrosine kinase functions in pigment cell development. PLoS Genet. 2008;4:e1000026.
17. Kunugi H, Hashimoto R, Okada T, Hori H, Nakabayashi T, Baba A, et al. Possible association between nonsynonymous polymorphisms of the anaplastic lymphoma kinase (ALK) gene and schizophrenia in a Japanese population. J Neural Transm. 2006;113:1569–73.
18. Li N, Nakamura K, Jiang Y, Tsurui H, Matsuoka S, Abe M, et al. Gain-of-function polymorphism in mouse and human Ltk: implications for the pathogenesis of systemic lupus erythematosus. Hum Mol Genet. 2004;13:171–9.
19. Honda H, Harada K, Komuro I, Terasaki F, Ueno H, Tanaka Y, et al. Heart-specific activation of LTK results in cardiac hypertrophy, cardiomyocyte degeneration and gene reprogramming in transgenic mice. Oncogene. 1999;18:3821–30.
20. Degoutin J, Brunet-de Carvalho N, Cifuentes-Diaz C, Vigny M. ALK (Anaplastic Lymphoma Kinase) expression in DRG neurons and its involvement in neuron-Schwann cells interaction. Eur J Neurosci. 2009;29:275–86.
21. Hurley SP, Clary DO, Copie V, Lefcort F. Anaplastic lymphoma kinase is dynamically expressed on subsets of motor neurons and in the peripheral nervous system. J Comp Neurol. 2006;495:202–12.
22. Pulford K, Lamant L, Morris SW, Butler LH, Wood KM, Stroud D, et al. Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. Blood. 1997;89:1394–404.
23. Vernersson E, Khoo NK, Henriksson ML, Roos G, Palmer RH, Hallberg B. Characterization of the expression of the ALK receptor tyrosine kinase in mice. Gene Expr Patterns. 2006;6:448–61.
24. Yan X, Kennedy CR, Tilkens SB, Wiedemeier O, Guan H, Park JI, et al. Cooperative crosstalk between neuroblastoma subtypes confers resistance to anaplastic lymphoma kinase inhibition. Genes Cancer. 2011;2:538–49.
25. Loren CE, Englund C, Grabbe C, Hallberg B, Hunter T, Palmer RH. A crucial role for the anaplastic lymphoma kinase receptor tyrosine kinase in gut development in Drosophila melanogaster. EMBO Rep. 2003;4:781–6.
26. Englund C, Loren CE, Grabbe C, Varshney GK, Deleuil F, Hallberg B, et al. Jeb signals through the Alk receptor tyrosine kinase to drive visceral muscle fusion. Nature. 2003;425:512–6.
27. Lee HH, Norris A, Weiss JB, Frasch M. Jelly belly protein activates the receptor tyrosine kinase Alk to specify visceral muscle pioneers. Nature. 2003;425:507–12.
28. Stute C, Schimmelpfeng K, Renkawitz-Pohl R, Palmer RH, Holz A. Myoblast determination in the somatic and visceral mesoderm depends on Notch signalling as well as on milliways(mili(Alk)) as receptor for Jeb signalling. Development. 2004;131:743–54.
29. Shirinian M, Varshney G, Loren CE, Grabbe C, Palmer RH. Drosophila anaplastic lymphoma kinase regulates Dpp signalling in the developing embryonic gut. Differentiation. 2007;75:418–26.

30. Shirinian M, Popovic M, Grabbe C, Varshney G, Hugosson F, Bos H, et al. The Rap1 guanine nucleotide exchange factor C3G is required for preservation of larval muscle integrity in Drosophila melanogaster. PLoS One. 2010;5:e9403.
31. Varshney GK, Palmer RH. The bHLH transcription factor Hand is regulated by Alk in the Drosophila embryonic gut. Biochem Biophys Res Commun. 2006;351:839–46.
32. Bazigou E, Apitz H, Johansson J, Loren CE, Hirst EM, Chen PL, et al. Anterograde Jelly belly and Alk receptor tyrosine kinase signaling mediates retinal axon targeting in Drosophila. Cell. 2007;128:961–75.
33. Rohrbough J, Broadie K. Anterograde Jelly belly ligand to Alk receptor signaling at developing synapses is regulated by Mind the gap. Development. 2010;137:3523–33.
34. Gouzi JY, Moressis A, Walker JA, Apostolopoulou AA, Palmer RH, Bernards A, et al. The receptor tyrosine kinase Alk controls neurofibromin functions in Drosophila growth and learning. PLoS Genet. 2011;7:e1002281.
35. Cheng LY, Bailey AP, Leevers SJ, Ragan TJ, Driscoll PC, Gould AP. Anaplastic lymphoma kinase spares organ growth during nutrient restriction in Drosophila. Cell. 2011;146:435–47.
36. Lasek AW, Lim J, Kliethermes CL, Berger KH, Joslyn G, Brush G, et al. An evolutionary conserved role for anaplastic lymphoma kinase in behavioral responses to ethanol. PLoS One. 2011;6:e22636.
37. Foret S, Kucharski R, Pellegrini M, Feng S, Jacobsen SE, Robinson GE, et al. DNA methylation dynamics, metabolic fluxes, gene splicing, and alternative phenotypes in honey bees. Proc Natl Acad Sci USA. 2012;109:4968–73.
38. Inoue T, Thomas JH. Targets of TGF-beta signaling in *Caenorhabditis elegans* dauer formation. Dev Biol. 2000;217:192–204.
39. Reiner DJ, Ailion M, Thomas JH, Meyer BJ. C. elegans anaplastic lymphoma kinase ortholog SCD-2 controls dauer formation by modulating TGF-beta signaling. Curr Biol. 2008; 18:1101–9.
40. Ishihara T, Iino Y, Mohri A, Mori I, Gengyo-Ando K, Mitani S, et al. HEN-1, a secretory protein with an LDL receptor motif, regulates sensory integration and learning in *Caenorhabditis elegans*. Cell. 2002;109:639–49.
41. Schutzman JL, Borland CZ, Newman JC, Robinson MK, Kokel M, Stern MJ. The Caenorhabditis elegans EGL-15 signaling pathway implicates a DOS-like multisubstrate adaptor protein in fibroblast growth factor signal transduction. Mol Cell Biol. 2001;21:8104–16.
42. Watanabe N, Nagamatsu Y, Gengyo-Ando K, Mitani S, Ohshima Y. Control of body size by SMA-5, a homolog of MAP kinase BMK1/ERK5, in *C. elegans*. Development. 2005;132:3175–84.
43. Li YX, Wang Y, Hu YO, Zhong JX, Wang DY. Modulation of the assay system for the sensory integration of 2 sensory stimuli that inhibit each other in nematode Caenorhabditis elegans. Neurosci Bull. 2011;27:69–82.
44. Shinkai Y, Yamamoto Y, Fujiwara M, Tabata T, Murayama T, Hirotsu T, et al. Behavioral choice between conflicting alternatives is regulated by a receptor guanylyl cyclase, GCY-28, and a receptor tyrosine kinase, SCD-2, in AIA interneurons of Caenorhabditis elegans. J Neurosci. 2011;31:3007–15.
45. Shen LL, Wang Y, Wang DY. Involvement of genes required for synaptic function in aging control in C elegans. Neurosci Bull. 2007;23:21–9.
46. Yao S, Cheng M, Zhang Q, Wasik M, Kelsh R, Winkler C. Anaplastic lymphoma kinase is required for neurogenesis in the developing central nervous system of zebrafish. PLoS One. 2013; 8:e63757.
47. Zhu S, Lee JS, Guo F, Shin J, Perez-Atayde AR, Kutok JL, et al. Activated ALK collaborates with MYCN in neuroblastoma pathogenesis. Cancer Cell. 2012;21:362–73.
48. Bilsland JG, Wheeldon A, Mead A, Znamenskiy P, Almond S, Waters KA, et al. Behavioral and neurochemical alterations in mice deficient in anaplastic lymphoma kinase suggest therapeutic potential for psychiatric indications. Neuropsychopharmacology. 2008;33:685–700.
49. Lasek AW, Gesch J, Giorgetti F, Kharazia V, Heberlein U. Alk is a transcriptional target of LMO4 and ERalpha that promotes cocaine sensitization and reward. J Neurosci. 2011;31:14134–41.

50. Weiss JB, Xue C, Benice T, Xue L, Morris SW, Raber J. Anaplastic lymphoma kinase and leukocyte tyrosine kinase: functions and genetic interactions in learning, memory and adult neurogenesis. Pharmacol Biochem Behav. 2012;100:566–74.
51. Ou SH, Azada M, Dy J, Stiber JA. Asymptomatic profound sinus bradycardia (heart rate </=45) in non-small cell lung cancer patients treated with crizotinib. J Thorac Oncol. 2011;6:2135–7.
52. Weickhardt AJ, Rothman MS, Salian-Mehta S, Kiseljak-Vassiliades K, Oton AB, Doebele RC, et al. Rapid-onset hypogonadism secondary to crizotinib use in men with metastatic nonsmall cell lung cancer. Cancer. 2012;118:5302–9.
53. Camidge DR, Kono SA, Lu X, Okuyama S, Baron AE, Oton AB, et al. Anaplastic lymphoma kinase gene rearrangements in non-small cell lung cancer are associated with prolonged progression-free survival on pemetrexed. J Thorac Oncol. 2011;6:774–80.
54. Kwak EL, Bang Y-J, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med. 2010;363:1693–703.
55. Chun SG, Iyengar P, Gerber DE, Hogan RN, Timmerman RD. Optic neuropathy and blindness associated with Crizotinib for non-small-cell lung cancer with EML4-ALK translocation. J Clin Oncol. 2014;33:e25–6.
56. Gouzi JY, Moog-Lutz C, Vigny M, Brunet-de CN. Role of the subcellular localization of ALK tyrosine kinase domain in neuronal differentiation of PC12 cells. J Cell Sci. 2005;118:5811–23.
57. Moog-Lutz C, Degoutin J, Gouzi JY, Frobert Y, Brunet-de Carvalho N, Bureau J, et al. Activation and inhibition of anaplastic lymphoma kinase receptor tyrosine kinase by monoclonal antibodies and absence of agonist activity of pleiotrophin. J Biol Chem. 2005;280: 26039–48.
58. Motegi A, Fujimoto J, Kotani M, Sakuraba H, Yamamoto T. ALK receptor tyrosine kinase promotes cell growth and neurite outgrowth. J Cell Sci. 2004;117:3319–29.
59. Souttou B, Carvalho NB, Raulais D, Vigny M. Activation of anaplastic lymphoma kinase receptor tyrosine kinase induces neuronal differentiation through the mitogen-activated protein kinase pathway. J Biol Chem. 2001;276:9526–31.
60. Yang HL, Eriksson T, Vernersson E, Vigny M, Hallberg B, Palmer RH. The ligand Jelly Belly (Jeb) activates the Drosophila Alk RTK to drive PC12 cell differentiation, but is unable to activate the mouse ALK RTK. J Exp Zool B Mol Dev Evol. 2007;308:269–82.
61. Cazes A, Lopez-Delisle L, Tsarovina K, Pierre-Eugene C, De Preter K, Peuchmaur M, et al. Activated Alk triggers prolonged neurogenesis and Ret upregulation providing a therapeutic target in ALK-mutated neuroblastoma. Oncotarget. 2014;5:2688–702.
62. Degoutin J, Vigny M, Gouzi JY. ALK activation induces Shc and FRS2 recruitment: Signaling and phenotypic outcomes in PC12 cells differentiation. FEBS Lett. 2007;581:727–34.
63. Schonherr C, Yang HL, Vigny M, Palmer RH, Hallberg B. Anaplastic lymphoma kinase activates the small GTPase Rap1 via the Rap1-specific GEF C3G in both neuroblastoma and PC12 cells. Oncogene. 2010;29:2817–30.
64. Umapathy G, El Wakil A, Witek B, Chesler L, Danielson L, Deng X, et al. The kinase ALK stimulates the kinase ERK5 to promote the expression of the oncogene MYCN in neuroblastoma. Sci Signal. 2014;7:102.
65. Hasan MK, Nafady A, Takatori A, Kishida S, Ohira M, Suenaga Y, et al. ALK is a MYCN target gene and regulates cell migration and invasion in neuroblastoma. Sci Rep. 2013;3:3450.
66. Stein H, Mason DY, Gerdes J, O'Connor N, Wainscoat J, Pallesen G, et al. The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. Blood. 1985;66:848–58.
67. Agnarsson BA, Kadin ME. Ki-1 positive large cell lymphoma. A morphologic and immunologic study of 19 cases. Am J Surg Pathol. 1988;12:264–74.
68. Kadin ME, Sako D, Berliner N, Franklin W, Woda B, Borowitz M, et al. Childhood Ki-1 lymphoma presenting with skin lesions and peripheral lymphadenopathy. Blood. 1986;68:1042–9.

69. Amin HM, Lai R. Pathobiology of ALK+ anaplastic large-cell lymphoma. Blood. 2007;110: 2259–67.
70. Stein H, Foss HD, Durkop H, Marafioti T, Delsol G, Pulford K, et al. CD30(+) anaplastic large cell lymphoma: a review of its histopathologic, genetic, and clinical features. Blood. 2000;96:3681–95.
71. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editors. WHO classification of tumours of haematopoietic and lymphiod tissues. 4th ed. Lyon, France: IARC Press; 2008.
72. Falini B, Pileri S, Zinzani PL, Carbone A, Zagonel V, Wolf-Peeters C, et al. ALK+ lymphoma: clinico-pathological findings and outcome. Blood. 1999;93:2697–706.
73. Gascoyne RD, Aoun P, Wu D, Chhanabhai M, Skinnider BF, Greiner TC, et al. Prognostic significance of anaplastic lymphoma kinase (ALK) protein expression in adults with anaplastic large cell lymphoma. Blood. 1999;93:3913–21.
74. Savage KJ, Harris NL, Vose JM, Ullrich F, Jaffe ES, Connors JM, et al. ALK- anaplastic large-cell lymphoma is clinically and immunophenotypically different from both ALK+ ALCL and peripheral T-cell lymphoma, not otherwise specified: report from the International Peripheral T-Cell Lymphoma Project. Blood. 2008;111:5496–504.
75. Shiota M, Nakamura S, Ichinohasama R, Abe M, Akagi T, Takeshita M, et al. Anaplastic large cell lymphomas expressing the novel chimeric protein p80NPM/ALK: a distinct clinicopathologic entity. Blood. 1995;86:1954–60.
76. ten Berge RL, Meijer CJ, Dukers DF, Kummer JA, Bladergroen BA, Vos W, et al. Expression levels of apoptosis-related proteins predict clinical outcome in anaplastic large cell lymphoma. Blood. 2002;99:4540–6.
77. Khoury JD, Medeiros LJ, Rassidakis GZ, Yared MA, Tsioli P, Leventaki V, et al. Differential expression and clinical significance of tyrosine-phosphorylated STAT3 in ALK+ and ALK- anaplastic large cell lymphoma. Clin Cancer Res. 2003;9:3692–9.
78. Rassidakis GZ, Goy A, Medeiros LJ, Jiang Y, Thomaides A, Remache Y, et al. Prognostic significance of MUC-1 expression in systemic anaplastic large cell lymphoma. Clin Cancer Res. 2003;9:2213–20.
79. Schlette EJ, Medeiros LJ, Goy A, Lai R, Rassidakis GZ. Survivin expression predicts poorer prognosis in anaplastic large-cell lymphoma. J Clin Oncol. 2004;22:1682–8.
80. Maes B, Vanhentenrijk V, Wlodarska I, Cools J, Peeters B, Marynen P, et al. The NPM-ALK and the ATIC-ALK fusion genes can be detected in non-neoplastic cells. Am J Pathol. 2001;158:2185–93.
81. Trumper L, Pfreundschuh M, Bonin FV, Daus H. Detection of the t(2;5)-associated NPM/ALK fusion cDNA in peripheral blood cells of healthy individuals. Br J Haematol. 1998; 103:1138–44.
82. Cools J, Wlodarska I, Somers R, Mentens N, Pedeutour F, Maes B, et al. Identification of novel fusion partners of ALK, the anaplastic lymphoma kinase, in anaplastic large-cell lymphoma and inflammatory myofibroblastic tumor. Genes Chromosomes Cancer. 2002;34:354–62.
83. Colleoni GW, Bridge JA, Garicochea B, Liu J, Filippa DA, Ladanyi M. ATIC-ALK: a novel variant ALK gene fusion in anaplastic large cell lymphoma resulting from the recurrent cryptic chromosomal inversion, inv(2)(p23q35). Am J Pathol. 2000;156:781–9.
84. Ma Z, Cools J, Marynen P, Cui X, Siebert R, Gesk S, et al. Inv(2)(p23q35) in anaplastic large-cell lymphoma induces constitutive anaplastic lymphoma kinase (ALK) tyrosine kinase activation by fusion to ATIC, an enzyme involved in purine nucleotide biosynthesis. Blood. 2000;95:2144–9.
85. Hernandez L, Pinyol M, Hernandez S, Bea S, Pulford K, Rosenwald A, et al. TRK-fused gene (TFG) is a new partner of ALK in anaplastic large cell lymphoma producing two structurally different TFG-ALK translocations. Blood. 1999;94:3265–8.
86. Hernandez L, Bea S, Bellosillo B, Pinyol M, Falini B, Carbone A, et al. Diversity of genomic breakpoints in TFG-ALK translocations in anaplastic large cell lymphomas: identification of a new TFG-ALK(XL) chimeric gene with transforming activity. Am J Pathol. 2002;160:1487–94.

87. Tort F, Pinyol M, Pulford K, Roncador G, Hernandez L, Nayach I, et al. Molecular characterization of a new ALK translocation involving moesin (MSN-ALK) in anaplastic large cell lymphoma. Lab Invest. 2001;81:419–26.
88. Lamant L, Dastugue N, Pulford K, Delsol G, Mariame B. A new fusion gene TPM3-ALK in anaplastic large cell lymphoma created by a (1;2)(q25;p23) translocation. Blood. 1999; 93:3088–95.
89. Meech SJ, McGavran L, Odom LF, Liang X, Meltesen L, Gump J, et al. Unusual childhood extramedullary hematologic malignancy with natural killer cell properties that contains tropomyosin 4-anaplastic lymphoma kinase gene fusion. Blood. 2001;98:1209–16.
90. Siebert R, Gesk S, Harder L, Steinemann D, Grote W, Schlegelberger B, et al. Complex variant translocation t(1;2) with TPM3-ALK fusion due to cryptic ALK gene rearrangement in anaplastic large-cell lymphoma. Blood. 1999;94:3614–7.
91. Feldman AL, Vasmatzis G, Asmann YW, Davila J, Middha S, Eckloff BW, et al. Novel TRAF1-ALK fusion identified by deep RNA sequencing of anaplastic large cell lymphoma. Genes Chromosomes Cancer. 2013;52:1097–102.
92. Lamant L, Gascoyne RD, Duplantier MM, Armstrong F, Raghab A, Chhanabhai M, et al. Non-muscle myosin heavy chain (MYH9): a new partner fused to ALK in anaplastic large cell lymphoma. Genes Chromosomes Cancer. 2003;37:427–32.
93. Touriol C, Greenland C, Lamant L, Pulford K, Bernard F, Rousset T, et al. Further demonstration of the diversity of chromosomal changes involving 2p23 in ALK-positive lymphoma: 2 cases expressing ALK kinase fused to CLTCL (clathrin chain polypeptide-like). Blood. 2000;95:3204–7.
94. Gleason BC, Hornick JL. Inflammatory myofibroblastic tumours: where are we now? J Clin Pathol. 2008;61:428–37.
95. Coffin CM, Watterson J, Priest JR, Dehner LP. Extrapulmonary inflammatory myofibroblastic tumor (inflammatory pseudotumor). A clinicopathologic and immunohistochemical study of 84 cases. Am J Surg Pathol. 1995;19:859–72.
96. Janik JS, Janik JP, Lovell MA, Hendrickson RJ, Bensard DD, Greffe BS. Recurrent inflammatory pseudotumors in children. J Pediatr Surg. 2003;38:1491–5.
97. Griffin CA, Hawkins AL, Dvorak C, Henkle C, Ellingham T, Perlman EJ. Recurrent involvement of 2p23 in inflammatory myofibroblastic tumors. Cancer Res. 1999;59:2776–80.
98. Lawrence B, Perez-Atayde A, Hibbard MK, Rubin BP, Dal Cin P, Pinkus JL, et al. TPM3-ALK and TPM4-ALK oncogenes in inflammatory myofibroblastic tumors. Am J Pathol. 2000;157:377–84.
99. Debelenko LV, Arthur DC, Pack SD, Helman LJ, Schrump DS, Tsokos M. Identification of CARS-ALK fusion in primary and metastatic lesions of an inflammatory myofibroblastic tumor. Lab Invest. 2003;83:1255–65.
100. Debiec-Rychter M, Marynen P, Hagemeijer A, Pauwels P. ALK-ATIC fusion in urinary bladder inflammatory myofibroblastic tumor. Genes Chromosomes Cancer. 2003;38:187–90.
101. Panagopoulos I, Nilsson T, Domanski HA, Isaksson M, Lindblom P, Mertens F, et al. Fusion of the SEC31L1 and ALK genes in an inflammatory myofibroblastic tumor. Int J Cancer. 2006;118:1181–6.
102. Takeuchi K, Soda M, Togashi Y, Sugawara E, Hatano S, Asaka R, et al. Pulmonary inflammatory myofibroblastic tumor expressing a novel fusion, PPFIBP1-ALK: reappraisal of anti-ALK immunohistochemistry as a tool for novel ALK fusion identification. Clin Cancer Res. 2011;17:3341–8.
103. Ma Z, Hill DA, Collins MH, Morris SW, Sumegi J, Zhou M, et al. Fusion of ALK to the Ran-binding protein 2 (RANBP2) gene in inflammatory myofibroblastic tumor. Genes Chromosomes Cancer. 2003;37:98–105.
104. Bridge JA, Kanamori M, Ma Z, Pickering D, Hill DA, Lydiatt W, et al. Fusion of the ALK gene to the clathrin heavy chain gene, CLTC, in inflammatory myofibroblastic tumor. Am J Pathol. 2001;159:411–5.
105. Patel AS, Murphy KM, Hawkins AL, Cohen JS, Long PP, Perlman EJ, et al. RANBP2 and CLTC are involved in ALK rearrangements in inflammatory myofibroblastic tumors. Cancer Genet Cytogenet. 2007;176:107–14.

106. Cook JR, Dehner LP, Collins MH, Ma Z, Morris SW, Coffin CM, et al. Anaplastic lymphoma kinase (ALK) expression in the inflammatory myofibroblastic tumor: a comparative immunohistochemical study. Am J Surg Pathol. 2001;25:1364–71.
107. Chun YS, Wang L, Nascimento AG, Moir CR, Rodeberg DA. Pediatric inflammatory myofibroblastic tumor: anaplastic lymphoma kinase (ALK) expression and prognosis. Pediatr Blood Cancer. 2005;45:796–801.
108. Butrynski JE, D'Adamo DR, Hornick JL, Dal Cin P, Antonescu CR, Jhanwar SC, et al. Crizotinib in ALK-rearranged inflammatory myofibroblastic tumor. N Engl J Med. 2010;363:1727–33.
109. Sasaki T, Okuda K, Zheng W, Butrynski J, Capelletti M, Wang L, et al. The neuroblastoma-associated F1174L ALK mutation causes resistance to an ALK kinase inhibitor in ALK-translocated cancers. Cancer Res. 2010;70:10038–43.
110. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
111. Travis WD, Brambilla E, Müller-Hermelink HK, Harris CC. World Health Organisation Classification of Tumours; Pathology and gentics of tumours of the lung, pleura, thymus and heart. Lyon, France: IACR Press; 2004.
112. Ezzati M, Lopez AD. Estimates of global mortality attributable to smoking in 2000. Lancet. 2003;362:847–52.
113. Ezzati M, Henley SJ, Lopez AD, Thun MJ. Role of smoking in global and regional cancer epidemiology: current patterns and data needs. Int J Cancer. 2005;116:963–71.
114. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell. 2007;131:1190–203.
115. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature. 2007;448:561–6.
116. Sasaki T, Rodig SJ, Chirieac LR, Janne PA. The biology and treatment of EML4-ALK non-small cell lung cancer. Eur J Cancer. 2010;46:1773–80.
117. Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol. 2009;27:4247–53.
118. Suda K, Tomizawa K, Yatabe Y, Mitsudomi T. Lung cancers unrelated to smoking: characterized by single oncogene addiction? Int J Clin Oncol. 2011;16:294–305.
119. Yano T, Haro A, Shikada Y, Maruyama R, Maehara Y. Non-small cell lung cancer in never smokers as a representative 'non-smoking-associated lung cancer': epidemiology and clinical features. Int J Clin Oncol. 2011;16:287–93.
120. Clinical Lung Cancer Genome Project, Network Genomic Medicine. A genomics-based classification of human lung tumors. Sci Transl Med. 2013;5:209ra153.
121. Kris M, Johnson BE, Kwiatkowski DJ, Iafrate AJ, Wistuba II, Aronson SL, Engelman JA, Shyr Y, Khuri FR, Rudin CM, Garon EB, Pao W, Schiller JH, Haura EB, Shirai K, Giaccone G, Berry LD, Kugler K, Minna JD, Bunn PA. Identification of driver mutations in tumor specimens from 1,000 patients with lung adenocarcinoma: The NCI's Lung Cancer Mutation Consortium (LCMC). J Clin Oncol. 2011;29, CRA7506.
122. Pao W, Girard N. New driver mutations in non-small-cell lung cancer. Lancet Oncol. 2011;12:175–80.
123. Inamura K, Takeuchi K, Togashi Y, Hatano S, Ninomiya H, Motoi N, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. Mod Pathol. 2009;22:508–15.
124. Kim HR, Shim HS, Chung JH, Lee YJ, Hong YK, Rha SY, et al. Distinct clinical features and outcomes in never-smokers with nonsmall cell lung cancer who harbor EGFR or KRAS mutations or ALK rearrangement. Cancer. 2012;118:729–39.
125. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ, et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res. 2008;14:4275–83.

126. Soda M, Takada S, Takeuchi K, Choi YL, Enomoto M, Ueno T, et al. A mouse model for EML4-ALK-positive lung cancer. Proc Natl Acad Sci USA. 2008;105:19893–7.
127. Doebele RC, Pilling AB, Aisner D, Kutateladze TG, Le AT, Weickhardt AJ, et al. Mechanisms of resistance to Crizotinib in patients with ALK gene rearranged non-small cell lung cancer. Clin Cancer Res. 2012;18:1472–82.
128. Gainor JF, Shaw AT. Emerging paradigms in the development of resistance to tyrosine kinase inhibitors in lung cancer. J Clin Oncol. 2013;31:3987–96.
129. Miyanaga A, Shimizu K, Noro R, Seike M, Kitamura K, Kosaihira S, et al. Activity of EGFR-tyrosine kinase and ALK inhibitors for EML4-ALK-rearranged non-small-cell lung cancer harbored coexisting EGFR mutation. BMC Cancer. 2013;13:262.
130. Sasaki T, Koivunen J, Ogino A, Yanagita M, Nikiforow S, Zheng W, et al. A novel ALK secondary mutation and EGFR signaling cause resistance to ALK kinase inhibitors. Cancer Res. 2011;71:6051–60.
131. Durinck S, Ho C, Wang NJ, Liao W, Jakkula LR, Collisson EA, et al. Temporal dissection of tumorigenesis in primary cancers. Cancer Discov. 2011;1:137–43.
132. Han Y, Amin HM, Franko B, Frantz C, Shi X, Lai R. Loss of SHP1 enhances JAK3/STAT3 signaling and decreases proteosome degradation of JAK3 and NPM-ALK in ALK+ anaplastic large-cell lymphoma. Blood. 2006;108:2796–803.
133. Jung Y, Kim P, Jung Y, Keum J, Kim SN, Choi YS, et al. Discovery of ALK-PTPN3 gene fusion from human non-small cell lung carcinoma cell line using next generation RNA sequencing. Genes Chromosomes Cancer. 2012;51:590–7.
134. Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, et al. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res. 2009;15:3143–9.
135. Togashi Y, Soda M, Sakata S, Sugawara E, Hatano S, Asaka R, et al. KLC1-ALK: a novel fusion in lung cancer identified using a formalin-fixed paraffin-embedded tissue only. PLoS One. 2012;7:e31323.
136. Wong DW, Leung EL, Wong SK, Tin VP, Sihoe AD, Cheng LC, et al. A novel KIF5B-ALK variant in nonsmall cell lung cancer. Cancer. 2011;117:2709–18.
137. Sasaki T, Janne PA. New strategies for treatment of ALK-rearranged non-small cell lung cancers. Clin Cancer Res. 2011;17:7213–8.
138. Camidge DR, Theodoro M, Maxson DA, Skokan M, O'Brien T, Lu X, et al. Correlations between the percentage of tumor cells showing an ALK (anaplastic lymphoma kinase) gene rearrangement, ALK signal copy number, and response to crizotinib therapy in ALK fluorescence in situ hybridization-positive nonsmall cell lung cancer. Cancer. 2012;118:4486–94.
139. Kim H, Yoo SB, Choe JY, Paik JH, Xu X, Nitta H, et al. Detection of ALK gene rearrangement in non-small cell lung cancer: a comparison of fluorescence in situ hybridization and chromogenic in situ hybridization with correlation of ALK protein expression. J Thorac Oncol. 2011;6:1359–66.
140. Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res. 2008;14:6618–24.
141. Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. Blood. 2011;117:5019–32.
142. Delsol G, Lamant L, Mariame B, Pulford K, Dastugue N, Brousset P, et al. A new subtype of large B-cell lymphoma expressing the ALK kinase and lacking the 2; 5 translocation. Blood. 1997;89:1483–90.
143. Laurent C, Do C, Gascoyne RD, Lamant L, Ysebaert L, Laurent G, et al. Anaplastic lymphoma kinase-positive diffuse large B-cell lymphoma: a rare clinicopathologic entity with poor prognosis. J Clin Oncol. 2009;27:4211–6.
144. De Paepe P, Baens M, van Krieken H, Verhasselt B, Stul M, Simons A, et al. ALK activation by the CLTC-ALK fusion is a recurrent event in large B-cell lymphoma. Blood. 2003;102: 2638–41.

145. Gesk S, Gascoyne RD, Schnitzer B, Bakshi N, Janssen D, Klapper W, et al. ALK-positive diffuse large B-cell lymphoma with ALK-Clathrin fusion belongs to the spectrum of pediatric lymphomas. Leukemia. 2005;19:1839–40.
146. Reichard KK, McKenna RW, Kroft SH. ALK-positive diffuse large B-cell lymphoma: report of four cases and review of the literature. Mod Pathol. 2007;20:310–9.
147. Adam P, Katzenberger T, Seeberger H, Gattenlohner S, Wolf J, Steinlein C, et al. A case of a diffuse large B-cell lymphoma of plasmablastic type associated with the t(2;5)(p23;q35) chromosome translocation. Am J Surg Pathol. 2003;27:1473–6.
148. Onciu M, Behm FG, Downing JR, Shurtleff SA, Raimondi SC, Ma Z, et al. ALK-positive plasmablastic B-cell lymphoma with expression of the NPM-ALK fusion transcript: report of 2 cases. Blood. 2003;102:2642–4.
149. Takeuchi K, Soda M, Togashi Y, Ota Y, Sekiguchi Y, Hatano S, et al. Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma. Haematologica. 2011;96:464–7.
150. Van Roosbroeck K, Cools J, Dierickx D, Thomas J, Vandenberghe P, Stul M, et al. ALK-positive large B-cell lymphomas with cryptic SEC31A-ALK and NPM1-ALK fusions. Haematologica. 2010;95:509–13.
151. Stachurski D, Miron PM, Al-Homsi S, Hutchinson L, Harris NL, Woda B, et al. Anaplastic lymphoma kinase-positive diffuse large B-cell lymphoma with a complex karyotype and cryptic 3′ ALK gene insertion to chromosome 4 q22–24. Hum Pathol. 2007;38:940–5.
152. Debelenko LV, Raimondi SC, Daw N, Shivakumar BR, Huang D, Nelson M, et al. Renal cell carcinoma with novel VCL-ALK fusion: new representative of ALK-associated tumor spectrum. Mod Pathol. 2011;24:430–42.
153. Marino-Enriquez A, Ou WB, Weldon CB, Fletcher JA, Perez-Atayde AR. ALK rearrangement in sickle cell trait-associated renal medullary carcinoma. Genes Chromosomes Cancer. 2011;50:146–53.
154. Sugawara E, Togashi Y, Kuroda N, Sakata S, Hatano S, Asaka R, et al. Identification of anaplastic lymphoma kinase fusions in renal cancer: large-scale immunohistochemical screening by the intercalated antibody-enhanced polymer method. Cancer. 2012;118:4427–37.
155. Kelly LM, Barila G, Liu P, Evdokimova VN, Trivedi S, Panebianco F, et al. Identification of the transforming STRN-ALK fusion as a potential therapeutic target in the aggressive forms of thyroid cancer. Proc Natl Acad Sci USA. 2014;111:4233–8.
156. Perot G, Soubeyran I, Ribeiro A, Bonhomme B, Savagner F, Boutet-Bouzamondo N, et al. Identification of a recurrent STRN/ALK fusion in thyroid carcinomas. PLoS One. 2014;9:e87170.
157. Onoda T, Kanno M, Sato H, Takahashi N, Izumino H, Ohta H, et al. Identification of novel ALK rearrangement A2M-ALK in a neonate with fetal lung interstitial tumor. Genes Chromosomes Cancer. 2014;53:865–74.
158. Lim JH, Jang S, Park CJ, Cho YU, Lee JH, Lee KH, et al. RANBP2-ALK fusion combined with monosomy 7 in acute myelomonocytic leukemia. Cancer Genet. 2014;207:40–5.
159. Tokuda K, Eguchi-Ishimae M, Yagi C, Kawabe M, Moritani K, Niiya T, et al. CLTC-ALK fusion as a primary event in congenital blastic plasmacytoid dendritic cell neoplasm. Genes Chromosomes Cancer. 2014;53:78–89.
160. Du XL, Hu H, Lin DC, Xia SH, Shen XM, Zhang Y, et al. Proteomic profiling of proteins dysregulted in Chinese esophageal squamous cell carcinoma. J Mol Med (Berl). 2007; 85:863–75.
161. Jazii FR, Najafi Z, Malekzadeh R, Conrads TP, Ziaee AA, Abnet C, et al. Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer. World J Gastroenterol. 2006;12:7104–12.
162. Lin E, Li L, Guan Y, Soriano R, Rivers CS, Mohan S, et al. Exon array profiling detects EML4-ALK fusion in breast, colorectal, and non-small cell lung cancers. Mol Cancer Res. 2009;7:1466–76.
163. Kim SM, Kim MJ, Jung HA, Sun JM, Choi YL, Ko YH et al. The presence of ALK translocation in sarcomatoid carcinoma of head and neck and treatment effect of crizotinib: a case series. Head Neck. 2015;37(5):E66–9. doi: 10.1002/hed.23884. Epub 2015 Mar 29.

164. Bavi P, Jehan Z, Bu R, Prabhakaran S, Al-Sanea N, Al-Dayel F, et al. ALK gene amplification is associated with poor prognosis in colorectal carcinoma. Br J Cancer. 2013;109:2735–43.
165. Dirks WG, Fahnrich S, Lis Y, Becker E, MacLeod RA, Drexler HG. Expression and functional analysis of the anaplastic lymphoma kinase (ALK) gene in tumor cell lines. Int J Cancer. 2002;100:49–56.
166. Lamant L, Pulford K, Bischof D, Morris SW, Mason DY, Delsol G, et al. Expression of the ALK tyrosine kinase gene in neuroblastoma. Am J Pathol. 2000;156:1711–21.
167. Miyake I, Hakomori Y, Shinohara A, Gamou T, Saito M, Iwamatsu A, et al. Activation of anaplastic lymphoma kinase is responsible for hyperphosphorylation of ShcC in neuroblastoma cell lines. Oncogene. 2002;21:5823–34.
168. Miyake I, Hakomori Y, Misu Y, Nakadate H, Matsuura N, Sakamoto M, et al. Domain-specific function of ShcC docking protein in neuroblastoma cells. Oncogene. 2005;24:3206–15.
169. Osajima-Hakomori Y, Miyake I, Ohira M, Nakagawara A, Nakagawa A, Sakai R. Biological role of anaplastic lymphoma kinase in neuroblastoma. Am J Pathol. 2005;167:213–22.
170. Salido M, Pijuan L, Martinez-Aviles L, Galvan AB, Canadas I, Rovira A, et al. Increased ALK gene copy number and amplification are frequent in non-small cell lung cancer. J Thorac Oncol. 2011;6:21–7.
171. Passoni L, Longo L, Collini P, Coluccia AM, Bozzi F, Podda M, et al. Mutation-independent anaplastic lymphoma kinase overexpression in poor prognosis neuroblastoma patients. Cancer Res. 2009;69:7338–46.
172. Schulte JH, Bachmann HS, Brockmeyer B, Depreter K, Oberthur A, Ackermann S, et al. High ALK receptor tyrosine kinase expression supersedes ALK mutation as a determining factor of an unfavorable phenotype in primary neuroblastoma. Clin Cancer Res. 2011;17:5082–92.
173. Pejovic T, Pande NT, Mori M, Mhawech-Fauceglia P, Harrington C, Mongoue-Tchokote S, et al. Expression profiling of the ovarian surface kinome reveals candidate genes for early neoplastic changes. Transl Oncol. 2009;2:341–9.
174. Chun MG, Mao JH, Chiu CW, Balmain A, Hanahan D. Polymorphic genetic control of tumor invasion in a mouse model of pancreatic neuroendocrine carcinogenesis. Proc Natl Acad Sci USA. 2010;107:17268–73.
175. Maris JM. Recent advances in neuroblastoma. N Engl J Med. 2010;362:2202–11.
176. Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med. 1999;341:1165–73.
177. De Brouwer S, De Preter K, Kumps C, Zabrocki P, Porcu M, Westerhout EM, et al. Meta-analysis of neuroblastomas reveals a skewed ALK mutation spectrum in tumors with MYCN amplification. Clin Cancer Res. 2010;16:4353–62.
178. Janoueix-Lerosey I, Schleiermacher G, Michels E, Mosseri V, Ribeiro A, Lequin D, et al. Overall genomic pattern is a predictor of outcome in neuroblastoma. J Clin Oncol. 2009;27:1026–33.
179. Michels E, Vandesompele J, De Preter K, Hoebeeck J, Vermeulen J, Schramm A, et al. ArrayCGH-based classification of neuroblastoma into genomic subgroups. Genes Chromosomes Cancer. 2007;46:1098–108.
180. Vandesompele J, Baudis M, De Preter K, Van Roy N, Ambros P, Bown N, et al. Unequivocal delineation of clinicogenetic subgroups and development of a new model for improved outcome prediction in neuroblastoma. J Clin Oncol. 2005;23:2280–99.
181. Caren H, Abel F, Kogner P, Martinsson T. High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours. Biochem J. 2008;416:153–9.
182. Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M, et al. Oncogenic mutations of ALK kinase in neuroblastoma. Nature. 2008;455:971–4.
183. George RE, Sanda T, Hanna M, Frohling S, Luther 2nd W, Zhang J, et al. Activating mutations in ALK provide a therapeutic target in neuroblastoma. Nature. 2008;455:975–8.

184. Janoueix-Lerosey I, Lequin D, Brugieres L, Ribeiro A, de Pontual L, Combaret V, et al. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. Nature. 2008;455:967–70.
185. Martinsson T, Eriksson T, Abrahamsson J, Caren H, Hansson M, Kogner P, et al. Appearance of the novel activating F1174S ALK mutation in neuroblastoma correlates with aggressive tumour progression and unresponsiveness to therapy. Cancer Res. 2011;71:98–105.
186. Mosse YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, et al. Identification of ALK as a major familial neuroblastoma predisposition gene. Nature. 2008;455:930–5.
187. Monclair T, Brodeur GM, Ambros PF, Brisse HJ, Cecchetto G, Holmes K, et al. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. J Clin Oncol. 2009;27:298–303.
188. Bachetti T, Di Paolo D, Di Lascio S, Mirisola V, Brignole C, Bellotti M, et al. PHOX2B-mediated regulation of ALK expression: in vitro identification of a functional relationship between two genes involved in neuroblastoma. PLoS One. 2010;5.
189. de Pontual L, Kettaneh D, Gordon CT, Oufadem M, Boddaert N, Lees M, et al. Germline gain-of-function mutations of ALK disrupt central nervous system development. Hum Mutat. 2011;32:272–6.
190. Schleiermacher G, Javanmardi N, Bernard V, Leroy Q, Cappo J, Rio Frio T, et al. Emergence of new ALK mutations at relapse of neuroblastoma. J Clin Oncol. 2014;32:2727–34.
191. Schonherr C, Ruuth K, Kamaraj S, Wang CL, Yang HL, Combaret V, et al. Anaplastic Lymphoma Kinase (ALK) regulates initiation of transcription of MYCN in neuroblastoma cells. Oncogene. 2012;31:5193–200.
192. Liu Z, Thiele CJ. ALK and MYCN: when two oncogenes are better than one. Cancer Cell. 2012;21:325–6.
193. Berry T, Luther W, Bhatnagar N, Jamin Y, Poon E, Sanda T, et al. The ALK(F1174L) mutation potentiates the oncogenic activity of MYCN in neuroblastoma. Cancer Cell. 2012;22:117–30.
194. Heukamp LC, Thor T, Schramm A, De Preter K, Kumps C, De Wilde B, et al. Targeted expression of mutated ALK induces neuroblastoma in transgenic mice. Sci Transl Med. 2012;4:141.
195. Chand D, Yamazaki Y, Ruuth K, Schonherr C, Martinsson T, Kogner P, et al. Cell and Drosophila model systems define three classes of ALK mutations in neuroblastoma. Dis Model Mech. 2012;6:373–82.
196. Schonherr C, Ruuth K, Yamazaki Y, Eriksson T, Christensen J, Palmer RH, et al. Activating ALK mutations found in neuroblastoma are inhibited by Crizotinib and NVP-TAE684. Biochem J. 2011;440:405–13.
197. Bourdeaut F, Ferrand S, Brugieres L, Hilbert M, Ribeiro A, Lacroix L, et al. ALK germline mutations in patients with neuroblastoma: a rare and weakly penetrant syndrome. Eur J Hum Genet. 2012;20:291–7.
198. Schonherr C, Ruuth K, Eriksson T, Yamazaki Y, Ottmann C, Combaret V, et al. The neuroblastoma ALK(I1250T) mutation is a kinase-dead RTK in vitro and in vivo. Transl Oncol. 2011;4:258–65.
199. Heuckmann JM, Holzel M, Sos ML, Heynck S, Balke-Want H, Koker M, et al. ALK mutations conferring differential resistance to structurally diverse ALK inhibitors. Clin Cancer Res. 2011;17:7394–401.
200. Wang YW, Tu PH, Lin KT, Lin SC, Ko JY, Jou YS. Identification of oncogenic point mutations and hyperphosphorylation of anaplastic lymphoma kinase in lung cancer. Neoplasia. 2011;13:704–15.
201. McDuff FK, Lim SV, Dalbay M, Turner SD. Assessment of the transforming potential of novel anaplastic lymphoma kinase point mutants. Mol Carcinog. 2011;52:79–83.
202. Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B, et al. Mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers. Sci Transl Med. 2012;4:120ra17.

203. Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. N Engl J Med. 2010;363:1734–9.
204. Katayama R, Friboulet L, Koike S, Lockerman EL, Khan TM, Gainor JF, et al. Two novel ALK mutations mediate acquired resistance to the next-generation ALK inhibitor alectinib. Clin Cancer Res. 2014;20:5686–96.
205. Ou SH, Klempner SJ, Greenbowe JR, Azada M, Schrock AB, Ali SM, et al. Identification of a novel HIP1-ALK fusion variant in non-small-cell lung cancer (NSCLC) and discovery of ALK I1171 (I1171N/S) mutations in two ALK-rearranged NSCLC patients with resistance to alectinib. J Thorac Oncol. 2014;9:1821–5.
206. Toyokawa G, Hirai F, Inamasu E, Yoshida T, Nosaki K, Takenaka T, et al. Secondary mutations at I1171 in the ALK gene confer resistance to both crizotinib and alectinib. J Thorac Oncol. 2014;9:e86–87.
207. Murugan AK, Xing M. Anaplastic thyroid cancers harbor novel oncogenic mutations of the ALK gene. Cancer Res. 2011;71:4403–11.
208. Fransson S, Hansson M, Ruuth K, Djos A, Berbegall A, Javanmardi N, et al. Intragenic anaplastic lymphoma kinase (ALK) rearrangements: translocations as a novel mechanism of ALK activation in neuroblastoma tumors. Genes Chromosomes Cancer. 2015;54:99–109.
209. Okubo J, Takita J, Chen Y, Oki K, Nishimura R, Kato M, et al. Aberrant activation of ALK kinase by a novel truncated form ALK protein in neuroblastoma. Oncogene. 2012;31:4667–76.
210. Boeva V, Jouannet S, Daveau R, Combaret V, Pierre-Eugene C, Cazes A, et al. Breakpoint features of genomic rearrangements in neuroblastoma with unbalanced translocations and chromothripsis. PLoS One. 2013;8:e72182.
211. Bresler SC, Wood AC, Haglund EA, Courtright J, Belcastro LT, Plegaria JS, et al. Differential inhibitor sensitivity of anaplastic lymphoma kinase variants found in neuroblastoma. Sci Transl Med. 2011;3:108ra114.
212. Muller-Tidow C, Diederichs S, Bulk E, Pohle T, Steffen B, Schwable J, et al. Identification of metastasis-associated receptor tyrosine kinases in non-small cell lung cancer. Cancer Res. 2005;65:1778–82.
213. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature. 2008;455:1069–75.
214. Roll JD, Reuther GW. ALK-activating homologous mutations in LTK induce cellular transformation. PLoS One. 2012;7:e31733.
215. Zhang H, Pao LI, Zhou A, Brace AD, Halenbeck R, Hsu AW, et al. Deorphanization of the human leukocyte tyrosine kinase (LTK) receptor by a signaling screen of the extracellular proteome. Proc Natl Acad Sci USA. 2014;111:15741–5.
216. Vishwamitra D, Li Y, Wilson D, Manshouri R, Curry CV, Shi B, et al. MicroRNA-96 is a post-transcriptional suppressor of anaplastic lymphoma kinase expression. Am J Pathol. 2012;180:1772–80.
217. Mazot P, Cazes A, Boutterin MC, Figueiredo A, Raynal V, Combaret V, et al. The constitutive activity of the ALK mutated at positions F1174 or R1275 impairs receptor trafficking. Oncogene. 2011;30:2017–25.
218. Del Grosso F, De Mariano M, Passoni L, Luksch R, Tonini GP, Longo L. Inhibition of N-linked glycosylation impairs ALK phosphorylation and disrupts pro-survival signaling in neuroblastoma cell lines. BMC Cancer. 2011;11:525.
219. Bossi RT, Saccardo MB, Ardini E, Menichincheri M, Rusconi L, Magnaghi P, et al. Crystal structures of anaplastic lymphoma kinase in complex with ATP competitive inhibitors. Biochemistry. 2010;49:6813–25.
220. Lee CC, Jia Y, Li N, Sun X, Ng K, Ambing E, et al. Crystal structure of the anaplastic lymphoma kinase (ALK) catalytic domain. Biochem J. 2010;430:425–37.
221. Donella-Deana A, Marin O, Cesaro L, Gunby RH, Ferrarese A, Coluccia AM, et al. Unique substrate specificity of anaplastic lymphoma kinase (ALK): development of phosphoacceptor peptides for the assay of ALK activity. Biochemistry. 2005;44:8533–42.
222. Tartari CJ, Gunby RH, Coluccia AM, Sottocornola R, Cimbro B, Scapozza L, et al. Characterization of some molecular mechanisms governing autoactivation of the catalytic domain of the anaplastic lymphoma kinase. J Biol Chem. 2008;283:3743–50.

223. Wang P, Wu F, Ma Y, Li L, Lai R, Young LC. Functional characterization of the kinase activation loop in nucleophosmin (NPM)-anaplastic lymphoma kinase (ALK) using tandem affinity purification and liquid chromatography-mass spectrometry. J Biol Chem. 2010;285:95–103.
224. Chikamori M, Fujimoto J, Tokai-Nishizumi N, Yamamoto T. Identification of multiple SNT-binding sites on NPM-ALK oncoprotein and their involvement in cell transformation. Oncogene. 2007;26:2950–4.
225. Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, et al. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol. 2005;23:94–101.
226. Englund C, Birve A, Falileeva L, Grabbe C, Palmer RH. Miple1 and miple2 encode a family of MK/PTN homologues in Drosophila melanogaster. Dev Genes Evol. 2006;216:10–8.
227. Stoica GE, Kuo A, Aigner A, Sunitha I, Souttou B, Malerczyk C, et al. Identification of anaplastic lymphoma kinase as a receptor for the growth factor pleiotrophin. J Biol Chem. 2001;276:16772–9.
228. Stoica GE, Kuo A, Powers C, Bowden ET, Sale EB, Riegel AT, et al. Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types. J Biol Chem. 2002;277:35990–8.
229. Mathivet T, Mazot P, Vigny M. In contrast to agonist monoclonal antibodies, both C-terminal truncated form and full length form of Pleiotrophin failed to activate vertebrate ALK (anaplastic lymphoma kinase)? Cell Signal. 2007;19:2434–43.
230. Mourali J, Benard A, Lourenco FC, Monnet C, Greenland C, Moog-Lutz C, et al. Anaplastic lymphoma kinase is a dependence receptor whose proapoptotic functions are activated by caspase cleavage. Mol Cell Biol. 2006;26:6209–22.
231. Maeda N, Nishiwaki T, Shintani T, Hamanaka H, Noda M. 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase zeta/RPTPbeta, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). J Biol Chem. 1996;271:21446–52.
232. Meng K, Rodriguez-Pena A, Dimitrov T, Chen W, Yamin M, Noda M, et al. Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. Proc Natl Acad Sci USA. 2000;97:2603–8.
233. Muramatsu H, Zou K, Sakaguchi N, Ikematsu S, Sakuma S, Muramatsu T. LDL receptor-related protein as a component of the midkine receptor. Biochem Biophys Res Commun. 2000;270:936–41.
234. Muramatsu T. Midkine, a heparin-binding cytokine with multiple roles in development, repair and diseases. Proc Jpn Acad Ser B Phys Biol Sci. 2010;86:410–25.
235. Nakanishi T, Kadomatsu K, Okamoto T, Ichihara-Tanaka K, Kojima T, Saito H, et al. Expression of syndecan-1 and -3 during embryogenesis of the central nervous system in relation to binding with midkine. J Biochem. 1997;121:197–205.
236. Raulo E, Chernousov MA, Carey DJ, Nolo R, Rauvala H. Isolation of a neuronal cell surface receptor of heparin binding growth-associated molecule (HB-GAM). Identification as N-syndecan (syndecan-3). J Biol Chem. 1994;269:12999–3004.
237. Perez-Pinera P, Zhang W, Chang Y, Vega JA, Deuel TF. Anaplastic lymphoma kinase is activated through the pleiotrophin/receptor protein-tyrosine phosphatase beta/zeta signaling pathway: an alternative mechanism of receptor tyrosine kinase activation. J Biol Chem. 2007;282:28683–90.
238. Hugosson F, Sjogren C, Birve A, Hedlund L, Eriksson T, Palmer RH. The Drosophila midkine/pleiotrophin homologues Miple1 and Miple2 affect adult lifespan but are dispensable for Alk signaling during embryonic gut formation. PLoS One. 2014;9:e112250.
239. Piccinini G, Bacchiocchi R, Serresi M, Vivani C, Rossetti S, Gennaretti C, et al. A ligand-inducible epidermal growth factor receptor/anaplastic lymphoma kinase chimera promotes mitogenesis and transforming properties in 3T3 cells. J Biol Chem. 2002;277:22231–9.
240. Tomiyama A, Uekita T, Kamata R, Sasaki K, Takita J, Ohira M, et al. Flotillin-1 regulates oncogenic signaling in neuroblastoma cells by regulating ALK membrane association. Cancer Res. 2014;74:3790–801.

241. Vogt K, Mellor J, Tong G, Nicoll R. The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron. 2000;26:187–96.
242. Bennasroune A, Mazot P, Boutterin MC, Vigny M. Activation of the orphan receptor tyrosine kinase ALK by zinc. Biochem Biophys Res Commun. 2010;398:702–6.
243. Barreca A, Lasorsa E, Riera L, Machiorlatti R, Piva R, Ponzoni M, et al. Anaplastic lymphoma kinase in human cancer. J Mol Endocrinol. 2011;47:R11–23.
244. Chiarle R, Martinengo C, Mastini C, Ambrogio C, D'Escamard V, Forni G, et al. The anaplastic lymphoma kinase is an effective oncoantigen for lymphoma vaccination. Nat Med. 2008;14:676–80.
245. Palmer RH, Vernersson E, Grabbe C, Hallberg B. Anaplastic lymphoma kinase: signalling in development and disease. Biochem J. 2009;420:345–61.
246. Martinengo C, Poggio T, Menotti M, Scalzo MS, Mastini C, Ambrogio C, et al. ALK-dependent control of hypoxia-inducible factors mediates tumor growth and metastasis. Cancer Res. 2014;74:6094–106.
247. Bai RY, Dieter P, Peschel C, Morris SW, Duyster J. Nucleophosmin-anaplastic lymphoma kinase of large-cell anaplastic lymphoma is a constitutively active tyrosine kinase that utilizes phospholipase C-gamma to mediate its mitogenicity. Mol Cell Biol. 1998;18:6951–61.
248. Amin HM, McDonnell TJ, Ma Y, Lin Q, Fujio Y, Kunisada K, et al. Selective inhibition of STAT3 induces apoptosis and G(1) cell cycle arrest in ALK-positive anaplastic large cell lymphoma. Oncogene. 2004;23:5426–34.
249. Galkin AV, Melnick JS, Kim S, Hood TL, Li N, Li L, et al. Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK. Proc Natl Acad Sci USA. 2007;104:270–5.
250. Marzec M, Kasprzycka M, Ptasznik A, Wlodarski P, Zhang Q, Odum N, et al. Inhibition of ALK enzymatic activity in T-cell lymphoma cells induces apoptosis and suppresses proliferation and STAT3 phosphorylation independently of Jak3. Lab Invest. 2005;85:1544–54.
251. Wan W, Albom MS, Lu L, Quail MR, Becknell NC, Weinberg LR, et al. Anaplastic lymphoma kinase activity is essential for the proliferation and survival of anaplastic large-cell lymphoma cells. Blood. 2006;107:1617–23.
252. Zamo A, Chiarle R, Piva R, Howes J, Fan Y, Chilosi M, et al. Anaplastic lymphoma kinase (ALK) activates Stat3 and protects hematopoietic cells from cell death. Oncogene. 2002;21:1038–47.
253. Zhang Q, Raghunath PN, Xue L, Majewski M, Carpentieri DF, Odum N, et al. Multilevel dysregulation of STAT3 activation in anaplastic lymphoma kinase-positive T/null-cell lymphoma. J Immunol. 2002;168:466–74.
254. Amin HM, Medeiros LJ, Ma Y, Feretzaki M, Das P, Leventaki V, et al. Inhibition of JAK3 induces apoptosis and decreases anaplastic lymphoma kinase activity in anaplastic large cell lymphoma. Oncogene. 2003;22:5399–407.
255. Shi X, Franko B, Frantz C, Amin HM, Lai R. JSI-124 (cucurbitacin I) inhibits Janus kinase-3/signal transducer and activator of transcription-3 signalling, downregulates nucleophosmin-anaplastic lymphoma kinase (ALK), and induces apoptosis in ALK-positive anaplastic large cell lymphoma cells. Br J Haematol. 2006;135:26–32.
256. Hegazy SA, Wang P, Anand M, Ingham RJ, Gelebart P, Lai R. The tyrosine 343 residue of nucleophosmin (NPM)-anaplastic lymphoma kinase (ALK) is important for its interaction with SHP1, a cytoplasmic tyrosine phosphatase with tumor suppressor functions. J Biol Chem. 2010;285:19813–20.
257. Khoury JD, Rassidakis GZ, Medeiros LJ, Amin HM, Lai R. Methylation of SHP1 gene and loss of SHP1 protein expression are frequent in systemic anaplastic large cell lymphoma. Blood. 2004;104:1580–1.
258. Nieborowska-Skorska M, Slupianek A, Xue L, Zhang Q, Raghunath PN, Hoser G, et al. Role of signal transducer and activator of transcription 5 in nucleophosmin/anaplastic lymphoma kinase-mediated malignant transformation of lymphoid cells. Cancer Res. 2001;61:6517–23.

259. Zhang Q, Wang HY, Liu X, Wasik MA. STAT5A is epigenetically silenced by the tyrosine kinase NPM1-ALK and acts as a tumor suppressor by reciprocally inhibiting NPM1-ALK expression. Nat Med. 2007;13:1341–8.
260. Bai RY, Ouyang T, Miething C, Morris SW, Peschel C, Duyster J. Nucleophosmin-anaplastic lymphoma kinase associated with anaplastic large-cell lymphoma activates the phosphatidylinositol 3-kinase/Akt antiapoptotic signaling pathway. Blood. 2000;96:4319–27.
261. Fujimoto J, Shiota M, Iwahara T, Seki N, Satoh H, Mori S, et al. Characterization of the transforming activity of p80, a hyperphosphorylated protein in a Ki-1 lymphoma cell line with chromosomal translocation t(2;5). Proc Natl Acad Sci USA. 1996;93:4181–6.
262. Polgar D, Leisser C, Maier S, Strasser S, Ruger B, Dettke M, et al. Truncated ALK derived from chromosomal translocation t(2;5)(p23;q35) binds to the SH3 domain of p85-PI3K. Mutat Res. 2005;570:9–15.
263. Slupianek A, Nieborowska-Skorska M, Hoser G, Morrione A, Majewski M, Xue L, et al. Role of phosphatidylinositol 3-kinase-Akt pathway in nucleophosmin/anaplastic lymphoma kinase-mediated lymphomagenesis. Cancer Res. 2001;61:2194–9.
264. McDonnell SR, Hwang SR, Basrur V, Conlon KP, Fermin D, Wey E, et al. NPM-ALK signals through glycogen synthase kinase 3beta to promote oncogenesis. Oncogene. 2011;31:3733–40.
265. Singh RR, Cho-Vega JH, Davuluri Y, Ma S, Kasbidi F, Milito C, et al. Sonic hedgehog signaling pathway is activated in ALK-positive anaplastic large cell lymphoma. Cancer Res. 2009;69:2550–8.
266. Gu TL, Tothova Z, Scheijen B, Griffin JD, Gilliland DG, Sternberg DW. NPM-ALK fusion kinase of anaplastic large-cell lymphoma regulates survival and proliferative signaling through modulation of FOXO3a. Blood. 2004;103:4622–9.
267. Rassidakis GZ, Feretzaki M, Atwell C, Grammatikakis I, Lin Q, Lai R, et al. Inhibition of Akt increases p27Kip1 levels and induces cell cycle arrest in anaplastic large cell lymphoma. Blood. 2005;105:827–9.
268. Marzec M, Kasprzycka M, Liu X, El-Salem M, Halasa K, Raghunath PN, et al. Oncogenic tyrosine kinase NPM/ALK induces activation of the rapamycin-sensitive mTOR signaling pathway. Oncogene. 2007;26:5606–14.
269. Vega F, Medeiros LJ, Leventaki V, Atwell C, Cho-Vega JH, Tian L, et al. Activation of mammalian target of rapamycin signaling pathway contributes to tumor cell survival in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma. Cancer Res. 2006;66:6589–97.
270. Gu L, Gao J, Li Q, Zhu YP, Jia CS, Fu RY, et al. Rapamycin reverses NPM-ALK-induced glucocorticoid resistance in lymphoid tumor cells by inhibiting mTOR signaling pathway, enhancing G1 cell cycle arrest and apoptosis. Leukemia. 2008;22:2091–6.
271. Crockett DK, Lin Z, Elenitoba-Johnson KS, Lim MS. Identification of NPM-ALK interacting proteins by tandem mass spectrometry. Oncogene. 2004;23:2617–29.
272. Riera L, Lasorsa E, Ambrogio C, Surrenti N, Voena C, Chiarle R. Involvement of Grb2 adaptor protein in nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-mediated signaling and anaplastic large cell lymphoma growth. J Biol Chem. 2010;285:26441–50.
273. Ouyang T, Bai RY, Bassermann F, von Klitzing C, Klumpen S, Miething C, et al. Identification and characterization of a nuclear interacting partner of anaplastic lymphoma kinase (NIPA). J Biol Chem. 2003;278:30028–36.
274. Bassermann F, von Klitzing C, Munch S, Bai RY, Kawaguchi H, Morris SW, et al. NIPA defines an SCF-type mammalian E3 ligase that regulates mitotic entry. Cell. 2005;122:45–57.
275. Ambrogio C, Voena C, Manazza AD, Martinengo C, Costa C, Kirchhausen T, et al. The anaplastic lymphoma kinase controls cell shape and growth of anaplastic large cell lymphoma through Cdc42 activation. Cancer Res. 2008;68:8899–907.
276. Colomba A, Courilleau D, Ramel D, Billadeau DD, Espinos E, Delsol G, et al. Activation of Rac1 and the exchange factor Vav3 are involved in NPM-ALK signaling in anaplastic large cell lymphomas. Oncogene. 2008;27:2728–36.
277. Ambrogio C, Voena C, Manazza AD, Piva R, Riera L, Barberis L, et al. p130Cas mediates the transforming properties of the anaplastic lymphoma kinase. Blood. 2005;106:3907–16.

278. Cussac D, Greenland C, Roche S, Bai RY, Duyster J, Morris SW, et al. Nucleophosmin-anaplastic lymphoma kinase of anaplastic large-cell lymphoma recruits, activates, and uses pp60c-src to mediate its mitogenicity. Blood. 2004;103:1464–71.
279. Voena C, Conte C, Ambrogio C, Boeri Erba E, Boccalatte F, Mohammed S, et al. The tyrosine phosphatase Shp2 interacts with NPM-ALK and regulates anaplastic lymphoma cell growth and migration. Cancer Res. 2007;67:4278–86.
280. Dupuis-Coronas S, Lagarrigue F, Ramel D, Chicanne G, Saland E, Gaits-Iacovoni F, et al. The nucleophosmin-anaplastic lymphoma kinase oncogene interacts, activates, and uses the kinase PIKfyve to increase invasiveness. J Biol Chem. 2011;286:32105–14.
281. Leventaki V, Drakos E, Medeiros LJ, Lim MS, Elenitoba-Johnson KS, Claret FX, et al. NPM-ALK oncogenic kinase promotes cell-cycle progression through activation of JNK/cJun signaling in anaplastic large-cell lymphoma. Blood. 2007;110:1621–30.
282. Turner SD, Tooze R, Maclennan K, Alexander DR. Vav-promoter regulated oncogenic fusion protein NPM-ALK in transgenic mice causes B-cell lymphomas with hyperactive Jun kinase. Oncogene. 2003;22:7750–61.
283. Cui YX, Kerby A, McDuff FK, Ye H, Turner SD. NPM-ALK inhibits the p53 tumor suppressor pathway in an MDM2 and JNK-dependent manner. Blood. 2009;113:5217–27.
284. Boccalatte FE, Voena C, Riganti C, Bosia A, D'Amico L, Riera L, et al. The enzymatic activity of 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase is enhanced by NPM-ALK: new insights in ALK-mediated pathogenesis and the treatment of ALCL. Blood. 2009;113:2776–90.
285. Cussac D, Pichereaux C, Colomba A, Capilla F, Pont F, Gaits-Iacovoni F, et al. Proteomic analysis of anaplastic lymphoma cell lines: identification of potential tumour markers. Proteomics. 2006;6:3210–22.
286. Galietta A, Gunby RH, Redaelli S, Stano P, Carniti C, Bachi A, et al. NPM/ALK binds and phosphorylates the RNA/DNA-binding protein PSF in anaplastic large-cell lymphoma. Blood. 2007;110:2600–9.
287. Lim MS, Carlson ML, Crockett DK, Fillmore GC, Abbott DR, Elenitoba-Johnson OF, et al. The proteomic signature of NPM/ALK reveals deregulation of multiple cellular pathways. Blood. 2009;114:1585–95.
288. Sjostrom C, Seiler C, Crockett DK, Tripp SR, Elenitoba Johnson KS, Lim MS. Global proteome profiling of NPM/ALK-positive anaplastic large cell lymphoma. Exp Hematol. 2007;35:1240–8.
289. Wu F, Wang P, Young LC, Lai R, Li L. Proteome-wide identification of novel binding partners to the oncogenic fusion gene protein, NPM-ALK, using tandem affinity purification and mass spectrometry. Am J Pathol. 2009;174:361–70.
290. Young LC, Bone KM, Wang P, Wu F, Adam BA, Hegazy S, et al. Fusion tyrosine kinase NPM-ALK deregulates MSH2 and suppresses DNA mismatch repair function novel insights into a potent oncoprotein. Am J Pathol. 2011;179:411–21.
291. Lamant L, de Reynies A, Duplantier MM, Rickman DS, Sabourdy F, Giuriato S, et al. Gene-expression profiling of systemic anaplastic large-cell lymphoma reveals differences based on ALK status and two distinct morphologic ALK+ subtypes. Blood. 2007;109:2156–64.
292. Piva R, Chiarle R, Manazza AD, Taulli R, Simmons W, Ambrogio C, et al. Ablation of oncogenic ALK is a viable therapeutic approach for anaplastic large-cell lymphomas. Blood. 2006;107:689–97.
293. Pearson JD, Lee JK, Bacani JT, Lai R, Ingham RJ. NPM-ALK and the JunB transcription factor regulate the expression of cytotoxic molecules in ALK-positive, anaplastic large cell lymphoma. Int J Clin Exp Pathol. 2011;4:124–33.
294. Piva R, Pellegrino E, Mattioli M, Agnelli L, Lombardi L, Boccalatte F, et al. Functional validation of the anaplastic lymphoma kinase signature identifies CEBPB and BCL2A1 as critical target genes. J Clin Invest. 2006;116:3171–82.
295. Staber PB, Vesely P, Haq N, Ott RG, Funato K, Bambach I, et al. The oncoprotein NPM-ALK of anaplastic large-cell lymphoma induces JUNB transcription via ERK1/2 and JunB translation via mTOR signaling. Blood. 2007;110:3374–83.

296. Dejean E, Renalier MH, Foisseau M, Agirre X, Joseph N, de Paiva GR, et al. Hypoxia-microRNA-16 downregulation induces VEGF expression in anaplastic lymphoma kinase (ALK)-positive anaplastic large-cell lymphomas. Leukemia. 2011;25:1882–90.
297. Desjobert C, Renalier MH, Bergalet J, Dejean E, Joseph N, Kruczynski A, et al. MiR-29a down-regulation in ALK-positive anaplastic large cell lymphomas contributes to apoptosis blockade through MCL-1 overexpression. Blood. 2011;117:6627–37.
298. Matsuyama H, Suzuki HI, Nishimori H, Noguchi M, Yao T, Komatsu N, et al. MiR-135b mediates NPM-ALK-driven oncogenicity and renders IL-17-producing immunophenotype to anaplastic large cell lymphoma. Blood. 2011;118:6881–92.
299. Trinei M, Lanfrancone L, Campo E, Pulford K, Mason DY, Pelicci PG, et al. A new variant anaplastic lymphoma kinase (ALK)-fusion protein (ATIC-ALK) in a case of ALK-positive anaplastic large cell lymphoma. Cancer Res. 2000;60:793–8.
300. Armstrong F, Duplantier MM, Trempat P, Hieblot C, Lamant L, Espinos E, et al. Differential effects of X-ALK fusion proteins on proliferation, transformation, and invasion properties of NIH3T3 cells. Oncogene. 2004;23:6071–82.
301. Chen Z, Sasaki T, Tan X, Carretero J, Shimamura T, Li D, et al. Inhibition of ALK, PI3K/MEK, and HSP90 in murine lung adenocarcinoma induced by EML4-ALK fusion oncogene. Cancer Res. 2010;70:9827–36.
302. Takezawa K, Okamoto I, Nishio K, Janne PA, Nakagawa K. Role of ERK-BIM and STAT3-survivin signaling pathways in ALK inhibitor-induced apoptosis in EML4-ALK-positive lung cancer. Clin Cancer Res. 2011;17:2140–8.
303. Maru Y, Hirai H, Takaku F. Human ltk: gene structure and preferential expression in human leukemic cells. Oncogene Res. 1990;5:199–204.
304. Snijders AJ, Haase VH, Bernards A. Four tissue-specific mouse ltk mRNAs predict tyrosine kinases that differ upstream of their transmembrane segment. Oncogene. 1993;8:27–35.
305. Toyoshima H, Kozutsumi H, Maru Y, Hagiwara K, Furuya A, Mioh H, et al. Differently spliced cDNAs of human leukocyte tyrosine kinase receptor tyrosine kinase predict receptor proteins with and without a tyrosine kinase domain and a soluble receptor protein. Proc Natl Acad Sci USA. 1993;90:5404–8.
306. Kozutsumi H, Toyoshima H, Hagiwara K, Furuya A, Mioh H, Hanai N, et al. Identification of the human ltk gene product in placenta and hematopoietic cell lines. Biochem Biophys Res Commun. 1993;190:674–9.
307. Ueno H, Hirano N, Kozutsumi H, Sasaki K, Tanaka T, Yazaki Y, et al. An epidermal growth factor receptor-leukocyte tyrosine kinase chimeric receptor generates ligand-dependent growth signals through the Ras signaling pathway. J Biol Chem. 1995;270:20135–42.
308. Ueno H, Sasaki K, Kozutsumi H, Miyagawa K, Mitani K, Yazaki Y, et al. Growth and survival signals transmitted via two distinct NPXY motifs within leukocyte tyrosine kinase, an insulin receptor-related tyrosine kinase. J Biol Chem. 1996;271:27707–14.
309. Ueno H, Honda H, Nakamoto T, Yamagata T, Sasaki K, Miyagawa K, et al. The phosphatidylinositol 3′ kinase pathway is required for the survival signal of leukocyte tyrosine kinase. Oncogene. 1997;14:3067–72.
310. Yamada S, Nomura T, Takano K, Fujita S, Miyake M, Miyake J. Expression of a chimeric CSF1R-LTK mediates ligand-dependent neurite outgrowth. Neuroreport. 2008;19:1733–8.
311. Christensen JG, Zou HY, Arango ME, Li Q, Lee JH, McDonnell SR, et al. Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. Mol Cancer Ther. 2007;6:3314–22.
312. Shaw AT, Yeap BY, Solomon BJ, Riely GJ, Gainor J, Engelman JA, et al. Effect of crizotinib on overall survival in patients with advanced non-small-cell lung cancer harbouring ALK gene rearrangement: a retrospective analysis. Lancet Oncol. 2011;12:1004–12.
313. Shaw AT, Kim DW, Nakagawa K, Seto T, Crino L, Ahn MJ, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. N Engl J Med. 2013;368:2385–94.
314. Hallberg B, Palmer RH. Crizotinib–latest champion in the cancer wars? N Engl J Med. 2010;363:1760–2.

315. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc Natl Acad Sci USA. 2008;105:2070–5.
316. Sakamoto H, Tsukaguchi T, Hiroshima S, Kodama T, Kobayashi T, Fukami TA, et al. CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. Cancer Cell. 2011;19:679–90.
317. Katayama R, Khan TM, Benes C, Lifshits E, Ebi H, Rivera VM, et al. Therapeutic strategies to overcome crizotinib resistance in non-small cell lung cancers harboring the fusion oncogene EML4-ALK. Proc Natl Acad Sci USA. 2011;108:7535–40.
318. Sequist LV, Gettinger S, Senzer NN, Martins RG, Janne PA, Lilenbaum R, et al. Activity of IPI-504, a novel heat-shock protein 90 inhibitor, in patients with molecularly defined non-small-cell lung cancer. J Clin Oncol. 2010;28:4953–60.
319. Kuo YW, Wu SG, Ho CC, Shih JY. Good response to gefitinib in lung adenocarcinoma harboring coexisting EML4-ALK fusion gene and EGFR mutation. J Thorac Oncol. 2010;5:2039–40.
320. Tiseo M, Gelsomino F, Boggiani D, Bortesi B, Bartolotti M, Bozzetti C, et al. EGFR and EML4-ALK gene mutations in NSCLC: a case report of erlotinib-resistant patient with both concomitant mutations. Lung Cancer. 2011;71:241–3.
321. Martelli MP, Sozzi G, Hernandez L, Pettirossi V, Navarro A, Conte D, et al. EML4-ALK rearrangement in non-small cell lung cancer and non-tumor lung tissues. Am J Pathol. 2009;174:661–70.
322. Hallberg B, Palmer RH. ALK and NSCLC: targeted therapy with ALK inhibitors. F1000 Med Rep. 2011;3:21.
323. Dhillon S, Clark M. Ceritinib: first global approval. Drugs. 2014;74:1285–91.
324. McKeage K. Alectinib: a review of its use in advanced ALK-rearranged non-small cell lung cancer. Drugs. 2015;75:75–82.
325. Shaw AT, Kim DW, Mehra R, Tan DS, Felip E, Chow LQ, et al. Ceritinib in ALK-rearranged non-small-cell lung cancer. N Engl J Med. 2014;370:1189–97.
326. Lovly CM, Heuckmann JM, de Stanchina E, Chen H, Thomas RK, Liang C, et al. Insights into ALK-driven cancers revealed through development of novel ALK tyrosine kinase inhibitors. Cancer Res. 2011;71:4920–31.
327. Huang Q, Johnson TW, Bailey S, Brooun A, Bunker KD, Burke BJ, et al. Design of potent and selective inhibitors to overcome clinical anaplastic lymphoma kinase mutations resistant to crizotinib. J Med Chem. 2014;57:1170–87.
328. Johnson TW, Richardson PF, Bailey S, Brooun A, Burke BJ, Collins MR, et al. Discovery of (10R)-7-amino-12-fluoro-2,10,16-trimethyl-15-oxo-10,15,16,17-tetrahydro-2H-8,4-(metheno)pyrazolo[4,3-h][2,5,11]-benzoxadiazacyclotetradecine-3-carbonitrile (PF-06463922), a macrocyclic inhibitor of anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1) with preclinical brain exposure and broad-spectrum potency against ALK-resistant mutations. J Med Chem. 2014;57:4720–44.
329. Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L. Treatment of HER2-positive breast cancer: current status and future perspectives. Nat Rev Clin Oncol. 2012;9:16–32.
330. Hudis CA. Trastuzumab–mechanism of action and use in clinical practice. N Engl J Med. 2007;357:39–51.
331. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344:783–92.
332. Mazot P, Cazes A, Dingli F, Degoutin J, Irinopoulou T, Boutterin MC, et al. Internalization and down-regulation of the ALK receptor in neuroblastoma cell lines upon monoclonal antibodies treatment. PLoS One. 2012;7:e33581.
333. Carpenter EL, Haglund EA, Mace EM, Deng D, Martinez D, Wood AC, et al. Antibody targeting of anaplastic lymphoma kinase induces cytotoxicity of human neuroblastoma. Oncogene. 2012;31:4859–67.

334. Mosse YP, Balis FM, Lim MS, Laliberte J, Voss SD, Fox E, Bagatell R, Weigel B, Adamson PC, Ingle AM, Ahern CH, Blaney S. Efficacy of crizotinib in children with relapsed/refractory ALK-driven tumors including anaplastic large cell lymphoma and neuroblastoma: A Children's Oncology Group phase I consortium study. J Clin Oncol. 2012;30(15 Suppl):9500 (May 20 Supplement).
335. Moore NF, Azarova AM, Bhatnagar N, Ross KN, Drake LE, Frumm S, et al. Molecular rationale for the use of PI3K/AKT/mTOR pathway inhibitors in combination with crizotinib in ALK-mutated neuroblastoma. Oncotarget. 2014;5:8737–49.
336. Neckers L, Workman P. Hsp90 molecular chaperone inhibitors: are we there yet? Clin Cancer Res. 2012;18:64–76.
337. Bonvini P, Gastaldi T, Falini B, Rosolen A. Nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), a novel Hsp90-client tyrosine kinase: down-regulation of NPM-ALK expression and tyrosine phosphorylation in ALK(+) CD30(+) lymphoma cells by the Hsp90 antagonist 17-allylamino,17-demethoxygeldanamycin. Cancer Res. 2002;62:1559–66.
338. Schumacher JA, Crockett DK, Elenitoba-Johnson KS, Lim MS. Proteome-wide changes induced by the Hsp90 inhibitor, geldanamycin in anaplastic large cell lymphoma cells. Proteomics. 2007;7:2603–16.
339. Ge J, Normant E, Porter JR, Ali JA, Dembski MS, Gao Y, et al. Design, synthesis, and biological evaluation of hydroquinone derivatives of 17-amino-17-demethoxygeldanamycin as potent, water-soluble inhibitors of Hsp90. J Med Chem. 2006;49:4606–15.
340. Sydor JR, Normant E, Pien CS, Porter JR, Ge J, Grenier L, et al. Development of 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504), an anti-cancer agent directed against Hsp90. Proc Natl Acad Sci USA. 2006;103:17408–13.
341. Normant E, Paez G, West KA, Lim AR, Slocum KL, Tunkey C, et al. The Hsp90 inhibitor IPI-504 rapidly lowers EML4-ALK levels and induces tumor regression in ALK-driven NSCLC models. Oncogene. 2011;30:2581–6.
342. Di Paolo D, Ambrogio C, Pastorino F, Brignole C, Martinengo C, Carosio R, et al. Selective therapeutic targeting of the anaplastic lymphoma kinase with liposomal siRNA induces apoptosis and inhibits angiogenesis in neuroblastoma. Mol Ther. 2011;19:2201–12.
343. Di Paolo D, Brignole C, Pastorino F, Carosio R, Zorzoli A, Rossi M, et al. Neuroblastoma-targeted nanoparticles entrapping siRNA specifically knockdown ALK. Mol Ther. 2011; 19:1131–40.
344. Shim MS, Kwon YJ. Efficient and targeted delivery of siRNA in vivo. FEBS J. 2010; 277:4814–27.
345. Ito M, Zhao N, Zeng Z, Chang CC, Zu Y. Synergistic growth inhibition of anaplastic large cell lymphoma cells by combining cellular ALK gene silencing and a low dose of the kinase inhibitor U0126. Cancer Gene Ther. 2010;17:633–44.

Chapter 2
The TAM Receptor Family

Greg Lemke

Abbreviations

AC	Apoptotic cell
DC	Dendritic cell
Gla	Gamma-carboxylated glutamic acid
IFNAR	Type I IFN receptor
PR	Photoreceptor
PRR	Pattern recognition receptor
PtdSer	Phosphatidylserine
RPE	Retinal pigment epithelium
SHBG	Sex hormone binding globulin
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
TKO	Triple knockout

2.1 Introduction to the TAM Receptor Tyrosine Kinase Family

The TAM receptors—Tyro3, Axl, and Mer—are an unusual family of receptor tyrosine kinases [1]. As a group, they play no essential role in embryonic development but rather are specialized to function as regulators of cell and tissue homeostasis in fully differentiated organ systems that are subject to continuous renewal throughout adult

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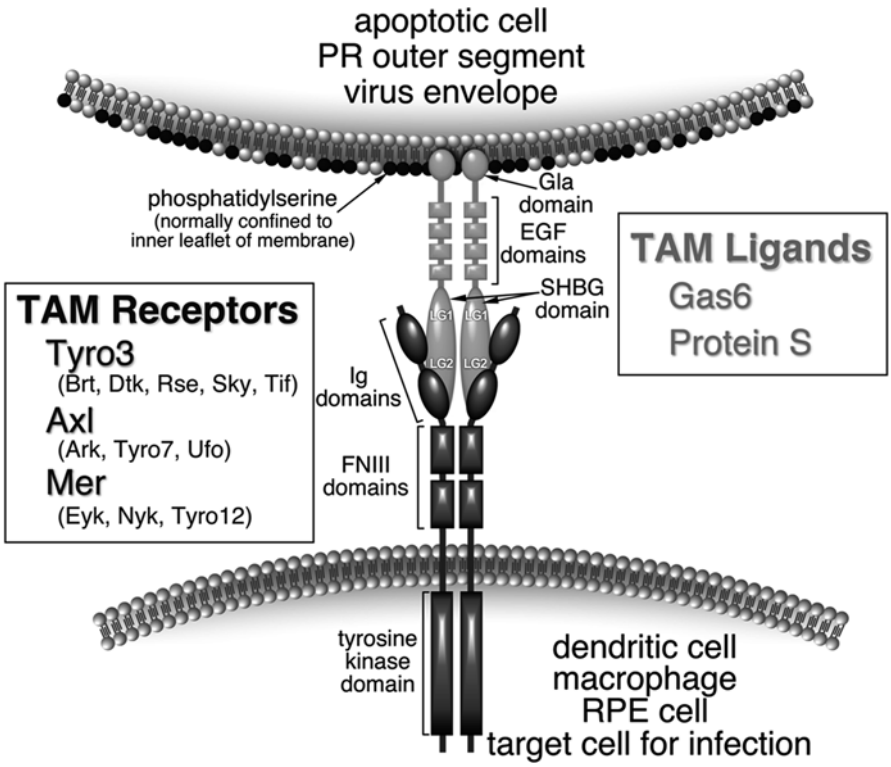

Fig. 2.1 TAM receptors and ligands. The *TAM* receptors are *Tyro3* [3, 6]—also designated Brt [188], Dtk [189], Rse [190], Sky [191], and Tif [192]; *Axl* [4]—also designated Ark [193], Tyro7 [3], and Ufo [194]; and *Mer* [5]—also designated Eyk [146], Nyk [195], and Tyro12 [3]. The TAMs are expressed by cells of the mature immune, nervous, vascular, and reproductive systems. The TAM ligands are Gas6 and Protein S (ProS). The C-terminal SHBG domains of the ligands bind to the immunoglobulin (Ig) domains of the receptors, induce dimerization, and activate the TAM tyrosine kinases. When γ-carboxylated in a vitamin-K-dependent reaction, the N-terminal Gla domains of the dimeric ligands bind to the phospholipid phosphatidylserine expressed on the surface on an apposed apoptotic cell or enveloped virus. Adapted from [85]

life. These include the mature immune, reproductive, hematopoietic, vascular, and nervous systems. The name of the family was coined in 2006, from the first letter of its three members—Tyro3, Axl, and Mer [2]. Since the TAMs were identified independently by multiple investigators, they appear in the early literature under several alternative names (see Fig. 2.1), but Tyro3, Axl, and Mer (officially c-Mer or MerTK for the protein, *Mertk* for the gene) have now been adopted as the NCBI designations.

The TAMs were among the last of the 58 RTKs to be identified: they were discovered and grouped into a distinct family of orphan RTKs, based on PCR amplification of conserved regions of their kinase domains, in 1991 [3]. The subsequent cloning of full-length cDNAs for Axl [4], Mer [5], and Tyro3 [6] confirmed their

segregation into this structurally distinctive family [7]. The two ligands that bind and activate the TAMs—Gas6 and Protein S—were identified only in 1995 [8–12].

To a significant extent, deciphering the biological roles of the TAM receptors required the derivation of mouse loss-of-function mutants [13, 14]. The fact that *Tyro3*$^{-/-}$, *Axl*$^{-/-}$, and *Mertk*$^{-/-}$ mice are all viable and fertile permitted the generation of a complete TAM mutant series that included all possible double mutants and even triple mutants that lack all three receptors [14]. Remarkably, these *Tyro3*$^{-/-}$*Axl*$^{-/-}$ *Mertk*$^{-/-}$ mice—so-called TAM TKOs—are viable for more than a year in a standard mouse colony, and for the first week or so after birth, are largely indistinguishable from their wild-type counterparts [14]. Since RTKs often play essential roles in embryonic development, even single loss-of-function mutations in many RTK genes result in an embryonic lethal phenotype [15–18]. The postnatal viability of mice in which an entire RTK family is ablated is therefore highly unusual. Their viability notwithstanding, the TAM mutants go on to develop a plethora of disease phenotypes, some of them debilitating [2, 13, 14, 19–21]. Without exception, these phenotypes are degenerative in nature and reflect the loss of TAM signaling activities in tissues that are subject to regular challenge and renewal.

2.1.1 TAM Receptor Structure and Signaling Features

The extracellular domains of TAM receptors are composed of two structural modules that are used repeatedly in other RTK ectodomains, but that are configured in a defining two-plus-two combination in the TAMs (Fig. 2.1). The amino-terminal regions of these ectodomains carry tandem immunoglobulin-related domains that mediate ligand binding [22–24], which are followed by tandem fibronectin type III repeats [4–6, 25, 26]. All three TAM receptors have a single transmembrane domain, and all carry a catalytically competent protein-tyrosine kinase (Fig. 2.1). In the complete vertebrate "kinome," the TAMs are most closely related to Met, the receptor for hepatocyte growth factor, and Ron, the receptor for macrophage-stimulating protein [7].

In many cells, the activation of the Tyro3, Axl, or Mer tyrosine kinase is coupled to the downstream activation of the phosphoinositide 3 kinase (PI3K)/AKT pathway. Most of this downstream PI3K signaling is nucleated through a TAM-autophosphorylated Grb2 binding site, which is located 18 residues downstream of the kinase domain in all three TAMs [27–36] (Fig. 2.2). Coupling to phospholipase C, ERK1/2, Ras, and MAP kinase activation have also been described in many different cells [37–39]. These TAM-activated signaling pathways (Fig. 2.2) operate in all TAM-expressing cells. Macrophages, dendritic cells, and other sentinel cells of the immune system, however, also express cytokine receptors—such as the type I interferon (IFN) receptor—that are directly coupled to the TAM receptors. In these cells, the TAM-activated PI3K/AKT pathway is often dominated by a stronger TAM-activated JAK/STAT signaling pathway (Fig. 2.2) [26, 40, 41]. Differential TAM activation of PI3K/AKT versus JAK/STAT signaling may be important for the differential activation of distinct TAM-regulated bioactivities.

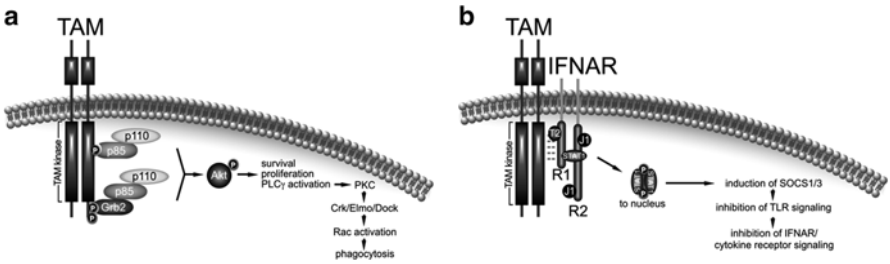

Fig. 2.2 TAM receptor signaling pathways. (**a**) Free TAMs. Activated TAM proteins drive a conventional RTK signaling pathway that is dominated by the phosphorylation and activation of Akt. The positions of major tyrosine autophosphorylation sites shared between Tyro3, Axl, and Mer are indicated (P). The tyrosine immediately downstream of the kinase domain (Y821 in human Axl) is bound by the SH2 domain of Grb2, which recruits the p85 subunit of PI3 kinase through an SH3 (Grb2)-proline-rich domain (p85) interaction. Alternatively, p85 can bind this phosphotyrosine directly using its own SH2 domain. P85 also binds to the indicated phosphotyrosine within the kinase domain (see, for example, [36]). Mobilization of the joint p85/p110 PI3K complex results in the downstream phosphorylation and activation of Akt. Mer activation has also been found to drive the downstream activation of PLCγ, by a mechanism that is not delineated biochemically [34]. These pathways are required for cell survival and the mobilization of the actin cytoskeleton that is required for the engulfment of ACs by phagocytes. (**b**) TAM receptors complexed with the type I interferon receptor (IFNAR). In dendritic cells, the TAM receptor Axl—when activated by the binding of a TAM ligand—forms a co-immunoprecipitable complex specifically with the R1 (or α) chain of the IFNAR [40]. This may be associated with the activation of Jak1 (J1) [41]. Direct activation of the hybrid TAM-IFNAR receptor by the addition of Gas6 leads to the rapid tyrosine phosphorylation and activation of Stat1. This dimeric transcription factor then translocates to the nucleus, where it drives the expression of the cytoplasmic cytokine inhibitors SOCS1 and 3. This pathway, which often obscures the PI3K–AKT pathway diagrammed in **a**, is required for the inhibition of inflammatory responses in dendritic cells [26, 40]

TAM receptors are among the last RTKs to have appeared during evolution [7, 42]. There are no TAM representatives in either *Drosophila* or *C. elegans*. Interestingly, a single Gas6/Protein S-like ligand gene is first seen in the genomes of pre-vertebrate urochordates such as *Ciona*. Quite remarkably, these urochordates genomes also contain a second gene in which a TAM-like tyrosine kinase is linked, via a single transmembrane domain, to a TAM-*ligand*-like "Gla domain" [26, 43]. As discussed below, this TAM ligand-kinase chimera could function as a direct receptor for the phospholipid phosphatidylserine. The appearance of the TAM–like tyrosine kinase in urochordates is coincident with the first appearance of type I and type II cytokines (e.g., interferons) and their receptors.

2.1.2 TAM Ligand Structure and Receptor Specificity

The two TAM ligands—Gas6 and Protein S (gene designation *Pros1*) [8, 9, 44]—are large (~80 kD) proteins that are ~42 % identical in amino acid sequence and share the same multidomain arrangement (Fig. 2.1). They have two structural

features that are key to their activities. The first is a carboxy-terminal "sex hormone binding globulin" (SHBG) domain composed of two laminin G domains (Fig. 2.1). This SHBG domain binds to the Ig domains of the receptors, and induces their dimerization and subsequent kinase activation [22, 45–48]. The second is a so-called "Gla" domain positioned at the amino terminus of both ligands [8, 49, 50]. The SHBG and Gla domains are separated by four EGF-related domains.

The ~60-amino acid Gla domain is rich in glutamic acid residues whose gamma (γ carbons are post-translationally carboxylated in a vitamin K-dependent modification [51–53]. Gas6 and Protein S share Gla domains with several proteins of the blood coagulation cascade, including Prothrombin, Protein C, and Factors VII, IX, and X, [51, 52, 54, 55]; and in addition to acting as a TAM ligand, Protein S also functions, in a TAM-independent mechanism, as an anticoagulant in this cascade [55, 56].

Gamma-carboxylation allows Gla domains to bind, in a Ca^{2+}-dependent manner, to phosphatidylserine (PtdSer). In most cells, this phospholipid is confined to the inner, cytoplasm-facing leaflet of the plasma membrane [57]. In activated platelets and apoptotic cells, the "flippases" that maintain this membrane asymmetry are disabled, and a set of "scramblases" are activated [57–59], such that PtdSer is displayed on the extracellular membrane surface as well. For apoptotic cells (ACs), PtdSer is among the most potent of the so-called "eat-me" signals by which these dead cells are recognized by phagocytes [60]. Gla-domain-containing proteins can therefore bind to the surface of ACs. As discussed below, the interaction of the amino-terminal Gla domains of Gas6 and Protein S with a PtdSer-containing membrane (Fig. 2.1) is a crucial feature of their activation of TAM receptors.

Gas6 and Protein S bind to TAM receptors as dimers, and multimerization appears to be required for TAM activation [61]. Apart from this, receptor–ligand pairing relationships and signaling interactions for the TAM system have only recently been addressed definitively [24]. We do not know the extent to which Protein S and Gas6 may heterodimerize, and if this occurs, how receptor binding and activation profiles of the heterodimer may differ from those of Gas6 or Protein S homodimers. Similarly, the extent to which individual TAM receptors may heterodimerize in different cellular settings in which two or more receptors are co-expressed is also poorly understood. However, it is now clear that, in multiple settings, Tyro3, Axl, and Mer can all effectively signal as homodimeric receptors that bind just one ligand dimer [24, 62, 63].

The preponderance of evidence overwhelmingly indicates that Gas6 functions as a ligand for all three TAM receptors [8–11, 24, 26, 63–65]. Full-length recombinant Gas6 strongly activates the kinase activity of all three receptors at low nanomolar concentrations when assayed in cell-based systems (Fig. 2.3) [24]. However, recent work shows that the Gas6-Axl relationship is unique. Firstly, Axl is completely dependent on Gas6 for its activation, in that it does not bind and cannot be activated by Protein S [24, 65]. At the same time, the stable expression of Gas6 in many tissues in vivo is dependent on the co-expression of Axl in these same tissues: in *Axl*$^{-/-}$ mice, but not *Mertk*$^{-/-}$ or *Tyro3*$^{-/-}$ mice, Gas6 protein expression in the spleen, lung, liver, and other tissues is entirely lost, even though expression of *Gas6* mRNA

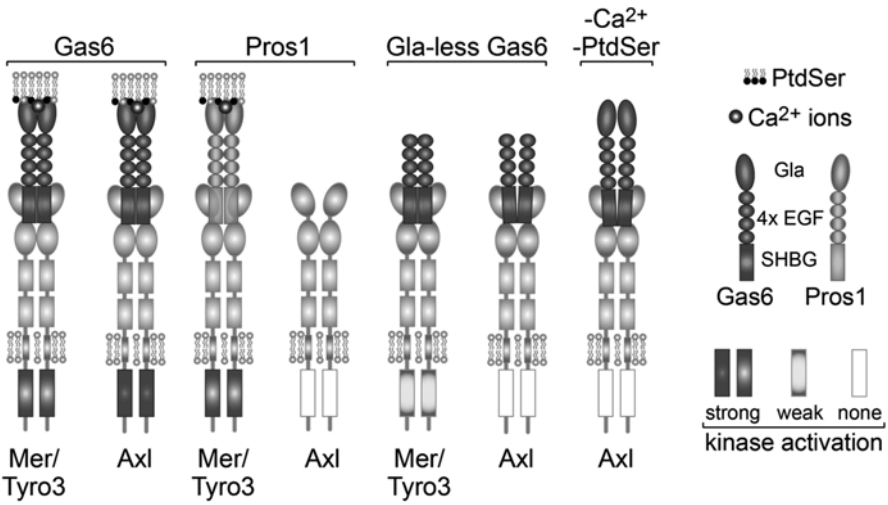

Fig. 2.3 TAM Receptor–ligand rules of engagement. Gas6 activates all three TAM receptors independently. Pros1 activates Tyro3 and Mer but not Axl. Optimal activation of any receptor by either ligand requires the simultaneous presence of the phospholipid phosphatidylserine (PtdSer), which binds to the Gla domain of the ligands, and calcium ions (Ca^{2+}). Gla-less Gas6 is dead as an Axl ligand and weak as a Mer and Tyro3 ligand. Its Axl-bound orientation is therefore schematized differently from Tyro3- and Mer-bound Gla-less Gas6. Adapted from [24]

is maintained at normal levels [63]. Even more remarkable is the fact that Gas6 appears to be constitutively and specifically bound to Axl in these tissues—without significant activation of the receptor [63]. Full activation of Axl requires, in addition, the exposure of PtdSer to which the Gla domain of Gas6 binds, and even further, the presence of extracellular Ca^{2+}, which is required for productive interaction between the Gas6 Gla domain and PtdSer (Fig. 2.3) [24]. Gas6 that lacks its Gla domain is significantly weakened as a Tyro3 and Mer ligand, but is completely inactivated as an Axl ligand [24]. These and related observations suggest that the Axl–Gas6 pair is in fact best viewed as a PtdSer receptor [24, 63], something that urochordates may be able to accomplish with a single chimeric protein [43, 66].

One additional important feature of the Axl–Gas6 pair relates to the proteolytic processing of Axl subsequent to its activation. Upon activation, the Axl "ectodomain" is proteolytically cleaved from the rest of the receptor [67–69]. This can occur subsequent to Gas6 binding, in which case a soluble Axl (sAxl)–Gas6 complex is generated. However, it can also occur subsequent to activation of the receptor by cross-linking anti-Axl antibodies, in the absence of Gas6 [63]. Essentially all of the very low level of Gas6 that normally appears in blood is thought to be complexed to sAxl [70]. Interestingly, elevated blood levels of sAxl appear to be a very good biomarker for inflammation generally, in that they have been reported to mark a variety of human disease and trauma states, including aortic aneurysm, lupus flares, pneumonia infection, preeclampsia, coronary bypass, insulin resistance, and

limb ischemia [71–77]. In each of these settings, the generation of a soluble Axl–Gas6 complex may be triggered by the induced cellular exposure of PtdSer.

In contrast to Gas6, Protein S is a selective TAM ligand that binds and activates Tyro3 and Mer, with no affinity for Axl (Fig. 2.3) [2, 8, 24, 26, 61, 65, 78]. The selectivity of Protein S for Tyro3 over Axl is set by the two Ig-like domains of the receptor [24]. An important additional difference between Protein S and Gas6 relates to their sites of expression. Protein S is abundant in the blood, being present at ~300 nM in the human circulation [56], whereas Gas6 is nearly absent. As noted above, the very low levels of Gas6 in the circulation (0.02–0.2 nM) are almost entirely bound to soluble (cleaved) Axl extracellular domain. Whereas conventional *Pros1* mouse knockouts display an embryonic lethal phenotype that results from exuberant blood coagulation, reflecting the essential TAM-independent function of Protein S in this process [56], conventional *Gas6* knockouts are superficially normal and have normal life spans [79].

The extent to which Gas6 and/or Protein S make specific contributions to TAM receptor signaling in biological settings in vivo has only recently begun to be dissected genetically. The first of these takes place in the eye, where retinal pigment epithelial (RPE) cells perform a daily TAM-dependent phagocytotic excision of the distal ends of photoreceptor outer segments [80, 81]. The second such setting is the testes, where tens of millions of apoptotic germ cells must clear cleared, also in a TAM-dependent process, from the seminiferous tubule at each cycle of spermatogenesis [14, 24, 82]. Both of these recent genetic analysis of differential TAM ligand contributions are discussed in detail below.

In several cell types, TAM signaling appears to be autocrine/paracrine, in that a TAM-positive cell has often been found to express Protein S and/or Gas6 [2, 14, 40]. As noted above, Protein S is expressed at high levels in the blood, into which it is secreted by hepatocytes and endothelial cells [56]. Tyro3- and Mer-expressing cells (and viruses, see below) that transit through the circulation are therefore exposed to saturating levels of this ligand. In the immune system, an important source of Protein S is activated T cells [83, 84]. The general "rules of engagement" for TAM receptors and ligands are summarized in Fig. 2.3.

2.2 The Role of the TAM Receptor Tyrosine Kinase Family in Adult Physiology

2.2.1 TAM Mediation of the Phagocytosis of Apoptotic Cells

TAM receptor signaling plays a critical role in the phagocytosis of apoptotic cells (ACs) and membranes in mature tissues [14, 21, 26, 63, 85]. In this process, a TAM ligand, Gas6 or Protein S, serves as a "bridge" that links a TAM receptor, expressed on the surface of the phagocyte, to PtdSer displayed on the surface of the AC that will be engulfed [63, 86, 87] (Fig. 2.1). At the same time, this ligand must activate the tyrosine kinase activity of the TAM receptor for the process of phagocytosis to

go forward [21, 34, 63, 85, 88, 89]. "Bridging" in the absence of TAM kinase activation and kinase activation in the absence of bridging are both insufficient to promote AC phagocytosis [63].

The first phenotype described in the TAM TKOs was male infertility, which results from the degenerative death of nearly all germ cells in the testes [14]. This cell death is due to a failure in the clearance of ACs from the seminiferous tubules of the testes [14] and reflects the loss of TAM receptor function in Sertoli cells [14, 24, 82, 90]. These somatic support cells are phagocytes: among their most important roles is the PtdSer-dependent clearance of the extraordinary number of apoptotic germ cells that are generated during meiosis [91]. It has been estimated that more than half of the meiotic population dies during each cycle of mammalian spermatogenesis, and so the clearance of these AC corpses by Sertoli cells is critical. This process is TAM-dependent: Sertoli cells express all three TAMs and both TAM ligands, and in the absence of TAM signaling, the phagocytosis of apoptotic germ cells in the testes is markedly attenuated [14, 24].

A related and similarly strong phenotype is evident in the eyes of *Mertk*$^{-/-}$ mutants. These mutants are born with normal retinae, but by 2 months after birth most of their photoreceptors (PRs) have died [2, 14, 19, 62]. This is a nonautonomous phenotype, in that PRs do not express the TAMs. Rather, both Mer and Tyro3 are expressed by cells of the retinal pigment epithelium (RPE) [2]. Like Sertoli cells, RPE cells are phagocytes [92]. Unlike Sertoli cells, however, they do not engulf ACs, but rather engulf and excise only part of a living cell—the distal ends (tips) of PR outer segments. These outer segments (OS) are the rhodopsin-containing organelles in which light is detected. PRs synthesize and insert new membrane at the proximal base of their OS every day, and the distal tips of these organelles are phagocytized by RPE cells—also on a daily basis, for a few hours just after subjective dawn—in order to maintain a constant OS length [2, 93–95]. Extracellularly displayed PtdSer is localized specifically to these distal tips only during the time of phagocytosis [96]. In *Mertk*$^{-/-}$ mice, RPE cells differentiate normally but fail to perform this phagocytosis, which leads to the death of nearly all PRs [19, 97]. Consistent with the phenotype of the *Mertk*$^{-/-}$ mice, more than a dozen distinct pathogenic sequence variants in the *Mertk* gene have been found to lead to inherited forms of retinitis pigmentosa and retinal dystrophy in humans [98, 99].

Genetic studies have recently been carried out to dissect the relative contributions of Gas6 and Protein S to TAM-dependent phagocytosis in both the testes and the retina. In the latter, retinal inactivation of either the *Gas6* or *Pros1* gene alone results in no PR degeneration and an essentially normal mouse retina, whereas inactivation of both ligand genes results in severe PR degeneration that fully phenocopies that seen in the *Mertk*$^{-/-}$ mice [2, 62]. Even only half the normal level of just a single TAM ligand—e.g., half the normal level of Protein S and no Gas6—is sufficient to yield a retina with a normal number of PRs [62]. A normal retina is also observed in *Tyro3*$^{-/-}$*Gas6*$^{-/-}$ double knockouts, in which the only possible signaling configuration is Protein S activating Mer [24] (RPE cells do not express Axl [2, 24, 62]). Together, these results demonstrate that Gas6 and Protein S are functionally redundant ligands for the Mer receptor expressed by RPE cells in the eye.

In the testes, where all three TAM receptors are expressed by phagocytic Sertoli cells [14], Mer again plays a predominant role, as *Mertk*$^{-/-}$ single mouse knockouts display a clear accumulation of apoptotic germ cells relative to wild type, whereas *Tyro3*$^{-/-}$ and *Axl*$^{-/-}$ mice display no AC accumulation [24]. That notwithstanding, loss of Tyro3 in addition to Mer makes the situation much worse, and *Tyro3*$^{-/-}$*Axl*$^{-/-}$ *Mertk*$^{-/-}$ triple mutants display the greatest AC accumulation of all [24]. As for the retina, only half the normal level of only a single TAM ligand is sufficient to support a normal level of homeostatic phagocytosis [24].

The TAMs play similarly critical roles in AC clearance by macrophages and other phagocytes of the immune system [21]. In humans, more than a million ACs, primarily erythrocytes and neutrophils, are generated every second (several hundred billion cells per day), but these dead cells are nearly impossible to detect [100]. This is because they are immediately cleared by macrophages and other phagocytes. In many settings, these phagocytes rely on the "eat-me" signal PtdSer to recognize dead cells as engulfment targets [60]. Incomplete phagocytosis of ACs leads to the accumulation of secondary necrotic cells, which constitute a major source of autoantigens. Not surprisingly then, defects in phagocytosis are associated with the development of human autoimmune diseases [101, 102] and are prominent features of the phenotypes of TAM mouse mutants [21, 85, 103–106]. Axl and Mer are the two most important TAM receptors for the engulfment of ACs by macrophages and dendritic cells [21, 106]. Recent studies indicate, however, that these two receptors are specialized to function in two very different environments, with Mer mediating normal, homeostatic phagocytosis in tolerogenic settings, and Axl operating in inflammatory environments brought on by infection or tissue trauma [63]. In those settings in which AC phagocytosis is Axl dependent, it is also Gas6 dependent, whereas Mer-dependent AC phagocytosis can be stimulated by both Gas6 and Protein S [63].

2.2.2 TAM Inhibition of the Innate Immune Response

TAM receptors also play a key role in the feedback inhibition of the innate immune response to pathogens, a regulatory activity that has been studied in macrophages and dendritic cells (DCs) [40]. These sentinel cells use pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), to detect invariant molecular patterns, such as lipopolysaccharide and double-stranded RNA, which are associated with bacteria, viruses, and other pathogens [107, 108]. Activation of PRRs leads to the production of proinflammatory cytokines such as type I interferons [109]. Although these cytokines are required to combat infection, they are powerful agents that must be controlled after the innate immune response is mobilized, since unrestrained cytokine signaling results in chronic inflammation [110].

In DCs, the *Axl* gene is induced by TLR activation and subsequently by type I IFNs through a JAK-Stat1-dependent mechanism [40]. Upregulated Axl protein then binds to and co-opts the type I IFN receptor (IFNAR) by forming a complex

with the R1 chain of this receptor (Fig. 2.2). In so doing, Axl switches the IFNAR signaling modality from proinflammatory to immunosuppressive, by driving the activation of the genes encoding the suppressor of cytokine signaling (SOCS) proteins 1 and 3 [40, 111]. An SH2 domain of these cytoplasmic inhibitors binds to phosphotyrosine residues in JAK kinases that are associated with the IFNAR and other cytokine receptors (and to phosphorylated tyrosine within the receptors themselves). The N-terminal regions of SOCS1 and SOCS3 also contain a kinase-inhibitory region that acts as a JAK pseudo-substrate [111, 112]. Through these and related mechanisms, the induced SOCS proteins, whose expression in DCs is largely dependent on activation of the TAM-IFNAR complex, terminate the inflammatory response to pathogens [40].

This pathway is a fundamental inhibitor of inflammation. The induction of SOCS1 and 3 by type I IFNs is markedly blunted in Axl-deficient DCs, and their induction by direct activation of the TAM receptors is equally dependent on the presence of both the IFNAR and associated Stat1 [40]. The TAM and IFNAR receptor systems are thus co-dependent with respect to immunosuppression [26, 40, 113] (Fig. 2.3). The provision of an immune stimulus—e.g., through activation of TLR4 with LPS—to a TAM-deficient cell or mouse inevitably leads to a hyper-elevated inflammatory response [13, 20, 40]. This means that deficiencies in TAM signaling are always associated with sustained immune activation. Immunosuppression of inflammatory activation in DCs requires both type IFNs and a TAM ligand [40]. Although DCs themselves produce some of these ligands [63], recent work indicates that an important source of Protein S is activated T cells, the cells with which DCs normally interact during antigen presentation [84].

2.3 The Role of the TAM Receptor Tyrosine Kinase Family in Human Disease

2.3.1 TAM Receptors and Autoimmune Disease

Mouse mutants in TAM genes eventually develop a severe autoimmune disease [20, 21, 114–117], which is particularly prominent in $Axl^{-/-}Mer^{-/-}$ double mutants and in TAM TKOs [20] and has clinical features of both systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in humans. TAM mutant mice also display elevated titers of antibodies to auto-antigens such as double-stranded DNA, phospholipids, and ribonucleoproteins [20, 21, 117]. Defects in the clearance of ACs and unabated type I IFN signaling—both of which are consequences of TAM deficiency—are also both thought to drive the development of human autoimmune diseases, including SLE, RA, and inflammatory bowel diseases (IBDs) [86, 101, 118]. Correspondingly, several lines of evidence suggest that diminished TAM signaling may contribute to human autoimmunity [114]. There is an anecdotal medical literature that ties low circulating levels of Protein S to IBDs [119–122], and a much larger literature that establishes an association between low Protein S and lupus

(SLE) [122–124]. A recent analysis of a large patient cohort found that levels of free protein S were significantly lower in SLE patients with a history of serositis, neurologic disorder, hematologic disorder, and immunologic disorder, and that low Protein S levels were correlated with other disease-associated risk factors [125]. Polymorphisms in the *Mertk* gene have been tied to SLE [126], and a clear genetic link to Mer has also been made with respect to the development of Multiple Sclerosis (MS). Here, a large genome-wide association study identified polymorphisms in the *Mertk* gene as risk factors for the development of MS [127, 128].

Immunosuppressive glucocorticoids (GCs), such as prednisone and prednisolone, have recently been shown to potentiate Mer signaling. One well-described activity of GCs is their ability to stimulate the phagocytosis of ACs by macrophages [129]. Agonists for the liver-X-receptor (LXR) family of nuclear hormone receptors display this same activity [130], and the ability of both GCs and LXR agonists to stimulate macrophage phagocytosis of ACs has been shown to be entirely dependent on their ability to upregulate expression of Mer [63, 130, 131].

2.3.2 TAM Receptors and Viral Infection

In a process termed "apoptotic mimicry" [132, 133], the "eat-me" signal PtdSer is displayed on the membrane surface of enveloped viruses, including vaccinia virus, cytomegalovirus, Lassa fever virus, Dengue virus, and HIV [134–138]. This allows these viruses to use Gas6 and Protein S as "bridging molecules" that link virus particles to TAM receptors on the surface of the cells they will infect (Fig. 2.1).

Tyro3, Axl, and Mer have been found to function as entry factors for the Ebola/Marburg family of hemorrhagic fever filoviruses [139, 140]. Similar results have been obtained using infection of cells with lentiviral vectors pseudotyped with Sindbis virus, vesicular stomatitis virus, Ebola, and Marburg virus glycoproteins [137, 141], and for infection by the flaviviruses Dengue virus and West Nile virus [137, 138]. All of these experiments identify Gas6 and Protein S as bridging factors that link PtdSer on the viral envelope to a TAM receptor on the target cell. Depending on culture conditions, introduction of TAM cDNAs potentiates virus titers in infection-resistant cell lines $\geq$50-fold. In general, these findings have been interpreted to suggest that TAM receptors serve as docking sites for TAM-ligand-bound virus particles. However, mutational and kinase inhibitor analyses also indicate that tyrosine kinase activity is required for Axl potentiation of Ebola infection [140] and Axl and Tyro3 potentiation of Dengue and West Nile virus infection [137, 138], and so active TAM signaling appears to be required for the potentiation of virus infection. Given that (i) Axl activation suppresses type IFN signaling in DCs and macrophages [40, 113, 115], (2) type I IFNs are strong antiviral agents [142], and (3) suppression of type I IFN signaling is a mechanism that viruses often exploit as a means of immune evasion [142–144], it was perhaps not surprising that investigators found that the production of type I IFN mRNAs was much higher, and that of SOCS1 and SOCS3 mRNAs much lower, in TAM TKO DCs infected with retroviruses than

in wild-type DCs infected with the same viruses [137]. An anti-Axl antibody has recently been reported to be effective in the treatment of mouse pulmonary infections with influenza and respiratory syncytial virus (RSV) [145]. Together, these findings suggest that the activation of TAM receptor signaling by viruses may be an effective mechanism of viral immune evasion.

2.3.3 TAM Receptors and Cancer

Full-length cDNAs for Axl and Mer were first cloned from myeloid leukemia and lymphoblastoid lines, respectively [4, 5], and a truncated form of Mer (designated v-eyk) was identified initially as an avian retroviral oncogene [146, 147]. Over the ensuing decades, several hundred papers have linked TAM receptor and ligand over- or mis-expression to various forms of cancer [148, 149], and the analysis of TAM expression—and in particular, Axl expression—in cancer is now an exploding area of investigation. For the most part, studies have reported overexpression or upregulation of Axl, Mer, Tyro3, and/or Gas6. Consistent with the divergent regulation of Mer and Axl in tolerogenic versus inflammatory settings [63], a search of the TCGA cancer genome database reveals that tumor upregulation of TAM expression generally involves upregulation of Axl or Mer or Tyro3, but rarely more than one of these receptors at the same time. In many settings, a definitive demonstration that overexpression is causal for particular features of cancer development has not been made. Elevated expression of TAM signaling components has been reported for leukemias [5, 150, 151], gliomas [39, 152], colorectal carcinomas [153], breast cancers [154, 155], gastrointestinal stromal tumors [156], hepatocellular carcinoma [157], melanoma [158–160], thyroid cancer [161], pancreatic adenocarcinoma [162], and prostate cancer [163, 164], among others.

Expression of Axl is correlated with an adverse prognosis in acute myeloid leukemia [165], glioblastoma multiforme [152], pancreatic cancer [160], and esophageal adenocarcinoma [166]. Axl upregulation is also a driving feature of targeted (e.g., EGFR-targeted) therapy resistance in breast cancer and leukemias [151, 167, 168]. In these settings, Axl and/or Gas6 expression is often primarily associated with tumor metastasis, rather than growth of the primary tumor [155, 162, 169]. A small molecule inhibitor of the Axl tyrosine kinase has shown efficacy with respect to a reduced metastatic burden, rather than primary tumor growth, in mouse models of breast cancer metastasis [170]. Similarly, Axl "decoy receptors" composed of the ligand-binding region of the Axl extracellular domain linked to immunoglobulin Fc and designed to bind elevated levels of Gas6 have shown efficacy in inhibiting metastasis in mouse xenograft tumor models [169, 171]. The link between TAM receptor (Axl) expression and tumor metastasis is interesting in light of the importance of Axl and Tyro3 in the migration of gonadotropin-releasing hormone (GnRH) neurons from the olfactory placode to hypothalamus of the brain [172, 173]. This migration of GnRH neurons involves Gas6 activation of the same downstream signaling pathways—PI3 kinase, ERK1/2, and Rac via Ras [172, 174, 175]—that

are engaged downstream of TAMs in tumor cells. Given the potent immunosuppressive activities of Mer and Axl, an increasingly supported hypothesis is that elevated TAM, especially Mer, expression in tumor-associated macrophages may be important in suppressing the normal immune response to tumor cells [149, 176, 177]. All of the work cited above indicates that the inhibition of TAM signaling—via small molecule kinase inhibitors, antibodies, or receptor decoys—in the context of cancer development and metastasis is very definitely a "growth area" for translational medicine and application.

2.3.4 TAM Receptors and the Vasculature

TAM signaling also functions in the regulation of blood vessel integrity and permeability. The TAM ligands Gas6 and Protein S were initially purified from aortic endothelial cells [8], and studies using conditional Protein S knockouts have demonstrated that vascular endothelial cells are a major source of the Protein S that appears in the circulation [56]. Axl and Tyro3 are also expressed by the vascular smooth muscle cells that surround these endothelia, and Gas6 and Protein S have potent trophic effects on these cells [33, 178–182].

Blood vessel damage leads to the upregulation of both Axl and Gas6 expression [183], and differential regulation of Axl, Mer, Gas6, and Protein S has been reported in human atherosclerotic plaques [184]. Defects in the clearance of apoptotic cells from these plaques are linked to progression of advanced atherosclerotic lesions, and the role of compromised TAM signaling in cardiovascular disease is a subject of active study [104, 185]. Mutant mice with a 50 % reduction in Protein S display vessel breaches, with leakage of blood into the parenchyma of tissues [56]. Protein S has also been linked to vascular integrity in the brain: Protein S, signaling through Tyro3 expressed in brain microvascular endothelial cells, has been implicated in maintenance of the blood–brain barrier and has been found to ameliorate blood–brain barrier disruption brought on by ischemia [186].

In addition to these direct activities, TAM signaling has been shown to affect vascular integrity indirectly, through the regulation of platelet function. Loss of one or more TAM receptors inhibits the stabilization of platelet aggregates, in part by reducing platelet granule secretion. Gas6 activates PI3K/Akt signaling in platelets and stimulates tyrosine phosphorylation of β3 integrin, thereby amplifying thrombus formation [79, 187].

2.4 Tyro3

Gene The *Tyro3* gene is located on chromosome 15 (at 15q15) in humans and on chromosome 2 (at 2 67.1 cM) in the mouse. The human gene spans ~20 kb and is split into 19 exons.

Protein The Tyro3 protein precursor (with signal sequence) ranges from 880 to 890 amino acids across vertebrate species and is composed of the domains diagrammed in Fig. 2.1.

Ligand Tyro3 is bound and activated by both Protein S and Gas6. Full receptor activation requires the presence of ligand, the phospholipid phosphatidylserine, and calcium.

Activation and Signaling The principal signaling pathways activated downstream of ligand-induced Tyro3 dimerization are reported to be the PI3 kinase/Akt pathway.

2.5 Axl

Gene The *Axl* gene is located on chromosome 19 (at 19q13.1) in humans and on chromosome 7 (at 7 6.0 cM) in the mouse. The human gene spans ~42.5 kb and is split into 19 exons.

Protein The Axl protein precursor (with signal sequence) ranges from 879 to 894 amino acids across vertebrate species and is composed of the domains diagrammed in Fig. 2.1.

Ligand Gas6 is the only ligand for Axl, and Protein S is unable to activate this TAM receptor. Axl activation requires the presence of Gas6, the phospholipid phosphatidylserine, and calcium.

Activation and Signaling As highlighted in Fig. 2.2, the principal signaling pathways activated downstream of ligand-induced Axl dimerization in cells that do not express cytokine receptors are reported to be the PI3 kinase/Akt pathway. In cells expressing the type I IFN receptor (and probably other cytokine receptors), this pathway is dominated and obscured by JAK-STAT signaling pathway.

2.6 Mer

Gene The *Mertk* gene is located on chromosome 2 (at 2q14.1) in humans and on chromosome 2 in the mouse. (The mouse *Mertk* and *Tyro3* genes are close enough to frequently segregate together.) The human gene spans ~131 kb and is split into 19 exons.

Protein The Mer protein precursor (with signal sequence) ranges from 975 to 999 amino acids across vertebrate species and is composed of the domains diagrammed in Fig. 2.1. It is appreciably larger than Tyro3 and Axl, due to an extension (relative to the other TAMs) at its amino terminus.

Ligand Both Gas6 and Protein S function as ligands for Mer. Full receptor activation requires the presence of ligand, the phospholipid phosphatidylserine, and calcium.

Activation and Signaling As highlighted in Fig. 2.2, the principal signaling pathways activated downstream of ligand-induced Mer dimerization in cells that do not express cytokine receptors are reported to be the PI3 kinase/Akt pathway. In cells expressing the type I IFN receptor (and probably other cytokine receptors) this pathway is dominated and obscured by JAK-STAT signaling pathway.

Receptor at a glance

	Tyro3	Axl	Mer
Chromosome location (human)	15q15 NC_000015.9 (41851220..41871536)	19q13.1 NC_000019.9 (41725108..41767671)	2q14.1 NC_000002.11 (112656191..112786945)
Gene size (bp, human)	20,316	42,563	130,754
Exons (human)	19	19	19
Amino acid number (human)	890	894	999
KDa (human, SDS-PAGE)	~120	~120	~150–175
Domains	2Ig, 2 FN3, TK	2Ig, 2 FN3, TK	2Ig, 2 FN3, TK
Ligands	Protein S, Gas6	Gas6	Protein S, Gas6
Known dimerizing partners	No non-TAM described	Type I IFNAR	No non-TAM described
Pathways activated	Multiple (Inc. PI3K/pAKT)	Multiple (Inc. PI3K/pAKT, JAK/STAT)	Multiple (Inc. PI3K/pAKT, JAK/STAT)
Tissues expressed	Multiple (See text)	Multiple (See text)	Multiple (See text)
Human diseases	Several probable (Understudy)	Cancer, multiple prob. (See text)	Cancer, multiple prob. (See text)
Knockout mouse phenotypes	Multiple (See text)	Multiple (See text)	Multiple (See text)

References

1. Lemke G. Biology of the TAM receptors. Cold Spring Harb Perspect. 2013;5(11):a009076. doi:10.1101/cshperspect.a009076.
2. Prasad D, Rothlin CV, Burrola P, Burstyn-Cohen T, Lu Q, Garcia de Frutos P, et al. TAM receptor function in the retinal pigment epithelium. Mol Cell Neurosci. 2006;33(1):96–108.
3. Lai C, Lemke G. An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. Neuron. 1991;6(5):691–704.

4. O'Bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, et al. axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Mol Cell Biol. 1991;11(10):5016–31.
5. Graham DK, Dawson TL, Mullaney DL, Snodgrass HR, Earp HS. Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. Cell Growth Differ. 1994;5(6):647–57.
6. Lai C, Gore M, Lemke G. Structure, expression, and activity of Tyro 3, a neural adhesion-related receptor tyrosine kinase. Oncogene. 1994;9:2567–78.
7. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298(5600):1912–34.
8. Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, et al. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. Cell. 1995;80(4):661–70.
9. Mark MR, Chen J, Hammonds RG, Sadick M, Godowsk PJ. Characterization of Gas6, a member of the superfamily of G domain-containing proteins, as a ligand for Rse and Axl. J Biol Chem. 1996;271(16):9785–9.
10. Nagata K, Ohashi K, Nakano T, Arita H, Zong C, Hanafusa H, et al. Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. J Biol Chem. 1996;271(47):30022–7.
11. Ohashi K, Nagata K, Toshima J, Nakano T, Arita H, Tsuda H, et al. Stimulation of sky receptor tyrosine kinase by the product of growth arrest-specific gene 6. J Biol Chem. 1995; 270(39):22681–4.
12. Varnum BC, Young C, Elliott G, Garcia A, Bartley TD, Fridell YW, et al. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Nature. 1995;373(6515):623–6.
13. Camenisch TD, Koller BH, Earp HS, Matsushima GK. A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. J Immunol. 1999;162(6):3498–503.
14. Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagranda F, et al. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. Nature. 1999;398(6729):723–8.
15. Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, et al. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature. 1995;378(6555): 390–4.
16. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature. 1995;378(6555):394–8.
17. Soriano P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development. 1997;124(14):2691–700.
18. Arman E, Haffner-Krausz R, Chen Y, Heath JK, Lonai P. Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc Natl Acad Sci USA. 1998;95(9):5082–7.
19. Duncan HJ, LaVail MM, Yasumura D, Matthes MT, Yang H, Trautmann N, et al. An RCS-like retinal dystrophy phenotype in mer knockout mice. Invest Ophthalmol Vis Sci. 2003;44:826–38.
20. Lu Q, Lemke G. Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. Science. 2001;293:306–11.
21. Scott RS, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature. 2001;411(6834):207–11.
22. Sasaki T, Knyazev PG, Clout NJ, Cheburkin Y, Gohring W, Ullrich A, et al. Structural basis for Gas6-Axl signalling. EMBO J. 2006;25(1):80–7.
23. Heiring C, Dahlback B, Muller YA. Ligand recognition and homophilic interactions in Tyro3: structural insights into the Axl/Tyro3 receptor tyrosine kinase family. J Biol Chem. 2004;279(8):6952–8.
24. Lew ED, Oh J, Burrola PG, Lax I, Zagórska A, Través PG, et al. Differential TAM receptor-ligand-phospholipid interactions delimit differential TAM bioactivities. eLife. 2014;3:e03385.

25. Lango Allen H, Estrada K, Lettre G, Berndt SI, Weedon MN, Rivadeneira F, et al. Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature. 2010;467(7317):832–8.
26. Lemke G, Rothlin CV. Immunobiology of the TAM receptors. Nat Rev Immunol. 2008;8(5):327–36. PMC2856445.
27. Ling L, Templeton D, Kung HJ. Identification of the major autophosphorylation sites of Nyk/Mer, an NCAM-related receptor tyrosine kinase. J Biol Chem. 1996;271(31):18355–62.
28. Braunger J, Schleithoff L, Schulz AS, Kessler H, Lammers R, Ullrich A, et al. Intracellular signaling of the Ufo/Axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking-site. Oncogene. 1997;14(22):2619–31.
29. Goruppi S, Ruaro E, Varnum B, Schneider C. Requirement of phosphatidylinositol 3-kinase-dependent pathway and Src for Gas6-Axl mitogenic and survival activities in NIH 3T3 fibroblasts. Mol Cell Biol. 1997;17(8):4442–53.
30. Ming Cao W, Murao K, Imachi H, Sato M, Nakano T, Kodama T, et al. Phosphatidylinositol 3-OH kinase-Akt/protein kinase B pathway mediates Gas6 induction of scavenger receptor a in immortalized human vascular smooth muscle cell line. Arterioscler Thromb Vasc Biol. 2001;21(10):1592–7.
31. Lan Z, Wu H, Li W, Wu S, Lu L, Xu M, et al. Transforming activity of receptor tyrosine kinase Tyro3 is mediated, at least in part, by the PI3 kinase-signaling pathway. Blood. 2000; 95(2):633–8.
32. Georgescu MM, Kirsch KH, Shishido T, Zong C, Hanafusa H. Biological effects of c-Mer receptor tyrosine kinase in hematopoietic cells depend on the Grb2 binding site in the receptor and activation of NF-kappaB. Mol Cell Biol. 1999;19(2):1171–81.
33. Son BK, Kozaki K, Iijima K, Eto M, Nakano T, Akishita M, et al. Gas6/Axl-PI3K/Akt pathway plays a central role in the effect of statins on inorganic phosphate-induced calcification of vascular smooth muscle cells. Eur J Pharmacol. 2007;556(1–3):1–8.
34. Tibrewal N, Wu Y, D'Mello V, Akakura R, George TC, Varnum B, et al. Autophosphorylation docking site Tyr-867 in Mer receptor tyrosine kinase allows for dissociation of multiple signaling pathways for phagocytosis of apoptotic cells and down-modulation of lipopolysaccharide-inducible NF-kappaB transcriptional activation. J Biol Chem. 2008; 283(6):3618–27.
35. Fridell YW, Jin Y, Quilliam LA, Burchert A, McCloskey P, Spizz G, et al. Differential activation of the Ras/extracellular-signal-regulated protein kinase pathway is responsible for the biological consequences induced by the Axl receptor tyrosine kinase. Mol Cell Biol. 1996;16(1):135–45.
36. Weinger JG, Gohari P, Yan Y, Backer JM, Varnum B, Shafit-Zagardo B. In brain, Axl recruits Grb2 and the p85 regulatory subunit of PI3 kinase; in vitro mutagenesis defines the requisite binding sites for downstream Akt activation. J Neurochem. 2008;106(1):134–46.
37. Lijnen HR, Christiaens V, Scroyen L. Growth arrest-specific protein 6 receptor antagonism impairs adipocyte differentiation and adipose tissue development in mice. J Pharmacol Exp Ther. 2011;337(2):457–64.
38. Ou WB, Corson JM, Flynn DL, Lu WP, Wise SC, Bueno R, et al. AXL regulates mesothelioma proliferation and invasiveness. Oncogene. 2011;30(14):1643–52.
39. Keating AK, Kim GK, Jones AE, Donson AM, Ware K, Mulcahy JM, et al. Inhibition of Mer and Axl receptor tyrosine kinases in astrocytoma cells leads to increased apoptosis and improved chemosensitivity. Mol Cancer Ther. 2010;9(5):1298–307.
40. Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G. TAM receptors are pleiotropic inhibitors of the innate immune response. Cell. 2007;131(6):1124–36.
41. Zong C, Yan R, August A, Darnell Jr JE, Hanafusa H. Unique signal transduction of Eyk: constitutive stimulation of the JAK-STAT pathway by an oncogenic receptor-type tyrosine kinase. EMBO J. 1996;15(17):4515–25.
42. Manning G, Plowman GD, Hunter T, Sudarsanam S. Evolution of protein kinase signaling from yeast to man. Trends Biochem Sci. 2002;27(10):514–20.

43. Kulman JD, Harris JE, Nakazawa N, Ogasawara M, Satake M, Davie EW. Vitamin K-dependent proteins in Ciona intestinalis, a basal chordate lacking a blood coagulation cascade. Proc Natl Acad Sci USA. 2006;103(43):15794–9.
44. Manfioletti G, Brancolini C, Avanzi G, Schneider C. The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. Mol Cell Biol. 1993;13(8):4976–85.
45. Nyberg P, He X, Hardig Y, Dahlback B, Garcia de Frutos P. Stimulation of Sky tyrosine phosphorylation by bovine protein S–domains involved in the receptor-ligand interaction. Eur J Biochem. 1997;246(1):147–54.
46. Tanabe K, Nagata K, Ohashi K, Nakano T, Arita H, Mizuno K. Roles of gamma-carboxylation and a sex hormone-binding globulin-like domain in receptor-binding and in biological activities of Gas6. FEBS Lett. 1997;408(3):306–10.
47. Evenas P, Dahlback B, Garcia de Frutos P. The first laminin G-type domain in the SHBG-like region of protein S contains residues essential for activation of the receptor tyrosine kinase sky. Biol Chem. 2000;381(3):199–209.
48. Sasaki T, Knyazev PG, Cheburkin Y, Gohring W, Tisi D, Ullrich A, et al. Crystal structure of a C-terminal fragment of growth arrest-specific protein Gas6. Receptor tyrosine kinase activation by laminin G-like domains. J Biol Chem. 2002;277(46):44164–70.
49. Rajotte I, Hasanbasic I, Blostein M. Gas6-mediated signaling is dependent on the engagement of its gamma-carboxyglutamic acid domain with phosphatidylserine. Biochem Biophys Res Commun. 2008;376(1):70–3.
50. Ishimoto Y, Ohashi K, Mizuno K, Nakano T. Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. J Biochem. 2000;127(3):411–7.
51. Huang M, Rigby AC, Morelli X, Grant MA, Huang G, Furie B, et al. Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins. Nat Struct Biol. 2003;10(9):751–6.
52. Bandyopadhyay PK. Vitamin K-dependent gamma-glutamylcarboxylation: an ancient post-translational modification. Vitam Horm. 2008;78:157–84.
53. Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. Nature. 2004;427(6974):541–4.
54. Stafford DW. The vitamin K cycle. J Thromb Haemost. 2005;3(8):1873–8.
55. Dahlback B. Blood coagulation. Lancet. 2000;355(9215):1627–32.
56. Burstyn-Cohen T, Heeb MJ, Lemke G. Lack of protein S in mice causes embryonic lethal coagulopathy and vascular dysgenesis. J Clin Invest. 2009;119(10):2942–53. PMC2752078.
57. van Meer G, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol. 2008;9(2):112–24.
58. Suzuki J, Fujii T, Imao T, Ishihara K, Kuba H, Nagata S. Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. J Biol Chem. 2013;288(19): 13305–16.
59. Segawa K, Kurata S, Yanagihashi Y, Brummelkamp TR, Matsuda F, Nagata S. Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. Science. 2014;344(6188):1164–8.
60. Ravichandran KS. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. J Exp Med. 2010;207(9):1807–17.
61. Uehara H, Shacter E. Auto-oxidation and oligomerization of protein S on the apoptotic cell surface is required for Mer tyrosine kinase-mediated phagocytosis of apoptotic cells. J Immunol. 2008;180(4):2522–30.
62. Burstyn-Cohen T, Lew ED, Traves PG, Burrola PG, Hash JC, Lemke G. Genetic dissection of TAM receptor-ligand interaction in retinal pigment epithelial cell phagocytosis. Neuron. 2012;76(6):1123–32.
63. Zagórska A, Través PG, Lew ED, Dransfield I, Lemke G. Diversification of TAM receptor tyrosine kinase function. Nat Immunol. 2014;15(10):920–8.

64. Chen J, Carey K, Godowski PJ. Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. Oncogene. 1997;14(17):2033–9.
65. Tsou WI, Nguyen KQ, Calarese DA, Garforth SJ, Antes AL, Smirnov SV, et al. Receptor tyrosine kinases, TYRO3, AXL and MER, demonstrate distinct patterns and complex regulation of ligand-induced activation. J Biol Chem. 2014;289:25750–63.
66. Wang CP, Yagi K, Lin PJ, Jin DY, Makabe KW, Stafford DW. Identification of a gene encoding a typical gamma-carboxyglutamic acid domain in the tunicate Halocynthia roretzi. J Thromb Haemost. 2003;1(1):118–23.
67. Costa M, Bellosta P, Basilico C. Cleavage and release of a soluble form of the receptor tyrosine kinase ARK in vitro and in vivo. J Cell Physiol. 1996;168(3):737–44.
68. O'Bryan JP, Fridell YW, Koski R, Varnum B, Liu ET. The transforming receptor tyrosine kinase, Axl, is post-translationally regulated by proteolytic cleavage. J Biol Chem. 1995;270(2):551–7.
69. Wilhelm I, Nagyoszi P, Farkas AE, Couraud PO, Romero IA, Weksler B, et al. Hyperosmotic stress induces Axl activation and cleavage in cerebral endothelial cells. J Neurochem. 2008;107(1):116–26.
70. Ekman C, Stenhoff J, Dahlback B. Gas6 is complexed to the soluble tyrosine kinase receptor Axl in human blood. J Thromb Haemost. 2010;8(4):838–44.
71. Ekman C, Gottsater A, Lindblad B, Dahlback B. Plasma concentrations of Gas6 and soluble Axl correlate with disease and predict mortality in patients with critical limb ischemia. Clin Biochem. 2010;43(10–11):873–6.
72. Ekman C, Site DF, Gottsater A, Lindblad B, Dahlback B. Plasma concentrations of growth arrest specific protein 6 and the soluble form of its tyrosine kinase receptor Axl as markers of large abdominal aortic aneurysms. Clin Biochem. 2010;43(1–2):110–4.
73. Hsiao FC, Lin YF, Hsieh PS, Chu NF, Shieh YS, Hsieh CH, et al. Circulating growth arrest-specific 6 protein is associated with adiposity, systemic inflammation, and insulin resistance among overweight and obese adolescents. J Clin Endocrinol Metab. 2013;98(2):E267–74.
74. Ko CP, Yu YL, Hsiao PC, Yang SF, Yeh CB. Plasma levels of soluble Axl correlate with severity of community-acquired pneumonia. Mol Med Rep. 2014;9(4):1400–4.
75. Lee CH, Shieh YS, Tsai CS, Hung YJ, Tsai YT, Lin CY. Plasma concentrations predict aortic expression of growth-arrest-specific protein 6 in patients undergoing coronary artery bypass grafting. PLoS One. 2013;8(11):e79452.
76. Liu X, Gong Y, Jia J, Bai Y, Gui S, Wang T, et al. Plasma concentrations of sAxl are associated with severe preeclampsia. Clin Biochem. 2014;47(3):173–6.
77. Zhu H, Sun X, Zhu L, Hu F, Shi L, Fan C, et al. Different expression patterns and clinical significance of mAxl and sAxl in systemic lupus erythematosus. Lupus. 2014;23:624–34.
78. Zhong Z, Wang Y, Guo H, Sagare A, Fernandez JA, Bell RD, et al. Protein S protects neurons from excitotoxic injury by activating the TAM receptor Tyro3-phosphatidylinositol 3-kinase-Akt pathway through its sex hormone-binding globulin-like region. J Neurosci. 2010;30(46):15521–34.
79. Angelillo-Scherrer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, et al. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med. 2001;7(2):215–21.
80. Kevany BM, Palczewski K. Phagocytosis of retinal rod and cone photoreceptors. Physiology (Bethesda). 2010;25(1):8–15.
81. Strauss O. The retinal pigment epithelium in visual function. Physiol Rev. 2005;85(3):845–81.
82. Chen Y, Wang H, Qi N, Wu H, Xiong W, Ma J, et al. Functions of TAM RTKs in regulating spermatogenesis and male fertility in mice. Reproduction. 2009;138(4):655–66.
83. Smiley ST, Boyer SN, Heeb MJ, Griffin JH, Grusby MJ. Protein S is inducible by interleukin 4 in T cells and inhibits lymphoid cell procoagulant activity. Proc Natl Acad Sci USA. 1997;94(21):11484–9.
84. Carrera Silva EA, Chan PY, Joannas L, Errasti AE, Gagliani N, Bosurgi L, et al. T cell-derived protein S engages TAM receptor signaling in dendritic cells to control the magnitude of the immune response. Immunity. 2013;39(1):160–70.

85. Lemke G, Burstyn-Cohen T. TAM receptors and the clearance of apoptotic cells. Ann NY Acad Sci. 2010;1209:23–9. PMC3061224.
86. Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. Cell. 2010;140(5):619–30.
87. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. Trends Cell Biol. 2006;16(4):189–97.
88. Mahajan NP, Earp HS. An SH2 domain-dependent, phosphotyrosine-independent interaction between Vav1 and the Mer receptor tyrosine kinase: a mechanism for localizing guanine nucleotide-exchange factor action. J Biol Chem. 2003;278(43):42596–603.
89. Todt JC, Hu B, Curtis JL. The scavenger receptor SR-A I/II (CD204) signals via the receptor tyrosine kinase Mertk during apoptotic cell uptake by murine macrophages. J Leukoc Biol. 2008;84(2):510–8.
90. Sun B, Qi N, Shang T, Wu H, Deng T, Han D. Sertoli cell-initiated testicular innate immune response through toll-like receptor-3 activation is negatively regulated by Tyro3, Axl, and mer receptors. Endocrinology. 2010;151(6):2886–97.
91. Kawasaki Y, Nakagawa A, Nagaosa K, Shiratsuchi A, Nakanishi Y. Phosphatidylserine binding of class B scavenger receptor type I, a phagocytosis receptor of testicular sertoli cells. J Biol Chem. 2002;277(30):27559–66.
92. Sparrow JR, Hicks D, Hamel CP. The retinal pigment epithelium in health and disease. Curr Mol Med. 2010;10(9):802–23.
93. Coleman JA, Kwok MC, Molday RS. Localization, purification, and functional reconstitution of the P4-ATPase Atp8a2, a phosphatidylserine flippase in photoreceptor disc membranes. J Biol Chem. 2009;284(47):32670–9.
94. Nandrot EF, Dufour EM. Mertk in daily retinal phagocytosis: a history in the making. Adv Exp Med Biol. 2010;664:133–40.
95. Strick DJ, Feng W, Vollrath D. Mertk drives myosin II redistribution during retinal pigment epithelial phagocytosis. Invest Ophthalmol Vis Sci. 2009;50(5):2427–35.
96. Ruggiero L, Connor MP, Chen J, Langen R, Finnemann SC. Diurnal, localized exposure of phosphatidylserine by rod outer segment tips in wild-type but not Itgb5-/- or Mfge8-/- mouse retina. Proc Natl Acad Sci USA. 2012;109(21):8145–8.
97. Feng W, Yasumura D, Matthes MT, LaVail MM, Vollrath D. Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. J Biol Chem. 2002;277(19):17016–22.
98. Gal A, Li Y, Thompson DA, Weir J, Orth U, Jacobson SG, et al. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nat Genet. 2000;26(3):270–1.
99. Ostergaard E, Duno M, Batbayli M, Vilhelmsen K, Rosenberg T. A novel MERTK deletion is a common founder mutation in the Faroe Islands and is responsible for a high proportion of retinitis pigmentosa cases. Mol Vis. 2011;17:1485–92.
100. Ravichandran KS. Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. Immunity. 2011;35(4):445–55.
101. Gaipl US, Munoz LE, Grossmayer G, Lauber K, Franz S, Sarter K, et al. Clearance deficiency and systemic lupus erythematosus (SLE). J Autoimmun. 2007;28(2–3):114–21.
102. Shao WH, Cohen PL. Disturbances of apoptotic cell clearance in systemic lupus erythematosus. Arthritis Res Ther. 2011;13(1):202.
103. Shao WH, Zhen Y, Eisenberg RA, Cohen PL. The Mer receptor tyrosine kinase is expressed on discrete macrophage subpopulations and mainly uses Gas6 as its ligand for uptake of apoptotic cells. Clin Immunol. 2009;133(1):138–44.
104. Ait-Oufella H, Pouresmail V, Simon T, Blanc-Brude O, Kinugawa K, Merval R, et al. Defective mer receptor tyrosine kinase signaling in bone marrow cells promotes apoptotic cell accumulation and accelerates atherosclerosis. Arterioscler Thromb Vasc Biol. 2008;28(8):1429–31.
105. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice. Arterioscler Thromb Vasc Biol. 2008;28(8):1421–8.

106. Seitz HM, Camenisch TD, Lemke G, Earp HS, Matsushima GK. Macrophages and dendritic cells use different Axl/Mertk/Tyro3 receptors in clearance of apoptotic cells. J Immunol. 2007;178(9):5635–42.
107. Akira S. TLR signaling. Curr Top Microbiol Immunol. 2006;311:1–16.
108. Beutler B, Jiang Z, Georgel P, Crozat K, Croker B, Rutschmann S, et al. Genetic analysis of host resistance: toll-like receptor signaling and immunity at large. Annu Rev Immunol. 2006;24:353–89.
109. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004;5(10):987–95.
110. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. Nat Rev Immunol. 2006;6(11):823–35.
111. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol. 2007;7(6):454–65.
112. Croker BA, Kiu H, Nicholson SE. SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol. 2008;19(4):414–22.
113. Sharif MN, Sosic D, Rothlin CV, Kelly E, Lemke G, Olson EN, et al. Twist mediates suppression of inflammation by type I IFNs and Ax1. J Exp Med. 2006;203(8):1891–901.
114. Rothlin CV, Lemke G. TAM receptor signaling and autoimmune disease. Curr Opin Immunol. 2010;22(6):740–6. PMC2997887.
115. Shao WH, Kuan AP, Wang C, Abraham V, Waldman MA, Vogelgesang A, et al. Disrupted Mer receptor tyrosine kinase expression leads to enhanced MZ B-cell responses. J Autoimmun. 2010;35(4):368–74.
116. Wallet MA, Sen P, Flores RR, Wang Y, Yi Z, Huang Y, et al. MerTK is required for apoptotic cell-induced T cell tolerance. J Exp Med. 2008;205(1):219–32.
117. Radic M, Shah K, Zhang W, Lu Q, Lemke G, Hilliard GM. Heterogeneous nuclear ribonucleoprotein P2 is an autoantibody target in mice deficient for Mer, Axl, and Tyro3 receptor tyrosine kinases. J Immunol. 2006;176(1):68–74.
118. Ronnblom L, Pascual V. The innate immune system in SLE: type I interferons and dendritic cells. Lupus. 2008;17(5):394–9.
119. Diakou M, Kostadima V, Giannopoulos S, Zikou AK, Argyropoulou MI, Kyritsis AP. Cerebral venous thrombosis in an adolescent with ulcerative colitis. Brain Dev. 2011;33(1):49–51.
120. Cakal B, Gokmen A, Yalinkilic M, Cakal E, Ayaz S, Nadir I, et al. Natural anticoagulant protein levels in Turkish patients with inflammatory bowel disease. Blood Coagul Fibrinolysis. 2010;21(2):118–21.
121. Zezos P, Papaioannou G, Nikolaidis N, Vasiliadis T, Giouleme O, Evgenidis N. Thrombophilic abnormalities of natural anticoagulants in patients with ulcerative colitis. Hepatogastroenterology. 2007;54(77):1417–21.
122. Song KS, Park YS, Kim HK. Prevalence of anti-protein S antibodies in patients with systemic lupus erythematosus. Arthritis Rheum. 2000;43(3):557–60.
123. Meesters EW, Hansen H, Spronk HM, Hamulyak K, Rosing J, Rowshani AT, et al. The inflammation and coagulation cross-talk in patients with systemic lupus erythematosus. Blood Coagul Fibrinolysis. 2007;18(1):21–8.
124. Brouwer JL, Bijl M, Veeger NJ, Kluin-Nelemans HC, van der Meer J. The contribution of inherited and acquired thrombophilic defects, alone or combined with antiphospholipid antibodies, to venous and arterial thromboembolism in patients with systemic lupus erythematosus. Blood. 2004;104(1):143–8.
125. Suh CH, Hilliard B, Li S, Merrill JT, Cohen PL. TAM receptor ligands in lupus: protein S but not Gas6 levels reflect disease activity in systemic lupus erythematosus. Arthritis Res Ther. 2010;12(4):R146.
126. Cheong HS, Lee SO, Choi CB, Sung YK, Shin HD, Bae SC. MERTK polymorphisms associated with risk of haematological disorders among Korean SLE patients. Rheumatology (Oxford). 2007;46(2):209–14.
127. Ma GZ, Stankovich J, Kilpatrick TJ, Binder MD, Field J. Polymorphisms in the receptor tyrosine kinase MERTK gene are associated with multiple sclerosis susceptibility. PLoS One. 2011;6(2):e16964.

128. Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;476(7359):214–9.
129. Liu Y, Cousin JM, Hughes J, Van Damme J, Seckl JR, Haslett C, et al. Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. J Immunol. 1999;162(6):3639–46.
130. A-Gonzalez N, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, et al. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. Immunity. 2009;31(2):245–58.
131. McColl A, Bournazos S, Franz S, Perretti M, Morgan BP, Haslett C, et al. Glucocorticoids induce protein S-dependent phagocytosis of apoptotic neutrophils by human macrophages. J Immunol. 2009;183(3):2167–75.
132. Mercer J. Viral apoptotic mimicry party: P.S. Bring your own Gas6. Cell Host Microbe. 2011;9(4):255–7.
133. Mercer J, Helenius A. Apoptotic mimicry: phosphatidylserine-mediated macropinocytosis of vaccinia virus. Ann N Y Acad Sci. 2010;1209:49–55.
134. Mercer J, Helenius A. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. Science. 2008;320(5875):531–5.
135. Soares MM, King SW, Thorpe PE. Targeting inside-out phosphatidylserine as a therapeutic strategy for viral diseases. Nat Med. 2008;14(12):1357–62.
136. Callahan MK, Popernack PM, Tsutsui S, Truong L, Schlegel RA, Henderson AJ. Phosphatidylserine on HIV envelope is a cofactor for infection of monocytic cells. J Immunol. 2003;170(9):4840–5.
137. Bhattacharyya S, Zagorska A, Lew ED, Shrestha B, Rothlin CV, Naughton J, et al. Enveloped viruses disable innate immune responses in dendritic cells by direct activation of TAM receptors. Cell Host Microbe. 2013;14(2):136–47.
138. Meertens L, Carnec X, Perera Lecoin M, Ramdasi R, Guivel-Benhassine F, Lew E, et al. TIM and TAM receptors mediate dengue virus infection. Cell Host Microbe. 2012;12:544–57. PMC3572209.
139. Shimojima M, Takada A, Ebihara H, Neumann G, Fujioka K, Irimura T, et al. Tyro3 family-mediated cell entry of Ebola and Marburg viruses. J Virol. 2006;80(20):10109–16.
140. Shimojima M, Ikeda Y, Kawaoka Y. The mechanism of Axl-mediated Ebola virus infection. J Infect Dis. 2007;196 Suppl 2:S259–63.
141. Morizono K, Xie Y, Olafsen T, Lee B, Dasgupta A, Wu AM, et al. The soluble serum protein Gas6 bridges virion envelope phosphatidylserine to the TAM receptor tyrosine kinase Axl to mediate viral entry. Cell Host Microbe. 2011;9(4):286–98.
142. Diamond MS. Evasion of innate and adaptive immunity by flaviviruses. Immunol Cell Biol. 2003;81:196–206.
143. Versteeg GA, Garcia-Sastre A. Viral tricks to grid-lock the type I interferon system. Curr Opin Microbiol. 2010;13(4):508–16.
144. Bonjardim CA, Ferreira PC, Kroon EG. Interferons: signaling, antiviral and viral evasion. Immunol Lett. 2009;122(1):1–11.
145. Shibata T, Habiel DM, Coelho AL, Kunkel SL, Lukacs NW, Hogaboam CM. Axl receptor blockade ameliorates pulmonary pathology resulting from primary viral infection and viral exacerbation of asthma. J Immunol. 2014;192(8):3569–81.
146. Jia R, Hanafusa H. The proto-oncogene of v-eyk (v-ryk) is a novel receptor-type protein tyrosine kinase with extracellular Ig/GN-III domains. J Biol Chem. 1994;269(3):1839–44.
147. Jia R, Mayer BJ, Hanafusa T, Hanafusa H. A novel oncogene, v-ryk, encoding a truncated receptor tyrosine kinase is transduced into the RPL30 virus without loss of viral sequences. J Virol. 1992;66(10):5975–87.
148. Verma A, Warner SL, Vankayalapati H, Bearss DJ, Sharma S. Targeting axl and mer kinases in cancer. Mol Cancer Ther. 2011;10(10):1763–73.
149. Linger RM, Keating AK, Earp HS, Graham DK. TAM receptor tyrosine kinases: biologic functions, signaling, and potential therapeutic targeting in human cancer. Adv Cancer Res. 2008;100:35–83.

150. Graham DK, Salzberg DB, Kurtzberg J, Sather S, Matsushima GK, Keating AK, et al. Ectopic expression of the proto-oncogene Mer in pediatric T-cell acute lymphoblastic leukemia. Clin Cancer Res. 2006;12(9):2662–9.
151. Hong CC, Lay JD, Huang JS, Cheng AL, Tang JL, Lin MT, et al. Receptor tyrosine kinase AXL is induced by chemotherapy drugs and overexpression of AXL confers drug resistance in acute myeloid leukemia. Cancer Lett. 2008;268(2):314–24.
152. Hutterer M, Knyazev P, Abate A, Reschke M, Maier H, Stefanova N, et al. Axl and growth arrest-specific gene 6 are frequently overexpressed in human gliomas and predict poor prognosis in patients with glioblastoma multiforme. Clin Cancer Res. 2008;14(1):130–8.
153. Craven RJ, Xu LH, Weiner TM, Fridell YW, Dent GA, Srivastava S, et al. Receptor tyrosine kinases expressed in metastatic colon cancer. Int J Cancer. 1995;60(6):791–7.
154. Berclaz G, Altermatt HJ, Rohrbach V, Kieffer I, Dreher E, Andres AC. Estrogen dependent expression of the receptor tyrosine kinase axl in normal and malignant human breast. Ann Oncol. 2001;12(6):819–24.
155. Gjerdrum C, Tiron C, Hoiby T, Stefansson I, Haugen H, Sandal T, et al. Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. Proc Natl Acad Sci USA. 2010;107(3):1124–9.
156. Mahadevan D, Cooke L, Riley C, Swart R, Simons B, Della Croce K, et al. A novel tyrosine kinase switch is a mechanism of imatinib resistance in gastrointestinal stromal tumors. Oncogene. 2007;26(27):3909–19.
157. He L, Zhang J, Jiang L, Jin C, Zhao Y, Yang G, et al. Differential expression of Axl in hepatocellular carcinoma and correlation with tumor lymphatic metastasis. Mol Carcinog. 2010;49(10):882–91.
158. Zhu S, Wurdak H, Wang Y, Galkin A, Tao H, Li J, et al. A genomic screen identifies TYRO3 as a MITF regulator in melanoma. Proc Natl Acad Sci USA. 2009;106(40):17025–30.
159. Quong RY, Bickford ST, Ing YL, Terman B, Herlyn M, Lassam NJ. Protein kinases in normal and transformed melanocytes. Melanoma Res. 1994;4(5):313–9.
160. Koorstra JB, Karikari CA, Feldmann G, Bisht S, Rojas PL, Offerhaus GJ, et al. The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. Cancer Biol Ther. 2009;8(7):618–26.
161. Avilla E, Guarino V, Visciano C, Liotti F, Svelto M, Krishnamoorthy G, et al. Activation of TYRO3/AXL tyrosine kinase receptors in thyroid cancer. Cancer Res. 2011;71(5):1792–804.
162. Song X, Wang H, Logsdon CD, Rashid A, Fleming JB, Abbruzzese JL, et al. Overexpression of receptor tyrosine kinase Axl promotes tumor cell invasion and survival in pancreatic ductal adenocarcinoma. Cancer. 2010;117(4):734–43.
163. Wu YM, Robinson DR, Kung HJ. Signal pathways in up-regulation of chemokines by tyrosine kinase MER/NYK in prostate cancer cells. Cancer Res. 2004;64(20):7311–20.
164. Sainaghi PP, Castello L, Bergamasco L, Galletti M, Bellosta P, Avanzi GC. Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor. J Cell Physiol. 2005;204(1):36–44.
165. Rochlitz C, Lohri A, Bacchi M, Schmidt M, Nagel S, Fopp M, et al. Axl expression is associated with adverse prognosis and with expression of Bcl-2 and CD34 in de novo acute myeloid leukemia (AML): results from a multicenter trial of the Swiss Group for Clinical Cancer Research (SAKK). Leukemia. 1999;13(9):1352–8.
166. Hector A, Montgomery EA, Karikari C, Canto M, Dunbar KB, Wang JS, et al. The Axl receptor tyrosine kinase is an adverse prognostic factor and a therapeutic target in esophageal adenocarcinoma. Cancer Biol Ther. 2010;10(10):1009–18.
167. Meyer AS, Miller MA, Gertler FB, Lauffenburger DA. The receptor AXL diversifies EGFR signaling and limits the response to EGFR-targeted inhibitors in triple-negative breast cancer cells. Sci Signal. 2013;6(287):ra66.
168. Zhang Z, Lee JC, Lin L, Olivas V, Au V, LaFramboise T, et al. Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. Nat Genet. 2012;44(8):852–60.
169. Rankin EB, Fuh KC, Castellini L, Viswanathan K, Finger EC, Diep AN, et al. Direct regulation of GAS6/AXL signaling by HIF promotes renal metastasis through SRC and MET. Proc Natl Acad Sci USA. 2014;111(37):13373–8.

170. Holland SJ, Pan A, Franci C, Hu Y, Chang B, Li W, et al. R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. Cancer Res. 2010;70(4):1544–54.
171. Kariolis MS, Miao YR, Jones 2nd DS, Kapur S, Mathews II, Giaccia AJ, et al. An engineered Axl 'decoy receptor' effectively silences the Gas6-Axl signaling axis. Nat Chem Biol. 2014;10(11):977–83.
172. Allen MP, Linseman DA, Udo H, Xu M, Schaack JB, Varnum B, et al. Novel mechanism for gonadotropin-releasing hormone neuronal migration involving Gas6/Ark signaling to p38 mitogen-activated protein kinase. Mol Cell Biol. 2002;22(2):599–613.
173. Pierce A, Bliesner B, Xu M, Nielsen-Preiss S, Lemke G, Tobet S, et al. Axl and Tyro3 modulate female reproduction by influencing gonadotropin-releasing hormone neuron survival and migration. Mol Endocrinol. 2008;22(11):2481–95.
174. Nielsen-Preiss SM, Allen MP, Xu M, Linseman DA, Pawlowski JE, Bouchard RJ, et al. Adhesion-related kinase induction of migration requires phosphatidylinositol-3-kinase and ras stimulation of rac activity in immortalized gonadotropin-releasing hormone neuronal cells. Endocrinology. 2007;148(6):2806–14.
175. Allen MP, Xu M, Linseman DA, Pawlowski JE, Bokoch GM, Heidenreich KA, et al. Adhesion-related kinase repression of gonadotropin-releasing hormone gene expression requires Rac activation of the extracellular signal-regulated kinase pathway. J Biol Chem. 2002;277(41):38133–40.
176. Cook RS, Jacobsen KM, Wofford AM, DeRyckere D, Stanford J, Prieto AL, et al. MerTK inhibition in tumor leukocytes decreases tumor growth and metastasis. J Clin Invest. 2013;123(8):3231–42.
177. Linger RM, Lee-Sherick AB, DeRyckere D, Cohen RA, Jacobsen KM, McGranahan A, et al. Mer receptor tyrosine kinase is a therapeutic target in pre-B-cell acute lymphoblastic leukemia. Blood. 2013;122(9):1599–609.
178. Fridell YW, Villa Jr J, Attar EC, Liu ET. GAS6 induces Axl-mediated chemotaxis of vascular smooth muscle cells. J Biol Chem. 1998;273(12):7123–6.
179. Melaragno MG, Fridell YW, Berk BC. The Gas6/Axl system: a novel regulator of vascular cell function. Trends Cardiovasc Med. 1999;9(8):250–3.
180. Collett GD, Sage AP, Kirton JP, Alexander MY, Gilmore AP, Canfield AE. Axl/phosphatidylinositol 3-kinase signaling inhibits mineral deposition by vascular smooth muscle cells. Circ Res. 2007;100(4):502–9.
181. Gasic GP, Arenas CP, Gasic TB, Gasic GJ. Coagulation factors X, Xa, and protein S as potent mitogens of cultured aortic smooth muscle cells. Proc Natl Acad Sci USA. 1992;89(6):2317–20.
182. Cavet ME, Smolock EM, Ozturk OH, World C, Pang J, Konishi A, et al. Gas6-axl receptor signaling is regulated by glucose in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2008;28(5):886–91.
183. Melaragno MG, Wuthrich DA, Poppa V, Gill D, Lindner V, Berk BC, et al. Increased expression of Axl tyrosine kinase after vascular injury and regulation by G protein-coupled receptor agonists in rats. Circ Res. 1998;83(7):697–704.
184. Hurtado B, Munoz X, Recarte-Pelz P, Garcia N, Luque A, Krupinski J, et al. Expression of the vitamin K-dependent proteins GAS6 and protein S and the TAM receptor tyrosine kinases in human atherosclerotic carotid plaques. Thromb Haemost. 2011;105(5):873–82.
185. Thorp EB. Mechanisms of failed apoptotic cell clearance by phagocyte subsets in cardiovascular disease. Apoptosis. 2010;15(9):1124–36.
186. Zhu D, Wang Y, Singh I, Bell RD, Deane R, Zhong Z, et al. Protein S controls hypoxic/ischemic blood-brain barrier disruption through the TAM receptor Tyro3 and sphingosine 1-phosphate receptor. Blood. 2010;115(23):4963–72.
187. Angelillo-Scherrer A, Burnier L, Flores N, Savi P, DeMol M, Schaeffer P, et al. Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. J Clin Invest. 2005;115(2):237–46.

188. Fujimoto J, Yamamoto T. brt, a mouse gene encoding a novel receptor-type protein-tyrosine kinase, is preferentially expressed in the brain. Oncogene. 1994;9(3):693–8.
189. Crosier PS, Lewis PM, Hall LR, Vitas MR, Morris CM, Beier DR, et al. Isolation of a receptor tyrosine kinase (DTK) from embryonic stem cells: structure, genetic mapping and analysis of expression. Growth Factors. 1994;11(2):125–36.
190. Mark MR, Scadden DT, Wang Z, Gu Q, Goddard A, Godowski PJ. rse, a novel receptor-type tyrosine kinase with homology to Axl/Ufo, is expressed at high levels in the brain. J Biol Chem. 1994;269(14):10720–8.
191. Ohashi K, Mizuno K, Kuma K, Miyata T, Nakamura T. Cloning of the cDNA for a novel receptor tyrosine kinase, sky, predominantly expressed in brain. Oncogene. 1994;9(3):699–705.
192. Dai W, Pan H, Hassanain H, Gupta SL, Murphy Jr MJ. Molecular cloning of a novel receptor tyrosine kinase, tif, highly expressed in human ovary and testis. Oncogene. 1994;9(3):975–9.
193. Rescigno J, Mansukhani A, Basilico C. A putative receptor tyrosine kinase with unique structural topology. Oncogene. 1991;6(10):1909–13.
194. Janssen JW, Schulz AS, Steenvoorden AC, Schmidberger M, Strehl S, Ambros PF, et al. A novel putative tyrosine kinase receptor with oncogenic potential. Oncogene. 1991;6(11):2113–20.
195. Ling L, Kung HJ. Mitogenic signals and transforming potential of Nyk, a newly identified neural cell adhesion molecule-related receptor tyrosine kinase. Mol Cell Biol. 1995;15(12):6582–92.

Chapter 3
The DDR Receptor Family

Birgit Leitinger

3.1 Introduction to the DDR Receptor Tyrosine Kinase Family

The discoidin domain receptors (DDRs), DDR1 and DDR2, were cloned in the early 1990s but remained orphan receptors until 1997 when two independent groups reported them to be receptors for a number of collagen types [1, 2]. Collagens are key components of the extracellular matrices that provide structural support for connective tissues. In addition to providing a physical scaffold, collagens are involved in controlling fundamental cellular functions, including growth, differentiation and morphogenesis, through their interactions with matrix receptors. As collagen receptors, the DDRs regulate the interactions of cells with their surrounding matrices and belong to a structurally diverse group of transmembrane receptors which also include a subfamily of β1 integrins [3].

The DDRs are characterised by the presence of a discoidin homology (DS) domain in their ectodomains through which they interact with collagen. DS domains derive their name from their homology to *Dictyostelium discoideum* discoidin I. Both DDRs are widely expressed in different tissues during development and postnatally. In common with many RTKs, the DDRs control cell proliferation, differentiation, adhesion and migration [3–5]. In addition to these functions, the DDRs regulate extracellular matrix remodelling by controlling the expression and activity of matrix metalloproteinases (MMPs). By activating MMPs, which degrade extracellular matrices, the DDRs cooperate with the proteolytic machinery of cells and can mediate cell invasion. Dysregulation of expression or function of the DDRs is associated with a wide range of human diseases, ranging from fibrotic disorders of

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several organs to arthritis and atherosclerosis, as well as several types of cancer. Compared with the majority of RTKs, however, there are considerable gaps in our knowledge about the biological functions of DDR signalling in physiological and pathological processes, and relatively little is known about their mechanism of activation, signal attenuation, pathway activation or the genes that they regulate [5]. As outlined below, the DDRs have several unusual features that distinguish them from conventional RTKs, and mechanistic insight obtained from other RTKs cannot readily be transferred to the DDRs. Many aspects of basic DDR biology thus remain to be elucidated.

3.2 The Role of the DDR Receptor Tyrosine Kinase Family in Embryonic Development

Relatively little is known about the role of DDRs in adult physiology, but targeted deletion of the *Ddr* genes in mice revealed DDR functions in embryonic development. In early development, the DDR1 protein is found predominantly in neuroectodermal cells, whereas it is more widely expressed in adult tissues. In adult mice, DDR1 is found in epithelial cells, in particular in the brain, mammary gland, lung, kidney and gastrointestinal tract, as well as in smooth muscle cells, oligodendrocytes and macrophages [4]. Mice that lack DDR1 exhibit normal embryonic and postnatal development, but female fertility is strongly impaired, with a large percentage of female knockout mice being infertile due to blastocyst implantation failure [6]. DDR1$^{-/-}$ mice are significantly smaller than their littermates, and those females that are fertile display a mammary gland morphogenesis defect that results in the failure to secrete milk [6, 7]. The morphogenesis defect displays improper differentiation of mammary gland alveoli, abnormal branching, as well as hyperproliferative ducts and excessive collagen deposition [6]. DDR1$^{-/-}$ mice were also found to suffer from hearing loss [8]. Furthermore, DDR1 was shown to maintain glomerular architecture in the kidney, as ablation of DDR1 led to abnormal glomerular basement membranes [9]. Thus, DDR1 mediates tissue architecture and collagen deposition in a number of different organs.

DDR2 is mostly expressed in mesenchymal cells [10] and found in the skin, heart, skeletal muscle, lung, brain, kidney and connective tissue. DDR2 expression during embryo development is not well characterised. One study found DDR2 expression in skin fibroblasts to be constant during gestation [11], while another study examined DDR2 expression in the embryonic heart [12]. Two types of mice that lack DDR2 were obtained, both of which exhibit dwarfism. Targeted deletion of *Ddr2* resulted in shortened long bones, which was due to reduced chondrocyte proliferation [13]. A spontaneous mutation resulting in deletion of most of the *Ddr2* gene was characterised by Kano et al. (2008) [14]. These mice, termed *slie*, are additionally infertile owing to gonadal dysregulation. Since it was not reported whether the targeted *Ddr2* deletion leads to infertile mice [13], it is not clear whether

additional defects in the *slie* mice, other than lack of DDR2, are responsible for the observed infertility.

A function for DDR2 in human skeletal growth was uncovered through a rare human genetic disorder named spondylo-meta-epiphyseal dysplasia with short limbs and abnormal calcifications (SMED-SL). This autosomal recessive chondrodysplasia is characterised by disproportionally short stature, bone abnormalities and premature calcifications. Several *DDR2* missense mutations and a splice site mutation were shown to cause SMED-SL [15–17]. The combined insights from mouse and human genetics show that DDR2 is a key regulator of bone growth and that it is involved in at least two aspects of this process: it not only controls chondrocyte maturation in endochondral ossification [18] but also osteoblast differentiation in intramembranous ossification [18, 19].

3.3 The Role of the DDR Receptor Tyrosine Kinase Family in Human Disease

DDR function has been linked to a wide variety of human disorders, including fibrotic disorders of several organs, wound healing, atherosclerosis, arthritis and various cancers [5, 20]. The roles of the DDRs in disease progression have been explored in different mouse models of disease. Since both DDRs are associated with pathogenic changes in a wide range of diseases, the DDRs are promising candidates for therapeutic targets.

3.3.1 Fibrosis of Kidney and Lung

Both DDRs are very highly expressed in the adult human kidney [10]. DDR1 was shown to mediate inflammation and fibrosis in mouse models of hypertension-induced renal disease [21], Alport syndrome [22] and obstructive nephropathy [23]. Collectively, these studies suggest that DDR1 may be a major regulator of fibrotic responses in the kidney. DDR1 has also been linked to lung fibrosis in a mouse model of the disease [24], and in vitro studies are consistent with DDR1 playing a role in lung epithelial repair [25], but further studies are needed to clarify the role of DDR1 in the pathogenesis of lung fibrosis.

3.3.2 DDR2 and Liver Fibrosis

DDR2 has been linked to liver fibrosis in animal models. Induction of liver fibrosis by acute or chronic injury resulted in upregulation of DDR2 expression and signalling in stellate cells of the liver [26, 27]. Increased DDR2 expression was also

observed in primary and alcoholic liver fibrosis [28–30]. Consistent with a pathogenic role in liver fibrosis, DDR2 silencing decreased alcohol-induced liver fibrosis in a model for early-stage liver disease [30]. However, a study using DDR2-deficient mice showed enhanced liver fibrosis after chronic injury in DDR2-deficient mice compared with wild-type mice, implying that the function of DDR2 in normal liver might be to suppress fibrosis under chronic injury conditions [27].

3.3.3 DDR2 and Wound Healing

DDR2 is involved in cutaneous wound healing, where it regulates fibroblast responses during tissue injury. This conclusion was drawn from studies of DDR2$^{-/-}$ mice, which are defective in wound responses, displaying defective recruitment of skin fibroblasts and wounds with decreased tensile strength [13, 31].

3.3.4 DDR2 and Arthritis

DDR2 function has been linked to both osteoarthritis and rheumatoid arthritis. DDR2 overexpression, which leads to induction of MMP-13, a key enzyme contributing to cartilage degeneration, was found to be an early event in the pathogenesis of osteoarthritis and rheumatoid arthritis. DDR2 overexpression was seen in mouse models of different forms of osteoarthritis, as well as in human samples from osteoarthritis and rheumatoid arthritis patients [32–36]. Reduced expression of DDR2 in mouse models of osteoarthritis attenuates cartilage degeneration, suggesting that DDR2 may be a valid target for drug development in osteoarthritis treatment [37].

3.3.5 DDRs and Cancer

Both DDRs are overexpressed in a variety of cancers, with most of the available data coming from studies on DDR1, which is particularly found in carcinomas (reviewed by [38]). In certain cancer types, mutated DDRs have been discovered, but the functional significance of these mutations on tumourigenesis is not clear. High levels of DDR1 expression has been detected in many cancers, including breast, brain, ovarian, lung, oesophageal and liver cancers, and for some of these cancers, DDR1 expression was correlated with poor outcome. In addition to solid tumours, DDR expression has also been observed to be upregulated in haematological cancers, where DDR1 and DDR2 are associated with leukaemias and lymphomas, respectively. The best evidence that DDRs are associated with tumour progression is available for lung cancer. Upregulated DDR1 was found in non-small cell lung cancer (NSCLC) [39], where several somatic DDR1 mutations were also detected [40–42]. Several studies reported that DDR1 expression was related to

poor patient survival [43–45]. Furthermore, global screening of the phosphoproteome in NSCLC identified DDR1 as one of the most highly phosphorylated RTKs [46]. In a mouse model of lung cancer, inhibition of DDR1 reduced bone metastasis [44]. Together, these data suggest that DDR1 may be a novel therapeutic target for NSCLC. DDR2, on the other hand, has been associated with squamous cell lung cancer where oncogenic DDR2 mutations were discovered [47]. Thus, both DDRs seem to be involved in the pathogenesis of lung cancer. DDR2 has also been shown to play a key role in aggressive breast cancer [48, 49], with the molecular mechanism of DDR2-mediated metastasis involving DDR2-induced stabilisation of the transcription factor SNAIL1 [48, 50].

While more studies are required to establish the contribution of the DDRs to cancer progression and patient survival, accumulating evidence suggests that the DDRs represent novel drug targets for the treatment of various cancers [38]. The existing anticancer drugs imatinib, nilotinib and dasatinib exhibit potent anti-DDR activity [51, 52] and could be exploited in the treatment of those cancers where the DDRs contribute to disease progression.

3.4 DDR1

3.4.1 DDR1 Gene

The human *DDR1* gene maps to chromosome 6 (6p21.3), between the HLA-E and HLA-C genes at the major histocompatibility complex locus [53, 54]. The DDR1 gene spans ~24 kb and contains 17 exons [55]. Exons 1–8 encode the extracellular domain; the transmembrane domain is encoded by exon 9. Exons 10–12 encode the cytosolic juxtamembrane domain, with the remaining exons predominantly coding for the kinase domain. Alternative splicing yields five different gene products, with different cytoplasmic regions (see below). The two most abundant DDR1 isoforms, DDR1a and DDR1b, are generated by alternative splicing of exons 10–12, with DDR1a lacking exon 11 and DDR1b containing exons 10, 11 and 12 [10]. DDR1c, on the other hand, contains an additional 18 bp relative to DDR1b, due to the presence of an additional, cryptic splice acceptor site 5′ to the preferred splice site at the intron/exon boundary of exon 14 [55]. This results in the addition of six amino acids to the kinase domain. Further alternative splicing leads to two kinase-deficient variants: DDR1d, which lacks exons 11 and 12, and DDR1e, which misses the first half of exon 10 in addition to lacking exons 11 and 12 [56].

3.4.1.1 Transcriptional Regulation

The *DDR1* gene structure has not been explored in detail. The promoter region contains a functional consensus binding site for the tumour suppressor p53 [57, 58]. DDR1 expression can be induced in a p53-dependent manner by genotoxic stress, such as ionising radiation or chemotherapy [57–59]. Moreover, the DNA repair

protein XRCC3, which is induced by genotoxic stress, can upregulate DDR1 expression by an as yet undefined mechanism [60]. The *DDR1* gene also contains a hnRNP A2 response element sequence, which may be involved in alternative splicing and nuclear export of DDR1 mRNA in oligodendrocytes [61]. DDR1 expression is downregulated during induction of epithelial–mesenchymal transition (EMT) [62]. Consistent with this, the *DDR1* promoter contains a binding site for the EMT-associated transcription factor Zeb1 [63] and was shown to be a target for Zeb1 in breast epithelial cells undergoing H-Ras-induced EMT [64]. DDR1 expression can also be posttranscriptionally regulated by microRNAs: levels of microRNA-199a-5p and microRNA-199b-5p inversely correlate with DDR1 expression in human hepatocellular carcinoma and colorectal carcinoma cells and in acute myeloid leukaemia, respectively [65–67].

The Ras/Raf/ERK signalling pathway is one of the signalling pathways that can regulate transcription of DDR1. For example, the T-cell receptor can induce DDR1 expression in human T cells through Ras/Raf/ERK and protein kinase C-dependent pathways [68]. Moreover, in primary lung fibroblasts, DDR1 expression can be induced by collagen I, through DDR2 activation, in an ERK1/2-dependent manner [69]. In certain cell types, DDR1 activation can also positively regulate its own expression. For example, in MCF7 breast and HCT116 colon carcinoma cells, DDR1 activation results in Ras/Raf/ERK signalling, which induces further DDR1 expression [58]. However, in most cases, the upstream signals that regulate DDR1 transcription have not yet been defined.

3.4.2 DDR1 Protein

3.4.2.1 Amino Acid Sequence

Five isoforms of DDR1, resulting from alternative splicing, have been described [56]. All DDR1 isoforms have common extracellular and transmembrane domains but differ in the cytoplasmic region. The longest isoform, termed DDR1c, contains 919 amino acids. The most common isoforms, DDR1a and DDR1b, lack six amino acids in the kinase domain with respect to DDR1c. DDR1a additionally lacks 37 amino acids in the intracellular juxtamembrane region (Fig. 3.1). DDR1d and DDR1e are truncated proteins without functional kinase domains, either lacking the entire kinase domain or parts of the juxtamembrane domain and the ATP binding site (Fig. 3.1).

3.4.2.2 Processing

Many transmembrane proteins, including RTKs, can be processed by proteases to release their extracellular domains. The cleavage, or “shedding”, of ectodomains can terminate receptor signalling, since the functional ectodomains are depleted

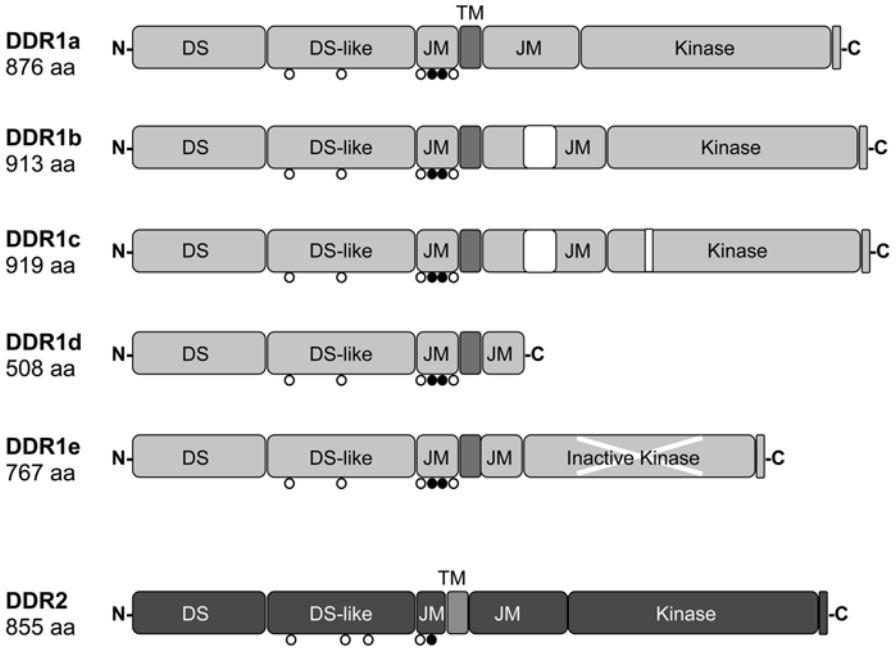

Fig. 3.1 Schematic representation of the domain organisation in DDR1 and DDR2. The ectodomains of both receptors contain an N-terminal DS domain of ~160 amino acids, followed by a DS-like domain of ~180 amino acids and a juxtamembrane region of ~50 (DDR1) or ~30 (DDR2) amino acids. The transmembrane domains are followed by large cytosolic juxtamembrane domains (up to ~170 amino acids in DDR1, ~140 amino acids in DDR2), the kinase domains of ~300 amino acids and short C-terminal tails (8 and 6 amino acids in DDR1 and DDR2, respectively). For DDR1, the five known isoforms (DDR1a–e) are depicted. The additional 37-amino acid region in the juxtamembrane regions of DDR1b and DDR1c, with respect to DDR1a, is highlighted in white. The extra 6 amino acids in the kinase domain of DDR1c are also indicated in *white*. DDR1d is a truncated isoform that lacks part of the cytosolic juxtamembrane region and the kinase domain. DDR1e is an inactive isoform, lacking part of the cytosolic juxtamembrane region and the ATP binding biding site in the kinase domain. *White circles* indicate predicted N-glycosylation sites; *black circles* indicate putative O-glycosylation sites. *DS* discoidin homology, *JM* juxtamembrane, *TM* transmembrane

from the cell surface. A proportion of DDR1 receptors undergoes ectodomain shedding upon prolonged (overnight) incubation with collagen [70, 71], but the functional significance of this process is not clear. The DDR1 ectodomain is most likely proteolytically processed close to the transmembrane domain, by a zinc-dependent metalloproteinase [70, 71], but the sheddase and the cleavage site remain to be defined. In addition to collagen-induced DDR1 shedding, constitutive shedding of the DDR1 ectodomain has been observed [10, 72]. This reaction is mediated by transmembrane MMPs (MT1-MMP, MT2-MMP, MT3-MMP) and occurs at two cleavage sites, 9 and 19 amino acids away from the transmembrane domain, respectively [72]. However, the biological function of the shed ectodomain and the remnant receptor fragment remains to be established.

3.4.2.3 Domain Structure

DDR1 has two globular domains in its extracellular region, an N-terminal discoidin (DS) domain followed by a second discoidin-like (DS-like) domain [73, 74] (Fig. 3.1). There are approximately 50 amino acids, which are predicted to be unstructured [75], that make up the extracellular juxtamembrane region. The ligand-binding DS domain is homologous to the protein discoidin 1 from the slime mould *Dictyostelium discoideum*, and related domains are also present in a number of secreted (e.g. blood coagulation factors V and VIII) and transmembrane proteins (e.g. neuropilin) [76]. The DDR1 DS domain is highly similar to the DDR2 DS domain and adopts a β-barrel structure with eight strands arranged in two antiparallel β-sheets [77] (see also Fig. 3.4). The N- and C-termini are connected by a disulfide bridge and are located at the bottom of the barrel. At the top of the barrel, there are five protruding loops which create a trench that can accommodate a collagen triple helix [78]. Despite little sequence conservation, the DDR1 DS-like domain adopts an eight-stranded β-barrel fold similar to the DS domain, with five additional strands protruding between the β1 and β2 strands [73].

The DDR1 transmembrane domain connects the extracellular juxtamembrane region to an unusually large intracellular juxtamembrane region of 169 amino acids (DDR1b isoform), which is followed by the kinase domain and a short C-terminal tail of eight amino acids.

3.4.2.4 Posttranslational Modification

Apart from glycosylation, no other posttranslational modifications have been described for DDR1. During biosynthesis, DDR1 undergoes N-glycosylation [79]. In addition to four predicted N-glycosylation sites, DDR1 has two predicted O-glycosylation sites in its extracellular domain (Fig. 3.1).

3.4.2.5 Phosphorylation Sites and Known Functions

Like other RTKs, DDR1 becomes phosphorylated on tyrosine residues after collagen binding, and these phosphorylation sites serve as docking sites for Src homology-2 (SH2) and phosphotyrosine-binding (PTB) domain-containing proteins. In full-length DDR1 (DDR1b and DDR1c isoforms), there are 15 tyrosine residues (13 in DDR1a) within the intracellular juxtamembrane and kinase domains that can potentially undergo phosphorylation upon receptor activation (see Fig. 3.2). The first signalling protein to be identified as a DDR1 interaction partner was the adaptor ShcA, which associates via its PTB domain with the alternatively spliced Tyr-513 that is present in DDR1b and DDR1c but not in DDR1a [1]. Signalling molecules that interact with DDR1 via their SH2 domains include the phosphatase Shp2, C-terminal Src tyrosine kinase and the adaptor Nck2 [80–82]. Furthermore, the structural subunit p85a of the phosphoinositide-3 kinase associates directly with

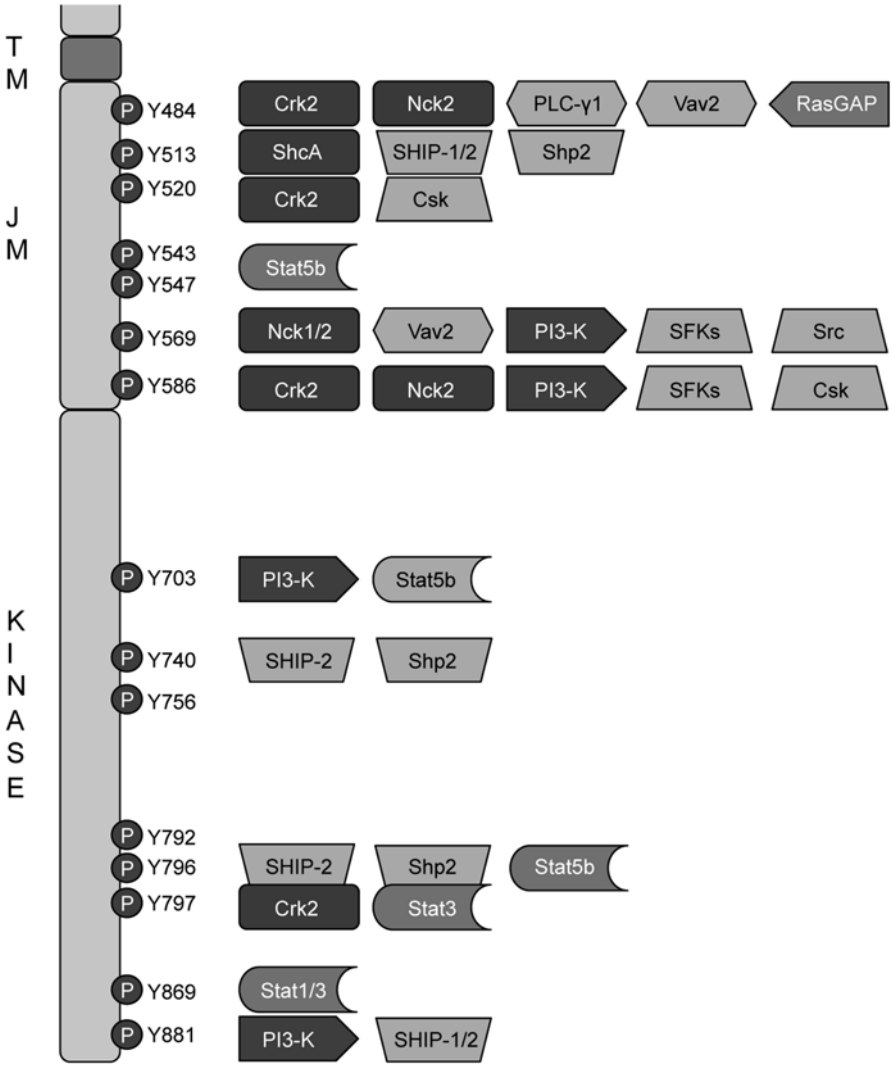

Fig. 3.2 Interaction map of phosphotyrosine-mediated DDR1 interactions. The indicated amino acid numbers refer to the DDR1b isoform. The figure summarises results obtained mainly from phosphotyrosine peptide pull-downs in human placenta tissue [84]. Only proteins with SH2 or PTB domains are shown. Crk2, adaptor protein Crk2; Nck1/2, adaptor protein Nck1/2; PLC-γ1, phospholipase C γ1; Vav2, guanine nucleotide exchange factor Vav2; RasGAP, negative regulator of Ras; ShcA, SH2 containing transforming protein A; SHIP1/2, SH2 containing inositol polyphosphate 5-phosphatase 1/2; Shp2, SH2 containing protein tyrosine phosphatase 2; Csk, C-terminal Src kinase; Stat1/3/5b, signal transducer and activator of transcription 1/3/5b; PI3-K, phosphoinositide 3-kinase; SFKs, Src family tyrosine kinases (Yes, Lyn, Fyn)

DDR1 [83]. A proteomics study that used the phosphatase inhibitor pervanadate to induce phosphorylation of DDR1 identified numerous signalling proteins and their docking sites on the DDR1 cytoplasmic tail [84]. A phosphopeptide library comprising all 15 cytoplasmic DDR1b tyrosines in phosphorylated form identified ~35 proteins as potential DDR1 interaction partners [84]. These proteins include RasGAP, a negative regulator of Ras, the guanine exchange factors Vav2 and Vav3, the adaptor Crk2 and the phosphatase SHIP-2 as partners for Tyr-484; PI-3 kinase as a partner for Tyr-881; and members of the STAT (signal transducer and activator of transcription) family as interaction partners for Tyr-869 (see Fig. 3.2). At present, very little information exists about the functional consequences of DDR1 interaction partners binding to the various docking sites, and it remains largely unknown how the DDR1 signalling partners are biochemically linked to specific cell regulatory functions.

3.4.3 DDR1 Ligands

DDR1 is activated by a number of different collagen types. Collagens are major components of extracellular matrices, and the 28 different collagens collectively represent the most abundant proteins in vertebrates. Collagens play fundamental roles in defining biomechanical properties of tissues but are also intimately involved in controlling cell behaviour. Cells interact with collagens through various receptor systems, including integrins and members of the immunoglobulin superfamily [3]. Collagens can act as DDR1 ligands only in their native, triple-helical conformation, since DDR1 does not recognise heat-denatured collagen (gelatin) [1, 85].

3.4.3.1 Collagen Structure

Collagens are characterised by their triple-helical structure. All collagens are composed of three polypeptide chains that are termed α chains and contain repeating glycine-X-X′ motifs. Amino acids in positions X and X′ can be any amino acid but are often proline and 4-hydroxyproline (O), respectively [86]. The three α chains form a right-handed triple-helical structure by coiling around each another with a one-residue stagger between chains. The triple helix is tightly packed and resembles a stiff cable (Fig. 3.3a). This tight packing requires every third amino acid to be a glycine. The three chains are held together by interchain hydrogen bonds, and the helix is stabilised in part through hydroxylation of prolines in the X′ position.

Vertebrates have 28 collagen types, resulting from assembly of specific combinations of 46 distinct α chains [87, 88]. Collagens can be homotrimers (containing three identical α chains) or heterotrimers made up of two or three different α chains. In tissues, most collagens form supramolecular assemblies, such as fibrils and fibres or planar sheetlike networks. Collagen fibrils and fibres are formed by lateral associations of individual triple helices (Fig. 3.3b, c). The most abundant collagens are the fibril-forming collagens, types I–III [89]. The basement membrane collagen type IV is the

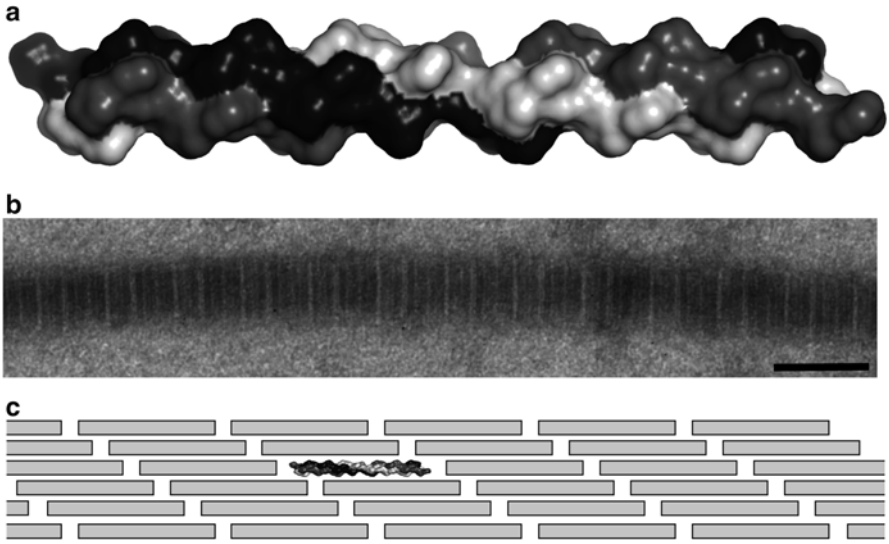

Fig. 3.3 Collagen structure. (**a**) Surface representation of a collagen triple helix, represented by the collagen-like peptide $(PPG)_9$ [138]. The three α chains are wound around each other with a one amino acid stagger between chains. (**b**) Electron micrograph of a heterotypic collagen fibril isolated from human articular cartilage (courtesy of Dr Uwe Hansen, University Hospital Münster, Germany). *Scale bar*, 100 nm. (**c**) Schematic diagram showing the lateral association of individual collagen triple helices in a collagen fibril

prototype of network-forming collagens. Additional families include transmembrane collagens and fibril-associated collagens with interruptions in their triple helices.

3.4.3.2 Collagen Cleavage

In each collagen α chain, the triple-helical collagenous domains, termed COL domains, are flanked by noncollagenous domains (the so-called propeptides) [89]. In some collagens, these noncollagenous domains remain uncleaved, but in the fibril-forming collagens, these domains are proteolytically cleaved once the trimerised molecules are secreted from cells. Removal of the propeptides decreases solubility of the collagens, allowing lateral association of triple helices into the macromolecular fibrils and fibres that are major constituents of connective tissues.

3.4.3.3 Collagen Specificity of DDR1

DDR1 displays a broad collagen-binding specificity, with fibrillar collagens constituting the main DDR1 ligands [1, 2]. In addition to fibrillar collagens, DDR1 can also be activated by the basement membrane collagen IV [1, 2]. Furthermore, DDR1 on vascular smooth muscle cells can use collagen VIII as its ligand [90].

Similar to collagen-binding integrins, DDR1 recognises specific amino acid motifs in collagen. A high affinity DDR1 motif, GVMGFO (O is hydroxyproline), was uncovered in studies utilising libraries of overlapping triple-helical peptides, the so-called Collagen Toolkits [91, 92]. Triple-helical peptides encompassing the GVMGFO motif, which cannot assemble into higher molecular structures, are able to induce DDR1 activation (autophosphorylation), indicating that the supramolecular structure of collagen is not required for DDR1 transmembrane signalling. The GVMGFO motif is present in collagens I–III but not in non-fibrillar collagens. The nature of the DDR1-binding motifs contained in collagen IV or VIII remains to be elucidated.

3.4.4 DDR1 Activation and Signalling

3.4.4.1 Dimerisation

Unlike most RTKs, which are generally thought to be monomeric in the absence of ligand and to be activated by ligand-induced dimerisation, DDR1 forms stable ligand-independent dimers on the cell membrane [75, 93–95]. DDR1 dimers were observed on the cell membrane and in the biosynthetic pathway [93]. Furthermore, cysteine mutagenesis of extracellular residues close to the transmembrane resulted in a high degree of covalent cross-linking, strongly suggesting that dimerisation is constitutive [75]. No single DDR1 subdomain was found to be solely responsible for DDR1 dimerisation, but the transmembrane helices showed very strong self-association in a bacterial reporter system [93]. The propensity for strong self-interaction of the DDR1 transmembrane helices was confirmed in a systematic study that compared the ability for self-interactions of all RTK transmembrane domains [96]. How collagen binding to the DDR1 DS domain leads to activation of the cytosolic kinase domain is not known and remains to be established. Collagen-induced conformational changes within the dimer are an unlikely mechanism since the extracellular juxtamembrane region was found to be exceptionally flexible [75]. Collagen-induced DDR1 clustering has been observed in one study [95], but it is not clear whether or how this process is related to receptor activation. Moreover, the exact composition of the ligand-induced higher-order DDR1 oligomers remains undefined. Furthermore, since DDR1 can be activated by short collagen-like peptides (see above), DDR1 clustering by multivalent collagen assemblies does not appear to be essential for receptor activation.

3.4.4.2 Phosphorylation

Like all RTKs, DDR1 undergoes receptor autophosphorylation upon ligand binding, but, compared with the rapid response of typical RTKs to their soluble ligands, in DDR1, this process is unusually slow and sustained [1, 2]. Maximal activation (phosphorylation) is often achieved only hours after stimulation with collagen and can remain detectable for up to several days poststimulation. There are cell type-dependent differences in activation kinetics. For example, DDR1 in human

embryonic kidney cells is maximally phosphorylated 60–90 min after collagen stimulation, whereas it takes several hours for a strong phosphorylation signal to appear in certain cancer cell lines [1, 2, 83]. Intriguingly, phosphorylation of DDR1 by Src seems to be required for full phosphorylation of DDR1 [97, 98]. The biochemical and cellular mechanisms behind the slow activation kinetics of DDR1 remain unknown.

3.4.4.3 Pathway Activation

Little information exists about specific signalling pathways activated by collagen-induced DDR1 signalling and their biological consequences. DDR1 can activate signalling through the mitogen-activated protein (MAP) kinase pathway, with activation of certain MAP kinase family members dependent on the cellular context. Thus, DDR1-dependent extracellular signal-regulated kinase (ERK)1/2 activation was observed in smooth muscle cells, in mammary epithelial cells and in megakaryocytes [98–100], whereas in mesangial cells, the function of DDR1 might be to suppress ERK1/2 activation [101]. In pancreatic cancer cells as well as in adipose stromal cells, DDR1 can signal via c-Jun N-terminal kinase (JNK) [102, 103]. Exposure of breast and colon cancer cells to genotoxic stress results in DDR1-mediated prosurvival signals via the Ras/MEK pathway, but the identity of the activated MAP kinase is not known [58].

DDR1 can also activate STAT signalling in a cell type-dependent manner. In mammary epithelial cells, DDR1 regulates lactation via Stat5 activation [7]. In contrast, DDR1 signalling leads to suppression of Stat1 and Stat3 activation in MDCK cells [81].

3.4.4.4 Major Genes Regulated

No systematic microarray studies have been performed to identify transcriptional targets of DDR1 signalling. We therefore only have limited information about the genes that are regulated by DDR1. Several studies, using knockout mice or human carcinoma cells, have shown that DDR1 signalling affects expression of MMPs, including MMP-2, MMP-9 and MMP-10 [104, 105], but whether the genes encoding these proteases are direct transcriptional targets of DDR1 is not clear. Other genes reported to be upregulated by DDR1 include those for monocyte chemotactic protein (MCP)-1 in macrophages [52, 106], N-cadherin in epithelial cells [102] and cyclooxygenase 2 in fibroblasts [59].

3.4.4.5 Crosstalk with Other Receptor Systems

The major collagen receptors expressed by most cell types belong to the integrin receptor family [3]. Collagen-induced DDR1 activation is independent of collagen-binding integrins [107], but DDR1 activation can lead to downstream signalling that intersects with integrin-induced pathways. In MDCK cells, DDR1 suppresses

integrin α2β1-mediated functions, such as cell spreading and cell migration [81, 108]. Specifically, DDR1 signalling inhibits cell spreading by blocking Cdc42 activity, which is initiated through integrin signalling via focal adhesion kinase [108]. The molecular mechanism of DDR1-dependent suppression of integrin α2β1-mediated cell migration involves DDR1 binding to the phosphatase Shp2, thereby suppressing integrin α2β1-mediated phosphorylation of Stat1 and Stat3 [81]. In contrast to the situation in MDCK cells, in pancreatic cancer cells, the cooperation of DDR1 and integrin α2β1 is required for collagen-induced EMT [102]. In these cells, DDR1 and integrin α2β1 signals coordinate to activate JNK, which in turn enhances N-cadherin expression, leading to cell scattering. DDR1 and integrin α2β1 have also been observed to cooperate in mouse embryonic stem cells to promote self-renewal through cell cycle regulation [109]. While DDR1 was found to negatively regulate cell adhesion in MDCK cells [81], in many other cell types, DDR1 seems to promote cell adhesion [5]. In human embryonic kidney cells, DDR1 was found to promote cell adhesion to collagen by enhancing the activity of the collagen-binding integrins α1β1 and α2β1 [110]. Thus, DDR1 and integrins can either antagonise one another or play cooperative roles, in a cell type- and context-dependent manner.

Not much information exists about crosstalk of DDR1 with receptor systems other than integrins. A study in colon cancer cells demonstrated that DDR1 activation leads to intracellular activation of the Notch signalling pathway [111]. Collagen-induced DDR1 signalling generates the intracellular form of Notch 1, which translocates into the nucleus and upregulates prosurvival genes. In breast cancer cells, it was observed that DDR1 receives positive input from the Wnt5a/Frizzled pathway [97, 112], but how this pathway is integrated with collagen-induced DDR1 activation is not clear.

3.4.4.6 Collagen-Independent Functions of DDR1

DDR1 has been shown to promote cell migration and/or invasion of many cell types (e.g. [90, 113–116]). The molecular mechanisms underlying collagen-induced DDR1-mediated cell migration and invasion remain largely undefined, but the pro-invasive activity of the DDRs may be ascribed to their ability to upregulate MMP expression, which is expected to contribute to the degradation of matrix components. However, a study using squamous carcinoma cells described a novel role for DDR1 acting in a collagen-independent function. DDR1 was found to be required for collective cancer cell migration and invasion [117]. In this system, DDR1 regulates actomyosin contractility by interacting with the cell polarity regulators Par3 and Par6. DDR1 was localised to cell–cell contacts, where it may act as a cell–cell adhesion receptor, rather than as a matrix receptor. Localisation to cell–cell contacts requires E-cadherin function [117, 118]. DDR1, in turn, can regulate E-cadherin function by increasing the stability of E-cadherin at the cell surface, which promotes cell–cell adhesion [119, 120]. A DDR1 ligand for the cell–cell adhesion role of DDR1 has not been identified. Another collagen-independent function for DDR1 was found in human adipose stromal cells grown in 3D collagen matrices, where DDR1 induces aromatase production [103]. Under these experimental conditions, DDR1 activation may occur via a putative sensor for matrix compliance [103].

3.4.5 DDR1 Internalisation, Processing and Attenuation

Unlike for other RTKs, little information is available on how DDR1 signalling is attenuated, and the mechanisms of DDR1 trafficking and dynamics in cellular membranes have only partly been explored. Two studies examined DDR1 internalisation after collagen incubation. Using GFP-tagged DDR1, Mihai et al. (2009) performed experiments that suggested that collagen exposure leads to rapid receptor aggregation, followed by internalisation of DDR1 into early endosomes [95]. However, whether internalisation is followed by receptor recycling to the plasma membrane, as suggested by the authors, or by degradation of DDR1 has not been properly addressed. In another study, internalised DDR1b was detected biochemically in a reversible biotinylation assay [121], but the specific cellular compartment of internalised DDR1 was not analysed. Further cell biological studies will thus be required to define whether DDR1 signalling can be initiated from endosomes. Furthermore, given that collagen stimulation leads to prolonged receptor activation (autophosphorylation) of up to several days in some cellular systems, how DDR1 signalling is switched off remains a mystery.

3.4.6 Unique Features of DDR1

DDR1 and DDR2 share several features that distinguish them from conventional RTKs. Among RTKs, the DDRs are the only receptors that are activated by an extracellular matrix protein, collagen. The DDRs are thus uniquely placed to mediate cell–matrix interactions and transduce signals from the extracellular matrix. Another unusual feature is that the DDRs seem not to be activated by ligand-induced dimerisation but are already dimers in the absence of ligand. Finally, while the DDRs, like typical RTKs, undergo receptor autophosphorylation upon ligand binding, they do so with unusually slow kinetics, and phosphorylated receptors are detectable for prolonged times.

3.5 DDR2

3.5.1 DDR2 Gene

Little is known about the genomic structure of the *DDR2* gene. The human *DDR2* gene maps to chromosome 1 (1q23.3) [122] and is composed of 18 exons, of which exons 3–18 are coding exons. The extracellular domain is encoded by exons 3–10, the transmembrane domain by exon 11. Exons 12 and 13 encode the cytosolic juxtamembrane domain, with the remaining exons predominantly coding for the kinase domain. No alternatively spliced isoforms of DDR2 have been described.

3.5.1.1 Transcriptional Regulation

DDR2 expression is regulated by different transcription factors in a cell type-dependent manner. For example, in rat vascular smooth muscle cells, DDR2 expression can be increased by hypoxia or hyperbaric oxygen, which increases Myc-Max DNA-binding activity in the *DDR2* promoter [123, 124]. During osteogenic differentiation, the ATF4 transcription factor binds to a CCAAT/enhancer binding site in the *DDR2* promoter, which induces DDR2 transcription [19]. DDR2 mRNA is also upregulated in hepatic stellate cells during liver injury [26]. In these cells, DDR2 mRNA can be downregulated by microRNA-29b, which targets collagen I, suggesting a relationship between collagen I expression and DDR2 expression [125].

3.5.2 DDR2 Protein

3.5.2.1 Amino Acid Sequence

DDR1 and DDR2 share a high degree of amino acid conservation, with the same overall domain structure. In contrast to DDR1, for which five isoforms have been identified, only one isoform of DDR2 has been described which contains 855 amino acids (Fig. 3.1).

3.5.2.2 Processing

Unlike for DDR1, whose ectodomain can be cleaved upon prolonged collagen incubation (see Sect. 3.4.2), no reports on shedding of the DDR2 ectodomain exist, and no other forms of protein processing have been described to date.

3.5.2.3 Domain Structure

DDR2 has the same overall protein architecture as DDR1, with two globular domains in its extracellular region: an N-terminal DS domain followed by a second DS-like domain [126] (Fig. 3.1). The DS and DS-like domains of the DDRs are highly similar, but their extracellular juxtamembrane domains are not well conserved [74]. Like the DDR1 DS domain, the DDR2 DS domain adopts an 8-stranded β-barrel structure in which the N- and C-termini are connected by a disulfide bridge at the bottom of the barrel [77] (see Fig. 3.4). The collagen-binding loops at the top of the barrel are highly conserved in DDR1 and DDR2 [78], consistent with both receptors binding to fibrillar collagens. Several residues at the periphery of the GVMGFO peptide-binding interface are not conserved in DDR1, and these amino acids are responsible for the distinct collagen-binding specificity of the DDRs. Replacing these residues in DDR2 with the corresponding DDR1 residues created a DDR2 construct which was able to bind collagen IV [92].

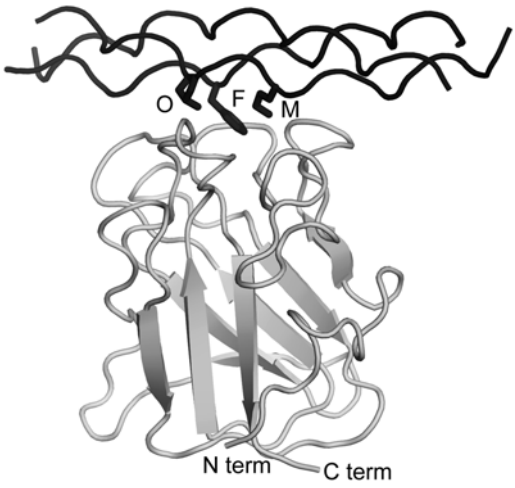

Fig. 3.4 Cartoon drawing of the crystal structure of the DDR2 DS domain bound to a collagen peptide [78]. The DS domain is shown in *light grey*, the collagen triple helix in *dark grey*. The side chains of the collagen residues that are involved in DDR2 binding are shown and labelled (O is hydroxyproline). The N- and C-termini at the *bottom* of the DS domain are indicated

The DDR2 transmembrane domain is very similar to the DDR1 transmembrane domain and connects the extracellular juxtamembrane domain to the intracellular juxtamembrane domain of about 140 amino acids. The intracellular juxtamembrane domains are less well conserved, but the C-terminal kinase domains are again highly similar. Like DDR1, DDR2 has a short C-terminal tail of six amino acids.

3.5.2.4 Posttranslational Modification

As is the case for DDR1, apart from glycosylation, no other posttranslational modifications have been described for DDR2. DDR2 undergoes N-glycosylation during biosynthesis [16] and has four predicted N- and one predicted O-glycosylation sites in its extracellular domain (Fig. 3.1).

3.5.2.5 Phosphorylation Sites and Known Functions

Very limited information is available on signalling proteins that interact with phosphorylated tyrosines on activated DDR2. The DDR2 cytoplasmic domain contains 14 tyrosine residues, of which only Tyr-471 has been shown to be a docking site for a DDR2 interaction partner, ShcA [127, 128]. In contrast to its interaction with activated DDR1b, which occurs via the ShcA PTB binding domain, ShcA interacts with DDR2 via its SH2 domain. The functional consequences of ShcA binding to DDR2 have not been described, and little information exists on other intracellular DDR2 signalling partners that directly interact with the receptor. A phosphoproteomic study observed collagen-induced phosphorylation at two sites in the kinase domain (Tyr684 and Tyr813) [129]. This study also found Tyr471 in the intracellular juxtamembrane region to be constitutively phosphorylated.

3.5.3 DDR2 Ligands

DDR2, like DDR1, is activated by a number of different collagen types (see Sect. 3.4.3 for a description of collagen structure and collagen cleavage). Both DDRs share the ability to be activated by fibrillar collagen types [1, 2], but they have distinct preferences for certain collagen types. Only DDR1 binds the basement membrane collagen type IV [1, 2], whereas DDR2 seems to be the preferred receptor for collagen II [130] and collagen X [131]. It is not known whether DDR2 can bind collagen VIII, a ligand for DDR1.

DDR2, like DDR1, binds the GVMGFO motif in collagens I–III, and transmembrane signalling can be induced with triple-helical peptides containing this binding motif [132]. DDR2 has additional binding motifs in collagens II and III [92, 132], but their exact sequences have not yet been determined.

3.5.4 DDR2 Activation and Signalling

Given the high degree of similarity between DDR1 and DDR2, we can assume that they employ similar mechanisms of receptor activation and signalling. Specific DDR2 features are summarised below. At present, not much information exists about the interplay between DDR2 and other receptor systems, but like DDR1, DDR2 signalling enhances cell adhesion via collagen-binding integrins [110]. DDR2 signalling has also been shown to crosstalk with insulin signalling [133], but the mechanistic details have not been explored. No studies have been published so far on DDR2 internalisation or signal attenuation.

3.5.4.1 Dimerisation

Similar to DDR1, DDR2 forms ligand-independent stable dimers on the cell membrane and in the biosynthetic pathway [93]. The transmembrane domains of the DDRs are highly conserved, and, like for DDR1, a strong propensity for self-interaction of the DDR2 transmembrane helices was found in a study that compared self-interactions of all RTK transmembrane domains [96]. Due to their high degree of amino acid conservation and structural similarities, it is reasonable to hypothesise that DDR1 and DDR2 employ a similar mechanism of transmembrane signalling. However, the molecular details of this process are as yet poorly defined, and no studies have addressed this specifically for DDR2.

3.5.4.2 Phosphorylation

DDR2, like DDR1, undergoes receptor autophosphorylation upon ligand binding, with unusually slow and sustained kinetics [1, 2]. While the cellular mechanisms behind this slow activation process are not understood, two studies have shown that

maximal DDR2 phosphorylation is dependent on the tyrosine kinase Src [127, 128]. Based on these studies, a model emerged that suggests that ligand binding promotes Src to phosphorylate tyrosines in the DDR2 activation loop, which in turn stimulates intramolecular autophosphorylation of additional tyrosine residues. These phosphorylated tyrosines then promote DDR2 binding to ShcA and other, as yet unidentified, cytoplasmic signalling partners. DDR2-Src interactions may thus play crucial roles in initiating DDR2 signalling.

3.5.4.3 Pathway Activation

Limited information exists about specific signalling pathways downstream of collagen-induced DDR2 signalling and their biological consequences. Like DDR1, DDR2 can activate signalling through the MAP kinase pathway, in a cell type and context-dependent manner. In fibroblasts, osteoblasts, chondrocytes and breast cancer cells, DDR2 has been shown to signal via ERK1/2 [18, 48, 69, 134, 135]. However, conflicting data suggest that DDR2 uses p38 MAP kinase rather than ERK1/2 to activate the transcription factor Runx2 in osteoblasts [19]. In chondrocytes, DDR2 can also use JNK; however, the relative contributions of DDR2 and integrin α1β1 to this pathway are not clear [135]. DDR2 can also transmit signals through the Janus kinase (JAK)/STAT pathway by using JAK2 [69]. A global phosphoproteomic study of collagen-induced DDR2 signalling networks identified potential downstream effectors of DDR2, including SHP-2, Nck1, the Src family kinase Lyn, phospholipase C-like2 and phosphatidylinositol-4-phosphate 3 kinase [133]. However, it is not clear whether these effectors interact directly with specific phosphotyrosine sites on DDR2.

3.5.4.4 Major Genes Regulated

As is the case for DDR1, limited information is available on transcriptional targets of DDR2 signalling, but it is clear that in a number of cellular systems, DDR2 regulates expression of MMPs, including MMP-1 [1], MMP-2 [136], MMP-10 [69] and MMP-13 [137]. At least for MMP-2 and MMP-13, it is clear that DDR2 signalling affects their gene promoter activities [134, 136]. In human lung fibroblasts, DDR2 signalling regulates the expression of the genes for DDR1, the bone morphogenetic protein BMP-2 and MCP-1 [69], but the molecular mechanism involved in the regulation of BMP-2 and MCP-1 expression remains sketchy. In osteoblasts, DDR2 activates osteoblast-specific gene expression for gene products that are required for osteoblast differentiation, including osteocalcin and osteopontin [18, 19]. These genes are transcriptional targets of the master transcription factor Runx2, whose activity (phosphorylation) is controlled in turn by DDR2 [18].

3.5.5 Unique Features of DDR2

DDR2 shares the same unusual features that distinguish DDR1 from other RTKs. These are listed under Sect. 3.4.6.

Receptor at a glance: DDR1

Chromosome location	Chromosome 6 (6p21.3)
Gene size (bp)	23,736 bp
Number of exons	17
mRNA size (5′, ORF,3′)	3,738 b (GenBank entry L11315.1)
Amino acid number	919 amino acids (DDR1c, largest isoform)
kDa	~120 kD
Posttranslational modifications	N- and O-glycosylation
Domains	Two globular domains in the extracellular region: DS domain and DS-like domain, transmembrane domain, large cytosolic juxtamembrane domain, kinase domain
Ligands	Fibrillar collagens, collagen IV, collagen VIII
Known dimerising partners	Not defined
Pathways activated	MAP kinase pathway, STAT signalling
Tissues expressed	Widely expressed, with high levels in brain, mammary gland, lung, kidney, gastrointestinal tract
Human diseases	Kidney fibrosis, lung fibrosis, atherosclerosis, many types of cancer
Knockout mouse phenotype	Mammary gland defect, lactational defect, thickening of glomerular basement membrane, impaired blastocyst implantation into uterine wall, loss of auditory function

Receptor at a glance: DDR2

Chromosome location	Chromosome 1 (1q23.3)
Gene size (bp)	149,093 bp
Number of exons	18
mRNA size (5′, ORF,3′)	3,263 b (GenBank entry BCO52998.2)
Amino acid number	855 amino acids
kDa	~125 kD
Posttranslational modifications	N- and O-glycosylation
Domains	Two globular domains in the extracellular region: DS domain and DS-like domain, transmembrane domain, large cytosolic juxtamembrane domain, kinase domain
Ligands	Fibrillar collagens, collagen X

(continued)

Known dimerising partners	None defined
Pathways activated	MAP kinase pathway, JAK/STAT pathway
Tissues expressed	Widely expressed, highest levels in skeletal muscle, skin, kidney and lung
Human diseases	Liver fibrosis, wound healing, osteoarthritis, rheumatoid arthritis, several types of cancer
Knockout mouse phenotype	Dwarfism, shorter long bones due to chondrocyte proliferation defect, impaired dermal wound healing

References

1. Vogel W, Gish GD, Alves F, Pawson T. The discoidin domain receptor tyrosine kinases are activated by collagen. Mol Cell. 1997;1(1):13–23.
2. Shrivastava A, Radziejewski C, Campbell E, Kovac L, McGlynn M, Ryan TE, et al. An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. Mol Cell. 1997;1(1):25–34.
3. Leitinger B. Transmembrane collagen receptors. Annu Rev Cell Dev Biol. 2011;27:265–90.
4. Vogel WF, Abdulhussein R, Ford CE. Sensing extracellular matrix: an update on discoidin domain receptor function. Cell Signal. 2006;18(8):1108–16.
5. Leitinger B. Discoidin domain receptor functions in physiological and pathological conditions. Int Rev Cell Mol Biol. 2014;310:39–87.
6. Vogel WF, Aszodi A, Alves F, Pawson T. Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development. Mol Cell Biol. 2001;21(8):2906–17.
7. Faraci-Orf E, McFadden C, Vogel WF. DDR1 signaling is essential to sustain Stat5 function during lactogenesis. J Cell Biochem. 2006;97(1):109–21.
8. Meyer zum Gottesberge AM, Gross O, Becker-Lendzian U, Massing T, Vogel WF. Inner ear defects and hearing loss in mice lacking the collagen receptor DDR1. Lab Invest. 2008; 88(1):27–37.
9. Gross O, Beirowski B, Harvey SJ, McFadden C, Chen D, Tam S, et al. DDR1-deficient mice show localized subepithelial GBM thickening with focal loss of slit diaphragms and proteinuria. Kidney Int. 2004;66(1):102–11.
10. Alves F, Vogel W, Mossie K, Millauer B, Hofler H, Ullrich A. Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer. Oncogene. 1995;10(3):609–18.
11. Chin GS, Lee S, Hsu M, Liu W, Kim WJ, Levinson H, et al. Discoidin domain receptors and their ligand, collagen, are temporally regulated in fetal rat fibroblasts in vitro. Plast Reconstr Surg. 2001;107(3):769–76.
12. Morales MO, Price RL, Goldsmith EC. Expression of discoidin domain receptor 2 (DDR2) in the developing heart. Microsc Microanal. 2005;11(3):260–7.
13. Labrador JP, Azcoitia V, Tuckermann J, Lin C, Olaso E, Manes S, et al. The collagen receptor DDR2 regulates proliferation and its elimination leads to dwarfism. EMBO Rep. 2001; 2(5):446–52.
14. Kano K, Marin de Evsikova C, Young J, Wnek C, Maddatu TP, Nishina PM, et al. A novel dwarfism with gonadal dysfunction due to loss-of-function allele of the collagen receptor gene, DDR2, in the mouse. Mol Endocrinol. 2008;22(8):1866–80.
15. Bargal R, Cormier-Daire V, Ben-Neriah Z, Le Merrer M, Sosna J, Melki J, et al. Mutations in DDR2 gene cause SMED with short limbs and abnormal calcifications. Am J Hum Genet. 2009;84(1):80–4.

16. Ali BR, Xu H, Akawi NA, John A, Karuvantevida NS, Langer R, et al. Trafficking defects and loss of ligand binding are the underlying causes of all reported DDR2 missense mutations found in SMED-SL patients. Hum Mol Genet. 2010;19(11):2239–50.
17. Al-Kindi A, Kizhakkedath P, Xu H, John A, Sayegh AA, Ganesh A, et al. A novel mutation in DDR2 causing spondylo-meta-epiphyseal dysplasia with short limbs and abnormal calcifications (SMED-SL) results in defective intra-cellular trafficking. BMC Med Genet. 2014;15:42.
18. Zhang Y, Su J, Yu J, Bu X, Ren T, Liu X, et al. An essential role of discoidin domain receptor 2 (DDR2) in osteoblast differentiation and chondrocyte maturation via modulation of Runx2 activation. J Bone Miner Res. 2011;26(3):604–17.
19. Lin KL, Chou CH, Hsieh SC, Hwa SY, Lee MT, Wang FF. Transcriptional upregulation of DDR2 by ATF4 facilitates osteoblastic differentiation through p38 MAPK-mediated Runx2 activation. J Bone Miner Res. 2010;25(11):2489–503.
20. Borza CM, Pozzi A. Discoidin domain receptors in disease. Matrix Biol. 2014;34:185–92.
21. Flamant M, Placier S, Rodenas A, Curat CA, Vogel WF, Chatziantoniou C, et al. Discoidin domain receptor 1 null mice are protected against hypertension-induced renal disease. J Am Soc Nephrol. 2006;17(12):3374–81.
22. Gross O, Girgert R, Beirowski B, Kretzler M, Kang HG, Kruegel J, et al. Loss of collagen-receptor DDR1 delays renal fibrosis in hereditary type IV collagen disease. Matrix Biol. 2010;29(5):346–56.
23. Guerrot D, Kerroch M, Placier S, Vandermeersch S, Trivin C, Mael-Ainin M, et al. Discoidin domain receptor 1 is a major mediator of inflammation and fibrosis in obstructive nephropathy. Am J Pathol. 2011;179(1):83–91.
24. Avivi-Green C, Singal M, Vogel WF. Discoidin domain receptor 1-deficient mice are resistant to bleomycin-induced lung fibrosis. Am J Respir Crit Care Med. 2006;174(4):420–7.
25. Roberts ME, Magowan L, Hall IP, Johnson SR. Discoidin domain receptor 1 regulates bronchial epithelial repair and matrix metalloproteinase production. Eur Respir J. 2011;37(6):1482–93.
26. Olaso E, Ikeda K, Eng FJ, Xu L, Wang LH, Lin HC, et al. DDR2 receptor promotes MMP-2-mediated proliferation and invasion by hepatic stellate cells. J Clin Invest. 2001;108(9):1369–78.
27. Olaso E, Arteta B, Benedicto A, Crende O, Friedman SL. Loss of discoidin domain receptor 2 promotes hepatic fibrosis after chronic carbon tetrachloride through altered paracrine interactions between hepatic stellate cells and liver-associated macrophages. Am J Pathol. 2011;179(6):2894–904.
28. Mao TK, Kimura Y, Kenny TP, Branchi A, Gishi RG, Van de Water J, et al. Elevated expression of tyrosine kinase DDR2 in primary biliary cirrhosis. Autoimmunity. 2002;35(8):521–9.
29. Zhang XH, Yan M, Liu L, Wu TJ, Ma LL, Wang LX. Expression of discoidin domain receptors (DDR2) in alcoholic liver fibrosis in rats. Arch Med Res. 2010;41(8):586–92.
30. Luo Z, Liu H, Sun X, Guo R, Cui R, Ma X, et al. RNA interference against discoidin domain receptor 2 ameliorates alcoholic liver disease in rats. PLoS One. 2013;8(2):e55860.
31. Olaso E, Lin HC, Wang LH, Friedman SL. Impaired dermal wound healing in discoidin domain receptor 2-deficient mice associated with defective extracellular matrix remodeling. Fibrogenesis Tissue Repair. 2011;4(1):5.
32. Hu K, Xu L, Cao L, Flahiff CM, Brussiau J, Ho K, et al. Pathogenesis of osteoarthritis-like changes in the joints of mice deficient in type IX collagen. Arthritis Rheum. 2006;54(9):2891–900.
33. Li Y, Xu L, Olsen BR. Lessons from genetic forms of osteoarthritis for the pathogenesis of the disease. Osteoarthritis Cartilage. 2007;15(10):1101–5.
34. Sunk IG, Bobacz K, Hofstaetter JG, Amoyo L, Soleiman A, Smolen J, et al. Increased expression of discoidin domain receptor 2 is linked to the degree of cartilage damage in human knee joints: a potential role in osteoarthritis pathogenesis. Arthritis Rheum. 2007;56(11):3685–92.
35. Xu L, Peng H, Glasson S, Lee PL, Hu K, Ijiri K, et al. Increased expression of the collagen receptor discoidin domain receptor 2 in articular cartilage as a key event in the pathogenesis of osteoarthritis. Arthritis Rheum. 2007;56(8):2663–73.

36. Holt DW, Henderson ML, Stockdale CE, Farrell JT, Kooyman DL, Bridgewater LC, et al. Osteoarthritis-like changes in the heterozygous sedc mouse associated with the HtrA1-Ddr2-Mmp-13 degradative pathway: a new model of osteoarthritis. Osteoarthritis Cartilage. 2012; 20(5):430–9.
37. Xu L, Servais J, Polur I, Kim D, Lee PL, Chung K, et al. Attenuation of osteoarthritis progression by reduction of discoidin domain receptor 2 in mice. Arthritis Rheum. 2010;62(9):2736–44.
38. Valiathan RR, Marco M, Leitinger B, Kleer CG, Fridman R. Discoidin domain receptor tyrosine kinases: new players in cancer progression. Cancer Metastasis Rev. 2012;31(1–2):295–321.
39. Ford CE, Lau SK, Zhu CQ, Andersson T, Tsao MS, Vogel WF. Expression and mutation analysis of the discoidin domain receptors 1 and 2 in non-small cell lung carcinoma. Br J Cancer. 2007;96(5):808–14.
40. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature. 2008;455(7216):1069–75.
41. Kan Z, Jaiswal BS, Stinson J, Janakiraman V, Bhatt D, Stern HM, et al. Diverse somatic mutation patterns and pathway alterations in human cancers. Nature. 2010;466(7308):869–73.
42. Davies H, Hunter C, Smith R, Stephens P, Greenman C, Bignell G, et al. Somatic mutations of the protein kinase gene family in human lung cancer. Cancer Res. 2005;65(17):7591–5.
43. Yang SH, Baek HA, Lee HJ, Park HS, Jang KY, Kang MJ, et al. Discoidin domain receptor 1 is associated with poor prognosis of non-small cell lung carcinomas. Oncol Rep. 2010;24(2):311–9.
44. Valencia K, Ormazabal C, Zandueta C, Luis-Ravelo D, Anton I, Pajares MJ, et al. Inhibition of collagen receptor discoidin domain receptor-1 (DDR1) reduces cell survival, homing, and colonization in lung cancer bone metastasis. Clin Cancer Res. 2012;18(4):969–80.
45. Miao L, Zhu S, Wang Y, Li Y, Ding J, Dai J, et al. Discoidin domain receptor 1 is associated with poor prognosis of non-small cell lung cancer and promotes cell invasion via epithelial-to-mesenchymal transition. Med Oncol. 2013;30(3):626.
46. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell. 2007;131(6):1190–203.
47. Hammerman PS, Sos ML, Ramos AH, Xu C, Dutt A, Zhou W, et al. Mutations in the DDR2 kinase gene identify a novel therapeutic target in squamous cell lung cancer. Cancer Discov. 2011;1(1):78–89.
48. Zhang K, Corsa CA, Ponik SM, Prior JL, Piwnica-Worms D, Eliceiri KW, et al. The collagen receptor discoidin domain receptor 2 stabilizes SNAIL1 to facilitate breast cancer metastasis. Nat Cell Biol. 2013;15(6):677–87.
49. Ren T, Zhang J, Liu X, Yao L. Increased expression of discoidin domain receptor 2 (DDR2): a novel independent prognostic marker of worse outcome in breast cancer patients. Med Oncol. 2013;30(1):397.
50. Ren T, Zhang W, Liu X, Zhao H, Zhang J, Li X, et al. Discoidin domain receptor 2 (DDR2) promotes breast cancer cell metastasis and the mechanism implicates epithelial-mesenchymal transition programme under hypoxia. J Pathol. 2014;234(4):526–37.
51. Rix U, Hantschel O, Durnberger G, Remsing Rix LL, Planyavsky M, Fernbach NV, et al. Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. Blood. 2007;110(12):4055–63.
52. Day E, Waters B, Spiegel K, Alnadaf T, Manley PW, Buchdunger E, et al. Inhibition of collagen-induced discoidin domain receptor 1 and 2 activation by imatinib, nilotinib and dasatinib. Eur J Pharmacol. 2008;599(1–3):44–53.
53. Perez JL, Shen X, Finkernagel S, Sciorra L, Jenkins NA, Gilbert DJ, et al. Identification and chromosomal mapping of a receptor tyrosine kinase with a putative phospholipid binding sequence in its ectodomain. Oncogene. 1994;9(1):211–9.
54. Edelhoff S, Sweetser DA, Disteche CM. Mapping of the NEP receptor tyrosine kinase gene to human chromosome 6p21.3 and mouse chromosome 17C. Genomics. 1995;25(1):309–11.
55. Playford MP, Butler RJ, Wang XC, Katso RM, Cooke IE, Ganesan TS. The genomic structure of discoidin receptor tyrosine kinase. Genome Res. 1996;6(7):620–7.

56. Alves F, Saupe S, Ledwon M, Schaub F, Hiddemann W, Vogel WF. Identification of two novel, kinase-deficient variants of discoidin domain receptor 1: differential expression in human colon cancer cell lines. FASEB J. 2001;15(7):1321–3.
57. Sakuma S, Saya H, Tada M, Nakao M, Fujiwara T, Roth JA, et al. Receptor protein tyrosine kinase DDR is up-regulated by p53 protein. FEBS Lett. 1996;398(2–3):165–9.
58. Ongusaha PP, Kim JI, Fang L, Wong TW, Yancopoulos GD, Aaronson SA, et al. p53 induction and activation of DDR1 kinase counteract p53-mediated apoptosis and influence p53 regulation through a positive feedback loop. EMBO J. 2003;22(6):1289–301.
59. Das S, Ongusaha PP, Yang YS, Park JM, Aaronson SA, Lee SW. Discoidin domain receptor 1 receptor tyrosine kinase induces cyclooxygenase-2 and promotes chemoresistance through nuclear factor-{kappa}b pathway activation. Cancer Res. 2006;66(16):8123–30.
60. Martinez-Marignac VL, Rodrigue A, Davidson D, Couillard M, Al-Moustafa AE, Abramovitz M, et al. The effect of a DNA repair gene on cellular invasiveness: XRCC3 over-expression in breast cancer cells. PLoS One. 2011;6(1):e16394.
61. Roig B, Moyano S, Martorell L, Costas J, Vilella E. The Discoidin domain receptor 1 gene has a functional A2RE sequence. J Neurochem. 2012;120(3):408–18.
62. Maeyama M, Koga H, Selvendiran K, Yanagimoto C, Hanada S, Taniguchi E, et al. Switching in discoid domain receptor expressions in SLUG-induced epithelial-mesenchymal transition. Cancer. 2008;113(10):2823–31.
63. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci USA. 2010;107(35):15449–54.
64. Koh M, Woo Y, Valiathan RR, Jung HY, Park SY, Kim YN, et al. Discoidin domain receptor 1 is a novel transcriptional target of ZEB1 in breast epithelial cells undergoing H-Ras-induced epithelial to mesenchymal transition. Int J Cancer. 2015;136(6):E508–20.
65. Shen Q, Cicinnati VR, Zhang X, Iacob S, Weber F, Sotiropoulos GC, et al. Role of microRNA-199a-5p and discoidin domain receptor 1 in human hepatocellular carcinoma invasion. Mol Cancer. 2010;9:227.
66. Favreau AJ, Cross EL, Sathyanarayana P. mir-199b-5p directly targets PODXL and DDR1 and decreased levels of miR-199b-5p correlate with elevated expressions of PODXL and DDR1 in acute myeloid leukemia. Am J Hematol. 2012;87(4):442–6.
67. Hu Y, Liu J, Jiang B, Chen J, Fu Z, Bai F, et al. MiR-199a-5p loss up-regulated DDR1 aggravated colorectal cancer by activating epithelial-to-mesenchymal transition related signaling. Dig Dis Sci. 2014;59(9):2163–72.
68. Chetoui N, El Azreq MA, Boisvert M, Bergeron ME, Aoudjit F. Discoidin domain receptor 1 expression in activated T cells is regulated by the ERK MAP kinase signaling pathway. J Cell Biochem. 2011;112(12):3666–74.
69. Ruiz PA, Jarai G. Collagen I induces discoidin domain receptor (DDR) 1 expression through DDR2 and a JAK2-ERK1/2-mediated mechanism in primary human lung fibroblasts. J Biol Chem. 2011;286(15):12912–23.
70. Vogel WF. Ligand-induced shedding of discoidin domain receptor 1. FEBS Lett. 2002; 514(2–3):175–80.
71. Slack BE, Siniaia MS, Blusztajn JK. Collagen type I selectively activates ectodomain shedding of the discoidin domain receptor 1: involvement of Src tyrosine kinase. J Cell Biochem. 2006;98(3):672–84.
72. Fu HL, Sohail A, Valiathan RR, Wasinski BD, Kumarasiri M, Mahasenan KV, et al. Shedding of discoidin domain receptor 1 by membrane-type matrix metalloproteinases. J Biol Chem. 2013;288(17):12114–29.
73. Carafoli F, Mayer MC, Shiraishi K, Pecheva MA, Chan LY, Nan R, et al. Structure of the discoidin domain receptor 1 extracellular region bound to an inhibitory Fab fragment reveals features important for signaling. Structure. 2012;20(4):688–97.
74. Carafoli F, Hohenester E. Collagen recognition and transmembrane signalling by discoidin domain receptors. Biochim Biophys Acta. 2013;1834(10):2187–94.

75. Xu H, Abe T, Liu JK, Zalivina I, Hohenester E, Leitinger B. Normal activation of discoidin domain receptor 1 mutants with disulfide cross-links, insertions, or deletions in the extracellular juxtamembrane region: mechanistic implications. J Biol Chem. 2014;289(19):13565–74.
76. Baumgartner S, Hofmann K, Chiquet-Ehrismann R, Bucher P. The discoidin domain family revisited: new members from prokaryotes and a homology-based fold prediction. Protein Sci. 1998;7(7):1626–31.
77. Ichikawa O, Osawa M, Nishida N, Goshima N, Nomura N, Shimada I. Structural basis of the collagen-binding mode of discoidin domain receptor 2. EMBO J. 2007;26(18):4168–76.
78. Carafoli F, Bihan D, Stathopoulos S, Konitsiotis AD, Kvansakul M, Farndale RW, et al. Crystallographic insight into collagen recognition by discoidin domain receptor 2. Structure. 2009;17(12):1573–81.
79. Curat CA, Eck M, Dervillez X, Vogel WF. Mapping of epitopes in discoidin domain receptor 1 critical for collagen binding. J Biol Chem. 2001;276(49):45952–8.
80. Koo DH, McFadden C, Huang Y, Abdulhussein R, Friese-Hamim M, Vogel WF. Pinpointing phosphotyrosine-dependent interactions downstream of the collagen receptor DDR1. FEBS Lett. 2006;580(1):15–22.
81. Wang CZ, Su HW, Hsu YC, Shen MR, Tang MJ. A discoidin domain receptor 1/SHP-2 signaling complex inhibits {alpha}2beta1-integrin-mediated signal transducers and activators of transcription 1/3 activation and cell migration. Mol Biol Cell. 2006;17(6):2839–52.
82. Yang G, Li Q, Ren S, Lu X, Fang L, Zhou W, et al. Proteomic, functional and motif-based analysis of C-terminal Src kinase-interacting proteins. Proteomics. 2009;9(21):4944–61 [Research Support, Non-U.S. Gov't].
83. L'Hote CG, Thomas PH, Ganesan TS. Functional analysis of discoidin domain receptor 1: effect of adhesion on DDR1 phosphorylation. FASEB J. 2002;16(2):234–6.
84. Lemeer S, Bluwstein A, Wu Z, Leberfinger J, Muller K, Kramer K, et al. Phosphotyrosine mediated protein interactions of the discoidin domain receptor 1. J Proteomics. 2012; 75(12):3465–77.
85. Leitinger B. Molecular analysis of collagen binding by the human discoidin domain receptors, DDR1 and DDR2. Identification of collagen binding sites in DDR2. J Biol Chem. 2003;278(19):16761–9.
86. Brodsky B, Persikov AV. Molecular structure of the collagen triple helix. Adv Protein Chem. 2005;70:301–39.
87. Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet. 2004;20(1):33–43.
88. Shoulders MD, Raines RT. Collagen structure and stability. Annu Rev Biochem. 2009;78:929–58.
89. Kadler KE, Baldock C, Bella J, Boot-Handford RP. Collagens at a glance. J Cell Sci. 2007;120(Pt 12):1955–8.
90. Hou G, Vogel W, Bendeck MP. The discoidin domain receptor tyrosine kinase DDR1 in arterial wound repair. J Clin Invest. 2001;107(6):727–35.
91. Farndale RW, Lisman T, Bihan D, Hamaia S, Smerling CS, Pugh N, et al. Cell-collagen interactions: the use of peptide Toolkits to investigate collagen-receptor interactions. Biochem Soc Trans. 2008;36(Pt 2):241–50.
92. Xu H, Raynal N, Stathopoulos S, Myllyharju J, Farndale RW, Leitinger B. Collagen binding specificity of the discoidin domain receptors: binding sites on collagens II and III and molecular determinants for collagen IV recognition by DDR1. Matrix Biol. 2011;30(1):16–26.
93. Noordeen NA, Carafoli F, Hohenester E, Horton MA, Leitinger B. A transmembrane leucine zipper is required for activation of the dimeric receptor tyrosine kinase DDR1. J Biol Chem. 2006;281(32):22744–51.
94. Abdulhussein R, Koo DH, Vogel WF. Identification of disulfide-linked dimers of the receptor tyrosine kinase DDR1. J Biol Chem. 2008;283(18):12026–33.
95. Mihai C, Chotani M, Elton TS, Agarwal G. Mapping of DDR1 distribution and oligomerization on the cell surface by FRET microscopy. J Mol Biol. 2009;385(2):432–45.

96. Finger C, Escher C, Schneider D. The single transmembrane domains of human receptor tyrosine kinases encode self-interactions. Sci Signal. 2009;2(89):ra56.
97. Dejmek J, Dib K, Jonsson M, Andersson T. Wnt-5a and G-protein signaling are required for collagen-induced DDR1 receptor activation and normal mammary cell adhesion. Int J Cancer. 2003;103(3):344–51.
98. Lu KK, Trcka D, Bendeck MP. Collagen stimulates discoidin domain receptor 1-mediated migration of smooth muscle cells through Src. Cardiovasc Pathol. 2011;20(2):71–6.
99. Hilton HN, Stanford PM, Harris J, Oakes SR, Kaplan W, Daly RJ, et al. KIBRA interacts with discoidin domain receptor 1 to modulate collagen-induced signalling. Biochim Biophys Acta. 2008;1783(3):383–93.
100. Abbonante V, Gruppi C, Rubel D, Gross O, Moratti R, Balduini A. Discoidin domain receptor 1 protein is a novel modulator of megakaryocyte-collagen interactions. J Biol Chem. 2013;288(23):16738–46.
101. Curat CA, Vogel WF. Discoidin domain receptor 1 controls growth and adhesion of mesangial cells. J Am Soc Nephrol. 2002;13(11):2648–56.
102. Shintani Y, Fukumoto Y, Chaika N, Svoboda R, Wheelock MJ, Johnson KR. Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1. J Cell Biol. 2008;180(6):1277–89.
103. Ghosh S, Ashcraft K, Jahid MJ, April C, Ghajar CM, Ruan J, et al. Regulation of adipose oestrogen output by mechanical stress. Nat Commun. 2013;4:1821.
104. Hou G, Vogel WF, Bendeck MP. Tyrosine kinase activity of discoidin domain receptor 1 is necessary for smooth muscle cell migration and matrix metalloproteinase expression. Circ Res. 2002;90(11):1147–9.
105. Faraci E, Eck M, Gerstmayer B, Bosio A, Vogel WF. An extracellular matrix-specific microarray allowed the identification of target genes downstream of discoidin domain receptors. Matrix Biol. 2003;22(4):373–81.
106. Franco C, Hou G, Ahmad PJ, Fu EY, Koh L, Vogel WF, et al. Discoidin domain receptor 1 (DDR1) deletion decreases atherosclerosis by accelerating matrix accumulation and reducing inflammation in low-density lipoprotein receptor-deficient mice. Circ Res. 2008;102(10): 1202–11.
107. Vogel W, Brakebusch C, Fassler R, Alves F, Ruggiero F, Pawson T. Discoidin domain receptor 1 is activated independently of b1 integrin. J Biol Chem. 2000;275(8):5779–84.
108. Yeh YC, Wang CZ, Tang MJ. Discoidin domain receptor 1 activation suppresses alpha2beta1 integrin-dependent cell spreading through inhibition of Cdc42 activity. J Cell Physiol. 2009;218(1):146–56.
109. Suh HN, Han HJ. Collagen I regulates the self-renewal of mouse embryonic stem cells through alpha2beta1 integrin- and DDR1-dependent Bmi-1. J Cell Physiol. 2011; 226(12):3422–32.
110. Xu H, Bihan D, Chang F, Huang PH, Farndale RW, Leitinger B. Discoidin domain receptors promote alpha1beta1- and alpha2beta1-integrin mediated cell adhesion to collagen by enhancing integrin activation. PLoS One. 2012;7(12):e52209.
111. Kim HG, Hwang SY, Aaronson SA, Mandinova A, Lee SW. DDR1 receptor tyrosine kinase promotes prosurvival pathway through Notch1 activation. J Biol Chem. 2011;286(20): 17672–81.
112. Jonsson M, Andersson T. Repression of Wnt-5a impairs DDR1 phosphorylation and modifies adhesion and migration of mammary cells. J Cell Sci. 2001;114(Pt 11):2043–53.
113. Kamohara H, Yamashiro S, Galligan C, Yoshimura T. Discoidin domain receptor 1 isoform-a (DDR1a) promotes migration of leukocytes in three-dimensional collagen lattices. FASEB J. 2001;15(14):2724–6.
114. Ram R, Lorente G, Nikolich K, Urfer R, Foehr E, Nagavarapu U. Discoidin domain receptor-1a (DDR1a) promotes glioma cell invasion and adhesion in association with matrix metalloproteinase-2. J Neurooncol. 2006;76(3):239–48.
115. Yoshida D, Teramoto A. Enhancement of pituitary adenoma cell invasion and adhesion is mediated by discoidin domain receptor-1. J Neurooncol. 2007;82:29–40.

116. Huang Y, Arora P, McCulloch CA, Vogel WF. The collagen receptor DDR1 regulates cell spreading and motility by associating with myosin IIA. J Cell Sci. 2009;122(Pt 10):1637–46.
117. Hidalgo-Carcedo C, Hooper S, Chaudhry SI, Williamson P, Harrington K, Leitinger B, et al. Collective cell migration requires suppression of actomyosin at cell-cell contacts mediated by DDR1 and the cell polarity regulators Par3 and Par6. Nat Cell Biol. 2011;13(1):49–58.
118. Wang CZ, Yeh YC, Tang MJ. DDR1/E-cadherin complex regulates the activation of DDR1 and cell spreading. Am J Physiol Cell Physiol. 2009;297(2):C419–29.
119. Yeh YC, Wu CC, Wang YK, Tang MJ. DDR1 triggers epithelial cell differentiation by promoting cell adhesion through stabilization of E-cadherin. Mol Biol Cell. 2011;22(7):940–53.
120. Eswaramoorthy R, Wang CK, Chen WC, Tang MJ, Ho ML, Hwang CC, et al. DDR1 regulates the stabilization of cell surface E-cadherin and E-cadherin-mediated cell aggregation. J Cell Physiol. 2010;224(2):387–97.
121. Fu HL, Valiathan RR, Payne L, Kumarasiri M, Mahasenan KV, Mobashery S, et al. Glycosylation at ASN211 regulates the activation state of the discoidin domain receptor 1 (DDR1). J Biol Chem. 2014;289(13):9275–87.
122. Karn T, Holtrich U, Brauninger A, Bohme B, Wolf G, Rubsamen-Waigmann H, et al. Structure, expression and chromosomal mapping of TKT from man and mouse: a new subclass of receptor tyrosine kinases with a factor VIII-like domain. Oncogene. 1993;8(12):3433–40.
123. Chen SC, Wang BW, Wang DL, Shyu KG. Hypoxia induces discoidin domain receptor-2 expression via the p38 pathway in vascular smooth muscle cells to increase their migration. Biochem Biophys Res Commun. 2008;374(4):662–7.
124. Shyu KG, Wang BW, Chang H. Hyperbaric oxygen activates discoidin domain receptor 2 via tumour necrosis factor-alpha and the p38 MAPK pathway to increase vascular smooth muscle cell migration through matrix metalloproteinase 2. Clin Sci (Lond). 2009;116(7):575–83.
125. Sekiya Y, Ogawa T, Yoshizato K, Ikeda K, Kawada N. Suppression of hepatic stellate cell activation by microRNA-29b. Biochem Biophys Res Commun. 2011;412(1):74–9.
126. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010; 141(7):1117–34.
127. Yang K, Kim JH, Kim HJ, Park IS, Kim IY, Yang BS. Tyrosine 740 phosphorylation of discoidin domain receptor 2 by Src stimulates intramolecular autophosphorylation and Shc signaling complex formation. J Biol Chem. 2005;280(47):39058–66.
128. Ikeda K, Wang LH, Torres R, Zhao H, Olaso E, Eng FJ, et al. Discoidin domain receptor 2 interacts with Src and Shc following its activation by type I collagen. J Biol Chem. 2002;277(21):19206–12.
129. Iwai LK, Payne LS, Luczynski MT, Chang F, Xu H, Clinton RW, et al. Phosphoproteomics of collagen receptor networks reveals SHP-2 phosphorylation downstream of wild-type DDR2 and its lung cancer mutants. Biochem J. 2013;454(3):501–13.
130. Leitinger B, Steplewski A, Fertala A. The D2 period of collagen II contains a specific binding site for the human discoidin domain receptor, DDR2. J Mol Biol. 2004;344(4):993–1003.
131. Leitinger B, Kwan AP. The discoidin domain receptor DDR2 is a receptor for type X collagen. Matrix Biol. 2006;25(6):355–64.
132. Konitsiotis AD, Raynal N, Bihan D, Hohenester E, Farndale RW, Leitinger B. Characterization of high affinity binding motifs for the discoidin domain receptor DDR2 in collagen. J Biol Chem. 2008;283(11):6861–8.
133. Iwai LK, Chang F, Huang PH. Phosphoproteomic analysis identifies insulin enhancement of discoidin domain receptor 2 phosphorylation. Cell Adh Migr. 2013;7(2):161–4.
134. Su J, Yu J, Ren T, Zhang W, Zhang Y, Liu X, et al. Discoidin domain receptor 2 is associated with the increased expression of matrix metalloproteinase-13 in synovial fibroblasts of rheumatoid arthritis. Mol Cell Biochem. 2009;330(1–2):141–52.
135. Vonk LA, Doulabi BZ, Huang C, Helder MN, Everts V, Bank RA. Collagen-induced expression of collagenase-3 by primary chondrocytes is mediated by integrin 1 and discoidin domain receptor 2: a protein kinase C-dependent pathway. Rheumatology. 2011;50(3):463–72.
136. Olaso E, Labrador JP, Wang L, Ikeda K, Eng FJ, Klein R, et al. Discoidin domain receptor 2 regulates fibroblast proliferation and migration through the extracellular matrix in association

with transcriptional activation of matrix metalloproteinase-2. J Biol Chem. 2002; 277(5):3606–13.
137. Xu L, Peng H, Wu D, Hu K, Goldring MB, Olsen BR, et al. Activation of the discoidin domain receptor 2 induces expression of matrix metalloproteinase 13 associated with osteoarthritis in mice. J Biol Chem. 2005;280(1):548–55.
138. Okuyama K, Hongo C, Wu G, Mizuno K, Noguchi K, Ebisuzaki S, et al. High-resolution structures of collagen-like peptides [(Pro-Pro-Gly)4-Xaa-Yaa-Gly-(Pro-Pro-Gly)4]: implications for triple-helix hydration and Hyp(X) puckering. Biopolymers. 2009;91(5):361–72.

Chapter 4
The EGFR/ERBB Receptor Family

Fresia Pareja, Gur Pines, and Yosef Yarden

4.1 Introduction to the EGFR/ERBB Subfamily of Receptor Tyrosine Kinases

Half a century has elapsed since Stanley Cohen identified the epidermal growth factor (EGF), on the basis of his observation that injection of purified submaxillary gland extracts into newborn mice induced premature opening of eyelids as well as an earlier eruption of teeth [1]. Years later, using radioactively labeled EGF as a tool, the receptor for this growth factor, the epidermal growth factor receptor (EGFR), was identified [2]. In an effort to unravel the interplay between EGF and its receptor, Cohen and colleagues showed that binding of EGF to membrane preparations from human epidermoid carcinoma cells elicited tyrosine phosphorylation of EGFR [3]. In this sense, the effects of EGF stimulation emulated the impact of Rous sarcoma virus (RSV) transformation [4], suggesting an oncogenic role for EGFR. Later on, a close similarity was demonstrated between the amino acid sequence of EGFR and v-erb-B, a retroviral oncogene structurally close to src, the transforming gene of RSV. Moreover, the genes that encode EGFR and src were found to display high

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degree of similarity [5, 6]. Several lines of evidence implicated this receptor in oncogenesis in tissues from a variety of origins. EGFR expression was found to be elevated in brain tumors and in squamous cell carcinomas [7–9], and this was associated with increased metastasis and poor prognosis in breast cancer patients [10]. Likewise, high levels of EGFR were detected in a battery of tumoral cell lines from ovarian, cervical, and renal origin [11]. As EGFR emerged as a suitable target of anticancer therapy, therapeutic strategies were devised. Anti-EGFR monoclonal antibodies inhibited EGF-elicited phosphorylation and cellular proliferation [12] and suppressed growth of human epidermoid tumors in athymic mice [13].

Later on, other members of the ERBB family of receptors were discovered. A transforming protein with high degree of homology to EGFR was described, first in rodents and afterwards also in humans, and designated NEU, ERBB2, or HER2 [14–16]. Amplification of the ERBB2/HER2 was thereafter recognized as a frequent event in breast cancer, associated with disease relapse and poor patient survival [17]. However, despite extensive homology to EGFR, later studies suggested that this receptor lacks as ligand and functions as a signal amplifier [18]. A few years after the discovery of HER2, a third member was identified, HER3/ERBB3 [19]. Uniquely, it was demonstrated that this member of the ERBB family of receptors lacks an intrinsic tyrosine kinase activity [20]. ERBB4, which showed high expression levels in tumor cell lines of diverse origins, was the last receptor of this group to be identified and closed the list of the ERBB family [21].

The ERBB and EGF families are represented in *Caenorhabditis elegans* by a single receptor, Let-23, and a single ligand, Lin-12 [22]. A series of gene duplication events likely led the evolution of four receptors and eleven ligands in mammals [23]. This created a layered signaling network, which is characterized by ligand-induced formation of homo- and heterodimers, followed by autophosphorylation of the receptors and transphosphorylation of multiple downstream targets. This leads to the activation of several kinase cascades and downstream transcription factors and ultimately to the orchestration of an array of cellular processes [24].

4.2 The Role of the EGFR/ERBB Family in Embryonic Development and in Adult Physiology

Since its discovery, the ERBB family research branched into several fields, including the association of these receptors with malignancies and their typical role in embryogenesis and in tissue homeostasis. The developmental role of the EGFR ortholog, LET-23, in the nematode *Caenorhabditis elegans*, is best characterized in vulval formation and patterning through several fate-determining processes [25]. In the fruit fly *Drosophila melanogaster*, EGFR is involved in directing many cell fate choices, cell division, survival, and migration [26] and is also responsible for axial polarity [27]. In zebra fish, *Danio rerio,* ERBB proteins were shown to be required for skin pigmentation [28] and proper heart development [29]. The critical role of the ERBB family in mammalian embryonic development is demonstrated by early lethality of knockout mice. EGFR-deficient mice either die in mid-gestation due to placenta

defects, or they survive for a short time after birth and suffer from impaired epithelial development in multiple organs, including the skin, gastrointestinal tract, and lungs [30, 31]. ERBB2 null mice die at mid-gestation due to neural crest and motor nerve defects and trabeculae malformation in the heart [32], similar to ERBB4-depleted mice [33]. ERBB3 mutant embryos die at a later stage and they lack Schwann cell precursors, which results in the death of motor and sensory neurons [34].

The ERBB family also has an important role in postnatal development of organs such as the mammary gland. Both EGFR and ERBB2 are expressed in the mammary gland in all developmental stages, and mice expressing a mutated EGFR, with a dramatically reduced kinase activity, promote defective ductal growth and impaired maternal lactation [35]. Additionally, ERBB2 and ERBB4 were shown to be essential for lobuloalveolar formation and milk protein secretion [36, 37]. In addition to their developmental roles, the ERBB receptors are required for the maintenance of some adult tissues. EGFR is normally expressed in rapidly proliferating epithelial cells, including the lungs, skin, and gastrointestinal tract [38]. ERBB2 expression is detected in endocervical and endometrial cells, in thyroid C cells, and in sebaceous glands of the skin [39]. The ERBB3 and ERBB4 receptors were identified in the gastrointestinal, reproductive, respiratory, and urinary tracts as well as in the skin, endocrine, and nervous systems [40, 41].

4.3 The Role of EGFR Receptor Tyrosine Kinase Family in Human Diseases

The ERBB family and its multiple EGF-like ligands are richly involved in human cancer. A frequent involvement entails autocrine and paracrine loops, often associated with overexpression of the respective receptor or ligand [42]. In addition, multiple mutations affect all four ERBB proteins in human tumors, as summarized in Table 4.1. Yet, the ERBB family is involved in other, nonmalignant pathologies. NRG1 and to a lesser extent one of its receptors, ERBB4, were shown to be important candidates for schizophrenia susceptibility, with some suggesting that alterations in the NRG1-ERBB signaling pathway could account for dysregulation of the glutamatergic and dopaminergic system in this disease [43]. Additionally, it has been reported that some ERBB4 splice isoforms are significantly elevated in patients with schizophrenia [44]. Similarly, a variant of NRG was reported to diminish autoimmune demyelination of oligodendrocytes and to enhance remyelination in a chronic relapsing model for multiple sclerosis in mice, suggesting a potential treatment modality [45]. Another disease in which ERBB signaling might play a role is psoriasis. Aberrant activation of the EGFR by TGFalpha was shown to be important in the pathogenesis of psoriatic epidermal hyperplasia [44], and EGFR activation was suggested to promote psoriasis through disrupting the pro- and antiapoptotic balance toward hyper proliferation [46]. Additionally, amphiregulin elevated mRNA levels were found in psoriatic epidermis, relative to healthy tissue, and anti-amphiregulin antibodies reduced skin thickness of transplanted

Table 4.1 ERBB mutations in human tumors

Receptor	Domain	Mutation and amino acid sequence affected	Disease type	References
EGFR	Extracellular	N-terminal truncation	Brain (GBM)	[384–388]
		Deletion of exons 14–15 (EGFRvII)		
		Deletion of exons 2–7 (EGFRvIII)	Brain (GBM), lung, breast, ovarian, and other cancers	
		Point mutations (e.g., R108K, A289V)	Brain (GBM)	
	Kinase	Deletions within the segment of amino acids 746–759	Lung (NSCLC) and breast cancer (triple negative)	[186, 189, 389–391]
		Point mutations in exons 18 and 21 (e.g., L858R, G718S)		
		Insertion (D770InsNPG)	Lung (NSCLC)	
		Point mutation in exon 20, conferring gefitinib resistance (T790M)		
		Duplication	Brain (GBM)	
	Carboxyl tail	Deletions of exons 25–27 (EGFRvIVa), 26–27 (EGFRvIVb), and 27	Brain (GBM)	[386, 392–394]
		C-terminal truncation (EGFRvV)		
ERBB2/ HER2	Kinase	Insertion at position 774 (AYVM) or 776 (YVMA)	Lung (NSCLC)	[395–397]
		Point mutations at exons 18–21 (e.g., L755S, V777L/M)	Lung (NSCLC), breast carcinomas, gastric and colorectal cancer	
ERBB3	Ligand binding	Missense mutation resulting in a short, 399aa protein	Lethal congenital contractural syndrome type 2 (LCCS2)	[398]
ERBB4	Ligand binding	Point mutations in the Furin-like domain (e.g., T244R, Y285C)	Lung adenocarcinoma	[399]
	Kinase	Point mutations in exons 18–21 and 23 (e.g., V721I, P854G)	Lung (NSCLC) breast carcinoma, gastric and colorectal cancers	[400]
	All domains	Additional point mutations in multiple sites along the gene (e.g., Y111H, E452K, E836K)	Melanoma	[401]

The following abbreviations are used: *GBM* glioblastoma multiforme, *NSCLC* non-small cell lung cancer

psoriatic skin in mice [47]. Another ligand, NRG-1, was shown to significantly attenuate lesion formation following vascular injury and was suggested as a potential therapy for restenosis and atherosclerosis [48]. The underlying mechanism seems to involve inhibition of atherogenesis and macrophage foam cell formation [49].

4.4 EGFR (ERBB1)

4.4.1 The EGFR Gene

4.4.1.1 Promoter Structure

The promoter of EGFR contains neither TATA nor CCAAT boxes but has a high GC content and some CCGCCC and TCCTCCTCC repeats [50]. There are six transcriptional start sites, with the most 5′ start site being highly active in vivo. The promoter was shown to bind many transcription factors, including SP1 (with multiple binding sites [51]), EGR-1 [52], TCF, ETF1/2, GCF, ETF, p53 (both wild type and mutant) [53], and AP-2 [54]. The promoter region was also shown to be methylated in some cancers, an event that silences EGFR expression and hence voids any benefit from EGFR-specific therapy [55].

4.4.1.2 Transcriptional Regulation

EGFR was shown to be transcriptionally induced by beta-estradiol, phorbol, EGF, TGFbeta, progestins, cAMP, retinoic acid, vitamin D, thyroid hormone, and dexamethasone [54]. TGFalpha was shown to induce EGFR mRNA production in pancreatic cancer cells [56]. Additionally, EGFR transcription was found to depend on the amount of CA nucleotide repeats within intron 1 of the EGFR gene. This transcriptional regulation mechanism is inherited and controls the amount of EGFR mRNA produced, with a negative correlation between these repeats and pre-mRNA synthesis [57].

4.4.2 The EGFR Protein

4.4.2.1 Processing

EGFR is initially produced as a precursor, which undergoes cleavage of its amino-terminal signal and several glycosylation steps. The maturation process is initiated upon EGFR entry into the lumen of the endoplasmic reticulum (ER) and involves transfer of carbohydrate moieties to specific asparagine residues. The carbohydrate

side chains are then processed in the ER and in the Golgi network to produce a mature glycoprotein, which is exported through the secretory machinery to the plasma membrane [58]. In addition to asparagine-directed glycosylation, EGFR was reported to undergo fucosylation [59] and sialylation [60], side chain modifications critical for its activity.

4.4.2.2 Domain Structure

EGFR was the first RTK to be characterized [61], and it shares with the later discovered ERBB members a characteristic domain structure (see Fig. 4.1), including an extracellular ligand-binding region, that comprises two leucine-rich repeat domains (denoted I and III) and two cysteine-rich domains (II and IV), a single

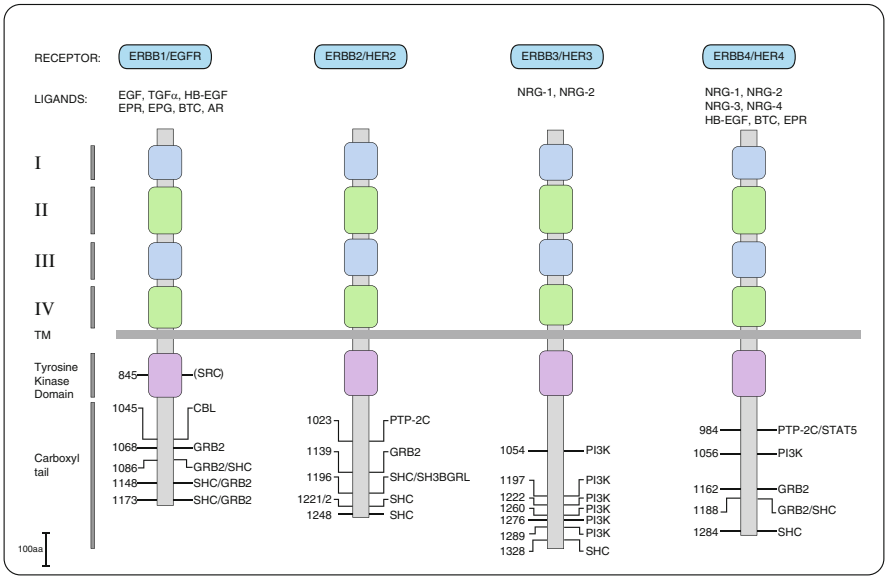

Fig. 4.1 Linear representation of the ERBB family of receptors. Receptors and domains are drawn to scale and aligned at the transmembrane domains. The respective ligands and the major phosphotyrosine sites (partial list) are indicated, along with their predicted binding partners (note that SRC was reported to phosphorylate EGFR on tyrosine number 845). The transmembrane domain predictions were based on http://www.cbs.dtu.dk/services/TMHMM/ and other domain predictions were based on http://www.ebi.ac.uk/Tools/pfa/iprscan/. The tyrosine phosphosites are based on published data [129]. *TGFalpha* transforming growth factor alpha, *HB-EGF* heparin-binding EGF-like growth factor, *EPR* epiregulin, *EPG* epigen, *BTC* betacellulin, *AR* amphiregulin, *NRG* neuregulin, *SRC* sarcoma viral oncogene homologue, *CBL* casitas B-lineage lymphoma proto-oncogene, *GRB2* growth factor receptor-bound protein 2, *SHC* SRC homology domain containing, *STAT5* signal transducers and activators of transcription, *PTP 2C* protein-tyrosine phosphatase 2C, *SH3BGRL* SH3 domain-binding glutamic acid-rich protein like, *PI3K* phosphoinositide-3-kinase. ERBB3 has two additional GRB2 sites at positions 1199 and 1262, which were not included due to space limitations

membrane-spanning region, an intracellular bilobular tyrosine kinase domain, and a carboxyl-terminal tail harboring multiple phosphorylation sites [62]. Note that domain II serves as a receptor dimerization site, which stabilizes homodimers of EGFR, as well as heterodimers with the other three ERBB proteins. Despite the common domain structure of all ERBBs, it is notable that two members of the family are peculiar; ERBB2 lacks the ligand-binding domain but carries an evolutionary conserved kinase domain, whereas ERBB3 retains the ligand-binding ability, but its kinase function is impaired [18, 20].

4.4.2.3 Posttranslational Modifications

EGFR undergoes phosphorylation on multiple serine, threonine, and tyrosine sites, as well as ubiquitination, neddylation, and acetylation. The major modifications are reviewed below.

Phosphorylation Sites and Known Functions The major target of the activated kinase of EGFR is the receptor itself and its dimer partner. The EGFR carboxyl terminus is rich in tyrosine residues that serve as phosphorylation targets (see Fig. 4.1). These phosphotyrosines serve as docking sites for adaptor proteins and signaling molecules that are responsible for the downstream signal propagation. The major EGFR phosphotyrosine sites are Y845, Y1045, Y1068, Y1086, Y1148, and Y1173.

EGFR Ubiquitination Upon EGFR activation, phosphorylated tyrosine residues act as docking sites for multiple adaptors, such as the three CBL proteins. These are ring-finger-containing E3 ubiquitin ligases, which recruit E2 conjugating enzymes and mediate EGFR ubiquitination [63, 64]. The interaction between CBL and EGFR can occur in a direct or an indirect fashion. CBL proteins possess a tyrosine kinase binding (TKB) domain, which allows their interaction with the phosphorylated EGFR, via the phosphorylated form of tyrosine residue 1045 (Fig. 4.1) [63, 65]. Alternatively, GRB2 harbors an SH2 domain, which makes contact with phosphotyrosine residues of EGFR, and two SH3 domains able to bind c-CBL and CBL-b [65, 66]. While initial studies showed that decoration of EGFRs with mono-ubiquitin moieties was sufficient for internalization [67, 68], later mass spectrometry studies revealed that EGFR is mono- and poly-ubiquitinated, mainly by means of K63 ubiquitin chains [69]. Furthermore, upon EGF stimulation, CBL modifies lysine residues in the tyrosine kinase domain of EGFR with the ubiquitin-like molecule Nedd8, which promotes subsequent ubiquitination [70].

Deubiquitinating Enzymes and Phosphatases Deubiquitinating enzymes (DUBs) that target EGFR oppose the action of CBL. AMSH, UBPY, Cezanne-1, and USP18 are DUBs that deubiquitinate this receptor. While USP18 regulates EGFR synthesis [71], AMSH, UBPY, and Cezanne-1 regulate its endocytic trafficking. AMSH silencing enhanced EGFR degradation in some studies [72]. In mice, UBPY deficiency caused embryonic lethality and fatal liver failure in adulthood,

along with decreased levels of RTKs, such as EGFR [73]. Cezanne-1, which was shown to inhibit EGFR degradation upon EGF stimulation, is amplified in a large proportion of breast tumors, and its mRNA abundance predicts relatively short patient survival [74]. Another layer of regulation is attained by protein-tyrosine phosphatases (PTPs). RPTP sigma dephosphorylates EGFR, resulting in signal attenuation [75]. PTPN1 and PTPN2 are other examples of PTPs targeting EGFR [76, 77]. DEP-1, a transmembrane PTP, is frequently deleted in human cancers from diverse origins, such as the colon, lung, breast, and thyroid [78, 79]. Recently, DEP-1 was found to dephosphorylate EGFR and play a tumor suppressor role [80].

4.4.3 EGFR Ligands

4.4.3.1 Autocrine, Paracrine, Juxtacrine, and Extracrine Modes of Signaling

Constant intercellular communication is necessary to achieve homeostasis. Growth factors are released to the extracellular environment and interact with receptors on the cell of its origin or stimulate a neighboring cell, namely, autocrine and paracrine modes, respectively. A recent study showed, using a 3D culture model, that signaling in breast cancer cells was dependent on an autocrine loop involving a protease, ADAM17, and cleavage of two direct ligands of EGFR, amphiregulin and TGFalpha, but this loop did not exist in non-tumoral cells [81]. Moreover, the expression of this protease and TGFalpha correlated with a relatively poor prognosis in breast cancer. Another form of ligand interaction, coined juxtacrine signaling, was unveiled when pro-TGFalpha, the membrane-anchored precursor form, was shown to bind to EGFR on adjacent cells, promoting cell proliferation [82]. Later on, juxtacrine signaling was found to involve other ligands. In canine kidney cells, stimulation of EGFR with a non-cleavable mutant of the membrane-bound precursor of HB-EGF rescued cells from anoikis and regulated the protein composition of tight junctions, in a way that elevated transepithelial resistance [83]. The membrane precursor of amphiregulin interacts with EGFR in a juxtacrine way, while the cleaved soluble form of the ligand plays a pivotal role in autocrine activation of EGFRs in human mammary epithelial cells [84]. Recently, a novel EGFR signaling mode was identified. Cancer cells of breast and colorectal origin release exosomes harboring amphiregulin, TGFalpha, and HB-EGF. Exosomal EGFR ligands are displayed in a signaling competent orientation, ready to interact with receptors in the target cell. Amphiregulin harbored in exosomes induced invasiveness in breast cancer cells. These data suggest that exosomes can be regarded as EGFR ligand signaling scaffold, mediating the extracrine (exosomal targeted receptor activation) signaling mode [85].

4.4.3.2 Ligands Specific to EGFR

The EGF-like family of growth factors comprises eleven members (see Fig. 4.1), which differ in receptor specificity and affinity. Some ligands bind exclusively to one receptor, such as EGF, TGFalpha, amphiregulin, and epigen, which interact solely with EGFR, or neuregulins (NRG) 3 and 4, which bind only with ERBB4. Other ligands recognize more than one receptor, such as HB-EGF, epiregulin, and betacellulin that bind to both EGFR and ERBB4 (see their description under ERBB4) and NRG 1 and 2, which bind both ERBB3 and ERBB4 [86]. Mature ERBB ligands share a characteristic sequence, necessary for interaction with receptors, named the EGF motif. This region is a cysteine-rich domain, where six cysteine residues form three disulfide bonds [87].

EGF This prototypical member of the family of ligands was the first one to be discovered. It is peculiar because it is synthesized as a very large precursor, pro-EGF, a 1,207-amino acid-long membrane-anchored polypeptide, and proteolytic processing of pro-EGF results in the 53-amino acid mature EGF. A systematic study aimed at characterizing ligand processing implicated ADAM10 as the protease that processes this ligand [88]. Interestingly, the cytoplasmic domain of EGF (proEGF-cyt) modulates microtubule dynamics in thyroid cancer cells [89].

TGF Alpha Transforming growth factor alpha is a 50-amino acid ligand, derived from a 160-amino acid length precursor polypeptide [90]. Processing of this ligand is performed predominantly by ADAMS17/TACE (tumor necrosis factor alpha-converting enzyme) [91].

Amphiregulin The precursor of amphiregulin (AR) is a transmembrane protein of 252 amino acids, which following cleavage produces the 78–84-amino acid-long mature form [92]. Recently it was demonstrated that AR plays a protective role against Fas-mediated liver injury in a mouse model and is involved in the early phases of liver regeneration [93, 94]. In addition, the amphiregulin gene is a transcriptional target of YAP, the main effector of the Hippo pathway, mediating cell proliferation and migration [95].

Epigen Epigen is synthesized as a 152-amino acid membrane-bound precursor, and it is cleaved to produce a 72-amino acid peptide by ADAM17 [96]. Even though epigen has as lower receptor-binding affinity than EGF, it displays a stronger mitogenic activity [97].

EGF-Like Growth Factors Encoded by Poxviruses The family of poxviruses, which cause an array of pathologies both in humans and animals, utilizes EGF-like growth factors as virulence factors. For instance, Shope fibroma growth factor (SFGF), myxoma growth factor (MGF), and vaccinia growth factor (VGF) are encoded by Shope fibroma, myxoma, and vaccinia viruses, respectively [98, 99]. These growth factors display distinct specificity for different members of the ERBB family of receptors. While VGF binds mainly to ERBB1 homodimers and MGF to ERBB2-ERBB3 heterodimers, SFGF displays a more promiscuous pattern of

interactions, binding to the ERBB2-ERBB3 pair and to all ERBB-1-containing dimers [100]. Despite having a lower affinity for their receptors than their mammalian counterparts, they display a higher mitogenic potential. This might be explained by the fact that interaction with their cognate receptors is coupled to a relatively weak downregulation and endocytosis.

4.4.3.3 Ligand Cleavage

EGFR Transactivation Growth factors belonging to the EGF family are synthesized as transmembrane precursor molecules; hence shedding of the ectodomain by proteases is a key step that determines ligand availability. Early studies showed that agonists of certain G-protein-coupled receptors lead to EGFR tyrosine phosphorylation, by a phenomenon called transactivation [101]. Later on, in vivo experiments showed that mice expressing a mutant ADAM17 exhibited developmental abnormalities reminiscent of mice deficient in TGFalpha [91]. Nowadays, it is widely accepted that metalloproteases belonging to a disintegrin and metalloprotease (ADAM) and matrix metalloproteases (MMP) families are accountable for this phenomenon [102].

Processing of Ligands: Role of Proteases Ligand ectodomain cleavage can be elicited by a multiplicity of stimuli, such as WNT binding to Frizzled [103] or estradiol binding to the estrogen receptor [104]. Two proteases have been thoroughly studied, ADAM17 (tumor necrosis factor-alpha converting enzyme; TACE), which is involved in the processing of TGFalpha, HB-EGF, amphiregulin, epiregulin, and epigen, and ADAM10, which is accountable for ectodomain shedding of proEGF and betacellulin [105]. Recent studies implicate several ADAM members in cancer pathogenesis [106]. For instance, expression of ADAM17 correlates with poor prognosis in breast cancer patients [81], and overexpression of ADAM 9 enhanced metastatic potential of non-small cell lung carcinoma cells to the brain [107]. Different EGF-like ligands stimulating a given receptor can elicit dissimilar effects. At equipotent concentrations, amphiregulin induced redistribution of E-cadherin and a cellular spindle-like conformation in MDKC cells, while TGFalpha did not [108]. Amphiregulin produced more extensive invasive and migratory phenotype than EGF in MCF10 cells [84]. Variations in affinity and potency, and perhaps also intrinsic ligand properties, might be responsible for these divergent cellular outcomes. Moreover, profiling of the expression of various ligands in breast cancer showed that EGF and amphiregulin were associated with smaller tumors and lower-grade histology, whereas high expression of TGFalpha, HB-EGF, and NRG-2 correlated with an aggressive disease course, exemplifying how differential ligand expression can exert an impact on prognosis [109].

4.4.4 EGFR Activation and Signaling

4.4.4.1 EGFR Dimerization

Ligand-induced receptor dimerization was originally discovered with EGF and EGFR [110, 111] and later extended to all RTKs [112]. Nevertheless, there is evidence suggesting that receptors of the ERBB family are present in a pre-dimerized or an oligomerized state in the absence of ligands [113, 114]. Stimulation of these receptors with growth factors promotes the formation of an array of homo- and heterodimers, which leads to the activation of the tyrosine kinase domain, subsequent tyrosine phosphorylation in the C-terminal tails, and recruitment of downstream signaling molecules [110]. For some RTKs, such as KIT, dimerization is mediated by a dimeric ligand, which binds to two receptors, facilitating dimer formation [115, 116]. In the case of ERBB receptors, ligands do not directly take part in the dimerization interface; instead, dimer formation is mediated by the receptors themselves. A crystal structure of the extracellular region of EGFR in an inactive state showed that in the absence of ligand, the receptor is in a "tethered" autoinhibitory conformation, in which the dimerization arm of domain II is obscured by intramolecular interactions between domains II and IV [117, 118]. Upon ligand binding, conformational changes ensue, which lead to the uncovering of the dimerization arm. Studies, representing the crystal structure of EGF or TGFalpha bound to the extracellular region of EGFR, showed that these ligands bind simultaneously to domains I and III [119, 120]. By bringing these domains together, ligand binding causes the receptor to acquire an extended configuration, which uncovers the dimerization arm of domain II and poises the receptor to interact with another one. Interestingly, crystal structure studies revealed that HER2/ERBB2 displays an activated conformation, similar to the ligand-bound configuration of other ERBB receptors, which could explain why EEBB2 lacks a ligand, and is the preferred dimerization partner of other ERBBs [121, 122]. Conformational changes of the extracellular domains are followed by asymmetric dimerization of kinase domains, by which the C-terminal lobe of the "activator" kinase domain converges with the N-terminal lobe of the "receiver" one and activates it [95, 123].

4.4.4.2 Pathway Activation

ERBB activation initiates multiple signal transduction pathways that regulate a plethora of biological outcomes, such as proliferation, differentiation, survival, adhesion, and migration (see signaling pathways in Fig. 4.2). The nature and kinetics of the elicited signal is tightly regulated at several points during the relay of information.

Signaling Adaptors Early studies showed that Src homology 2 (SH2) domains, small modules harbored in an array of intracytoplasmic signaling adaptors, recognize phosphorylated tyrosine residues of EGFR and PDGFR [124, 125]. Nowadays,

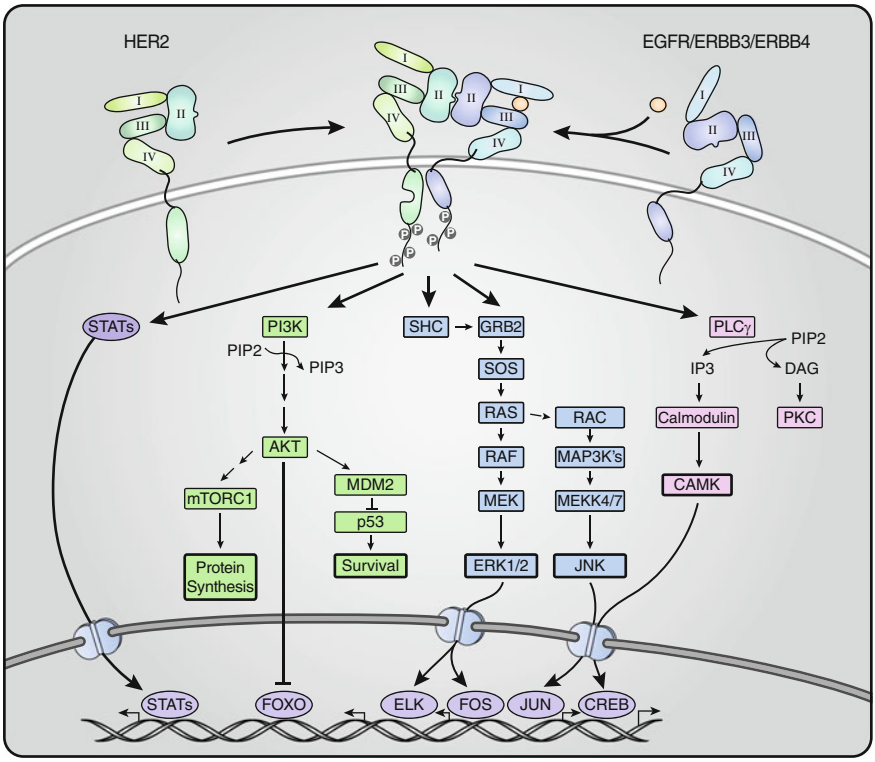

Fig. 4.2 Major signaling pathways activated by ERBB receptors. HER2 is shown on the *left* side and a generic (EGFR, ERBB3, or ERBB4) ERBB receptor dimer partner is depicted on the *right* side. Ligand stimulation instigates dimerization (center), auto- and transphosphorylation, as well as activation of multiple signaling pathways, which culminate in transcriptional regulation

SH2 and phosphotyrosine-binding (PTB) domains are recognized as key mediators of phosphorylation-dependent protein interactions. The domains bind the phosphorylated tyrosine in a specific sequence context, determined by few surrounding amino acids [126], and subsequently trigger the activation of downstream signaling cascades. The networks that are mainly activated comprise PLCgamma, phosphatidylinositol 3-kinase (PI3K), and STAT signaling pathways, as well as several canonical mitogen-activated protein kinase (MAPK) cascades [127]. SH2 and PTB domains allow repositioning of proteins that contain them and link EGFR tyrosine phosphorylation with downstream signaling molecules. For instance, binding of PLCgamma to activated EGFRs, by means of its SH2 domain, allows its translocation to the plasma membrane where it meets its substrate, phosphoinositol 4,5 bisphosphate (PIP_2). The SH2 domain of GRB2 interacts with phosphorylated tyrosines of EGFR, while the SH3 domains bind to SOS (or to CBL), which is a GTP exchange factor of RAS, to activate the MAPK pathway [128]. A recent proteomic study aimed to systematically determine the molecules that interact with all tyrosine

residues of the four ERBB receptors. The results showed that each receptor possesses a distinct predilection for a set of interactors, and that each binding partner targets several docking sites on each receptor [129]. Interestingly, EGFR and ERBB4 displayed comparable patterns of interaction, implying that they might trigger analogous signaling events. Moreover, ERBB3 was shown to harbor 6 docking sites for PI3K, consistent with the propensity of this receptor to ignite this particular signaling cascade.

PLCgamma Pathway Phospholipase Cgamma recognizes phosphotyrosine residues of an activated receptor via its SH2 domain. This leads to PLCgamma phosphorylation and activation, along with recruitment to the plasma membrane, where its substrate, phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2), is located [130]. Breakdown of PIP2 by PLCgamma produces the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). The latter, a calcium mobilizer, promotes release of Ca^{2+} from the endoplasmic reticulum, whereas DAG recruits and activates several protein kinase C isoforms (PKC), a calcium dependent kinase, which in turn phosphorylates multiple downstream targets [131].

The PI3K-to-AKT Pathway The PI3K/AKT pathway can be sparked by ERBB receptors either directly or indirectly. The p85 regulatory subunit of PI3K interacts with phosphorylated residues of activated receptors by means of its SH2 domain, which allows translocation of this kinase to the cell membrane and its subsequent activation. Alternatively, PI3K can be activated by RAS [132]. Subsequently, the p110 catalytic subunit of PI3K phosphorylates PIP2, thereby producing phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3) in the inner aspect of the plasma membrane [133]. PI(3,4,5)P3 recruits to the plasma membrane proteins that harbor a PH domain, such as PDK1, and PKB, called also AKT. Several studies demonstrated that PDK1 phosphorylates AKT, rendering it active, and consequently, AKT acts on downstream targets [134]. Using an analysis of single-cell protein concentrations in MCF10A cells, rather than population averages, one group showed cell-to-cell variability in PI3K activity. EGF stimulation elicited a bimodal response of AKT activation, which was correlated with cellular PI3K protein levels, such that only cells with elevated PI3K in a population activate AKT [135]. This might serve as a protective mechanism against senescence and cancer.

Signal Transducer and Activator of Transcription Pathway Signal transducer and activator of transcription (STATs) are transcription factors, which in their inactive state reside as monomers in the cytoplasm. Diverse RTKs can phosphorylate and activate them, triggering their dimerization, which is dependent on the interaction between the SH2 domain of one monomer and a phosphorylated tyrosine in the C-terminal portion of the other one [136]. Translocation to the nucleus ensues, where STATs promote transcription of target genes [137] or interact with other transcriptional regulators like c-FOS and c-JUN [138]. The EGF signaling pathway leads to the activation of STAT1, STAT3, and STAT5, which play a role in cancer [139]. For instance, STAT3 has been implicated in the induction of MMP-1 by EGF, which is necessary for tumor formation of bladder cancer cells in mice [140].

PTPN9, a phosphatase with tumor suppressor attributes, impairs ERBB2 signaling by the downregulation of STAT3 and STAT5 [135].

The MAPK Pathways The mitogen-activated protein kinase (MAPK) pathways show a layered structure, where three tiers of kinases are phosphorylated sequentially, ultimately resulting in the phosphorylation of a multiplicity of targets, located in different subcellular compartments but mainly in the nucleus [141]. Thus, an MAPK is activated by MAPK-kinase (MAPKK), which in turn is the target of a MAPKKK (or MAP3K). Oftentimes, additional layers exist, such as MAP4K, a component upstream of MAP3K, and MAPKAPK, which can be activated by MAPK [142]. There are four recognized MAPK cascades, which can be triggered by members of the ERBB family of receptors. They are named according to their MAPK module: ERK1/2, JNK, p38, and ERK5.

ERK1/2 Following the activation of the small GTPase RAS by RTKs, a phosphorylation cascade ensues. RAS recruits MAP3K/RAF to the cell membrane and activates them. Subsequently, serine phosphorylation of the activation loop of the downstream MAPKK (MEK) take place [143]. Thereafter, activation of ERK1/2 occurs as a result of threonine and tyrosine phosphorylation of the Thr-Glu-Tyr motif [144]. Later on, ERK1/2 target an array of substrates in diverse subcellular compartments, such as AP-1 in the nucleus.

The JNK Pathway JNK proteins were originally identified as protein kinases that phosphorylate c-JUN in cells exposed to UV-radiation [145]. While the JNK cascade is predominantly triggered under stress conditions, it is nowadays known that it might as well be induced by growth factors [146]. A diversity of MAP4Ks and MAP3Ks bind to different scaffold proteins such as JIPs, which promote specificity and facilitate the signal transmission [147]. Ensuing activation of MAPKKs like MKK4 and MKK7 leads to tyrosine and threonine phosphorylation of JNK1-3 in their Thr-Pro-Tyr motif [148]. Activation of the JNK pathway leads to phosphorylation of transcription factors such as c-Jun, Jun-A, Jun-B, Elk, and ATF-2, among others [149].

The p38 Pathway p38alpha was originally described as a protein that undergoes rapid phosphorylation upon stimulation with lipopolysaccharides [150]. Currently, four splice variants of this protein are known. It is also known that the p38 cascade is primarily initiated by stress signals, but growth factors might ignite it as well. Diverse MAP3K molecules activate MAPKKs, such as MKK3 and MKK6, which afterward result in threonine and tyrosine p38 phosphorylation within the Thr-Gly-Tyr motif. Under stress conditions, p38 phosphorylates EGFR on multiple serine and threonine residues, to promote clathrin-mediated endocytosis of EGFR into RAB5 containing endosomes [151]. Therefore, by depriving the cell from mitogenic receptors on the membrane, p38-induced EGFR endocytosis promotes cell death

The ERK5 Cascade ERK5, also known as Big MAP kinase (Bmk-1), exhibits a higher molecular weight than other MAPKs and therefore its name. The ERK5 cascade is initiated by both stress signals and growth factors [152]. Upon stimulation,

MEKK2 and MEKK3 phosphorylate MEK5 at serine and threonine residues. Like other MAPKs, ERK5 is activated by means of threonine and tyrosine phosphorylation within its Thr-Glu-Tyr motif and targets several molecules, such as MYC, FOS, and the serum- and glucocorticoid-inducible kinase (SGK) [153].

4.4.5 Regulation of Transcription by ERBB Signaling

The robustness of the ERBB system is mainly attained by positive and negative feedback loops that control the biological outcomes, and these are often defective in cancer [154]. These regulatory systems can be classified using a temporal criterion as immediate or late. Immediate regulatory mechanisms encompass events occurring posttranslationally, such as phosphorylation, ubiquitination, downregulation of microRNAs (miRs), and receptor endocytosis, the latter being regarded as the major negative regulator of the system. Late regulatory loops entail de novo synthesis of new proteins and other components, as detailed below.

4.4.5.1 Regulation by microRNAs

Regulation by microRNAs (miRs) constitutes a recently described regulatory layer of signaling via the ERBB network. For instance, in retinal cells miR-7 and Yan display reciprocal suppression allowing mutually exclusive expression. Thus, Yan inhibits miR-7 in progenitor cells and miR-7 suppresses Yan in photoreceptor cells. Following EGF stimulation, EGFR elicits ERK-mediated degradation of Yan, derepressing transcription of miR-7 and promoting a photoreceptor differentiation [155]. A recent study using a genome-wide approach demonstrated that EGF stimulation of mammary cells is followed by a rapid decrease in the abundance of a group of 23 miRs, called immediate downregulated microRNAs (ID-miRs). In resting cells, ID-miRs suppress transcription of immediate early genes (IEG), such as FOS or EGR1, and upon stimulation the rapid decrease of ID-miRs allows upregulation of the IEGs [156]. Importantly, the ID-miR subset was found to be repressed in breast and in brain tumors.

4.4.5.2 Immediate Early Genes

Growth factor stimulation activates a complex transcriptional response, characterized by temporally defined waves of gene expression. The interplay among three different subsets of genes shapes the ultimate cellular outcome. In this way, transcripts induced up to 45 min after stimulation are termed immediate early genes (IEGs), the ones upregulated from 45 min to 2 h belong to the group of delayed early genes (DEGs), and lastly, the family of secondary response genes (SRGs) comprise the transcripts that are elevated after 2 h. IEGs were initially described as

a group of mRNAs that were upregulated in BALB/c 3T3 fibroblasts after serum or growth factor stimulation [157]. The group includes genes like the AP-1 components FOS and JUN and early growth response 1 (EGR1) gene. Since IEGs are rapidly induced, their promoters are ready for activation, which is achieved by diverse mechanisms. For instance, it was recently shown that in the unstimulated state, promoters of IEGs are in a permissive state; thus, they display positive histone modifications and they preassemble RNA polymerase II [158]. Under unstimulated conditions, the DSIF/NELF complex negatively regulates IEG transcription, by stalling Pol II at promoter proximal regions. Upon stimulation, NELF detachment allows prompt IEG induction [159].

4.4.5.3 Delayed Early Genes

Following the rapid surge of IEG, a fast downregulation of this cluster of genes ensues. An analysis of the kinetics of transcriptional events triggered by growth factors showed that the decline of the IEG wave is controlled by another group of transcripts called delayed early genes (DEGs) [160]. The DEG subset comprises molecules like dual specificity phosphatases (DUSPs) as well as DNA- and RNA-binding proteins. Some DEGs are induced by IEGs; examples of this scenario are dual specificity phosphatases (DUSPs) that are transcriptionally induced by IEGs [161] and restrain signaling by MAPK pathways. Zfp36 is an RNA-binding protein belonging to the DEG subset. It binds to AU-rich elements in the 3′ untranslated region (UTR) of unstable mRNAs to promote their exosomal degradation [162]. In line with their tumor suppressor role, DEGs are significantly repressed in a variety of epithelial malignancies [160].

4.4.5.4 Secondary Response Genes

Secondary response genes encode transcripts involved in processes that modify cellular phenotypes, such as the epithelial to mesenchymal transition (EMT). Upon growth factor stimulation, there is an induction of transcription factors, such as Snail, Slug, ZEB-1, Twist, and Goosecoid, which ultimately lead to the repression of E-cadherin [163–166] and in some cases also to the upregulation of N-cadherin [167]. Members of the miR-200 and miR-205 families regulate the expression of ZEB1 and SIP1, transcriptional repressors of E-cadherin. Downregulation of these microRNAs induces EMT upon growth factor stimulation, and their expression is attenuated in invasive breast cancer of the mesenchymal phenotype [168]. Within this context, stimulation with EGF also leads to a reorganization of the actin cytoskeleton. Upon exposure of mammary cells to EGF, the expression of tensin-3 is downregulated, while an upregulation of Cten takes place, resulting in collapse of stress fibers and the promotion of cell motility [127].

4.4.6 EGFR Endocytosis and Signaling from Endosomes

4.4.6.1 Receptor Endocytosis

RTKs like ERBB proteins can undertake several internalization routes, which involve clathrin-independent endocytosis (CIE) and clathrin-mediated endocytosis (CME), which is the preferred endocytic route for ERBBs. Well-described CIE portals include caveolin-mediated endocytosis, which like CME is dependent on dynamin, and endocytic routes reliant on cortical actin, such as macropinocytosis [169]. It was demonstrated that following stimulation with low doses of EGF, EGFR is internalized primarily via the clathrin-dependent way, whereas stimulation with high EGF doses drives internalization through a clathrin-independent route [170]. The first step of CME is the nucleation of clathrin-coated pits, which entails recruitment of AP2 and clathrin triskelia at phosphatidylinositol-4,5-biphosphate (PIP2)-rich membrane microdomains. Similar protein complexes, such as AP-1, AP-3, and AP-4, were shown to be associated with clathrin-coated pits [171]. Alongside, a wealth of adaptors, named clathrin-associated sorting proteins (CLASPs), are engaged in this scaffolding process. Accordingly, molecules such as epsins, Eps15, stonin, and disabled2 contribute to the recognition of cargo, some of which make use of ubiquitin-binding domains, which recognize the ubiquitinated form of EGFR [172]. Later, the GTPase dynamin closes the base of the nascent vesicle, and its radial twisting, along with longitudinal tension produced by the actin cytoskeleton, severs the bud from the plasma membrane [173]. Following disassembly of the clathrin lattice, which is assisted by auxilin and Hsc70 [174, 175], endocytic vesicles fuse with early endosomes that later mature into multivesicular bodies (MVBs). Afterwards, MVBs fuse with lysosomes to enable degradation of their contents. Trafficking of endocytic cargo is assisted by an array of molecules, such as RAB GTPases, phosphoinositides, and endosomal sorting complexes required for transport (ESCRTs). The four ESCRTs are multiprotein complexes that aid in the formation of MVBs and harbor a multiplicity of ubiquitin-binding domains, which recognize ubiquitinated cargo and mediate its trafficking [176].

4.4.6.2 Signaling from Endosomes

While EGFR downregulation is regarded mainly as a desensitizing mechanism, depriving the cell from functional receptors on the cell surface, there is evidence suggesting that signaling can be generated in endosomal platforms. Early studies demonstrated the presence of active EGFRs, along with various downstream effectors, such as SHC, GRB2, and SOS in endosomes, and this was linked to RAS activation [177, 178]. Later on, using a dominant-negative mutant of dynamin (K44A), it was shown that cells defective in clathrin-dependent receptor endocytosis exhibited suppression of MAPK pathway activation. Nonetheless, the exact contribution of endosomal signaling is yet to be fully understood. For example, upon activation of the EGFR, a pool of MEK2 accumulated in a subset of endosomes.

However, activated MEK was detected only at the plasma membrane and not in endosomes, and MEK2-containing endosomes did not contain active EGFRs, suggesting that the recruitment of MEK2 to endosomes can be a part of the negative feedback regulation of the EGFR-MAPK pathway [179].

4.4.7 Unique Features of EGFR

4.4.7.1 Cross Talk with Other Receptor Systems

As mentioned above, EGFR can be trans-activated by GPCRs, which activate metalloproteinases to release active EGF-like ligands. Several similar cross talk pathways exist: a cross talk was reported between an oncogenic mutant form of EGFR, EGFRvIII, and c-MET, the hepatocyte growth factor receptor, and a significant effect was shown upon dual inhibition of both receptors [180]. EGFR was also shown to be trans-activated by growth hormone (GH), with the Janus kinase (JAK) as a mediator [181]. Specifically, growth-hormone-induced activation of MAPK requires phosphorylation of tyrosines on EGFR, but not its own intrinsic tyrosine kinase activity. Thus, the role of EGFR in signaling by GH is to be phosphorylated by JAK2, thereby providing docking sites for GRB2 and activating MAPK. Similarly, autocrine secretion of prolactin stimulates tyrosine phosphorylation of HER2 by JAK2, which provides docking sites for GRB2 and stimulates the RAS-MAPK cascade [182].

4.4.7.2 EGFR Activation in Cancer

Recent sequencing efforts of the genomes of a large spectrum of human tumors have repeatedly confirmed that the gene encoding EGFR is one of the most frequently mutated genes in non-hematopoietic cancer (see Table 4.1). Importantly, all EGFR mutants are characterized by an intact, or hyperactivated, kinase domain. Elevated EGFR levels were originally reported in specimens of carcinomas of the lung and head/neck tumors [8]. Later studies confirmed overexpression of EGFR, with or without gene amplification, in a larger spectrum of carcinomas, and in some cancers overexpression can serve as an indicator for recurrence or for shorter patient survival. An important example is provided by brain tumors of glial origin: EGFR gene amplification occurs in approximately 50 % of high-grade gliomas. In addition, large and small deletions often associate with gene amplification in glioblastoma [183]. The discovery of point mutations within the kinase domain of EGFR of non-small cell lung cancer was motivated by the results of international clinical trials that tested EGFR's specific kinase inhibitors, which showed that patients enrolled in Japan had significantly higher response rates [184]. Following this and similar observations, several groups reported activating mutations within the kinase domain of EGFR; these mutations often associate with patient response to kinase inhibitors [185–188]. Unfortunately, patients treated with EGFR kinase inhibitors show initial responses but their tumors eventually progress. Analyses of patients

with acquired resistance to the drugs discovered that progressing tumors contained, in addition to a primary drug-sensitive mutation, a secondary, resistance-conferring mutation leading to a substitution of threonine for methionine at position 790 [189]. Despite the high frequency of EGFR's genetic aberrations, it seems that the more frequent mode of EGFR activation in tumors entails autocrine loops. In these cases, tumor cells co-express both EGFR and one or more EGF-like ligands, leading to deregulated receptor activation, which might reduce patient response to conventional and targeted cancer therapies [190].

4.4.7.3 Cancer Therapeutic Strategies Targeting EGFR

The mAb cetuximab is a human-mouse anti-EGFR chimeric antibody, which has shown efficacy in colorectal cancer, in combination with chemotherapy [191], and in head and neck cancer, in combination with radiotherapy (Fig. 4.3) [192, 193].

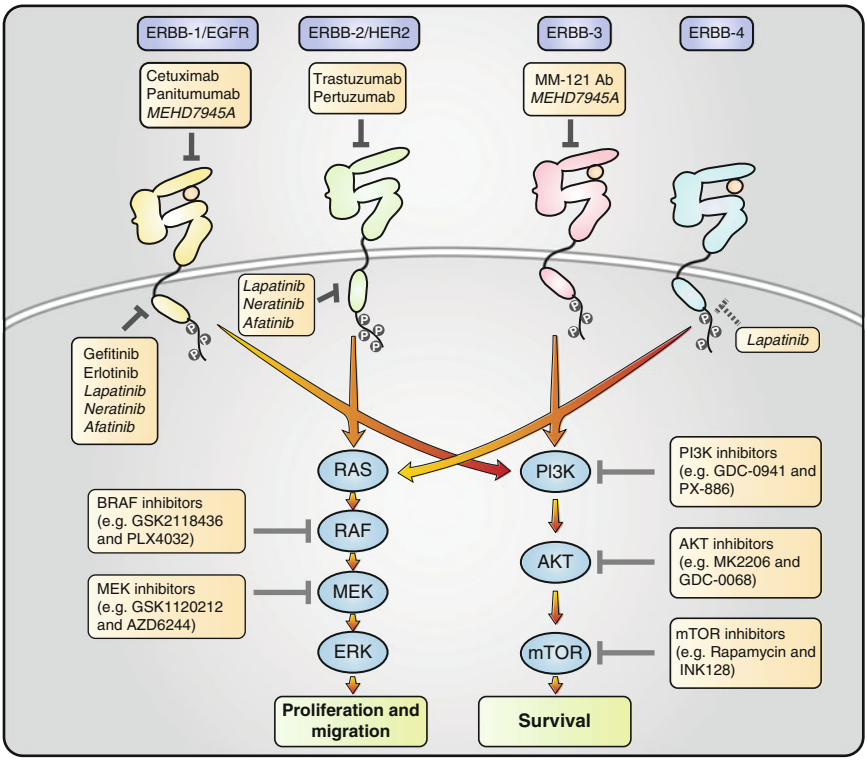

Fig. 4.3 Molecular-targeted agents intercepting ERBB receptors and downstream effectors. The ERBB family of receptors is illustrated, as well as the major components of the MAPK and PI3K pathways. *Yellow boxes* highlight specific drugs, some still under ongoing clinical trials. Drugs in *italics* indicate the existence of more than one molecular target (e.g., lapatinib intercepting EGFR and HER2). Note that the suffix *mab* refers to monoclonal antibodies and the suffix *nib* refers to kinase inhibitors

This antibody displaces EGF and other ligands of EGFR, as well as prevents receptor dimerization and downstream signaling. Panitumumab is a fully human antibody specific to EGFR, which is effective and well tolerated in colorectal cancer. Interestingly, the more recently developed anti-EGFR antibody, nimotuzumab, is unique; treatment of patients is characterized by the absence of severe adverse effects (e.g., skin rash), which commonly associate with cetuximab and panitumumab [194]. Low toxicity of nimotuzumab might be due to intermediate affinity and incomplete abrogation of the active conformation of EGFR [195]. Several EGFR kinase inhibitors have been developed, but unlike monoclonal antibodies, their target specificity is strictly concentration dependent. The first inhibitor, called erbstatin, was identified in the medium of Gram-positive bacteria [196]. Later inhibitors were designed according to the backbone of erbstatin, and they were able to block proliferation of EGFR-overexpressing cells [197]. Early preclinical studies using a quinazoline-based inhibitor, called gefitinib, demonstrated inhibition of several types of tumor xenografts. Following trials in lung cancer [198], gefitinib was approved for non-small cell lung cancer patients. Another inhibitor, erlotinib, is approved for treatment of both lung and pancreatic cancer. Yet a third inhibitor, lapatinib, blocks both EGFR and HER2 in vitro and in patients [199]. Following clinical trials that showed efficacy of lapatinib in combination with another drug, capecitabine, the drug was approved for treatment of HER2 positive breast cancer patients [200].

4.5 HER2/ERBB2

Pioneering studies showed that the introduction of DNA of cell lines derived from ethylnitrosourea-induced rat neuro/glioblastomas resulted in the transformation of NIH-3T3 fibroblasts. This work led to the discovery of a 185 kDa cell-surface phosphoprotein, closely related to EGFR, termed NEU [201]. Independently other groups cloned a novel RTK with high similarity to EGFR [14, 15], which was found to be amplified in human adenocarcinoma of the salivary gland [202]. Later on, chromosomal mapping studies revealed that this novel RTK demonstrated to correspond to NEU, HER2, or ERBB2 [203]. Chromosomal mapping determined that the HER2 locus is located on human chromosome 17 at q21, which was found to be amplified in gastric cancer and in mammary cancer cell lines [204, 205]. Later analysis of 189 breast cancer specimens revealed that one third of human breast tumors displayed an amplification of the HER2 gene and a corresponding overexpression of the HER2/ERBB2 protein [17]. Moreover, amplification of HER2 was a predictor of overall survival in breast cancer patients. Following HER2's identification, the hunt for its putative ligand was led by several groups [206–208]; however, further studies confirmed that the ligands they isolated, called neuregulins or heregulins, were actually ERBB3 and ERBB4 ligands that activated HER2 by means of heterodimerization (see below). Structural studies of HER2 confirmed that no direct ligand binding is required to achieve an active conformation that allows dimerization and subsequent signaling [121, 122].

4.5.1 The HER2 Gene

4.5.1.1 Promoter Structure

The human ERBB2 promoter includes typical TATA (nucleotides −22 to −26) and CCAAT (−71 to −75) boxes [209]. In addition to the major start site at +1, a weaker upstream site is centered at −69 and is activated mainly in ERBB2-overexpressing cells. The two main transcription factors that were shown to bind to the promoter and to be essential for its activity are AP-2 (GCTGCAGGC) at −213 to −221 and ETS (GAGGAA) at −33 to −28 [210]. Importantly, ETS and also Sp1 are induced by HER2/ERBB2 activation, which enables a positive feedback loop [211].

4.5.1.2 The HER2 Amplicon

The GRB7 gene, which resides in a chromosomal location close to HER2, was found to be amplified in concert with HER2 in breast cancer cell lines and also overexpressed in breast tumors [212]. Later on, systematic surveys of genes in the 17q12 amplicon in breast cancer unveiled a subset of transcripts consistently displaying an increased copy number and expression in 17q12-amplified cell lines. Thus, the smallest region of amplification (SRA), which harbored HER2, spanned 280 kb and contained GRB7, MLN64, and PNMT [213, 214]. Nowadays it is known that the HER2 amplicon is quite complex and encompasses many other genes. HER2 amplification is accompanied by severe chromosome 17 rearrangements [215]. For instance, the TOP2A gene, which codes for a topoisomerase, is amplified in 40 % of breast tumors where an amplification of HER2 is often observed [216]. Importantly, TOP2A is a molecular target of anthracyclines and is associated with a favorable outcome to anthracycline-based adjuvant chemotherapy in HER2-amplified breast cancer [217, 218]. Recently a novel SRA including HER2 and TOP2A was confirmed, which besides TOP2A contained four additional genes, CASC3, CDC6, RARA, and SMARCE1 [219], but the contribution of these genes to HER2-mediated tumorigenicity remains open. Interestingly, amplification of the 17q11–q12 region is present in a wide array of human cancers from diverse tissue origin, such as the ovary [220], endometrium [221], prostate [222], and stomach [223].

4.5.1.3 Transcriptional Regulation of HER2

Analysis of the HER2 gene locus in mammary cancer cell lines showed that mRNA and protein overexpression occurs also in the absence of gene amplification, suggesting the existence of additional regulatory mechanisms [224]. PEA3, a DNA-binding protein encoded by a gene of the ETS family, binds to the HER2 promoter and downregulates its transcription. Moreover, intratumoral injection of PEA3 in

mice harboring HER2 overexpressing tumors inhibited growth of the xenografts [225]. Conversely, Foxp3 inactivates the HER2 promoter; tumors that lacked the wild-type Foxp3 allele overexpressed HER2, and Foxp3 is frequently deleted or downregulated in human breast cancer [226]. Congruently, female mice heterozygous for a Foxp3 mutation displayed high propensity to develop malignancies, particularly breast cancer. Other transcriptional modulators of HER2 exist, such as GATA4, which transcriptionally represses HER2, but HER2 activates GATA4 in breast cancer cells [227]. The 5′ UTR of HER2 has an upstream open reading frame (uORF), which controls translation of the coding region. In mammary cancer cells overexpressing HER2, an interplay between the 5′ and 3′ UTR of HER2 modulates translation of the transcript; the 3′UTR containing a translational derepression element (TDE) overrides the inhibitory activity of the uORF in the 5′UTR [228].

4.5.2 The HER2 Protein

The overall HER2 domain structure is similar to that of EGFR (see Fig. 4.1). The major difference is the ligand-binding domain, which cannot bind any known EGF-like ligand. HER2's main phosphorylation sites are Y1023, Y1139, Y1196, Y1221/2, and Y1248, with the last three being docking sites for the SHC adaptor protein (see Fig. 4.1). Approximately 50 % of HER2-overexpressing tumors exhibit enhanced expression of truncated HER2 proteins lacking most of the extracellular portion. These are generated by alternative translation initiation or by ADAM10-mediated proteolytic cleavage of the extracellular domain, resulting in a 95 kDa carboxyl-terminal receptor fragment (p95) [229–235]. Importantly, these variants exhibit enhanced transforming potential in animal models [232]. Another HER2 variant, HER2Δ16, likely generated by alternative mRNA splicing, lacks a small part of the extracellular domain. HER2Δ16 potently couples to downstream signaling pathways and correspondingly exhibits transforming activity [236].

4.5.3 HER2 Activation and Signaling

Due to its nonautonomous nature, HER2 cannot form homodimers, and it can only heterodimerize with other ERBB family members (Fig. 4.4). As described above, the ligand-binding domain of HER2 adopts a conformation similar to the active, ligand-bound EGFR. This conformation allows HER2 to interact and dimerize with other ERBB receptors without ligand binding. By using a variety of experimental strategies, such as intracellular antibodies, to block the delivery of HER2 to the cell surface or by expressing selected pairs of ERBB proteins in ERBB-naïve myeloid cells, it became clear that HER2 enhances signaling instigated when ligands activate the other three ERBB proteins [237–242]. Interestingly, the most potent dimerization partner of HER2 is the second nonautonomous member ERBB3 [243],

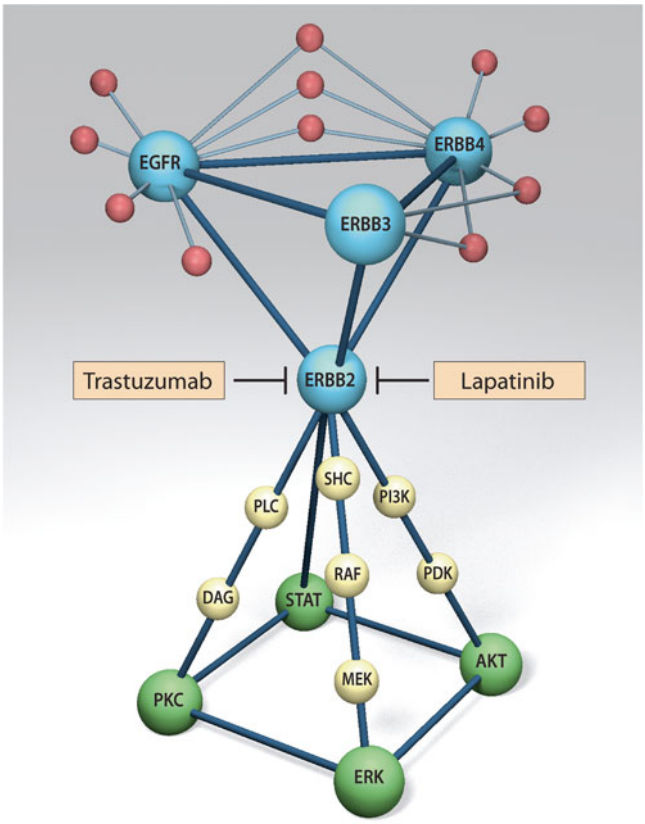

Fig. 4.4 Centrality of HER2/ERBB2. The variant ERBB network of tumors overexpressing HER2 (e.g., significant fractions of breast and gastric cancer) is schematically presented. *Red balls* indicate ERBB ligands, *blue balls* indicate ERBB receptors, and *yellow* and *green balls* represent downstream signaling pathways. Due to overexpression, HER2 becomes the preferred heterodimer partner of the other three ERBB proteins. However, this centrality of HER2 leads also to network fragility: HER2 targeting, either by an antibody like trastuzumab or by a tyrosine kinase inhibitor, such as lapatinib, might intercept the majority of growth factor signals, thereby repress HER2-overexpressing tumors

establishing a potent "oncogenic unit" [242]. Co-expression of the two proteins reconstitutes a high-affinity receptor for neuregulins [244]. In addition, within ERBB3-ERBB2 heterodimers, the cytoplasmic tail of ERBB3 strongly activates the phosphatidylinositol 3′-kinase (PI3K) and the AKT survival pathway, whereas HER2 signals through the ERK/MAPK pathway. This combination of signaling pathways enhances cell proliferation and evasion of apoptosis [245, 246]. The ability of HER2 to enhance growth factor signaling in the context of heterodimers has been attributed to both augmentation of signal generation and delay of signal desensitization. Thus, HER2 not only enhances ligand-binding affinity by decreasing the rate of ligand dissociation from heterodimers [247], but it also broadens the

repertoire of activating ligands of a specific heterodimer. For example, within heterodimers, ERBB3 can bind EGF [248]. In addition, unlike other RTKs, HER2 only weakly interacts with CBL and undergoes ubiquitination [249]; hence heterodimers display a tendency to recycle rather than undergo sorting for degradation in lysosomes [250, 251].

4.5.4 Nuclear Translocation of HER2 and Other Family Members

Several lines of evidence propose that HER2, as well as other family members, might bypass the membrane and cytoplasmic signaling cascades and shortcut to the nucleus, to regulate gene expression. Nuclear shuttling refers to both full-length receptors [252–254] and the truncated forms of HER2 [229] and ERBB4 [255]. ERBB proteins can serve either as nuclear shuttles for transcription factors or they can directly associate with nuclear proteins [254, 256–262]. Nuclear translocation can be instigated by direct ligand binding [252, 254, 263–265], transmodulation [266, 267], or ectodomain cleavage [255]. In addition, shuttling to the nucleus seems to involve receptor endocytosis [268, 269] and the endoplasmic reticulum-associated degradation system [270, 271]. On the target side, nuclear entry may involve the nuclear import machinery, since all ERBB proteins exhibit a nuclear localization sequence (NLS) in their intracellular domain [252, 257, 258, 272, 273], and their import depends upon nuclear transport receptors [266, 268, 269]. Once in the nucleus, ERBBs can act as transcriptional co-activators, corepressors, or binders of proteins involved in DNA synthesis and repair [254, 256–262]. For example, nuclear EGFR might activate the transcription of cyclin D1, c-MYB, and COX-2, genes associated with cell proliferation and the nitric oxide pathway [257].

4.5.5 Unique Features of HER2: Anti-HER2 Cancer Therapy

Unlike other ERBB family members, the mature form of ERBB2 was found to be a robust client of the heat shock protein 90 (HSP90). HSP90 inhibition results in a dramatic reduction of HER2/ERBB2 levels due to decreased stability [274, 275], but so far this unique feature of HER2 has not been translated to a drug targeting HER2-overexpressing tumors. Importantly, other efforts to pharmacologically target HER2 in cancer have been quite successful. Initial studies showed that administration of an anti-HER2 antibody inhibited HER2-transformed murine fibroblasts [276]. This was followed by the development of a monoclonal antibody, targeting HER2's extracellular domain, which inhibited proliferation of human mammary cancer cells overexpressing HER2 [277]. Subsequently, the murine monoclonal antibody was humanized [278]. Clinical trials that made use of the humanized antibody, called trastuzumab, demonstrated efficacy in HER2-amplified metastatic breast cancer, which led to the approval of trastuzumab in this setting [279, 280].

Especially effective is the application of this antibody in first-line chemotherapy [281] and also in the neoadjuvant setting [282]. More recent trials showed that trastuzumab in combination with chemotherapy can confer improved survival in patients with HER2-amplified gastric or gastroesophageal junction cancer [283]. Unlike trastuzumab, which is specific to HER2, the small molecule GW572016/lapatinib was shown to dually inhibit EGFR and HER2 (see Fig. 4.3). Treatment with lapatinib also reduced ERK and AKT activation in cell lines and in human tumor xenografts [284]. A clinical trial involving patients with metastatic malignancies overexpressing EGFR and/or HER2 proved that the use lapatinib was associated with a positive response [199]. Moreover, the addition of lapatinib to chemotherapy was proven to be superior to chemotherapy alone in patients with HER2-positive metastatic breast cancer [200], which led to the approval of lapatinib in combination with chemotherapy in this group of patients [285].

4.6 ERBB3

More than 20 years ago, two groups reported the cloning of a novel member of the ERBB family of receptors, called HER3 or ERBB3, which was found to be overexpressed in breast cancer cell lines [19, 286]. Afterwards, a study that expressed bovine ERBB3 in insect cells showed that this receptor displays an impaired tyrosine kinase activity [20]. Crystal structure studies of the ERBB3 kinase domain confirmed locking in an inactive conformation [95]. Nevertheless, this receptor can act as a partner within heterodimers. Accordingly, ERBB3 was found to be overexpressed with other members of the ERBB family in various malignancies, such as breast cancer and bladder cancer [287, 288].

4.6.1 The ERBB3 Gene

4.6.1.1 Promoter Structure

Cloning and sequencing of the human ERBB3 promoter revealed that it is GC rich, lacks a TATA box, and harbors multiple transcription start sites. DNase I footprinting showed the existence of binding sites for several transcription factors, such as AP-2. Indeed, AP-2 promotes high ERBB3 expression in mammary carcinoma cells [289]. Later on, it was shown that all three AP-2 isoforms were capable of transactivating the ERBB3 promoter in AP-2-deficient HepG2 cells [290]. Shortly after, studies, in which AP-2Δ, a dominant-negative variant of AP-2, was overexpressed, confirmed these findings. Transfection of AP-2Δ inhibited ERBB3 promoter activity and transcription, which was coupled with decreased cellular proliferation [291]. Another study demonstrated that estradiol inhibited ERBB3 expression in breast cancer cells, while estrogen inhibition enhanced it [54].

4.6.1.2 mRNA Structure

The ERBB3 gene is located in chromosome 12q13. This 23.2 kb gene is transcribed as a 5.8 kb product [19]. Multiple alternative transcripts have been identified. A 1.4 kb transcript was identified in MKN45 gastric cancer cells. The first 140 amino acids encoded by this alternative transcript are identical to the N-terminus of the full-length ERBB3, but the following 43 amino acids are different [292]. The ratio between the 1.4 kb and the larger ERBB3 transcripts was found to be low in ERBB3-overexpressing cells and high in cells expressing low levels of this receptor, implying that transcription of short ERBB3 products inhibits synthesis of full-length transcripts. Later on, four other ERBB3 transcripts, of 1.6, 1.7, 2.1, and 2.3 kb, were isolated from ovarian carcinoma cell lines. It was subsequently shown that truncated ERBB3 transcripts, such as p85-soluble ERBB3, binds NRGs with high affinity, and therefore the secreted protein inhibits binding of the ligand to cell-surface ERBB3 [293].

4.6.1.3 Transcriptional Regulation

Suppression of ERBB2 phosphorylation by lapatinib is hindered by an upregulation of ERBB3, which is mediated by FOXO3a. The transcription factor FOXO3a has 3 putative binding sites in the promoter of ERBB3. Downregulation of FOXO3 is coupled to defective upregulation of ERBB3 mRNA following lapatinib treatment [294]. ZNF217 is another transcription factor implicated in the transcriptional regulation of ERBB3. ZNF217 induces ERBB3 gene expression and is amplified in 20 % of breast tumors [295]. A nuclear variant of ERBB3, named $ERBB_{80kDa}$, has been described. $ERBB3_{80kDa}$ lacks the extracellular domain and regulates cellular proliferation through the binding with the promoter of Cyclin D1, enhancing its expression. $p14^{ARF}$, a tumor suppressor, binds $ERBB3_{80kDa}$ and sequesters it in the nucleolus, hence inhibiting its interaction with the cyclin D promoter [296]. MicroRNAs participate as well in the regulation of ERBB3 signaling. For instance, miR-205 expression is decreased in breast tumors. It directly targets and inhibits ERBB3 expression, with the subsequent interference of the PI3K/AKT pathway [297].

4.6.2 The ERBB3 Protein

4.6.2.1 Amino Acid Sequence

The protein encoded by the human ERBB3 gene is 1,342-amino acid long and can be found in [19].

Domain Structure

Like other members of the ERBB family, ERBB3 possesses an extracellular ligand-binding domain, a transmembrane domain, and an intracellular kinase domain. The extracellular region has four subdomains (I–IV) (See Fig. 4.1). Crystallization of the un-liganded extracellular domain of ERBB3 showed that domains II and IV interact via a short hairpin loop of domain II, which restrains the interaction of domain I and III, the ligand-binding domains, and maintains ERBB3 in a locked conformation [298]. Interestingly, domain I of the extracellular region of ERBB3 has a stronger contribution to ligand binding than domain III, contrary to EGFR, in which domain III is the dominant one. Domain I of the extracellular region of ERBB3 harbors a high-affinity site for ligand binding at a low pH, which might compensate for the weak interactions of NRGs with domain III [299].

Posttranslational Modifications

A yeast two-hybrid screen identified Nrdp1 (neuregulin receptor degradation protein-1), a ring-finger, B-box, coiled-coil (RBCC) protein that interacts with ERBB3. Nrdp1 controls the steady-state levels of ERBB3, ubiquitinating this receptor in the absence of growth factors [300]. Nrdp1 undergoes self-ubiquitination and is degraded by the proteasome. The deubiquitinating enzyme USP8 was identified as an interactor of Nrdp1 by affinity chromatography. Upon stimulation of cells with NRG-1, threonine phosphorylation of USP8 ensues, which mediates its stability. Subsequently, USP8 deubiquitinates Nrdp1, rescuing it from degradation [301]. In MCF7 mammary cancer cells, silencing of USP8 or Nrdp1 prevents degradation of ERBB3 following NRG-1 stimulation [302].

Phosphorylation Sites and Their Functions

Synthetic phosphopeptide analysis unveiled six binding sites for the p85 subunit of PI3K harbored in the C-terminal domain of ERBB3, namely, Y1054, Y1197, Y1222, Y1260, Y1276, and Y1289 (see Fig. 4.1). The same study showed that phosphorylated Y1328, contained in the motif NPXY, serves as a binding site for SHC [303]. An early study identified Y1199 as the main GRB7-binding site and Y1262 as a minor GRB7-binding site [304]. Nevertheless, an analysis using quantitative proteomics showed that Y1199 and Y1262 are interaction sites for GRB2 instead [129]. ERBB3 harbors three YEY motifs, where two tyrosines are separated by a glutamic acid residue, i.e., Y1197 and Y1199, Y1222 and Y1224, and Y1260 and 1262. However, the identity of their common interactors, if any, remains unknown [129].

4.6.3 ERBB3 Ligands

Neuregulin-1 was at first identified in the medium of RAS-transformed cells as a protein that can induce phosphorylation of ERBB2 [208]. Later on, NRG-2, NRG-3, and NRG-4 were identified [305–307]. While NRG-1 and NRG-2 interact with ERBB3 and ERBB4, NRG-3 and NRG-4 bind exclusively to ERBB4. Due to alternative splicing and promoters, there is an array of NRG isoforms. Studies using mice with mutations that abolish different isoforms showed that they have distinct functions [308]. Specific isoforms play critical roles in the development of heart and neural tissues and have been linked with pathologies like breast cancer, schizophrenia, and multiple sclerosis [309]. Some neuregulins are synthesized as transmembrane proteins, called proNRGs. These precursors are cleaved and processed by metalloproteases, producing truncated segments bound to the cell membrane. The precursor of NRGbeta (isoform 2a) was found to be partially resistant to cleavage, while the 4a isoform was processed in a more efficient manner. The processing of proNRGalpha2c was defective in fibroblasts derived from mice devoid of an active TACE/ADAM17, implying that this metalloprotease is involved in the processing of NRGs [310]. Expression of ADAM19 (Meltrin beta) and expression of NRGs occurred at the same stages of mouse embryogenesis in the dorsal root ganglia. Moreover, overexpression of the wild-type ADAM19 enhanced secretion of soluble NRGs, while transfection of a dominant-negative form of this protein inhibited NRGs processing [311].

4.6.4 ERBB3 Activation and Signaling

4.6.4.1 ERBB3 Dimerization

Tumor cells that overexpress ERBB2 frequently show elevated tyrosine phosphorylation of ERBB3, and inducible silencing of ERBB2 in SKBR3 mammary cancer cells led to decreased phosphotyrosine content of ERBB3 [312]. Especially critical are ERBB2/ERBB3 heterodimers, which play an important role in breast cancer. Inhibition of ERBB3 expression with E3, an artificial transcription factor, demonstrated that ERBB2 requires ERBB3 to induce breast cancer cell proliferation [242]. Likewise, ERBB3 knockdown in HER2 overexpressing cells inhibited tumor growth of xenografts [313]. Heterodimerization of ERBB3 with EGFR [314] and with ERBB4 has also been suggested. It was demonstrated that intestinal epithelial cells of mice with intestine-specific ERBB3 knockout showed inhibition of ERBB4 expression. Moreover, inhibition of ERBB3 led to a loss of ERBB4 expression in colon cancer cells and to subsequent apoptosis [315].

4.6.4.2 Phosphorylation

Because the kinase activity of ERBB3 is extremely weak [316], it depends on other ERBB family members. Although ERBB3 dimerizes preferentially with ERBB2 [239], coupling with EGFR and ERBB4 also occurs. Among other kinases that alter ERBB3 phosphorylation status is SRC. c-SRC overexpression enhances tyrosine phosphorylation of ERBB2 and ERBB3 and triggers downstream signaling in mammary cancer cells [317]. The intracellular BRK tyrosine kinase, implicated in breast oncogenesis, was also shown to interact with ERBB3 and phosphorylate it, followed by enhanced signaling via the PI3K/AKT pathway [318].

4.6.4.3 Pathway Activation

Inactivation of HER2/ERBB2 in breast cancer cells results in loss of activity of ERBB3, along with the attenuation of MAPK and PKB/AKT signaling, hinting that ERBB2/ERBB3 dimers play a key role in the ERBB signaling network [312]. The carboxyl-terminal domain of ERBB3 possesses a motif capable of interaction with the SH2 domain of p85-PI3K. Indeed, following EGF stimulation of A431 cells, which express EGFR and ERBB3, PI3K co-precipitated preferentially with ERBB3 [319]. Consistent with these findings, ERBB3 was found to be efficiently coupled with the PI3K signaling pathway, in line with the potent PI3K activation triggered by ERBB2/ERBB3 heterodimers [320]. In addition, genetic and pharmacologic ablation of ERBB3 inhibits the growth of PI3K/AKT-dependent mammary tumors in mice [321]. SHC constitutes another binding partner of ERBB3 [303, 322]. NRG stimulation of NIH-3T3 murine fibroblasts ectopically expressing ERBB3 led to signaling through the MAPK and PI3K/AKT pathways. Mutation of the SHC-binding site of ERBB3 inhibited stimulation of the MAPK pathway [323]. Association of ERBB3 with other signaling molecules, such as BRK, c-SRC, ABL1, ABL2, and RASA1N, has also been reported [322, 324].

4.6.4.4 Cross Talk with Other Receptor Systems

Cross talk between ERBB3 and the interferon alpha signaling complex has been demonstrated. IFNalpha-induced ERBB3 phosphorylation has been shown in the KAS-6/1 multiple myeloma cell line. Moreover, depletion of ERBB3 in these cells inhibited cell proliferation upon stimulation with IFNalpha. Nevertheless, a physical association between ERBB3 and IFNalpha receptor could not be demonstrated [325]. Shortly after, it was shown that IFNalpha-induced ErBB3 transactivation is mediated by the Janus kinase family members Tyk2 and Jak1 [326].

4.6.5 ERBB3 Internalization, Processing, and Attenuation

Unlike EGFR, ligand-activated ERBB3 is directed to a short endocytic pathway leading to recycling rather than to degradation in lysosomes [327, 328]. Nevertheless, following stimulation with NRG, the deubiquitinating enzyme USP8 is stabilized, leading to accumulation of Nrdp1, an E3 ubiquitin ligase, and subsequent ERBB3 ubiquitination and degradation [302]. Furthermore, analysis of human breast tumors overexpressing ERBB3 showed a decreased Nrdp1 expression [329]. Other molecules potentiate ERBB3 signaling by altering its endocytic trafficking. For instance, Muc4, a transmembrane mucin, induces translocation of ERBB2/HER2 and ERBB3 to the cell membrane by suppressing their internalization [330].

4.6.6 Unique Features of ERBB3

4.6.6.1 ERBB3 and Cancer

ERBB3 is involved in malignancies of diverse tissue origins. Immunohistochemical analysis of 130 primary and 87 metastatic melanomas demonstrated a high expression of ERBB3 in these tumors [331]. Moreover, high ERBB3 expression predicted a short patient survival. Treatment with monoclonal antibodies directed toward this receptor inhibited NRG-induced transformation of melanoma cell lines. Similarly, ERBB3 has been implicated in colon cancer. ERBB3 mRNA was identified in 55 % of human colorectal carcinomas, compared to 22 % of normal colon mucosa specimens [332]. Additionally, high expression of ERBB3 in colonic tumors correlated with a shorter patient survival [333]. A systematic analysis of all RTKs in NSCLC tumors showed that high ERBB3 expression can predict decreased survival [334]. Likewise, an autocrine loop involving NRG1 and ERBB3 was identified in ovarian cancer cell lines and tumors [335]. Ablation of this loop led to longer survival times in an ovarian cancer mouse model [336]. Pilocytic astrocytoma and radiation-induced pediatric glioblastomas are among other malignancies associated with ERBB3 overt signaling [337].

4.6.6.2 Therapeutic Implications of ERBB3

Along with immunological attempts to target ERBB3 in tumors due to its ability to drive proliferation and migration of tumor cells [338], ERBB3 is attractive because it appears to play pivotal roles in the development of drug resistance in a variety of malignancies. For example, TKIs display limited activity on HER2-amplified breast tumors, due to relocalization of ERBB3 to the membrane, where phosphorylation occurs, along with reduced receptor dephosphorylation. Consistent with these findings, silencing of ERBB3 restored response to TKIs [339]. Another report showed that a TKI-sensitive lung cancer cell line acquired resistance to this therapy, by means of MET amplification and activation of ERBB3 signaling [340].

4.7 ERBB4

4.7.1 The ERBB4 Gene

4.7.1.1 Promoter Structure

ERBB4 expression correlates with estrogen receptor (ER) expression in breast cancer. Accordingly, ERBB4 was found to be an estrogen-inducible gene. Moreover, three potential estrogen response elements (ERE) are located within the ERBB4 promoter. Estrogen leads to association of ER with the ERBB4 intracellular domain, thereby establishing an ERBB4 autocrine loop regulated by estrogen [341]. An analysis of the ERBB4 promoter identified a novel promoter polymorphism that appears to increase the risk for breast and colorectal cancer [342]. On the other hand, a CpG island has been identified in the ERBB4 promoter, and there is an inverse correlation between ERBB4 expression and promoter methylation. Elevated ERBB4 promoter methylation was associated with worse prognosis of breast cancer patients. These findings suggest that ERBB4 might play a tumor suppressor role, which is inhibited in mammary tumors by promoter hypermethylation [343].

4.7.1.2 mRNA Structure

The ERBB4 gene is located in the long arm of chromosome 2q33.3-34 [344] and it spans 1.16 Mb and 28 exons [345]. ERBB4 is subject to alternative splicing, and four splicing variants have been described. JM-a and JM-b have dissimilar extracellular juxtamembrane domains, which differ by the inclusion of exons 16 and 15b, respectively. Exon 16 of JM-a encodes a 23-residue sequence, which harbors a TACE cleavage site, while exon 15b of JM-b encodes a 13-amino acid sequence which lacks the TACE cleavage site [346]. The ERBB4 CYT isoforms originate from the inclusion or exclusion of exon 26. The CYT-1 variant harbors, while CYT-2 lacks a 16-residue sequence of the cytoplasmic tail of ERBB4, which constitutes a binding site for PI3K. Tissues like the heart or breast express preferentially the ERBB4 CYT-1 variant, while the CYT-2 variant is the dominant form in neural tissues and in kidney [347].

4.7.1.3 Transcriptional Regulation

Silencing of KRAB-associated protein 1 (Kap1) by shRNA was associated with increased levels for ERBB4, both at the mRNA and protein levels, which identified Kap1 as a corepressor of ERBB4 transcription. The interplay between Kap1 and ERBB4 regulates the stabilization of Mdm2 and subsequently of p53 [348]. The WW domain harboring Yes-associated protein (YAP) interacts with ERBB4 and serves as a co-transcriptional activator for ERBB4 C-terminal fragment (CTF). Such interaction is mediated by the WW domain of YAP and a carboxyl-terminal

PPXY motif of ERBB4. Two isoforms of YAP exist, YAP1 and YAP2, with one and two WW domains, respectively. YAP2 exerts a higher activation activity than YAP1 [349]. WWOX, a WW domain-containing oxidoreductase, interacts with ERBB4 and opposes the action of YAP [350].

4.7.2 The ERBB4 Protein

The fourth member of the ERBB family was identified in 1990. ERBB4 encodes a 180 kD protein primarily expressed in breast cancer cell lines and the normal skeletal muscle, heart, and brain [21].

4.7.2.1 Processing of ERBB4

The four ERBB4 isoforms, JM-a, JM-b, CYT-1, and CYT-2, differ either in their extracellular domains or in their C-terminal cytoplasmic tails [346]. The JM-a isoform is cleaved by TACE, while the JM-b isoform undergoes no known processing [351]. Ectodomain shedding of ERBB4 is followed by intramembrane proteolysis by a gamma secretase, followed by the production of an intracellular domain (ICD) [352]. The ICD translocates to the nucleus, where it regulates transcription of target genes [255]. The JM-a isoforms, which can be cleaved by TACE, were found to be overexpressed together with TACE in breast tumors. Overexpression of the JM-a CYT-2 receptor was associated with higher ERBB4 phosphorylation and breast cancer cell proliferation [353]. ERBB4 ectodomain was found in 75 % of breast tumors versus 18 % of paired normal tissues, indicating that ectodomain shedding is increased in breast malignancies [354].

4.7.2.2 Domain Structure

Like other ERBBs, ERBB4 is composed of an extracellular domain harboring the ligand-binding site, a juxtamembrane, a transmembrane, and an intracellular domain (see Fig. 4.1). The intracellular region harbors the tyrosine kinase domain and multiple phosphotyrosine sites accountable for the interactions with molecules containing SH2 and PTB domains. The CYT-1 variant contains a PI3K-binding site, while the CYT-2 variant lacks it. The ICD harbors also 3 PPXY motifs, which are responsible for the interaction with WW-containing proteins, such as YAP [355].

4.7.2.3 Posttranslational Modifications

ERBB4 stability is regulated by posttranslational modifications, such as ubiquitination. Several E3 ligases targeting this receptor have been identified, such as AIP4/Itch, a WW domain-containing E3 ligase, which ubiquitinates ERBB4, leading to

its degradation [356]. WWP1 is also involved in the regulation of ERBB4 levels; however, it preferentially modifies the CYT-1 variants of ERBB4 [357]. An siRNA screen of all WWP1 family members identified HECW1/NEDL1 as a novel E3 involved in the regulation of ERBB4 [358]. ERBB4 possesses multiple binding sites for GRB2 or common GRB2/SHC sites, such as phosphorylated Y1162, Y1188, Y1202, Y1208, Y1221, Y1242, and Y1268 (see Fig. 4.1). The Y733 and Y1284 phosphosites were SHC specific. The ERBB4 phosphotyrosine interaction partners show a high degree of overlap with those of EGFR. ERBB4 and EGFR interact with STAT5, with Y984 being the recruitment site in the case of ERBB4. One PI3K-binding site, Y1056, is present in ERBB4 [129].

4.7.3 ERBB4-Specific Ligands

4.7.3.1 Ligand Structure and Cleavage

ERBB4 interacts with a multitude of ligands: HB-EGF, epiregulin, and betacellulin bind both ERBB4 and EGFR. While NRG-1 and NRG-2 can engage with both ERBB3 and ERBB4, NRG-3 and NRG-4 exclusively bind with ERBB4.

HB-EGF The heparin-binding EGF-like growth factor is an 86-amino acid-long product of processing of a 206-amino acid-long transmembrane precursor [359]. ADAM9, ADAM10, ADAM12, and ADAM17 have been implicated in HB-EGF processing [87]. Interestingly, pro-HB-EGF acts as a diphtheria toxin receptor [360]. Monoclonal antibodies targeting the EGF-like domain of this ligand suppress processing and inhibit cell proliferation [361].

Epiregulin This 46-amino acid peptide is cleaved by ADAM17 from a 162-residue-long transmembrane precursor [362]. Besides its well-described role in oncogenesis, this growth factor plays roles in other pathologies. Epiregulin controls homeostasis of human epidermal keratinocytes, and mice lacking this growth factor develop chronic dermatitis [363, 364]. A recent study showed that epiregulin expression is induced by *M. tuberculosis* in a MYD88- and TLR2-dependent manner. Moreover, a polymorphism of epiregulin was associated with a higher susceptibility for *M. tuberculosis* infection [365].

Betacellulin Betacellulin (BTC) was originally identified in medium conditioned by pancreatic tumor cells derived from mice [366]. It is produced as a 178-amino acid-long precursor, pro-BTC, which is processed to an 80-amino acid mature form by ADAM10 [367]. It was originally shown to have mitogenic action on vascular smooth muscle and retinal pigment epithelial cells. Recently, studies have shown that betacellulin is a potent angiogenesis inducer and can promote proliferation of pancreatic beta cells by activation of ERBB1 and ERBB2 [368]. In vivo studies showed that betacellulin stimulates neurogenesis and neural stem cell proliferation in mice [369].

4.7.4 ERBB4 Activation and Signaling

4.7.4.1 Dimerization

ERBB4 is capable of forming homo- and heterodimers with the three other ERBB receptors. ERBB4 heterodimerization with ERBB2 increases its response to ligand stimulation. Thus, it was shown that co-expression of ERBB2 and ERBB4 in 32D cells, which do not express ERBB receptors endogenously, enhanced their mitogenic response to EGF and TGFalpha stimulation [370]. Likewise, co-expression of ERBB4 and EGFR in NIH3T3 murine fibroblasts induced their transformation [371]. Interestingly, loss of ERBB3 in the intestinal epithelium of mice and in human colon cancer cells can lead to depletion of ERBB4 and subsequent apoptosis, suggesting that the ERBB3-ERBB4 heterodimer contributes to colon oncogenesis [315].

4.7.4.2 Phosphorylation

Upon ligand binding, ERBB4 can be autophosphorylated or transphosphorylated by other members of the ERBB family. Nevertheless, phosphorylation of the ERBB4 JM-a CYT-2 variant can occur in a ligand-independent manner [353]. Expression of constitutively active H-, K-, and N-Ras in PC12 cells induced ERBB4 phosphorylation in a ligand-independent way. However, using a kinase-inactive ERBB4 mutant, it was demonstrated that ERBB4 phosphorylation was still dependent on an intact kinase activity of ERBB4 [372].

4.7.4.3 Pathway Activation

Conditional ablation of ERBB4 in the mammary epithelium resulted in the inhibition of STAT5 activation and consequently in reduced expression of STAT5-regulated genes, such as WAP and Csnb [373]. Nuclear translocation of STAT5A depends on nuclear shuttling of the ERBB4's ICD. These findings suggest that the ICD acts as a nuclear chaperone for STAT5A [273]. Importantly, the different isoforms of ERBB4 display signaling differences. For example, the CYT-2 variant, which lacks the PI3K-binding site, is able to induce cellular proliferation, but not chemotaxis or survival [374]. On the other hand, the CYT-1 variant was shown to enhance survival of medulloblastoma cells by the activation of PI3K/AKT signaling pathway [375].

4.7.4.4 Cross Talk with Other Receptor Systems

A proteomic analysis identified ERBB2 and ERBB4 as interactors of the endothelin receptor type A (ET_A), a GPCR. Upon stimulation of myocytes with the GPCR agonist endothelin-1 (ET-1), inhibition of ERBB4 and AKT phosphorylation, both

induced by NRG-1beta, took place. Moreover, stimulation with ET-1 abolished the negative inotropic action of NRG1beta, thus enhancing the heart contractility [376]. Co-localization of ERBB4's ICD and ERalpha was evidenced in the nucleus. Moreover, the ICD enhances ERalpha-mediated transcription at ER-specific elements in neuronal and nonneuronal cells. In addition, estrogen stimulation triggers the interaction of extranuclear ERalpha and ERBB4 and induces nuclear translocation of the ICD [341].

4.7.5 ERBB4 Internalization and Attenuation

ERBB4 internalization was observed following NRG-1 stimulation of neurons and was shown to be dependent on the tyrosine kinase activity of ERBB4. Moreover, blockage of ERBB4 endocytosis inhibits NRG-1-driven phosphorylation of ERK and AKT in neurons [377]. The different ERBB4 isoforms display distinct endocytosis regulation. While CYT-1 variants were found to co-localize with RAB5- and RAB7-positive endosomes and are promptly endocytosed, CYT-2 variants undergo inefficient endocytosis. A PPXY domain, which is carried by the CYT-1 isoforms, but absent in CYT-2 variants, is required for ubiquitination and endocytosis of CYT-1 variants. The PPXY domain acts as the recruitment site for the WW domain E3 ligase Itch, which monoubiquitinates the CYT-1 isoforms [378].

4.7.6 Unique Features of ERBB4

Immunohistochemical analysis of thyroid tissue samples showed overexpression of ERBB3 and ERBB4 in papillary thyroid carcinoma [379]. ERBB4 was also shown to be overexpressed in ovarian granulosa tumor cells [380] and to play a role in breast carcinogenesis [378]. Furthermore, ERBB4 is frequently overexpressed together with ERBB2 in childhood ependymoma [381] and in medulloblastoma [382]. However, other studies showed that ERBB4 is downregulated in tumors. Indeed, overexpression of ERBB4 in breast cancer was found to be associated with increased patient survival [383]. Results on the prognostic significance of ERBB4 levels in breast cancer are conflicting [378]. Thus, contrary to other ERBB proteins, the prognostic significance of ERBB4 might be context dependent.

Receptor at a glance: EGFR

Chromosome location	7p12
Gene size (bp)	23,760 bases
mRNA size (5′, ORF, 3′)	5.616 bp, CDS:247-3,879 (NM_005228.3)
Amino acid number	1,210 aa
kDa	134,277 Da

(continued)

Posttranslational modifications	Phosphorylation, ubiquitination, acetylation, and neddylation
Domains	– Extracellular, ligand-binding domain – Transmembrane domain – Tyrosine kinase domain – Carboxyl-terminal tail
Ligands	EGF, TGFalpha, HB-EGF, epiregulin, epigen, betacellulin, and amphiregulin
Known dimerizing partners	EGFR, HER2/ERBB2, ERBB3, ERBB4
Pathways activated	MAPK, PI3K-AKT, STATs, PLCgamma
Tissues expressed	Breast, lung, skin, gastrointestinal system, brain, and more
Human diseases	Glioblastoma multiforme, lung and breast cancer, psoriasis
Knockout mouse phenotype	Embryonic lethality. Lung maturation abnormalities, skin defects, newborn mice with open eyes, abnormal heart and brain development

Receptor at a glance: ERBB2

Chromosome location	17q11.2-q12
Gene size (bp)	40,523 bases
mRNA size (5′, ORF, 3′)	4,624 bp, CDS: 239-4,006
Amino acid number	1,255 aa
kDa	137,910 Da
Posttranslational modifications	Phosphorylation, ubiquitination
Domains	– Extracellular domain – Transmembrane domain – Tyrosine kinase domain – Carboxyl-terminal tail
Ligands	None
Known dimerizing partners	EGFR, ERBB2, ERBB3, ERBB4
Pathways activated	MAPK, PI3K-AKT, STATs, PLCgamma
Tissues expressed	Gastrointestinal, respiratory, and genitourinary tracts, breast, skin, placenta
Human diseases	Ovarian, lung, breast, gastric, and colorectal carcinomas
Knockout mouse phenotype	Embryonic lethality, heart and neural abnormal development, dilated cardiomyopathy

Receptor at a glance: ERBB3

Chromosome location	12q13
Gene size (bp)	23.2 kb
mRNA size	5,765 bp, CDS: 277-4308 (NM_001982.3)
Amino acid number	1342 aa
kDa	148,098 Da
Posttranslational modifications	Phosphorylation, ubiquitination

(continued)

Domains	– Extracellular ligand-binding region – Transmembrane domain – Tyrosine kinase domain – Carboxyl-terminal tail
Ligands	NRG-1, NRG-2
Known dimerizing partners	EGFR, ERBB2, ERBB3, ERBB4
Pathways activated	SHC, PI3K-AKT
Tissues expressed	Gastrointestinal, reproductive, respiratory, and urinary systems, nervous and endocrine systems, skin
Human diseases	Melanoma, colon cancer, breast cancer, NSCLC, ovarian cancer, lethal congenital contractural syndrome type 2 (LCCS2)
Knockout mouse phenotype	Embryonic lethality; neural crest cell migration defects; cardiac, cerebellar, and cranial ganglia defects; stomach and pancreas defective development; abnormal breast morphogenesis

Receptor at a glance: ERBB4

Chromosome location	2q33.3-34
Gene size (bp)	1.16 Mb
mRNA size (5′, ORF, 3′)	11,941 bp, CDS: 99-4025 (JM-a/CYT1) (NM_005235.2)
Amino acid number	1,308 aa
kDa	146,808 Da
Posttranslational modifications	Phosphorylation, ubiquitination
Domains	– Extracellular ligand-binding region – Transmembrane domain – Tyrosine kinase domain – Carboxyl-terminal tail
Ligands	NRG-1, NRG-2, NRG-3, NRG-4, HB-EGF, betacellulin, epiregulin
Known dimerizing partners	EGFR, ERBB2, ERBB3, ERBB4
Pathways activated	MAPK, PI3K-AKT, STATs, PLC-Gamma
Tissues expressed	Brain, skeletal muscle, heart, parathyroid, spleen, testis, breast, and kidney
Human diseases	Breast cancer, schizophrenia, medulloblastoma, lung cancer, gastric and colorectal cancer, and melanoma
Knockout mouse phenotype	Embryonic lethality, neural crest cell migration defects with abnormal heart development, abnormal architecture of cranial nerves, defective mammary gland morphogenesis and maturation, and bronchopulmonary dysplasia

References

1. Cohen S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J Biol Chem. 1962;237:1555–62.
2. Das M, Miyakawa T, Fox CF, Pruss RM, Aharonov A, Herschman HR. Specific radiolabeling of a cell surface receptor for epidermal growth factor. Proc Natl Acad Sci USA. 1977;74(7):2790–4.

3. Ushiro H, Cohen S. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. J Biol Chem. 1980;255(18):8363–5. Epub 1980/09/25.
4. Brugge JS, Erikson E, Erikson RL. The specific interaction of the Rous sarcoma virus transforming protein, pp60src, with two cellular proteins. Cell. 1981;25(2):363–72. Epub 1981/08/01.
5. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, et al. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature. 1984;307(5951):521–7.
6. Yamamoto T, Nishida T, Miyajima N, Kawai S, Ooi T, Toyoshima K. The erbB gene of avian erythroblastosis virus is a member of the src gene family. Cell. 1983;35(1):71–8.
7. Ozanne B, Richards CS, Hendler F, Burns D, Gusterson B. Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. J Pathol. 1986;149(1):9–14. Epub 1986/05/01.
8. Hendler FJ, Ozanne BW. Human squamous cell lung cancers express increased epidermal growth factor receptors. J Clin Invest. 1984;74(2):647–51. Epub 1984/08/01.
9. Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, et al. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature. 1985;313(5998):144–7.
10. Sainsbury JR, Farndon JR, Harris AL, Sherbet GV. Epidermal growth factor receptors on human breast cancers. Br J Surg. 1985;72(3):186–8.
11. Xu YH, Richert N, Ito S, Merlino GT, Pastan I. Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. Proc Natl Acad Sci USA. 1984;81(23):7308–12.
12. Sato JD, Kawamoto T, Le AD, Mendelsohn J, Polikoff J, Sato GH. Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. Mol Biol Med. 1983;1(5):511–29. Epub 1983/12/01.
13. Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. Cancer Res. 1984;44(3):1002–7. Epub 1984/03/01.
14. Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science. 1985;230(4730):1132–9.
15. Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, et al. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. Nature. 1986;319(6050):230–4.
16. Bargmann CI, Hung MC, Weinberg RA. The neu oncogene encodes an epidermal growth factor receptor-related protein. Nature. 1986;319(6050):226–30. Epub 1986/01/16.
17. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235(4785):177–82.
18. Klapper LN, Glathe S, Vaisman N, Hynes NE, Andrews GC, Sela M, et al. The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. Proc Natl Acad Sci USA. 1999;96(9):4995–5000. Epub 1999/04/29.
19. Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA. Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc Natl Acad Sci USA. 1989;86(23):9193–7. Epub 1989/12/01.
20. Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway 3rd KL. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci USA. 1994;91(17):8132–6. Epub 1994/08/16.

21. Plowman GD, Culouscou JM, Whitney GS, Green JM, Carlton GW, Foy L, et al. Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. Proc Natl Acad Sci USA. 1993;90(5):1746–50. Epub 1993/03/01.
22. Aroian RV, Koga M, Mendel JE, Ohshima Y, Sternberg PW. The let-23 gene necessary for Caenorhabditis elegans vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature. 1990;348(6303):693–9. Epub 1990/12/20.
23. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol. 2006;7(7):505–16.
24. Citri A, Alroy I, Lavi S, Rubin C, Xu W, Grammatikakis N, et al. Drug-induced ubiquitylation and degradation of ErbB receptor tyrosine kinases: implications for cancer therapy. EMBO J. 2002;21(10):2407–17. Epub 2002/05/15.
25. Moghal N, Sternberg PW. The epidermal growth factor system in Caenorhabditis elegans. Exp Cell Res. 2003;284(1):150–9.
26. Shilo BZ. Signaling by the Drosophila epidermal growth factor receptor pathway during development. Exp Cell Res. 2003;284(1):140–9. Epub 2003/03/22.
27. Lynch JA, Peel AD, Drechsler A, Averof M, Roth S. EGF signaling and the origin of axial polarity among the insects. Curr Biol. 2010;20(11):1042–7.
28. Budi EH, Patterson LB, Parichy DM. Embryonic requirements for ErbB signaling in neural crest development and adult pigment pattern formation. Development. 2008; 135(15):2603–14.
29. Goishi K, Lee P, Davidson AJ, Nishi E, Zon LI, Klagsbrun M. Inhibition of Zebrafish epidermal growth factor receptor activity results in cardiovascular defects. Mech Dev. 2003;120(7):811–22.
30. Sibilia M, Wagner EF. Strain-dependent epithelial defects in mice lacking the EGF receptor. Science. 1995;269(5221):234–8.
31. Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, et al. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature. 1995; 376(6538):337–41.
32. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature. 1995;378(6555):394–8. Epub 1995/11/23.
33. Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, et al. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature. 1995; 378(6555):390–4. Epub 1995/11/23.
34. Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C. Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature. 1997;389(6652):725–30. Epub 1997/10/24 21:29.
35. Fowler KJ, Walker F, Alexander W, Hibbs ML, Nice EC, Bohmer RM, et al. A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. Proc Natl Acad Sci USA. 1995;92(5):1465–9.
36. Jones FE, Stern DF. Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation. Oncogene. 1999; 18(23):3481–90.
37. Jones FE, Welte T, Fu XY, Stern DF. ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation. J Cell Biol. 1999;147(1):77–88.
38. Real FX, Rettig WJ, Chesa PG, Melamed MR, Old LJ, Mendelsohn J. Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation. Cancer Res. 1986;46(9):4726–31.
39. De Potter CR, Van Daele S, Van de Vijver MJ, Pauwels C, Maertens G, De Boever J, et al. The expression of the neu oncogene product in breast lesions and in normal fetal and adult human tissues. Histopathology. 1989;15(4):351–62.
40. Prigent SA, Lemoine NR, Hughes CM, Plowman GD, Selden C, Gullick WJ. Expression of the c-erbB-3 protein in normal human adult and fetal tissues. Oncogene. 1992;7(7):1273–8.

41. Srinivasan R, Poulsom R, Hurst HC, Gullick WJ. Expression of the c-erbB-4/HER4 protein and mRNA in normal human fetal and adult tissues and in a survey of nine solid tumour types. J Pathol. 1998;185(3):236–45.
42. Sporn MB, Todaro GJ. Autocrine secretion and malignant transformation of cells. N Engl J Med. 1980;303(15):878–80. Epub 1980/10/09.
43. Buonanno A. The neuregulin signaling pathway and schizophrenia: from genes to synapses and neural circuits. Brain Res Bull. 2010;83(3–4):122–31. Epub 2010/08/07.
44. Law AJ, Kleinman JE, Weinberger DR, Weickert CS. Disease-associated intronic variants in the ErbB4 gene are related to altered ErbB4 splice-variant expression in the brain in schizophrenia. Hum Mol Genet. 2007;16(2):129–41. Epub 2006/12/14.
45. Cannella B, Hoban CJ, Gao YL, Garcia-Arenas R, Lawson D, Marchionni M, et al. The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model for multiple sclerosis. Proc Natl Acad Sci USA. 1998;95(17):10100–5. Epub 1998/08/26.
46. Jost M, Class R, Kari C, Jensen PJ, Rodeck U. A central role of Bcl-X(L) in the regulation of keratinocyte survival by autocrine EGFR ligands. J Invest Dermatol. 1999;112(4):443–9. Epub 1999/04/14.
47. Bhagavathula N, Nerusu KC, Fisher GJ, Liu G, Thakur AB, Gemmell L, et al. Amphiregulin and epidermal hyperplasia: amphiregulin is required to maintain the psoriatic phenotype of human skin grafts on severe combined immunodeficient mice. Am J Pathol. 2005;166(4):1009–16. Epub 2005/03/29.
48. Clement CM, Thomas LK, Mou Y, Croslan DR, Gibbons GH, Ford BD. Neuregulin-1 attenuates neointimal formation following vascular injury and inhibits the proliferation of vascular smooth muscle cells. J Vasc Res. 2007;44(4):303–12. Epub 2007/04/18.
49. Xu G, Watanabe T, Iso Y, Koba S, Sakai T, Nagashima M, et al. Preventive effects of heregulin-beta1 on macrophage foam cell formation and atherosclerosis. Circ Res. 2009;105(5):500–10. Epub 2009/08/01.
50. Ishii S, Xu YH, Stratton RH, Roe BA, Merlino GT, Pastan I. Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc Natl Acad Sci USA. 1985;82(15):4920–4.
51. Schuch R, Agelopoulos K, Neumann A, Brandt B, Burger H, Korsching E. Site-specific chromatin immunoprecipitation: a selective method to individually analyze neighboring transcription factor binding sites in vivo. BMC Res Notes. 2012;5:109.
52. Nishi H, Nishi KH, Johnson AC. Early growth response-1 gene mediates up-regulation of epidermal growth factor receptor expression during hypoxia. Cancer Res. 2002; 62(3):827–34.
53. Ludes-Meyers JH, Subler MA, Shivakumar CV, Munoz RM, Jiang P, Bigger JE, et al. Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol Cell Biol. 1996;16(11):6009–19.
54. Bates NP, Hurst HC. Transcriptional regulation of type I receptor tyrosine kinases in the mammary gland. J Mammary Gland Biol Neoplasia. 1997;2(2):153–63.
55. Scartozzi M, Bearzi I, Mandolesi A, Giampieri R, Faloppi L, Galizia E, et al. Epidermal growth factor receptor (EGFR) gene promoter methylation and cetuximab treatment in colorectal cancer patients. Br J Cancer. 2011;104(11):1786–90.
56. Schmiegel W, Roeder C, Schmielau J, Rodeck U, Kalthoff H. Tumor necrosis factor alpha induces the expression of transforming growth factor alpha and the epidermal growth factor receptor in human pancreatic cancer cells. Proc Natl Acad Sci USA. 1993;90(3):863–7.
57. Brandt B, Meyer-Staeckling S, Schmidt H, Agelopoulos K, Buerger H. Mechanisms of egfr gene transcription modulation: relationship to cancer risk and therapy response. Clin Cancer Res. 2006;12(24):7252–60.
58. Contessa JN, Bhojani MS, Freeze HH, Rehemtulla A, Lawrence TS. Inhibition of N-linked glycosylation disrupts receptor tyrosine kinase signaling in tumor cells. Cancer Res. 2008;68(10):3803–9.

59. Wang X, Gu J, Ihara H, Miyoshi E, Honke K, Taniguchi N. Core fucosylation regulates epidermal growth factor receptor-mediated intracellular signaling. J Biol Chem. 2006;281(5):2572–7.
60. Liu YC, Yen HY, Chen CY, Chen CH, Cheng PF, Juan YH, et al. Sialylation and fucosylation of epidermal growth factor receptor suppress its dimerization and activation in lung cancer cells. Proc Natl Acad Sci USA. 2011;108(28):11332–7.
61. Carpenter G, Cohen S. Epidermal growth factor. J Biol Chem. 1990;265(14):7709–12. Epub 1990/05/15.
62. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2(2):127–37. Epub 2001/03/17.
63. Levkowitz G, Waterman H, Ettenberg SA, Katz M, Tsygankov AY, Alroy I, et al. Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell. 1999;4(6):1029–40.
64. Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science. 1999;286(5438):309–12. Epub 1999/10/09.
65. Waterman H, Katz M, Rubin C, Shtiegman K, Lavi S, Elson A, et al. A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. EMBO J. 2002;21(3):303–13. Epub 2002/02/02.
66. Huang F, Sorkin A. Growth factor receptor binding protein 2-mediated recruitment of the RING domain of Cbl to the epidermal growth factor receptor is essential and sufficient to support receptor endocytosis. Mol Biol Cell. 2005;16(3):1268–81.
67. Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol. 2003;5(5):461–6.
68. Mosesson Y, Shtiegman K, Katz M, Zwang Y, Vereb G, Szollosi J, et al. Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. J Biol Chem. 2003;278(24):21323–6.
69. Huang F, Kirkpatrick D, Jiang X, Gygi S, Sorkin A. Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. Mol Cell. 2006;21(6):737–48.
70. Oved S, Mosesson Y, Zwang Y, Santonico E, Shtiegman K, Marmor MD, et al. Conjugation to Nedd8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases. J Biol Chem. 2006;281(31):21640–51. Epub 2006/06/01.
71. Duex JE, Sorkin A. RNA interference screen identifies Usp18 as a regulator of epidermal growth factor receptor synthesis. Mol Biol Cell. 2009;20(6):1833–44. Epub 2009/01/23.
72. McCullough J, Clague MJ, Urbe S. AMSH is an endosome-associated ubiquitin isopeptidase. J Cell Biol. 2004;166(4):487–92. Epub 2004/08/18.
73. Niendorf S, Oksche A, Kisser A, Lohler J, Prinz M, Schorle H, et al. Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking in vivo. Mol Cell Biol. 2007;27(13):5029–39. Epub 2007/04/25.
74. Pareja F, Ferraro DA, Rubin C, Cohen-Dvashi H, Zhang F, Aulmann S, et al. Deubiquitination of EGFR by Cezanne-1 contributes to cancer progression. Oncogene. 2011;31:4599–608. Epub 2011/12/20.
75. Suarez Pestana E, Tenev T, Gross S, Stoyanov B, Ogata M, Bohmer FD. The transmembrane protein tyrosine phosphatase RPTPsigma modulates signaling of the epidermal growth factor receptor in A431 cells. Oncogene. 1999;18(28):4069–79.
76. Flint AJ, Tiganis T, Barford D, Tonks NK. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. Proc Natl Acad Sci USA. 1997;94(5):1680–5.
77. Liu F, Chernoff J. Protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor. Biochem J. 1997;327(Pt 1):139–45. Epub 1997/11/14.

78. Iuliano R, Trapasso F, Le Pera I, Schepis F, Sama I, Clodomiro A, et al. An adenovirus carrying the rat protein tyrosine phosphatase eta suppresses the growth of human thyroid carcinoma cell lines in vitro and in vivo. Cancer Res. 2003;63(4):882–6.
79. Ruivenkamp CA, van Wezel T, Zanon C, Stassen AP, Vlcek C, Csikos T, et al. Ptprj is a candidate for the mouse colon-cancer susceptibility locus Scc1 and is frequently deleted in human cancers. Nat Genet. 2002;31(3):295–300.
80. Tarcic G, Boguslavsky SK, Wakim J, Kiuchi T, Liu A, Reinitz F, et al. An unbiased screen identifies DEP-1 tumor suppressor as a phosphatase controlling EGFR endocytosis. Curr Biol. 2009;19(21):1788–98. Epub 2009/10/20.
81. Kenny PA, Bissell MJ. Targeting TACE-dependent EGFR ligand shedding in breast cancer. J Clin Invest. 2007;117(2):337–45.
82. Anklesaria P, Teixido J, Laiho M, Pierce JH, Greenberger JS, Massague J. Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor alpha to epidermal growth factor receptors promotes cell proliferation. Proc Natl Acad Sci USA. 1990;87(9):3289–93.
83. Singh AB, Sugimoto K, Dhawan P, Harris RC. Juxtacrine activation of EGFR regulates claudin expression and increases transepithelial resistance. Am J Physiol Cell Physiol. 2007;293(5):C1660–8. Epub 2007/09/15.
84. Willmarth NE, Ethier SP. Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells. J Biol Chem. 2006;281(49):37728–37.
85. Higginbotham JN, Demory Beckler M, Gephart JD, Franklin JL, Bogatcheva G, Kremers GJ, et al. Amphiregulin exosomes increase cancer cell invasion. Curr Biol. 2011;21(9):779–86. Epub 2011/04/26.
86. Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. Curr Opin Cell Biol. 2009;21(2):177–84. Epub 2009/02/12.
87. Harris RC, Chung E, Coffey RJ. EGF receptor ligands. Exp Cell Res. 2003;284(1):2–13.
88. Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J, et al. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol. 2004;164(5):769–79. Epub 2004/03/03.
89. Pyka J, Glogowska A, Dralle H, Hoang-Vu C, Klonisch T. Cytoplasmic domain of proEGF affects distribution and post-translational modification of microtubuli and increases microtubule-associated proteins 1b and 2 production in human thyroid carcinoma cells. Cancer Res. 2005;65(4):1343–51. Epub 2005/03/01.
90. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV. Human transforming growth factor-alpha: precursor structure and expression in E coli. Cell. 1984;38(1):287–97.
91. Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, et al. An essential role for ectodomain shedding in mammalian development. Science. 1998;282(5392):1281–4.
92. Brown JR, Nigh E, Lee RJ, Ye H, Thompson MA, Saudou F, et al. Fos family members induce cell cycle entry by activating cyclin D1. Mol Cell Biol. 1998;18(9):5609–19.
93. Berasain C, Garcia-Trevijano ER, Castillo J, Erroba E, Lee DC, Prieto J, et al. Amphiregulin: an early trigger of liver regeneration in mice. Gastroenterology. 2005;128(2):424–32. Epub 2005/02/03.
94. Berasain C, Garcia-Trevijano ER, Castillo J, Erroba E, Santamaria M, Lee DC, et al. Novel role for amphiregulin in protection from liver injury. J Biol Chem. 2005;280(19):19012–20. Epub 2005/03/09.
95. Jura N, Endres NF, Engel K, Deindl S, Das R, Lamers MH, et al. Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. Cell. 2009;137(7):1293–307. Epub 2009/07/01.
96. Sahin U, Blobel CP. Ectodomain shedding of the EGF-receptor ligand epigen is mediated by ADAM17. FEBS Lett. 2007;581(1):41–4. Epub 2006/12/16.
97. Kochupurakkal BS, Harari D, Di-Segni A, Maik-Rachline G, Lyass L, Gur G, et al. Epigen, the last ligand of ErbB receptors, reveals intricate relationships between affinity and mitogenicity. J Biol Chem. 2005;280(9):8503–12.

98. Chang W, Upton C, Hu SL, Purchio AF, McFadden G. The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similarity to those encoding epidermal growth factor and transforming growth factor alpha. Mol Cell Biol. 1987;7(1):535–40. Epub 1987/01/01.
99. Blomquist MC, Hunt LT, Barker WC. Vaccinia virus 19-kilodalton protein: relationship to several mammalian proteins, including two growth factors. Proc Natl Acad Sci USA. 1984;81(23):7363–7. Epub 1984/12/01.
100. Tzahar E, Moyer JD, Waterman H, Barbacci EG, Bao J, Levkowitz G, et al. Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network. EMBO J. 1998;17(20):5948–63. Epub 1998/10/17.
101. Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature. 1996;379(6565):557–60.
102. Higashiyama S, Iwabuki H, Morimoto C, Hieda M, Inoue H, Matsushita N. Membrane-anchored growth factors, the epidermal growth factor family: beyond receptor ligands. Cancer Sci. 2008;99(2):214–20.
103. Civenni G, Holbro T, Hynes NE. Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells. EMBO Rep. 2003;4(2):166–71.
104. Razandi M, Pedram A, Park ST, Levin ER. Proximal events in signaling by plasma membrane estrogen receptors. J Biol Chem. 2003;278(4):2701–12. Epub 2002/11/08.
105. Blobel CP, Carpenter G, Freeman M. The role of protease activity in ErbB biology. Exp Cell Res. 2009;315(4):671–82. Epub 2008/11/18.
106. Mochizuki S, Okada Y. ADAMs in cancer cell proliferation and progression. Cancer Sci. 2007;98(5):621–8. Epub 2007/03/16.
107. Shintani Y, Higashiyama S, Ohta M, Hirabayashi H, Yamamoto S, Yoshimasu T, et al. Overexpression of ADAM9 in non-small cell lung cancer correlates with brain metastasis. Cancer Res. 2004;64(12):4190–6. Epub 2004/06/19.
108. Chung E, Graves-Deal R, Franklin JL, Coffey RJ. Differential effects of amphiregulin and TGF-alpha on the morphology of MDCK cells. Exp Cell Res. 2005;309(1):149–60.
109. Revillion F, Lhotellier V, Hornez L, Bonneterre J, Peyrat JP. ErbB/HER ligands in human breast cancer, and relationships with their receptors, the bio-pathological features and prognosis. Ann Oncol. 2008;19(1):73–80. Epub 2007/10/27.
110. Yarden Y, Schlessinger J. Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. Biochemistry. 1987;26(5):1443–51. Epub 1987/03/10.
111. Yarden Y, Schlessinger J. Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. Biochemistry. 1987;26(5):1434–42. Epub 1987/03/10.
112. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010; 141(7):1117–34. Epub 2010/07/07.
113. Gadella Jr TW, Jovin TM. Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. J Cell Biol. 1995;129(6):1543–58.
114. Clayton AH, Walker F, Orchard SG, Henderson C, Fuchs D, Rothacker J, et al. Ligand-induced dimer-tetramer transition during the activation of the cell surface epidermal growth factor receptor-A multidimensional microscopy analysis. J Biol Chem. 2005;280(34):30392–9. Epub 2005/07/05.
115. Liu P, Sudhaharan T, Koh RM, Hwang LC, Ahmed S, Maruyama IN, et al. Investigation of the dimerization of proteins from the epidermal growth factor receptor family by single wavelength fluorescence cross-correlation spectroscopy. Biophys J. 2007;93(2):684–98.
116. Wiesmann C, Ultsch MH, Bass SH, de Vos AM. Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. Nature. 1999;401(6749):184–8. Epub 1999/09/18.
117. Ferguson KM, Berger MB, Mendrola JM, Cho HS, Leahy DJ, Lemmon MA. EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol Cell. 2003;11(2):507–17.

118. Burgess AW, Cho HS, Eigenbrot C, Ferguson KM, Garrett TP, Leahy DJ, et al. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell. 2003;12(3):541–52.
119. Ogiso H, Ishitani R, Nureki O, Fukai S, Yamanaka M, Kim JH, et al. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. Cell. 2002;110(6):775–87.
120. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, et al. Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. Cell. 2002;110(6):763–73.
121. Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney Jr DW, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature. 2003;421(6924):756–60.
122. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, et al. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. Mol Cell. 2003;11(2):495–505.
123. Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell. 2006;125(6):1137–49.
124. Anderson D, Koch CA, Grey L, Ellis C, Moran MF, Pawson T. Binding of SH2 domains of phospholipase C gamma 1, GAP, and Src to activated growth factor receptors. Science. 1990;250(4983):979–82. Epub 1990/11/16.
125. Pawson T. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. Cell. 2004;116(2):191–203. Epub 2004/01/28.
126. Kavanaugh WM, Turck CW, Williams LT. PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. Science. 1995;268(5214):1177–9. Epub 1995/05/26.
127. Katz M, Amit I, Citri A, Shay T, Carvalho S, Lavi S, et al. A reciprocal tensin-3-cten switch mediates EGF-driven mammary cell migration. Nat Cell Biol. 2007;9(8):961–9. Epub 2007/07/24.
128. Schlessinger J, Lemmon MA. SH2 and PTB domains in tyrosine kinase signaling. Sci STKE. 2003;2003(191):RE12. Epub 2003/07/17.
129. Schulze WX, Deng L, Mann M. Phosphotyrosine interactome of the ErbB-receptor kinase family. Mol Syst Biol. 2005;1:2005.0008.
130. Margolis B, Li N, Koch A, Mohammadi M, Hurwitz DR, Zilberstein A, et al. The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma. EMBO J. 1990;9(13):4375–80. Epub 1990/12/01.
131. Bunney TD, Katan M. PLC regulation: emerging pictures for molecular mechanisms. Trends Biochem Sci. 2011;36(2):88–96. Epub 2010/09/28.
132. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature. 1994;370(6490):527–32. Epub 1994/08/18.
133. Dillon RL, Brown ST, Ling C, Shioda T, Muller WJ. An EGR2/CITED1 transcription factor complex and the 14-3-3sigma tumor suppressor are involved in regulating ErbB2 expression in a transgenic-mouse model of human breast cancer. Mol Cell Biol. 2007;27(24):8648–57.
134. Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, et al. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science. 1998;279(5351):710–4. Epub 1998/02/21.
135. Yuan TL, Wulf G, Burga L, Cantley LC. Cell-to-cell variability in PI3K protein level regulates PI3K-AKT pathway activity in cell populations. Curr Biol. 2011;21(3):173–83. Epub 2011/01/25.
136. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell Jr JE, Kuriyan J. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. Cell. 1998;93(5):827–39. Epub 1998/06/18.

137. Schindler C, Shuai K, Prezioso VR, Darnell Jr JE. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science. 1992;257(5071):809–13. Epub 1992/08/17.
138. Shuai K. Modulation of STAT signaling by STAT-interacting proteins. Oncogene. 2000;19(21):2638–44. Epub 2000/06/13.
139. Quesnelle KM, Boehm AL, Grandis JR. STAT-mediated EGFR signaling in cancer. J Cell Biochem. 2007;102(2):311–9.
140. Itoh M, Murata T, Suzuki T, Shindoh M, Nakajima K, Imai K, et al. Requirement of STAT3 activation for maximal collagenase-1 (MMP-1) induction by epidermal growth factor and malignant characteristics in T24 bladder cancer cells. Oncogene. 2006;25(8):1195–204. Epub 2005/10/06.
141. Yao Z, Seger R. The ERK signaling cascade–views from different subcellular compartments. Biofactors. 2009;35(5):407–16. Epub 2009/07/01.
142. Plotnikov A, Zehorai E, Procaccia S, Seger R. The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. Biochim Biophys Acta. 2011;1813(9):1619–33. Epub 2010/12/21.
143. Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR, et al. Raf-1 activates MAP kinase-kinase. Nature. 1992;358(6385):417–21. Epub 1992/07/30.
144. Seger R, Krebs EG. The MAPK signaling cascade. FASEB J. 1995;9(9):726–35. Epub 1995/06/01.
145. Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. 1993;7(11):2135–48. Epub 1993/11/01.
146. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol. 2007;19(2):142–9. Epub 2007/02/17.
147. Whitmarsh AJ. The JIP, family of MAPK scaffold proteins. Biochem Soc Trans. 2006;34(Pt 5):828–32. Epub 2006/10/21.
148. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell. 2000;103(2):239–52. Epub 2000/11/01.
149. Kawasaki H, Schiltz L, Chiu R, Itakura K, Taira K, Nakatani Y, et al. ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. Nature. 2000;405(6783):195–200. Epub 2000/05/23.
150. Han J, Luby-Phelps K, Das B, Shu X, Xia Y, Mosteller RD, et al. Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. Science. 1998;279(5350):558–60. Epub 1998/02/07.
151. Zwang Y, Yarden Y. p38 MAP kinase mediates stress-induced internalization of EGFR: implications for cancer chemotherapy. EMBO J. 2006;25(18):4195–206. Epub 2006/08/26.
152. Kato Y, Tapping RI, Huang S, Watson MH, Ulevitch RJ, Lee JD. Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. Nature. 1998;395(6703):713–6. Epub 1998/10/28.
153. Hayashi M, Tapping RI, Chao TH, Lo JF, King CC, Yang Y, et al. BMK1 mediates growth factor-induced cell proliferation through direct cellular activation of serum and glucocorticoid-inducible kinase. J Biol Chem. 2001;276(12):8631–4. Epub 2001/03/20.
154. Avraham R, Yarden Y. Feedback regulation of EGFR signalling: decision making by early and delayed loops. Nat Rev Mol Cell Biol. 2011;12(2):104–17. Epub 2011/01/22.
155. Li X, Carthew RW. A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the Drosophila eye. Cell. 2005;123(7):1267–77.
156. Avraham R, Sas-Chen A, Manor O, Steinfeld I, Shalgi R, Tarcic G, et al. EGF decreases the abundance of microRNAs that restrain oncogenic transcription factors. Sci Signal. 2010;3(124):ra43. Epub 2010/06/03.
157. Lau LF, Nathans D. Expression of a set of growth-related immediate early genes in BALB/c 3 T3 cells: coordinate regulation with c-fos or c-myc. Proc Natl Acad Sci USA. 1987;84(5):1182–6. Epub 1987/03/01.

158. Hargreaves DC, Horng T, Medzhitov R. Control of inducible gene expression by signal-dependent transcriptional elongation. Cell. 2009;138(1):129–45. Epub 2009/07/15.
159. Fujita T, Piuz I, Schlegel W. Negative elongation factor NELF controls transcription of immediate early genes in a stimulus-specific manner. Exp Cell Res. 2009;315(2):274–84. Epub 2008/11/19.
160. Amit I, Citri A, Shay T, Lu Y, Katz M, Zhang F, et al. A module of negative feedback regulators defines growth factor signaling. Nat Genet. 2007;39(4):503–12. Epub 2007/02/27.
161. Farooq A, Zhou MM. Structure and regulation of MAPK phosphatases. Cell Signal. 2004;16(7):769–79. Epub 2004/04/30.
162. Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, et al. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell. 2001;107(4):451–64. Epub 2001/11/24.
163. Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, et al. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell. 2001;7(6):1267–78. Epub 2001/06/30.
164. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development. 2005;132(14):3151–61. Epub 2005/06/29.
165. Medici D, Hay ED, Olsen BR. Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. Mol Biol Cell. 2008;19(11):4875–87. Epub 2008/09/19.
166. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. 2004;117(7):927–39. Epub 2004/06/24.
167. Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol. 1999;147(3):631–44. Epub 1999/11/05.
168. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10(5):593–601.
169. Donaldson JG, Porat-Shliom N, Cohen LA. Clathrin-independent endocytosis: a unique platform for cell signaling and PM remodeling. Cell Signal. 2009;21(1):1–6. Epub 2008/07/24.
170. Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, et al. Clathrin-independent endocytosis of ubiquitinated cargos. Proc Natl Acad Sci USA. 2005; 102(8):2760–5.
171. Robinson MS. Adaptable adaptors for coated vesicles. Trends Cell Biol. 2004;14(4):167–74. Epub 2004/04/07.
172. Zwang Y, Yarden Y. Systems biology of growth factor-induced receptor endocytosis. Traffic. 2009;10(4):349–63. Epub 2009/02/03.
173. Sever S, Damke H, Schmid SL. Dynamin: GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. J Cell Biol. 2000;150(5):1137–48. Epub 2000/09/07.
174. Hannan LA, Newmyer SL, Schmid SL. ATP- and cytosol-dependent release of adaptor proteins from clathrin-coated vesicles: a dual role for Hsc70. Mol Biol Cell. 1998;9(8):2217–29. Epub 1998/08/07.
175. Massol RH, Boll W, Griffin AM, Kirchhausen T. A burst of auxilin recruitment determines the onset of clathrin-coated vesicle uncoating. Proc Natl Acad Sci USA. 2006;103(27):10265–70. Epub 2006/06/27.
176. Raiborg C, Stenmark H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature. 2009;458(7237):445–52. Epub 2009/03/28.
177. Di Guglielmo GM, Baass PC, Ou WJ, Posner BI, Bergeron JJ. Compartmentalization of SHC, GRB2 and mSOS, and hyperphosphorylation of Raf-1 by EGF but not insulin in liver parenchyma. EMBO J. 1994;13(18):4269–77. Epub 1994/09/15.

178. Jiang X, Sorkin A. Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. Mol Biol Cell. 2002;13(5):1522–35. Epub 2002/05/15.
179. Galperin E, Sorkin A. Endosomal targeting of MEK2 requires RAF, MEK kinase activity and clathrin-dependent endocytosis. Traffic. 2008;9(10):1776–90. Epub 2008/07/29.
180. Huang PH, Mukasa A, Bonavia R, Flynn RA, Brewer ZE, Cavenee WK, et al. Quantitative analysis of EGFRvIII cellular signaling networks reveals a combinatorial therapeutic strategy for glioblastoma. Proc Natl Acad Sci USA. 2007;104(31):12867–72.
181. Yamauchi T, Ueki K, Tobe K, Tamemoto H, Sekine N, Wada M, et al. Growth hormone-induced tyrosine phosphorylation of EGF receptor as an essential element leading to MAP kinase activation and gene expression. Endocr J. 1998;45(Suppl):S27–31.
182. Yamauchi T, Yamauchi N, Ueki K, Sugiyama T, Waki H, Miki H, et al. Constitutive tyrosine phosphorylation of ErbB-2 via Jak2 by autocrine secretion of prolactin in human breast cancer. J Biol Chem. 2000;275(43):33937–44.
183. Sugawa N, Ekstrand AJ, James CD, Collins VP. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. Proc Natl Acad Sci USA. 1990;87(21):8602–6.
184. Miller VA, Kris MG, Shah N, Patel J, Azzoli C, Gomez J, et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. J Clin Oncol. 2004;22(6):1103–9. Epub 2004/03/17.
185. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004;350(21):2129–39.
186. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004; 304(5676):1497–500.
187. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA. 2004;101(36):13306–11.
188. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science. 2004;305(5687):1163–7.
189. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med. 2005;2(3):e73.
190. Wheeler DL, Huang S, Kruser TJ, Nechrebecki MM, Armstrong EA, Benavente S, et al. Mechanisms of acquired resistance to cetuximab: role of HER (ErbB) family members. Oncogene. 2008;27(28):3944–56. Epub 2008/02/26.
191. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med. 2004;351(4):337–45. Epub 2004/07/23.
192. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006;354(6):567–78.
193. Vermorken JB, Trigo J, Hitt R, Koralewski P, Diaz-Rubio E, Rolland F, et al. Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. J Clin Oncol. 2007;25(16):2171–7. Epub 2007/06/01.
194. Perez-Soler R, Delord JP, Halpern A, Kelly K, Krueger J, Sureda BM, et al. HER1/EGFR inhibitor-associated rash: future directions for management and investigation outcomes from the HER1/EGFR inhibitor rash management forum. Oncologist. 2005;10(5):345–56. Epub 2005/04/27.
195. Talavera A, Friemann R, Gomez-Puerta S, Martinez-Fleites C, Garrido G, Rabasa A, et al. Nimotuzumab, an antitumor antibody that targets the epidermal growth factor receptor,

blocks ligand binding while permitting the active receptor conformation. Cancer Res. 2009;69(14):5851–9. Epub 2009/07/09.
196. Umezawa H, Imoto M, Sawa T, Isshiki K, Matsuda N, Uchida T, et al. Studies on a new epidermal growth factor-receptor kinase inhibitor, erbstatin, produced by MH435-hF3. J Antibiot (Tokyo). 1986;39(1):170–3.
197. Yaish P, Gazit A, Gilon C, Levitzki A. Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. Science. 1988;242(4880):933–5.
198. Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. J Clin Oncol. 2003;21(12):2237–46.
199. Spector NL, Xia W, Burris 3rd H, Hurwitz H, Dees EC, Dowlati A, et al. Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. J Clin Oncol. 2005;23(11):2502–12.
200. Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med. 2006; 355(26):2733–43.
201. Schechter AL, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, et al. The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature. 1984;312(5994):513–6. Epub 1984/12/06.
202. Semba K, Kamata N, Toyoshima K, Yamamoto T. A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA. 1985;82(19):6497–501.
203. Schechter AL, Hung MC, Vaidyanathan L, Weinberg RA, Yang-Feng TL, Francke U, et al. The neu gene: an erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science. 1985;229(4717):976–8. Epub 1985/09/06.
204. Fukushige S, Matsubara K, Yoshida M, Sasaki M, Suzuki T, Semba K, et al. Localization of a novel v-erbB-related gene, c-erbB-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol Cell Biol. 1986;6(3):955–8. Epub 1986/03/01.
205. King CR, Kraus MH, Aaronson SA. Amplification of a novel v-erbB-related gene in a human mammary carcinoma. Science. 1985;229(4717):974–6.
206. Yarden Y, Weinberg RA. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the neu protooncogene. Proc Natl Acad Sci USA. 1989;86(9):3179–83. Epub 1989/05/01.
207. Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, et al. Identification of heregulin, a specific activator of p185erbB2. Science. 1992;256(5060):1205–10. Epub 1992/05/22.
208. Peles E, Bacus SS, Koski RA, Lu HS, Wen D, Ogden SG, et al. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell. 1992;69(1):205–16. Epub 1992/04/03.
209. Ishii S, Imamoto F, Yamanashi Y, Toyoshima K, Yamamoto T. Characterization of the promoter region of the human c-erbB-2 protooncogene. Proc Natl Acad Sci USA. 1987;84(13):4374–8.
210. Hurst HC. Update on HER-2 as a target for cancer therapy: the ERBB2 promoter and its exploitation for cancer treatment. Breast Cancer Res. 2001;3(6):395–8.
211. Park JW, Neve RM, Szollosi J, Benz CC. Unraveling the biologic and clinical complexities of HER2. Clin Breast Cancer. 2008;8(5):392–401.
212. Stein D, Wu J, Fuqua SA, Roonprapunt C, Yajnik V, D'Eustachio P, et al. The SH2 domain protein GRB-7 is co-amplified, overexpressed and in a tight complex with HER2 in breast cancer. EMBO J. 1994;13(6):1331–40. Epub 1994/03/15.
213. Kauraniemi P, Barlund M, Monni O, Kallioniemi A. New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. Cancer Res. 2001;61(22):8235–40. Epub 2001/11/24.

214. Kauraniemi P, Kuukasjarvi T, Sauter G, Kallioniemi A. Amplification of a 280-kilobase core region at the ERBB2 locus leads to activation of two hypothetical proteins in breast cancer. Am J Pathol. 2003;163(5):1979–84. Epub 2003/10/28.
215. Orsetti B, Nugoli M, Cervera N, Lasorsa L, Chuchana P, Ursule L, et al. Genomic and expression profiling of chromosome 17 in breast cancer reveals complex patterns of alterations and novel candidate genes. Cancer Res. 2004;64(18):6453–60. Epub 2004/09/18.
216. Jarvinen TA, Tanner M, Barlund M, Borg A, Isola J. Characterization of topoisomerase II alpha gene amplification and deletion in breast cancer. Genes Chromosomes Cancer. 1999;26(2):142–50. Epub 1999/09/01.
217. Tanner B, Hasenclever D, Stern K, Schormann W, Bezler M, Hermes M, et al. ErbB-3 predicts survival in ovarian cancer. J Clin Oncol. 2006;24(26):4317–23. Epub 2006/08/10.
218. Arriola E, Lambros MB, Jones C, Dexter T, Mackay A, Tan DS, et al. Evaluation of Phi29-based whole-genome amplification for microarray-based comparative genomic hybridisation. Lab Invest. 2007;87(1):75–83. Epub 2006/12/16.
219. Arriola E, Marchio C, Tan DS, Drury SC, Lambros MB, Natrajan R, et al. Genomic analysis of the HER2/TOP2A amplicon in breast cancer and breast cancer cell lines. Lab Invest. 2008;88(5):491–503. Epub 2008/03/12.
220. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science. 1989;244(4905):707–12. Epub 1989/05/12.
221. Rolitsky CD, Theil KS, McGaughy VR, Copeland LJ, Niemann TH. HER-2/neu amplification and overexpression in endometrial carcinoma. Int J Gynecol Pathol. 1999;18(2):138–43. Epub 1999/04/15.
222. Ross JS, Sheehan CE, Hayner-Buchan AM, Ambros RA, Kallakury BV, Kaufman Jr RP, et al. Prognostic significance of HER-2/neu gene amplification status by fluorescence in situ hybridization of prostate carcinoma. Cancer. 1997;79(11):2162–70. Epub 1997/06/01.
223. Nakajima M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, et al. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer. 1999;85(9):1894–902. Epub 1999/05/01.
224. Kraus MH, Popescu NC, Amsbaugh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J. 1987;6(3):605–10. Epub 1987/03/01.
225. Xing X, Wang SC, Xia W, Zou Y, Shao R, Kwong KY, et al. The ets protein PEA3 suppresses HER-2/neu overexpression and inhibits tumorigenesis. Nat Med. 2000;6(2):189–95.
226. Zuo T, Wang L, Morrison C, Chang X, Zhang H, Li W, et al. FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. Cell. 2007;129(7):1275–86.
227. Hua G, Zhu B, Rosa F, Deblon N, Adelaide J, Kahn-Perles B, et al. A negative feedback regulatory loop associates the tyrosine kinase receptor ERBB2 and the transcription factor GATA4 in breast cancer cells. Mol Cancer Res. 2009;7(3):402–14. Epub 2009/03/12.
228. Mehta A, Trotta CR, Peltz SW. Derepression of the Her-2 uORF is mediated by a novel post-transcriptional control mechanism in cancer cells. Genes Dev. 2006;20(8):939–53. Epub 2006/04/07.
229. Anido J, Scaltriti M, Bech Serra JJ, Santiago Josefat B, Todo FR, Baselga J, et al. Biosynthesis of tumorigenic HER2 C-terminal fragments by alternative initiation of translation. EMBO J. 2006;25(13):3234–44.
230. Scaltriti M, Rojo F, Ocana A, Anido J, Guzman M, Cortes J, et al. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. J Natl Cancer Inst. 2007;99(8):628–38.
231. Codony-Servat J, Albanell J, Lopez-Talavera JC, Arribas J, Baselga J. Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. Cancer Res. 1999;59(6):1196–201.

232. Pedersen K, Angelini PD, Laos S, Bach-Faig A, Cunningham MP, Ferrer-Ramon C, et al. A naturally occurring HER2 carboxy-terminal fragment promotes mammary tumor growth and metastasis. Mol Cell Biol. 2009;29(12):3319–31. Epub 2009/04/15.
233. Xia W, Liu LH, Ho P, Spector NL. Truncated ErbB2 receptor (p95ErbB2) is regulated by heregulin through heterodimer formation with ErbB3 yet remains sensitive to the dual EGFR/ErbB2 kinase inhibitor GW572016. Oncogene. 2004;23(3):646–53. Epub 2004/01/23.
234. Saez R, Molina MA, Ramsey EE, Rojo F, Keenan EJ, Albanell J, et al. p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. Clin Cancer Res. 2006;12(2):424–31. Epub 2006/01/24.
235. Hudelist G, Kostler WJ, Attems J, Czerwenka K, Muller R, Manavi M, et al. Her-2/neu-triggered intracellular tyrosine kinase activation: in vivo relevance of ligand-independent activation mechanisms and impact upon the efficacy of trastuzumab-based treatment. Br J Cancer. 2003;89(6):983–91.
236. Siegel PM, Ryan ED, Cardiff RD, Muller WJ. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. EMBO J. 1999;18(8):2149–64.
237. Pinkas-Kramarski R, Eilam R, Alroy I, Levkowitz G, Lonai P, Yarden Y. Differential expression of NDF/neuregulin receptors ErbB-3 and ErbB-4 and involvement in inhibition of neuronal differentiation. Oncogene. 1997;15(23):2803–15. Epub 1998/01/07.
238. Riese 2nd DJ, van Raaij TM, Plowman GD, Andrews GC, Stern DF. The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. Mol Cell Biol. 1995;15(10):5770–6. Epub 1995/10/01.
239. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol. 1996;16(10):5276–87.
240. Karunagaran D, Tzahar E, Beerli RR, Chen X, Graus-Porta D, Ratzkin BJ, et al. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. EMBO J. 1996;15(2):254–64.
241. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J. 1997;16(7):1647–55.
242. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas 3rd CF, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci USA. 2003;100(15):8933–8. Epub 2003/07/11.
243. Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. Exp Cell Res. 2003;284(1):54–65. Epub 2003/03/22.
244. Sliwkowski MX, Schaefer G, Akita RW, Lofgren JA, Fitzpatrick VD, Nuijens A, et al. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J Biol Chem. 1994;269(20):14661–5.
245. Alimandi M, Romano A, Curia MC, Muraro R, Fedi P, Aaronson SA, et al. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene. 1995;10(9):1813–21. Epub 1995/05/04.
246. Wallasch C, Weiss FU, Niederfellner G, Jallal B, Issing W, Ullrich A. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. EMBO J. 1995;14(17):4267–75. Epub 1995/09/01.
247. Ben-Levy R, Peles E, Goldman-Michael R, Yarden Y. An oncogenic point mutation confers high affinity ligand binding to the neu receptor. Implications for the generation of site heterogeneity. J Biol Chem. 1992;267(24):17304–13. Epub 1992/08/25.
248. Pinkas-Kramarski R, Lenferink AE, Bacus SS, Lyass L, van de Poll ML, Klapper LN, et al. The oncogenic ErbB-2/ErbB-3 heterodimer is a surrogate receptor of the epidermal growth factor and betacellulin. Oncogene. 1998;16(10):1249–58. Epub 1998/04/18.
249. Klapper LN, Waterman H, Sela M, Yarden Y. Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. Cancer Res. 2000;60(13):3384–8.

250. Lenferink AE, Pinkas-Kramarski R, van de Poll ML, van Vugt MJ, Klapper LN, Tzahar E, et al. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. EMBO J. 1998;17(12):3385–97.
251. Worthylake R, Opresko LK, Wiley HS. ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. J Biol Chem. 1999;274(13):8865–74.
252. Offterdinger M, Schofer C, Weipoltshammer K, Grunt TW. c-erbB-3: a nuclear protein in mammary epithelial cells. J Cell Biol. 2002;157(6):929–39.
253. Xie Y, Hung MC. Nuclear localization of p185neu tyrosine kinase and its association with transcriptional transactivation. Biochem Biophys Res Commun. 1994;203(3):1589–98.
254. Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol. 2001;3(9):802–8.
255. Ni CY, Murphy MP, Golde TE, Carpenter G. Gamma-secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science. 2001;294(5549):2179–81.
256. Wells A, Marti U. Signalling shortcuts: cell-surface receptors in the nucleus? Nat Rev Mol Cell Biol. 2002;3(9):697–702.
257. Lo HW, Hsu SC, Ali-Seyed M, Gunduz M, Xia W, Wei Y, et al. Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. Cancer Cell. 2005;7(6):575–89.
258. Wang SC, Lien HC, Xia W, Chen IF, Lo HW, Wang Z, et al. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. Cancer Cell. 2004; 6(3):251–61.
259. Wang SC, Nakajima Y, Yu YL, Xia W, Chen CT, Yang CC, et al. Tyrosine phosphorylation controls PCNA function through protein stability. Nat Cell Biol. 2006;8(12):1359–68.
260. Bandyopadhyay D, Mandal M, Adam L, Mendelsohn J, Kumar R. Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. J Biol Chem. 1998;273(3):1568–73.
261. Rodemann HP, Dittmann K, Toulany M. Radiation-induced EGFR-signaling and control of DNA-damage repair. Int J Radiat Biol. 2007;83(11–12):781–91.
262. Klein C, Gensburger C, Freyermuth S, Nair BC, Labourdette G, Malviya AN. A 120 kDa nuclear phospholipase Cgamma1 protein fragment is stimulated in vivo by EGF signal phosphorylating nuclear membrane EGFR. Biochemistry. 2004;43(50):15873–83.
263. Grasl-Kraupp B, Schausberger E, Hufnagl K, Gerner C, Low-Baselli A, Rossmanith W, et al. A novel mechanism for mitogenic signaling via pro-transforming growth factor alpha within hepatocyte nuclei. Hepatology. 2002;35(6):1372–80.
264. Raper SE, Burwen SJ, Barker ME, Jones AL. Translocation of epidermal growth factor to the hepatocyte nucleus during rat liver regeneration. Gastroenterology. 1987;92(5 Pt 1):1243–50.
265. Schausberger E, Eferl R, Parzefall W, Chabicovsky M, Breit P, Wagner EF, et al. Induction of DNA synthesis in primary mouse hepatocytes is associated with nuclear pro-transforming growth factor alpha and erbb-1 and is independent of c-jun. Carcinogenesis. 2003;24(5):835–41.
266. Dittmann K, Mayer C, Fehrenbacher B, Schaller M, Raju U, Milas L, et al. Radiation-induced epidermal growth factor receptor nuclear import is linked to activation of DNA-dependent protein kinase. J Biol Chem. 2005;280(35):31182–9.
267. Wanner G, Mayer C, Kehlbach R, Rodemann HP, Dittmann K. Activation of protein kinase Cepsilon stimulates DNA-repair via epidermal growth factor receptor nuclear accumulation. Radiother Oncol. 2008;86:383–90.
268. Giri DK, Ali-Seyed M, Li LY, Lee DF, Ling P, Bartholomeusz G, et al. Endosomal transport of ErbB-2: mechanism for nuclear entry of the cell surface receptor. Mol Cell Biol. 2005;25(24):11005–18.
269. Lo HW, Ali-Seyed M, Wu Y, Bartholomeusz G, Hsu SC, Hung MC. Nuclear-cytoplasmic transport of EGFR involves receptor endocytosis, importin beta1 and CRM1. J Cell Biochem. 2006;98(6):1570–83. Epub 2006/03/23.

270. Tsai B, Ye Y, Rapoport TA. Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. Nat Rev Mol Cell Biol. 2002;3(4):246–55.
271. Liao HJ, Carpenter G. Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. Mol Biol Cell. 2007;18(3):1064–72.
272. Hsu SC, Hung MC. Characterization of a novel tripartite nuclear localization sequence in the EGFR family. J Biol Chem. 2007;282(14):10432–40.
273. Williams CC, Allison JG, Vidal GA, Burow ME, Beckman BS, Marrero L, et al. The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone. J Cell Biol. 2004;167(3):469–78.
274. Citri A, Gan J, Mosesson Y, Vereb G, Szollosi J, Yarden Y. Hsp90 restrains ErbB-2/HER2 signalling by limiting heterodimer formation. EMBO Rep. 2004;5(12):1165–70.
275. Mimnaugh EG, Chavany C, Neckers L. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. J Biol Chem. 1996;271(37):22796–801.
276. Drebin JA, Link VC, Stern DF, Weinberg RA, Greene MI. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell. 1985;41(3):697–706. Epub 1985/07/01.
277. Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol. 1989;9(3):1165–72. Epub 1989/03/01.
278. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA. 1992;89(10):4285–9.
279. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol. 1999;17(9):2639–48.
280. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol. 2002;20(3):719–26.
281. Slamon D, Pegram M. Rationale for trastuzumab (Herceptin) in adjuvant breast cancer trials. Semin Oncol. 2001;28(1 Suppl 3):13–9.
282. Untch M, Rezai M, Loibl S, Fasching PA, Huober J, Tesch H, et al. Neoadjuvant treatment with trastuzumab in HER2-positive breast cancer: results from the GeparQuattro study. J Clin Oncol. 2010;28(12):2024–31.
283. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet. 2010;376(9742):687–97. Epub 2010/08/24.
284. Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. Oncogene. 2002;21(41):6255–63. Epub 2002/09/06.
285. Ryan Q, Ibrahim A, Cohen MH, Johnson J, Ko CW, Sridhara R, et al. FDA drug approval summary: lapatinib in combination with capecitabine for previously treated metastatic breast cancer that overexpresses HER-2. Oncologist. 2008;13(10):1114–9.
286. Plowman GD, Whitney GS, Neubauer MG, Green JM, McDonald VL, Todaro GJ, et al. Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proc Natl Acad Sci USA. 1990;87(13):4905–9. Epub 1990/07/01.
287. Naidu R, Yadav M, Nair S, Kutty MK. Expression of c-erbB3 protein in primary breast carcinomas. Br J Cancer. 1998;78(10):1385–90. Epub 1998/11/21.
288. Chow NH, Chan SH, Tzai TS, Ho CL, Liu HS. Expression profiles of ErbB family receptors and prognosis in primary transitional cell carcinoma of the urinary bladder. Clin Cancer Res. 2001;7(7):1957–62. Epub 2001/07/13.

289. Skinner A, Hurst HC. Transcriptional regulation of the c-erbB-3 gene in human breast carcinoma cell lines. Oncogene. 1993;8(12):3393–401.
290. Zhu CH, Huang Y, Oberley LW, Domann FE. A family of AP-2 proteins down-regulate manganese superoxide dismutase expression. J Biol Chem. 2001;276(17):14407–13. Epub 2001/03/30.
291. Zhu CH, Domann FE. Dominant negative interference of transcription factor AP-2 causes inhibition of ErbB-3 expression and suppresses malignant cell growth. Breast Cancer Res Treat. 2002;71(1):47–57.
292. Katoh M, Yazaki Y, Sugimura T, Terada M. c-erbB3 gene encodes secreted as well as transmembrane receptor tyrosine kinase. Biochem Biophys Res Commun. 1993;192(3):1189–97. Epub 1993/05/14.
293. Lee H, Akita RW, Sliwkowski MX, Maihle NJ. A naturally occurring secreted human ErbB3 receptor isoform inhibits heregulin-stimulated activation of ErbB2, ErbB3, and ErbB4. Cancer Res. 2001;61(11):4467–73. Epub 2001/06/05.
294. Garrett JT, Arteaga CL. Resistance to HER2-directed antibodies and tyrosine kinase inhibitors: mechanisms and clinical implications. Cancer Biol Ther. 2011;11(9):793–800. Epub 2011/02/11.
295. Krig SR, Miller JK, Frietze S, Beckett LA, Neve RM, Farnham PJ, et al. ZNF217, a candidate breast cancer oncogene amplified at 20q13, regulates expression of the ErbB3 receptor tyrosine kinase in breast cancer cells. Oncogene. 2010;29(40):5500–10. Epub 2010/07/28.
296. Andrique L, Fauvin D, El Maassarani M, Colasson H, Vannier B, Seite P. ErbB3(80 kDa), a nuclear variant of the ErbB3 receptor, binds to the Cyclin D1 promoter to activate cell proliferation but is negatively controlled by p14ARF. Cell Signal. 2012;24(5):1074–85. Epub 2012/01/21.
297. Iorio MV, Casalini P, Piovan C, Di Leva G, Merlo A, Triulzi T, et al. microRNA-205 regulates HER3 in human breast cancer. Cancer Res. 2009;69(6):2195–200. Epub 2009/03/12.
298. Cho HS, Leahy DJ. Structure of the extracellular region of HER3 reveals an interdomain tether. Science. 2002;297(5585):1330–3.
299. Kani K, Park E, Landgraf R. The extracellular domains of ErbB3 retain high ligand binding affinity at endosome pH and in the locked conformation. Biochemistry. 2005;44(48):15842–57. Epub 2005/11/30.
300. Diamonti AJ, Guy PM, Ivanof C, Wong K, Sweeney C, Carraway 3rd KL. An RBCC protein implicated in maintenance of steady-state neuregulin receptor levels. Proc Natl Acad Sci USA. 2002;99(5):2866–71. Epub 2002/02/28.
301. Wu X, Yen L, Irwin L, Sweeney C, Carraway 3rd KL. Stabilization of the E3 ubiquitin ligase Nrdp1 by the deubiquitinating enzyme USP8. Mol Cell Biol. 2004;24(17):7748–57. Epub 2004/08/18.
302. Cao Z, Wu X, Yen L, Sweeney C, Carraway 3rd KL. Neuregulin-induced ErbB3 downregulation is mediated by a protein stability cascade involving the E3 ubiquitin ligase Nrdp1. Mol Cell Biol. 2007;27(6):2180–8. Epub 2007/01/11.
303. Prigent SA, Gullick WJ. Identification of c-erbB-3 binding sites for phosphatidylinositol 3′-kinase and SHC using an EGF receptor/c-erbB-3 chimera. EMBO J. 1994;13(12):2831–41. Epub 1994/06/15.
304. Fiddes RJ, Campbell DH, Janes PW, Sivertsen SP, Sasaki H, Wallasch C, et al. Analysis of Grb7 recruitment by heregulin-activated erbB receptors reveals a novel target selectivity for erbB3. J Biol Chem. 1998;273(13):7717–24. Epub 1998/04/29.
305. Carraway 3rd KL, Weber JL, Unger MJ, Ledesma J, Yu N, Gassmann M, et al. Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. Nature. 1997;387(6632):512–6.
306. Zhang D, Sliwkowski MX, Mark M, Frantz G, Akita R, Sun Y, et al. Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4. Proc Natl Acad Sci USA. 1997;94(18):9562–7.
307. Harari D, Tzahar E, Romano J, Shelly M, Pierce JH, Andrews GC, et al. Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. Oncogene. 1999;18(17):2681–9. Epub 1999/05/29.

308. Meyer D, Yamaai T, Garratt A, Riethmacher-Sonnenberg E, Kane D, Theill LE, et al. Isoform-specific expression and function of neuregulin. Development. 1997;124(18):3575–86. Epub 1997/10/28.
309. Falls DL. Neuregulins: functions, forms, and signaling strategies. Exp Cell Res. 2003;284(1):14–30.
310. Montero JC, Yuste L, Diaz-Rodriguez E, Esparis-Ogando A, Pandiella A. Differential shedding of transmembrane neuregulin isoforms by the tumor necrosis factor-alpha-converting enzyme. Mol Cell Neurosci. 2000;16(5):631–48. Epub 2000/11/21.
311. Shirakabe K, Wakatsuki S, Kurisaki T, Fujisawa-Sehara A. Roles of Meltrin beta /ADAM19 in the processing of neuregulin. J Biol Chem. 2001;276(12):9352–8. Epub 2000/12/26.
312. Neve RM, Sutterluty H, Pullen N, Lane HA, Daly JM, Krek W, et al. Effects of oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. Oncogene. 2000;19(13):1647–56. Epub 2000/04/14.
313. Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, et al. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Res. 2008;68(14):5878–87. Epub 2008/07/18.
314. Verveer PJ, Wouters FS, Reynolds AR, Bastiaens PI. Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. Science. 2000;290(5496):1567–70. Epub 2000/11/25.
315. Lee D, Yu M, Lee E, Kim H, Yang Y, Kim K, et al. Tumor-specific apoptosis caused by deletion of the ERBB3 pseudo-kinase in mouse intestinal epithelium. J Clin Invest. 2009;119(9):2702–13. Epub 2009/08/20.
316. Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. Proc Natl Acad Sci USA. 2010;107(17):7692–7.
317. Ishizawar RC, Miyake T, Parsons SJ. c-Src modulates ErbB2 and ErbB3 heterocomplex formation and function. Oncogene. 2007;26(24):3503–10. Epub 2006/12/19.
318. Kamalati T, Jolin HE, Fry MJ, Crompton MR. Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via erbB3 phosphorylation. Oncogene. 2000;19(48):5471–6. Epub 2000/12/15.
319. Soltoff SP, Carraway 3rd KL, Prigent SA, Gullick WG, Cantley LC. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol Cell Biol. 1994;14(6):3550–8. Epub 1994/06/01.
320. Fedi P, Pierce JH, di Fiore PP, Kraus MH. Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase C gamma or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. Mol Cell Biol. 1994;14(1):492–500. Epub 1994/01/01.
321. Cook RS, Garrett JT, Sanchez V, Stanford JC, Young C, Chakrabarty A, et al. ErbB3 ablation impairs PI3K/Akt-dependent mammary tumorigenesis. Cancer Res. 2011;71(11):3941–51. Epub 2011/04/13.
322. Jones RB, Gordus A, Krall JA, MacBeath G. A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature. 2006;439(7073):168–74.
323. Vijapurkar U, Kim MS, Koland JG. Roles of mitogen-activated protein kinase and phosphoinositide 3′-kinase in ErbB2/ErbB3 coreceptor-mediated heregulin signaling. Exp Cell Res. 2003;284(2):291–302. Epub 2003/03/26.
324. Contessa JN, Abell A, Mikkelsen RB, Valerie K, Schmidt-Ullrich RK. Compensatory ErbB3/c-Src signaling enhances carcinoma cell survival to ionizing radiation. Breast Cancer Res Treat. 2006;95(1):17–27. Epub 2005/11/04.
325. Walters DK, French JD, Arendt BK, Jelinek DF. Atypical expression of ErbB3 in myeloma cells: cross-talk between ErbB3 and the interferon-alpha signaling complex. Oncogene. 2003;22(23):3598–607. Epub 2003/06/06.
326. Walters DK, Jelinek DF. A role for Janus kinases in crosstalk between ErbB3 and the interferon-alpha signaling complex in myeloma cells. Oncogene. 2004;23(6):1197–205. Epub 2003/12/03.

327. Waterman H, Alroy I, Strano S, Seger R, Yarden Y. The C-terminus of the kinase-defective neuregulin receptor ErbB-3 confers mitogenic superiority and dictates endocytic routing. EMBO J. 1999;18(12):3348–58.
328. Waterman H, Sabanai I, Geiger B, Yarden Y. Alternative intracellular routing of ErbB receptors may determine signaling potency. J Biol Chem. 1998;273(22):13819–27. Epub 1998/06/05.
329. Yen L, Cao Z, Wu X, Ingalla ER, Baron C, Young LJ, et al. Loss of Nrdp1 enhances ErbB2/ErbB3-dependent breast tumor cell growth. Cancer Res. 2006;66(23):11279–86. Epub 2006/12/06.
330. Funes M, Miller JK, Lai C, Carraway 3rd KL, Sweeney C. The mucin Muc4 potentiates neuregulin signaling by increasing the cell-surface populations of ErbB2 and ErbB3. J Biol Chem. 2006;281(28):19310–9. Epub 2006/05/13.
331. Reschke M, Mihic-Probst D, van der Horst EH, Knyazev P, Wild PJ, Hutterer M, et al. HER3 is a determinant for poor prognosis in melanoma. Clin Cancer Res. 2008;14(16):5188–97. Epub 2008/08/14.
332. Ciardiello F, Kim N, Saeki T, Dono R, Persico MG, Plowman GD, et al. Differential expression of epidermal growth factor-related proteins in human colorectal tumors. Proc Natl Acad Sci USA. 1991;88(17):7792–6. Epub 1991/09/01.
333. Kapitanovic S, Radosevic S, Slade N, Kapitanovic M, Andelinovic S, Ferencic Z, et al. Expression of erbB-3 protein in colorectal adenocarcinoma: correlation with poor survival. J Cancer Res Clin Oncol. 2000;126(4):205–11. Epub 2000/04/27.
334. Muller-Tidow C, Diederichs S, Bulk E, Pohle T, Steffen B, Schwable J, et al. Identification of metastasis-associated receptor tyrosine kinases in non-small cell lung cancer. Cancer Res. 2005;65(5):1778–82. Epub 2005/03/09.
335. Mills GB, Yarden Y. The rebirth of a phoenix: ovarian cancers are addicted to ErbB-3. Cancer Cell. 2010;17(3):217–8. Epub 2010/03/17.
336. Sheng Q, Liu X, Fleming E, Yuan K, Piao H, Chen J, et al. An activated ErbB3/NRG1 autocrine loop supports in vivo proliferation in ovarian cancer cells. Cancer Cell. 2010;17(3):298–310. Epub 2010/03/17.
337. Addo-Yobo SO, Straessle J, Anwar A, Donson AM, Kleinschmidt-Demasters BK, Foreman NK. Paired overexpression of ErbB3 and Sox10 in pilocytic astrocytoma. J Neuropathol Exp Neurol. 2006;65(8):769–75. Epub 2006/08/10.
338. Schoeberl B, Faber AC, Li D, Liang MC, Crosby K, Onsum M, et al. An ErbB3 antibody, MM-121, is active in cancers with ligand-dependent activation. Cancer Res. 2010; 70(6):2485–94.
339. Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM, et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature. 2007;445(7126):437–41. Epub 2007/01/09.
340. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007;316(5827):1039–43.
341. Zhu Y, Sullivan LL, Nair SS, Williams CC, Pandey AK, Marrero L, et al. Coregulation of estrogen receptor by ERBB4/HER4 establishes a growth-promoting autocrine signal in breast tumor cells. Cancer Res. 2006;66(16):7991–8.
342. Rokavec M, Justenhoven C, Schroth W, Istrate MA, Haas S, Fischer HP, et al. A novel polymorphism in the promoter region of ERBB4 is associated with breast and colorectal cancer risk. Clin Cancer Res. 2007;13(24):7506–14. Epub 2007/12/21.
343. Das PM, Thor AD, Edgerton SM, Barry SK, Chen DF, Jones FE. Reactivation of epigenetically silenced HER4/ERBB4 results in apoptosis of breast tumor cells. Oncogene. 2010;29(37):5214–9. Epub 2010/07/07.
344. Zimonjic DB, Alimandi M, Miki T, Popescu NC, Kraus MH. Localization of the human HER4/erbB-4 gene to chromosome 2. Oncogene. 1995;10(6):1235–7. Epub 1995/03/16.

345. Tan W, Dean M, Law AJ. Molecular cloning and characterization of the human ErbB4 gene: identification of novel splice isoforms in the developing and adult brain. PLoS One. 2010;5(9):e12924. Epub 2010/10/05.
346. Elenius K, Corfas G, Paul S, Choi CJ, Rio C, Plowman GD, et al. A novel juxtamembrane domain isoform of HER4/ErbB4. Isoform-specific tissue distribution and differential processing in response to phorbol ester. J Biol Chem. 1997;272(42):26761–8.
347. Elenius K, Choi CJ, Paul S, Santiestevan E, Nishi E, Klagsbrun M. Characterization of a naturally occurring ErbB4 isoform that does not bind or activate phosphatidyl inositol 3-kinase. Oncogene. 1999;18(16):2607–15. Epub 1999/06/03.
348. Gilmore-Hebert M, Ramabhadran R, Stern DF. Interactions of ErbB4 and Kap1 connect the growth factor and DNA damage response pathways. Mol Cancer Res. 2010;8(10):1388–98. Epub 2010/09/23.
349. Komuro A, Nagai M, Navin NE, Sudol M. WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. J Biol Chem. 2003;278(35):33334–41.
350. Aqeilan RI, Donati V, Palamarchuk A, Trapasso F, Kaou M, Pekarsky Y, et al. WW domain-containing proteins, WWOX and YAP, compete for interaction with ErbB-4 and modulate its transcriptional function. Cancer Res. 2005;65(15):6764–72.
351. Rio C, Buxbaum JD, Peschon JJ, Corfas G. Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. J Biol Chem. 2000;275(14):10379–87.
352. Lee HJ, Jung KM, Huang YZ, Bennett LB, Lee JS, Mei L, et al. Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4. J Biol Chem. 2002;277(8):6318–23. Epub 2001/12/14.
353. Maatta JA, Sundvall M, Junttila TT, Peri L, Laine VJ, Isola J, et al. Proteolytic cleavage and phosphorylation of a tumor-associated ErbB4 isoform promote ligand-independent survival and cancer cell growth. Mol Biol Cell. 2006;17(1):67–79.
354. Hollmen M, Maatta JA, Bald L, Sliwkowski MX, Elenius K. Suppression of breast cancer cell growth by a monoclonal antibody targeting cleavable ErbB4 isoforms. Oncogene. 2009;28(10):1309–19. Epub 2009/01/20.
355. Chuu CP, Chen RY, Barkinge JL, Ciaccio MF, Jones RB. Systems-level analysis of ErbB4 signaling in breast cancer: a laboratory to clinical perspective. Mol Cancer Res. 2008;6(6):885–91. Epub 2008/06/24.
356. Omerovic J, Santangelo L, Puggioni EM, Marrocco J, Dall'Armi C, Palumbo C, et al. The E3 ligase Aip4/Itch ubiquitinates and targets ErbB-4 for degradation. FASEB J. 2007;21(11):2849–62. Epub 2007/04/28.
357. Feng SM, Muraoka-Cook RS, Hunter D, Sandahl MA, Caskey LS, Miyazawa K, et al. The E3 ubiquitin ligase WWP1 selectively targets HER4 and its proteolytically derived signaling isoforms for degradation. Mol Cell Biol. 2009;29(3):892–906. Epub 2008/12/03.
358. Li Y, Zhou Z, Alimandi M, Chen C. WW domain containing E3 ubiquitin protein ligase 1 targets the full-length ErbB4 for ubiquitin-mediated degradation in breast cancer. Oncogene. 2009;28:2948–58.
359. Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science. 1991;251(4996):936–9. Epub 1991/02/22.
360. Naglich JG, Metherall JE, Russell DW, Eidels L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. Cell. 1992;69(6):1051–61. Epub 1992/06/12.
361. Hamaoka M, Chinen I, Murata T, Takashima S, Iwamoto R, Mekada E. Anti-human HB-EGF monoclonal antibodies inhibiting ectodomain shedding of HB-EGF and diphtheria toxin binding. J Biochem. 2010;148(1):55–69. Epub 2010/03/25.
362. Sunnarborg SW, Hinkle CL, Stevenson M, Russell WE, Raska CS, Peschon JJ, et al. Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. J Biol Chem. 2002;277(15):12838–45. Epub 2002/02/02.

363. Shirakata Y, Komurasaki T, Toyoda H, Hanakawa Y, Yamasaki K, Tokumaru S, et al. Epiregulin, a novel member of the epidermal growth factor family, is an autocrine growth factor in normal human keratinocytes. J Biol Chem. 2000;275(8):5748–53. Epub 2000/02/22.
364. Shirasawa S, Sugiyama S, Baba I, Inokuchi J, Sekine S, Ogino K, et al. Dermatitis due to epiregulin deficiency and a critical role of epiregulin in immune-related responses of keratinocyte and macrophage. Proc Natl Acad Sci USA. 2004;101(38):13921–6. Epub 2004/09/15.
365. Thuong NT, Hawn TR, Chau TT, Bang ND, Yen NT, Thwaites GE, et al. Epiregulin (EREG) variation is associated with susceptibility to tuberculosis. Genes Immun. 2012;13(3):275–81. Epub 2011/12/16.
366. Shing Y, Christofori G, Hanahan D, Ono Y, Sasada R, Igarashi K, et al. Betacellulin: a mitogen from pancreatic beta cell tumors. Science. 1993;259(5101):1604–7. Epub 1993/03/12.
367. Sanderson MP, Erickson SN, Gough PJ, Garton KJ, Wille PT, Raines EW, et al. ADAM10 mediates ectodomain shedding of the betacellulin precursor activated by p-aminophenylmercuric acetate and extracellular calcium influx. J Biol Chem. 2005;280(3):1826–37. Epub 2004/10/28.
368. Oh YS, Shin S, Lee YJ, Kim EH, Jun HS. Betacellulin-induced beta cell proliferation and regeneration is mediated by activation of ErbB-1 and ErbB-2 receptors. PLoS One. 2011;6(8):e23894. Epub 2011/09/08.
369. Gomez-Gaviro MV, Scott CE, Sesay AK, Matheu A, Booth S, Galichet C, et al. Betacellulin promotes cell proliferation in the neural stem cell niche and stimulates neurogenesis. Proc Natl Acad Sci USA. 2012;109(4):1317–22. Epub 2012/01/11.
370. Wang LM, Kuo A, Alimandi M, Veri MC, Lee CC, Kapoor V, et al. ErbB2 expression increases the spectrum and potency of ligand-mediated signal transduction through ErbB4. Proc Natl Acad Sci USA. 1998;95(12):6809–14. Epub 1998/06/17.
371. Zhang K, Sun J, Liu N, Wen D, Chang D, Thomason A, et al. Transformation of NIH 3 T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. J Biol Chem. 1996;271(7):3884–90. Epub 1996/02/16.
372. Tal-Or P, Erlich S, Porat-Shliom N, Goldshmit Y, Ben-Baruch G, Shaharabani E, et al. Ligand-independent regulation of ErbB4 receptor phosphorylation by activated Ras. J Cell Biochem. 2006;98(6):1482–94. Epub 2006/03/07.
373. Long W, Wagner KU, Lloyd KC, Binart N, Shillingford JM, Hennighausen L, et al. Impaired differentiation and lactational failure of Erbb4-deficient mammary glands identify ERBB4 as an obligate mediator of STAT5. Development. 2003;130(21):5257–68. Epub 2003/09/05.
374. Kainulainen V, Sundvall M, Maatta JA, Santiestevan E, Klagsbrun M, Elenius K. A natural ErbB4 isoform that does not activate phosphoinositide 3-kinase mediates proliferation but not survival or chemotaxis. J Biol Chem. 2000;275(12):8641–9.
375. Di Marcotullio L, Ferretti E, De Smaele E, Screpanti I, Gulino A. Suppressors of hedgehog signaling: Linking aberrant development of neural progenitors and tumorigenesis. Mol Neurobiol. 2006;34(3):193–204. Epub 2007/02/20.
376. Chung KY, Walker JW. Interaction and inhibitory cross-talk between endothelin and ErbB receptors in the adult heart. Mol Pharmacol. 2007;71(6):1494–502. Epub 2007/03/03.
377. Liu Y, Tao YM, Woo RS, Xiong WC, Mei L. Stimulated ErbB4 internalization is necessary for neuregulin signaling in neurons. Biochem Biophys Res Commun. 2007;354(2):505–10. Epub 2007/01/26.
378. Sundvall M, Korhonen A, Paatero I, Gaudio E, Melino G, Croce CM, et al. Isoform-specific monoubiquitination, endocytosis, and degradation of alternatively spliced ErbB4 isoforms. Proc Natl Acad Sci USA. 2008;105(11):4162–7. Epub 2008/03/13.
379. Haugen DR, Akslen LA, Varhaug JE, Lillehaug JR. Expression of c-erbB-3 and c-erbB-4 proteins in papillary thyroid carcinomas. Cancer Res. 1996;56(6):1184–8. Epub 1996/03/15.
380. Furger C, Fiddes RJ, Quinn DI, Bova RJ, Daly RJ, Sutherland RL. Granulosa cell tumors express erbB4 and are sensitive to the cytotoxic action of heregulin-beta2/PE40. Cancer Res. 1998;58(9):1773–8. Epub 1998/05/15.
381. Gilbertson RJ, Bentley L, Hernan R, Junttila TT, Frank AJ, Haapasalo H, et al. ERBB receptor signaling promotes ependymoma cell proliferation and represents a potential novel therapeutic target for this disease. Clin Cancer Res. 2002;8(10):3054–64. Epub 2002/10/11.

382. Gilbertson RJ, Perry RH, Kelly PJ, Pearson AD, Lunec J. Prognostic significance of HER2 and HER4 coexpression in childhood medulloblastoma. Cancer Res. 1997;57(15):3272–80. Epub 1997/08/01.
383. Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett JM. Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer. J Pathol. 2003;200(3):290–7.
384. Moscatello DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zoltick PW, et al. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res. 1995;55(23):5536–9.
385. Wong AJ, Ruppert JM, Bigner SH, Grzeschik CH, Humphrey PA, Bigner DS, et al. Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proc Natl Acad Sci USA. 1992;89(7):2965–9.
386. Ekstrand AJ, Sugawa N, James CD, Collins VP. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. Proc Natl Acad Sci USA. 1992;89(10):4309–13.
387. Humphrey PA, Gangarosa LM, Wong AJ, Archer GE, Lund-Johansen M, Bjerkvig R, et al. Deletion-mutant epidermal growth factor receptor in human gliomas: effects of type II mutation on receptor function. Biochem Biophys Res Commun. 1991;178(3):1413–20.
388. Lee JC, Vivanco I, Beroukhim R, Huang JH, Feng WL, DeBiasi RM, et al. Epidermal growth factor receptor activation in glioblastoma through novel missense mutations in the extracellular domain. PLoS Med. 2006;3(12):e485.
389. Greulich H, Chen TH, Feng W, Janne PA, Alvarez JV, Zappaterra M, et al. Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. PLoS Med. 2005;2(11):e313.
390. Teng YH, Tan WJ, Thike AA, Cheok PY, Tse GM, Wong NS, et al. Mutations in the epidermal growth factor receptor (EGFR) gene in triple negative breast cancer: possible implications for targeted therapy. Breast Cancer Res. 2011;13(2):R35.
391. Ozer BH, Wiepz GJ, Bertics PJ. Activity and cellular localization of an oncogenic glioblastoma multiforme-associated EGF receptor mutant possessing a duplicated kinase domain. Oncogene. 2010;29(6):855–64.
392. Frederick L, Wang XY, Eley G, James CD. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. Cancer Res. 2000;60(5):1383–7.
393. Pines G, Huang PH, Zwang Y, White FM, Yarden Y. EGFRvIV: a previously uncharacterized oncogenic mutant reveals a kinase autoinhibitory mechanism. Oncogene. 2010;29:5850–60.
394. Cho J, Pastorino S, Zeng Q, Xu X, Johnson W, Vandenberg S, et al. Glioblastoma-derived Epidermal Growth Factor Receptor (EGFR) carboxyl-terminal deletion mutants are transforming and are sensitive to EGFR-directed therapies. Cancer Res. 2011;71:7587–96.
395. Stephens P, Hunter C, Bignell G, Edkins S, Davies H, Teague J, et al. Lung cancer: intragenic ERBB2 kinase mutations in tumours. Nature. 2004;431(7008):525–6.
396. Shigematsu H, Takahashi T, Nomura M, Majmudar K, Suzuki M, Lee H, et al. Somatic mutations of the HER2 kinase domain in lung adenocarcinomas. Cancer Res. 2005; 65(5):1642–6.
397. Lee JW, Soung YH, Seo SH, Kim SY, Park CH, Wang YP, et al. Somatic mutations of ERBB2 kinase domain in gastric, colorectal, and breast carcinomas. Clin Cancer Res. 2006; 12(1):57–61.
398. Narkis G, Ofir R, Manor E, Landau D, Elbedour K, Birk OS. Lethal congenital contractural syndrome type 2 (LCCS2) is caused by a mutation in ERBB3 (Her3), a modulator of the phosphatidylinositol-3-kinase/Akt pathway. Am J Hum Genet. 2007;81(3):589–95.
399. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature. 2008;455(7216):1069–75.
400. Soung YH, Lee JW, Kim SY, Wang YP, Jo KH, Moon SW, et al. Somatic mutations of the ERBB4 kinase domain in human cancers. Int J Cancer. 2006;118(6):1426–9.
401. Prickett TD, Agrawal NS, Wei X, Yates KE, Lin JC, Wunderlich JR, et al. Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. Nat Genet. 2009;41(10):1127–32.

Chapter 5
The Eph Receptor Family

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5.1 Introduction to The Eph Receptor Tyrosine Kinase Family

Eph receptors, named after their expression in an *e*rythropoietin-*p*roducing human *h*epatocellular carcinoma cell line, represent the largest family of receptor tyrosine kinases (RTKs) in the animal kingdom. They are divided into two subclasses, A and B. In mammals, nine EphA (EphA1-8 and EphA10; EphA9 is exclusively avian and will not be discussed) and five EphB (EphB1-B4 and EphB6; EphB5 is also specific to the chick and will not be discussed) receptors have been characterized [1]. Structurally, all Eph receptors are highly similar. The extracellular part of Eph receptors contains a globular ligand-binding domain [2–4], a cysteine-rich region, and two fibronectin type III domains (FN1 and FN2). FN2 is followed by a transmembrane helix, and an intracellular part consisting of a juxtamembrane region with several conserved tyrosine residues, a tyrosine kinase domain, a sterile-α motif (SAM) protein–protein interaction domain, and a C-terminal Psd-95, Dlg, and ZO1 domain (PDZ)-binding motif (Fig. 5.1a). The kinase domains of one receptor from each class (EphA10 and EphB6) lack residues that are essential for catalytic activity, indicating that these two receptors might not function by phosphorylating

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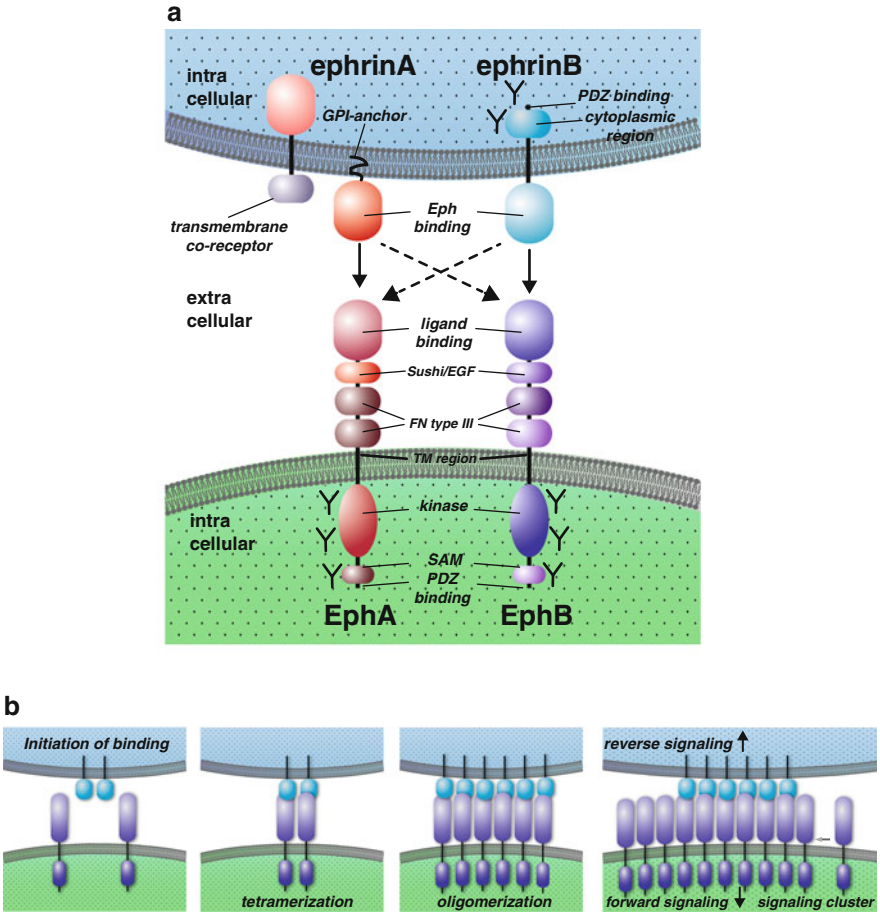

Fig. 5.1 Domain Organization of Eph Receptors and Ephrin Ligands, Cluster Formation, and Bidirectional Signaling. (**a**) Structural features of Ephs and ephrins. The Eph receptor is comprised of a ligand-binding domain, a cysteine-rich region including sushi and EGF domains, two fibronectin type III (FN) domains, a transmembrane region (TM), a kinase domain, a C-terminal SAM domain, and a PDZ-binding motif. Tyrosine phosphorylation sites are indicated with Ys. Typically, ephrinA ligands bind to EphA receptors and ephrinB ligands to EphB receptors however limited cross-talk between the two subclasses exists (*dashed arrows*). EphrinA ligands are GPI anchored, whereas ephrinB has a TM domain, a PDZ-binding motif and conserved tyrosines that are phosphorylated upon activation. Eph/ephrin signaling can be bidirectional. GPI-anchored ephrinAs signal by *cis* interacting with transmembrane co-receptors, such as TrkB, p75, and Ret. (**b**) Binding of ephrin ligand to Eph receptor in *trans* between two opposing cells initiates signaling and formation of heterotetramers which further aggregate into higher-order clusters. Cluster formation is required for physiological signaling in both the ephrin ligand (reverse) and the Eph receptor (forward) expressing cells. Eph–Eph *cis* interactions allow the lateral expansion of clustering and enhance signaling within the cells

cytoplasmic target proteins. The subdivision into EphA and EphB receptor classes was initially based on similarities in the extracellular sequences, but also corresponds to the binding preference for either the five glycosyl phosphatidylinositol (GPI) anchor-linked ephrinA ligands (ephrinA1–A5 that bind predominantly A-type receptors) or the three transmembrane ephrinB ligands (ephrinB1–B3 that bind predominantly B-type receptors). Receptor–ligand interactions within A or B class have considerably varying binding affinities [1, 5]; however, there is also a limited degree of promiscuous cross-binding between members of the two classes.

Eph/ephrin interaction requires cell-to-cell contact since both are anchored to the plasma membrane (Fig. 5.1b). In contrast to classical RTKs, Eph/ephrin binding leads to signal transduction that propagates bidirectionally into both the Eph receptor (in a process known as forward signaling) and the ephrin ligand (reverse signaling) expressing cells [6–8]. For ephrinBs, the signal transduction involves phosphorylation of several highly conserved tyrosine residues in the cytoplasmic domain, and the binding of several cytoplasmic adaptor proteins to the C terminal PDZ-binding motif. Although ephrinAs lack a cytoplasmic domain, they are capable of signaling, either by interacting with co-receptors or by forming plasma membrane microdomains [9–11] (Fig. 5.1a). The neurotrophin receptors TrkB, p75, and the receptor tyrosine kinase Ret serve as such co-receptors [12–14]. Another distinctive feature of Eph receptors is that functional signaling first requires dimerization and activation of the kinase domain followed by a progressive assembly of the Eph/ephrin complexes into larger clusters, the size of which depends on the densities of Eph receptors and ephrins on the cell surface [15–17] (Fig. 5.1b). In addition to the binding of Eph/ephrin molecules in *trans, cis* interactions between receptors and ligands expressed in the same cell have been reported. *Cis* binding does not lead to active signaling but rather seems to interfere with receptor activation by the ephrin ligand presented on surrounding cells. The *cis*-inhibition model can explain how partially overlapping expression of EphAs and ephrinAs can generate a gradient of active receptors in the developing visual system [18–20]. Finally, some Eph receptors can be proteolytically cleaved, a process that regulates Eph expression on the cell surface and initiates signaling by the released intracellular domain [21–25].

The variety of processes that are influenced by Eph receptors, which include roles in development, physiology, as well as pathology, is remarkable; as is the sophistication of their mechanism of action and the diversity of their signaling output. Eph signals have widespread effects on the actin cytoskeleton, cell–substrate adhesion, intercellular junctions, cell shape, and cell movement. Eph receptor function contributes to the regulation and the assembly of cells in tissues, and to the modulation of cell fate and morphology. Eph/ephrin interaction between cells in contact can be stabilized to mediate adhesion, or broken to mediate repulsion. Thus, sorting and segregation of mixed Eph- and ephrin-expressing cell subpopulations is a major role that has been observed in a variety of biological processes. In this context, cells will migrate in order to minimize Eph/ephrin interactions, so that Eph- and ephrin-expressing cells preferentially end up in separate tissue domains [26–31]. Eph receptor signaling and their role in development, physiology, and disease will be further discussed in detail throughout this chapter.

5.2 The Role of the Eph Receptor Tyrosine Kinase Family in Embryonic Development and Adult Physiology

5.2.1 Embryonic Development

5.2.1.1 Gastrulation and Somitogenesis

Gastrulation Vertebrate gastrulation involves specification and coordinated morphogenetic movements of large cell populations that will give rise to the ectodermal, mesodermal, and endodermal germ layers during early development. In frog embryos, this process begins with invagination of mesodermal cells and epiboly of the non-involuting ectodermal cells. The involuting mesoderm undergoes convergent extension (CE) movements which establish the anterior–posterior axis of the embryo. CE movements are generated by the polarization of mesodermal cells followed by coordinated cell migration toward the dorsal side of the gastrula. Mesodermal cells use the ectoderm as a substrate for migration. While the boundary between mesoderm and ectoderm is strictly kept, repeated cycles of cell attachment and detachment are observed. In Xenopus, loss of EphA4 function results in aberrant gastrulation movements, which are due to selective inhibition of tissue constriction and separation. At the cellular level, antisense morpholino knockdown of EphA4 impairs cell polarization and migratory activity of gastrulating cells but not cell fate specification. While EphA4 is expressed in involuting mesodermal cells, one of its cognate ligands, ephrinA1, is expressed in a complementary manner in non-involuting ectodermal cells. EphA4 controls tissue separation by regulating RhoA GTPase activity and Wnt signaling [32]. Earlier work implicated Wnt signaling, EphB1, and the formin-homology protein Daam1 in CE movements in Zebrafish embryos [33]. A complex consisting of EphB receptors and the noncanonical Wnt signaling components Daam1 and Disheveled-2, when removed from the cell surface by endocytosis, induces cell repulsion followed by the initiation of CE movements. A link to Wnt signaling was demonstrated, but the exact molecular nature of the interactions remains unclear.

A more recent study described a new mechanism of mesoderm/ectoderm separation that involves Eph/ephrin signaling at the interface. Transient attachment of mesodermal cells to the ectoderm induces EphB/ephrinB signaling that leads to temporary detachment, thereby allowing cells to migrate along the boundary. When cells are apart, the repulsive signal decays and cells emit protrusions and reestablish contacts (Fig. 5.2). Cell contact initiates the next round of EphB/ephrinB signaling. Multiple ephrinBs and EphBs are expressed on each side of the boundary. EphB-forward signaling via Rho GTPases seems to be required [34]. Further in development, the dorsal mesoderm separates into the notochord and presomitic mesoderm and, during convergent extension movements, adhesion at their boundaries decreases dramatically. This allows the cells to “slide” against each other. The decreased adhesion is not caused by downregulation of adhesion molecules, but rather by blebbing-like behavior of the cells along the boundary that inhibits cadherin clustering. Cell blebbing

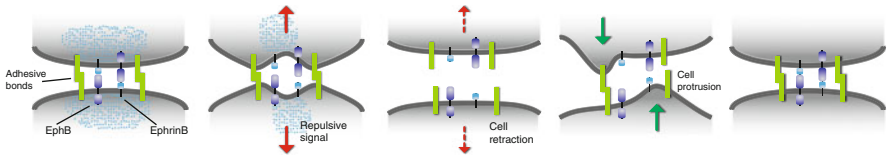

Fig. 5.2 EphB/ephrinB Signaling During Gastrulation. Model for tissue separation during gastrulation. Mesodermal and ectodermal cells go through cycles of attachment and detachment. Attachment of cells at the boundary via adhesion molecules triggers EphB/ephrinB signaling, which induces repulsion and detachment. This allows cells to migrate along the boundary. When cells are separated, the repulsive signal from EphB receptors decays and cells grow protrusions, and re-establish contacts. Contact initiates the next round of EphB/ephrinB signaling

derives from Eph/ephrin-induced changes in actomyosin-driven contractility [35]. EphA4 and EphB4 in presomitic mesoderm and ephrinB2 in the notochord were shown to be contact cues that control separation at the boundary.

Somitogenesis Somitogenesis is used as a model to study the molecular mechanisms underlying segmentation and boundary formation. When somites form in the presomitic mesoderm, an intersomitic boundary emerges and the cells on each side of this boundary undergo mesenchymal-to-epithelial transition (MET). EphA4 signaling in zebrafish is important in establishing cell polarity during MET of the paraxial mesoderm, required for somite formation [36–38]. In chick somitogenesis, one of the regulators that induce EphA4 expression is the bHLH-transcription factor cMeso-1, the homologue of mouse Mesp2. cMeso-1 upregulates EphA4 in the cells located posteriorly to the forming boundary. This in turn activates ephrinB2 reverse signaling in the anteriorly opposed cells. Moreover, phosphorylation of tyrosine residues on ephrinB2 represses Cdc42 GTPase, leading to gap formation and cell autonomous MET [39].

Somitogenesis also depends on the segmental assembly of extracellular matrix (ECM) and integrin clustering at the nascent somite boundary. Integrin transmembrane proteins can be activated by cytoplasmic signals in a process called "inside-out" signaling by inducing a conformational change to a high-affinity ligand binding state. EphA4 *trans* interaction at the somite boundary induces ephrinB2 reverse signaling, which is sufficient to initiate integrinα5 clustering and ECM assembly specifically at the boundary [40].

5.2.1.2 Neural Development

The development of the nervous system starts with the formation of the neural plate, a specialized area of the ectoderm which folds to form the neural tube in a process called neurulation. Regionalization of the neural tube then leads to the formation of the main structures of the central nervous system, whose development proceeds according to the same basic principles. Neurons are generated from progenitor cells in the proliferative zones, undergo differentiation, and migrate from their place of

birth to their defined locations where they start growing axons and dendrites. The axons then extend toward their target tissue, directed by guidance cues along the way. Within the target tissue, they recognize and form synaptic contacts with their partner cells. Inappropriate or excessive contacts are then pruned, leading to the refinement of neuronal connectivity map. Different members of the Eph family are involved in all these major steps of neuronal development. Cell-contact-dependent Eph/ephrin interactions are required to mediate cell sorting, migration, and establishment of selective connections between neurons.

Early Morphogenesis and Patterning During neurulation, ephinA5 and EphA7 are expressed at the dorsal edge of neural folds, and their interaction is required for neural tube closure. Interestingly, both full-length and truncated isoforms of EphA7 are present. The short splice variants, devoid of the kinase domain, inhibit phosphorylation and repulsive signaling of the co-expressed full-length EphA7 and make the ephrinA5/EphA7 interaction adhesive. Deletion of ephrinA5 in the mouse leads to defects of neural tube closure, in some cases resulting in anencephaly [41].

As in several other tissues, in the developing nervous system Eph receptors play a role in segmentation and boundary formation by restricting cell intermingling. For example, segmentation of the developing hindbrain results in the formation of rhombomeres, which are defined by restricted expression of several genes and are important for the orderly formation of cranial nerves and specification of neural crest cells. Ephs and ephrins show a complementary expression pattern in the hindbrain, with EphA4, EphB2, and EphB3 receptors restricted to odd-numbered rhombomeres (r3 and r5), while the ligands ephrinB1, ephrinB2, and ephrinB3 are found in even-numbered rhombomeres (r2/r4/r6) [42–45]. Studies in Zebrafish and Xenopus embryos showed that overexpression of a dominant-negative truncated EphA4 receptor as well as morpholino knockdowns of EphA4 and ephrinB2 all lead to disruption of rhombomere boundaries, with expression of r3/r5 markers spreading into even-numbered rhombomeres [46, 47]. Eph/ephrin signaling at the rhombomere boundaries appears to operate bidirectionally, so that Eph-forward signaling mediates cell sorting in odd-numbered rhombomeres, while ephrin reverse signaling performs this function in even-numbered rhombomeres [31]. These findings indicate that repulsive interactions between ephrins and Ephs restrict movement and intermingling of cells between neighboring segments and thereby stabilize rhombomere boundaries. In addition to these repulsive effects, Eph receptors might contribute to rhombomere formation by mediating adhesion within the odd-numbered rhombomeres [47]. Furthermore, EphA4 has been implicated in Zebrafish forebrain patterning, where it is expressed in the developing diencephalon and required for the formation of the boundary between the diencephalon and the eye field. Overexpression of a truncated version of EphA4 disrupted this boundary, leading to an increase in the size of the eye field at the expense of diencephalic structures [48].

Neural crest cell (NCC) migration, which also follows a segmental pattern, occurs in parallel to hindbrain patterning. NCCs are a group of cells that derive from the dorsal neural tube, migrate ventrally along stereotypic trajectories, and give rise to a number of different cell types in the periphery, including sensory and sympathetic

ganglia, as well as bones of the skull. A role of Ephs in separating the streams of migrating NCCs has been demonstrated in Xenopus embryos. Expression of EphA4 and EphB2 is observed in the NCCs of the third branchial arch, whereas ephrinB2 is present in the adjacent second arch NCCs. Overexpression of ephrinB2 or truncated dominant-negative variants of EphA4 and EphB2 both resulted in disturbed migration of third arch NCCs, which invaded neighboring second and forth arch territories [49]. Experiments in chick embryos indicated that Ephs might also be involved in trunk NCC migration through the somites. EphB3-expressing trunk NCCs migrate through the rostral half of the somitic sclerotome, avoiding the caudal half where ephrinB1 is present [50, 51].

Proliferation, Migration, and Sorting of Neurons Several studies have implicated Ephs in regulating neurogenesis and cell death in the developing cortex. Overactivation of EphA7 signaling in cortical progenitors by conditional transgenic overexpression of ephrinA5 resulted in enhanced apoptosis of neuronal progenitors and as a consequence a dramatic decrease in cortical size. Conversely, cell death was suppressed and cortical size increased in EphA7 knockout mice [52]. In contrast to the apoptosis-promoting effects of EphA7, EphA4 signaling stimulates proliferation of cortical progenitors, as demonstrated by diminished cortical size and reduced BrdU incorporation in EphA4 knockout mice. Since ephrinB1 is strongly expressed in progenitor cells and has proliferation-promoting effects, it was proposed to be the ligand for EphA4 in this system [53].

Eph/ephrin-mediated contact repulsion also contributes to the dispersion of Cajal-Retzius (CR) that are born in discrete regions of the pallium, from which they migrate tangentially to colonize the entire cortex [54]. Once CR cells reach their cortical marginal zone destination, they secrete reelin which besides binding its receptors from the LDL protein family also interacts with Ephs and ephrins. Mice deficient in ephrinBs or EphBs display typical reeler-like changes in migration of neurons in cerebral cortex and hippocampus, respectively [55, 56].

Newborn cortical neurons migrate radially from the proliferative zone to the cortical plate, where they form vertically oriented cortical columns, the main information-processing units in the cortex. A fraction of neurons diverge laterally and integrate into neighboring columns, a process that requires forward EphA/ephrinA signaling. Interfering with ephrin expression by knockout of multiple ephrin genes impairs the lateral intermixing of neuronal clones and disrupts the organization of columns, while overexpression of EphAs leads to formation of columns of multiclonal nature [57]. EphrinB1 also controls the columnar distribution of cortical pyramidal neurons by inhibiting their tangential migration [58]. Further work will be required to tease apart the relative contributions of ephrinAs and ephrinBs in this process.

Finally, patterning of the striatum involves sorting of cells between two morphologically and functionally distinct compartments, matrix and striosomes. EphrinA5 is predominantly expressed in striosomes, while EphA4 is selectively enriched in the later developing matrix cells. Experiments with organotypic striatal slices and analysis of EphA4 knockout mutants revealed a role for EphA4/ephrin signaling in striatal compartmentalization [30].

Axon Guidance The roles of Ephs in axon guidance are among the most diverse and well-studied functions for this receptor family. In different neuronal populations, Ephs have been implicated in gradient mapping, midline crossing, and binary pathway choices. Eph/ephrin interactions in *trans* can lead to repulsive or attractive responses, mediated by forward or reverse signaling. In addition, *cis*-binding of receptors and ligands co-expressed on the same axons seems to regulate their signaling output (Fig. 5.3a, b).

Axon Guidance in the Brain Establishment of thalamocortical and corticothalamic projections represents an example of gradient mapping. EphrinAs and EphAs are expressed in complementary gradients in the developing cortex and thalamus and regulate reciprocal wiring of these structures. The development of thalamocortical connections proceeds in several steps: The axons undergo initial sorting in the ventral telencephalon, where the broad areal map of the cortex is defined, and later topographic maps are also formed within the cortical areas. EphA/ephrinA interactions are required for specifying both the inter-areal and intra-areal topography [59–62]. Furthermore, studies of several EphA4 signaling mutants revealed that EphA4 kinase activity regulation is required for thalamocortical mapping, while the noncatalytic intracellular domains are dispensable [63, 64]. The formation of corticothalamic projections proceeds independently of thalamocortical mapping, but also relies on EphA/ephrinA interactions, as shown by EphA7 overexpression and knockdown in the cortex by in utero electroporation [65]. EphB receptors have also been implicated in thalamocortical axon guidance. EphB1- and EphB2-forward signaling and the ephrinB1 ligand are required during the early navigation of subpopulations of thalamic fibers in the ventral telencephalon, and the misguided thalamic fibers appear to influence the reciprocal cortical fibers guidance. These results are consistent with the "handshake hypothesis" that proposes that cofasciculation of specific thalamic and cortical axons controls proper cortical and thalamic interconnectivity [66].

In the developing hippocampus, multiple EphA receptors are also expressed in a gradient, whereas ephrinA gradients are detected in the lateral septum, a major target region of hippocampal axons [67–69]. Transgenic expression of a truncated dominant-negative EphA5 mutant resulted in mistargeting of hippocamposeptal projections [69]. Several Ephs have been implicated in the formation of the major brain commissures connecting the two hemispheres, the anterior commissure (AC) and corpus callosum (CC). In a large fraction of EphA4 null mutants, a total agenesis of AC was observed with both the anterior and posterior branches (aAC and pAC, respectively) missing [70]. In contrast, in the absence of EphB2 only pAC failed to form, due to misguidance of pAC axons into the ventral forebrain [71]. EphrinB ligand expression is detected in the AC axons, whereas EphA4 and EphB2 receptors are expressed in the adjacent tissue. Knockins of EphA4 and EphB2 signaling mutants rescued the AC guidance defects, pointing to a role of ephrinB reverse signaling in AC formation [70, 71]. MRI analysis of AC structure in various mouse mutants further suggested that EphA4 prevents the intermingling of pAC axons with the aAC bundle and that both EphA4 and EphB2 act synergistically to prevent aAC fibers from projecting along the pAC trajectory [72].

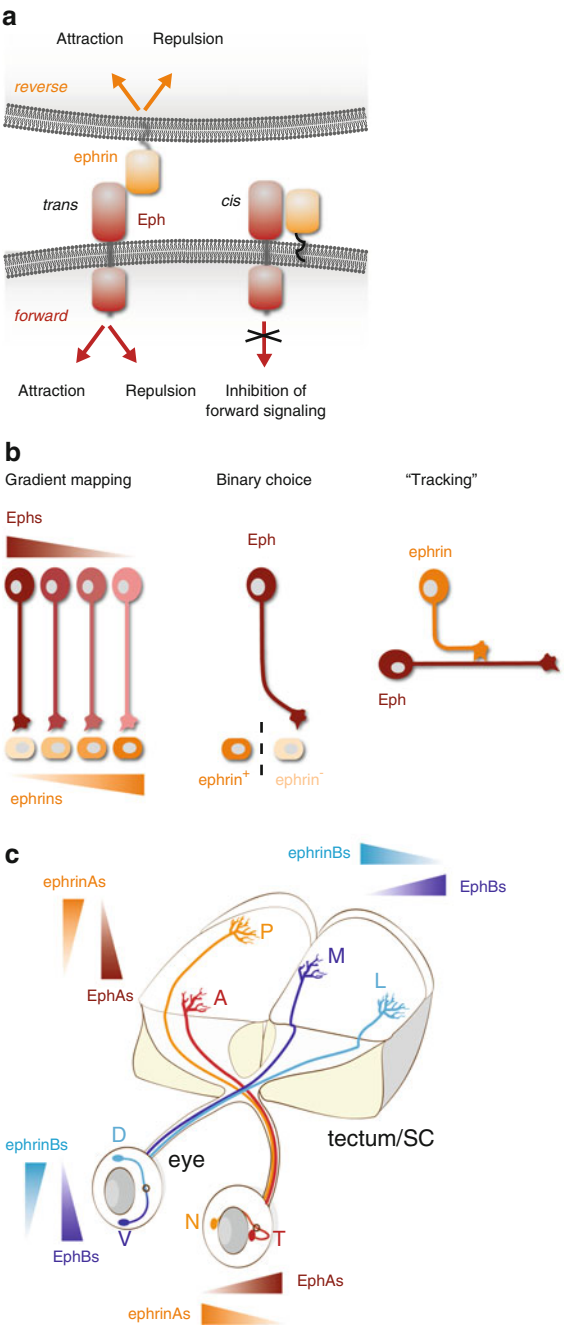

Fig. 5.3 Ephrin/Eph Signaling in Axon Guidance. (**a**) Axon guidance choices can be mediated by forward and reverse Eph/ephrin signaling, both of which can lead either to attraction or repulsion. In addition to *trans*-binding of ligands and receptors expressed on opposing cells, *cis* interactions can occur between Eph receptors and ephrins on the same membrane, leading to the inhibition of signaling. (**b**) Eph receptors and ephrins mediate various types of guidance decisions: topographic mapping of axonal projections along a continuous gradient of a guidance cue (*left*), binary choices between areas with high and low ligand expression (*middle*), and axon–axons interactions where one type of axons closely follows the trajectory of the other type (*right*). (**c**) Gradient expression patterns of Eph receptors and ephrins in the retina and tectum/superior colliculus (SC) and topographic mapping of retinotectal projections. Axons with high abundance of Ephs (e.g. from temporal retina) target regions with low abundance of ephrins (e.g., anterior tectum/SC). *D* dorsal, *V* ventral, *N* nasal, *T* temporal, *A* anterior, *P* posterior, *M* medial, *L* lateral

In the case of CC, expression of multiple Eph receptors and ephrin ligands was detected both in the callosal fibers and in the adjacent tissues at the midline. EphrinB3 mutants and several EphB receptor knockouts were reported to show defects in CC formation. The severity of the phenotype in different mutants varied from mild hypoplasia to total agenesis of CC and was generally higher in double knockouts, suggesting complex genetic interactions between several ephrins and Ephs in callosal axon guidance. In the most severe cases, callosal axons failed to cross the midline and accumulated on both sides within so-called Probst's bundles [73, 74]. Delayed growth and guidance errors of CC projections were also found in transgenic mice expressing a truncated version of EphA5 receptor lacking the intracellular domain, which presumably acted as a dominant-negative isoform and interfered with the activation of endogenous EphAs [75]. Interestingly, projections from different parts of the cortex are arranged in an ordered manner within the CC, so that axons from the medial cortex occupy a more dorsal position, whereas those from the lateral cortex are found more ventrally. In vitro experiments with cortical explants and in vivo knockdown in mice demonstrated that EphA3 receptor is required for this segregation as well as for correct pathfinding of callosal axons [76].

Finally, a defect in midline guidance was also observed in EphA8 knockout mice, where a group of commissural fibers connecting the superior colliculus with the contralateral inferior colliculus was found misguided into the ipsilateral spinal cord [77].

Axon Guidance in the Spinal Cord EphA4 is required for the development of the corticospinal tract (CST), which is thought to be important for fine movement control. CST fibers start from layer V pyramidal neurons, cross the midline in the medulla, and descend along the contralateral side of the spinal cord. In the absence of EphA4-forward signaling, many CST axons abnormally recross the spinal cord midline and exhibit premature branching [70, 78–80]. In addition, EphA4-forward signaling is also required for the guidance of an ascending ipsilateral dorsal spinal tract [80]. The dorsal funiculus of the spinal cord, which contains the CST along with several other fiber tracts, has an altered shallow shape in EphA4 knockouts [70, 78, 80]. All of these defects are also observed in mice lacking ephrinB3, which is expressed at the spinal cord midline and serves as a repulsive signal for EphA4-expressing axons [81].

EphrinB3/EphA4 interaction is also important for the correct assembly of central pattern generators (CPG), local circuits of spinal interneurons controlling coordinated limb locomotion during walking. In the absence of EphA4 signaling, the disturbed wiring of the CPG leads to synchronous activation of motor neurons on both sides of the spinal cord instead of the normal left–right alternation, causing the characteristic rabbit-like hopping gait of ephrinB3 and EphA4 null mutant mice [82]. This abnormality has been attributed to the mistargeting of a population of ipsilateral excitatory EphA4-expressing interneurons, which aberrantly cross the midline in EphA4 mutants and disturb the balance of excitatory and inhibitory signals in the CPG [82–84].

Forward ephrinB3/EphA4 signaling in cortical motor neurons and in the CPG is mediated by the RacGAP alpha2-chimaerin, which was shown to interact with

EphA4 and to mediate growth cone collapse in response to ephrins [85–88]. Alpha2-chimaerin knockout mice show phenotypes similar to ephrinB3 and EphA4 mutants, such as defects in CST guidance, dorsal funiculus shape, and CPG wiring [85–87]. The same impairments, as well as a reduction in the posterior branch of the AC, were observed in mice lacking Nck1 and Nck2 in the nervous system, indicating that Nck adaptor proteins also act downstream of EphA4 [89].

Axon Guidance in the Periphery Spinal sensory-motor circuits have proven a very useful system to study various modes of Eph/ephrin interactions in axon guidance. Ephs have a well-established role in the binary dorsal–ventral guidance of motor neuron projections to the limb. Limb muscles receive innervation from motor neurons situated within the lateral motor column (LMC) of the spinal cord. Motor neurons located in the medial subdivision of the LMC (LMC_M neurons) send their axons to the ventrally derived limb muscles, while the cells of the lateral subdivision (LMC_L neurons) innervate the dorsally derived muscles. EphA4 is enriched in LMC_L neurons, whereas ephrinAs are found in the ventral compartment of the limb [90–92]. Genetic deletion of EphA4 leads to LMC_L misprojections into the ventral limb [92], while overexpression of EphA4 in LMC_M cells causes some of the ventrally fated LMC_M axons to choose the dorsal trajectory [91, 93], suggesting that a repulsive ephrinA/EphA4 interaction is required for directing motor axons to the dorsal pathway in the limb. In mirror symmetry to the effects of EphA4 signaling in the LMC_L population, LMC_M neurons express EphB receptors, and are repelled from ephrinB2 in the dorsal limb mesenchyme [94]. Experiments in chick embryos suggested that Src family kinases are among the downstream effectors of both EphA4 and EphB signaling in motor neurons. Inhibition of Src family kinase activity attenuated the guidance errors caused by overexpression of EphA4 and EphB2 in LMC cells [95].

Motor neurons express ephrin ligands in addition to Eph receptors [90, 96, 97]. Intriguingly, co-expressed receptors and ligands were proposed to preferentially engage in *cis* or *trans* interactions in different motor neuron populations (Fig. 5.3a). In LMC_M neurons, ephrinAs are more abundant and bind EphAs in *cis*, preventing them from transmitting repulsive signals from ligands in *trans* and allowing the ingrowth of LMC_M axons into the ephrinA-rich ventral limb [97]. In contrast, in LMC_L neurons, ephrinAs and EphAs were shown to spatially segregate into different domains of the membrane, and to independently mediate parallel forward and reverse signaling [96, 97]. Reverse ephrinA signaling in motor neurons is attractive [96], and contributes to guiding LMC_L axons toward dorsally expressed EphAs in vivo [14, 98]. Extracellular proteolytic cleavage of EphA4 has been recently shown to also play a role in limb axon guidance by regulating the expression levels of EphA4 in the limb mesenchyme, where it can interact with ephrinAs in *cis* and thereby determine the effective concentration of the ligands available for inducing forward EphA signaling in axons [25]. Later in development, Eph/ephrin signaling might mediate positional mapping of motor axons within individual muscles, as muscle topography was disturbed in loss- and gain-of-function ephrinA mouse mutants [99].

The growth of motor axons has to be coordinated with the development of sensory fibers, which follow a very similar pattern of innervation and are bundled

together with motor axons within mixed nerves. Sensory-motor axon–axon interactions also largely rely on the Eph/ephrin system. Forward EphA signaling is required in motor axons innervating the axial musculature to segregate them from the neighboring ephrinA-expressing sensory fibers. Genetic deletion of EphA3 and EphA4 receptors leads to sensory-motor miswiring, with motor fibers invading the dorsal root ganglia [100]. Interestingly, the same signaling partners are then reused to guide sensory projections along the grid of the earlier developing motor nerves. Time-lapse imaging of cocultured sensory and motor neurons suggested that sensory axons track along the preestablished motor fibers toward their distal ends, and this interesting behavior is dependent on reverse ephrinA signaling, elicited by EphA3 and EphA4 expressed in motor axons (Fig. 5.3b). Genetic interference with reverse signaling therefore leads to a disrupted map of sensory projections [101].

Topographic Mapping in Sensory Systems Eph/ephrin signaling has been implicated in the development of topographic maps in several sensory modalities. The best-studied sensory system that relies on Eph/ephrin signaling is the retinotectal projection. Multiple Eph receptors are expressed in the retina: A low-nasal to high-temporal gradient of expression is observed for EphA5 and EphA6 in the mouse and EphA3 in the chick, while EphA4 is also detected, but does not show a gradient pattern [102–107]. The target tissues of retinal ganglion cell (RGC) axons, the superior colliculus (SC) in mammals and tectum in nonmammalian vertebrates, display a complementary low-anterior-to-high-posterior gradient of ephrinA2 and ephrinA5 [103–106, 108, 109] (Fig. 5.3c). High Eph-expressing temporal axons project to the anterior regions of the superior colliculus with low ephrin levels, whereas low Eph-expressing nasal axons map onto the ephrin-rich posterior regions, consistent with a repulsive EphA/ephrinA interaction (Fig. 5.3c). This axonal behavior can be recapitulated in vitro with the help of the stripe assay, where temporal RGC axons are repelled from stripes containing posterior tectal membranes or coated with ephrinAs, while nasal axons are not sensitive to these stripes [104, 106, 110, 111]. In ephrinA knockout mice, temporal axons form ectopic more posterior termination zones, and posterior SC membranes from ephrin mutants lose their repulsive activity toward temporal RGC axons in vitro [103, 104, 109, 112]. Changes in RGC axons' responsiveness to tectal membranes in vitro and/or disruption of the retinotectal map in vivo were also observed in mice overexpressing EphA6 in the retina and mouse mutants lacking the EphA5 intracellular domain, as well as in chick embryos overexpressing ephrinA2 in the tectum or a dominant-negative truncated version of EphA3 in the retina [111, 113, 114]. Interestingly, deletion of one copy of Tsc2 gene also led to a shift of RGC termination zones similar to the one observed in ephrinA mutants and changed the sensitivity of RGC axons to ephrinAs in vitro. These findings suggested that the effects of EphA/ephrinA signaling in the retinotectal system are at least in part mediated by the Tsc2–mTOR pathway [115].

Further studies demonstrated that retinocollicular mapping depends on relative rather than absolute differences in Eph signaling levels between RGCs [102, 116, 117]. Thus, overexpression of EphA3 in a subset of RGCs interspersed between the cells with endogenous amounts of receptors led to the duplication of the retinotopic map in the superior colliculus. Cells with elevated EphA levels formed an independent

map in the anterior (low ephrin) part of the superior colliculus, while the map established by axons with endogenous EphA levels was shifted posteriorly into the ephrin-rich region [102].

EphrinAs are also expressed in the retina in a nasotemporal countergradient to EphA receptors, while EphAs are detected in the SC in a high-anterior to low-posterior gradient reciprocal to the ephrinA ligands [18, 107, 118, 119] (Fig. 5.3c). On the one hand, there is evidence that ephrins present on RGC axons might convey a repulsive signal from Ephs in the target tissue. For instance, in EphA7 knockout mice, nasal (ephrinA-rich) axons form ectopic termination zones in the anterior SC. In agreement with the knockout phenotype, in vitro stripe assay demonstrated repulsion of retinal axons from EphA7 stripes [120]. On the other hand, ephrinAs were proposed to engage in a *cis* interaction with the co-expressed axonal EphAs, leading to inhibition of EphA-forward signaling. A countergradient of ephrinAs in the retina may therefore serve to enhance the differences in EphA activation in RGC axons along the nasal-temporal axis and thereby fine-tune their ephrin sensitivity [18, 19]. Current models of retinotopic mapping were recently challenged by the analysis of conditional knockout mice in which ephrinA5 was deleted selectively from the retina or the SC. This study demonstrated that axon–axon interactions play crucial roles in retino-collicular mapping [121]. These findings suggest that topographic maps form in part by the ability of retinal axons to self-organize into a map.

Guidance of RGC axons by Eph/ephrin reverse signaling seems to require endocytosis of Eph receptors. Transgenic mice expressing an endocytosis-deficient EphA8 mutant displayed an anterior shift of nasal axon projections, consistent with a diminished repulsion from EphAs in the anterior SC [122].

In a similar way to ephrinAs and EphAs, ephrinBs and EphBs are expressed in countergradients in RGCs, and their interactions mediate the mapping of the dorsal–ventral axis of the retina onto the lateral–medial axis of the SC. EphB receptors are enriched in the ventral retina and ephrinBs in the dorsal retina. In the SC, EphB expression is present, but does not follow a clear gradient pattern, while ephrinB1 is detected in a high-medial to low-lateral gradient [107, 118, 119, 123–127] (Fig. 5.3c). Gain- and loss-of-function genetic manipulations have suggested that both EphB/ephrinB forward and reverse signaling contribute to the medial-lateral guidance of RGC axons, probably by an attractive mechanism [123, 126–128], although one study suggested that ephrinB1 might have both attractive and repulsive properties depending on the dorsoventral origin of the axons [128]. In addition, EphB/ephrinB reverse signaling was reported to mediate an earlier step in the guidance of RGC axons, directing them toward the optic disc in the eye [125].

Interestingly, in binocular animals like primates the retinal expression pattern of Ephs and ephrins appears different from that in the commonly studied predominantly monocular model organisms. For example, in humans EphA5 and EphA6 are expressed in a bidirectional gradient along the nasal-temporal axis, peaking in the center of the retina and declining toward the periphery, while ephrinA5 shows a complementary pattern with high levels at the periphery and low in the center. In the target tissue, the dorsal lateral geniculate nucleus, the distribution of EphAs and ephrinAs follows a single gradient, similar to the pattern described in the mouse [129].

While the great majority of RGC axons in the mouse cross the midline at the optic chiasm to innervate the contralateral SC, a small population of early-born RGCs located in the ventrotemporal crescent of the retina form ipsilateral projections. These cells express EphB1 and are sensitive to the repulsive action of ephrinB2, which is present at the chiasm midline during the development of the uncrossed projection [130]. Genetic deletion of EphB1 in mice led to a marked reduction in the number of ipsilateral axons, while precocious ectopic expression of ephrinB2 at the chiasm in Xenopus resulted in the opposite phenotype with increased numbers of uncrossed fibers [130, 131]. The crossed and uncrossed RGCs selectively express the transcription factors Islet2 and Zic2, respectively. Mice lacking Islet2 show an upregulation of EphB1 expression in the ventrotemporal retina and have an increased ipsilateral projection [132]. In contrast, in FoxD1 knockout mice that display a downregulation of Zic2 and EphB1, the ipsilateral part of optic tract is reduced [133]. A defect in the ipsilateral projection was also observed in mice deficient for the Rho GEFs Vav2 and Vav3, suggesting that these proteins mediate Eph-forward signaling in vivo [134].

In the vestibular system, ephrinB/EphB signaling is required for the guidance of efferent fibers innervating the inner ear. In EphB2 knockouts and EphB2/EphB3 double mutants, the development of these fibers is delayed, and some axons show guidance errors at the midline. It should be noted that the vestibular phenotypes (see also "Adult physiology") seem to be dependent on genetic background, as they were only observed in CD1 mice [135]. Peripheral axons from auditory spiral ganglion neurons (SGNs) form an elaborate series of radially and spirally oriented projections that relay sound stimuli to the brain. SGN fascicles project through the otic mesenchyme to form synapses within the cochlea. EphA4 expressed in the otic mesenchyme provides a cue that promotes the fasciculation of SGNs via binding to ephrinB2 on their surfaces [136]. EphA4-forward signaling was also shown to control the targeting of SGN afferent fibers to inner and outer hair cells expressing ephrinA5 [137].

Attractive EphA/ephrinA reverse signaling has been implicated in the guidance of olfactory and vomeronasal axons. Sensory neurons expressing the same odorant receptor are widely distributed within the olfactory sensory epithelium or vomeronasal organ, but their axons coalesce into one or several glomeruli with stereotypic positions within the olfactory bulb and accessory olfactory bulb, respectively. EphrinAs are expressed on olfactory sensory axons, showing different expression levels on axons with different olfactory receptor identity, while EphA5 is detected in the olfactory bulb [138]. Similarly, ephrinA5 is present on a subpopulation of vomeronasal axons, and EphA6 is detected in the accessory olfactory bulb, although in this case EphA6 is expressed in a gradient. Vomeronasal axons with high ephrinA levels map onto the EphA6-rich area of the accessory olfactory bulb, suggesting an attractive/adhesive interaction between EphAs and ephrinAs, which was confirmed in in vitro stripe assays [139]. Analysis of ephrinA mouse mutants and mice overexpressing ephrinA5 demonstrated that reverse ephrinA signaling is required in both olfactory and vomeronasal systems for correct glomerular map formation [138, 139]. Another study also found ephrinAs and EphAs among molecules differentially expressed in olfactory glomeruli, and furthermore demonstrated that their expression is activity regulated [140].

The involvement of Ephs in synapse formation and plasticity is described below in the section "Adult physiology."

5.2.1.3 Cardiovascular Development

The vascular system is hierarchically organized in arteries, veins, and capillaries. During development, some vascular structures, such as the dorsal aorta and the first primitive capillary plexus, are formed by vasculogenesis, the de novo assembly of vascular tubes by progenitor endothelial cells. Later, the capillary plexus is remodeled and new capillaries form by sprouting from preexisting blood vessels, processes that are summarized under the term angiogenesis.

The EphB4 receptor and its cognate ligand ephrinB2 are expressed very early and are required for vascular development. Knockout mice lacking either EphB2/EphB3 have severe defects in angiogenesis and in remodeling of the yolk sac vasculature and do not survive past embryonic development [141, 142]. EphB4 expression is highest in the venous endothelium, whereas ephrinB2 shows a complementary pattern, being most prominent in arterial endothelial cells [143, 144]. Since EphB4 and ephrinB2 are expressed in the primitive capillary plexus prior to remodeling, the current view is that ephrinB2/EphB4 signaling occurs at the arterial/venous boundary and mediates vascular remodeling in the early embryo (Fig. 5.4a). Endothelial cell-to-cell interactions via ephrins and Ephs are not restricted to the border between arteries and veins. Vascular defects in EphB2/EphB3 double knockouts and expression of EphB2 in mesenchyme adjacent to blood vessels suggest a requirement for Eph/ephrin signaling at the domain boundaries between endothelial cells and surrounding tissue [144]. The chemokine SDF-1 and EphB2/EphB4 receptors orchestrate endothelial cell movement and morphogenesis into capillary-like structures [145].

EphB4 and ephrinB2 regulate the formation of two major blood vessels that connect the heart with the vascular system: the dorsal aorta, which carries the blood from the heart to the periphery, and the cardinal vein, which returns the blood from the trunk to the heart. Work in zebrafish has challenged the prevailing view that these two major vessels form by vasculogenesis. Instead, early zebrafish embryos have only one common precursor vessel that co-expresses EphB4 (EphB4a) and ephrinB2 (ephrinB2a). Bidirectional repulsion between ephrinB2a- and EphB4a-expressing angioblasts induces ventral sprouting of EphB4a-positive angioblasts that eventually segregate from the dorsal aorta to form the cardinal vein (Fig. 5.4b) [146].

EphB4 is also expressed in lymphatic vessels that carry lymph unidirectionally toward the heart. EphB4 appears to act as a "ligand" for ephrinB2 whose activation drives lymphatic vessel sprouting during development [147, 148]. EphrinB2 acts in part by regulating the spatial activation of vascular endothelial growth factor (VEGF) receptor endocytosis and signaling [149, 150].

EphA2 is expressed in blood and lymphatic vessels in airways of juvenile mice. EphA2-deficient mice have fewer capillaries, a larger number of endothelial sprouts, and greater capillary diameters. This may be in part caused by defective coverage of vessels with pericytes which normally wrap around the endothelial cells, thereby

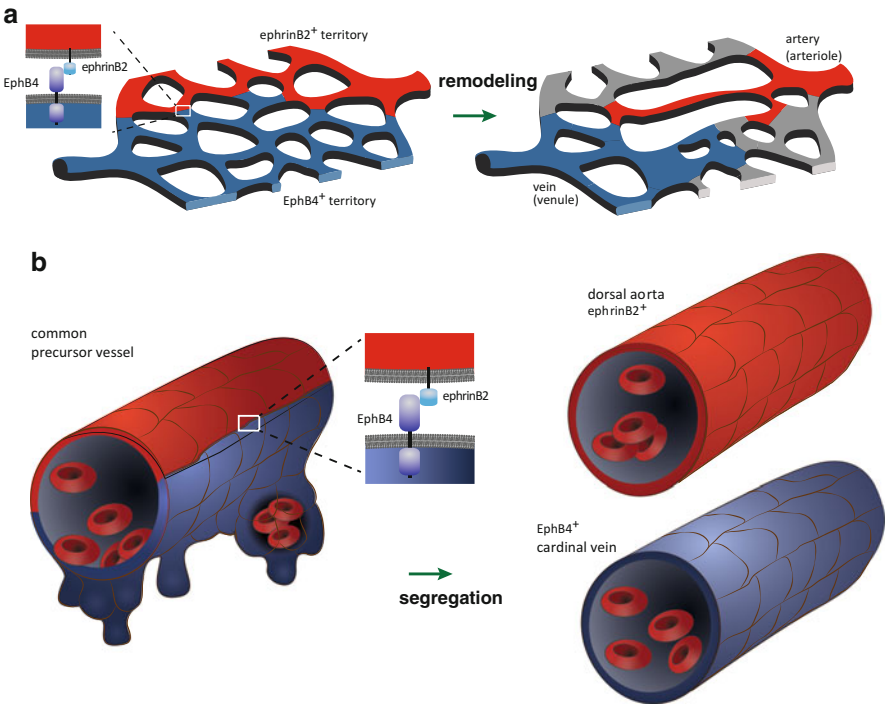

Fig. 5.4 EphB/ephrinB Signaling During Angiogenesis. (**a**) Model for EphB4/ephrinB2 function at the arterial/venous boundary. During vasculogenesis, early endothelial precursor cells assemble a primitive vascular network (plexus). Future arterial and venous territories are already marked by complementary expression of ephrinB2 and EphB4. EphB4/ephrinB2 signaling at the boundary initiates remodeling of the primitive plexus into a hierarchically organized vascular network. (**b**) In zebrafish embryos, the two major axial blood vessels, dorsal aorta and cardinal vein, develop from a single common precursor vessel. EphB4/ephrinB2 signaling in endothelial cells of the precursor vessel triggers EphB4-expressing cells (EphB4a) to migrate ventrally where they contribute in the assembly of the future cardinal vein

providing stability and regulating blood flow, amongst other functions. It was concluded that lack of EphA2 causes abnormal interactions between endothelial cells and pericytes [151].

EphA3 and its ligand ephrinA1 are expressed in adjacent cells in the developing heart in the so-called endocardial cushions. This mesenchyme-derived tissue undergoes morphological changes to result in the formation of the heart valves and septa. EphA3 knockouts have defects in the development of their atrial septa and endocardial cushions, and these abnormalities lead to the death of 75 % of homozygous EphA3 mutants [152].

During development, EphA4 is expressed by endothelial cells in the central nervous system. EphA4 null mice exhibit an abnormal CNS vascular structure in cerebral cortex and spinal cord, with disorganized branching and smaller diameter vessels [153].

5.2.1.4 Other Organ Development

Skeletal Patterning During development of the embryonic thorax, paired ribs extend from the vertebral column and fuse ventrally to form the sternum. The sternum eventually ossifies into segmented sternebrae. EphB2/EphB3 and ephrinB1 are involved in the assembly and segmentation of mesenchymal condensations and their differentiation into cartilage and bone. Double EphB2/EphB3 and ephrinB1 mouse mutants display skeletal abnormalities. In the thorax, sternocostal connections are arranged asymmetrically and sternebrae are fused. Heterozygous ephrinB1 female mice, in which expression of the X-linked *ephrinB1* gene is mosaic and ectopic EphB–ephrinB1 interactions take place, display additional bone malformations, such as preaxial polydactyly. These results point to an important role of EphB receptors in providing positional cues required for the normal morphogenesis of skeletal elements [27]. EphrinB1 loss-of-function mutations in humans cause similar developmental bone defects including craniofrontonasal syndrome (CFNS), with greater severity in heterozygous females than in hemizygous males [154].

Palate Formation The development of the secondary palate in mammals is a complex process which when disturbed results in the birth defect cleft palate. The process involves the formation of two palatal shelves, the elevation of these shelves to a horizontal position, and subsequent midline fusion. EphB2/EphB3 double mutant mice display a cleft palate phenotype [73]. Similarly, knockout mice lacking the Wnt kinase-dead receptor, Ryk, exhibit craniofacial defects including cleft palate. In mice, Ryk was shown to modulate EphB signaling, possibly by involving the scaffolding protein Af-6 (Afadin) [155]; however this was not reciprocated in human studies [156]. More recent work indicates that midline fusion at the palate requires EphB/ephrinB bidirectional signaling [157]. Similar mechanisms seem to underlie midline cell–cell adhesion and fusion events during urorectal development [158].

Pancreas The pancreas develops from an endoderm-derived protodifferentiated epithelium. EphB2 and EphB3 are expressed in the pancreatic epithelium, and double mutants display defects in the overall morphology and branching of the pancreas gland. A decrease of membrane-associated β-catenin in the outer cap cells was found in these mice, suggesting that EphB signaling regulates β-catenin expression and cellular localization. E-cadherin also seems to be decreased. In summary, EphB signaling plays a role in epithelial remodeling during pancreatic branching [159].

Thymus The development of the thymus occurs in several phases, comprising a postnatal T-cell developmental phase when bone marrow precursor cells enter the thymus and differentiate in a mature and compartmented thymic stroma. EphA4 knockout mice have an altered maturation of the thymic epithelium that results in defective T-cell development, with a decreased proportion of double-positive (CD4–CD8) cells [160].

Thyroid EphA4 is a regulator of postnatal thyroid morphogenesis and influences the development of the two major endocrine cell lineages of the differentiating gland, the follicular epithelium and the parafollicular C cells, which produce calcitonin. EphA4-forward signaling in the follicular epithelium and ephrin reverse signaling in the C cells are probably functionally important [161].

5.2.2 Adult Physiology

5.2.2.1 Nervous System

Synaptogenesis and Spine Morphogenesis Dendritic spines are small protrusions from neuronal dendrites that form the postsynaptic component of most excitatory synapses in the brain. The formation and remodeling of spines, processes that underlie synaptic development and plasticity, are regulated in part by Eph receptors. EphB signaling has been associated with the formation of glutamatergic synapses in dissociated neuronal cultures. EphB2 was found to interact directly with NMDA receptors (NMDAR), resulting in NMDAR recruitment to Eph receptor clusters [162, 163], and enhanced NMDAR-mediated Ca^{2+} influx and gene expression [164]. Knockdown experiments in dissociated neurons have shown that different EphB2 domains are required for different aspects of synapse formation. Trans-synaptic interaction with ephrinBs promotes presynaptic differentiation; the EphB2 ectodomain seems to be important for the interaction with NMDAR, whereas the EphB2 intracellular PDZ interacting motif regulates the localization of AMPA-type receptors (AMPAR) [165]. EphB function in synaptic differentiation has also been linked to the regulation of spine motility; EphB knockdown reduces filopodia motility hampering synaptogenesis [166]. Moreover, in vivo, EphB2 knockout mice display reduced hippocampal NMDAR-mediated currents and NR1 (GluN1) synaptic levels [167], supporting the role of EphB2 in the regulation of synaptic function. In the context of spine morphogenesis, there seems to be functional redundancy between EphB receptors (EphB1–B3), as synapse morphology appears to be normal in EphB2 knockout mice [167, 168], whereas EphB1, B2, B3 triple knockouts show reduced spine density in the hippocampus [169]. The defects seen in cortical neurons of EphB triple knockouts could, nonetheless, be rescued by postnatal re-expression of EphB2 in single neurons in slice cultures [165].

EphB receptors regulate spine morphogenesis by modulating the activity of Rho family GTPases, key regulators of actin dynamics that influence spine morphogenesis (Fig. 5.5a). In cultured hippocampal neurons, activation of EphB signaling by

Fig. 5.5 (continued) tyrosine phosphorylation of the heparin sulfate proteoglycan syndecan. EphB2 phosphorylates the RhoA GTPase ephexin5 thereby triggering its degradation via the ubiquitin pathway. Ephexin5 expression suppresses synapse development by activating the small G protein RhoA that functions to antagonize the effects of Rac1. (**b**) EphrinA3 expressed on astrocytes activates EphA4 on the postsynaptic neuron and restricts the growth of dendritic spines. EphA4-forward signaling involves tyrosine phosphorylation and subsequent activation of the serine/threonine kinase Cdk5, which then activates the RhoA-specific GEF ephexin1 via serine phosphorylation, leading to increased RhoA activity. EphA4 activation inhibits the integrin pathway, disrupting integrin-mediated attachment to the extracellular matrix. Mechanistically, EphA4 signaling causes the disassembly and inactivation of integrin signaling complexes, reducing tyrosine phosphorylation of the downstream targets Cas, Fyn, and Pyk2. EphA4 activation also causes the actin depolymerization factor cofilin to dissociate from the plasma membrane upon activation of PLCγ, leading to actin depolymerization and spine retraction. EphA signaling has also been proposed to induce spine formation via a different mechanism. Upon binding of ephrinAs, EphA4 is cleaved by γ-secretase. The EphA4 intracellular domain (ICD) then activates Rac1, thus inducing spine formation

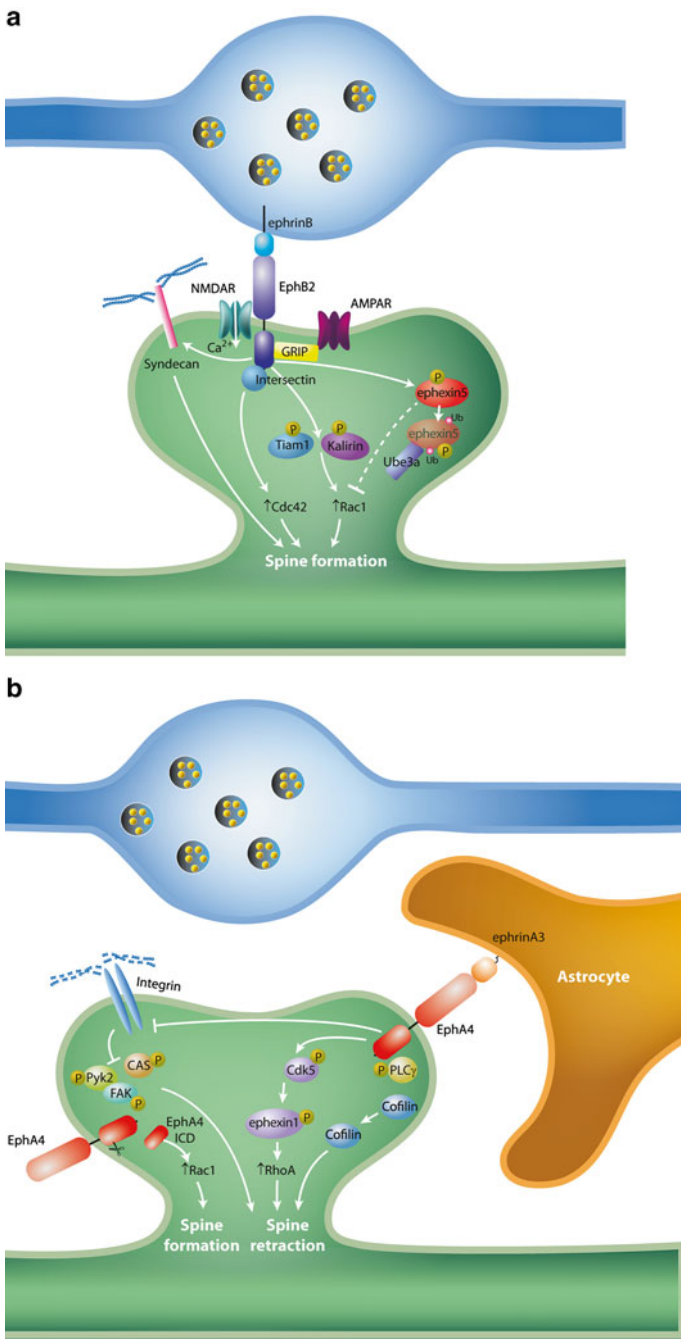

Fig. 5.5 Signal transduction mechanisms of Eph receptor forward signaling in spine formation and morphology. (**a**) EphBs in the dendritic spines are thought to be activated by presynaptic ephrinBs. EphB-forward signaling promotes spine growth by increasing the activity of the Rho GTPases Rac1 and Cdc42 through regulation of the GEFs Intersectin, Kalirin and Tiam1. Activation of postsynaptic EphB2 receptor also leads to clustering of glutamatergic ion channels via direct interaction (NMDA receptor) or indirectly through the PDZ scaffold protein GRIP (AMPA receptor). EphB2 receptor may also have a global effect on synapse formation by facilitating NMDA receptor-mediated Ca^{2+} influx and gene expression. In addition, the formation of mature spines involves

ephrinBs induces phosphorylation and clustering of Kalirin and Tiam1, Rac1-specific GEFs. Expression of catalytically inactive Kalirin, dominant-negative Tiam1, and dominant-negative Rac1, as well as knockdown of Tiam1, inhibits ephrinB-dependent spine development [170, 171]. Activated Rac1 acts on P21-activated kinase (PAK), which is required downstream of EphB receptors for spine and synapse formation [166, 170]. EphB2 also interacts with and activates the GEF intersectin-1, which in turn activates another Rho GTPase, Cdc42 [172]. It was also shown that upon binding of ephrinBs, EphB2 phosphorylates the RhoA GTPase ephexin5, thereby triggering its degradation. Ephexin5 normally suppresses synapse development by activating RhoA, which antagonizes Rac1 GTPase function [173]. Finally, activation of EphB2 results in the phosphorylation of the heparin sulfate proteoglycan syndecan-2, a known regulator of spine morphogenesis [174]. Paradoxically, EphB tyrosine kinase activity does not seem to be required for the formation or maintenance of functional synapses, as shown by acutely blocking tyrosine kinase activity of EphB1,B2,B3 in triple knock-in mice, suggesting a possible role for cytoplasmic domain oligomerization and other forms of protein–protein interactions in this process [175].

Whereas EphB signaling promotes spine growth, EphA signaling appears to have the opposite effect, preventing excessive spine growth. Activation of EphA4 signaling by ephrinA3 in hippocampal slice cultures decreases spine length and density. In addition, EphA4 and ephrinA3 knockouts display longer and disorganized spines [176, 177]. EphA4 seems to regulate spine morphology through a repulsive interaction with ephrinA3 expressed in glial cells.

As in the case of EphB2, the modulation of spine retraction by EphA4 signaling involves the regulation of Rho GTPases (Fig. 5.5b). In hippocampal brain slices, stimulation with ephrinAs results in the recruitment and activation of cyclin-dependent kinase 5 (Cdk5) to EphA4. Cdk5 activates ephexin1, a RhoA-specific GEF, which promotes RhoA activity and thereby regulates actin reorganization in spines [178]. EphA4 was also shown to regulate the activity levels of Ras proteins Rap1 and Rap2, two GTPases involved in the regulation of spine morphology. The EphA4 C-terminus binds to the PDZ domain of the GTPase-activating protein spine-associated RapGAP (SPAR). In a neuronal cell line, ephrinA stimulation was shown to induce SPAR-dependent inactivation of Rap1 and Rap2 [179]. Furthermore, in hippocampal slices EphA4 was shown to interact with and activate phospholipase Cγ (PLCγ) through its juxtamembrane tyrosines, leading to the reduction of the cofilin pool that is associated with the membrane. This leads to the release of cofilin into the cytoplasm and its binding to actin filaments, triggering their depolymerization [180]. Moreover, EphA4 signaling causes the inactivation of integrin signaling by decreasing the phosphorylation of Crk-associated substrate (Cas), focal adhesion kinase (FAK), and proline-rich tyrosine kinase 2 (Pyk2)[181], leading to a reduction of adhesion to the extracellular matrix. Furthermore, EphA4 was shown to be an activity-dependent substrate of γ-secretase. Cleavage by γ-secretase results in the release of the EphA4 intracellular domain, which induces the formation of dendritic spines in hippocampal cultured neurons through activation of Rac1 [22].

In summary, there is good evidence that EphBs and EphA4 signal cell-autonomously to induce spine morphogenesis and synapse formation. Reverse signaling by ephrinBs,

not discussed in this chapter, is also crucial for synapse formation and plasticity (for reviews, see [182, 183]).

Synaptic Plasticity Eph receptors are largely known for their involvement in brain development, but they are also expressed in the adult central nervous system, where their involvement in synaptic plasticity, learning and memory has also emerged. The storage of information in the brain during learning and memory requires persistent alterations in the strength of synaptic transmission, a property best known as synaptic plasticity. A widely studied form of synaptic plasticity is long-term potentiation (LTP). LTP is defined as a lasting increase in synaptic strength induced by high-frequency electrical stimulation. In vertebrates, LTP has most extensively been studied in the hippocampus. It is generally agreed that LTP in the mossy fibers, which connect granule cells of the dentate gyrus with pyramidal CA3 neurons in the hippocampus, is NMDAR independent and expressed presynaptically as an increased probability of neurotransmitter release. Mossy fiber LTP was reduced when postsynaptic neurons were treated with a peptide that disrupts the interaction between the EphB2 PDZ-binding motif and scaffolding protein GRIP1, arguing that postsynaptic mechanisms dependent on EphB2–PDZ binding interactions might contribute to LTP induction. Mossy fiber LTP was also reduced by the extracellular application of soluble ephrinBs, which block EphB/ephrin interactions, confirming that a trans-synaptic Eph/ephrin signal is required for the presynaptic changes that underlie potentiation [184].

LTP in the Schaffer collaterals (CA3-CA1), unlike in the mossy fibers, is postsynaptic and dependent on NMDAR. EphB2 and EphA4 knockout mice show impairments in both LTP and LTD (long-term depression). Interestingly, C-terminally truncated EphB2 and EphA4 receptors consisting of ecto- and transmembrane domains rescued these defects, indicating that the kinase domains and noncatalytic modules such as the PDZ-binding motifs are dispensable [167, 168, 185]. EphB2 might regulate synaptic plasticity through the ability to interact with and modulate NMDAR function [164, 167, 168, 185]. Alternatively, Eph receptors could act as ligands at this synapse, inducing reverse signaling through postsynaptic ephrinBs. In agreement, ephrinB2 mutants lacking tyrosine phosphorylation sites or the PDZ-binding motif show defects in long-term potentiation [186]. EphA4 in the postsynaptic neuron has been proposed to modulate LTP by interacting with ephrinA3 expressed in astrocytes. Disruption of EphA4/ephrinA3 signaling leads to an upregulation of glial glutamate transporters and an increase of glutamate uptake by astrocytes [176, 187].

Eph receptors also seem to be required for synaptic plasticity in other brain regions. EphA4 knockout mice have impaired amygdala LTP. Rin1, a Rab5 GEF, was shown to influence EphA4 endocytosis and thereby control EphA4 surface distribution and plasticity in the amygdala [188]. In addition, EphB2 in the amygdala regulates stress-induced plasticity and anxiety-like behavior. It was shown that upon stress EphB2 is cleaved by the serine protease neuropsin, causing dissociation of EphB2 from NMDA receptors. The dynamic interaction of EphB2 and GluN1 results in increased excitatory synaptic currents and enhanced behavioral signatures of anxiety [23].

Changes in synaptic plasticity often translate into behavioral defects in learning and memory. EphB2 and ephrinA3 knockout mice have deficiencies in some forms of spatial learning [168, 176]. Other less well-studied Eph receptors may also be involved in learning and memory formation. For example, EphA6 knockout mice display learning and memory impairments [189] and EphA5 knockouts show alterations in aggressive behavior, which correlate with altered concentrations of the neurotransmitter serotonin in the hypothalamus [190].

Synaptic plasticity, the modulation of the synapse strength, may also be accompanied by structural plasticity, the formation and elimination of synapses. Hippocampal mossy fiber terminal arborizations (TAs) exhibit prominent structural plasticity in early postnatal development, a process requiring EphA4. Interference with EphA4 function by a short peptide in organotypic slice culture experiments increased the number and reduced remodeling of TAs [191]. In cortical neuronal cultures, overexpression of EphA4 by in utero electroporation seems to be associated with an increased network activity, accompanied by an increase in mature spines in a cell-autonomous manner [192].

Homeostatic synaptic plasticity is a compensatory mechanism of synaptic strength regulation to counterbalance excessive excitation or inhibition in the neuronal network activity. A study in the Drosophila neuromuscular junction reported a requirement for Eph signaling in motor neurons for homeostatic control of synaptic transmission, where an impairment of postsynaptic activity leads to upregulation of presynaptic neurotransmitter release. Eph receptor signaling regulates the presynaptic $Ca_v2.1$ calcium channel through the Rho GEF ephexin and the Rho GTPase Cdc42, thus enhancing presynaptic calcium influx and neurotransmitter release [193]. In cultured hippocampal neurons, EphA4 is activated by elevated synaptic activity and is required for the scaling down of the network activity. EphA4 activation leads to a reduction of the amplitude of miniature excitatory postsynaptic currents (mEPSC), by targeting AMPAR subunits for degradation through the ubiquitin pathway [194].

Regeneration Damage in the adult CNS usually results in very limited regeneration of lesioned axons, which are inhibited by the environment of the injured site. The glial environment of the adult CNS, which includes inhibitory molecules like myelin, as well as the formation of astroglial scarring, have been implicated in inhibition of axonal regeneration. In addition to these myelin components, repulsive guidance molecules with roles in axon pathfinding during development, such as the Eph receptors, have also been implicated as inhibitors of axon repair in the adult, but in some situations they may provide guidance cues in the reestablishment of connections. Upregulation of multiple Eph receptors has been detected at sites of nervous system injury [195–197]. In *C. elegans,* signaling by the Eph receptor VAB-1 was shown to increase guidance errors of regenerating mechanosensory axons after laser axotomy [198]. On the other hand, EphB3 expressed in the macrophages recruited to the injured mouse optic nerve is necessary for the re-extension and sprouting of the injured RGC axons, which express ephrinB3 [199]. Furthermore, the interplay between EphB2 expressed in Schwann cells and ephrinB2 expressed

in fibroblasts after sciatic nerve transection mediates the segregation of the two cell types, promoting the migration of the Schwann cells and axonal regrowth [200].

The EphA4 receptor is emerging as an inhibitor of nerve regeneration. After spinal cord injury (SCI), EphA4 accumulates in corticospinal tract axons and reactive astrocytes, and its ligand ephrinB2 is upregulated in astrocytes in the glial scar [201–203]. EphA4 knockout mice show functional regrowth of corticospinal and rubrospinal tract fibers after spinal cord hemisection, attributed to reduced astrocytic gliosis [202]. Administration of blockers of EphA4 following SCI was also shown to promote axonal regeneration and functional recovery [204, 205]. Work by another group, however, did not provide evidence for reduction of fibrotic scar formation in EphA4 mutants following SCI [206]. Furthermore, prevention of EphA4 upregulation at the lesion site after SCI by injection of antisense oligonucleotides did not induce functional motor recovery but instead led to increased chronic pain [203].

5.2.2.2 Cardiovascular System

The assembly of blood vessels involves the recruitment of endothelial cells, supporting mural cells such as pericytes that cover blood vessel capillaries and post-capillary venules, as well as vascular smooth muscle cells (VSMC) that are associated with arteries and larger veins. Despite their well-described role during development (see above), less is known about the involvement of Eph receptors and ephrins in physiological adult angiogenesis. In the retinal vasculature, which has been used as a model of postnatal angiogenesis, EphB4 overexpression favors the formation of large blood vessels, disorganized branching, and reduced vascular permeability [207]. These effects are mainly mediated by ephrinB reverse signaling. EphrinB2 expression is maintained in adult arteries and arterial smooth muscle cells at sites of neovascularization [208, 209], but a functional role for this ligand has yet to be demonstrated. EphB4 expression persists in adult veins and determines their identity. Venous adaptation to the arterial environment, which is observed in vein grafts, is characterized by thickening of the venous walls and loss of EphB4 expression. EphrinB2-Fc stimulation of adult vein grafts or adenoviral induced EphB4 overexpression prevented venous remodeling by inducing EphB4-forward signaling and subsequent binding and phosphorylation of caveolin-1. In addition, vein grafts derived from EphB4 heterozygous, EphB2 kinase-dead, or Cav-1 null mutant mice failed to inhibit wall thickening [210]. Thus, stimulation of EphB4 during vein graft adaptation promotes retention of venous identity by preventing vein wall thickening. EphA4 is also highly expressed throughout adulthood in VSMCs whose contractility regulates vascular tone to maintain blood circulation. Upon stimulation with ephrinA1, EphA4 phosphorylation enhances Vsm-RhoGEF activity of RhoA and promotes the assembly of actin stress fibers [211]. This suggests that upon activation, EphA4 regulates VSMC contractility, vascular tone, and blood pressure via RhoA activation.

The role of Eph/ephrin signaling in tumor angiogenesis is controversial, with opposite functions proposed depending on the experimental model used. For example,

EphB4 reverse signaling negatively regulates the formation of blood vasculature in malignant brain tumor models by inducing circumferential vessel growth instead of branching angiogenesis and by reducing the permeability of the tumor vascular system [207]. However, overexpression of kinase-dead EphB4 in breast cancer cells demonstrates that this receptor acts as a positive cue to ephrinB2-expressing endothelial cells and promotes tumor vascularization [212]. Furthermore, EphA2 and its ligand, ephrinA1, are also expressed by tumor endothelial cells [213]. It was shown that EphA2 deficiency inhibits tumor angiogenesis in a mouse model of mammary adenocarcinoma [214], while ephrinA1 promotes angiogenesis-dependent metastasis [215].

EphB signaling plays an important role in stabilizing and promoting thrombus growth [216–218]. Vascular injury induces the initiation of thrombus formation and involves platelet activation and aggregation as well as fibrin clot formation. EphA4, EphB1, and ephrinB1 are expressed on the surface of circulating human platelets. Activation of integrin-dependent sustained contacts between platelets allows interactions between Ephs and ephrinB1. Eph/ephrin signals induce the phosphorylation of integrin αIIbβ3, facilitating binding to myosin. This subsequently promotes clot retraction which is thought to enhance thrombus stability and to prevent its premature dissolution.

5.2.2.3 Other Systems

Immune System Certain T-cell functions depend on the expression of the EphB6 receptor. In vitro assays in Jurkat cells revealed that both proliferation and lymphokine production are reduced in EphB6 null mutant T cells. In vivo, the response to T-cell receptor stimulation is compromised in mice deficient for EphB6 [219]. EphB6-mediated T-cell co-stimulation seems to be triggered by ephrinB1, -B2, and -B3. A requirement for ephrinB1 and ephrinB2 in T-cell activation has been indeed demonstrated. EphA receptors and their ligands are also expressed by T cells and are thought to reduce apoptosis by inhibiting T-cell receptor signaling [220, 221]. While expression of Eph receptors and ephrins has been detected in B lymphocytes, their function in these cells is unclear [222].

Pancreas Physiology Communication between endocrine β cells in the pancreas is required to inhibit basal insulin secretion during fasting periods, as well as to enhance glucose-stimulated insulin release after food intake. EphA5 receptor and ephrinA5 ligand are co-expressed in β cells. When blood glucose levels are low, EphA5 receptor forward signaling prevents insulin release from secretory granules, whereas upon increased glucose concentration, EphA5 receptor is dephosphorylated. Under these conditions, reverse ephrinA5 signaling prevails, inducing insulin secretion from β cells [223]. This observation may have therapeutic implications for treatment of diabetes.

Intestinal Epithelium Eph/ephrin signaling is an important regulator of the intestinal epithelium architecture. The innermost layer of the intestinal tube is an epithelium

layer which is folded into invaginations called crypts. The base of each crypt is populated by a limited number of active stem cells which continuously regenerate the epithelium, comprised of mucosecreting, absorptive, enteroendocrine, and Paneth cells [224]. The first three cell types migrate upward toward the lumen as they undergo terminal differentiation, whereas Paneth cells unlike the other cell types remain at the base of the crypts. Wnt signaling which is active in a gradient, with the highest activity at the crypt bottom, regulates cell renewal in the crypt and promotes β-catenin/Tcf-driven transcription in intestinal stem cells (ISCs) and Paneth cells (PCs) localized within this niche [225, 226]. Expression of EphB2 and EphB3 receptors in crypts is induced by Wnt signaling [26]. EphB2 is highly expressed in ISCs and in a gradient manner along the villus–crypt axis with the highest expression at the bottom of the crypt, whereas EphB3 expression is restricted to ISCs and PCs at the bottommost of the crypt. Conversely, ephrinB1 and ephrinB2 ligands that are negatively regulated by β-catenin/Tcf activity show highest levels in differentiated cells [26]. EphB/ephrinB signaling is pivotal in establishing cell compartments and in organizing ordered migration of epithelial cells along the crypt–villus axis. In EphB3 null mice and intestine-specific ephrinB1 mutant mice, localization of PCs is no longer restricted to the crypt base; instead PCs migrate upward and are found dispersed throughout the epithelium [26, 227]. In EphB2/EphB3 double knockout mice, ISCs intermingle instead of undergoing unidirectional upward migration, and the boundary between the proliferative and differentiated cell compartments is lost. Furthermore, these mice show decreased proliferation in the intestinal crypts despite a paradoxical increase in the number of ISCs within the stem cell niche as a result of PCs mislocalization [228].

Other Epithelial Tissue Eph receptor forward signaling is also involved in the maintenance of the cellular architecture of other epithelia. For example, EphA2, EphB4, and ephrinB2 are highly expressed in the mammary gland epithelium and are regulated by estrogens [229]. EphB4 receptor overexpression throughout the different stages of adult mammary development results in delayed proliferation and incomplete branching of the ductal tree during the pubertal and pregnancy phases, as well as in retarded cell apoptosis at the time of post-lactational gland reduction [230]. Furthermore, EphB2 and ephrinB1 display partially overlapping expression patterns along the nephron. EphB2 activation induces cell retraction and adhesion to the substrate, suggesting a possible involvement in the regulation of the permeability of tubule cells in the renal medulla [231], yet further in vivo experiments are still lacking to support this hypothesis.

Bone Homeostasis The coupling of bone resorption and bone formation is critical during the normal process of bone remodeling, which is necessary for skeletal growth and replacement of damaged and/or aged bone. Dysregulation of this coupling results in the development of a range of bone diseases including osteoporosis, rheumatoid arthritis, and metastatic cancer. Interaction between EphB4 receptor and its cognate ephrinB2 ligand seems to be a key component regulating the switch between resorption and formation. EphB4 is expressed on osteoblasts whereas ephrinB2 on osteoclasts [232]. Forward signaling through EphB4 stimulates bone

formation, whereas reverse signaling through ephrinB2 inhibits bone resorption. With the exception of an implication in cancer bone metastasis, the role of the EphA /ephrinA family has not been investigated [233].

Vestibular Function EphB2 and EphB3 null mice exhibit a circling behavior pointing to a role for these receptors in vestibular function. EphB2 receptor is specifically expressed in the single-cell layer of dark cells within the vestibular epithelium and its cognate ligand ephrinB2 is highly expressed in adjacent transitional cells. Upon activation, EphB2 receptor co-clusters with aquaporins and anion exchangers through a molecular bridge formed by Pick-1 and Syntenin and thus regulates endolymph production and vestibular function [135].

5.2.3 Stem Cells

5.2.3.1 Embryonic Stem Cells

Early embryonic stem cells (ESCs) harbor the capacity for unlimited self-renewal and differentiation into any cell type that constitute the hallmarks of pluripotency. In early embryos, cells in the inner cell mass (ICM) are pluripotent and develop into the cells that form the tissues and the germ cells of the adult organism. The ICM cells persist only transiently as they undergo differentiation and become progressively restricted in their developmental potential. Various Eph receptors have been found to be highly expressed in totipotent cells derived from the ICM (EphA1, EphA2, EphA3, EphA4, EphB2, and EphB4) [234], but little is known about either their role in self-renewal or their engagement in various differentiation pathways. EphB4, a receptor mainly involved in the organization of blood vessels, was found to modify the rate and magnitude of ESCs' differentiation into mesodermal layer cells [235]. EphB4 knockout ES cells displayed impaired hemangioblast, blood cell, cardiomyocyte, and vascular differentiation. Re-expression of full-length EphB4 was able to restore the cardiomyocyte development in EphB4 null ES cells, while a truncated EphB4 lacking the intracellular domain failed to do so, suggesting that EphB4-forward signaling is essential for the development of cardiomyocytes [236]. In addition, decreased expression of mesoderm-associated genes in EphB4 null ICM suggests that EphB4 is a key regulator of the response to mesoderm induction signals. Emerging proof has highlighted the role of Eph signaling in influencing cell fate in various stages of development. Further in vitro analysis of the various null or signaling mutant alleles for Eph receptors could give greater insight into their role in ESC proliferation, differentiation, and fate determination.

5.2.3.2 Adult Stem Cells

Emerging evidence points to a role of Eph signaling in the regulation of the balance between adult stem cell renewal and differentiation and fate determination. Ephs and ephrins are commonly expressed in adult stem cell niches. Most studies to date have

focused on the intestinal crypt and the nervous system, although an increasing number of studies also implicate Ephs in other organs. EphB4 and ephrinB2 are expressed in a complementary pattern in the mammary gland [237], where they are implicated in the development of the mammary epithelium [230]. Moreover, hair follicle bulge stem cells express high levels of EphA4, EphB4, and ephrinB1[238], and EphA2 and ephrinA1 are expressed in a complementary pattern in the epidermis allowing for receptor–ligand interaction only at the proliferative basal layer of the epidermis [239]. Both the A and B Eph classes negatively regulate proliferation of hair follicle and epidermal progenitor cells in the adult mouse [240], although it is unclear whether this is mediated by forward or reverse signaling.

Stem cell maintenance and progenitor cell proliferation in the intestinal epithelium are regulated by canonical Wnt signaling involving β-catenin and Tcf/LEF family transcription factors [225, 241]. Within the intestinal crypt, Wnt pathway is active in a gradient, with the highest activity at the crypt bottom (described above). Postmitotic Paneth cells preferentially express EphB3, whereas proliferating progenitor cells are positive for EphB2. In the absence of EphB2 and EphB3, stem cells are no longer confined to the lateral crypts but scattered along the villus–crypt axis [228]. In addition to its role in cell sorting of the stem cell population from postmitotic neighboring cells in the intestinal epithelium, EphB2 promotes progenitor cell proliferation in a kinase activity and ligand-dependent manner, by activating Abl–cyclinD1 pathway and cell cycle reentry [228].

Active adult neurogenesis, a process of generating functional neurons from adult neural precursors, is spatially restricted to two neurogenic brain regions, the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, where new dentate granule cells are generated, and the subventricular zone (SVZ) of the lateral ventricles, where new neurons are generated and then migrate through the rostral migratory stream to the olfactory bulb to become interneurons [242]. Both SGZ and SVZ display complementary expression patterns of Eph receptors and ephrin ligands. Progenitor cells and neuroblasts in SVZ express ephrinA2, whereas quiescent ependymal cells, as well as some GFAP-positive putative stem cells, express EphA7. EphA7 induces ephrinA2 reverse signaling, negatively regulating adult neural progenitor cell proliferation [243, 244]. Moreover, cells in the SVZ express all three ephrinB ligands, as well as EphB1, EphB2, and EphB3. Blocking the interaction between ephrinBs and Eph receptors as well as genetic ablation of ephrinB3 leads to an increase in the number of dividing cells in the SVZ [244–246]. Furthermore, EphB3 represses proliferation of progenitor cells in the SVZ by increasing the expression of p53, a negative regulator of cell renewal [247]. Thus, both the A and B receptor classes negatively regulate neural progenitor proliferation in the adult SVZ. Hippocampal progenitor cells present in the SGZ of the dentate gyrus express EphB1 and EphB2 receptors. EphB-forward signaling positively regulates neurogenesis and migration of progenitor cells when stimulated with ephrinB3 expressed by mature granule cells in the dentate gyrus inner molecular layer [248]. Decreased neurogenesis in the dentate gyrus of ephrinA5 null mice indicates a similar role for EphA receptors [249]. In addition to influencing the progenitor cells in the adult brain, EphB-forward signaling also acts as a key regulator of the main stem cell niche in the lateral ventricle walls [250]. However, during forebrain

neurogenesis, EphA7-forward signaling was shown to have a direct pro-apoptotic effect on embryonic cortical progenitors without influencing their proliferation rate [52]. Furthermore, EphA4 signaling has been implicated in maintaining neural stem cells in an undifferentiated state and in regulating the number of adult neuroblasts in the SVZ [251].

5.3 The Role of the Eph Receptor Tyrosine Kinase Family in Human Disease

5.3.1 Cancer

EphA1 was first cloned from hepatocellular carcinoma cell line [252], and Eph receptors have subsequently been implicated in many different cancers, including breast and colorectal cancer, neuroblastoma, glioma, myeloma, and lymphomas (for a comprehensive list, see [253]). Given their role in cell adhesion, migration, boundary formation, and positioning, it is not surprising that changes in Eph expression or function correlate with tumor progression and metastasis. Despite this, the complex and at times contradictory role of Eph receptors in cancer has yet to be clearly defined. The response appears to be specific for Eph type, tumor type, local microenvironment, and tumor stage. Given their involvement in many different cancer types, Eph receptors provide a valid drug target option; however, their complex role produces a challenging caveat to overcome.

5.3.1.1 Eph Expression in Cancer

Changes in Eph receptor expression, both overexpression and downregulation, seem to play a significant role in cancer progression, depending on cell type and context. EphA2 is the most studied Eph receptor in cancer. It is overexpressed in many different cancer types, in particular in malignant tumors originating in breast, skin, and colorectal tissue [253]. In breast cancer cells, EphA2 overexpression has been proposed to occur through deregulation of the negative feedback loop with the Ras–Raf–MAPK pathway. Ligand-induced EphA2 activation blocks Ras activity, while EphA2 is itself a transcriptional target of Ras. Interestingly, MAPK activity downregulated ephrinA1 expression, an effect that contradicts the negative feedback loop seen for the receptor [254–256]. Alternatively, EphB6 has been seen to be downregulated in breast cancer, resulting in increased invasive properties [257]. Restoring EphB6 levels promoted actin-induced spreading and cell–cell attachment, thus reducing the invasiveness. The kinase-defective EphB6 signaling is mediated by EphB4 interaction, leading to activation of the Abl kinase c-Cbl [258].

In colon carcinomas high expression of both EphA2 and EphA4 has been correlated with the formation of metastasis and significantly shorter overall survival periods [259, 260]. EphB2-4 and EphA1 receptor expression levels in colorectal

tumors, however, change from high to low in correlation with a transition from adenoma to a metastatic state and are associated with poor patient survival. Adenomas are formed by activation of Wnt/β-catenin signaling that results in ectopic crypt progenitor state-like cells, inducing expression of genes including EphB2 and EphB3 (see above, Fig. 5.6a). The expression of Eph receptors inhibits tumor

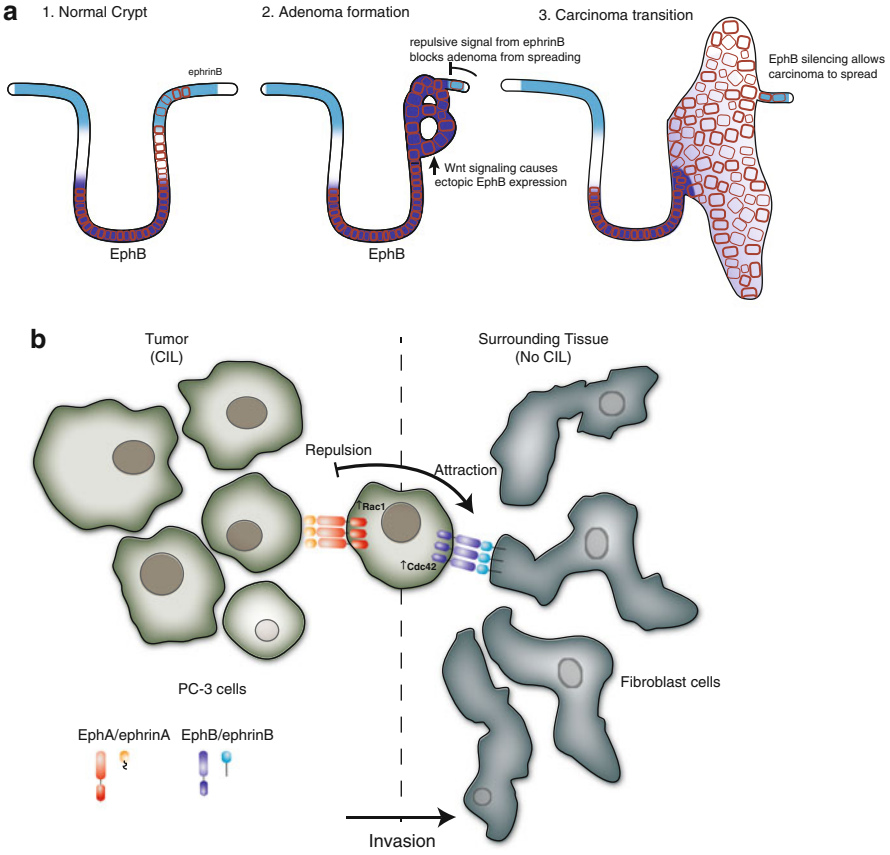

Fig. 5.6 Eph/ephrin signaling in cancer. (**a**) The role of EphB in the formation of intestinal adenomas and the transition to carcinomas. [1] In normal tissue, EphB expression is restricted to progenitor cells at the base of the crypt, whereas ephrinB-expressing differentiated epithelial cells are located at the crypt–villus boundary. EphB/ephrinB interactions are required to position the cells in the intestine. Activation of ectopic Wnt signaling results in EphB expression and adenoma formation on the walls of the crypt [2]. EphrinB expression across the top of the crypt induces EphB repulsive signaling and inhibits cell metastasis. Loss of EphB expression in the proliferating cells removes the repulsive signal and allows for carcinoma formation [3]. (**b**) A model for invasion of prostate tumor cells into the surrounding non-tumor tissue. PC-3 tumor cells express both EphA and EphB receptors, as well as ephrinA ligand. Homotypic cell interactions between PC-3 cells activate the Rho GTPase pathway via EphA–ephrinA signaling, inducing contact inhibition of locomotion (CIL) resulting in cell repulsion. When the PC-3 cells come into contact with the surrounding fibroblasts that express high amounts of ephrinB2, however, EphB signaling blocks CIL by inducing cell–cell attraction via Cdc42 activation, thus promoting tissue invasion

growth by activating repulsive signals. The decrease in Eph expression upon transition to the malignant state is therefore necessary, but surprising given the high Wnt/β-catenin activity. This contradiction has been explained epigenetically, with hypermethylation of the promoter region of the receptor suppressing its expression (Fig. 5.6a). As with other tumors, re-expressing the receptor in cells with low levels reduced tumor progression potential [261–264]. Interestingly, another study showed that although there was a decrease in EphB2 expression upon transition from adenomatous polyp to metastatic cancer, EphB4 expression increased and correlated with poor prognosis. The change in expression was due to a switch in the Wnt pathway transcriptional coactivators from p300, which regulates EphB2 expression, to cyclic AMP-responsive element binding protein (CBP) that induces EphB4 expression [265].

As with human studies, mouse models indicate differing levels of Eph receptors result in different outcomes for cancer development depending on cellular context. For example, EphA2 has been shown to complex with ErbB2 and enhance Ras–MAPK signaling and Rho GTPase activity. Ablation of EphA2 in mouse mammary epithelium reduced tumor initiation and metastases when ErbB2 was overexpressed, whereas tumor progression increased in mice overexpressing the polyomavirus middle T antigen [214]. In the case of skin carcinogenesis, tumors in EphA2 null mice grew faster and were twice as likely to show invasive malignant progression. In wild-type mice EphA2 and ephrinA1 are co-expressed in the basal layer of the epidermis, and in vitro treatment of EphA2-expressing cells with ephrinA1 suppressed proliferation. Despite this suppressive function, as with humans, EphA2 is often overexpressed in mouse tumor models [239], thus indicating the importance of the local environment and potential ligand-independent mode of action.

In colorectal cancer, mouse models show that reduced EphB4 expression greatly increases the rate of tumor growth, supporting the findings that EphB4 is down regulated in many human colon cancers (see above) [261, 266]. Loss of EphB3 and EphB4 allows for intermingling of cell layers within the intestinal epithelium that are normally separated by ephrinB1–EphB boundary formation, thus removing the cell invasion inhibition present in the wild-type situation [79, 227]. Moreover, low expression of EphB4 correlated with changes in transcription of genes related to cell proliferation, including many growth factors, extracellular matrix reorganization proteins, and cell attachment proteins [266].

Aside from expression levels of the Eph receptors, somatic mutations within the receptor are common and can have a profound effect in tumor progression. For example, EphA3, EphB2, and EphB6 have all been described with multiple different mutations in a range of unrelated cancer types. Many mutations were found to be at sites that disrupted kinase activity, ligand binding, or localization of the receptor to the cell membrane, thereby implying a ligand-induced, kinase-dependent role for the receptors in cancer suppression [267–269].

5.3.1.2 The Role of Ephrins in Eph-Induced Cancer Progression

Interestingly, Eph-induced cancer progression is often independent of ligand interaction, and in some cases ligand-induced receptor activation can be considered anti-mitogenic. Ligand-activated Eph receptors have been shown to suppress oncogenic signaling pathways, such as the AKT and Ras–Raf–MAPK pathways [239, 254, 270, 271]. Another possibility for ligand-induced reduction of EphA activity is by endocytic removal of the receptor cluster from the cell membrane [254]. This response requires a spatial rearrangement of the individual Eph–ephrin dimers into higher-order clusters prior to internalization. By inhibiting this rearrangement, the Eph receptor remains on the cell surface. Molecularly this can occur due to the different recruitment properties of receptor–ligand dimers compared to higher-order clusters. For example, dimers are unable to recruit the metalloprotease ADAM10, which cleaves the ephrin from the opposing cell and potentially allows endocytosis after EphA2 cluster formation [272]. Importantly, a strong correlation was observed between invasiveness of breast cancer cell lines and their ability to cluster EphA2, and moreover, problems with receptor spatial rearrangement also correlated with expression levels of proteins in MAPK, ErbB, and other pathways already implemented in EphA2-induced invasiveness [272].

How Eph receptors promote tumorigenesis in a ligand-independent way is still unresolved. Ligand-independent EphA2 promotion of migration of glioma cells requires AKT-dependent phosphorylation on serine 897 of the carboxy tail of the receptor, and a non-phosphorylatable mutation of this site, or exposure to ephrinA1 abolished the response [270]. In breast cancer cells, EphA2 has been shown to act downstream of the EGF receptor independently of ephrin stimulation. This has been shown to occur by an interaction between EphA2 and the Rho GEF, Ephexin4, which in turn activates another Rho GEF, ELMO2, and the Rac GEF Dock4 at the tips of cortactin protrusions, causing the cells to grow toward an EGF stimulus [273].

Ligand-independent activation is not restricted to EphA family members. In breast cancer cells, although EphB4 expression is often increased, ephrinB2 levels are found to be reduced. Stimulation by ephrinB2, acting via the Abl–Crk pathway, and independently of Src and MAPK pathways, inhibits tumor growth both in vitro and in vivo [212, 274]. Other studies, however, have shown that EphB4 is stimulated by ephrinB2 in breast epithelial cells and knockdown of the receptor decreased survival, increased apoptosis via TRIAL, decreased migration, and inhibited tumor growth in vivo [275]. This illustrates that ephrin activation of Ephs still plays a major role in certain cancer types. In prostate tumors, where EphB4 has been shown to be increased, ephrinB2 was expressed in a subset of surrounding tissue including smooth muscle, vascular endothelial cells, and nerve fibers. The interaction between ephrinB2 expressed in nonmalignant fibroblasts and EphB3 and EphB4 expressed in prostate cancer (PC) cells promoted cell invasion by a loss of contact inhibition of locomotion via activation of the Cdc42 GTPase pathway. Conversely, cell–cell contact inhibition occurs between homotypic tumor cells via EphAs/ephrinAs and

the ROCK signaling pathway, further promoting metastasis (Fig. 5.6b). The relative ratio between ephrinA and ephrinB on the contacted cell thereby regulates tumor invasion [276].

Ligand–receptor induced promotion of metastasis has also been described to occur by an increased adherence of EphB4-expressing tumor cells and ephrinB2-expressing tissue in the surrounding area [277]. Full-length EphB4-expressing A375 melanoma cells bound strongly to HUVEC cells expressing ephrinB2. Moreover, A375 intravenously injected into mice homed into tissues that express ephrinB2 in the vasculature, such as lungs. They did not migrate into tissue where ephrinB2 was not expressed, such as the heart, despite its extreme vascularization. These effects were reduced when cells expressing a C-terminally truncated form of EphB4 were injected, suggesting a requirement for an active receptor. Again, these effects rely on specific cellular environment. Xenografts of B16 melanoma cells that overexpress EphB4 suppressed tumor growth. In these tumors, arterial vasculature endogenously expressing ephrinB2 was reduced, whereas venous vessels that do not express ephrinB2 were unaffected. In vitro experiments showed that the ephrinB2-expressing cells died via apoptosis; however the EphB-positive cells were unaffected [278].

5.3.1.3 Targeting Ephs for Research and Disease Treatment

As overexpression of Eph receptors is a common trait in many tumors, researchers have looked at the outcome of reducing their levels or activity, not only to clarify the role of the receptor in tumor progression but also to see if they are valid druggable targets [279]. RNAi of EphA2 in melanoma cell lines resulted in a reduction in cellular viability, colony formation, and migration in vitro; and in vivo resulted in a loss of its tumorigenic potential [280]. Similarly, high EphA2-expressing head and neck squamous cell carcinoma (HNSCC) cells showed reduced viability following EphA2 RNAi treatment or exposure to ephrinA1 ligand, a result that was associated with a downregulation of AKT and ERK signaling [271]. EphA4 was found upregulated in the malignant transformation of prostatic intraepithelial neoplasia to invasive PC tumors. Downregulation of EphA4 by RNAi in PC cells led to attenuation of PC cell viability [281]. EphA4 is also upregulated in pancreatic ductal adenocarcinoma (PDAC) cells. Knocking down EphA4 expression by RNAi, attenuated PDAC cell viability, and constitutive expression of EphA4 in PDAC-derived cells led to a more rapid growth rate [282].

Small molecule inhibitors are a useful tool both for treatment of disease and to study protein function. To date only a few inhibitors have been described that specifically target Eph receptors (see [253]). Often the kinase activity is targeted or the ephrin binding domain is blocked, as has been described for EphA2, EphA4, and EphB4 [283, 284]. Interestingly, Eph receptors are also targeted by multi-kinase inhibitors, such as dasatinib, which along with inhibiting Src and Abl kinases, also blocks EphA2 kinase activity [285, 286]. How EphA2 regulates the efficacy of dasatinib treatment, both alone and in combination with paclitaxel and carboplatin, is currently the primary objective of a pilot and translational clinical study

(NSC #732517). In follicular lymphoma cells, it has been shown that a truncated splice variant of EphA7 (EphA7^{TR}) possesses tumor suppressor properties by potentially acting as a decoy receptor for EphA2 and thereby inhibiting its oncogenic signaling. Administration of purified EphA7^{TR} was able to inhibit xenografted tumor progression, and moreover, antibody fusion of EphA7^{TR} targeted the treatment to lymphomas in vivo [287]. Alternatively, activation, and subsequent downregulation of the receptor by Eph-specific monoclonal antibodies, has been studied to target tumor cells overexpressing Ephs while not affecting non-tumor tissue. For example, antibodies against the extracellular domain of EphA2 suppressed breast cancer cells' ability to colonize the surrounding area but did not affect the monolayer growth of nontransformed cells [288]. Moreover, administration of a cytotoxic compound linked to an ephrin ligand to patients provides novel mechanism of tumor-specific drug delivery to cancers where Eph receptors are overexpressed [289]. Finally, with the advent of nanotechnology, Eph receptors can be strategically targeted within a desired microenvironment by nanoparticles conjugated either directly to ephrins or to second-generation antagonists Shaw [290, 291]. Given the fine balance of Eph expression in cancer and the dire results seen in some cases when Eph receptors are downregulated, targeting Ephs will need to be precisely controlled to avoid detrimental effects; thus the development of nanotherapeutics provides a promising novel approach.

5.3.2 Neurological Disorders

Aberrant synaptic activity impairs cognitive functions, which is believed to be a major hallmark for several neurological disorders, such as neurodegenerative and psychiatric disorders. Given the prominent function of Eph receptors in the regulation of dendritic spine morphology and synaptic plasticity, their dysfunction may underlie cognitive impairments in neurological disorders.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive loss of cognitive functions. AD is associated with the accumulation of amyloid-β containing senile plaques due to the aberrant processing of the amyloid precursor protein (APP) by γ-secretase. A reduction in hippocampal EphA4 and EphB2 has been observed in a mouse model of AD, prior to the development of cognitive deficits [292]. In addition, in a mouse model for AD where mutant human APP is overexpressed, amyloid-β interacts with the FN domain of EphB2 leading to its proteasomal degradation. Reduction of cell surface EphB2 abundance leads to a decrease of NMDAR currents and impaired LTP. Conversely, overexpression of EphB2 in the APP mouse reversed the NMDAR-dependent LTP defect and the AD-associated behavioral and cognitive deficits [293]. Moreover, EphA4 was shown to be an activity-dependent substrate of γ-secretase, being processed in the intracellular domain. The EphA4 intracellular domain induces the formation of dendritic spines in hippocampal cultured neurons through activation of Rac1. EphA4 cleavage by γ-secretase was reduced in cells expressing mutant presenilin, which is

linked to familial AD, suggesting that the processing of EphA4 could affect the pathogenesis of AD [22].

The control of the levels of the neurotransmitter glutamate is crucial for brain physiology, since insufficient glutamate hinders synaptic transmission, while excessive glutamate can trigger excitotoxicity and cell death. Numerous neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) are accompanied by dysfunctions in glutamate transporters and abnormal concentrations of extracellular glutamate. Genetic deletion of the postsynaptic EphA4 or its ligand partner in astrocytes, ephrinA3, increases the expression of glial glutamate transporters and the uptake of glutamate from the synaptic cleft, thus impairing LTP in these animals. Conversely, the overexpression of ephrinA3 in astrocytes downregulates glutamate transporter levels, leading to dendrite degeneration and exacerbated susceptibility to epileptic seizures [187]. The amyotrophic lateral sclerosis type 8 protein, VAPB, was shown to be cleaved and secreted as a diffusible binding protein for EphA4 that antagonizes its interaction with ephrin. A VAPB mutation identified in ALS patients may increase EphA/ephrin signaling by inactivating the processing of VAPB [294]. Furthermore, inhibition of EphA4 signaling increased survival in mouse and rat models of ALS [295].

Angelman syndrome is a neurogenetic disorder that is characterized by developmental delay and neurological problems, caused by mutations in the maternally inherited E3 ubiquitin ligase (Ube3) gene. The RhoA guanine exchange factor ephexin5 is one of the substrates identified for Ube3. Ephexin5 activates RhoA which leads to suppression of excitatory synapses during development, until ephrinB binding to EphB2 receptor triggers ephexin5 to degradation. Ube3A is the ubiquitin ligase that controls EphB-mediated ephexin5 degradation raising the possibility that the cognitive defects in Angelman syndrome might result from increased levels of ephexin5. Consistent with this possibility, in a mouse model of Angelman syndrome in which the maternally inherited copy of Ube3A is deleted, the levels of ephexin5 expression are increased and neurons are insensitive to ephrinB treatment [173]. Ube3A is duplicated in some forms of Autism Spectrum Disorders, raising the possibility that altered levels of ephexin5 might also be relevant to the etiology of autism. In addition, EphA3 has been identified in a large homozygous haplotype mapping screen for genes associated with autism spectrum disorders [296].

Emerging evidence implicates the deregulation of Eph/ephrin signaling in aberrant synaptic functions associated with cognitive impairments in several neurological disorders. Sequencing of Eph receptor and ephrin genes may in future reveal mutations or polymorphisms that are involved in susceptibility to these diseases.

5.3.3 Viral Infection

Hepatitis C virus (HCV) is a major cause of liver disease for which new antiviral strategies are urgently needed. HCV entry is a multistep process involving viral glycoproteins and several cellular factors. Using a functional RNAi kinase screen,

both EGF receptor and EphA2 were identified as host cofactors for HCV entry. Blocking receptor kinase activity by inhibitors impaired infection by all major HCV variants in cell culture and in a human liver chimeric mouse model in vivo. Mechanistically, EGFR and EphA2 mediate HCV entry by regulating CD81-claudin-1 co-receptor association and viral glycoprotein-dependent membrane fusion [297].

5.4 Specifications of Individual Eph Receptors

5.4.1 EphA1

5.4.1.1 EphA1 Gene

- Promoter structure

  The EphA1 promoter structure is largely unknown.

- mRNA structure

  Human: 5′ UTR—1 to 87; 3′ UTR—3,019 to 3,363. Mouse: 5′ UTR—1 to 58; 3′ UTR—2,993 to 3,273. 18 exons. 7 splice variants are predicted for the human gene and 1 for the mouse gene (See note 1).

- Transcriptional regulation

  The EphA1 promoter is hypermethylated and EphA1 expression is downregulated in advanced colorectal cancer [253].

5.4.1.2 EphA1 Protein

- Amino acid sequence

  Human: Q60750. Mouse: P21709.

- Processing

  No information is available.

- Domain structure

  The domain structure of EphA1 protein is similar to other Eph receptors: The extracellular region includes an N-terminal ligand-binding domain (LBD), a cysteine-rich region, and two fibronectin type III domains (FN1 and FN2), followed by a transmembrane sequence. The intracellular part is composed of a juxtamembrane region, a tyrosine kinase domain, a sterile-α motif (SAM) domain, and a C-terminal PSD-95, Dlg, and ZO1 domain (PDZ)-binding motif.

- Posttranslational modifications

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

EphA1 is phosphorylated on tyrosine residues. Juxtamembrane Y599 and Y605 and active site Y781 (human) are likely phosphorylation sites (See note 3).

5.4.1.3 EphA1 Ligands

- Ligand structure

  EphA1 interacts with all GPI-anchored A-class ephrin ligands.

- Ligand cleavage

  No information is available.

5.4.1.4 EphA1 Activation and Signaling

- Dimerization

Heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. Higher-order cluster formation is required to induce biological responses and signaling.

- Phosphorylation

  Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

EphA1 directly binds integrin-linked kinase (ILK). This interaction requires the SAM domain of EphA1 and the ankyrin region of ILK. The interaction does not depend on EphA1 kinase activity, although it is triggered by ephrinA1 ligand binding. Activation of EphA1 kinase signaling inhibits ILK, leading to a reduction in Rac1 signaling and stimulation of RhoA–ROCK pathway, which regulates cell morphology and movement [298].

- Major genes regulated

  No transcriptional targets of EphA1 have been reported.

- Cross talk with other receptor systems

  No information is available.

5.4.1.5 EphA1 Internalization, Processing, and Attenuation

No information is available.

5.4.1.6 Unique Features of the EphA1 Receptor

No unique features.

5.4.2 EphA2

5.4.2.1 EphA2 Gene

- Promoter structure

A canonical cAMP-responsive element (CRE) was identified within the human EphA2 promoter. This regulatory element seems to be required for basal as well as Src kinase-induced activity of the EphA2 promoter [300].

- mRNA structure

Human: 5′ UTR—1 to 155; 3′ UTR—3,087–3,964. Mouse: 5′ UTR—1–113; 3′ UTR—3,048–3,913. 17 exons. 4 splice variants are predicted for the human and mouse genes (See note 1).

- Transcriptional regulation

EphA2 expression is enhanced by the Ras–Raf–MAPK pathway. EphA2 signaling in turn suppresses activation of Ras in response to growth factor treatment, creating a negative feedback loop that controls Ras signaling. In addition, the MAPK pathway negatively regulates ephrinA1 expression, and ephrinA1 in turn reduces EphA2 expression. This might explain the mutually exclusive expression of EphA2 and ephrinA1 in many breast cancer cell lines [254].

EphA2 is also regulated by the transcriptional repressor hypermethylated in cancer 1 (HIC1), which is known to be a tumor suppressor [301].

5.4.2.2 EphA2 Protein

- Amino acid sequence

Human: P29317. Mouse: Q03145.

- Processing

No information is available.

- Domain structure

The domain structure of EphA2 protein is similar to other Eph receptors (see Table "Receptor at a glance**:** EphA2").

The crystal structure of the complete ectodomain of EphA2 has been solved. It revealed that the cysteine-rich region of EphA2 consists of a Sushi (CCP) domain and an epidermal growth factor (EGF)-like domain. The EphA2 ectodomain displays an elongated architecture that is stabilized by *cis* interactions involving the ephrin ligand-binding domain and the Sushi domains [302, 303]. It was suggested that ephrin binding at "nucleation" points could trigger more widespread recruitment of EphA2 into signaling assemblies [302, 303].

- Posttranslational modifications

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

EphA2 is phosphorylated on tyrosine residues (Y588, Y594, Y735, Y771, Y930 in the human protein), and possibly on serine/threonine residues (S892, S897, T898, S899, S901 in the human protein) (see note 3). Phosphorylated residues Y588/587 (human/mouse) and Y594/593 (human/mouse) are required for binding Vav2 and Vav3 GEFs, while phosphorylated residues Y735/734 (human/mouse) and possibly Y930 are required for association with the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) [270, 304]. Mutations in the juxtamembrane (Y587F, Y593F, Y587E/Y593E), kinase domain (Y734F), or SAM domain (Y929F) tyrosines impaired ephrinA1-induced assembly of endothelial cells into vascular tubules in vitro. Furthermore, endothelial cells expressing these EphA2 point mutants failed to integrate into tumor vasculature in vivo [304].

Ligand-independent EphA2 phosphorylation on S897 by Akt stimulates motility in glioma and prostate cancer cells. EphrinA1 binding leads to S897 dephosphorylation, prevents Akt activation, and inhibits cell migration [270].

5.4.2.3 EphA2 Ligands

- Ligand structure

EphA2 interacts with all A-class ephrin ligands. EphrinA1 is the preferred ligand for EphA2.

- Ligand cleavage

Soluble monomeric ephrinA1 was found to be secreted by glioblastoma multiforme and breast adenocarcinoma cells. Surprisingly, this form of ephrinA1 appears to be functional, since it triggers EphA2 internalization and downregulation and suppresses the Ras–MAPK pathway. Moreover, monomeric soluble ephrinA1 is able to induce growth cone collapse in primary neurons [305].

5.4.2.4 EphA2 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. Higher-order array formation is required to induce biological responses and signaling.

- Phosphorylation

Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

EphA2 stimulation by ephrinA1 ligand suppresses Akt activation and inhibits migration of glioma and prostate cancer cells. Conversely, phosphorylation of EphA2 by Akt promotes cell migration and invasion. Hence, if cancer cells down-regulate ephrin expression and at the same time the PI3K/Akt pathway is activated, the EphA2 receptor converts from a tumor suppressor inhibiting migration to a tumor promoter driving malignant progression [270].

Association of EphA2 with Vav GEFs and p85 results in activation of Rac1 GTPase, which regulates cell migration [270, 304].

EphA2/EphrinA1 signaling leads to phosphorylation of p190 RhoGAP-A and subsequent suppression of RhoA. This causes dephosphorylation and inhibition of Ezrin, which is activated by Rho kinase. Ezrin is a known regulator of epithelial morphology and polarity, and its inactivation by Eph/ephrin signaling results in altered shape and increased polarization of kidney epithelial cells [306].

A positive feedback loop was proposed where EphA/ephrinA signaling is potentiated at E-cadherin-mediated cell contacts. Eph signaling in turn suppresses Arf6 activity, thereby strengthening E-cadherin-based cell adhesion and promoting the apical–basal polarization of epithelial cells [307].

Binding of EphA2 to the RhoGEF Ephexin4 was demonstrated in breast cancer cells. Activation of RhoG by Ephexin4 downstream of EphA2 enhances migration and stimulated invasive properties of a breast cancer cell line. This response requires the recruitment of the RhoG effector ELMO2 and the ELMO binding partner Dock4 into a ternary complex with EphA2. Dock4 activates Rac, which is known to play a crucial role in cancer cell migration by regulating the formation of cellular protrusions [273].

EphA2 signaling leads to inactivation of integrins and dephosphorylation of paxillin and focal adhesion kinase, inhibiting cell spreading, migration, and adhesion [308].

- Major genes regulated

No transcriptional targets of EphA2 have been reported.

- Cross talk with other receptor systems

No information is available.

5.4.2.5 EphA2 Internalization, Processing, and Attenuation

The interaction of EphA2 and the related EphA4 receptor with their cognate ephrin ligands can be specifically and competitively inhibited by dimethyl-pyrrole derivatives. These compounds prevent ephrin-induced phosphorylation of EphA4 and EphA2 receptors and efficiently inhibit the cellular responses to Eph signaling, such as EphA-dependent growth cone collapse in neurons and EphA2-induced changes in prostate cancer cell morphology [284]. In follicular lymphoma cells, EphA2 oncogenic signaling is attenuated by the binding of a truncated splice variant of EphA7, which acts as a tumor suppressor [287]. Furthermore, binding of the soluble monomeric ephrinA1 ectodomain was shown to induce EphA2 internalization and downregulation [305].

5.4.2.6 Unique Features of the EphA2 Receptor

EphA2 may be unique among other Eph receptors in its ability to respond to soluble, monomeric ephrinA1 [305]. The formation of extended arrays by EphA2 in complex with ephrinA5 appears to promote adhesive responses in contrast to the smaller EphA4 clusters that mediate repulsion [309].

5.4.3 EphA3

5.4.3.1 EphA3 Gene

- Promoter structure

The basal promoter of the human EphA3 gene is 86 bp long and located between −348 bp and −262 bp upstream of the transcription start site. In addition, a CpG-rich region with several DNA methylation sites was found downstream of the basal promoter [312].

- mRNA structure

Human: 5′ UTR—1–225, 3′ UTR—3,177–5,809. Mouse: 5′ UTR—1–111, 3′ UTR—3,066–5,659. 17 exons. 3 splice variants are predicted for the human gene and 1 for the mouse gene (See note 1).

- Transcriptional regulation

The methylation state of the CpG-rich region correlates with the levels of EphA3 gene expression. While no methylation was detected in normal adult human tissues, EphA3 was extensively methylated in a subset of samples from leukemia patients [312]. EphA3 DNA hypermethylation also occurs in colorectal cancers [313].

In the mouse limb, EphA3 expression was shown to be under reciprocal control of transcription factors of the Hox and Sall families. While EphA3 expression was

upregulated and expanded in the limbs of Hoxd13 and Hoxa13 mouse mutants [314, 315], it was downregulated in Sall1; Sall3 double knockout mice [315].

Two more homeobox-containing genes, SOHo1 and GH6, expressed in a complementary pattern to EphA3 in the developing chick retina, were shown to specifically repress EphA3 expression in this tissue [316]. In addition, chick brain factor-1 (CBF1) represses EphA3 in the nasal retina. Ectopic overexpression of CBF1 in the temporal retina led to downregulation of EphA3 [317].

In Jurkat cells, upregulation of EphA3 expression was observed in response to stimulation with CD28 and insulin-like growth factor-1 (IGF-1), as well as upon overexpression of IGF-1 receptor [318].

In neonatal rat cardiomyocytes, EphA3 transcription was downregulated upon exposure to interleukin-1β [319].

5.4.3.2 EphA3 Protein

- Amino acid sequence

  Human: P29320. Mouse: P29318.

- Processing

  No information is available.

- Domain structure

The domain structure of EphA3 is similar to other Eph receptors (see Table "Receptor at a glance**:** EphA3").

- Posttranslational modifications

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

EphA3 is phosphorylated on several tyrosine residues: Y596, Y602, Y779 (human and mouse). In melanoma cells and in Jurkat cells, EphA3 phosphorylation leads to the recruitment of the adaptor protein CrkII via its SH3 domain and to the activation of RhoA. The RhoA-dependent changes in cell morphology were delayed upon mutations of Y596 or Y602, and completely abolished when Y779 or all three tyrosine residues were mutated [320]. Phosphorylation on Tyr-602 also mediates the interaction with the SH2 domain of Nck1 [321].

5.4.3.3 EphA3 Ligands

- Ligand structure

  EphA3 interacts with all A-class ephrin ligands.

- Ligand cleavage

The metalloprotease ADAM10 was described to constitutively associate with EphA3. Upon binding of ephrinA5 to EphA3, the ligand is cleaved by ADAM10 located on the opposing membrane [322].

5.4.3.4 EphA3 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. Higher-order cluster formation is required to induce biological responses and signaling.

- Phosphorylation

Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

EphA3 was shown to activate RhoA via the recruitment of the adaptor CrkII, resulting in cell rounding, membrane blebbing, and de-adhesion [318, 320].

Phosphorylated EphA3 binds Src family kinases Src, Yes, and Fyn in chick embryonic retina [323].

EphA3 also interacts with Nck1, leading to the inhibition of cell migration and process outgrowth, as was demonstrated in HEK293 cells [321].

- Major genes regulated

No transcriptional targets of EphA3 have been reported.

- Cross talk with other receptor systems

No information is available.

5.4.3.5 EphA3 Internalization, Processing, and Attenuation

EphA3 is endocytosed upon ligand binding. A study in HEK293 cells showed that EphA3 interacts with and is dephosphorylated by the phosphatase PTP1B, which also controls EphA3 endocytosis and cells surface localization, and regulates the effects of EphA3 on cell morphology and cell sorting [324].

5.4.3.6 Unique Features of the EphA3 Receptor

EphA3 has been shown to heterodimerize with the EphB2 receptor [325].

5.4.4 EphA4

5.4.4.1 EphA4 Gene

- Promoter structure

  The EphA4 promoter structure is largely unknown.

- mRNA structure

Human: 5′UTR—1–57; 3′UTR—3,004–6,346. Mouse: 5′ UTR—1–57; 3′ UTR—3,019–6,328. 18 exons. 15 splice variants are predicted for the human gene and 2 for the mouse gene (See note 1).

- Transcriptional regulation

A somite-specific enhancer for EphA4 expression was identified. Mesp2, a bHLH transcription factor, was shown to bind directly to this enhancer of EphA4 [326]. A 470 bp enhancer element drives specific expression of EphA4 in rhombomers 3 and 5, where Krox-20 was shown to be the direct transcriptional activator of EphA4 [327]. Hoxa13 and Hoxd13 repress, while Sall1 and Sall3 activate EphA4 expression in the limb bud. Hox and Sall transcription factors compete for the binding of a common AT-rich sequence in the upstream region of EphA4; this mechanism could contribute to the mutual antagonistic function between Sall and Hox proteins [315]. Zic2 transcription factor binds to the EphA4 promoter immediately upstream of the transcription starting site, positively regulating EphA4 expression [328]. Pou3f4 binds to and positively regulates expression of EphA4 in otic mesenchyme cells [136].

5.4.4.2 EphA4 Protein

- Amino acid sequence

  Human: P54764. Mouse Q03137.

- Processing

EphA4 is a substrate of matrix metalloproteases and γ-secretase. The processed intracellular domain increases the number of dendritic spines [22], and EphA4 processing regulates the expression levels of the receptor itself [25].

- Domain structure

The domain structure of EphA4 is similar to other Eph receptors (see Table "Receptor at a glance**:** EphA4"). The crystal structure of the ligand-binding domain shows the same jellyroll β-sandwich architecture as described for EphB receptors [329]. Several studies have addressed the promiscuity of EphA4 in binding both A

and B ephrin ligands; one study has shown two specific residues as important contributors to the class specificity [330], while another study proposed that the crystal structure of the EphA4 ligand-binding domain in complex with ephrinA2 resembles other class A Eph receptors but on binding ephrinB2 assumes structural hallmarks of the class B Eph receptors [3]. The tyrosine kinase domain of EphA4, as well as a tight regulation of its activity (via the phosphorylation of the two juxtamembrane tyrosine residues), has been shown to be required in vivo for corticospinal tract guidance and thalamocortical topographic mapping [63, 64, 70]; but not for anterior commissure formation or Schaffer-Collateral LTP. The noncatalytic structural modules, such as the PDZ-binding motif (PBM) and the sterile-α motif (SAM) domain, have no required function described so far for EphA4 signaling in vivo [64, 70]. The crystal structure of the EphA4 SAM domain indicated its contribution to the formation of homodimers [331].

- Posttranslational modification

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

The juxtamembrane tyrosines Y596 and Y602 [332], the kinase domain Y779 [333], and SAM domain Y928 [334] are EphA4 phosphorylation sites. The two juxtamembrane tyrosine residues regulate kinase activity [70]. The proto-oncogene protein kinase Fyn binds to the Y602 juxtamembrane autophosphorylation site [332].

5.4.4.3 EphA4 Ligands

- Ligand structure

EphA4 binds with high affinity to ephrinAs, but is also able to bind ephrinBs. In addition, VAPB was shown to be a ligand for EphA4 [294]. However, this observation remains to be confirmed by others.

- Ligand cleavage

  VAPB is cleaved and secreted as a diffusible binding protein for EphA4 [294].

5.4.4.4 EphA4 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. The formation of higher-order clusters is required to induce biological responses and signaling [63]. The formation of small circular clusters by EphA4 in complex with ephrinA5 appears to promote repulsion in contrast to the larger EphA2 arrays that mediate adhesion [309].

- Phosphorylation

Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

The neuronal RacGAP α2-chimaerin and Nck1 and Nck2 SH2/SH3 adaptor proteins were identified as effectors for EphA4 in cortical and spinal motor circuits. EphA4 interacts with α2-chimaerin through its Src homology 2 domain. Activated EphA4 induces tyrosine phosphorylation of α2-chimaerin and enhances Rac1 GTPase activity. Biochemical evidence suggests that EphA4, Nck, and α2-chimaerin could function in a signaling pathway [85–89].

EphA4 signaling leads to tyrosine phosphorylation of ephexin1, a RhoA-GEF. Ephexin1 is required for EphA4-dependent axonal growth cone collapse [335, 336]. The association of EphA4 and ephexin1 was shown to be mediated through the activation of Cdk5, which promotes RhoA activity [178].

In vascular smooth muscle cells, EphA4 activation enhances Vsm-RhoGEF activity for RhoA and promotes the assembly of actin stress fibers [211].

EphA4 binds to the PDZ domain of the GTPase-activating protein spine-associated RapGAP (SPAR). EphA4 signaling induces SPAR-dependent inactivation of Rap1 and Rap2, two GTPases involved in the regulation of spine morphology. In vitro experiments in a neuronal cell line demonstrated that inactivation of Rap1 by SPAR is required for ephrinA-induced growth cone collapse, as well as for regulation of integrin-mediated cell adhesion [179].

EphA4 activation by ephrinA3 inhibits integrin signaling pathways that stabilize dendritic spines. EphA4 activation decreases tyrosine phosphorylation of the scaffolding protein Crk-associated substrate (Cas), the tyrosine kinase focal adhesion kinase (Fak), and the proline-rich tyrosine kinase 2 (Pyk2), and also reduces the association of Cas with the Src family kinase Fyn and the adaptor Crk [181]. Stimulation of EphA4 by ephrinA also leads to the recruitment and activation of phospholipase Cγ1 (PLCγ1). This interaction occurs through EphA4 juxtamembrane tyrosines and Src homology 2 domain of PLCγ1. EphA4 and PLCγ1 activity modulate the association of the actin depolymerizing/severing factor cofilin with the plasma membrane [180].

- Major genes regulated

No transcriptional targets of EphA4 have been reported.

- Cross talk with other receptor systems

EphA4 cooperates with Ret receptor signaling in the guidance of LMC_L axons in the limb [14, 337, 338].

EphA4 enhances FGFR signaling pathway in the proliferation and migration of glioblastoma cells [339].

5.4.4.5 EphA4 Internalization, Processing, and Attenuation

EphA4 is endocytosed upon ligand binding. Rin1, a postnatal brain-specific Rab5-GEF, mediates EphA4 endocytosis in amygdala neurons [188]. EphA4 was also shown to be cleaved by a metalloprotease extracellularly and subsequently by

γ-secretase intracellularly, enhancing the formation and maintenance of dendritic spines through the activation of the Rac signaling pathway [22].

5.4.4.6 Unique Features of the EphA4 Receptor

EphA4 is the most promiscuous receptor of the family, due to its property of being activated by both ephrinAs and ephrinBs.

5.4.5 EphA5

5.4.5.1 EphA5 Gene

- Promoter structure

  The mouse EphA5 promoter region contains two CpG islands, the first one stretching from −327 to +387 relative to the transcription initiation site, and the second one located in the coding region of exon 1 and extending into intron 1, from +557 to +1348 [340].

- mRNA structure

  Human: 5′ UTR—1–601, 3′ UTR—3,716–8,266. Mouse: 5′ UTR—1–416; 3′ UTR—3,084–4,298. 18 exons. 4 splice variants are predicted for the human gene, 12 for the mouse gene (See note 1).

- Transcriptional regulation

  In the mouse retina, increased methylation of the CpG island of the EphA5 promoter correlated with lower levels of receptor mRNA expression [340]. High methylation levels also correlated with a dramatic reduction in EphA5 mRNA in breast cancer cell lines and tissue samples from breast cancer patients [341].

  EphA5 transcription is regulated by cAMP, as was shown in a human neuroepithelioma cell line [342].

5.4.5.2 EphA5 Protein

- Amino acid sequence

  Human: P54756. Mouse: Q60629.

- Processing

  No information available.

- Domain structure

The domain structure of EphA5 is similar to other Eph receptors (see Table "Receptor at a glance**:** EphA5").

- Posttranslational modification

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

  Mouse EphA5 is phosphorylated on Y672 (human Y833) (see note 3).

5.4.5.3 EphA5 Ligands

- Ligand structure

  EphA5 interacts with all A-class ephrin ligands.

- Ligand cleavage

  No information available.

5.4.5.4 EphA5 Activation and Signaling

- Dimerization

Heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. Higher-order cluster formation is required to induce biological responses and signaling.

- Phosphorylation

  Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

In cholangiocytes (epithelial cells of the biliary ducts), EphA5 was shown to activate the Rho family GTPase Cdc42. This effect was abolished in the presence of the PI3K inhibitor wortmannin, suggesting that the EphA5-Cdc42 pathway requires PI3K activation [343].

- Major genes regulated

  No transcriptional targets of EphA5 have been reported.

- Cross talk with other receptor systems

  No information is available.

5.4.5.5 EphA5 Internalization, Processing, and Attenuation

No information is available.

5.4.5.6 Unique Features of the EphA5 Receptor

No unique features.

5.4.6 EphA6

5.4.6.1 EphA6 Gene

- Promoter structure

  The EphA6 promoter structure is largely unknown.

- mRNA structure

  Human: 5′ UTR—1–38; 3′ UTR—3,432–3,971. Mouse: 5′ UTR—no information; 3′ UTR—3,109–3,643. 18 exons. 11 splice variants are predicted for the human gene, 5 for the mouse gene (See note 1).

- Transcriptional regulation

  The Homeobox transcription factor Hoxa13 directly regulates EphA6 expression in the developing genital tubercle (GT) vasculature. GT chromatin immunoprecipitation revealed in vivo binding of Hoxa13 to a number of conserved *cis*-regulatory elements in the EphA6 promoter region. Moreover, activation of gene expression by Hoxa13 through the EphA6 gene-regulatory elements was demonstrated by in vitro experiments [344]. The transcription factor Foxd1 regulates EphA6 expression in the developing temporal retina and thereby contributes to topographic mapping of the visual system [114].

5.4.6.2 EphA6 Protein

- Amino acid sequence

  Human: Q9UF33. Mouse: Q62413.

- Processing

  No information is available.

- Domain structure

  The domain structure of EphA6 is similar to other Eph receptors (see Table "Receptor at a glance**:** EphA6").

- Posttranslational modification

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

EphA6 is probably phosphorylated on tyrosine residues (see note 3). No information is available about the functions of specific EphA6 phosphorylation sites.

5.4.6.3 EphA6 Ligands

- Ligand structure

  EphA6 interacts with all A-class ephrin ligands.

- Ligand cleavage

  No information is available.

5.4.6.4 EphA6 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. Higher-order cluster formation is probably required to induce biological responses and signaling.

- Phosphorylation

Receptor tyrosine phosphorylation is probably induced by binding of ligand dimers.

- Pathway activation

  No information on downstream signaling is available.

5.4.6.5 EphA6 Internalization, Processing, and Attenuation

No information is available.

5.4.6.6 Unique Features of the EphA6 Receptor

No unique features.

5.4.7 EphA7

5.4.7.1 EphA7 Gene

- Promoter structure

The EphA7 promoter was proposed to localize within ~2 kb upstream of the transcription start site [346]. Another study described a region of ~6 kb upstream of the start site, which contained the regulatory elements necessary to reproduce the endogenous expression pattern of EphA7, while truncated versions of this sequence proved insufficient [347].

- mRNA structure

Human: 5′ UTR—1–185, 3′ UTR—3,183–6,588. Mouse: 5′ UTR—1–253, 3′ UTR—3,251–6,746. 17 exons. 2 splice variants are predicted for the human gene, 8 for the mouse gene (See note 1).

- Transcriptional regulation

EphA7 is a direct target of the homeobox transcription factors Hoxa13 and Hoxd13 [344, 346]. A marked downregulation of EphA7 was observed in the forelimbs and in the genital tubercle vasculature of Hoxa13 knockout embryos [344, 348]. Moreover, several Hoxa13 and Hoxd13 binding sites were identified within the putative EphA7 promoter, and both Hox proteins were found to associate with the EphA7 promoter in vivo. Activation of EphA7 transcription by Hoxd13 and Hoxa13 was also demonstrated with luciferase assay [344, 346].

In addition, EphA7 transcriptional regulation was investigated in the developing cortex [347]. In this study, multiple putative binding sites for the transcription factor Pbx1 were described within the EphA7 promoter, and Pbx1 was found to bind directly to the EphA7 promoter in vivo.

EphA7 was also identified as a direct target of ALL1 proteins in acute leukemia. The ALL1 gene is known to be involved in chromosomal translocations, leading to the production of several ALL1 fusion proteins, which are associated with poor prognosis in acute leukemia patients. ALL1 fusion proteins bind to the EphA7 promoter and induce EphA7 transcription [349].

Downregulation of EphA7 expression due to promoter hypermethylation was observed in colorectal cancers [350].

5.4.7.2 EphA7 Protein

- Amino acid sequence

Human: Q15375. Mouse: Q61772.

In addition to the full-length protein, which is 998 amino acids long, C terminally truncated isoforms of EphA7 are also produced [41, 287, 351–353]. The two truncated membrane-bound isoforms described in the mouse contain 610 and 626 amino acids. The secreted EphA7 isoform found in human cancers contains 450 amino acids (See note 2).

- Processing

No information is available.

- Domain structure

The domain structure of full-length EphA7 is similar to other Eph receptors (see Table "Receptor at a glance**:** EphA7"). The truncated transmembrane mouse isoforms lack the kinase domain as well as the sterile-α motif and PDZ-binding motif. The secreted human isoform lacks the second fibronectin type III domain and all following domains.

- Posttranslational modification

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

EphA7 is phosphorylated on tyrosine residues. No information is available about the functions of specific EphA7 phosphorylation sites.

5.4.7.3 EphA7 Ligands

- Ligand structure

  EphA7 interacts with all A-class ephrin ligands.

- Ligand cleavage

  No information is available.

5.4.7.4 EphA7 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. Higher-order cluster formation is required to induce biological responses and signaling.

- Phosphorylation

  Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

In cortical progenitor cells, EphA7 signaling results in caspase 3 activation and induces apoptosis in vitro and in vivo [52]. ERK phosphorylation has also been observed downstream of EphA7 [349]. In addition, EphA7 interacts with the PDZ domain-containing proteins GRIP1, PICK1, and syntenin; this interaction requires the C-terminal PDZ-binding motif of EphA7 [354].

- Major genes regulated

  No transcriptional targets of EphA7 have been reported.

- Cross talk with other receptor systems

  No information is available.

5.4.7.5 EphA7 Internalization, Processing, and Attenuation

Interaction with truncated EphA7 isoforms suppresses full-length receptor phosphorylation [41].

5.4.7.6 Unique Features of the EphA7 Receptor

In addition to the full-length receptor, short kinase domain-deficient splice isoforms of EphA7 have been described [351, 352]. In the mouse, two membrane-bound truncated isoforms were detected in the neural folds. Interaction of these isoforms with the co-expressed full-length EphA7 inhibits phosphorylation of the latter and turns ephrinA5/EphA7 repulsion into adhesion, allowing for neural tube closure [41].

In addition, a secreted EphA7 isoform was described in human follicular lymphoma as well as in lung cancer cells [287, 353]. In lymphoma cells, secreted EphA7 was shown to act as a tumor suppressor by binding to and inhibiting oncogenic signaling of the EphA2 receptor [287].

5.4.8 EphA8

5.4.8.1 EphA8 Gene

- Promoter structure

The minimal EphA8 promoter lacks a TATA box and contains five copies of the putative binding sequence of the Sp1 transcription factor located upstream of the transcription start site. In addition, there is a CpG island spanning exon 1 and its flanking sequence [355].

- mRNA structure

Human: 5′ UTR—1–72; 3′ UTR—3,091–4,943. Mouse: 5′ UTR—1–69; 3′ UTR—3,085–4,713. 17 exons. 3 splice variants are predicted for the human gene, 1 splice variant for the mouse gene (See note 1).

- Transcriptional regulation

EphA8 enhancer region contains DNA binding sites for the TALE homeobox transcription factors Meis2 and Pbx1/2. In vitro experiments showed that Meis2 and Pbx2 synergistically bind to the EphA8 regulatory sequences and cooperate in activating EphA8 transcription. In vivo, overexpression of a dominant-negative form of Meis in the developing midbrain resulted in the downregulation of endogenous EphA8 [356].

5.4.8.2 EphA8 Protein

- Amino acid sequence

  Human: P29322. Mouse: O09127.

- Processing

  No information is available.

- Domain structure

The domain structure of EphA8 is similar to other Eph receptors (see Table "Receptor at a glance**:** EphA8").

- Posttranslational modification

  Phosphorylation, N-glycosylation, ubiquitination (See note 2).

- Phosphorylation sites and known functions

Mouse EphA8 is phosphorylated on Y615 in the juxtamembrane domain and Y838 in the kinase domain (Y616 and Y839 for the human). Phosphorylation of Y615 is important for the binding of Fyn via its SH2 domain [357].

5.4.8.3 EphA8 Ligands

- Ligand structure

  EphA8 interacts with A-class ephrin ligands.

- Ligand cleavage

  No information available.

5.4.8.4 EphA8 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. Higher-order cluster formation is required to induce biological responses and signaling.

- Phosphorylation

  Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

  Activation of Fyn downstream of EphA8 leads to reduced adhesion [357].

EphA8 also elicits sustained activation of the MAPK pathway in a ligand-independent manner. MAPK signaling was shown to be critical for EphA8-dependent neurite outgrowth in a neuronal cell line [358].

EphA8 interacts with two phosphotyrosine-binding domain-containing proteins, AIDA-1b and Odin, which both belong to the ankyrin repeat and sterile-α motif domain-containing (Anks) family. AIDA-1 and Odin directly associate with the juxtamembrane domain of EphA8; the binding is enhanced upon ephrin ligand stimulation and is required for ephrinA5/EphA8-induced inhibition of cell migration observed in HEK cells as well as ephrinA5/EphA8-dependent neurite retraction in a neuronal cell line [359].

The juxtamembrane segment of EphA8 associates with p100γ PI3-kinase. This interaction is dependent on ephrinA ligand binding, but seems to be independent of the EphA8 kinase domain, and leads to the activation of the integrin pathway, promoting cell adhesion to fibronectin via $\alpha_5\beta_1$ or β_3 integrins [360].

EphA8 interacts with the Rac-specific GEF Tiam1 and induces Rac activation [361].

- Major genes regulated

  No transcriptional targets of EphA8 have been reported.

- Cross talk with other receptor systems

  No information is available.

5.4.8.5 EphA8 Internalization, Processing, and Attenuation

EphA8 undergoes clathrin-dependent endocytosis upon ligand binding. This process requires the Rac-specific GEF Tiam1, which interacts with the juxtamembrane region of EphA8 [361]. Transgenic expression of an endocytosis-deficient EphA8 receptor in mice causes defects of retinotopic mapping, with an anterior shift of nasal axon projections, which is likely due to diminished repulsion from EphAs in the anterior SC [122].

Ubiquitination and proteosomal degradation of EphA8 is triggered by ephrinA ligand binding, which promotes EphA8 interaction with the ubiquitin E3 ligase c-Cbl. Anks family proteins, which also interact with EphA8, can directly bind ubiquitin and control the degradation of EphA8. Anks family member Odin was shown to interfere with c-Cbl binding to EphA8 and to increase the stability of the receptor, thereby enhancing its signaling [362].

5.4.8.6 Unique Features of the EphA8 Receptor

No unique features.

5.4.9 EphA10

5.4.9.1 EphA10 Gene

- Promoter structure

  The EphA10 promoter structure is largely unknown.

- mRNA structure

  Human: 5′ UTR—no information; 3′ UTR—3,028–5,425. Mouse: no information. 17 exons. 12 splice variants are predicted for the human gene, 3 for the mouse gene (See note 1).

- Transcriptional regulation

  No information is available.

5.4.9.2 EphA10 Protein

- Amino acid sequence

  Human: Q5JZY3. Mouse: Q8BYG9.

- Processing

  No information is available.

- Domain structure

  The domain structure of EphA10 is similar to other Eph receptors (see Table "Receptor at a glance**:** EphA10").

- Posttranslational modification

  N-Glycosylation (See note 2)

- Phosphorylation sites and known functions

  EphA10 is a kinase-dead receptor [363]. It is probably not phosphorylated (see note 3).

5.4.9.3 EphA8 Ligands

- Ligand structure

  EphA10 interacts with A-class ephrin ligands.

- Ligand cleavage

  No information is available.

5.4.9.4 EphA10 Activation and Signaling

- Dimerization

  No information is available.

- Phosphorylation

  EphA10 is probably not phosphorylated (see note 3).

- Pathway activation

  No information is available.

- Major genes regulated

  No transcriptional targets of EphA10 have been reported.

- Cross talk with other receptor systems

  No information is available.

5.4.9.5 EphA10 Internalization, Processing, and Attenuation

No information is available.

5.4.9.6 Unique Features of the EphA10 Receptor

Three putative EphA10 isoforms were identified: one soluble and two transmembrane isoforms. One of the latter isoforms lacked the sterile-α motif commonly found in Eph receptors [364].

5.4.10 EphB1

5.4.10.1 EphB1 Gene

- Promoter structure

  The EphB1 promoter structure is largely unknown.

- mRNA structure

  Human: 5′ UTR—1–370; 3′ UTR—3,326–4,672. Mouse: 5′ UTR—1–354; 3′ UTR—3,310–4,667. 16 exons. 12 splice variants are predicted for the human gene, 3 for the mouse gene (See note 1).

- Transcriptional regulation

  EphB1 expression in the ventrotemporal optic chiasm is induced by the zinc-finger transcription factor Zic2 [365].

5.4.10.2 EphB1 Protein

- Amino acid sequence

  Human: P54762. Mouse: Q8CBF3.

- Processing

  No information is available.

- Domain structure

The domain structure of EphB1 is similar to other Eph receptors (see Table "Receptor at a glance**:** EphB1").

- Posttranslational modification

  Phosphorlyation, N-glycosylation, ubiquitination (See note 2).

- Phosphorylation sites and known functions

As with other Eph receptors, EphB1 is activated by phosphorylation within the juxtamembrane domain on the conserved tyrosine Y594. EphB1 phosphorylation on Y928 is required for Grb7 recruitment via its SH2 domain. Mutation of either Y928 or juxtamembrane residue Y594, which reduces kinase activity, to phenylalanine resulted in reduced Grb7 binding, whereas EphB1 kinase dead completely prevented Grb7 interaction. EphB1 recruitment of Grb7 increases motility of fibroblast cells [366]. Phosphorylation on Y594 is also required for recruitment of both Nck and the focal adhesion protein paxillin to the receptor in a Src-dependent manner. Association of paxillin with EphB1 further recruits and activates Focal Adhesion Kinase (FAK), inducing cell migration [367]. Moreover, Nck association with EphB1 via phospho-Y594 also recruits Nck Interacting Kinase (NIK), p62 (doc), and RasGap, resulting in the activation of both c-Jun and integrin pathways [368, 369]. Phosphorylation of both EphB1 Y600 and Y778 are required for recruitment of Src and $p52^{Shc}$, thus activating the MAPK/ERK pathway [370].

5.4.10.3 EphB1 Ligands

- Ligand structure

EphB1 binds to all three ephrinB ligands. Unlike ephrinA, ephrinB ligands possess a transmembrane domain and an intracellular PDZ-binding motif. As with other EphB receptors, EphB1-induced ephrinB oligomerization can result in reverse signaling in the ephrinB-expressing cell.

- Ligand cleavage

EphrinB has been described to be cleaved in its transmembrane domain [371, 372], but no relationship to EphB1 receptor activation has been shown to date.

5.4.10.4 EphB1 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. EphB1 also dimerizes with EphB6, activating the kinase-dead receptor [373]. Higher-order cluster formation is required to induce biological responses and signaling.

- Phosphorylation

Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

Active EphB1 recruits c-Src and activates the MAPK/ERK signaling pathway [370]. Also, active EphB1 (and EphB2) recruits the SH2/SH3 binding protein Nck, which in turn recruits the c-Jun-activating kinase NIK, thus activating both the c-Jun and integrin pathways [368, 369]. In embryonic kidney cells, ephrinB-stimulated EphB1 was shown to promote cell adhesion via activation of the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin pathways [374]. Paxillin and FAK pathway is activated by EphB1 [367].

- Major genes regulated

No transcriptional targets of EphB1 have been reported.

- Cross talk with other receptor systems

No information is available.

5.4.10.5 EphB1 Internalization, Processing, and Attenuation

EphB1/ephrinB complexes are internalized by endocytosis. In Xenopus, EphB1 internalization has been associated with the noncanonical Wnt pathway via Daam1 protein and is involved in notochord formation during development [33]. EphB1 levels are also regulated by the lysosomal pathway. The receptor is ubiquitinated by the E3-ubiquitin ligase protein CBL. CBL is activated by phosphorylation by Src, which in turn is activated by ephrinB-induced EphB1. Active CBL binds to and targets EphB1 for degradation, creating a negative feedback loop regulating EphB1 levels [375].

5.4.10.6 Unique Features of the EphB1 Receptor

No unique features.

5.4.11 EphB2

5.4.11.1 EphB2 Gene

- Promoter structure

The human EphB2 promoter is located between −167 and +83 nucleotides in relation to the transcription start site [376]. A negative regulatory element in the −1174/-970 region and an enhancer element in the −425/-139 region have been characterized. A 22 bp sequence containing the c-Rel binding site is located between −1009 and −988. CpG islands of the EphB2 promoter are hypermethylated, and thus silenced, in prostate cancer samples; however, analysis in colon cancer and ovarian cancer samples revealed that EphB2 is not methylated [376].

- mRNA structure

Human: 5′ UTR—1–18; 3′ UTR—3,187–4,641. 17 exons. Mouse: 5′ UTR—1–126; 3′ UTR—3,091–4,804. 16 exons. 8 splice variants are predicted for the human gene and 6 for the mouse gene (See note 1).

- Transcriptional regulation

c-Rel transcription factor is responsible for directly silencing EphB2 transcription during the adenoma to carcinoma transformation in colorectal tumor progression [376]. Conversely β-catenin/TCF directly enhances the expression of EphB2 receptor downstream of Wnt signaling at the bottom part of the colonic crypt [26]. It has also been suggested that mVax2 homeodomain transcription factor [377, 378] and Smad3 component of TGF-beta signaling [379] positively regulate EphB2 expression in the developing retina and the colonic crypt, respectively. However, there is no evidence for their direct binding onto EphB2 promoter.

5.4.11.2 EphB2 Protein

- Amino acid sequence

Human: P29323 Mouse: P54763.

- Processing

Upon binding of ephrin, EphB2 extracellular domain is cleaved by metalloproteases, whereas the intracellular domain is cleaved by presenilin-dependent γ-secretase [380]. The intracellular EphB2 fragment released into the cytosol has intact tyrosine kinase activity, and was shown to directly phosphorylate NMDA receptors [381]. Neuropsin, a serine protease, was also shown to cleave the extracellular domain of EphB2 [23]. Finally, the Tissue Factor (TF)/serine protease factor VIIa (fVIIa)

complex cleaves EphB2 in its N-terminal ligand-binding domain, potentiating ephrinB-induced cell–cell repulsion [382].

- Domain structure

The domain structure of EphB2 receptor is similar to all Eph receptors (see Table "Receptor at a glance**:** EphB2"). Crystal structure of the amino-terminal ligand-binding domain revealed that the domain folds into a compact jellyroll β-sandwich composed of 11 anti-parallel β-strands [383]. In addition, X-ray crystal structure of the juxtamembrane region and the kinase domain revealed that the juxtamembrane region folds into a helical conformation, blocking the small lobe of the kinase domain and preventing it from adopting an activated conformation [384]. Phosphorylation of juxtamembrane tyrosines contributes to the dissociation of the juxtamembrane region from the kinase domain and the liberation of the phosphotyrosine sites for binding SH2 domain proteins. Monomeric and oligomeric crystal structures of the SAM domain revealed a possible role in the formation of large protein complexes and oligomerization of EphB2 receptor in large signaling clusters [385, 386].

- Posttranslational modifications

Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

Phosphorylation of the two juxtamembrane tyrosines Y604 and Y610 relieves autoinhibition of the receptor by disturbing the association of the juxtamembrane and kinase domains [384]. Phosphorylation of Y610 also mediates binding and activation of Src kinase [323]. Point mutations inactivating Y604 and Y610 showed that these two sites are dispensable for activation of the Ras/MAPK pathway and regulation of cell-cell adhesion [387]. Phosphorylation of Y750 leads to the activation of the catalytical function of the EphB2 kinase domain [388]. Crystallographic evidence suggests that Y979 and Y996, which are situated in the SAM domain, do not belong to its hydrophobic core [385, 386]. A possible role for the phosphotyrosines on the SAM domain is in the oligomerization of EphB2 receptor and the formation of signaling clusters.

In vivo point mutations indicated that the kinase activity of EphB2 controls the migration of progenitor cells situated in the dentate gyrus toward the dorsal half of the developing hippocampus that gives rise to the lateral suprapyramidal blade [389].

5.4.11.3 EphB2 Ligands

- Ligand structure

EphB2 receptor binds to all three transmembrane ephrinB ligands and it is the only member of subclass B receptors that can also bind to the GPI-anchored ephrinA5 ligand [5]. EphB2 is also activated by the glycoprotein Reelin [55].

- Ligand cleavage

  No information is available.

5.4.11.4 EphB2 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. EphB2 also dimerizes with EphA3 [325]. Higher-order cluster formation is required to induce biological responses and signaling [17].

- Phosphorylation

  Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

EphrinB-mediated EphB2 activation stimulates MAPK pathway, which in a positive feedback loop leads to the activation of hRas-ERK and to a further increase in Mek and/or ERK activity that in turn enhances the responsiveness of EphB2 to ephrinB stimulation [390]. However, in a different context EphB2 activation can also inhibit the oncogenic hRas–ERK signaling pathway [391], which in turn reduces EphB2 activation by ephrins. EphrinB1 stimulation can also reduce EphB2 activation by causing internalization and degradation of the receptor [392, 393].

Inactivation of R-Ras downstream of EphB2, probably via binding to SHEP-1 SH2 binding protein [394], can enhance glioma cell invasiveness most likely by modulating cell-to-substrate adhesion via R-Ras GTPase-Integrin [395, 396]. Abl/ Crk-Dock180, which are also activated downstream of EphB2, ultimately inhibit Rap1 and Rac1 GTPases [397] and thus migration and invasiveness, but in parallel increase cyclin-D1 levels [398], thereby enhancing proliferation. Cancer cell migration and invasion is probably also mediated by activation of Rac1 (via Kalirin and Tiam1) and Cdc42 (via Intersectin) upon EphB2 activation [399]. Furthermore, EphB2 can act as a tumor suppressor by increasing the expression of p110 PI3K subunit [398], by suppressing Rac1 and Rap1 activation via the Abl pathway, and by activating p120RasGAP that further inhibits H-Ras and R-Ras proliferation pathways [391, 397]. EphB2 can also directly activate ERK signaling and hence decrease integrin-mediated adhesion [390].

At the synapse, EphB2 activation increases Ca^{2+} influx by activating Src kinase, which phosphorylates and activates NMDA receptors [164]. Studies in neurons have implicated several nucleotide exchange factors for Rho GTPases, including Kalirin [170], Tiam1 [171], Intersectin [172], and ephexin5 [173], in EphB2-mediated spine formation. Focal adhesion kinase (FAK) is also involved downstream of EphB2 in the stabilization of mature dendritic spines [400, 401] by inhibiting cofilin activity via phosphorylation. Regulation of cofilin activity downstream of EphB2 also depends on the RhoA–ROCK–LimK1 pathway. Moreover,

EphB2 mediates morphological maturation of dendritic spines by binding, co-clustering, and phosphorylating syndecan-2 [174]. Activated Syndecan-2 further recruits Grip1 and Lin7 and induces spine maturation. In addition, Grip1–KIF5 interaction regulates the appropriate transport of EphB2 cargo to dendritic structures and co-clustering with GluR2 [402].

Disheveled in Xenopus is a necessary component of both EphB2 forward and ephrinB2 reverse signaling to mediate cell sorting, as seen in animal cap assays [403]. Src-mediated phosphorylation and activation of the adaptor protein Nck induces Disheveled activation that further promotes RhoA activity by Disheveled-associated activator of morphogenesis 1 (DAAM1) formin-homology protein [403].

- Major genes regulated

During nerve regeneration, EphB2-forward signaling in Schwann cells leads to Sox2 upregulation and relocation of N-cadherin to sites of cell–cell contact [200].

- Cross talk with other receptor systems

Links between EphB/ephrinB and Wnt signaling have been revealed in various systems. EphB2 receptor and Ryk, a Wnt receptor containing a kinase-dead domain, interact physically and function together in craniofacial development and axon guidance [155, 156]. Moreover, EphB2 receptor signals through components of noncanonical Wnt pathway [33]. This pathway is also involved in the removal of EphB2 clusters from the cell surface; however, Wnt canonical signaling induces the opposite effect, leading to EphB2 transcription and upregulation [26, 33].

EphB2-forward signaling during cell sorting processes communicates reciprocally with FGF signaling [390]. EphB2 signaling is negatively regulated by downstream effectors of FGF signaling, resulting in decreased cell repulsion behavior. FGFR activation leads to a downregulation of LAR phosphatase which is concurrent with an increased baseline of EphB2 phosphorylation. An additional component of the negative feedback loop regulating EphB2 signaling are FGFR1 gene targets sprouty2 and 4 that were shown to affect EphB2-dependent cell sorting behavior [390].

Numerous studies have focused on the cross talk between Eph and integrin signaling. In most cases EphB2 signaling counteracts and reduces integrin-mediated adhesion [396]. The point of convergence between the two signaling pathways appears to be at the level of cytoplasmic FAK, PI3K, and MAPK kinases and/or Rac1, Rho, Ras, and Rap1 GTPases.

5.4.11.5 EphB2 Internalization, Processing, and Attenuation

EphB2 is cleaved by metalloproteases, presenilin-dependent γ-secretase, and the serine protease neuropsin [23, 380]. Furthermore, bidirectional trans-endocytosis of Eph/ephrin complexes is a common mechanism used by cells to terminate Eph/ephrin interaction and mediate de-adhesion and repulsion [392, 404]. Vav2, a Rac1 GEF, was found to interact via the SH2 domain with EphB2 and to mediate its internalization [134].

5.4.11.6 Unique Features of the EphB2 Receptor

EphB2 is the only receptor of the B-subclass that was shown to bind both ephrinB and ephrinA ligands [5].

5.4.12 EphB3

5.4.12.1 EphB3 Gene

- Promoter structure

  The human EphB3 promoter has been mapped in the 1.3 kb region upstream the 5′ end of EphB3 (GeneCard database).

- mRNA structure

  Human: 5′UTR—1–452; 3′ UTR—3,450 to 4,236. Mouse: 5′ UTR—1–415; 3′ UTR—3,398–4,185. 16 exons. 3 splice variants are predicted for the human gene, 6 for the mouse gene (See note 1).

- Transcriptional regulation

  Achaete scute-like-2 transcription factor, a downstream effector of Wnt signaling, binds directly onto the EphB3 promoter and positively regulates its expression in the stem cell population at the bottom of the intestinal crypt [407]. Moreover, like for EphB2, β-catenin/TCF has been found to directly enhance the expression of EphB3 receptor downstream of Wnt signaling at the bottom part of the colonic crypt [26]. Smad3, a component of TGF-beta signaling, also regulates EphB3 expression in intestinal stem cells [379]. In addition, just like EphB2, during the progression of colorectal cancer, the EphB3 promoter seems to be hypermethylated and thus silenced via HDACs class I and III [264], whereas analysis of ovarian cancer samples showed that CpG islands in the EphB3 promoter are unmethylated [408]. In addition, data obtained by chromatin immunoprecipitation experiments demonstrated that the EphB3 promoter contains the following transcription factor binding sites: one POU3F1, two HNF-1s, three FoxD1s, two Egr-2s, one Preb-1, two Egr-1s, two Egr-4s, and one GR (GeneCards database).

5.4.12.2 EphB3 Protein

- Amino acid sequence

  Human: P54753. Mouse: P54754.

- Processing

  No information is available.

- Domain structure

The domain structure of EphB3 receptor is similar to other Eph receptors (see Table "Receptor at a glance**:** EphB3").

- Posttranslational modification

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

Tyrosine Y614, a major autophosphorylation site for EphB3, functions as a multi-docking site for SH2-domain-mediated interactions, such as RasGAP, Crk, and Fyn [409].

Phosphorylation of the tyrosines in the kinase domain (Y787, Y807) mediates the association with AF-6 Ras-binding protein via its PDZ domain [410].

5.4.12.3 EphB3 Ligands

- Ligand structure

  EphB3 receptor binds to all three transmembrane ephrinB ligands.

- Ligand cleavage

  No information is available.

5.4.12.4 EphB3 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. Higher-order cluster formation is required to induce biological responses and signaling.

- Phosphorylation

  Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

EphB3 signaling promotes shedding of E-cadherin that leads to an asymmetric localization of E-cadherin and to changes in cell affinity between Eph and ephrin-expressing cells [411]. EphB3 receptor can inhibit cell adhesion in a kinase-dependent manner and cell migration in a kinase-independent manner [412]. It suppresses integrin-mediated adhesion by inhibiting Rac1/Cdc42 signaling, which leads to a relative increase of RhoA signaling. Conversely, inhibition of migration that is independent of EphB3 catalytic activity is probably regulated by a distinct Rho GTPase pathway [412], possibly by an increase in Cdc42 signaling, as described in prostate cancer [276]. During cell sorting EphB3 signaling has also been associated with activating Ras–MAPK pathways. AF-6, a PDZ domain-containing protein normally associated with cell junctions, binds to EphB3 in a kinase activity-dependent manner, and also binds to

Ras [410]. Furthermore, AF-6 associates with Disheveled, thereby connecting EphB3 signaling with Wg/Wnt and Ras/MAPK pathways. In addition, RasGAP, Fyn, and Crk bind EphB3 via SH2-domain interactions on Y614 [409].

In adult neural stem cells, EphB3 in the subventricular zone activates p53 signaling pathway and thus suppresses cell proliferation and induces cell death of neural stem cells [247]. However, in malignant lymphocytes EphB3 signaling has the opposite effect, since it activates the Akt survival pathway and in parallel suppresses the Fas-induced apoptosis pathway [413].

EphB3 is also able to regulate gliotransmitters in astrocytes and hence influence synaptic plasticity. Upon activation in astrocytes, EphB3 interacts with PICK1 (protein interacting with C-kinase) and mediates PKC-α dephosphorylation to further activate the conversion of L-serine to D-serine by serine racemase and thereby enhance D-serine synthesis and release [414].

- Major genes regulated

  No transcriptional targets of EphB3 have been reported.

- Cross talk with other receptor systems

Synergistic interaction between the Wnt kinase dead receptor Ryk and EphB3 signaling regulates radial migration of neural progenitor cells in the cortex [415]. EphB3 signaling cross talk with E-cadherin influences cell sorting behavior. In epithelial cells, EphB3 co-clusters with the metalloprotease ADAM10 and E-cadherin at interfaces with ephrinB1-expressing cells. Upon EphB3 clustering ADAM10 is activated and sheds E-cadherin, influencing localization of cadherins and cell–cell affinities [411].

5.4.12.5 EphB3 Internalization, Processing, and Attenuation

Upregulated EphB3 surface expression in the palate of ephrinB1 null mutant mice suggested that ligand binding probably leads to internalization and degradation of the receptor in vivo [393].

5.4.12.6 Unique Features of the EphB3 Receptor

No unique features.

5.4.13 EphB4

5.4.13.1 EphB4 Gene

- Promoter structure

  The EphB4 promoter structure is largely unknown.

- mRNA structure

Human: 5′ UTR—1–469; 3′ UTR—3,434–4,329. Mouse: 5′ UTR—1–489; 3′ UTR—3,454–4,340. 17 axons. 9 splice variants are predicted for the human gene, 5 for the mouse gene.

- Transcriptional regulation

EphB4 expression is activated by CREB binding protein (CBP) downstream of Wnt signaling in colorectal cancer cells [265]; however, hypermethylation of the promoter region has also been shown to inhibit EphB4 expression in colorectal cancer [261].

5.4.13.2 EphB4 Protein

- Amino acid sequence

  Human: P54760. Mouse: P54761.

- Processing

  No information is available.

- Domain structure

The domain structure of EphB4 receptor is similar to other Eph receptors (see Table "Receptor at a glance**:** EphB4"). The crystal structure of the ligand-binding domain of EphB4 in a complex with the extracellular domain of its preferred ligand, ephrinB2, has been solved. The resulting structure revealed that the presence of a leucine at residue 95 of the receptor, where other Eph family members have an arginine, confers the high-affinity binding to ephrinB2 [417].

- Posttranslational modification

  Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

Mass spectrometry has shown multiple phosphorylation sites on serine, threonine, and tyrosine residues within the intracellular domain, including a cluster of three tyrosines and one threonine within the juxtamembrane domain (see note 3); however, no information is available about the functions of these specific EphB4 phosphorylation sites.

5.4.13.3 EphB4 Ligands

- Ligand structure

  EphB4 interacts with all B-class ephrin ligands, but ephrinB2 is its preferred ligand.

- Ligand cleavage

  No information is available.

5.4.13.4 EphB4 Activation and Signaling

- Dimerization

A heterotetramer is formed upon ligand binding. The heterotetramer consists of an ephrin dimer and a receptor dimer. EphB4 also dimerizes with EphB6, activating the kinase-dead receptor [258]. Higher-order cluster formation is required to induce biological responses and signaling.

- Phosphorylation

Receptor tyrosine phosphorylation is induced by binding of ligand dimers.

- Pathway activation

EphB4 activates several pathways including Cdc42/Rac1, resulting in cell collapse via reorganization of the actin network [404]. In endothelial cells, EphB4 activation leads to cell growth and increased migration by activating the PI3K/Akt pathway, a response that was dependent on Src activity [418]. By activating Cdc24 in prostate cancer cells, EphB4 is able to overcome contact inhibition, thus promoting invasion into surrounding tissue layers [276]. Finally, in a mouse xenograft model, active EphB4 was shown to inhibit cell viability and proliferation via Abl–Crk pathway [274].

- Major genes regulated

No transcriptional targets of EphB4 have been reported.

- Cross talk with other receptor systems

No information is available.

5.4.13.5 EphB4 Internalization, Processing, and Attenuation

EphB4/ephrinB2 complexes have been shown to endocytose, both in the forward and reverse direction. The forward endocytosis requires Rac1 signaling [404].

5.4.13.6 Unique Features of the EphB4 Receptor

No unique features.

5.4.14 EphB6

5.4.14.1 EphB6 Gene

- Promoter structure

The EphB6 promoter structure is largely unknown.

- mRNA structure

Human: 5′ UTR—1–787; 3′ UTR—3,853–4,043. 20 exons. Mouse: 5′ UTR—1–4,512; 3′ UTR—3,558–3,762. 18 exons. 10 splice variants are predicted for the human gene, 6 for the mouse gene.

- Transcriptional regulation

In non-small cell lung cancer, EphB6 promoter is hypermethylated, resulting in reduced expression which correlates with increased metastatic potential [419].

5.4.14.2 EphB6 Protein

- Amino acid sequence

Human: O15197. Mouse: O08644.

- Processing

No information is available.

- Domain structure

Although EphB6 contains the same general domain structure as other Eph receptors (see Table "Receptor at a glance**:** EphB6"), it is unusual in that it has amino acid substitutions in the kinase domain removing any kinase activity.

- Posttranslational modification

Phosphorylation, N-glycosylation (See note 2)

- Phosphorylation sites and known functions

EphB6 has been shown to be phosphorylated at Y635, Y644, Y645, and Y651 (see note 3).

5.4.14.3 EphB6 Ligands

- Ligand structure

EphB6 binds ephrinB1 and ephrinB2. EphB6 has a biphasic response to ephrinB2 stimulation depending on the level of ligand present. Low amounts of ephrinB2 lead to cell adhesion and promote migration, whereas higher concentrations result in repulsion and inhibition of migration via phosphorylation and activation of Src family kinases [420].

- Ligand cleavage

  No information is available.

5.4.14.4 EphB6 Activation and Signaling

- Dimerization

  EphB6 was shown to heterodimerize with EphB1 and EphB4 [258, 373]. Higher-order cluster formation is probably required to induce biological responses and signaling.

- Phosphorylation

  Activation of EphB6 requires phosphorylation by other Eph receptors that have kinase activity.

- Pathway activation

  In spite of the lack of kinase activity, EphB6 is a functional receptor. It constitutively binds the Src family kinase member Fyn [420]. Both EphB1 and EphB4 were shown to oligomerize with and activate EphB6, leading to recruitment and activation of the c-Cbl/Abl pathway [258, 373]. In T-lymphocytes, EphB6 activation suppressed the JNK pathway by inhibiting the small GTPase Rac1 [421].

- Major genes regulated

  No transcriptional targets of EphB6 have been reported.

- Cross talk with other receptor systems

  No information is available.

5.4.14.5 EphB6 Internalization, Processing, and Attenuation

Upon ephrinB activation, EphB6 is shown to be internalized along with EphB4 in clathrin-coated pits and degraded in the lysosomes [422].

5.4.14.6 Unique Features of the EphB6 Receptor

The kinase domain of EphB6 is nonfunctional; however, the receptor can be activated by EphB1 or EphB4-mediated phosphorylation [258, 373]. EphB6 also constitutively binds the Src family kinase Fyn [420].

Receptor at a glance: EphA1

Chromosome location	Human: chromosome 7: 143,087,382–143,105,985; reverse strand Mouse: chromosome 6: 42,308,486–42,323,267; reverse strand[a]
Gene size (bp)	Human: 18,604. Mouse: 14,782[a]
Intron/exon numbers	18 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—3,363; 5′ UTR—87; ORF—2,931; 3′UTR—345 Mouse: mRNA—3,273; 5′ UTR—58; ORF—2,934; 3′UTR—281[a]
Amino acid number	Human: 976. Mouse: 977[b]
kDa	Human: 108. Mouse: 109[b]
Posttranslational modifications	Tyrosine phosphorylation, N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	Ephrin-A1, −A3, −A4
Known dimerizing partners	EphA1
Pathways regulated	Inhibits Integrin-linked kinase; stimulates RhoA/ROCK
Tissues expressed	Adult: epithelial tissue elements including those found in skin, kidney, ureter, uterus, vagina [299]
Human Diseases	Risk locus for late-onset Alzheimer disease
Knockout Mouse phenotype	Highly penetrant kinky-tail phenotype due to the deformation of the most caudal tail structures, and reminiscent of the EphA2 knockout phenotype; partially penetrant failure in the process of uterovaginal canalization dependent on a pro-apoptotic mechanism [299]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphA2

Chromosome location	Human: chromosome 1: 16,450,832–16,482,582; reverse strand Mouse: chromosome 4: 140,857,155–140,885,299; forward strand[a]
Gene size (bp)	Human: 31,751. Mouse: 28,145[a]
Intron/exon numbers	17 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—3,964; 5′ UTR—155; ORF—2,931; 3′UTR—878 Mouse: mRNA—3,913; 5′ UTR—113; ORF—2,934; 3′UTR—866[a]
Amino acid number	Human: 976. Mouse: 977[b]
kDa	Human: 108. Mouse: 109[b]
Posttranslational modifications	Tyrosine phosphorylation, serine/threonine phosphorylation, N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	EphrinA1 (preferred ligand), −A2, −A3, −A4, −A5
Known dimerizing partners	EphA2

(continued)

Pathways regulated	Activates Rac1 and RhoG; inhibits PI3K/Akt and Rho/ROCK
Tissues expressed	During early mouse development, EphA2 is expressed in rhombomere 4 of the hindbrain [310], in distal regions of limb bud mesenchyme and various fetal epithelia [311]. In the adult EphA2 is expressed at low levels in epithelial tissue; highly upregulated in malignant cellular phenotypes including metastases
Human Diseases	EphA2 is associated with age-related cortical cataract
Knockout Mouse phenotype	EphA2 null mice develop skin tumors with an increased frequency and shortened latency. Moreover, tumors in homozygous knockout mice grow faster and are twice as likely to show invasive malignant progression [239]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphA3

Chromosome location	Human: chromosome 3: 89,156,674–89,531,284; forward strand Mouse: chromosome 16: 63,543,364–63,863,984; reverse strand[a]
Gene size (bp)	Human: 374,611. Mouse: 320,621[a]
Intron/exon numbers	17 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—5,809; 5′ UTR—225; ORF—2,952; 3′ UTR—2,632 Mouse: mRNA—5,659; 5′ UTR—111; ORF—2,595; 3′ UTR—2,593[a]
Amino acid number	Human: 983. Mouse: 984[a]
kDa	Human: 110. Mouse: 110[b]
Posttranslational modifications	Tyrosine phosphorylation, N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	EphrinA1, −A2, −A3, −A4, −A5
Known dimerizing partners	EphA3; EphB2 [325]
Pathways regulated	Activates RhoA
Tissues expressed	Expression is highest in the brain, also detected in testis. In the developing heart, EphA3 is expressed by mesenchymal cells of the endocardial cushions
Human diseases	Defects in EphA3 may be a cause of colorectal cancer. It was also identified in a homozygous haplotype mapping screen for genes associated with autism spectrum disorders
Knockout mouse phenotype	EphA3 mutants show defects in heart development, with hypoplasia of atrioventricular endocardial cushions. ~75 % of homozygous mutants die within 48 hours after birth due to cardiac dysfunction [152]. Survivors develop normally with no indications of cardiac abnormalities. In EphA3; EphA4 double knockouts, hypaxial motor nerves are misguided into the DRGs [100], and hypaxial sensory projections are disturbed [101]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphA4

Chromosome location	Human: chromosome 2: 222,282,747–222,438,922; reverse strand Mouse: chromosome 1: 77,363,760–77,511,663; reverse strand[a]
Gene size (bp)	Human: 156,176. Mouse: 147,904[a]
Intron/exon numbers	18 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—6,346; 5′ UTR—42; ORF—2,961; 3′ UTR—3,343 Mouse: mRNA—6,328; 5′ UTR—57; ORF—2,961; 3′ UTR—3,310[a]
Amino acid number	Human: 986. Mouse: 986[b]
kDa	Human: 110. Mouse: 110[b]
Posttranslational modifications	Tyrosine phosphorylation, N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	ephrinA1, -A2, -A3, -A4, -A5, -B2, and -B3
Known dimerizing partners	EphA4
Pathways regulated	Activates RhoA; inhibits Rac1, Rap1, Rap2, and integrin pathway. EphA4 intracellular domain activates Rac1
Tissues expressed	Developing nervous system: hindbrain, several neuronal subpopulations in spinal cord, cortex, hippocampus, striatum, thalamus, and retina. Developing cardiovascular system: CNS endothelial cells; neural crest cells; embryonic stem cells of the inner cell mass. Adult brain: hippocampus, amygdala, adult stem cells in SVZ; spinal cord; thyroid: follicular epithelium; kidney; lung; skeletal muscle; thymus; blood vessels: smooth muscle; platelets; stem cells of hair bulge. Cancer tumors: colon carcinoma, prostate tumors, pancreatic ductal adenocarcinoma
Human diseases	Differential expression of EphA4 is associated with metastatic melanoma, transition from prostatic intraepithelial neoplasia to invasive prostate cancer, and pancreatic ductal adenocarcinoma. Implicated as disease modifier in amyotrophic lateral sclerosis
Knockout Mouse phenotype	Axon guidance: Loss of coordination of limb movement associated with disruptions of central pattern generators; corticospinal tract; thalamocortical mapping; anterior commissure; limb motor neuron projection; retinotectal projection. Proliferation of cortical progenitors: diminished cortical size. Aberrant spine morphology. Defective Schaffer-collateral LTP and LTD. Impaired amygdala LTP. Abnormal CNS vascular structure. Defective T-cell development

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphA5

Chromosome location	Human: chromosome 4: 66,185,281–66,536,213; reverse strand Mouse: chromosome 5: 84,486,816–84,846,407; reverse strand[a]
Gene size (bp)	Human: 350,933. Mouse: 359,592[a]
Intron/exon numbers	18 exons[a]

(continued)

mRNA size (5′, ORF, 3′)	Human: mRNA—8,266; 5′ UTR—601; ORF—3,114; 3′ UTR—4,551 Mouse: mRNA—4,298; 5′ UTR—416; ORF—2,631; 3′ UTR—1,251[a]
Amino acid number	Human: 1,037. Mouse: 876[b]
kDa	Human: 115. Mouse: 97[b]
Posttranslational modifications	Tyrosine phosphorylation, N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	EphrinA1, −A2, −A3, −A4, −A5
Known dimerizing partners	EphA5
Pathways regulated	Activates Cdc42
Tissues expressed	Specifically expressed in the brain, with high levels in cortical neurons and cerebellar Purkinje cells. In addition, EphA5 is detected in the amygdala, medial septum, nucleus of the diagonal band, olfactory bulb, and retina. Outside the nervous system, EphA5 is expressed in pancreatic islet cells
Human diseases	
Knockout mouse phenotype	Homozygous mutant mice are overtly normal but show defects of retinotectal mapping, with temporal axons shifted posteriorly and nasal axons anteriorly [113]. EphA5 knockouts also have altered aggressive behavior [190]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphA6

Chromosome location	Human: chromosome 3: 96,533,425–97,471,304; forward strand Mouse: chromosome 16: 59,653,309–60,605,357; reverse strand[a]
Gene size (bp)	Human: 937,880. Mouse: 952,049[a]
Intron/exon numbers	18 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—3,971; 5′ UTR—38; ORF—3,393; 3′ UTR—540 Mouse: mRNA—3,643; ORF—3,108; 3′ UTR—535[a]
Amino acid number	Human: 1035. Mouse: 1035[b]
kDa	Human: 116. Mouse: 116[b]
Posttranslational modifications	Tyrosine phosphorylation[c], N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	EphrinA1, −A2, −A3, −A4, −A5
Known dimerizing partners	EphA6
Pathways regulated	No signaling pathways known

(continued)

Tissues expressed	During mouse development EphA6 is expressed in the accessory olfactory bulb (AOB), the site of axonal projections from the vomeronasal organ sensory neurons [139], and in retinal ganglion cells [107]. In adult mice, EphA6 is expressed predominantly in neurons in various neuronal populations [345]
Human diseases	
Knockout Mouse phenotype	Behavioral deficits specifically in tests of learning and memory [189]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)
[c]Phosphosite (http://www.phosphosite.org)

Receptor at a glance: EphA7

Chromosome location	Human: chromosome 6: 93,949,738–94,129,265; reverse strand Mouse: chromosome 4: 28,740,281–28,894,649; forward strand[a]
Gene size (bp)	Human: 179,507. Mouse: 154,369[a]
Intron/exon numbers	17 exons[a]
mRNA size (5′, ORF, 3′)	Human full-length isoform: mRNA—6,588; 5′ UTR—185, ORF—2,997; 3′ UTR—3406 Mouse full-length isoform: mRNA, 6,746; 5′ UTR—253; ORF—2,997; 3′ UTR—3,496[a]
Amino acid number	Human: full-length isoform—998; truncated isoform—450 Mouse: full-length isoform—998; truncated isoforms—610 and 626[b]
kDa	Human: full-length isoform—112; truncated isoform—51. Mouse: full-length isoform—112; truncated isoforms—68 and 70[b]
Posttranslational modifications	Tyrosine phosphorylation, N-glycosylation[b]
Domains	Full-length isoform: N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif. The truncated transmembrane mouse isoforms lack the kinase domain as well as the SAM domain and PDZ-binding motif. The secreted human isoform lacks the FN2 and all following domains
Ligands	EphrinA1, −A2, −A3, −A4, −A5
Known dimerizing partners	EphA7; EphA2 (shown for the secreted isoform of human EphA7) [287]
Pathways regulated	Activates caspase 3-dependent apoptosis; activates ERK
Tissues expressed	Widely expressed in the embryo. In adult, expression restricted to hippocampus, testis, and spleen. EphA7 truncated isoform is expressed in lymphoma and lung cancer
Human Diseases	
Knockout Mouse phenotype	Most of the mutants are viable and fertile and show no gross abnormalities. Retinotectal mapping defects were observed, with nasal axons forming ectopic termination zones in the anterior SC [120]. Cortical size is increased due to reduced apoptosis of progenitor cells, and 10 percent of the embryos display exencephalic overgrowth of forebrain tissues [52]. Some homozygous mutants display anencephaly, possibly due to defects of neural tube closure [41]. Mutants also exhibit increased proliferation of neural progenitor cells in the lateral ventricle wall of the adult brain [244]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphA8

Chromosome location	Human: Chromosome 1: 22,890,057–22,930,087 forward strand Mouse: Chromosome 4: 136,485,334–136,512,731 reverse strand[a]
Gene size (bp)	Human: 40,031. Mouse: 27,398[a]
Intron/exon numbers	17 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—4,943; 5′ UTR—72; ORF—3,018; 3′ UTR—1,853 Mouse: mRNA—4,713; 5′ UTR—69; ORF –3,015; 3′ UTR—1,629[a]
Amino acid number	Human: 1,005. Mouse: 1,004[b]
kDa	Human: 111. Mouse: 111[b]
Posttranslational modifications	Tyrosine phosphorylation, N-glycosylation, ubiquitination[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	EphrinA2, –A3, –A5
Known dimerizing partners	EphA8
Pathways regulated	Activates MAPK, Rac, p100γ PI3-kinase, and integrin pathways
Tissues expressed	Specifically expressed in the central nervous system. First detected at E10.5 with high levels near the midline region of the tectum and to a lower extent in discrete regions of hindbrain, in the dorsal horn of the spinal cord, and in the naso-lacrimal groove. The expression decreases at E12.5 and is barely detectable at E17.5. Not detected at postnatal stages
Human Diseases	
Knockout Mouse phenotype	Mice are viable and fertile, and mostly normal, but exhibit a defect in midline guidance of commissural fibers connecting the superior colliculus with the contralateral inferior colliculus, which misproject into the ipsilateral spinal cord [77]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphA10

Chromosome location	Human: chromosome 1: 38,179,552–38,230,805; reverse strand Mouse: chromosome 4: 124,558,143–124,595,044; forward strand[a]
Gene size (bp)	Human: 51,254. Mouse: 36,902[a]
Intron/exon numbers	17 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—5,425; 5′ UTR—no information; ORF—3,027; 3′ UTR—2,398 Mouse: no information[a]
Amino acid number	Human: 1,008. Mouse: 1,007[b]
kDa	Human: 110[b]. Mouse: 109[b]
Posttranslational modifications	N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain (lacking in one isoform), PDZ-binding motif
Ligands	EphrinA1, –A2, –A3, –A4, –A5

(continued)

Known dimerizing partners	No information
Pathways regulated	No information
Tissues expressed	Testis
Human Diseases	No information
Knockout Mouse phenotype	No information

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphB1

Chromosome location	Human: chromosome 3: 134,514,104–134,979,309; forward strand Mouse: chromosome 9: 101,824,458–102,257,023; reverse strand[a]
Gene size (bp)	Human: 465,206. Mouse: 432,566[a]
Intron/exon numbers	16 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—4,672; 5′UTR—370; ORF—2,955; 3′UTR—1,347 Mouse: mRNA—4,667; 5′UTR—354; ORF—2,955; 3′UTR—1358[a]
Amino acid number	Human: 984. Mouse: 984[b]
kDa	Human: 110 Mouse: 110[b]
Posttranslational modifications	Phosphorylation, N-glycosylation, ubiquitination[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	EphrinB1, −B2, −B3
Known dimerizing partners	EphB1, EphB6 [373]
Pathways regulated	MAPK/ERK, c-Jun, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin
Tissues expressed	Preferentially expressed in the brain
Human diseases	Implemented in different cancers
Knockout Mouse phenotype	Reduction of the ipsilateral retinotectal projection [130]. Reduced neural progenitors in the hippocampus [248]. EphB1; EphB2; EphB3-triple knockout display reduced spine density in the hippocampus [169]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphB2

Chromosome location	Human: chromosome 1: 23,037,458–23,241,818; forward strand Mouse: chromosome 4: 136,203,454–136,391,903; reverse strand[a]
Gene size (bp)	Human: 204,361. Mouse: 188,450[a]
Intron/exon numbers	Human: 17 exons. Mouse: 16 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—4,641; 5′ UTR—18; ORF—3,168; 3′ UTR—1,455 Mouse: mRNA—4,804; 5′ UTR—126; ORF—2,964; 3′UTR—1,714[a]
Amino acid number	Human: 1,055. Mouse: 994[b]

(continued)

kDa	Human: 117, Mouse: 111[b]
Posttranslational modifications	Tyrosine phosphorylation, possibly serine/threonine phosphorylation[c], N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	ephrinB1, −B2, −B3, −A5, Reelin
Known dimerizing partners	EphB2, EphA3[325]
Pathways regulated	p110-PI3K, Rap1, RhoA, Rac1, Cdc42, Erk, ROCK-LIMK1-cofilin
Tissues expressed	Endothelial cells in the vascular system, epithelium of intestinal colonic crypt, thymus. Nervous system: ventral midbrain, diencephalon, developing hindbrain, amygdala, cerebellum, subventricular zone walls, retinotectal system, motor neurons. Neural crest cells, inner ear epithelium, skeletal muscles
Human Diseases	Colorectal cancer, breast cancer, Alzheimer's Disease, anxiety
Knockout Mouse phenotype	Defects in ventral midbrain development [405], axon guidance errors at the midline [71], defective development of corpus callosum, cleft palate [73], defects in synaptic functions in the hippocampus, LTP and LTD impairment [168], defective dendritic spine morphogenesis [169], vascular defects [144], defective inner ear morphogenesis and circling behavior [135], defects in the morphology of the pancreas, urorectal development [158], thymus development [406], disorganized cell sorting in the intestinal epithelium [26], plasticity of adult stem cells [250], increased proliferation of stem cells in the SVZ [245]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)
[c]Phosphosite (http://www.phosphosite.org)

Receptor at a glance: EphB3

Chromosome location	Human: chromosome 3: 184,279,572–184,300,197; forward strand Mouse: chromosome 16: 21,204,828–21,223,377; forward strand[a]
Gene size (bp)	Human: 20,626. Mouse: 18,550[a]
Intron/exon numbers	16 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—4,236; 5′ UTR—452; ORF—2997; 3′ UTR—787 Mouse: mRNA—4,185; 5′ UTR—415; ORF—2,982; 3′ UTR—788[a]
Amino acid number	Human: 998. Mouse: 993[b]
kDa	Human: 110. Mouse: 110[b]
Posttranslational modifications	Tyrosine phosphorylation, Serine/Threonine phosphorylation[c], N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	ephrinB1, −B2, −B3
Known dimerizing partners	EphB3, EphB2

(continued)

Pathways regulated	ADAM-10-E-cadherin, RhoA, Rac1/Cdc42, AF-6-Ras GTPase/ Disheveled-Daam1, Fyn/Src-Crk-rasGAP-Ras GTPase, p53-cell proliferation, Akt-cell survival, PICK1-PKC-α-D-serine synthesis
Tissues expressed	Developing hindbrain and ventral midbrain [405], basal nuclei in the striatum, stem cells in the SVZ, retinal ganglion cells during development, Paneth cells and stem cells in the small and large intestine, pharynx, salivary glands, thymus, neural crest cells, inner ear efferent fibers, developing skeletal elements, secondary palate, pancreatic epithelium, macrophages, vestibular epithelium
Human Diseases	Colorectal cancer, prostate cancer, ovarian cancer
Knockout Mouse phenotype	Midline guidance errors [74], proliferation defects of the adult stem cells in the SVZ [247], vascular defects[144], skeletal abnormalities[27], cell migration in the intestinal epithelium, cleft palate [416], defective pancreatic branching [159], disturbed morphogenesis and regeneration of the intestinal epithelium [26, 228, 411], defective thymus development [406], affected development of the urogenital system [158]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)
[c]Phosphosite (http://www.phosphosite.org)

Receptor at a glance: EphB4

Chromosome location	Human: chromosome 7: 100,400,187–100,425,121; reverse strand Mouse: chromosome 5: 137,791,337–137,819,897; forward strand[a]
Gene size (bp)	Human: 24,935. Mouse: 28,561[a]
Intron/exon numbers	17 exons[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—4,329; 5′ UTR—469; ORF—2,964; 3′ UTR—896 Mouse: mRNA—4,340; 5′ UTR—489; ORF—2,964; 3′ UTR—887[a]
Amino acid number	Human: 987. Mouse: 987[b]
kDa	Human: 108. Mouse: 109[b]
Posttranslational modifications	Tyrosine phosphorylation, N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	All ephrinBs, but ephrinB2 is preferred
Known dimerizing partners	EphB4, EphB6 [258]
Pathways regulated	PI3K/Akt, Abl/Crk, RhoA, Cdc42, Rac1
Tissues expressed	Placenta, kidney, liver, lung, breast, pancreas, skeletal and heart muscle, lymph vessels, venous epithelium. Low levels in fetal brain, not expressed in adult brain
Human Diseases	Colorectal cancer, breast cancer
Knockout Mouse phenotype	Defects in angiogenesis, leading to embryonic lethality [141]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Receptor at a glance: EphB6

Chromosome location	Human: chromosome 7: 142,552,792–142,568,847; forward strand Mouse: chromosome 6: 41,555,481–41,570,508; forward strand[a]
Gene size (bp)	Human: 16,056. Mouse: 15,028[a]
Intron/exon numbers	Human: 20. Mouse: 18[a]
mRNA size (5′, ORF, 3′)	Human: mRNA—4,043; 5′UTR—787; ORF—3,066; 3′UTR—190 Mouse: mRNA—3,762; 5′UTR—512; ORF—3,045; 3′UTR—205[a]
Amino acid number	Human: 1,021. Mouse: 1,014[b]
kDa	Human: 111. Mouse: 110[b]
Posttranslational modifications	Tyrosine phosphorylation, N-glycosylation[b]
Domains	N-terminal ligand-binding domain (LBD), cysteine-rich region, two fibronectin type III domains (FN1 and FN2), single transmembrane helix, juxtamembrane region, inactive tyrosine kinase domain, sterile-α motif (SAM) domain, PDZ-binding motif
Ligands	ephrinB1, –B2
Known dimerizing partners	EphB6, EphB1, EphB4 [258, 373]
Pathways regulated	Activates c-Cbl/Abl pathway, inhibits JNK pathway
Tissues expressed	Brain, noninvasive breast carcinoma cell lines, pancreas
Human Diseases	Non-small cell lung cancer
Knockout Mouse phenotype	Compromised T-cell function, including proliferation and secretion, and reduced severity of experimental autoimmune encephalitis (EAE) when stimulated by $MOG_{33–55}$ [219]

[a]Ensembl (http://www.ensembl.org)
[b]UniProt (http://www.uniprot.org)

Notes

1. Ensembl (http://www.ensembl.org)
2. UniProt (http://www.uniprot.org)
3. Phosphosite (http://www.phosphosite.org)

References

1. Egea J, Klein R. Bidirectional Eph-ephrin signaling during axon guidance. Trends Cell Biol. 2007;17(5):230–8.
2. Labrador JP, Brambilla R, Klein R. The N-terminal globular domain of Eph receptors is sufficient for ligand binding and receptor signaling. EMBO J. 1997;16(13):3889–97.
3. Bowden TA, Aricescu AR, Nettleship JE, Siebold C, Rahman-Huq N, Owens RJ, et al. Structural plasticity of eph receptor A4 facilitates cross-class ephrin signaling. Structure. 2009;17(10):1386–97.
4. Himanen JP, Rajashankar KR, Lackmann M, Cowan CA, Henkemeyer M, Nikolov DB. Crystal structure of an Eph receptor-ephrin complex. Nature. 2001;414(6866):933–8.

5. Himanen JP, Chumley MJ, Lackmann M, Li C, Barton WA, Jeffrey PD, et al. Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. Nat Neurosci. 2004;7(5):501–9.
6. Flanagan JG, Vanderhaeghen P. The ephrins and Eph receptors in neural development. Annu Rev Neurosci. 1998;21:309–45.
7. Wilkinson DG. Multiple roles of EPH receptors and ephrins in neural development. Nat Rev Neurosci. 2001;2(3):155–64.
8. Kullander K, Klein R. Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol. 2002;3(7):475–86.
9. Davy A, Gale NW, Murray EW, Klinghoffer RA, Soriano P, Feuerstein C, et al. Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. Genes Dev. 1999;13(23):3125–35.
10. Davy A, Robbins SM. Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner. EMBO J. 2000;19(20):5396–405.
11. Holen HL, Shadidi M, Narvhus K, Kjosnes O, Tierens A, Aasheim HC. Signaling through ephrin-A ligand leads to activation of Src-family kinases, Akt phosphorylation, and inhibition of antigen receptor-induced apoptosis. J Leukoc Biol. 2008;84(4):1183–91.
12. Lim YS, McLaughlin T, Sung TC, Santiago A, Lee KF, O'Leary DD. p75(NTR) mediates ephrin-A reverse signaling required for axon repulsion and mapping. Neuron. 2008;59(5): 746–58.
13. Marler KJ, Becker-Barroso E, Martinez A, Llovera M, Wentzel C, Poopalasundaram S, et al. A TrkB/EphrinA interaction controls retinal axon branching and synaptogenesis. J Neurosci. 2008;28(48):12700–12.
14. Bonanomi D, Chivatakarn O, Bai G, Abdesselem H, Lettieri K, Marquardt T, et al. Ret is a multifunctional coreceptor that integrates diffusible- and contact-axon guidance signals. Cell. 2012;148(3):568–82.
15. Smith FM, Vearing C, Lackmann M, Treutlein H, Himanen J, Chen K, et al. Dissecting the EphA3/Ephrin-A5 interactions using a novel functional mutagenesis screen. J Biol Chem. 2004;279(10):9522–31.
16. Vearing CJ, Lackmann M. Eph receptor signalling; dimerisation just isn't enough. Growth Factors. 2005;23(1):67–76.
17. Schaupp A, Sabet O, Dudanova I, Ponserre M, Bastiaens P, Klein R. The composition of EphB2 clusters determines the strength in the cellular repulsion response. J Cell Biol. 2014;204(3):409–22.
18. Hornberger MR, Dutting D, Ciossek T, Yamada T, Handwerker C, Lang S, et al. Modulation of EphA receptor function by coexpressed ephrinA ligands on retinal ganglion cell axons. Neuron. 1999;22(4):731–42.
19. Carvalho RF, Beutler M, Marler KJ, Knoll B, Becker-Barroso E, Heintzmann R, et al. Silencing of EphA3 through a cis interaction with ephrinA5. Nat Neurosci. 2006;9(3):322–30.
20. Flanagan JG. Neural map specification by gradients. Curr Opin Neurobiol. 2006;16(1): 59–66.
21. Lin KT, Sloniowski S, Ethell DW, Ethell IM. Ephrin-B2-induced cleavage of EphB2 receptor is mediated by matrix metalloproteinases to trigger cell repulsion. J Biol Chem. 2008; 283(43):28969–79.
22. Inoue E, Deguchi-Tawarada M, Togawa A, Matsui C, Arita K, Katahira-Tayama S, et al. Synaptic activity prompts gamma-secretase-mediated cleavage of EphA4 and dendritic spine formation. J Cell Biol. 2009;185(3):551–64.
23. Attwood BK, Bourgognon JM, Patel S, Mucha M, Schiavon E, Skrzypiec AE, et al. Neuropsin cleaves EphB2 in the amygdala to control anxiety. Nature. 2011;473(7347):372–5.
24. Sugiyama N, Gucciardo E, Tatti O, Varjosalo M, Hyytiainen M, Gstaiger M, et al. EphA2 cleavage by MT1-MMP triggers single cancer cell invasion via homotypic cell repulsion. J Cell Biol. 2013;201(3):467–84.
25. Gatto G, Morales D, Kania A, Klein R. EphA4 receptor shedding regulates spinal motor axon guidance. Curr Biol. 2014;24:2355–65.

26. Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, et al. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell. 2002;111(2):251–63.
27. Compagni A, Logan M, Klein R, Adams RH. Control of skeletal patterning by ephrinB1-EphB interactions. Dev Cell. 2003;5(2):217–30.
28. Davy A, Aubin J, Soriano P. Ephrin-B1 forward and reverse signaling are required during mouse development. Genes Dev. 2004;18(5):572–83.
29. Jorgensen C, Sherman A, Chen GI, Pasculescu A, Poliakov A, Hsiung M, et al. Cell-specific information processing in segregating populations of Eph receptor ephrin-expressing cells. Science. 2009;326(5959):1502–9.
30. Passante L, Gaspard N, Degraeve M, Frisen J, Kullander K, De Maertelaer V, et al. Temporal regulation of ephrin/Eph signalling is required for the spatial patterning of the mammalian striatum. Development. 2008;135(19):3281–90.
31. Xu Q, Mellitzer G, Robinson V, Wilkinson DG. In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. Nature. 1999;399(6733):267–71.
32. Park EC, Cho GS, Kim GH, Choi SC, Han JK. The involvement of Eph-Ephrin signaling in tissue separation and convergence during Xenopus gastrulation movements. Dev Biol. 2011;350(2):441–50.
33. Kida YS, Sato T, Miyasaka KY, Suto A, Ogura T. Daam1 regulates the endocytosis of EphB during the convergent extension of the zebrafish notochord. Proc Natl Acad Sci USA. 2007;104(16):6708–13.
34. Rohani N, Canty L, Luu O, Fagotto F, Winklbauer R. EphrinB/EphB signaling controls embryonic germ layer separation by contact-induced cell detachment. PLoS Biol. 2011;9(3):e1000597.
35. Fagotto F, Rohani N, Touret AS, Li R. A molecular base for cell sorting at embryonic boundaries: contact inhibition of cadherin adhesion by ephrin/ Eph-dependent contractility. Dev Cell. 2013;27(1):72–87.
36. Barrios A, Poole RJ, Durbin L, Brennan C, Holder N, Wilson SW. Eph/Ephrin signaling regulates the mesenchymal-to-epithelial transition of the paraxial mesoderm during somite morphogenesis. Curr Biol. 2003;13(18):1571–82.
37. Durbin L, Brennan C, Shiomi K, Cooke J, Barrios A, Shanmugalingam S, et al. Eph signaling is required for segmentation and differentiation of the somites. Genes Dev. 1998; 12(19):3096–109.
38. Schmidt C, Christ B, Maden M, Brand-Saberi B, Patel K. Regulation of Epha4 expression in paraxial and lateral plate mesoderm by ectoderm-derived signals. Dev Dyn. 2001;220(4): 377–86.
39. Watanabe T, Sato Y, Saito D, Tadokoro R, Takahashi Y. EphrinB2 coordinates the formation of a morphological boundary and cell epithelialization during somite segmentation. Proc Natl Acad Sci USA. 2009;106(18):7467–72.
40. Julich D, Mould AP, Koper E, Holley SA. Control of extracellular matrix assembly along tissue boundaries via Integrin and Eph/Ephrin signaling. Development. 2009;136(17):2913–21.
41. Holmberg J, Clarke DL, Frisen J. Regulation of repulsion versus adhesion by different splice forms of an Eph receptor. Nature. 2000;408(6809):203–6.
42. Becker N, Seitanidou T, Murphy P, Mattei MG, Topilko P, Nieto MA, et al. Several receptor tyrosine kinase genes of the Eph family are segmentally expressed in the developing hindbrain. Mech Dev. 1994;47(1):3–17.
43. Nieto MA, Gilardi-Hebenstreit P, Charnay P, Wilkinson DG. A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. Development. 1992;116(4):1137–50.
44. Flenniken AM, Gale NW, Yancopoulos GD, Wilkinson DG. Distinct and overlapping expression patterns of ligands for Eph-related receptor tyrosine kinases during mouse embryogenesis. Dev Biol. 1996;179(2):382–401.
45. Gale NW, Flenniken A, Compton DC, Jenkins N, Copeland NG, Gilbert DJ, et al. Elk-L3, a novel transmembrane ligand for the Eph family of receptor tyrosine kinases, expressed in embryonic floor plate, roof plate and hindbrain segments. Oncogene. 1996;13(6):1343–52.

46. Xu Q, Alldus G, Holder N, Wilkinson DG. Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. Development. 1995;121(12):4005–16.
47. Cooke JE, Kemp HA, Moens CB. EphA4 is required for cell adhesion and rhombomere-boundary formation in the zebrafish. Curr Biol. 2005;15(6):536–42.
48. Xu Q, Alldus G, Macdonald R, Wilkinson DG, Holder N. Function of the Eph-related kinase rtk1 in patterning of the zebrafish forebrain. Nature. 1996;381(6580):319–22.
49. Smith A, Robinson V, Patel K, Wilkinson DG. The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. Curr Biol. 1997;7(8):561–70.
50. Krull CE, Lansford R, Gale NW, Collazo A, Marcelle C, Yancopoulos GD, et al. Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. Curr Biol. 1997;7(8):571–80.
51. Wang HU, Anderson DJ. Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. Neuron. 1997;18(3):383–96.
52. Depaepe V, Suarez-Gonzalez N, Dufour A, Passante L, Gorski JA, Jones KR, et al. Ephrin signalling controls brain size by regulating apoptosis of neural progenitors. Nature. 2005; 435(7046):1244–50.
53. North HA, Zhao X, Kolk SM, Clifford MA, Ziskind DM, Donoghue MJ. Promotion of proliferation in the developing cerebral cortex by EphA4 forward signaling. Development. 2009;136(14):2467–76.
54. Villar-Cervino V, Molano-Mazon M, Catchpole T, Valdeolmillos M, Henkemeyer M, Martinez LM, et al. Contact repulsion controls the dispersion and final distribution of Cajal-Retzius cells. Neuron. 2013;77(3):457–71.
55. Bouche E, Romero-Ortega MI, Henkemeyer M, Catchpole T, Leemhuis J, Frotscher M, et al. Reelin induces EphB activation. Cell Res. 2013;23(4):473–90.
56. Senturk A, Pfennig S, Weiss A, Burk K, Acker-Palmer A. Ephrin Bs are essential components of the Reelin pathway to regulate neuronal migration. Nature. 2011;472(7343): 356–60.
57. Torii M, Hashimoto-Torii K, Levitt P, Rakic P. Integration of neuronal clones in the radial cortical columns by EphA and ephrin-A signalling. Nature. 2009;461(7263):524–8.
58. Dimidschstein J, Passante L, Dufour A, van den Ameele J, Tiberi L, Hrechdakian T, et al. Ephrin-B1 controls the columnar distribution of cortical pyramidal neurons by restricting their tangential migration. Neuron. 2013;79(6):1123–35.
59. Vanderhaeghen P, Lu Q, Prakash N, Frisen J, Walsh CA, Frostig RD, et al. A mapping label required for normal scale of body representation in the cortex. Nat Neurosci. 2000; 3(4):358–65.
60. Uziel D, Muhlfriedel S, Zarbalis K, Wurst W, Levitt P, Bolz J. Miswiring of limbic thalamocortical projections in the absence of ephrin-A5. J Neurosci. 2002;22(21):9352–7.
61. Dufour A, Seibt J, Passante L, Depaepe V, Ciossek T, Frisen J, et al. Area specificity and topography of thalamocortical projections are controlled by ephrin/Eph genes. Neuron. 2003;39(3):453–65.
62. Prakash N, Vanderhaeghen P, Cohen-Cory S, Frisen J, Flanagan JG, Frostig RD. Malformation of the functional organization of somatosensory cortex in adult ephrin-A5 knock-out mice revealed by in vivo functional imaging. J Neurosci. 2000;20(15):5841–7.
63. Egea J, Nissen UV, Dufour A, Sahin M, Greer P, Kullander K, et al. Regulation of EphA 4 kinase activity is required for a subset of axon guidance decisions suggesting a key role for receptor clustering in Eph function. Neuron. 2005;47(4):515–28.
64. Dufour A, Egea J, Kullander K, Klein R, Vanderhaeghen P. Genetic analysis of EphA-dependent signaling mechanisms controlling topographic mapping in vivo. Development. 2006;133(22):4415–20.
65. Torii M, Levitt P. Dissociation of corticothalamic and thalamocortical axon targeting by an EphA7-mediated mechanism. Neuron. 2005;48(4):563–75.

66. Robichaux MA, Chenaux G, Ho HY, Soskis MJ, Dravis C, Kwan KY, et al. EphB receptor forward signaling regulates area-specific reciprocal thalamic and cortical axon pathfinding. Proc Natl Acad Sci USA. 2014;111(6):2188–93.
67. Gao PP, Zhang JH, Yokoyama M, Racey B, Dreyfus CF, Black IB, et al. Regulation of topographic projection in the brain: Elf-1 in the hippocamposeptal system. Proc Natl Acad Sci USA. 1996;93(20):11161–6.
68. Zhang JH, Cerretti DP, Yu T, Flanagan JG, Zhou R. Detection of ligands in regions anatomically connected to neurons expressing the Eph receptor Bsk: potential roles in neuron-target interaction. J Neurosci. 1996;16(22):7182–92.
69. Yue Y, Chen ZY, Gale NW, Blair-Flynn J, Hu TJ, Yue X, et al. Mistargeting hippocampal axons by expression of a truncated Eph receptor. Proc Natl Acad Sci USA. 2002;99(16):10777–82.
70. Kullander K, Mather NK, Diella F, Dottori M, Boyd AW, Klein R. Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. Neuron. 2001;29(1):73–84.
71. Henkemeyer M, Orioli D, Henderson JT, Saxton TM, Roder J, Pawson T, et al. Nuk controls pathfinding of commissural axons in the mammalian central nervous system. Cell. 1996;86(1):35–46.
72. Ho SK, Kovacevic N, Henkelman RM, Boyd A, Pawson T, Henderson JT. EphB2 and EphA4 receptors regulate formation of the principal inter-hemispheric tracts of the mammalian forebrain. Neuroscience. 2009;160(4):784–95.
73. Orioli D, Henkemeyer M, Lemke G, Klein R, Pawson T. Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. EMBO J. 1996;15(22): 6035–49.
74. Mendes SW, Henkemeyer M, Liebl DJ. Multiple Eph receptors and B-class ephrins regulate midline crossing of corpus callosum fibers in the developing mouse forebrain. J Neurosci. 2006;26(3):882–92.
75. Hu Z, Yue X, Shi G, Yue Y, Crockett DP, Blair-Flynn J, et al. Corpus callosum deficiency in transgenic mice expressing a truncated ephrin-A receptor. J Neurosci. 2003;23(34):10963–70.
76. Nishikimi M, Oishi K, Tabata H, Torii K, Nakajima K. Segregation and pathfinding of callosal axons through EphA3 signaling. J Neurosci. 2011;31(45):16251–60.
77. Park S, Frisen J, Barbacid M. Aberrant axonal projections in mice lacking EphA8 (Eek) tyrosine protein kinase receptors. EMBO J. 1997;16(11):3106–14.
78. Dottori M, Hartley L, Galea M, Paxinos G, Polizzotto M, Kilpatrick T, et al. EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract. Proc Natl Acad Sci USA. 1998;95(22):13248–53.
79. Canty AJ, Greferath U, Turnley AM, Murphy M. Eph tyrosine kinase receptor EphA4 is required for the topographic mapping of the corticospinal tract. Proc Natl Acad Sci USA. 2006;103(42):15629–34.
80. Paixao S, Balijepalli A, Serradj N, Niu J, Luo W, Martin JH, et al. EphrinB3/EphA4-mediated guidance of ascending and descending spinal tracts. Neuron. 2013;80(6):1407–20.
81. Kullander K, Croll SD, Zimmer M, Pan L, McClain J, Hughes V, et al. Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. Genes Dev. 2001;15(7):877–88.
82. Kullander K, Butt SJ, Lebret JM, Lundfald L, Restrepo CE, Rydstrom A, et al. Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. Science. 2003; 299(5614):1889–92.
83. Butt SJ, Lundfald L, Kiehn O. EphA4 defines a class of excitatory locomotor-related interneurons. Proc Natl Acad Sci USA. 2005;102(39):14098–103.
84. Restrepo CE, Margaryan G, Borgius L, Lundfald L, Sargsyan D, Kiehn O. Change in the balance of excitatory and inhibitory midline fiber crossing as an explanation for the hopping phenotype in EphA4 knockout mice. Eur J Neurosci. 2011;34(7):1102–12.
85. Iwasato T, Katoh H, Nishimaru H, Ishikawa Y, Inoue H, Saito YM, et al. Rac-GAP alpha-chimerin regulates motor-circuit formation as a key mediator of EphrinB3/EphA4 forward signaling. Cell. 2007;130(4):742–53.

86. Wegmeyer H, Egea J, Rabe N, Gezelius H, Filosa A, Enjin A, et al. EphA4-dependent axon guidance is mediated by the RacGAP alpha2-chimaerin. Neuron. 2007;55(5):756–67.
87. Beg AA, Sommer JE, Martin JH, Scheiffele P. Alpha2-Chimaerin is an essential EphA4 effector in the assembly of neuronal locomotor circuits. Neuron. 2007;55(5):768–78.
88. Shi L, Fu WY, Hung KW, Porchetta C, Hall C, Fu AK, et al. Alpha2-chimaerin interacts with EphA4 and regulates EphA4-dependent growth cone collapse. Proc Natl Acad Sci USA. 2007;104(41):16347–52.
89. Fawcett JP, Georgiou J, Ruston J, Bladt F, Sherman A, Warner N, et al. Nck adaptor proteins control the organization of neuronal circuits important for walking. Proc Natl Acad Sci USA. 2007;104(52):20973–8.
90. Iwamasa H, Ohta K, Yamada T, Ushijima K, Terasaki H, Tanaka H. Expression of Eph receptor tyrosine kinases and their ligands in chick embryonic motor neurons and hindlimb muscles. Dev Growth Differ. 1999;41(6):685–98.
91. Eberhart J, Swartz M, Koblar SA, Pasquale EB, Tanaka H, Krull CE. Expression of EphA4, ephrin-A2 and ephrin-A5 during axon outgrowth to the hindlimb indicates potential roles in pathfinding. Dev Neurosci. 2000;22(3):237–50.
92. Helmbacher F, Schneider-Maunoury S, Topilko P, Tiret L, Charnay P. Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. Development. 2000;127(15):3313–24.
93. Kania A, Jessell TM. Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A:EphA interactions. Neuron. 2003;38(4):581–96.
94. Luria V, Krawchuk D, Jessell TM, Laufer E, Kania A. Specification of motor axon trajectory by ephrin-B:EphB signaling: symmetrical control of axonal patterning in the developing limb. Neuron. 2008;60(6):1039–53.
95. Kao TJ, Palmesino E, Kania A. SRC family kinases are required for limb trajectory selection by spinal motor axons. J Neurosci. 2009;29(17):5690–700.
96. Marquardt T, Shirasaki R, Ghosh S, Andrews SE, Carter N, Hunter T, et al. Coexpressed EphA receptors and ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains. Cell. 2005;121(1):127–39.
97. Kao TJ, Kania A. Ephrin-mediated cis-attenuation of Eph receptor signaling is essential for spinal motor axon guidance. Neuron. 2011;71(1):76–91.
98. Dudanova I et al. Genetic evidence for a contribution of EphA:ephrinA reverse signaling to motor axon guidance. J Neurosci. 2012;32:5209–15.
99. Feng G, Laskowski MB, Feldheim DA, Wang H, Lewis R, Frisen J, et al. Roles for ephrins in positionally selective synaptogenesis between motor neurons and muscle fibers. Neuron. 2000;25(2):295–306.
100. Gallarda BW, Bonanomi D, Muller D, Brown A, Alaynick WA, Andrews SE, et al. Segregation of axial motor and sensory pathways via heterotypic trans-axonal signaling. Science. 2008;320(5873):233–6.
101. Wang L, Klein R, Zheng B, Marquardt T. Anatomical coupling of sensory and motor nerve trajectory via axon tracking. Neuron. 2011;71(2):263–77.
102. Brown A, Yates PA, Burrola P, Ortuno D, Vaidya A, Jessell TM, et al. Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling. Cell. 2000;102(1):77–88.
103. Feldheim DA, Vanderhaeghen P, Hansen MJ, Frisen J, Lu Q, Barbacid M, et al. Topographic guidance labels in a sensory projection to the forebrain. Neuron. 1998;21(6):1303–13.
104. Feldheim DA, Kim YI, Bergemann AD, Frisen J, Barbacid M, Flanagan JG. Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. Neuron. 2000;25(3):563–74.
105. Cheng HJ, Nakamoto M, Bergemann AD, Flanagan JG. Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell. 1995;82(3):371–81.
106. Monschau B, Kremoser C, Ohta K, Tanaka H, Kaneko T, Yamada T, et al. Shared and distinct functions of RAGS and ELF-1 in guiding retinal axons. EMBO J. 1997;16(6):1258–67.

107. Connor RJ, Menzel P, Pasquale EB. Expression and tyrosine phosphorylation of Eph receptors suggest multiple mechanisms in patterning of the visual system. Dev Biol. 1998;193(1):21–35.
108. Drescher U, Kremoser C, Handwerker C, Loschinger J, Noda M, Bonhoeffer F. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. Cell. 1995;82(3):359–70.
109. Frisen J, Yates PA, McLaughlin T, Friedman GC, O'Leary DD, Barbacid M. Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system. Neuron. 1998;20(2):235–43.
110. Walter J, Henke-Fahle S, Bonhoeffer F. Avoidance of posterior tectal membranes by temporal retinal axons. Development. 1987;101(4):909–13.
111. Nakamoto M, Cheng HJ, Friedman GC, McLaughlin T, Hansen MJ, Yoon CH, et al. Topographically specific effects of ELF-1 on retinal axon guidance in vitro and retinal axon mapping in vivo. Cell. 1996;86(5):755–66.
112. Pfeiffenberger C, Yamada J, Feldheim DA. Ephrin-As and patterned retinal activity act together in the development of topographic maps in the primary visual system. J Neurosci. 2006;26(50):12873–84.
113. Feldheim DA, Nakamoto M, Osterfield M, Gale NW, DeChiara TM, Rohatgi R, et al. Loss-of-function analysis of EphA receptors in retinotectal mapping. J Neurosci. 2004;24(10): 2542–50.
114. Carreres MI, Escalante A, Murillo B, Chauvin G, Gaspar P, Vegar C, et al. Transcription factor Foxd1 is required for the specification of the temporal retina in mammals. J Neurosci. 2011;31(15):5673–81.
115. Nie D, Di Nardo A, Han JM, Baharanyi H, Kramvis I, Huynh T, et al. Tsc2-Rheb signaling regulates EphA-mediated axon guidance. Nat Neurosci. 2010;13(2):163–72.
116. Reber M, Burrola P, Lemke G. A relative signalling model for the formation of a topographic neural map. Nature. 2004;431(7010):847–53.
117. Bevins N, Lemke G, Reber M. Genetic dissection of EphA receptor signaling dynamics during retinotopic mapping. J Neurosci. 2011;31(28):10302–10.
118. Marcus RC, Gale NW, Morrison ME, Mason CA, Yancopoulos GD. Eph family receptors and their ligands distribute in opposing gradients in the developing mouse retina. Dev Biol. 1996;180(2):786–9.
119. Holash JA, Soans C, Chong LD, Shao H, Dixit VM, Pasquale EB. Reciprocal expression of the Eph receptor Cek5 and its ligand(s) in the early retina. Dev Biol. 1997;182(2):256–69.
120. Rashid T, Upton AL, Blentic A, Ciossek T, Knoll B, Thompson ID, et al. Opposing gradients of ephrin-As and EphA7 in the superior colliculus are essential for topographic mapping in the mammalian visual system. Neuron. 2005;47(1):57–69.
121. Suetterlin P, Drescher U. Target-independent ephrinA/EphA-mediated axon-axon repulsion as a novel element in retinocollicular mapping. Neuron. 2014;84:740–52.
122. Yoo S, Kim Y, Noh H, Lee H, Park E, Park S. Endocytosis of EphA receptors is essential for the proper development of the retinocollicular topographic map. EMBO J. 2011;30(8): 1593–607.
123. Hindges R, McLaughlin T, Genoud N, Henkemeyer M, O'Leary DD. EphB forward signaling controls directional branch extension and arborization required for dorsal-ventral retinotopic mapping. Neuron. 2002;35(3):475–87.
124. Braisted JE, McLaughlin T, Wang HU, Friedman GC, Anderson DJ, O'Leary DD. Graded and lamina-specific distributions of ligands of EphB receptor tyrosine kinases in the developing retinotectal system. Dev Biol. 1997;191(1):14–28.
125. Birgbauer E, Cowan CA, Sretavan DW, Henkemeyer M. Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. Development. 2000;127(6):1231–41.
126. Mann F, Ray S, Harris W, Holt C. Topographic mapping in dorsoventral axis of the Xenopus retinotectal system depends on signaling through ephrin-B ligands. Neuron. 2002;35(3): 461–73.

127. Thakar S, Chenaux G, Henkemeyer M. Critical roles for EphB and ephrin-B bidirectional signalling in retinocollicular mapping. Nat Commun. 2011;2:431.
128. McLaughlin T, Hindges R, Yates PA, O'Leary DD. Bifunctional action of ephrin-B1 as a repellent and attractant to control bidirectional branch extension in dorsal-ventral retinotopic mapping. Development. 2003;130(11):2407–18.
129. Lambot MA, Depasse F, Noel JC, Vanderhaeghen P. Mapping labels in the human developing visual system and the evolution of binocular vision. J Neurosci. 2005;25(31):7232–7.
130. Williams SE, Mann F, Erskine L, Sakurai T, Wei S, Rossi DJ, et al. Ephrin-B2 and EphB1 mediate retinal axon divergence at the optic chiasm. Neuron. 2003;39(6):919–35.
131. Nakagawa S, Brennan C, Johnson KG, Shewan D, Harris WA, Holt CE. Ephrin-B regulates the Ipsilateral routing of retinal axons at the optic chiasm. Neuron. 2000;25(3):599–610.
132. Pak W, Hindges R, Lim YS, Pfaff SL, O'Leary DD. Magnitude of binocular vision controlled by islet-2 repression of a genetic program that specifies laterality of retinal axon pathfinding. Cell. 2004;119(4):567–78.
133. Herrera E, Marcus R, Li S, Williams SE, Erskine L, Lai E, et al. Foxd1 is required for proper formation of the optic chiasm. Development. 2004;131(22):5727–39.
134. Cowan CW, Shao YR, Sahin M, Shamah SM, Lin MZ, Greer PL, et al. Vav family GEFs link activated Ephs to endocytosis and axon guidance. Neuron. 2005;46(2):205–17.
135. Cowan CA, Yokoyama N, Bianchi LM, Henkemeyer M, Fritzsch B. EphB2 guides axons at the midline and is necessary for normal vestibular function. Neuron. 2000;26(2):417–30.
136. Coate TM, Raft S, Zhao X, Ryan AK, Crenshaw 3rd EB, Kelley MW. Otic mesenchyme cells regulate spiral ganglion axon fasciculation through a Pou3f4/EphA4 signaling pathway. Neuron. 2012;73(1):49–63.
137. Defourny J, Poirrier AL, Lallemend F, Mateo Sanchez S, Neef J, Vanderhaeghen P, et al. Ephrin-A5/EphA4 signalling controls specific afferent targeting to cochlear hair cells. Nat Commun. 2013;4:1438.
138. Cutforth T, Moring L, Mendelsohn M, Nemes A, Shah NM, Kim MM, et al. Axonal ephrin-As and odorant receptors: coordinate determination of the olfactory sensory map. Cell. 2003;114(3):311–22.
139. Knoll B, Zarbalis K, Wurst W, Drescher U. A role for the EphA family in the topographic targeting of vomeronasal axons. Development. 2001;128(6):895–906.
140. Serizawa S, Miyamichi K, Takeuchi H, Yamagishi Y, Suzuki M, Sakano H. A neuronal identity code for the odorant receptor-specific and activity-dependent axon sorting. Cell. 2006;127(5):1057–69.
141. Gerety SS, Wang HU, Chen ZF, Anderson DJ. Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Mol Cell. 1999;4(3):403–14.
142. Pitulescu ME, Adams RH. Eph/ephrin molecules—a hub for signaling and endocytosis. Genes Dev. 2010;24(22):2480–92.
143. Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell. 1998;93(5):741–53.
144. Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, et al. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev. 1999;13(3):295–306.
145. Salvucci O, de la Luz SM, Martina JA, McCormick PJ, Tosato G. EphB2 and EphB4 receptors forward signaling promotes SDF-1-induced endothelial cell chemotaxis and branching remodeling. Blood. 2006;108(9):2914–22.
146. Herbert SP, Huisken J, Kim TN, Feldman ME, Houseman BT, Wang RA, et al. Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. Science. 2009;326(5950):294–8.
147. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol. 2007;8(6):464–78.

148. Makinen T, Adams RH, Bailey J, Lu Q, Ziemiecki A, Alitalo K, et al. PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. Genes Dev. 2005; 19(3):397–410.
149. Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, et al. Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature. 2010;465(7297):487–91.
150. Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, et al. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature. 2010; 465(7297):483–6.
151. Okazaki T, Ni A, Baluk P, Ayeni OA, Kearley J, Coyle AJ, et al. Capillary defects and exaggerated inflammatory response in the airways of EphA2-deficient mice. Am J Pathol. 2009;174(6):2388–99.
152. Stephen LJ, Fawkes AL, Verhoeve A, Lemke G, Brown A. A critical role for the EphA3 receptor tyrosine kinase in heart development. Dev Biol. 2007;302(1):66–79.
153. Goldshmit Y, Galea MP, Bartlett PF, Turnley AM. EphA4 regulates central nervous system vascular formation. J Comp Neurol. 2006;497(6):864–75.
154. Twigg SR, Kan R, Babbs C, Bochukova EG, Robertson SP, Wall SA, et al. Mutations of ephrin-B1 (EFNB1), a marker of tissue boundary formation, cause craniofrontonasal syndrome. Proc Natl Acad Sci USA. 2004;101(23):8652–7.
155. Halford MM, Armes J, Buchert M, Meskenaite V, Grail D, Hibbs ML, et al. Ryk-deficient mice exhibit craniofacial defects associated with perturbed Eph receptor crosstalk. Nat Genet. 2000;25(4):414–8.
156. Trivier E, Ganesan TS. RYK, a catalytically inactive receptor tyrosine kinase, associates with EphB2 and EphB3 but does not interact with AF-6. J Biol Chem. 2002;277(25):23037–43.
157. Dravis C, Henkemeyer M. Ephrin-B reverse signaling controls septation events at the embryonic midline through separate tyrosine phosphorylation-independent signaling avenues. Dev Biol. 2011;355(1):138–51.
158. Dravis C, Yokoyama N, Chumley MJ, Cowan CA, Silvany RE, Shay J, et al. Bidirectional signaling mediated by ephrin-B2 and EphB2 controls urorectal development. Dev Biol. 2004;271(2):272–90.
159. Villasenor A, Chong DC, Henkemeyer M, Cleaver O. Epithelial dynamics of pancreatic branching morphogenesis. Development. 2010;137(24):4295–305.
160. Muñoz JJ, Alfaro D, García-Ceca J, Alonso-C LM, Jiménez E, Zapata A. Thymic alterations in EphA4-deficient mice. J Immunol. 2006;177(2):804–13.
161. Andersson L, Westerlund J, Liang S, Carlsson T, Amendola E, Fagman H, et al. Role of EphA4 receptor signaling in thyroid development: regulation of folliculogenesis and propagation of the C-cell lineage. Endocrinology. 2011;152(3):1154–64.
162. Dalva MB, Takasu MA, Lin MZ, Shamah SM, Hu L, Gale NW, et al. EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell. 2000;103(6): 945–56.
163. Nolt MJ, Lin Y, Hruska M, Murphy J, Sheffler-Colins SI, Kayser MS, et al. EphB controls NMDA receptor function and synaptic targeting in a subunit-specific manner. J Neurosci. 2011;31(14):5353–64.
164. Takasu MA, Dalva MB, Zigmond RE, Greenberg ME. Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. Science. 2002; 295(5554):491–5.
165. Kayser MS, McClelland AC, Hughes EG, Dalva MB. Intracellular and trans-synaptic regulation of glutamatergic synaptogenesis by EphB receptors. J Neurosci. 2006;26(47): 12152–64.
166. Kayser MS, Nolt MJ, Dalva MB. EphB receptors couple dendritic filopodia motility to synapse formation. Neuron. 2008;59(1):56–69.
167. Henderson JT, Georgiou J, Jia Z, Robertson J, Elowe S, Roder JC, et al. The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function. Neuron. 2001;32(6): 1041–56.

168. Grunwald IC, Korte M, Wolfer D, Wilkinson GA, Unsicker K, Lipp HP, et al. Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity. Neuron. 2001;32(6):1027–40.
169. Henkemeyer M, Itkis OS, Ngo M, Hickmott PW, Ethell IM. Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus. J Cell Biol. 2003;163(6):1313–26.
170. Penzes P, Beeser A, Chernoff J, Schiller MR, Eipper BA, Mains RE, et al. Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. Neuron. 2003;37(2):263–74.
171. Tolias KF, Bikoff JB, Kane CG, Tolias CS, Hu L, Greenberg ME. The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development. Proc Natl Acad Sci USA. 2007;104(17):7265–70.
172. Irie F, Yamaguchi Y. EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP. Nat Neurosci. 2002;5(11):1117–8.
173. Margolis SS, Salogiannis J, Lipton DM, Mandel-Brehm C, Wills ZP, Mardinly AR, et al. EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. Cell. 2010;143(3):442–55.
174. Ethell IM, Irie F, Kalo MS, Couchman JR, Pasquale EB, Yamaguchi Y. EphB/syndecan-2 signaling in dendritic spine morphogenesis. Neuron. 2001;31(6):1001–13.
175. Soskis MJ, Ho HY, Bloodgood BL, Robichaux MA, Malik AN, Ataman B, et al. A chemical genetic approach reveals distinct EphB signaling mechanisms during brain development. Nat Neurosci. 2012;15(12):1645–54.
176. Carmona MA, Murai KK, Wang L, Roberts AJ, Pasquale EB. Glial ephrin-A3 regulates hippocampal dendritic spine morphology and glutamate transport. Proc Natl Acad Sci USA. 2009;106(30):12524–9.
177. Murai KK, Nguyen LN, Irie F, Yamaguchi Y, Pasquale EB. Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling. Nat Neurosci. 2003;6(2):153–60.
178. Fu WY, Chen Y, Sahin M, Zhao XS, Shi L, Bikoff JB, et al. Cdk5 regulates EphA4-mediated dendritic spine retraction through an ephexin1-dependent mechanism. Nat Neurosci. 2007; 10(1):67–76.
179. Richter M, Murai KK, Bourgin C, Pak DT, Pasquale EB. The EphA4 receptor regulates neuronal morphology through SPAR-mediated inactivation of Rap GTPases. J Neurosci. 2007;27(51):14205–15.
180. Zhou L, Martinez SJ, Haber M, Jones EV, Bouvier D, Doucet G, et al. EphA4 signaling regulates phospholipase Cgamma1 activation, cofilin membrane association, and dendritic spine morphology. J Neurosci. 2007;27(19):5127–38.
181. Bourgin C, Murai KK, Richter M, Pasquale EB. The EphA4 receptor regulates dendritic spine remodeling by affecting beta1-integrin signaling pathways. J Cell Biol. 2007;178(7): 1295–307.
182. Lai KO, Ip NY. Synapse development and plasticity: roles of ephrin/Eph receptor signaling. Curr Opin Neurobiol. 2009;19(3):275–83.
183. Klein R. Eph/ephrin signaling in morphogenesis, neural development and plasticity. Curr Opin Cell Biol. 2004;16(5):580–9.
184. Contractor A, Rogers C, Maron C, Henkemeyer M, Swanson GT, Heinemann SF. Trans-synaptic Eph receptor-ephrin signaling in hippocampal mossy fiber LTP. Science. 2002; 296(5574):1864–9.
185. Grunwald IC, Korte M, Adelmann G, Plueck A, Kullander K, Adams RH, et al. Hippocampal plasticity requires postsynaptic ephrinBs. Nat Neurosci. 2004;7(1):33–40.
186. Bouzioukh F, Wilkinson GA, Adelmann G, Frotscher M, Stein V, Klein R. Tyrosine phosphorylation sites in ephrinB2 are required for hippocampal long-term potentiation but not long-term depression. J Neurosci. 2007;27(42):11279–88.
187. Filosa A, Paixao S, Honsek SD, Carmona MA, Becker L, Feddersen B, et al. Neuron-glia communication via EphA4/ephrin-A3 modulates LTP through glial glutamate transport. Nat Neurosci. 2009;12(10):1285–92.

188. Deininger K, Eder M, Kramer ER, Zieglgansberger W, Dodt HU, Dornmair K, et al. The Rab5 guanylate exchange factor Rin1 regulates endocytosis of the EphA4 receptor in mature excitatory neurons. Proc Natl Acad Sci USA. 2008;105(34):12539–44.
189. Savelieva KV, Rajan I, Baker KB, Vogel P, Jarman W, Allen M, et al. Learning and memory impairment in Eph receptor A6 knockout mice. Neurosci Lett. 2008;438(2):205–9.
190. Mamiya PC, Hennesy Z, Zhou R, Wagner GC. Changes in attack behavior and activity in EphA5 knockout mice. Brain Res. 2008;1205:91–9.
191. Galimberti I, Bednarek E, Donato F, Caroni P. EphA4 signaling in juveniles establishes topographic specificity of structural plasticity in the hippocampus. Neuron. 2010;65(5):627–42.
192. Clifford MA, Kanwal JK, Dzakpasu R, Donoghue MJ. EphA4 expression promotes network activity and spine maturation in cortical neuronal cultures. Neural Dev. 2011;6:21.
193. Frank CA, Pielage J, Davis GW. A presynaptic homeostatic signaling system composed of the Eph receptor, ephexin, Cdc42, and CaV2.1 calcium channels. Neuron. 2009;61(4):556–69.
194. Fu AKY, Hung KW, Fu WY, Shen C, Chen Y, Xia J, et al. APC(Cdh1) mediates EphA4-dependent downregulation of AMPA receptors in homeostatic plasticity. Nat Neurosci. 2011;14(2):181–U263.
195. Miranda JD, White LA, Marcillo AE, Willson CA, Jagid J, Whittemore SR. Induction of Eph B3 after spinal cord injury. Exp Neurol. 1999;156(1):218–22.
196. Willson CA, Irizarry-Ramirez M, Gaskins HE, Cruz-Orengo L, Figueroa JD, Whittemore SR, et al. Upregulation of EphA receptor expression in the injured adult rat spinal cord. Cell Transplant. 2002;11(3):229–39.
197. Willson CA, Miranda JD, Foster RD, Onifer SM, Whittemore SR. Transection of the adult rat spinal cord upregulates EphB3 receptor and ligand expression. Cell Transplant. 2003; 12(3):279–90.
198. Wu Z, Ghosh-Roy A, Yanik MF, Zhang JZ, Jin Y, Chisholm AD. Caenorhabditis elegans neuronal regeneration is influenced by life stage, ephrin signaling, and synaptic branching. Proc Natl Acad Sci USA. 2007;104(38):15132–7.
199. Liu X, Hawkes E, Ishimaru T, Tran T, Sretavan DW. EphB3: an endogenous mediator of adult axonal plasticity and regrowth after CNS injury. J Neurosci. 2006;26(12):3087–101.
200. Parrinello S, Napoli I, Ribeiro S, Digby PW, Fedorova M, Parkinson DB, et al. EphB signaling directs peripheral nerve regeneration through Sox2-dependent Schwann cell sorting. Cell. 2010;143(1):145–55.
201. Fabes J, Anderson P, Yanez-Munoz RJ, Thrasher A, Brennan C, Bolsover S. Accumulation of the inhibitory receptor EphA4 may prevent regeneration of corticospinal tract axons following lesion. Eur J Neurosci. 2006;23(7):1721–30.
202. Goldshmit Y, Galea MP, Wise G, Bartlett PF, Turnley AM. Axonal regeneration and lack of astrocytic gliosis in EphA4-deficient mice. J Neurosci. 2004;24(45):10064–73.
203. Cruz-Orengo L, Figueroa JD, Velazquez I, Torrado A, Ortiz C, Hernandez C, et al. Blocking EphA4 upregulation after spinal cord injury results in enhanced chronic pain. Exp Neurol. 2006;202(2):421–33.
204. Fabes J, Anderson P, Brennan C, Bolsover S. Regeneration-enhancing effects of EphA4 blocking peptide following corticospinal tract injury in adult rat spinal cord. Eur J Neurosci. 2007;26(9):2496–505.
205. Goldshmit Y, Spanevello MD, Tajouri S, Li L, Rogers F, Pearse M, et al. EphA4 blockers promote axonal regeneration and functional recovery following spinal cord injury in mice. PLoS One. 2011;6(9):e24636.
206. Herrmann JE, Shah RR, Chan AF, Zheng B. EphA4 deficient mice maintain astroglial-fibrotic scar formation after spinal cord injury. Exp Neurol. 2010;223(2):582–98.
207. Erber R, Eichelsbacher U, Powajbo V, Korn T, Djonov V, Lin J, et al. EphB4 controls blood vascular morphogenesis during postnatal angiogenesis. EMBO J. 2006;25(3): 628–41.
208. Gale NW, Baluk P, Pan L, Kwan M, Holash J, DeChiara TM, et al. Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev Biol. 2001;230(2):151–60.

209. Shin D, Garcia-Cardena G, Hayashi S, Gerety S, Asahara T, Stavrakis G, et al. Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. Dev Biol. 2001;230(2):139–50.
210. Muto A, Yi T, Harrison KD, Davalos A, Fancher TT, Ziegler KR, et al. Eph-B4 prevents venous adaptive remodeling in the adult arterial environment. J Exp Med. 2011; 208(3):561–75.
211. Ogita H, Kunimoto S, Kamioka Y, Sawa H, Masuda M, Mochizuki N. EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells. Circ Res. 2003;93(1):23–31.
212. Noren NK, Lu M, Freeman AL, Koolpe M, Pasquale EB. Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. Proc Natl Acad Sci USA. 2004; 101(15):5583–8.
213. Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL, Pasquale EB. The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. Oncogene. 2000;19(52):6043–52.
214. Brantley-Sieders DM, Zhuang G, Hicks D, Fang WB, Hwang Y, Cates JM, et al. The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. J Clin Invest. 2008;118(1):64–78.
215. Brantley-Sieders DM, Fang WB, Hwang Y, Hicks D, Chen J. Ephrin-A1 facilitates mammary tumor metastasis through an angiogenesis-dependent mechanism mediated by EphA receptor and vascular endothelial growth factor in mice. Cancer Res. 2006;66(21):10315–24.
216. Prevost N, Woulfe D, Tanaka T, Brass LF. Interactions between Eph kinases and ephrins provide a mechanism to support platelet aggregation once cell-to-cell contact has occurred. Proc Natl Acad Sci USA. 2002;99(14):9219–24.
217. Prevost N, Woulfe DS, Jiang H, Stalker TJ, Marchese P, Ruggeri ZM, et al. Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets. Proc Natl Acad Sci USA. 2005;102(28):9820–5.
218. Prevost N, Woulfe DS, Tognolini M, Tanaka T, Jian W, Fortna RR, et al. Signaling by ephrinB1 and Eph kinases in platelets promotes Rap1 activation, platelet adhesion, and aggregation via effector pathways that do not require phosphorylation of ephrinB1. Blood. 2004;103(4):1348–55.
219. Luo H, Yu G, Tremblay J, Wu J. EphB6-null mutation results in compromised T cell function. J Clin Invest. 2004;114(12):1762–73.
220. Freywald A, Sharfe N, Miller CD, Rashotte C, Roifman CM. EphA receptors inhibit anti-CD3-induced apoptosis in thymocytes. J Immunol. 2006;176(7):4066–74.
221. Sharfe N, Nikolic M, Cimpeon L, Van De Kratts A, Freywald A, Roifman CM. EphA and ephrin-A proteins regulate integrin-mediated T lymphocyte interactions. Mol Immunol. 2008;45(5):1208–20.
222. Aasheim HC, Munthe E, Funderud S, Smeland EB, Beiske K, Logtenberg T. A splice variant of human ephrin-A4 encodes a soluble molecule that is secreted by activated human B lymphocytes. Blood. 2000;95(1):221–30.
223. Konstantinova I, Nikolova G, Ohara-Imaizumi M, Meda P, Kucera T, Zarbalis K, et al. EphA-Ephrin-A-mediated beta cell communication regulates insulin secretion from pancreatic islets. Cell. 2007;129(2):359–70.
224. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;449(7165): 1003–7.
225. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell. 2002;111(2):241–50.
226. van Es JH, Jay P, Gregorieff A, van Gijn ME, Jonkheer S, Hatzis P, et al. Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nat Cell Biol. 2005;7(4):381–6.

227. Cortina C, Palomo-Ponce S, Iglesias M, Fernandez-Masip JL, Vivancos A, Whissell G, et al. EphB-ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells. Nat Genet. 2007;39(11):1376–83.
228. Holmberg J, Genander M, Halford MM, Anneren C, Sondell M, Chumley MJ, et al. EphB receptors coordinate migration and proliferation in the intestinal stem cell niche. Cell. 2006;125(6):1151–63.
229. Andres AC, Ziemiecki A. Eph and ephrin signaling in mammary gland morphogenesis and cancer. J Mammary Gland Biol Neoplasia. 2003;8(4):475–85.
230. Munarini N, Jager R, Abderhalden S, Zuercher G, Rohrbach V, Loercher S, et al. Altered mammary epithelial development, pattern formation and involution in transgenic mice expressing the EphB4 receptor tyrosine kinase. J Cell Sci. 2002;115(Pt 1):25–37.
231. Ogawa K, Wada H, Okada N, Harada I, Nakajima T, Pasquale EB, et al. EphB2 and ephrin-B1 expressed in the adult kidney regulate the cytoarchitecture of medullary tubule cells through Rho family GTPases. J Cell Sci. 2006;119(Pt 3):559–70.
232. Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, et al. Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. Cell Metab. 2006;4(2):111–21.
233. Edwards CM, Mundy GR. Eph receptors and ephrin signaling pathways: a role in bone homeostasis. Int J Med Sci. 2008;5(5):263–72.
234. Lickliter JD, Smith FM, Olsson JE, Mackwell KL, Boyd AW. Embryonic stem cells express multiple Eph-subfamily receptor tyrosine kinases. Proc Natl Acad Sci USA. 1996;93(1): 145–50.
235. Wang Z, Cohen K, Shao Y, Mole P, Dombkowski D, Scadden DT. Ephrin receptor, EphB4, regulates ES cell differentiation of primitive mammalian hemangioblasts, blood, cardiomyocytes, and blood vessels. Blood. 2004;103(1):100–9.
236. Chen K, Bai H, Liu Y, Hoyle D, Shen WF, Wu LQ, et al. EphB4 forward-signaling regulates cardiac progenitor development in mouse ES cells. J Cell Biochem. 2014;116:467–75.
237. Nikolova Z, Djonov V, Zuercher G, Andres AC, Ziemiecki A. Cell-type specific and estrogen dependent expression of the receptor tyrosine kinase EphB4 and its ligand ephrin-B2 during mammary gland morphogenesis. J Cell Sci. 1998;111(Pt 18):2741–51.
238. Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, et al. Defining the epithelial stem cell niche in skin. Science. 2004;303(5656):359–63.
239. Guo H, Miao H, Gerber L, Singh J, Denning MF, Gilliam AC, et al. Disruption of EphA2 receptor tyrosine kinase leads to increased susceptibility to carcinogenesis in mouse skin. Cancer Res. 2006;66(14):7050–8.
240. Genander M, Holmberg J, Frisen J. Ephrins negatively regulate cell proliferation in the epidermis and hair follicle. Stem Cells. 2010;28(7):1196–205.
241. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol. 2009;71:241–60.
242. Gage FH. Mammalian neural stem cells. Science. 2000;287(5457):1433–8.
243. Jiao JW, Feldheim DA, Chen DF. Ephrins as negative regulators of adult neurogenesis in diverse regions of the central nervous system. Proc Natl Acad Sci USA. 2008;105(25): 8778–83.
244. Holmberg J, Armulik A, Senti KA, Edoff K, Spalding K, Momma S, et al. Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis. Genes Dev. 2005;19(4):462–71.
245. Conover JC, Doetsch F, Garcia-Verdugo JM, Gale NW, Yancopoulos GD, Alvarez-Buylla A. Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. Nat Neurosci. 2000;3(11):1091–7.
246. Ricard J, Salinas J, Garcia L, Liebl DJ. EphrinB3 regulates cell proliferation and survival in adult neurogenesis. Mol Cell Neurosci. 2006;31(4):713–22.
247. Theus MH, Ricard J, Bethea JR, Liebl DJ. EphB3 limits the expansion of neural progenitor cells in the subventricular zone by regulating p53 during homeostasis and following traumatic brain injury. Stem Cells. 2010;28(7):1231–42.

248. Chumley MJ, Catchpole T, Silvany RE, Kernie SG, Henkemeyer M. EphB receptors regulate stem/progenitor cell proliferation, migration, and polarity during hippocampal neurogenesis. J Neurosci. 2007;27(49):13481–90.
249. Hara Y, Nomura T, Yoshizaki K, Frisen J, Osumi N. Impaired hippocampal neurogenesis and vascular formation in ephrin-A5-deficient mice. Stem Cells. 2010;28(5):974–83.
250. Nomura T, Goritz C, Catchpole T, Henkemeyer M, Frisen J. EphB signaling controls lineage plasticity of adult neural stem cell niche cells. Cell Stem Cell. 2010;7(6):730–43.
251. Khodosevich K, Watanabe Y, Monyer H. EphA4 preserves postnatal and adult neural stem cells in an undifferentiated state in vivo. J Cell Sci. 2011;124(Pt 8):1268–79.
252. Hirai H, Maru Y, Hagiwara K, Nishida J, Takaku F. A novel putative tyrosine kinase receptor encoded by the eph gene. Science. 1987;238(4834):1717–20.
253. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer. 2010;10(3):165–80.
254. Macrae M, Neve RM, Rodriguez-Viciana P, Haqq C, Yeh J, Chen C, et al. A conditional feedback loop regulates Ras activity through EphA2. Cancer Cell. 2005;8(2):111–8.
255. Miao H, Wei BR, Peehl DM, Li Q, Alexandrou T, Schelling JR, et al. Activation of EphA receptor tyrosine kinase inhibits the Ras/MAPK pathway. Nat Cell Biol. 2001;3(5):527–30.
256. Zelinski DP, Zantek ND, Stewart JC, Irizarry AR, Kinch MS. EphA2 overexpression causes tumorigenesis of mammary epithelial cells. Cancer Res. 2001;61(5):2301–6.
257. Fox BP, Kandpal RP. EphB6 receptor significantly alters invasiveness and other phenotypic characteristics of human breast carcinoma cells. Oncogene. 2009;28(14):1706–13.
258. Truitt L, Freywald T, DeCoteau J, Sharfe N, Freywald A. The EphB6 receptor cooperates with c-Cbl to regulate the behavior of breast cancer cells. Cancer Res. 2010;70(3):1141–53.
259. Oki M, Yamamoto H, Taniguchi H, Adachi Y, Imai K, Shinomura Y. Overexpression of the receptor tyrosine kinase EphA4 in human gastric cancers. World J Gastroenterol. 2008; 14(37):5650–6.
260. Saito T, Masuda N, Miyazaki T, Kanoh K, Suzuki H, Shimura T, et al. Expression of EphA2 and E-cadherin in colorectal cancer: correlation with cancer metastasis. Oncol Rep. 2004;11(3):605–11.
261. Batlle E, Bacani J, Begthel H, Jonkheer S, Gregorieff A, van de Born M, et al. EphB receptor activity suppresses colorectal cancer progression. Nature. 2005;435(7045):1126–30.
262. Davalos V, Dopeso H, Castano J, Wilson AJ, Vilardell F, Romero-Gimenez J, et al. EPHB4 and survival of colorectal cancer patients. Cancer Res. 2006;66(18):8943–8.
263. Herath NI, Doecke J, Spanevello MD, Leggett BA, Boyd AW. Epigenetic silencing of EphA1 expression in colorectal cancer is correlated with poor survival. Br J Cancer. 2009;100(7):1095–102.
264. Ronsch K, Jager M, Schopflin A, Danciu M, Lassmann S, Hecht A. Class I and III HDACs and loss of active chromatin features contribute to epigenetic silencing of CDX1 and EPHB tumor suppressor genes in colorectal cancer. Epigenetics. 2011;6(5):610–22.
265. Kumar SR, Scehnet JS, Ley EJ, Singh J, Krasnoperov V, Liu R, et al. Preferential induction of EphB4 over EphB2 and its implication in colorectal cancer progression. Cancer Res. 2009;69(9):3736–45.
266. Dopeso H, Mateo-Lozano S, Mazzolini R, Rodrigues P, Lagares-Tena L, Ceron J, et al. The receptor tyrosine kinase EPHB4 has tumor suppressor activities in intestinal tumorigenesis. Cancer Res. 2009;69(18):7430–8.
267. Lisabeth EM, Fernandez C, Pasquale EB. Cancer somatic mutations disrupt functions of the EphA3 receptor tyrosine kinase through multiple mechanisms. Biochemistry. 2012;51: 1464–75.
268. Prickett TD, Agrawal NS, Wei X, Yates KE, Lin JC, Wunderlich JR, et al. Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. Nat Genet. 2009; 41(10):1127–32.
269. Vail ME, Murone C, Tan A, Hii L, Abebe D, Janes PW, et al. Targeting EphA3 inhibits cancer growth by disrupting the tumor stromal microenvironment. Cancer Res. 2014;74(16):4470–81.

270. Miao H, Li DQ, Mukherjee A, Guo H, Petty A, Cutter J, et al. EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. Cancer Cell. 2009;16(1):9–20.
271. Wu Z, Doondeea JB, Moghaddas Gholami A, Janning MC, Lemeer S, Kramer K, et al. Quantitative chemical proteomics reveals new potential drug targets in head and neck cancer. Mol Cell Proteomics. 2011;10(12):M111.011635.
272. Salaita K, Nair PM, Petit RS, Neve RM, Das D, Gray JW, et al. Restriction of receptor movement alters cellular response: physical force sensing by EphA2. Science. 2010;327(5971):1380–5.
273. Hiramoto-Yamaki N, Takeuchi S, Ueda S, Harada K, Fujimoto S, Negishi M, et al. Ephexin4 and EphA2 mediate cell migration through a RhoG-dependent mechanism. J Cell Biol. 2010;190(3):461–77.
274. Noren NK, Foos G, Hauser CA, Pasquale EB. The EphB4 receptor suppresses breast cancer cell tumorigenicity through an Abl-Crk pathway. Nat Cell Biol. 2006;8(8):815–25.
275. Kumar SR, Singh J, Xia G, Krasnoperov V, Hassanieh L, Ley EJ, et al. Receptor tyrosine kinase EphB4 is a survival factor in breast cancer. Am J Pathol. 2006;169(1):279–93.
276. Astin JW, Batson J, Kadir S, Charlet J, Persad RA, Gillatt D, et al. Competition amongst Eph receptors regulates contact inhibition of locomotion and invasiveness in prostate cancer cells. Nat Cell Biol. 2010;12(12):1194–204.
277. Heroult M, Schaffner F, Pfaff D, Prahst C, Kirmse R, Kutschera S, et al. EphB4 promotes site-specific metastatic tumor cell dissemination by interacting with endothelial cell-expressed ephrinB2. Mol Cancer Res. 2010;8(10):1297–309.
278. Huang X, Yamada Y, Kidoya H, Naito H, Nagahama Y, Kong L, et al. EphB4 overexpression in B16 melanoma cells affects arterial-venous patterning in tumor angiogenesis. Cancer Res. 2007;67(20):9800–8.
279. Barquilla A, Pasquale EB. Eph receptors and ephrins: therapeutic opportunities. Annu Rev Pharmacol Toxicology. 2015;55:465–87.
280. Udayakumar D, Zhang G, Ji Z, Njauw CN, Mroz P, Tsao H. Epha2 is a critical oncogene in melanoma. Oncogene. 2011;30(50):4921–9.
281. Ashida S, Nakagawa H, Katagiri T, Furihata M, Iiizumi M, Anazawa Y, et al. Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs. Cancer Res. 2004; 64(17):5963–72.
282. Iiizumi M, Hosokawa M, Takehara A, Chung S, Nakamura T, Katagiri T, et al. EphA4 receptor, overexpressed in pancreatic ductal adenocarcinoma, promotes cancer cell growth. Cancer Sci. 2006;97(11):1211–6.
283. Martiny-Baron G, Holzer P, Billy E, Schnell C, Brueggen J, Ferretti M, et al. The small molecule specific EphB4 kinase inhibitor NVP-BHG712 inhibits VEGF driven angiogenesis. Angiogenesis. 2010;13(3):259–67.
284. Noberini R, Koolpe M, Peddibhotla S, Dahl R, Su Y, Cosford ND, et al. Small molecules can selectively inhibit ephrin binding to the EphA4 and EphA2 receptors. J Biol Chem. 2008;283(43):29461–72.
285. Chang Q, Jorgensen C, Pawson T, Hedley DW. Effects of dasatinib on EphA2 receptor tyrosine kinase activity and downstream signalling in pancreatic cancer. Br J Cancer. 2008; 99(7):1074–82.
286. Huang J, Hu W, Bottsford-Miller J, Liu T, Han HD, Zand B, et al. Cross-talk between EphA2 and BRaf/CRaf is a key determinant of response to Dasatinib. Clin Cancer Res. 2014; 20(7):1846–55.
287. Oricchio E, Nanjangud G, Wolfe AL, Schatz JH, Mavrakis KJ, Jiang M, et al. The eph-receptor a7 is a soluble tumor suppressor for follicular lymphoma. Cell. 2011;147(3): 554–64.
288. Carles-Kinch K, Kilpatrick KE, Stewart JC, Kinch MS. Antibody targeting of the EphA2 tyrosine kinase inhibits malignant cell behavior. Cancer Res. 2002;62(10):2840–7.

289. Wykosky J, Gibo DM, Debinski W. A novel, potent, and specific ephrinA1-based cytotoxin against EphA2 receptor expressing tumor cells. Mol Cancer Ther. 2007;6(12 Pt 1):3208–18.
290. Shaw A, Lundin V, Petrova E, Fordos F, Benson E, Al-Amin A, et al. Spatial control of membrane receptor function using ligand nanocalipers. Nat Methods. 2014;11(8):841–6.
291. You J, Zhang R, Xiong C, Zhong M, Melancon M, Gupta S, et al. Effective photothermal chemotherapy using doxorubicin-loaded gold nanospheres that target EphB4 receptors in tumors. Cancer Res. 2012;72(18):4777–86.
292. Simon AM, de Maturana RL, Ricobaraza A, Escribano L, Schiapparelli L, Cuadrado-Tejedor M, et al. Early changes in hippocampal Eph receptors precede the onset of memory decline in mouse models of Alzheimer's disease. J Alzheimers Dis. 2009;17(4):773–86.
293. Cisse M, Halabisky B, Harris J, Devidze N, Dubal DB, Sun B, et al. Reversing EphB2 depletion rescues cognitive functions in Alzheimer model. Nature. 2011;469(7328):47–52.
294. Tsuda H, Han SM, Yang Y, Tong C, Lin YQ, Mohan K, et al. The amyotrophic lateral sclerosis 8 protein VAPB is cleaved, secreted, and acts as a ligand for Eph receptors. Cell. 2008; 133(6):963–77.
295. Van Hoecke A, Schoonaert L, Lemmens R, Timmers M, Staats KA, Laird AS, et al. EPHA4 is a disease modifier of amyotrophic lateral sclerosis in animal models and in humans. Nat Med. 2012;18(9):1418–22.
296. Casey JP, Magalhaes T, Conroy JM, Regan R, Shah N, Anney R, et al. A novel approach of homozygous haplotype sharing identifies candidate genes in autism spectrum disorder. Hum Genet. 2011;131:565–79.
297. Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, et al. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nat Med. 2011;17(5):589–95.
298. Yamazaki T, Masuda J, Omori T, Usui R, Akiyama H, Maru Y. EphA1 interacts with integrin-linked kinase and regulates cell morphology and motility. J Cell Sci. 2009;122(Pt 2):243–55.
299. Duffy SL, Coulthard MG, Spanevello MD, Herath NI, Yeadon TM, McCarron JK, et al. Generation and characterization of EphA1 receptor tyrosine kinase reporter knockout mice. Genesis. 2008;46(10):553–61.
300. Du X, Baldwin C, Hooker E, Glorion P, Lemay S. Basal and Src kinase-mediated activation of the EphA2 promoter requires a cAMP-responsive element but is CREB-independent. J Cell Biochem. 2011;112(5):1268–76.
301. Foveau B, Boulay G, Pinte S, Van Rechem C, Rood BR, Leprince D. The receptor tyrosine kinase EphA2 is a direct target gene of hypermethylated in cancer 1 (HIC1). J Biol Chem. 2012;287(8):5366–78.
302. Himanen JP, Yermekbayeva L, Janes PW, Walker JR, Xu K, Atapattu L, et al. Architecture of Eph receptor clusters. Proc Natl Acad Sci USA. 2010;107(24):10860–5.
303. Seiradake E, Harlos K, Sutton G, Aricescu AR, Jones EY. An extracellular steric seeding mechanism for Eph-ephrin signaling platform assembly. Nat Struct Mol Biol. 2010; 17(4):398–402.
304. Fang WB, Brantley-Sieders DM, Hwang Y, Ham AJ, Chen J. Identification and functional analysis of phosphorylated tyrosine residues within EphA2 receptor tyrosine kinase. J Biol Chem. 2008;283(23):16017–26.
305. Wykosky J, Palma E, Gibo DM, Ringler S, Turner CP, Debinski W. Soluble monomeric EphrinA1 is released from tumor cells and is a functional ligand for the EphA2 receptor. Oncogene. 2008;27(58):7260–73.
306. Wakayama Y, Miura K, Sabe H, Mochizuki N. EphrinA1-EphA2 signal induces compaction and polarization of Madin-Darby canine kidney cells by inactivating Ezrin through negative regulation of RhoA. J Biol Chem. 2011;286(51):44243–53.
307. Miura K, Nam JM, Kojima C, Mochizuki N, Sabe H. EphA2 engages Git1 to suppress Arf6 activity modulating epithelial cell-cell contacts. Mol Biol Cell. 2009;20(7):1949–59.
308. Miao H, Burnett E, Kinch M, Simon E, Wang B. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. Nat Cell Biol. 2000;2(2):62–9.

309. Seiradake E, Schaupp A, del Toro RD, Kaufmann R, Mitakidis N, Harlos K, et al. Structurally encoded intraclass differences in EphA clusters drive distinct cell responses. Nat Struct Mol Biol. 2013;20(8):958–64.
310. Ruiz JC, Robertson EJ. The expression of the receptor-protein tyrosine kinase gene, eck, is highly restricted during early mouse development. Mech Dev. 1994;46(2):87–100.
311. Ganju P, Shigemoto K, Brennan J, Entwistle A, Reith AD. The Eck receptor tyrosine kinase is implicated in pattern formation during gastrulation, hindbrain segmentation and limb development. Oncogene. 1994;9(6):1613–24.
312. Dottori M, Down M, Huttmann A, Fitzpatrick DR, Boyd AW. Cloning and characterization of EphA3 (Hek) gene promoter: DNA methylation regulates expression in hematopoietic tumor cells. Blood. 1999;94(7):2477–86.
313. Hinoue T, Weisenberger DJ, Pan F, Campan M, Kim M, Young J, et al. Analysis of the association between CIMP and BRAF in colorectal cancer by DNA methylation profiling. PLoS One. 2009;4(12):e8357.
314. Cobb J, Duboule D. Comparative analysis of genes downstream of the Hoxd cluster in developing digits and external genitalia. Development. 2005;132(13):3055–67.
315. Kawakami Y, Uchiyama Y, Rodriguez Esteban C, Inenaga T, Koyano-Nakagawa N, Kawakami H, et al. Sall genes regulate region-specific morphogenesis in the mouse limb by modulating Hox activities. Development. 2009;136(4):585–94.
316. Schulte D, Cepko CL. Two homeobox genes define the domain of EphA3 expression in the developing chick retina. Development. 2000;127(23):5033–45.
317. Takahashi H, Shintani T, Sakuta H, Noda M. CBF1 controls the retinotectal topographical map along the anteroposterior axis through multiple mechanisms. Development. 2003; 130(21):5203–15.
318. Smith LM, Walsh PT, Rudiger T, Cotter TG, Mc Carthy TV, Marx A, et al. EphA3 is induced by CD28 and IGF-1 and regulates cell adhesion. Exp Cell Res. 2004;292(2):295–303.
319. Li YY, McTiernan CF, Feldman AM. IL-1 beta alters the expression of the receptor tyrosine kinase gene r-EphA3 in neonatal rat cardiomyocytes. Am J Physiol. 1998;274(1 Pt 2):H331–41.
320. Lawrenson ID, Wimmer-Kleikamp SH, Lock P, Schoenwaelder SM, Down M, Boyd AW, et al. Ephrin-A5 induces rounding, blebbing and de-adhesion of EphA3-expressing 293 T and melanoma cells by CrkII and Rho-mediated signalling. J Cell Sci. 2002;115(Pt 5):1059–72.
321. Hu T, Shi G, Larose L, Rivera GM, Mayer BJ, Zhou R. Regulation of process retraction and cell migration by EphA3 is mediated by the adaptor protein Nck1. Biochemistry. 2009; 48(27):6369–78.
322. Janes PW, Saha N, Barton WA, Kolev MV, Wimmer-Kleikamp SH, Nievergall E, et al. Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. Cell. 2005;123(2):291–304.
323. Zisch AH, Kalo MS, Chong LD, Pasquale EB. Complex formation between EphB2 and Src requires phosphorylation of tyrosine 611 in the EphB2 juxtamembrane region. Oncogene. 1998;16(20):2657–70.
324. Nievergall E, Janes PW, Stegmayer C, Vail ME, Haj FG, Teng SW, et al. PTP1B regulates Eph receptor function and trafficking. J Cell Biol. 2010;191(6):1189–203.
325. Janes PW, Griesshaber B, Atapattu L, Nievergall E, Hii LL, Mensinga A, et al. Eph receptor function is modulated by heterooligomerization of A and B type Eph receptors. J Cell Biol. 2011;195:1033–45.
326. Nakajima Y, Morimoto M, Takahashi Y, Koseki H, Saga Y. Identification of Epha4 enhancer required for segmental expression and the regulation by Mesp2. Development. 2006;133(13):2517–25.
327. Theil T, Frain M, Gilardi-Hebenstreit P, Flenniken A, Charnay P, Wilkinson DG. Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. Development. 1998;125(3):443–52.
328. Escalante A, Murillo B, Morenilla-Palao C, Klar A, Herrera E. Zic2-dependent axon midline avoidance controls the formation of major ipsilateral tracts in the CNS. Neuron. 2013; 80(6):1392–406.

329. Qin H, Shi J, Noberini R, Pasquale EB, Song J. Crystal structure and NMR binding reveal that two small molecule antagonists target the high affinity ephrin-binding channel of the EphA4 receptor. J Biol Chem. 2008;283(43):29473–84.
330. Qin H, Noberini R, Huan X, Shi J, Pasquale EB, Song J. Structural characterization of the EphA4-Ephrin-B2 complex reveals new features enabling Eph-ephrin binding promiscuity. J Biol Chem. 2010;285(1):644–54.
331. Stapleton D, Balan I, Pawson T, Sicheri F. The crystal structure of an Eph receptor SAM domain reveals a mechanism for modular dimerization. Nat Struct Biol. 1999;6(1):44–9.
332. Ellis C, Kasmi F, Ganju P, Walls E, Panayotou G, Reith AD. A juxtamembrane autophosphorylation site in the Eph family receptor tyrosine kinase, Sek, mediates high affinity interaction with p59fyn. Oncogene. 1996;12(8):1727–36.
333. Binns KL, Taylor PP, Sicheri F, Pawson T, Holland SJ. Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors. Mol Cell Biol. 2000;20(13):4791–805.
334. Park EK, Warner N, Bong YS, Stapleton D, Maeda R, Pawson T, et al. Ectopic EphA4 receptor induces posterior protrusions via FGF signaling in Xenopus embryos. Mol Biol Cell. 2004;15(4):1647–55.
335. Sahin M, Greer PL, Lin MZ, Poucher H, Eberhart J, Schmidt S, et al. Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse. Neuron. 2005;46(2):191–204.
336. Shamah SM, Lin MZ, Goldberg JL, Estrach S, Sahin M, Hu L, et al. EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor Ephexin. Cell. 2001;105(2):233–44.
337. Dudanova I, Gatto G, Klein R. GDNF acts as a chemoattractant to support ephrinA-induced repulsion of limb motor axons. Curr Biol. 2010;20(23):2150–6.
338. Kramer ER, Knott L, Su F, Dessaud E, Krull CE, Helmbacher F, et al. Cooperation between GDNF/Ret and ephrinA/EphA4 signals for motor-axon pathway selection in the limb. Neuron. 2006;50(1):35–47.
339. Fukai J, Yokote H, Yamanaka R, Arao T, Nishio K, Itakura T. EphA4 promotes cell proliferation and migration through a novel EphA4-FGFR1 signaling pathway in the human glioma U251 cell line. Mol Cancer Ther. 2008;7(9):2768–78.
340. Petkova TD, Seigel GM, Otteson DC. A role for DNA methylation in regulation of EphA5 receptor expression in the mouse retina. Vision Res. 2011;51(2):260–8.
341. Fu DY, Wang ZM, Wang BL, Chen L, Yang WT, Shen ZZ, et al. Frequent epigenetic inactivation of the receptor tyrosine kinase EphA5 by promoter methylation in human breast cancer. Hum Pathol. 2010;41(1):48–58.
342. Jassen AK, Yang H, Miller GM, Calder E, Madras BK. Receptor regulation of gene expression of axon guidance molecules: implications for adaptation. Mol Pharmacol. 2006;70(1):71–7.
343. Fukushima K, Ueno Y, Inoue J, Kanno N, Shimosegawa T. Filopodia formation via a specific Eph family member and PI3K in immortalized cholangiocytes. Am J Physiol Gastrointest Liver Physiol. 2006;291(5):G812–9.
344. Shaut CA, Saneyoshi C, Morgan EA, Knosp WM, Sexton DR, Stadler HS. HOXA13 directly regulates EphA6 and EphA7 expression in the genital tubercle vascular endothelia. Dev Dyn. 2007;236(4):951–60.
345. Maisonpierre PC, Barrezueta NX, Yancopoulos GD. Ehk-1 and Ehk-2: two novel members of the Eph receptor-like tyrosine kinase family with distinctive structures and neuronal expression. Oncogene. 1993;8(12):3277–88.
346. Salsi V, Zappavigna V. Hoxd13 and Hoxa13 directly control the expression of the EphA7 Ephrin tyrosine kinase receptor in developing limbs. J Biol Chem. 2006;281(4):1992–9.
347. Pietri S, Dimidschstein J, Tiberi L, Sotiropoulou PA, Bilheu A, Goffinet A, et al. Transcriptional Mechanisms of EphA7 Gene Expression in the Developing Cerebral Cortex. Cereb Cortex. 2011;27:1678–89.
348. Stadler HS, Higgins KM, Capecchi MR. Loss of Eph-receptor expression correlates with loss of cell adhesion and chondrogenic capacity in Hoxa13 mutant limbs. Development. 2001; 128(21):4177–88.

349. Nakanishi H, Nakamura T, Canaani E, Croce CM. ALL1 fusion proteins induce deregulation of EphA7 and ERK phosphorylation in human acute leukemias. Proc Natl Acad Sci USA. 2007;104(36):14442–7.
350. Wang J, Kataoka H, Suzuki M, Sato N, Nakamura R, Tao H, et al. Downregulation of EphA7 by hypermethylation in colorectal cancer. Oncogene. 2005;24(36):5637–47.
351. Ciossek T, Millauer B, Ullrich A. Identification of alternatively spliced mRNAs encoding variants of MDK1, a novel receptor tyrosine kinase expressed in the murine nervous system. Oncogene. 1995;10(1):97–108.
352. Valenzuela DM, Rojas E, Griffiths JA, Compton DL, Gisser M, Ip NY, et al. Identification of full-length and truncated forms of Ehk-3, a novel member of the Eph receptor tyrosine kinase family. Oncogene. 1995;10(8):1573–80.
353. Tsuboi M, Mori H, Bunai T, Kageyama S, Suzuki M, Okudela K, et al. Secreted form of EphA7 in lung cancer. Int J Oncol. 2010;36(3):635–40.
354. Torres R, Firestein BL, Dong H, Staudinger J, Olson EN, Huganir RL, et al. PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands. Neuron. 1998;21(6):1453–63.
355. Jeong J, Choi S, Gu C, Lee H, Park S. Genomic structure and promoter analysis of the mouse EphA8 receptor tyrosine kinase gene. DNA Cell Biol. 2000;19(5):291–300.
356. Shim S, Kim Y, Shin J, Kim J, Park S. Regulation of EphA8 gene expression by TALE homeobox transcription factors during development of the mesencephalon. Mol Cell Biol. 2007;27(5):1614–30.
357. Choi S, Park S. Phosphorylation at Tyr-838 in the kinase domain of EphA8 modulates Fyn binding to the Tyr-615 site by enhancing tyrosine kinase activity. Oncogene. 1999; 18(39):5413–22.
358. Gu C, Shim S, Shin J, Kim J, Park J, Han K, et al. The EphA8 receptor induces sustained MAP kinase activation to promote neurite outgrowth in neuronal cells. Oncogene. 2005;24(26):4243–56.
359. Shin J, Gu C, Park E, Park S. Identification of phosphotyrosine binding domain-containing proteins as novel downstream targets of the EphA8 signaling function. Mol Cell Biol. 2007;27(23):8113–26.
360. Gu C, Park S. The EphA8 receptor regulates integrin activity through p110gamma phosphatidylinositol-3 kinase in a tyrosine kinase activity-independent manner. Mol Cell Biol. 2001;21(14):4579–97.
361. Yoo S, Shin J, Park S. EphA8-ephrinA5 signaling and clathrin-mediated endocytosis is regulated by Tiam-1, a Rac-specific guanine nucleotide exchange factor. Mol Cells. 2010; 29(6):603–9.
362. Kim J, Lee H, Kim Y, Yoo S, Park E, Park S. The SAM domains of Anks family proteins are critically involved in modulating the degradation of EphA receptors. Mol Cell Biol. 2010;30(7):1582–92.
363. Truitt L, Freywald A. Dancing with the dead: Eph receptors and their kinase-null partners. Biochem Cell Biol. 2011;89(2):115–29.
364. Aasheim HC, Patzke S, Hjorthaug HS, Finne EF. Characterization of a novel Eph receptor tyrosine kinase, EphA10, expressed in testis. Biochim Biophys Acta. 2005;1723(1–3):1–7.
365. Garcia-Frigola C, Carreres MI, Vegar C, Mason C, Herrera E. Zic2 promotes axonal divergence at the optic chiasm midline by EphB1-dependent and -independent mechanisms. Development. 2008;135(10):1833–41.
366. Han DC, Shen TL, Miao H, Wang B, Guan JL. EphB1 associates with Grb7 and regulates cell migration. J Biol Chem. 2002;277(47):45655–61.
367. Vindis C, Teli T, Cerretti DP, Turner CE, Huynh-Do U. EphB1-mediated cell migration requires the phosphorylation of paxillin at Tyr-31/Tyr-118. J Biol Chem. 2004;279(27):27965–70.
368. Becker E, Huynh-Do U, Holland S, Pawson T, Daniel TO, Skolnik EY. Nck-interacting Ste20 kinase couples Eph receptors to c-Jun N-terminal kinase and integrin activation. Mol Cell Biol. 2000;20(5):1537–45.

369. Stein E, Huynh-Do U, Lane AA, Cerretti DP, Daniel TO. Nck recruitment to Eph receptor, EphB1/ELK, couples ligand activation to c-Jun kinase. J Biol Chem. 1998;273(3):1303–8.
370. Vindis C, Cerretti DP, Daniel TO, Huynh-Do U. EphB1 recruits c-Src and p52Shc to activate MAPK/ERK and promote chemotaxis. J Cell Biol. 2003;162(4):661–71.
371. Georgakopoulos A, Litterst C, Ghersi E, Baki L, Xu C, Serban G, et al. Metalloproteinase/Presenilin1 processing of ephrinB regulates EphB-induced Src phosphorylation and signaling. EMBO J. 2006;25(6):1242–52.
372. Pascall JC, Brown KD. Intramembrane cleavage of ephrinB3 by the human rhomboid family protease, RHBDL2. Biochem Biophys Res Commun. 2004;317(1):244–52.
373. Freywald A, Sharfe N, Roifman CM. The kinase-null EphB6 receptor undergoes transphosphorylation in a complex with EphB1. J Biol Chem. 2002;277(6):3823–8.
374. Huynh-Do U, Stein E, Lane AA, Liu H, Cerretti DP, Daniel TO. Surface densities of ephrin-B1 determine EphB1-coupled activation of cell attachment through alphavbeta3 and alpha5beta1 integrins. EMBO J. 1999;18(8):2165–73.
375. Fasen K, Cerretti DP, Huynh-Do U. Ligand binding induces Cbl-dependent EphB1 receptor degradation through the lysosomal pathway. Traffic. 2008;9(2):251–66.
376. Fu T, Li P, Wang H, He Y, Luo D, Zhang A, et al. c-Rel is a transcriptional repressor of EPHB2 in colorectal cancer. J Pathol. 2009;219(1):103–13.
377. Mui SH, Hindges R, O'Leary DD, Lemke G, Bertuzzi S. The homeodomain protein Vax2 patterns the dorsoventral and nasotemporal axes of the eye. Development. 2002; 129(3):797–804.
378. Schulte D, Furukawa T, Peters MA, Kozak CA, Cepko CL. Misexpression of the Emx-related homeobox genes cVax and mVax2 ventralizes the retina and perturbs the retinotectal map. Neuron. 1999;24(3):541–53.
379. Furukawa K, Sato T, Katsuno T, Nakagawa T, Noguchi Y, Tokumasa A, et al. Smad3 contributes to positioning of proliferating cells in colonic crypts by inducing EphB receptor protein expression. Biochem Biophys Res Commun. 2011;405(4):521–6.
380. Litterst C, Georgakopoulos A, Shioi J, Ghersi E, Wisniewski T, Wang R, et al. Ligand binding and calcium influx induce distinct ectodomain/gamma-secretase-processing pathways of EphB2 receptor. J Biol Chem. 2007;282(22):16155–63.
381. Xu J, Litterst C, Georgakopoulos A, Zaganas I, Robakis NK. Peptide EphB2/CTF2 generated by the gamma-secretase processing of EphB2 receptor promotes tyrosine phosphorylation and cell surface localization of N-methyl-D-aspartate receptors. J Biol Chem. 2009; 284(40):27220–8.
382. Eriksson O, Ramstrom M, Hornaeus K, Bergquist J, Mokhtari D, Siegbahn A. The Eph tyrosine kinase receptors EphB2 and EphA2 are novel proteolytic substrates of tissue factor/coagulation factor VIIa. J Biol Chem. 2014;289:32379–91.
383. Himanen JP, Henkemeyer M, Nikolov DB. Crystal structure of the ligand-binding domain of the receptor tyrosine kinase EphB2. Nature. 1998;396(6710):486–91.
384. Wybenga-Groot LE, Baskin B, Ong SH, Tong J, Pawson T, Sicheri F. Structural basis for autoinhibition of the Ephb2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region. Cell. 2001;106(6):745–57.
385. Thanos CD, Faham S, Goodwill KE, Cascio D, Phillips M, Bowie JU. Monomeric structure of the human EphB2 sterile alpha motif domain. J Biol Chem. 1999;274(52):37301–6.
386. Thanos CD, Goodwill KE, Bowie JU. Oligomeric structure of the human EphB2 receptor SAM domain. Science. 1999;283(5403):833–6.
387. Zisch AH, Pazzagli C, Freeman AL, Schneller M, Hadman M, Smith JW, et al. Replacing two conserved tyrosines of the EphB2 receptor with glutamic acid prevents binding of SH2 domains without abrogating kinase activity and biological responses. Oncogene. 2000; 19(2):177–87.
388. Wiesner S, Wybenga-Groot LE, Warner N, Lin H, Pawson T, Forman-Kay JD, et al. A change in conformational dynamics underlies the activation of Eph receptor tyrosine kinases. EMBO J. 2006;25(19):4686–96.

389. Catchpole T, Henkemeyer M. EphB2 tyrosine kinase-dependent forward signaling in migration of neuronal progenitors that populate and form a distinct region of the dentate niche. J Neurosci. 2011;31(32):11472–83.
390. Poliakov A, Cotrina ML, Pasini A, Wilkinson DG. Regulation of EphB2 activation and cell repulsion by feedback control of the MAPK pathway. J Cell Biol. 2008;183(5):933–47.
391. Dail M, Richter M, Godement P, Pasquale EB. Eph receptors inactivate R-Ras through different mechanisms to achieve cell repulsion. J Cell Sci. 2006;119(Pt 7):1244–54.
392. Zimmer M, Palmer A, Kohler J, Klein R. EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion. Nat Cell Biol. 2003;5(10):869–78.
393. Bush JO, Soriano P. Ephrin-B1 forward signaling regulates craniofacial morphogenesis by controlling cell proliferation across Eph-ephrin boundaries. Genes Dev. 2010;24(18): 2068–80.
394. Dodelet VC, Pazzagli C, Zisch AH, Hauser CA, Pasquale EB. A novel signaling intermediate, SHEP1, directly couples Eph receptors to R-Ras and Rap1A. J Biol Chem. 1999;274(45):31941–6.
395. Nakada M, Niska JA, Tran NL, McDonough WS, Berens ME. EphB2/R-Ras signaling regulates glioma cell adhesion, growth, and invasion. Am J Pathol. 2005;167(2):565–76.
396. Zou JX, Wang B, Kalo MS, Zisch AH, Pasquale EB, Ruoslahti E. An Eph receptor regulates integrin activity through R-Ras. Proc Natl Acad Sci USA. 1999;96(24):13813–8.
397. Elowe S, Holland SJ, Kulkarni S, Pawson T. Downregulation of the Ras-mitogen-activated protein kinase pathway by the EphB2 receptor tyrosine kinase is required for ephrin-induced neurite retraction. Mol Cell Biol. 2001;21(21):7429–41.
398. Genander M, Halford MM, Xu NJ, Eriksson M, Yu Z, Qiu Z, et al. Dissociation of EphB2 signaling pathways mediating progenitor cell proliferation and tumor suppression. Cell. 2009;139(4):679–92.
399. Pasquale EB. Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol. 2005;6(6):462–75.
400. Darie CC, Deinhardt K, Zhang G, Cardasis HS, Chao MV, Neubert TA. Identifying transient protein-protein interactions in EphB2 signaling by Blue Native PAGE and Mass Spectrometry. Proteomics. 2011;11:4514–28.
401. Shi Y, Pontrello CG, DeFea KA, Reichardt LF, Ethell IM. Focal adhesion kinase acts downstream of EphB receptors to maintain mature dendritic spines by regulating cofilin activity. J Neurosci. 2009;29(25):8129–42.
402. Hoogenraad CC, Milstein AD, Ethell IM, Henkemeyer M, Sheng M. GRIP1 controls dendrite morphogenesis by regulating EphB receptor trafficking. Nat Neurosci. 2005;8(7):906–15.
403. Tanaka M, Kamo T, Ota S, Sugimura H. Association of Dishevelled with Eph tyrosine kinase receptor and ephrin mediates cell repulsion. EMBO J. 2003;22(4):847–58.
404. Marston DJ, Dickinson S, Nobes CD. Rac-dependent trans-endocytosis of ephrinBs regulates Eph-ephrin contact repulsion. Nat Cell Biol. 2003;5(10):879–88.
405. Altick AL, Dravis C, Bowdler T, Henkemeyer M, Mastick GS. EphB receptor tyrosine kinases control morphological development of the ventral midbrain. Mech Dev. 2005; 122(4):501–12.
406. Garcia-Ceca J, Jimenez E, Alfaro D, Cejalvo T, Munoz JJ, Zapata AG. Cell-autonomous role of EphB2 and EphB3 receptors in the thymic epithelial cell organization. Eur J Immunol. 2009;39(10):2916–24.
407. van der Flier LG, van Gijn ME, Hatzis P, Kujala P, Haegebarth A, Stange DE, et al. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell. 2009; 136(5):903–12.
408. Wu Q, Lind GE, Aasheim HC, Micci F, Silins I, Trope CG, et al. The EPH receptor Bs (EPHBs) promoters are unmethylated in colon and ovarian cancers. Epigenetics. 2007; 2(4):237–43.
409. Hock B, Bohme B, Karn T, Feller S, Rubsamen-Waigmann H, Strebhardt K. Tyrosine-614, the major autophosphorylation site of the receptor tyrosine kinase HEK2, functions as multidocking site for SH2-domain mediated interactions. Oncogene. 1998;17(2):255–60.

410. Hock B, Bohme B, Karn T, Yamamoto T, Kaibuchi K, Holtrich U, et al. PDZ-domain-mediated interaction of the Eph-related receptor tyrosine kinase EphB3 and the ras-binding protein AF6 depends on the kinase activity of the receptor. Proc Natl Acad Sci USA. 1998;95(17):9779–84.
411. Solanas G, Cortina C, Sevillano M, Batlle E. Cleavage of E-cadherin by ADAM10 mediates epithelial cell sorting downstream of EphB signalling. Nat Cell Biol. 2011;13(9):1100–7.
412. Miao H, Strebhardt K, Pasquale EB, Shen TL, Guan JL, Wang B. Inhibition of integrin-mediated cell adhesion but not directional cell migration requires catalytic activity of EphB3 receptor tyrosine kinase. Role of Rho family small GTPases. J Biol Chem. 2005; 280(2):923–32.
413. Maddigan A, Truitt L, Arsenault R, Freywald T, Allonby O, Dean J, et al. EphB receptors trigger Akt activation and suppress Fas receptor-induced apoptosis in malignant T lymphocytes. J Immunol. 2011;187(11):5983–94.
414. Zhuang Z, Yang B, Theus MH, Sick JT, Bethea JR, Sick TJ, et al. EphrinBs regulate D-serine synthesis and release in astrocytes. J Neurosci. 2010;30(47):16015–24.
415. Kamitori K, Tanaka M, Okuno-Hirasawa T, Kohsaka S. Receptor related to tyrosine kinase RYK regulates cell migration during cortical development. Biochem Biophys Res Commun. 2005;330(2):446–53.
416. Risley M, Garrod D, Henkemeyer M, McLean W. EphB2 and EphB3 forward signalling are required for palate development. Mech Dev. 2009;126(3–4):230–9.
417. Chrencik JE, Brooun A, Kraus ML, Recht MI, Kolatkar AR, Han GW, et al. Structural and biophysical characterization of the EphB4*ephrinB2 protein-protein interaction and receptor specificity. J Biol Chem. 2006;281(38):28185–92.
418. Steinle JJ, Meininger CJ, Forough R, Wu G, Wu MH, Granger HJ. Eph B4 receptor signaling mediates endothelial cell migration and proliferation via the phosphatidylinositol 3-kinase pathway. J Biol Chem. 2002;277(46):43830–5.
419. Yu J, Bulk E, Ji P, Hascher A, Tang M, Metzger R, et al. The EPHB6 receptor tyrosine kinase is a metastasis suppressor that is frequently silenced by promoter DNA hypermethylation in non-small cell lung cancer. Clin Cancer Res. 2010;16(8):2275–83.
420. Matsuoka H, Obama H, Kelly ML, Matsui T, Nakamoto M. Biphasic functions of the kinase-defective Ephb6 receptor in cell adhesion and migration. J Biol Chem. 2005; 280(32):29355–63.
421. Freywald A, Sharfe N, Rashotte C, Grunberger T, Roifman CM. The EphB6 receptor inhibits JNK activation in T lymphocytes and modulates T cell receptor-mediated responses. J Biol Chem. 2003;278(12):10150–6.
422. Allonby O, El Zawily AM, Freywald T, Mousseau DD, Chlan J, Anderson D, et al. Ligand stimulation induces clathrin- and Rab5-dependent downregulation of the kinase-dead EphB6 receptor preceded by the disruption of EphB6-Hsp90 interaction. Cell Signal. 2014; 26(12):2645–57.

Chapter 6
The FGFR Receptor Family

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Abbreviations

ADAM	A Disintegrin And Metalloprotease
CAD	Coronary Artery Disease
CBL	Casitas B-lineage Lymphoma Protein
CLR-1	Cryptic Loci Regulator
c-MYC	Cellular-Myelocytomatosis Oncogene
COSMIC	Catalogue of Somatic Mutations in Cancer
CS	Chondroitin Sulphate
DAG	Diacylglycerol
DOF	Downstream of FGFR
EGL	Egg Laying Abnormal
EOC	Epithelial Ovarian Cancer
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FGFRL1	FGFR-Like 1
FRS2	Fibroblast Growth Factor Receptor Substrate 2
GAB1	GRB2-Associated Binding protein 1
GAG	Glycosaminoglycan
GAS	Gamma-Activated Site
GEF	Guanine Exchange Factor

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GRB2	Growth Factor Receptor-Bound Protein 2
HS	Heparan Sulphate
HSPG	Heparan Sulphate Proteoglycan
IGF	Insulin-like Growth Factor
INFS	Integrative Nuclear FGFR1 Signalling
IP_3	Inositol trisphosphate
JAK	Janus Kinase
KDR	Kinase Insert Domain Receptor
LADD	Lacrimo-Auriculo-Dento-Digital
LET-756	Lethal Protein 756
MAPK	Mitogen-Activated Protein Kinase
MEK	ERK Kinase
MMP	Metalloprotease
MPS	Myeloproliferative Syndrome
NBR1	Neighbor of BRCA1
NCAM	Neural Cell Adhesion Molecule
NLS	Nuclear Localisation Signal
PI3K	Phosphoinositide-3 Kinase
PIP_2	Phosphatidyl-inositol-4, 5-bisphosphate
PIP_3	Phosphatidyl-inositol (3, 4, 5)-trisphosphate
PKC	Protein Kinase C
PLCγ	Phospholipase C γ
PTB	Phosphotyrosine Binding
Rab5	Ras-Related Proteins in Brain 5
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat Sarcoma
RhoG	Ras Homology Growth-Related
RTK	Receptor Tyrosine Kinase
S4	Syndecan 4
SEF	Similar Expression to FGF
SH2	Src Homology 2
SH3	SRC Homology 3
SNP	Single Nucelotide Polymorphism
SOS	Son of Sevenless
SPRED	Sprouty-Related Enabled/Vasodilator-stimulated Phosphoprotein Homology 1 Domain-Containing Protein
SPRY	Sprouty
STAT	Signal Transducer and Activator of Transcription
TGFβ	Transforming Growth Factor β
TM	Transmembrane
VEGFR	Vascular Endothelial Growth Factor Receptor
XFLRT3	Xenopus Fibronectin Leucine-Rich Transmembrane Protein 3

6.1 Introduction

Fibroblast growth factors (FGFs) exert their cellular effects by interacting with FGF receptors (FGFRs) in a complex with heparan sulphate (HS) [1]. FGFRs, a class of receptor tyrosine kinase (RTK), dimerise and undergo transphosphorylation of the kinase domain upon ligand binding [2], leading to the recruitment of adapter proteins and initiating downstream signalling.

The extended FGF family is composed of 22 members, varying in size from 17 to 34 kDa. All members share a conserved 120 amino acid sequence and show 16–65 % sequence homology [3]. However, only eighteen FGFs signal via FGFR interactions (FGF1–10 and 16–23), while FGF11–14, which lack a signal peptide, act in an intracellular manner. Thus, many consider the FGF family to comprise only 18 members. Furthermore, although they are numbered from 1 to 23, FGF15 is the mouse ortholog of human FGF19. Each ligand binds to FGFRs with varying specificity; some are promiscuous, for example FGF1, and bind to multiple receptors, while others, like FGF7, bind only to one receptor isoform [4] (Fig. 6.1).

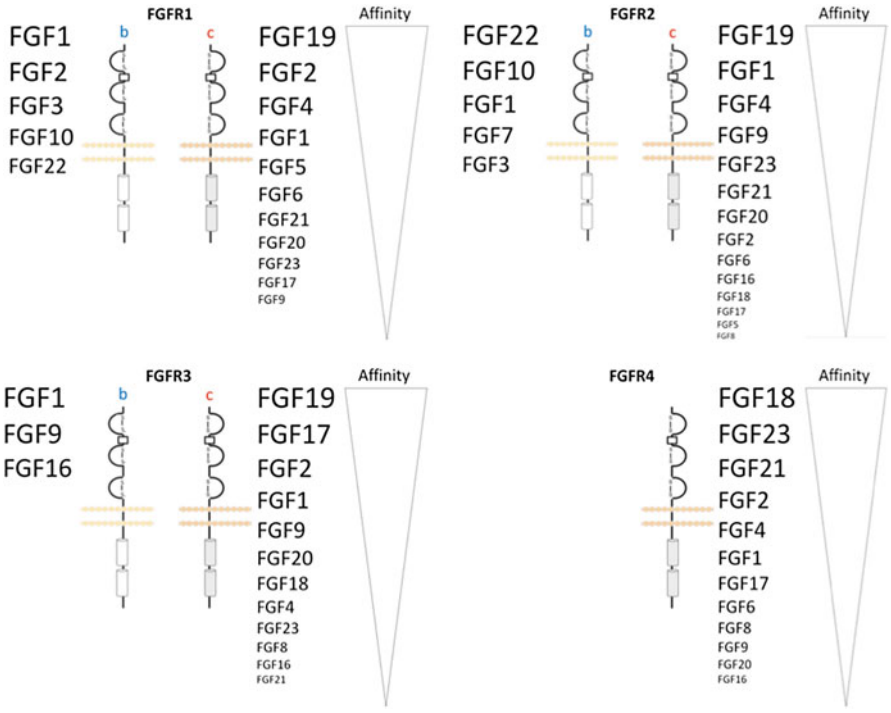

Fig. 6.1 FGFR isoform ligand specificity. Each receptor isoform has varying affinities for the FGF family of ligands. This variation is dictated by alternative splicing of the receptor. Binding affinities taken from [4, 5]

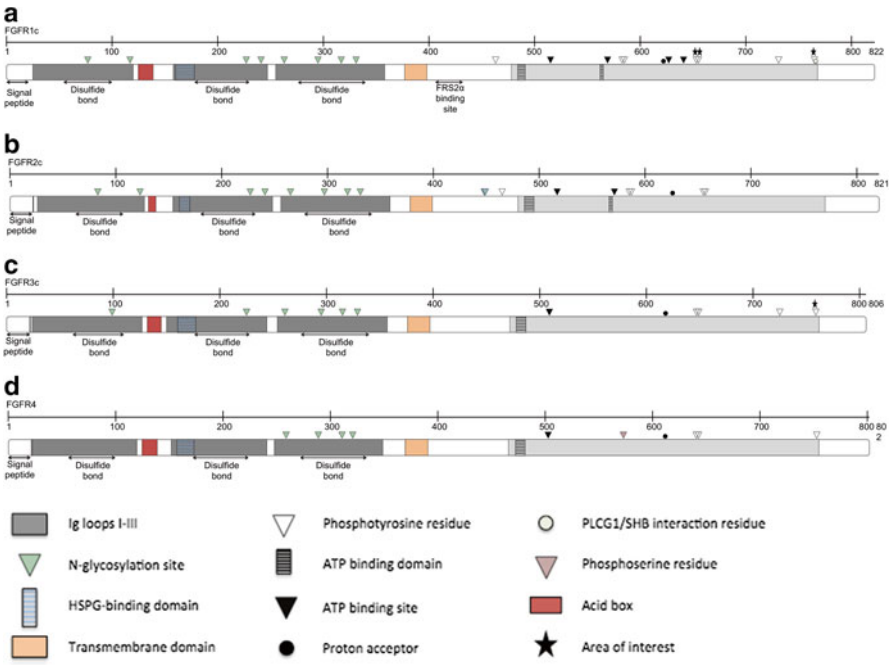

Fig. 6.2 Schematic representation of FGFR1–4. FGFR1–4 contain a variety of defined structural domains, some of which are highly conserved across the receptors and their individual isoforms. The receptors are encoded by genes found on chromosomes 8, 10, 4 and 5, respectively. Three isoforms, termed a, b and c, exist for FGFR1 and 2; only isoforms b and c function in a signalling capacity. FGFR3 has two isoforms, b and c, while only one isoform of FGFR4 exists. These isoforms are generated through alternative splicing. Only one isoform of each receptor is shown. Amino acid residue numbers are indicated at the top of each panel, including a 21–22 amino acid signal sequence (UniProt accession: P11362, P21802, P22607, P22455, respectively)

There are seven signalling receptors, encoded by four *FGFR* genes, *FGFR1–4* [6]. FGFRs 1–3 have highly conserved intron/exon boundaries [4] (Fig. 6.2).

Alternative splicing of exons 8 and 9, encoding IgIII of FGFR1–3, results in translation of two distinct isoforms capable of signal transduction. These isoforms are termed IIIb and IIIc, depending on which exons are spliced out (Fig. 6.3). This third Ig loop encodes the ligand binding domain; alternative splicing of this region is responsible for ligand binding specificity (Fig. 6.1). A third isoform exists for FGFR1 and 2, termed IIIa. This variant results in a truncated, secreted protein, which is unable to transduce a signal and may have an autoinhibitory role in FGF signalling, possibly by sequestering ligands [8]. FGFR4 is distinct in that it has only one isoform, homologous to the IIIc variant of FGFR1–3 [9].

Receptor expression is generally cell type specific, for example IIIb and IIIc isoforms of FGFR1 and 2 are expressed in epithelial and mesenchymal cells, respectively [10, 11]. However, as shall be discussed later, this cell type specificity can change when FGFRs are associated with diseases such as cancer.

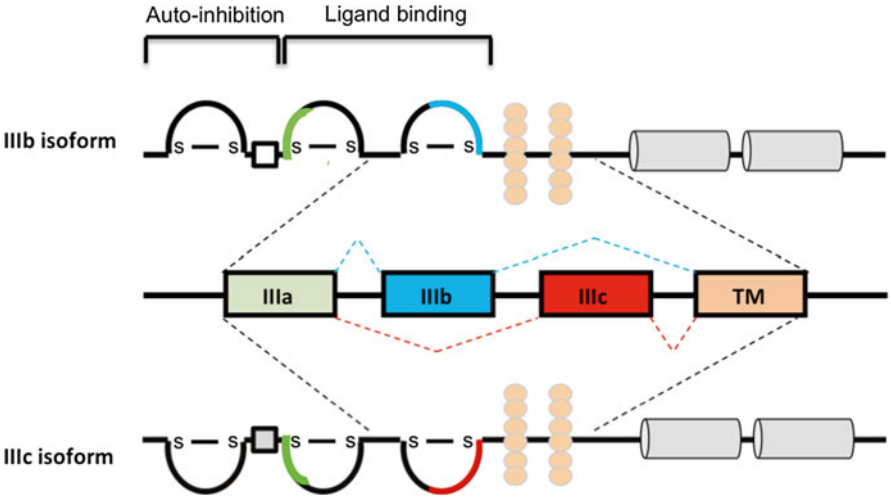

Fig. 6.3 FGFR structure, control of ligand specificity and receptor autoinhibition via alternative splicing. Each receptor monomer is comprised of an extracellular domain including three Ig loops, IgI, IgII and IgIII (also referred to as D1, D2 and D3, respectively), an acid box in the IgI–IgII linker region (represented by a *white box*), a transmembrane domain and an intracellular split kinase domain. Disulphide bonds are present in each Ig loop. IgI and the acid box are involved in autoinhibition of the receptor, while IgII and IgIII are involved in ligand binding. The HS-binding site lies in IgII, indicated in *green*. Ligand binding specificity is generated by alternative splicing of the IgIII domain. The first half of IgIII is encoded by an invariant exon (IIIa), which is spliced to either exon IIIb or IIIc (represented in blue and red, respectively), both of which splice to the exon that encodes the transmembrane domain (TM) region. Epithelial tissues predominantly express the IIIb isoform and mesenchymal tissues express IIIc. FGFR4 is expressed as a single isoform that is paralogous to FGFR-IIIc. An additional alternative splicing event can occur leading to the deletion of exons coding for IgI and/or the acid box/linker region. This leads to loss of receptor autoinhibition [7]

6.2 FGF:FGFR:HS Complex

Heparin, used in vitro as the model heparan sulphate (HS), is a member of the HS family of proteoglycans (HSPGs) and has been used to establish the necessity of HS binding in FGF:FGFR:HS complex formation [12]. This acidic molecule resembles the highly sulphated saccharide chains of HS [13]. Upon binding to FGFs/FGFRs, HS saccharide chains induce a conformational change. The length of the saccharide chain is important in FGF–FGFR interactions. Ornitz and colleagues reported interaction of a dodecasaccharide with both high- and low-affinity heparin-binding sites of ligands and showed that octasaccharides, thought to be the smallest saccharides with biological activity in FGF–FGFR interactions, could only engage the low-affinity binding sites of the ligand [14]. However, others have postulated that smaller chains, including hexasaccharides and disaccharides, may have biological activity [13, 15]. The heparin-binding residues found in the IgII loop of FGFRs (Fig. 6.2)

are highly conserved [16], while heparin-binding residues of FGFs are diverse. Because of this, different FGFs require various HS sulphation patterns and/or length of chains for their optimum activity. Variability of HS sulphation patterns and length across cell types has an effect on FGF–FGFR interactions and may be a mediator of the biological activity of FGFRs [13–15, 17].

Another highly sulphated glycosaminoglycan (GAG), chondroitin sulphate (CS), is also able to interact with FGFs and FGFRs to promote complex formation. Studies have shown that insufficient synthesis of GAGs, which are assembled in the Golgi, impairs FGF/FGFR signalling capabilities [18]. The sulphation pattern and chain length of GAGs is so variable that there may be tissue- and even cell-specific GAG chains with varying specificities for ligands and receptors [19]. The difference in these chains may be of particular importance in the regulation of FGF/FGFR signalling. Work by a number of groups has also shown that variations in GAG sequences capable of interacting with FGFs and FGFRs can both inhibit or facilitate FGF signalling [17, 20–23].

A widely accepted model of FGFR interactions [16] proposed a complex of FGF:FGFR:HS in a 2:2:2 ratio (Fig. 6.4a). Two independent FGF:FGFR:HS ternary complexes are formed in a 1:1:1 ratio via HS binding to both receptor and ligand.

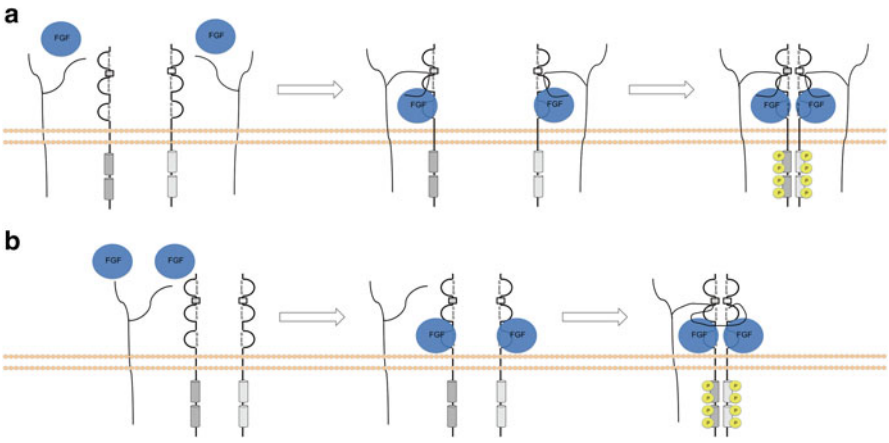

Fig. 6.4 Alternative FGF:FGFR:HS models. The basic structure of the FGF:FGFR complex comprises two receptor molecules and two ligands. Two models are presented which differ in the number of heparan sulphate proteoglycans (HSPGs) they contain. Dimerisation occurs upon ligand binding, leading to autophosphorylation of the kinase domain and subsequent downstream signalling. (**a**) FGF:FGFR:HS 2:2:2 model. First presented by Schlessinger et al. [16], and taken to be the most biologically relevant, this model proposes a symmetrical dimer utilising two HSPGs which bind ligands, bringing them into close proximity with the receptor, facilitating dimerisation. The HS chains also bind to the heparan-binding site of the IgII loop of the receptor to form a complete, active molecule capable of autophosphorylation and subsequent phosphorylation of signalling molecules. (**b**) FGF:FGFR:HS 2:2:1 model. Proposed by Pellegrini et al. [24], this dimer is formed using only one HSPG which binds both ligands necessary for each receptor monomer. Dimerisation occurs with the HS chain binding to both ligands and receptors, leading to signalling cascade activation

They bind via receptor interactions, as well as interactions between the ligand in one complex and the receptor in another, thus forming a stable, symmetrical dimer. Direct ligand–ligand interactions are not observed. This FGF-FGFR complex can only be formed in the presence of HS. In summary, stabilisation of the dimer is through the following interactions: receptor–ligand, receptor–HS, ligand–HS and receptor–receptor.

A second model [24] proposed FGF:FGFR:HS complex formation in a 2:2:1 ratio (Fig. 6.4b). Crystal structure analysis of FGFR2–FGF1 interactions showed a central heparin molecule linking two ligands and two receptor molecules. In this model, each ligand binds to a receptor monomer with heparin interacting with both ligands but only one receptor molecule. Two 1:1 FGF:FGFR complexes are joined to form a dimer via interactions with one HS chain.

6.3 Signalling Pathways

Upon dimerisation, reciprocal phosphorylation of the tyrosine kinase domains of the receptors occurs. These phosphorylated receptors are then able to act as docking sites for intracellular proteins, leading to activation of signalling cascades (Fig. 6.5) [25–27]. This autophosphorylation occurs in a specific order; 'first-phase' phosphorylation increases the catalytic activity of the kinase after ligand binding, while 'second-phase' phosphorylation creates phosphotyrosine-binding sites for docking molecules containing Src homology-2 (SH2) and phosphotyrosine-binding (PTB) domains [25, 28]. From this, four signalling pathways can be activated: MAP Kinase (MAPK), PI3K/AKT, PLCγ and STAT [25]. The key difference between FGFRs in signalling is the strength of their tyrosine kinase activity; their target proteins are the same [29].

The lipid-anchored adapter protein FRS2 plays an integral role in the MAPK and PI3K/AKT pathways. FRS2α binds to the receptor via its PTB domain [30, 31] and undergoes phosphorylation. GRB2, another adapter molecule, is then recruited to FRS2α. From this point, two FGF-induced signalling pathways can be activated:

6.3.1 Phosphoinositide-3 Kinase

GRB2/FRS2α binds to and phosphorylates GAB1 via the SH3 domain of GRB2 [32]. This FRS2α/GRB2/GAB1 complex recruits PI3K via the SH2 domain of its p85 subunit. Activated PI3K produces phosphatidyl-inositol (3, 4, 5)-trisphosphate (PIP3), resulting in activation of the AKT pathway. Anti-apoptotic signalling, as well as cell growth and proliferation, is then initiated [32].

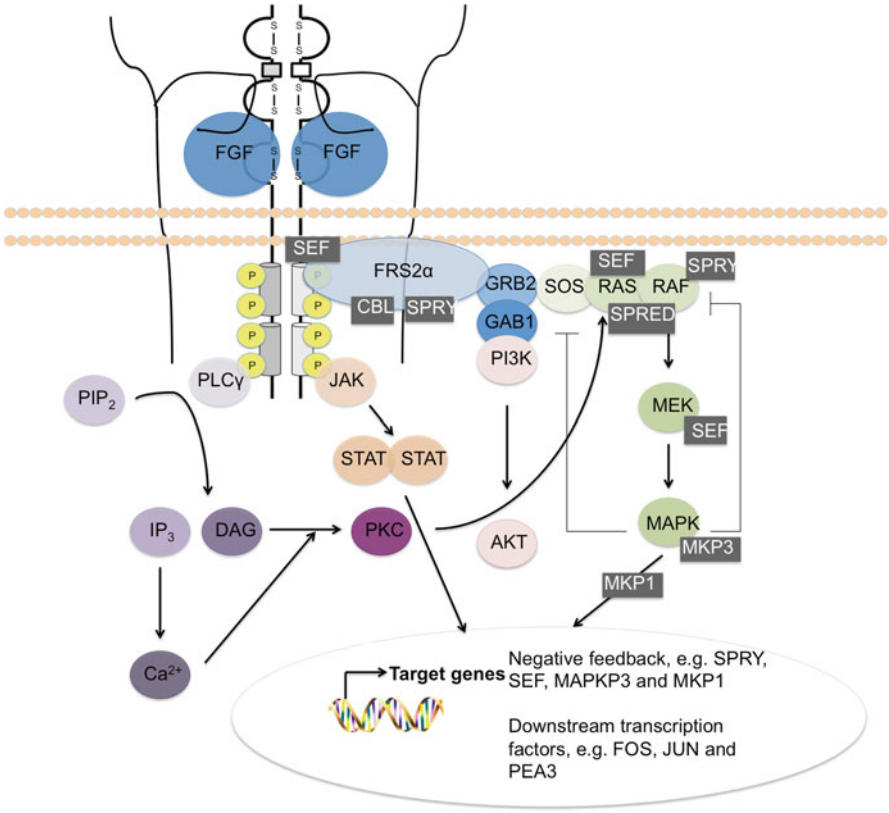

Fig. 6.5 FGF:FGFR-induced downstream signalling. Ligand-receptor binding induces four signalling cascades: MAPK, PI3K/AKT, PLCγ and STAT. These pathways comprise a series of phosphorylation events, culminating in the regulation of target genes, which dictate cellular processes, for example proliferation and migration

6.3.2 Mitogen-Activated Protein Kinase

Activation of the mitogen-activated protein kinase (MAPK) pathway results in mitogenic activity and cell survival [33]. The MAPK pathway is initiated by RAS binding to the FGFR/FRS2α/GRB2/SOS complex. RAS then recruits and phosphorylates RAF, leading to phosphorylation of MEK (MAPK/ERK kinase) and subsequent phosphorylation and activation of MAPK [33]. MAPK, also known as Extracellular Signal-Regulated Kinase (ERK), is then able to activate transcription factors in the nucleus, for example c-MYC, and influence the cell cycle.

The PLCγ and STAT pathways are mediated through other mechanisms.

6.3.3 Phospholipase C γ

Autophosphorylation of FGFR residue Tyr766 in FGFR1 creates a specific binding site for the SH2 domain of phospholipase Cγ (PLCγ), leading to tyrosine phosphorylation of PLCγ [34]. Recruitment of PLCγ is aided by PIP_3, generated in response to PI3K stimulation [35]. Activation of PLCγ leads to cleavage of phosphotidyl-inositol-4, 5-bisphosphate (PIP_2) into the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) [36]. IP_3 then releases calcium stores from the endoplasmic reticulum (ER) [37]. Calcium ions, along with DAG, then activate protein kinase C (PKC). PKC is then able to phosphorylate RAF and activate the MAPK pathway.

6.3.4 Signal Transducer and Activator of Transcription

The STAT family of cytoplasmic transcription factors can be activated by non-receptor tyrosine kinases, the Janus Kinases (JAK), leading to cell proliferation, differentiation or apoptosis [38]. Upon FGFR dimerisation and autophosphorylation, JAKs are phosphorylated by the receptor, forming a FGFR/JAK complex. This acts as a docking site for STATs, which are in turn tyrosine phosphorylated in their SH2 domain [39]. STAT dimers form and translocate to the nucleus, where they bind to gamma-activated site (GAS) enhancers to activate or repress gene transcription [39].

6.4 Regulation of FGF Signalling

Regulation of FGF signalling is critical to ensure a balanced response to receptor stimulation. This occurs largely through four mechanisms:

6.4.1 Receptor Internalisation

CBL, a multidomain protein that possesses an intrinsic ubiquitin ligase activity [40], binds to the FRS2α/GRB2 complex via the SH3 domain of GRB2 and the proline-rich region of CBL. Recruitment of CBL to FRS2α leads to ubiquitination of both FGFR and FRS2α and therefore attenuation of FGFR-mediated signalling.

6.4.2 Receptor Cleavage

Numerous growth factor receptors undergo ectodomain shedding, a process known to downregulate signalling. Ectodomain shedding, or S1 cleavage, is a process of proteolytic cleavage either within or near the membrane by members of the metalloprotease (MMP) and A Disintegrin And Metalloproteinase (ADAM) family [41]. Induction of this cleavage occurs in response to receptor activation [42]. Cleavage within the transmembrane domain by γ-secretase, known as S2 cleavage, often follows. Together, these cleavage events are known as Regulated Intramembrane Proteolysis [43].

FGFR1 is cleaved by MMP2 [44], and FGFR2 can be targeted by ADAM9 or 15 [45, 46]. Cleavage of both receptors leads to attenuation of signalling via two main mechanisms: downregulation of the number of active receptors at the cell surface and generation of a soluble extracellular domain able to compete with membrane-bound receptors for ligand binding [47]. Interestingly, FGFR1 also can be cleaved intracellularly by the serine protease Granzyme B. Although this was reported initially as a means of cytotoxic T lymphocytes inducing target cell apoptosis [48], the process is hijacked in cancer cells to allow nuclear trafficking of the C-terminus of the receptor, which acts to regulate transcription of a pro-migratory gene signature [49].

FGFR3 is unique in that the S1 cleavage occurs in an endosomal compartment, where it is cleaved by an as yet unknown protease, rather than involving a member of the ADAM family [41]. S2 cleavage via γ-secretase then occurs, generating a soluble intracellular domain capable of trafficking to the nucleus. Here, the nuclear FGFR3 fragment may be responsible for novel interactions in addition to the well-established downstream signalling pathways of receptor activation.

6.4.3 Induction of Negative Regulators

The first identified negative regulator of FGFRs was sprouty (SPRY) [50], one of a family of four proteins. SPRYs are thought to act through one of two mechanisms. Firstly, they may interact with GRB2, interrupting the FRS2α/GRB2 complex and therefore decreasing signal transduction [51]. Alternatively, SPRY–RAF interactions may occur, preventing RAF phosphorylation and therefore inhibiting MAPK signalling [52].

MAPK signalling can also be inhibited by Sprouty-related Enabled/vasodilator-stimulated phosphoprotein Homology 1 Domain-containing proteins (SPRED1 and 2) [53]. SPRED proteins prevent RAF activation of MEK by forming a complex between RAS and RAF. Co-localisation of SPRED2 with the protein Neighbor of BRCA1 (NBR1) results in sequestration of FGFR and lysosomal degradation [54].

Similar Expression to FGF (SEF) proteins also negatively regulate FGF signalling via a number of mechanisms: targeted inhibition at or downstream of MEK [55]; inhibition of RAS activation, which also inhibits the PI3K pathway [56];

direct interaction with FGFR and subsequent inhibition of FGFR and FRS2α phosphorylation [56–58]; and blockage of ERK/MEK dissociation, where SEF acts as a spatial regulator of phospho-ERK migration to the nucleus [59].

Another mechanism of negative regulation is via direct phosphorylation of MAPK pathway proteins. For example, SOS and RAF are substrates of MAPK. Phosphorylation of SOS by MAPK disrupts interactions between SOS and GRB2. This decreases recruitment of SOS to the membrane and results in diminished RAS activation [60]. MAPK also phosphorylates RAF, reducing RAF kinase activity and therefore decreasing MEK and MAPK phosphorylation [61]. Induction of the MAPK pathway can also lead to attenuation of the PI3K/AKT pathway. Activation of MAPK leads to GAB1 phosphorylation. This decreases PI3K recruitment to GAB1, in turn reducing AKT pathway activation [62].

Alternative internal control mechanisms of FGF signalling exist, including autoinhibition of the receptor [16, 63, 64]. The FGFRs exist in 'closed' and 'open' conformation equilibrium [7]. The first Ig loop (IgI) and the IgI/IgII linker region containing the acid box, a glutamate, aspartate and serine-rich sequence [6], are responsible for formation of the 'closed', autoinhibited state. Spectroscopic investigations have shown the acid box engages in electrostatic interactions with the HS-binding site of the IgII loop, inhibiting receptor–HS interactions and, therefore, receptor activation. This then encourages intramolecular interactions between IgI and the ligand-binding sites of the IgII and IgIII loops, further aiding the acquisition of a closed conformation [65]. Alternative splicing of exons encoding the IgI and/or acid box region leads to enhanced affinity of the receptor for its ligand and HS, increasing downstream signalling [65]. Loss of this region has been implicated in cancer [66, 67]. This mechanism of autoinhibition supports FGF binding specificity of receptors as only specific ligands with high affinity for the receptors will overcome the inhibition and bind to the receptor.

6.4.4 Klotho Interactions

FGFRs can also interact with klotho family proteins. These senescence-related, single-pass transmembrane proteins function as FGF19 subfamily signalling cofactors. FGFs are split into seven subfamilies, with the FGF19 subfamily comprising FGF19, 21 and 23. These endocrine factors regulate metabolic processes [68]. The HS-binding sites of this subfamily differ greatly from other FGFs, reducing their affinity for HS [69, 70]. Because of this, they require Klotho as a cofactor, to signal through FGFRs. Klotho expression is confined to a limited number of tissues [71]. It is able to bind FGFR1c independent of HS binding and convert it into a FGF23 receptor in the kidney [72]. Mutations in klotho proteins or the FGF19 subfamily are associated with diseases including autosomal dominant hypophosphatemic rickets, premature ageing disorders and diabetes [73]. Klotho is able to actively compete with FGF2 for FGFR1c binding, therefore attenuating FGF2 signalling [72].

6.5 FGFRs in Development

The critical role played by FGFR signalling during embryogenesis is highlighted by its conservation throughout evolution, from invertebrates through to higher mammals. There are a number of reviews that provide exquisite detail on FGFR signalling in a wide range of model organisms, including *Caenorhabditis elegans* [74], *Drosophila melanogaster* [75] and vertebrates [76–78]. However, we highlight below some key findings in the major model organisms.

6.5.1 Caenorhabditis elegans

C. elegans has just one FGFR, EGL-15, which was identified in mutant screens as a result of its importance in the migration of hermaphrodite sex myoblasts [79]. The EGL-15 receptor is essential for sensing the chemoattractant FGF ligand EGL-17, expressed in the target gonad and vulva [80]. Another FGF ortholog, LET-756, which shows structural homology to the FGF-9 subfamily [81], is essential for larval viability [82]. Further elements of the downstream signalling pathway were elucidated with the identification of a receptor tyrosine phosphatase, CLR-1 [83], and components of the MAPK cascade [84], which are key to FGF signalling in the worm, as they are in other model organisms. Interestingly, the FGF co-receptor Klotho has two functional orthologs in the worm, and these are essential in mediating the longevity and stress resistance effects of EGL-15/EGL-17 signalling [85]. Beyond the scope of this chapter, there are a number of non-canonical FGFR interactions in the worm that are the subject of an elegant review elsewhere [74].

6.5.2 Drosophila melanogaster

The tracheal system in *Drosophila* has been a key system for the identification of aspects of the FGF signalling pathway over the past 30 years. Breathless—one of two FGFRs in the fly—regulates tracheal branching [86], acting in concert with its cognate FGF ligand, branchless [87], to activate downstream MAPK signalling [75]. Further genetic dissection of the branching process identified Sprouty as a negative regulator of FGFR signalling [50] and described how the Notch pathway interacts with FGFR signalling in controlling cell fate [88], although orthologs of other negative regulators of the FGFR pathway, Sef and XFLRT3, are not present in invertebrates [89].

A further FGFR ortholog, Heartless, was identified by virtue of its pivotal role in mesoderm migration and subsequent specification [90, 91], although its ligands, the FGF-8 orthologs Pyramus and Thisbe, were not identified until much later [92, 93]. Downstream of FGF signalling, a novel adapter, Dof, is critical for activating intracellular signalling [94], in much the same fashion that FRS2 acts in the vertebrate pathway.

6.5.3 *Zebrafish*

FGF signalling is an important factor in patterning the zebrafish embryo, interacting with signalling by TGF-β superfamily members to regulate mesoderm induction [95]. FGF signalling acts as a posteriorising factor driving trunk and tail development during anterior–posterior patterning [96], regulating downstream T-box family transcription factors Notail and Spadetail [97], and also regulates dorso-ventral patterning [98].

One of the main regulators of FGF signalling, SEF, was found first in zebrafish [57, 99], and fundamental understanding of the roles for FGF-8 signalling in neural development has been identified through the study of mutant zebrafish strains [100–102].

6.5.4 *Xenopus*

The first studies of FGF signalling in early development focused on its role as a competence factor, using *Xenopus* as a model system and showing that cell fate in the developing embryo was regulated by FGFs [103, 104]. FGF signalling was shown to be essential for cells to respond to mesoderm inducing TGF-β superfamily members [105, 106], and components of the entire pathway, from FGFRs to HSPGs to signal transduction proteins, have all been studied in detail in the frog [76]. Defects caused when FGFR signalling is inhibited, by small molecule inhibition [107], morpholino knockdown [108, 109] or expression of dominant negative receptor [105, 110, 111], confirm its fundamental importance in mesoderm induction, morphogenetic movements, neural induction, neuronal determination and anterior–posterior patterning.

6.5.5 *Chick*

The ability to manipulate and culture chick embryos has helped reveal several key roles for FGFR signalling, including elegant grafting studies showing the importance of FGFR activity in specifying and driving limb development [112, 113] and dynamic studies of presomitic mesoderm determination and subsequent somitogenesis [114–116]. Furthermore, FGFR signalling has been shown to act as a competence factor for neural induction [117, 118].

6.5.6 *Mouse*

All of the FGFs and FGFRs have been targeted using genetically modified mouse models, with approaches including germline deletion, conditional knockout and constitutive/inducible expression of either dominant negative or activating mutation

constructs. The phenotypes of FGF ligand knockout mice are summarised elsewhere [119]. Extensive studies have revealed key roles for FGFRs in development, homeostasis and disease, and these are detailed in Table 'FGFR1–4 at a glance'.

6.6 FGFR1

FGFR1 is used as the model receptor in the majority of studies and many of the findings are relevant to all FGFRs.

Syndecan 4 (S4), a transmembrane proteoglycan with extracellular HS chains, can regulate FGFR1 signalling, as well as signal independently as a growth factor receptor, to initiate cell adhesion and migration [120]. Recent work has hypothesised S4 could also be involved in FGFR1 trafficking [121]. S4 has a PDZ-binding domain, which is capable of activating the small GTPase, RhoG. RhoG is kept in a complex with S4 in its inactive form. Upon FGF binding to FGFR1, aided by the HS chains of S4, a ligand–receptor–S4 complex is formed. Signalling pathways of the individual receptors are initiated, for example MAPK from FGFR1. Upon this complex formation, RhoG is released from S4 and is activated by guanine exchange factors (GEFs). This induces membrane ruffling, leading to macropinocytosis of the complete FGF–FGFR–S4 complex. Trafficking of the internalised complex is dependent on another small GTPase, Rab5. When Rab5 function is absent, the vesicles cannot mature and become functional signalling endosomes. In this scenario, the MAPK pathway is not activated. However, when Rab5 activity is restored and localises to the macropinosome containing the FGF-FGFR-S4 complex, maturation of the vesicle is facilitated and MAPK signalling is activated. When S4 is absent, RhoG activity is high, leading to increased macropinocytosis and therefore receptor internalisation. S4 controls the rate of FGF–FGFR–S4 complex macropinocytosis; overactive S4 and Rab5 can lead to inadequate attenuation of the MAPK signal leading to continuous downstream signalling effects, for example cell migration. Hence, a novel method of FGFR1 MAPK signalling regulation via S4-medated trafficking is proposed.

Nuclear localisation of both FGFs and FGFRs has been reported in a number of cell lines and tissues [122]. The mechanism of nuclear translocation of FGFR1 has recently been elucidated by Chioni & Grose.

Studies have also shown Importin β is involved in FGFR1 nuclear translocation [123]. It is proposed that this occurs via the Integrative Nuclear FGFR1 Signalling (INFS) pathway [124]. FGFR1 is released from the cytoplasmic membrane into the cytosol. As it does not contain a nuclear localisation signal (NLS), FGFR1 associates with Importin β, a carrier protein that does. FGFR1 can then be transported into the nucleus where it is able to influence expression of, for example, C-JUN.

6.7 FGFR2

Developmental disorders are commonly associated with FGFR mutations, including Kallmann and Lacrimo-Auriculo-Dento-Digital (LADD) syndromes [125]. In skeletal disorders, for example, Crouzon, Pfiffer and Jackson–Weiss syndromes, receptor

mutations tend to cluster in the linker region, connecting IgII and IgIII, and in both IgIII and the IgIII-transmembrane domain linker, functioning by either promoting receptor dimerisation or altering ligand–receptor specificity. Mutations in two conserved cysteine residues in IgIII of FGFR2 are commonly found in these skeletal disorders [33]. These cysteine residues usually function by linking to another cysteine in IgIII of the receptor via intramolecular bonds. Substitution of this amino acid with another creates an unpaired cysteine residue able to form an intermolecular disulphide bridge, leading to receptor dimerisation and therefore activation.

The craniosynostosis syndrome, Apert syndrome, depends on FGFR2 mutations. Gain-of-function changes in the highly conserved residues S252 and P253 of the IgII and IgIII linker of FGFR2 result in a change in ligand binding specificity [126, 127]. These are the cause of the majority of Apert syndrome cases [128, 129]. This has been further shown in mouse models; S252W FGFR2c mutants showed activation of the c isoform of the receptor by mesenchymally expressed FGF7, while FGFR2b was activated by FGFs associated with epithelial expression [130]. It is also possible that S252W and P253R mutations lead to the modified receptor remaining on the cell membrane for an extended period of time, rather than undergoing rapid endocytosis into the lysosomes like its wild-type counterpart. Downstream signalling pathways are affected, leading to increased ERK phosphorylation and therefore increased cell proliferation and migration capabilities, as well as premature differentiation [131].

Mouse modelling of Apert syndrome has shown that a soluble, truncated FGFR2 isoform is upregulated and influences FGF1-FGFR2 binding. This glycosylated IIIa-TM isoform is generated by direct splicing of exon 7 (IIIa) to exon 10 (TM), generating a premature stop codon three amino acids into the TM exon [8]. This loss-of-function mutation can thus negatively regulate FGF signalling.

A number of cancers have been found to contain somatic mutations identical to germ line mutations in FGFRs associated with developmental disorders. For example, FGFR2 mutations commonly seen in Apert syndrome and Pfeiffer are frequently identified in endometrial cancer [132], for example S252W and N550K, both of which result in receptor activation. The S252W mutation resides in the linker region between IgII and IgIII, the area responsible for providing key contacts with the ligand. This increases the binding affinity of the receptor for a range of FGFs while also leading to violation of ligand specificity of the receptor isoforms [133].

Other FGFR2 mutations in endometrial cancer include S373C and Y376C, which result in gain of a cysteine residue, allowing formation of intermolecular disulphide bonds [134]. This leads to constitutive receptor dimerisation and therefore downstream signalling. Although these findings were established using FGFR2c functional studies, it is known FGFR3 contains paralogous mutations.

6.8 FGFR3

FGFR3 is mutated in a range of developmental and skeletal disorders and is the most frequently mutated FGFR in cancer, as noted by the extensive list in the Catalogue of Somatic Mutations in Cancer (COSMIC) database. Gain-of-function FGFR3 mutations are involved in the most severe form of dwarfism in humans,

thanatrophoric dysplasia types I and II [135, 136] and achondroplasia [137, 138]. Similar somatic mutations have been found in bladder and cervical cancer, amongst others, where they are believed to have a positive effect on proliferation and inhibit apoptosis [139].

The FGFR3 germline mutation, A391E, is known to cause abnormal cranium growth and is responsible for Crouzon syndrome [140]. This mutation is also found in bladder cancer [141]. A391E leads to stabilisation of the transmembrane domain of the dimerised receptor independent of ligand binding [142] and is therefore responsible for ligand-independent receptor activation [143].

6.9 FGFR4

FGFR4 has a diverse range of roles, from involvement in the vascular system to regulation of hepatic bile acid and lipid metabolism [144, 145]. Recently, a single nucleotide polymorphism (SNP) in FGFR4 has been identified which is thought to have both positive and negative prognostic value in different diseases. This SNP (rs351855) results in a glycine-arginine change (G388R) in the transmembrane domain, leading to increased receptor stability and sustained receptor activation [146].

FGFR4 is expressed at high levels in coronary artery disease (CAD). Investigation of the SNP status of CAD patients in a Chinese population study showed this SNP is low in CAD patients [145]. It is therefore thought that having this SNP may be beneficial, acting as a protective factor against CAD development in Asian populations. This SNP is also associated with poor prognosis in prostate and breast cancer [146, 147].

6.10 FGFRL1: The Fifth FGFR

A fifth member of the FGFR family has been discovered, Fibroblast Growth Factor Receptor Like 1 (FGFRL1). This protein, which exists as a homodimer consisting of the three characteristic extracellular Ig-like domains, acid box between IgI and IgII and a transmembrane helix, differs from the classic receptors in that it has no intracellular tyrosine kinase domain [148–150]. Instead, the intracellular portion of FGFRL1 consists of only 100 residues including a histidine-rich sequence and a tandem tyrosine-based motif [148, 151, 152]. These two sequences function as signals for FGFRL1 trafficking from the plasma membrane to endosomes and lysosomes. Deletion of these sequences resulted in inefficient FGFRL1 internalisation and prolonged time at the plasma membrane [151].

Interactions with both FGFs and heparin have also been confirmed through dissociation studies [148]. FGFRL1 binds strongly to FGF3, 4, 8, 10 and 22 [153] and the affinity of FGF3 for FGFRL1 is at least one order of magnitude higher than the majority of FGFs for their receptors [151]. Affinity of this magnitude between FGFs and their receptors is only seen in mutant receptors in, for example, Pfeiffer, Apert and Muenke craniosynostosis syndromes [154]. The gain-of-function P253R mutation in Apert syndrome exactly matches an arginine residue at position 243 in

FGFRL1; this residue could be responsible for the high affinity of FGF3 for FGFRL1 [155]. Its interaction with HS is also stronger than that of classic FGFRs and heparin [156, 157]. FGFs bind FGFRL1 between IgII and IgIII domains while heparin binds at the basic region at the beginning of the IgII loop [149, 158]. Autoinhibition of FGFRL1 via the IgI loop also occurs and the protein can be post-transcriptionally modified on one of its four glycosylation sites.

As FGFRL1 does not contain a tyrosine kinase domain it is not able to signal in the classical FGFR fashion. Its signalling function is yet to be fully determined, but a number of theories have been postulated. Firstly, the receptor could have an inhibitory effect on FGF signalling by sequestering ligands and therefore preventing them binding to FGFR1–4 [148, 149, 153]. Secondly, FGFRL1 could aid in internalisation and degradation of the classic receptors by binding to the same HS chain as the signalling receptor and effectively dragging it into endosomes/lysosomes. Thirdly, the tandem tyrosine-based motif and histidine-rich sequence could act as a docking site for tyrosine phosphatases, which could act on the signalling receptors and therefore attenuate signalling.

Although signalling mechanisms are yet to be elucidated, FGFRL1 can affect multiple cellular behaviours, inhibiting cell proliferation, increasing cell differentiation, regulating cell–cell contact and inducing cell–cell and cell–matrix adhesion. FGFRL1 is often found at the site of cell–cell contact and it is thought it may mediate cell adhesion by interacting with HS expressed on other cells [157]. FGFRL1 is also thought to play a role in craniosynostosis diseases and mutations in the protein have been found in ovarian cancer [151, 159].

Although not itself a receptor tyrosine kinase, it is clear that FGFRL1 plays an important role in FGF/FGFR signalling. Though full understanding of this role is yet to be determined, it is important to consider this fifth member of the FGFR family when understanding the complexity of FGF signalling.

6.11 Disease

As discussed for each receptor individually, both germ line and somatic FGFR mutations are known to play a role in a range of diseases, most notably craniosynostosis dysplasia and cancer (Table 6.1). Given the ability of the FGF signalling pathway to initiate cell survival and proliferation, amongst other cellular responses, it is not surprising this pathway is hijacked in cancer cells. Mutations in FGFRs in cancer are generally indicative of a more malignant phenotype. The majority of these mutations are activating, resulting in increased proliferation, migration and angiogenesis. However, recent data suggest that loss-of-function FGFR mutations may play a role in the development of some cancers [160, 161].

In cancer, chromosomal translocations lead to expression of constitutively active fusion proteins in which the FGFR tyrosine kinase domain is fused downstream of a constitutive dimerisation domain from a fusion protein. This has been seen in myeloproliferative syndromes (MPS), amongst other malignancies [162]. FGFR1 fusion proteins are known to cause 8p11 myeloproliferative syndrome (EMS), a

Table 6.1 Disease associated with FGFR1–4

Receptor	Disease	Cancer
FGFR1	Pfeiffer syndrome Idiopathic hypogonadotrophic hypogonadism Kallman syndrome type 2 Osteoglophonic dysplasia Trigonocephaly non-syndromic Myeloproliferative syndrome (with ZNF198) Stem cell leukaemia lymphoma syndrome (with ZMYM2) Stem cell myeloproliferative disorder	Breast Pancreas Large intestine Lung Prostate Stomach
FGFR2	Crouzon syndrome Apert syndrome Pfeiffer syndrome Jackson–Weiss syndrome Antley–Bixler Unclassified Beare–Stevenson syndrome Saethre–Chotzen Crouzon with acanthosis nigricans Kallmann syndrome LADD	Endometrium Ovary Melanoma Bone Breast Cervix Kidney Large intestine Lung Pancreas Prostate Stomach Central nervous system Haematopietic and lymphoid tissue
FGFR3	Thanatophoric dysplasia type I Thanatophoric dysplasia type II Platyspondylic lethal skeletal dysplasia, San Diego type Craniosynostosis associated or without other limb malformations Achondroplasia Crouzon with acanthosis nigricans Hypochondroplasia Severe achondroplasia with development delay and acanthosis nigricans Camptodactyly, tall stature and hearing loss syndrome (CATSHL)	Prostate Skin Urinary tract Haematopoietic and lymphoid tissue Vulva Pancreas Large intestine Lung Ovary Testis Cervix Central nervous system Upper aerodigestive tract Multiple myeloma Bladder cancer Cervical cancer Peripheral T-cell lymphoma

(continued)

Table 6.1 (continued)

Receptor	Disease	Cancer
FGFR4	Coronary heart disease	Breast Ovarian cancer Prostate cancer Breast cancer Central nervous system Haematopietic and lymphoid tissue Kidney Large intestine Lung Ovary Pancreas Pleura Prostate Salivary gland Skin Stomach Testis Upper aerodigestive tract Urinary tract

form of MPS [163]. These fusion proteins are known to cause constitutive tyrosine kinase activation of FGFR1 while also leading to signalling independent of FRS2. Fusion proteins containing the FGFR3 kinase domain are also associated with multiple myeloma and peripheral T-cell lymphoma [33].

Other cancers associated with FGFR signalling deregulation include breast cancer, where FGFR1 and FGFR2 are amplified in approximately 10 and 2 % of breast cancers, respectively [164, 165]. Approximately 10 % of melanoma cases have FGFR2 mutations [160]. Interestingly, functional analysis has shown these mutations in melanomas result in loss of function of the receptor. The mutation spectrum, characteristic of those induced by UV radiation, includes 20 missense mutations occurring at conserved residues in FGFR2. Receptor loss of function due to this mutation is caused by loss of ligand binding affinity, impaired receptor dimerisation and decreased kinase activity.

FGFRs can also be involved in cellular transformation by interacting with other proteins. For example, in epithelial ovarian cancer (EOC), Neural Cell Adhesion Molecule (NCAM) is unregulated and promotes malignancy via interaction with FGFR [166]. However, as NCAM is known to inhibit FGF2–FGFR binding [167], NCAM/FGFR interactions increase malignancy via inhibition of normal FGF–FGFR interactions. FGF2/FGFR and NCAM/FGFR interactions therefore stimulate different receptor-mediated responses in EOC; NCAM/FGFR leads to increased cell migration, while FGF2/FGFR leads to increased proliferation. It is also possible the varying receptor interactions cause differential regulation of receptor trafficking to the endosome, resulting in different cellular responses and signalling kinetics [168]. Mouse models have shown that targeted abolition of NCAM/FGFR interaction with

FGFR1–4 at a glance

	FGFR1	FGFR2	FGFR3	FGFR4
Alternative names	BFGFR, FLT2, FGFBR, FLG, CEK, CD331, H2, H3, H4, KAL2, N-SAM, c-Fgr, HBGFR, FLJ14326	BEK, KGFR, KSAM, CD332, BFR-1, CEK3, CFD1, ECT1, TK14, TK25	CD333, JTK4, ACH, CEK2, HBGFR	CD334, JTK2, TKF
Chromosome location	8p12	10q26	4p16.3	5q35.1-qter
Gene size (bp)	57,696	120,128	15,560	11,206
Intron/exon number	18 exons	18 exons	17 exons	18 exons
mRNA size (5′, ORF, 3′)	Up to ~5900 bp	Up to ~4250 bp	Up to ~4150 bp	Up to ~3120 bp
Amino acid number	Up to 853	Up to 830	Up to 808	Up to 802
Protein size	Up to 95 kDa	Up to 93 kDa	Up to 88 kDa	88 kDa
Post-translational modifications	Autophosphorylated, ubiquitinated, N-glycosylated	Autophosphorylated, ubiquitinated, N-glycosylated	Autophosphorylated, ubiquitinated, N-glycosylated	Autophosphorylated, ubiquitinated, N-glycosylated
Domains	Up to three Ig domains, transmembrane domain, tyrosine kinase domain	Up to three Ig domains, transmembrane domain, tyrosine kinase domain	Up to three Ig domains, transmembrane domain, tyrosine kinase domain	Up to three Ig domains, transmembrane domain, tyrosine kinase domain
Pathways activated	MAPK, PI3K/AKT, PLCγ, STAT	MAPK, PI3K/AKT, PLCγ, STAT	MAPK, PI3K/AKT, PLCγ, STAT	MAPK, PI3K/AKT, PLCγ, STAT
Knockout mouse phenotype	FGFR1$^{-/-}$ embryonic lethal around gastrulation	FGFR2$^{-/-}$ embryonic lethal around gastrulation	FGFR3 mutant alleles show skeletal phenotypes and hearing defects	FGFR4 null mice show no overt phenotype barring a reduction in body weight
	FGFR1-IIIb$^{-/-}$ no phenotype	FGFR2-IIIb$^{-/-}$ multiple defects in organogenesis	Conditional knockout available	
	Conditional knockouts show multiple phenotypes in brain, limb and bone	Isoform specific conditional knockouts exist for IIIb, showing multiple phenotypes, and for IIIc, mimicking IIIb activity in Apert Syndrome		
		Conditional knockout of entire gene gives multiple phenotypes		
References	[174–189]	[187, 189–201]	[202–208]	[208–210]

a monoclonal antibody results in elimination of metastatic dissemination of EOC. This has been shown via NCAM/FGFR1 interaction studies. However, as NCAM binds FGFR2 and FGFR4 [169], interaction of NCAM with multiple FGFRs may increase malignancy of EOC [166].

The high rate of FGFR mutation in a range of diseases makes this family of proteins a potential therapeutic target. Numerous studies have shown the benefits of FGFR knockdown and inhibition in cancer cell lines where the result is, for example, a decrease in cell proliferation [170]. However, translating this into a therapy for patients has proven difficult. Even specific FGFR inhibitors have off-target effects.

The most clinically advanced FGFR inhibitors to date are mixed kinase inhibitors, targeting the kinase domain of receptors to prevent downstream signalling. These include Dovitinib [171] and SU6668 [172]. However, their anti-FGFR activity is often weak, leading to investigation of more potent FGFR inhibitors. One such inhibitor currently in phase I clinical trials is AZD4547 [173]. This pyrazoloamide derivative targets FGFR1, 2 and 3 and resulted in cell growth inhibition versus cancer cell lines with known FGFR mutations and induces apoptosis. However, even this inhibitor has off-target effects, e.g. selectivity against VEGFR2 (also known as Kinase insert Domain Receptor, KDR), Insulin-like Growth Factor (IGF), PI3K and AKT, although this off-target inhibition is much lower than that of FGFRs. Such inhibitors still need more investigation, but the possibilities of potential FGFR inhibition are an exciting field of cancer therapeutics.

References

1. Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell. 1991;64:841–8.
2. Coughlin SR, Barr PJ, Cousens LS, Fretto LJ, Williams LT. Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. J Biol Chem. 1988;263:988–93.
3. Ornitz DM, Itoh N. Fibroblast growth factors. Genome Biol. 2001; 2: REVIEWS3005.
4. Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, Gao G, Goldfarb M. Receptor specificity of the fibroblast growth factor family. J Biol Chem. 1996;271:15292–7.
5. Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M, Ornitz DM. Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J Biol Chem. 2006;281:15694–700.
6. Johnson DE, Williams LT. Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res. 1993;60:1–41.
7. Kalinina J, Dutta K, Ilghari D, Beenken A, Goetz R, Eliseenkova AV, Cowburn D, Mohammadi M. The alternatively spliced acid box region plays a key role in FGF receptor autoinhibition. Structure. 2012;20:77–88.
8. Wheldon LM, Khodabukus N, Patey SJ, Smith TG, Heath JK, Hajihosseini MK. Identification and characterization of an inhibitory fibroblast growth factor receptor 2 (FGFR2) molecule, up-regulated in an Apert Syndrome mouse model. Biochem J. 2011;436:71–81.
9. Vainikka S, Partanen J, Bellosta P, Coulier F, Birnbaum D, Basilico C, Jaye M, Alitalo K. Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction. EMBO J. 1992;11:4273–80.

10. Orr-Urtreger A, Bedford M, Burakova T, Arman E, Zimmer Y, Yayon A, Givol D, Lonai P. Developmental localization of the splicing alternatives of fibroblast growth-factor receptor-2 (FGFR2). Dev Biol. 1993;158:475–86.
11. Yan G, Fukabori Y, McBride G, Nikolaropolous S, McKeehan WL. Exon switching and activation of stromal and embryonic fibroblast growth factor (FGF)-FGF receptor genes in prostate epithelial cells accompany stromal independence and malignancy. Mol Cell Biol. 1993;13:4513–22.
12. Lindahl U, Hook M. Glycosaminoglycans and their binding to biological macromolecules. Annu Rev Biochem. 1978;47:385–417.
13. Gambarini AG, Miyamoto CA, Lima GA, Nader HB, Dietrich CP. Mitogenic activity of acidic fibroblast growth factor is enhanced by highly sulfated oligosaccharides derived from heparin and heparan sulfate. Mol Cell Biochem. 1993;124:121–9.
14. Ornitz DM, Yayon A, Flanagan JG, Svahn CM, Levi E, Leder P. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. Mol Cell Biol. 1992;12:240–7.
15. Ornitz DM, Herr AB, Nilsson M, Westman J, Svahn CM, Waksman G. FGF binding and FGF receptor activation by synthetic heparan-derived di- and trisaccharides. Science. 1995; 268:432–6.
16. Schlessinger J, Plotnikov AN, Ibrahimi OA, Eliseenkova AV, Yeh BK, Yayon A, Linhardt RJ, Mohammadi M. Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. Mol Cell. 2000;6:743–50.
17. Guimond SE, Turnbull JE. Fibroblast growth factor receptor signalling is dictated by specific heparan sulphate saccharides. Curr Biol. 1999;9:1343–6.
18. Ornitz DM. FGFs, heparan sulfate and FGFRs: complex interactions essential for development. Bioessays. 2000;22:108–12.
19. McDowell LM, Frazier BA, Studelska DR, Giljum K, Chen J, Liu J, Yu K, Ornitz DM, Zhang L. Inhibition or activation of Apert syndrome FGFR2 (S252W) signaling by specific glycosaminoglycans. J Biol Chem. 2006;281:6924–30.
20. Guimond S, Maccarana M, Olwin BB, Lindahl U, Rapraeger AC. Activating and inhibitory heparin sequences for FGF-2 (basic FGF). Distinct requirements for FGF-1, FGF-2, and FGF-4. J Biol Chem. 1993;268:23906–14.
21. Pye DA, Vives RR, Turnbull JE, Hyde P, Gallagher JT. Heparan sulfate oligosaccharides require 6-O-sulfation for promotion of basic fibroblast growth factor mitogenic activity. J Biol Chem. 1998;273:22936–42.
22. Taylor KR, Rudisill JA, Gallo RL. Structural and sequence motifs in dermatan sulfate for promoting fibroblast growth factor-2 (FGF-2) and FGF-7 activity. J Biol Chem. 2005; 280:5300–6.
23. Trowbridge JM, Rudisill JA, Ron D, Gallo RL. Dermatan sulfate binds and potentiates activity of keratinocyte growth factor (FGF-7). J Biol Chem. 2002;277:42815–20.
24. Pellegrini L, Burke DF, von Delft F, Mulloy B, Blundell TL. Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. Nature. 2000;407:1029–34.
25. Furdui CM, Lew ED, Schlessinger J, Anderson KS. Autophosphorylation of FGFR1 kinase is mediated by a sequential and precisely ordered reaction. Mol Cell. 2006;21:711–7.
26. Mohammadi M, Dionne CA, Li W, Li N, Spivak T, Honegger AM, Jaye M, Schlessinger J. Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. Nature. 1992;358:681–4.
27. Mohammadi M, Schlessinger J, Hubbard SR. Structure of the FGF receptor tyrosine kinase domain reveals a novel autoinhibitory mechanism. Cell. 1996;86:577–87.
28. Mohammadi M, Dikic I, Sorokin A, Burgess WH, Jaye M, Schlessinger J. Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. Mol Cell Biol. 1996; 16:977–89.
29. Raffioni S, Thomas D, Foehr ED, Thompson LM, Bradshaw RA. Comparison of the intracellular signaling responses by three chimeric fibroblast growth factor receptors in PC12 cells. Proc Natl Acad Sci USA. 1999;96:7178–83.

30. Dhalluin C, Yan KS, Plotnikova O, Lee KW, Zeng L, Kuti M, Mujtaba S, Goldfarb MP, Zhou MM. Structural basis of SNT PTB domain interactions with distinct neurotrophic receptors. Mol Cell. 2000;6:921–9.
31. Ong SH, Guy GR, Hadari YR, Laks S, Gotoh N, Schlessinger J, Lax I. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. Mol Cell Biol. 2000;20:979–89.
32. Gotoh N. Regulation of growth factor signaling by FRS2 family docking/scaffold adaptor proteins. Cancer Sci. 2008;99:1319–25.
33. Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 2005;16:139–49.
34. Mohammadi M, Honegger AM, Rotin D, Fischer R, Bellot F, Li W, Dionne CA, Jaye M, Rubinstein M, Schlessinger J. A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. Mol Cell Biol. 1991;11:5068–78.
35. Falasca M, Iurisci C, Carvelli A, Sacchetti A, Corda D. Release of the mitogen lysophosphatidylinositol from H-Ras-transformed fibroblasts; a possible mechanism of autocrine control of cell proliferation. Oncogene. 1998;16:2357–65.
36. Klint P, Claesson-Welsh L. Signal transduction by fibroblast growth factor receptors. Front Biosci. 1999;4:165–77.
37. Rameh LE, Rhee SG, Spokes K, Kazlauskas A, Cantley LC, Cantley LG. Phosphoinositide 3-kinase regulates phospholipase Cgamma-mediated calcium signaling. J Biol Chem. 1998;273:23750–7.
38. Ebong S, Yu CR, Carper DA, Chepelinsky AB, Egwuagu CE. Activation of STAT signaling pathways and induction of suppressors of cytokine signaling (SOCS) proteins in mammalian lens by growth factors. Invest Ophthalmol Vis Sci. 2004;45:872–8.
39. Darnell Jr JE. STATs and gene regulation. Science. 1997;277:1630–5.
40. Wang L, Rudert WA, Loutaev I, Roginskaya V, Corey SJ. Repression of c-Cbl leads to enhanced G-CSF Jak-STAT signaling without increased cell proliferation. Oncogene. 2002;21:5346–55.
41. Degnin CR, Laederich MB, Horton WA. Ligand activation leads to regulated intramembrane proteolysis of fibroblast growth factor receptor 3. Mol Biol Cell. 2011;22:3861–73.
42. Blobel CP, Carpenter G, Freeman M. The role of protease activity in ErbB biology. Exp Cell Res. 2009;315:671–82.
43. Brown MS, Ye J, Rawson RB, Goldstein JL. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell. 2000;100:391–8.
44. Levi E, Fridman R, Miao HQ, Ma YS, Yayon A, Vlodavsky I. Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1. Proc Natl Acad Sci USA. 1996;93:7069–74.
45. Maretzky T, Le Gall SM, Worpenberg-Pietruk S, Eder J, Overall CM, Huang XY, Poghosyan Z, Edwards DR, Blobel CP. Src stimulates fibroblast growth factor receptor-2 shedding by an ADAM15 splice variant linked to breast cancer. Cancer Res. 2009;69:4573–6.
46. Peduto L, Reuter VE, Shaffer DR, Scher HI, Blobel CP. Critical function for ADAM9 in mouse prostate cancer. Cancer Res. 2005;65:9312–9.
47. Ancot F, Foveau B, Lefebvre J, Leroy C, Tulasne D. Proteolytic cleavages give receptor tyrosine kinases the gift of ubiquity. Oncogene. 2009;28:2185–95.
48. Loeb CR, Harris JL, Craik CS. Granzyme B proteolyzes receptors important to proliferation and survival, tipping the balance toward apoptosis. J Biol Chem. 2006;281:28326–35.
49. Chioni AM, Grose R. FGFR1 cleavage and nuclear translocation regulates breast cancer cell behavior. J Cell Biol. 2012;197:801–17.
50. Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. Sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell. 1998;92:253–63.
51. Thisse B, Thisse C. Functions and regulations of fibroblast growth factor signaling during embryonic development. Dev Biol. 2005;287:390–402.

52. Sasaki A, Taketomi T, Kato R, Saeki K, Nonami A, Sasaki M, Kuriyama M, Saito N, Shibuya M, Yoshimura A. Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. Cell Cycle. 2003;2:281–2.
53. Wakioka T, Sasaki A, Kato R, Shouda T, Matsumoto A, Miyoshi K, Tsuneoka M, Komiya S, Baron R, Yoshimura A. Spred is a Sprouty-related suppressor of Ras signalling. Nature. 2001;412:647–51.
54. Mardakheh FK, Yekezare M, Machesky LM, Heath JK. Spred2 interaction with the late endosomal protein NBR1 down-regulates fibroblast growth factor receptor signaling. J Cell Biol. 2009;187:265–77.
55. Yang RB, Ng CK, Wasserman SM, Komuves LG, Gerritsen ME, Topper JN. A novel interleukin-17 receptor-like protein identified in human umbilical vein endothelial cells antagonizes basic fibroblast growth factor-induced signaling. J Biol Chem. 2003;278:33232–8.
56. Kovalenko D, Yang X, Nadeau RJ, Harkins LK, Friesel R. Sef inhibits fibroblast growth factor signaling by inhibiting FGFR1 tyrosine phosphorylation and subsequent ERK activation. J Biol Chem. 2003;278:14087–91.
57. Tsang M, Friesel R, Kudoh T, Dawid IB. Identification of Sef, a novel modulator of FGF signalling. Nat Cell Biol. 2002;4:165–9.
58. Xiong S, Zhao Q, Rong Z, Huang G, Huang Y, Chen P, Zhang S, Liu L, Chang Z. hSef inhibits PC-12 cell differentiation by interfering with Ras-mitogen-activated protein kinase MAPK signaling. J Biol Chem. 2003;278:50273–82.
59. Torii S, Kusakabe M, Yamamoto T, Maekawa M, Nishida E. Sef is a spatial regulator for Ras/MAP kinase signaling. Dev Cell. 2004;7:33–44.
60. Buday L, Warne PH, Downward J. Downregulation of the Ras activation pathway by MAP kinase phosphorylation of Sos. Oncogene. 1995;11:1327–31.
61. Ueki K, Matsuda S, Tobe K, Gotoh Y, Tamemoto H, Yachi M, Akanuma Y, Yazaki Y, Nishida E, Kadowaki T. Feedback regulation of mitogen-activated protein kinase kinase kinase activity of c-Raf-1 by insulin and phorbol ester stimulation. J Biol Chem. 1994;269:15756–61.
62. Gual P, Giordano S, Anguissola S, Parker PJ, Comoglio PM. Gab1 phosphorylation: a novel mechanism for negative regulation of HGF receptor signaling. Oncogene. 2001;20:156–66.
63. Plotnikov AN, Schlessinger J, Hubbard SR, Mohammadi M. Structural basis for FGF receptor dimerization and activation. Cell. 1999;98:641–50.
64. Stauber DJ, DiGabriele AD, Hendrickson WA. Structural interactions of fibroblast growth factor receptor with its ligands. Proc Natl Acad Sci USA. 2000;97:49–54.
65. Olsen SK, Ibrahimi OA, Raucci A, Zhang F, Eliseenkova AV, Yayon A, Basilico C, Linhardt RJ, Schlessinger J, Mohammadi M. Insights into the molecular basis for fibroblast growth factor receptor autoinhibition and ligand-binding promiscuity. Proc Natl Acad Sci USA. 2004;101:935–40.
66. Kobrin MS, Yamanaka Y, Friess H, Lopez ME, Korc M. Aberrant expression of type I fibroblast growth factor receptor in human pancreatic adenocarcinomas. Cancer Res. 1993;53:4741–4.
67. Mansson PE, Adams P, Kan M, McKeehan WL. Heparin-binding growth factor gene expression and receptor characteristics in normal rat prostate and two transplantable rat prostate tumors. Cancer Res. 1989;49:2485–94.
68. Itoh N, Ornitz DM. Evolution of the Fgf and Fgfr gene families. Trends Genet. 2004;20:563–9.
69. Goetz R, Beenken A, Ibrahimi OA, Kalinina J, Olsen SK, Eliseenkova AV, Xu C, Neubert TA, Zhang F, Linhardt RJ, Yu X, White KE, Inagaki T, Kliewer SA, Yamamoto M, Kurosu H, Ogawa Y, Kuro-o M, Lanske B, Razzaque MS, Mohammadi M. Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. Mol Cell Biol. 2007;27:3417–28.
70. Harmer NJ, Pellegrini L, Chirgadze D, Fernandez-Recio J, Blundell TL. The crystal structure of fibroblast growth factor (FGF) 19 reveals novel features of the FGF family and offers a structural basis for its unusual receptor affinity. Biochemistry. 2004;43:629–40.
71. Kurosu H, Kuro OM. The Klotho gene family as a regulator of endocrine fibroblast growth factors. Mol Cell Endocrinol. 2009;299:72–8.

72. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, Fujita T, Fukumoto S, Yamashita T. Klotho converts canonical FGF receptor into a specific receptor for FGF23. Nature. 2006;444:770–4.
73. Polanska UM, Edwards E, Fernig DG, Kinnunen TK. The cooperation of FGF receptor and Klotho is involved in excretory canal development and regulation of metabolic homeostasis in Caenorhabditis elegans. J Biol Chem. 2011;286:5657–66.
74. Polanska UM, Fernig DG, Kinnunen T. Extracellular interactome of the FGF receptor-ligand system: complexities and the relative simplicity of the worm. Dev Dyn. 2009;238:277–93.
75. Ghabrial A, Luschnig S, Metzstein MM, Krasnow MA. Branching morphogenesis of the Drosophila tracheal system. Annu Rev Cell Dev Biol. 2003;19:623–47.
76. Bottcher RT, Niehrs C. Fibroblast growth factor signaling during early vertebrate development. Endocr Rev. 2005;26:63–77.
77. Dorey K, Amaya E. FGF signalling: diverse roles during early vertebrate embryogenesis. Development. 2010;137:3731–42.
78. Huang P, Stern MJ. FGF signaling in flies and worms: more and more relevant to vertebrate biology. Cytokine Growth Factor Rev. 2005;16:151–8.
79. DeVore DL, Horvitz HR, Stern MJ. An FGF receptor signaling pathway is required for the normal-cell migrations of the sex myoblasts in C-elegans hermaphrodites. Cell. 1995; 83:611–20.
80. Burdine RD, Branda CS, Stern MJ. EGL-17(FGF) expression coordinates the attraction of the migrating sex myoblasts with vulval induction in C. elegans. Development. 1998;125:1083–93.
81. Popovici C, Conchonaud F, Birnbaum D, Roubin R. Functional phylogeny relates LET-756 to fibroblast growth factor 9. J Biol Chem. 2004;279:40146–52.
82. Roubin R, Naert K, Popovici C, Vatcher G, Coulier F, Thierry-Mieg J, Pontarotti P, Birnbaum D, Baillie D, Thierry-Mieg D. let-756, a C. elegans fgf essential for worm development. Oncogene. 1999;18:6741–7.
83. Kokel M, Borland CZ, DeLong L, Horvitz HR, Stern MJ. clr-1 encodes a receptor tyrosine phosphatase that negatively regulates an FGF receptor signaling pathway in Caenorhabditis elegans. Genes Dev. 1998;12:1425–37.
84. Borland CZ, Schutzman JL, Stern MJ. Fibroblast growth factor signaling in Caenorhabditis elegans. Bioessays. 2001;23:1120–30.
85. Chateau MT, Araiz C, Descamps S, Galas S. Klotho interferes with a novel FGF-signalling pathway and insulin/Igf-like signalling to improve longevity and stress resistance in Caenorhabditis elegans. Aging. 2010;2:567–81.
86. Glazer L, Shilo B-Z. The Drosophila FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. Genes Dev. 1991;5:697–705.
87. Sutherland D, Samakovlis C, Krasnow MA. branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. Cell. 1996;87:1091–101.
88. Ghabrial AS, Krasnow MA. Social interactions among epithelial cells during tracheal branching morphogenesis. Nature. 2006;441:746–9.
89. Tsang M, Dawid IB. Promotion and attenuation of FGF signaling through the Ras-MAPK pathway. Sci STKE. 2004:pe17.
90. Beiman M, Shilo BZ, Volk T. Heartless, a Drosophila FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. Genes Dev. 1996; 10:2993–3002.
91. Gisselbrecht S, Skeath JB, Doe CQ, Michelson AM. Heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the Drosophila embryo. Genes Dev. 1996;10:3003–17.
92. Gryzik T, Muller HA. FGF8-like1 and FGF8-like2 encode putative ligands of the FGF receptor Htl and are required for mesoderm migration in the Drosophila gastrula. Curr Biol. 2004;14:659–67.
93. Stathopoulos A, Tam B, Ronshaugen M, Frasch M, Levine M. pyramus and thisbe: FGF genes that pattern the mesoderm of Drosophila embryos. Genes Dev. 2004;18:687–99.

94. Vincent S, Wilson R, Coelho C, Affolter M, Leptin M. The Drosophila protein Dof is specifically required for FGF signaling. Mol Cell. 1998;2:515–25.
95. Rodaway A, Takeda H, Koshida S, Broadbent J, Price B, Smith JC, Patient R, Holder N. Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. Development. 1999;126:3067–78.
96. Griffin K, Patient R, Holder N. Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail. Development. 1995;121:2983–94.
97. Griffin KJ, Amacher SL, Kimmel CB, Kimelman D. Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. Development. 1998;125:3379–88.
98. Furthauer M, Thisse C, Thisse B. A role for FGF-8 in the dorsoventral patterning of the zebrafish gastrula. Development. 1997;124:4253–64.
99. Furthauer M, Lin W, Ang SL, Thisse B, Thisse C. Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. Nat Cell Biol. 2002;4:170–4.
100. Brand M, Heisenberg CP, Jiang YJ, Beuchle D, Lun K, Furutani-Seiki M, Granato M, Haffter P, Hammerschmidt M, Kane DA, Kelsh RN, Mullins MC, Odenthal J, van Eeden FJ, Nusslein-Volhard C. Mutations in zebrafish genes affecting the formation of the boundary between midbrain and hindbrain. Development. 1996;123:179–90.
101. Reifers F, Bohli H, Walsh EC, Crossley PH, Stainier DY, Brand M. Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development. 1998;125:2381–95.
102. Shanmugalingam S, Houart C, Picker A, Reifers F, Macdonald R, Barth A, Griffin K, Brand M, Wilson SW. Ace/Fgf8 is required for forebrain commissure formation and patterning of the telencephalon. Development. 2000;127:2549–61.
103. Kimelman D, Kirschner M. Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell. 1987;51:869–77.
104. Slack JM, Darlington BG, Heath JK, Godsave SF. Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. Nature. 1987;326:197–200.
105. Amaya E, Musci T, Kirschner M. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in xenopus embryos. Cell. 1991;66:257–70.
106. Smith JC, Price BM, Green JB, Weigel D, Herrmann BG. Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. Cell. 1991;67:79–87.
107. Sivak JM, Petersen LF, Amaya E. FGF signal interpretation is directed by Sprouty and Spred proteins during mesoderm formation. Dev Cell. 2005;8:689–701.
108. Fisher ME, Isaacs HV, Pownall ME. eFGF is required for activation of XmyoD expression in the myogenic cell lineage of Xenopus laevis. Development. 2002;129:1307–15.
109. Fletcher RB, Baker JC, Harland RM. FGF8 spliceforms mediate early mesoderm and posterior neural tissue formation in Xenopus. Development. 2006;133:1703–14.
110. Amaya E, Stein PA, Musci TJ, Kirschner MW. FGF signalling in the early specification of mesoderm in Xenopus. Development. 1993;118:477–87.
111. Hongo I, Kengaku M, Okamoto H. FGF signaling and the anterior neural induction in Xenopus. Dev Biol. 1999;216:561–81.
112. Cohn MJ, Izpisua-Belmonte JC, Abud H, Heath JK, Tickle C. Fibroblast growth factors induce additional limb development from the flank of chick embryos. Cell. 1995;80:739–46.
113. Laufer E, Nelson CE, Johnson RL, Morgan BA, Tabin C. Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. Cell. 1994;79:993–1003.
114. Benazeraf B, Francois P, Baker RE, Denans N, Little CD, Pourquie O. A random cell motility gradient downstream of FGF controls elongation of an amniote embryo. Nature. 2010;466:248–52.

115. Dubrulle J, McGrew MJ, Pourquie O. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. Cell. 2001; 106:219–32.
116. Dubrulle J, Pourquie O. fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. Nature. 2004;427:419–22.
117. Streit A, Berliner AJ, Papanayotou C, Sirulnik A, Stern CD. Initiation of neural induction by FGF signalling before gastrulation. Nature. 2000;406:74–8.
118. Wilson SI, Graziano E, Harland R, Jessell TM, Edlund T. An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. Curr Biol. 2000;10:421–9.
119. Itoh N, Ornitz DM. Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J Biochem. 2010;149:121–30.
120. Elfenbein A, Simons M. Auxiliary and autonomous proteoglycan signaling networks. Methods Enzymol. 2010;480:3–31.
121. Elfenbein A, Lanahan A, Zhou TX, Yamasaki A, Tkachenko E, Matsuda M, Simons M. Syndecan 4 regulates FGFR1 signaling in endothelial cells by directing macropinocytosis. Sci Signal. 2012;5:ra36.
122. Bryant DM, Stow JL. Nuclear translocation of cell-surface receptors: lessons from fibroblast growth factor. Traffic. 2005;6:947–54.
123. Reilly JF, Maher PA. Importin beta-mediated nuclear import of fibroblast growth factor receptor: role in cell proliferation. J Cell Biol. 2001;152:1307–12.
124. Stachowiak MK, Maher PA, Stachowiak EK. Integrative nuclear signaling in cell development–a role for FGF receptor-1. DNA Cell Biol. 2007;26:811–26.
125. Wilkie AO. Bad bones, absent smell, selfish testes: the pleiotropic consequences of human FGF receptor mutations. Cytokine Growth Factor Rev. 2005;16:187–203.
126. Ibrahimi OA, Eliseenkova AV, Plotnikov AN, Yu K, Ornitz DM, Mohammadi M. Structural basis for fibroblast growth factor receptor 2 activation in Apert syndrome. Proc Natl Acad Sci USA. 2001;98:7182–7.
127. Yu K, Herr AB, Waksman G, Ornitz DM. Loss of fibroblast growth factor receptor 2 ligand-binding specificity in Apert syndrome. Proc Natl Acad Sci USA. 2000;97:14536–41.
128. Oldridge M, Lunt PW, Zackai EH, McDonald-McGinn DM, Muenke M, Moloney DM, Twigg SR, Heath JK, Howard TD, Hoganson G, Gagnon DM, Jabs EW, Wilkie AO. Genotype-phenotype correlation for nucleotide substitutions in the IgII-IgIII linker of FGFR2. Hum Mol Genet. 1997;6:137–43.
129. Webster MK, Donoghue DJ. FGFR activation in skeletal disorders: too much of a good thing. Trends Genet. 1997;13:178–82.
130. Wang Y, Xiao R, Yang F, Karim BO, Iacovelli AJ, Cai J, Lerner CP, Richtsmeier JT, Leszl JM, Hill CA, Yu K, Ornitz DM, Elisseeff J, Huso DL, Jabs EW. Abnormalities in cartilage and bone development in the Apert syndrome FGFR2(+/S252W) mouse. Development. 2005;132:3537–48.
131. Ahmed Z, Schuller AC, Suhling K, Tregidgo C, Ladbury JE. Extracellular point mutations in FGFR2 elicit unexpected changes in intracellular signalling. Biochem J. 2008;413:37–49.
132. Pollock PM, Gartside MG, Dejeza LC, Powell MA, Mallon MA, Davies H, Mohammadi M, Futreal PA, Stratton MR, Trent JM, Goodfellow PJ. Frequent activating FGFR2 mutations in endometrial carcinomas parallel germline mutations associated with craniosynostosis and skeletal dysplasia syndromes. Oncogene. 2007;26:7158–62.
133. Greulich H, Pollock PM. Targeting mutant fibroblast growth factor receptors in cancer. Trends Mol Med. 2011;17:283–92.
134. Wilkie AO, Patey SJ, Kan SH, van den Ouweland AM, Hamel BC. FGFs, their receptors, and human limb malformations: clinical and molecular correlations. Am J Med Genet. 2002;112:266–78.
135. Rousseau F, el Ghouzzi V, Delezoide AL, Legeai-Mallet L, Le Merrer M, Munnich A, Bonaventure J. Missense FGFR3 mutations create cysteine residues in thanatophoric dwarfism type I (TD1). Hum Mol Genet. 1996;5:509–12.

136. Tavormina PL, Shiang R, Thompson LM, Zhu YZ, Wilkin DJ, Lachman RS, Wilcox WR, Rimoin DL, Cohn DH, Wasmuth JJ. Thanatophoric dysplasia (types I and II) caused by distinct mutations in fibroblast growth factor receptor 3. Nat Genet. 1995;9:321–8.
137. Rousseau F, Bonaventure J, Legeai-Mallet L, Pelet A, Rozet JM, Maroteaux P, Le Merrer M, Munnich A. Mutations of the fibroblast growth factor receptor-3 gene in achondroplasia. Horm Res. 1996;45:108–10.
138. Shiang R, Thompson LM, Zhu YZ, Church DM, Fielder TJ, Bocian M, Winokur ST, Wasmuth JJ. Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. Cell. 1994;78:335–42.
139. Knowles MA. Novel therapeutic targets in bladder cancer: mutation and expression of FGF receptors. Future Oncol. 2008;4:71–83.
140. Meyers GA, Orlow SJ, Munro IR, Przylepa KA, Jabs EW. Fibroblast growth factor receptor 3 (FGFR3) transmembrane mutation in Crouzon syndrome with acanthosis nigricans. Nat Genet. 1995;11:462–4.
141. van Rhijn BW, van Tilborg AA, Lurkin I, Bonaventure J, de Vries A, Thiery JP, van der Kwast TH, Zwarthoff EC, Radvanyi F. Novel fibroblast growth factor receptor 3 (FGFR3) mutations in bladder cancer previously identified in non-lethal skeletal disorders. Eur J Hum Genet. 2002;10:819–24.
142. Li E, You M, Hristova K. FGFR3 dimer stabilization due to a single amino acid pathogenic mutation. J Mol Biol. 2006;356:600–12.
143. Chen F, Degnin C, Laederich M, Horton WA, Hristova K. The A391E mutation enhances FGFR3 activation in the absence of ligand. Biochim Biophys Acta. 2011;1808:2045–50.
144. Chen Q, Jiang Y, An Y, Zhao N, Zhao Y, Yu C. Soluble FGFR4 extracellular domain inhibits FGF19-induced activation of FGFR4 signaling and prevents nonalcoholic fatty liver disease. Biochem Biophys Res Commun. 2011;409:651–6.
145. Zhu Q, Liu T. Fibroblast growth factor receptor 4 polymorphisms and coronary artery disease: a case control study. Mol Biol Rep. 2012;39:8679–85.
146. Wang J, Yu W, Cai Y, Ren C, Ittmann MM. Altered fibroblast growth factor receptor 4 stability promotes prostate cancer progression. Neoplasia. 2008;10:847–56.
147. Frullanti E, Berking C, Harbeck N, Jezequel P, Haugen A, Mawrin C, Parise Jr O, Sasaki H, Tsuchiya N, Dragani TA. Meta and pooled analyses of FGFR4 Gly388Arg polymorphism as a cancer prognostic factor. Eur J Cancer Prev. 2011;20:340–7.
148. Sleeman M, Fraser J, McDonald M, Yuan S, White D, Grandison P, Kumble K, Watson JD, Murison JG. Identification of a new fibroblast growth factor receptor, FGFR5. Gene. 2001;271:171–82.
149. Trueb B, Zhuang L, Taeschler S, Wiedemann M. Characterization of FGFRL1, a novel fibroblast growth factor (FGF) receptor preferentially expressed in skeletal tissues. J Biol Chem. 2003;278:33857–65.
150. Wiedemann M, Trueb B. Characterization of a novel protein (FGFRL1) from human cartilage related to FGF receptors. Genomics. 2000;69:275–9.
151. Rieckmann T, Zhuang L, Fluck CE, Trueb B. Characterization of the first FGFRL1 mutation identified in a craniosynostosis patient. Biochim Biophys Acta. 2009;1792:112–21.
152. Zhuang L, Karotki AV, Bruecker P, Trueb B. Comparison of the receptor FGFRL1 from sea urchins and humans illustrates evolution of a zinc binding motif in the intracellular domain. BMC Biochem. 2009;10:33.
153. Steinberg F, Zhuang L, Beyeler M, Kalin RE, Mullis PE, Brandli AW, Trueb B. The FGFRL1 receptor is shed from cell membranes, binds fibroblast growth factors (FGFs), and antagonizes FGF signaling in Xenopus embryos. J Biol Chem. 2010;285:2193–202.
154. Ibrahimi OA, Zhang F, Eliseenkova AV, Itoh N, Linhardt RJ, Mohammadi M. Biochemical analysis of pathogenic ligand-dependent FGFR2 mutations suggests distinct pathophysiological mechanisms for craniofacial and limb abnormalities. Hum Mol Genet. 2004;13:2313–24.
155. Trueb B. Biology of FGFRL1, the fifth fibroblast growth factor receptor. Cell Mol Life Sci. 2011;68:951–64.

156. Powell AK, Fernig DG, Turnbull JE. Fibroblast growth factor receptors 1 and 2 interact differently with heparin/heparan sulfate. Implications for dynamic assembly of a ternary signaling complex. J Biol Chem. 2002;277:28554–63.
157. Rieckmann T, Kotevic I, Trueb B. The cell surface receptor FGFRL1 forms constitutive dimers that promote cell adhesion. Exp Cell Res. 2008;314:1071–81.
158. Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. Nat Rev Drug Discov. 2009;8:235–53.
159. Schild C, Trueb B. Aberrant expression of FGFRL1, a novel FGF receptor, in ovarian tumors. Int J Mol Med. 2005;16:1169–73.
160. Gartside MG, Chen H, Ibrahimi OA, Byron SA, Curtis AV, Wellens CL, Bengston A, Yudt LM, Eliseenkova AV, Ma J, Curtin JA, Hyder P, Harper UL, Riedesel E, Mann GJ, Trent JM, Bastian BC, Meltzer PS, Mohammadi M, Pollock PM. Loss-of-function fibroblast growth factor receptor-2 mutations in melanoma. Mol Cancer Res. 2009;7:41–54.
161. Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer. 2010;10:116–29.
162. Grose R, Dickson C. Fibroblast growth factor signaling in tumorigenesis. Cytokine Growth Factor Rev. 2005;16:179–86.
163. Fioretos T, Panagopoulos I, Lassen C, Swedin A, Billstrom R, Isaksson M, Strombeck B, Olofsson T, Mitelman F, Johansson B. Fusion of the BCR and the fibroblast growth factor receptor-1 (FGFR1) genes as a result of t(8;22)(p11;q11) in a myeloproliferative disorder: the first fusion gene involving BCR but not ABL. Genes Chromosomes Cancer. 2001;32:302–10.
164. Courjal F, Cuny M, Simony-Lafontaine J, Louason G, Speiser P, Zeillinger R, Rodriguez C, Theillet C. Mapping of DNA amplifications at 15 chromosomal localizations in 1875 breast tumors: definition of phenotypic groups. Cancer Res. 1997;57:4360–7.
165. Turner N, Pearson A, Sharpe R, Lambros M, Geyer F, Lopez-Garcia MA, Natrajan R, Marchio C, Iorns E, Mackay A, Gillett C, Grigoriadis A, Tutt A, Reis-Filho JS, Ashworth A. FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. Cancer Res. 2010;70:2085–94.
166. Zecchini S, Bombardelli L, Decio A, Bianchi M, Mazzarol G, Sanguineti F, Aletti G, Maddaluno L, Berezin V, Bock E, Casadio C, Viale G, Colombo N, Giavazzi R, Cavallaro U. The adhesion molecule NCAM promotes ovarian cancer progression via FGFR signalling. EMBO Mol Med. 2011;3:480–94.
167. Francavilla C, Loeffler S, Piccini D, Kren A, Christofori G, Cavallaro U. Neural cell adhesion molecule regulates the cellular response to fibroblast growth factor. J Cell Sci. 2007; 120:4388–94.
168. Francavilla C, Cattaneo P, Berezin V, Bock E, Ami D, de Marco A, Christofori G, Cavallaro U. The binding of NCAM to FGFR1 induces a specific cellular response mediated by receptor trafficking. J Cell Biol. 2009;187:1101–16.
169. Christensen C, Lauridsen JB, Berezin V, Bock E, Kiselyov VV. The neural cell adhesion molecule binds to fibroblast growth factor receptor 2. FEBS Lett. 2006;580:3386–90.
170. Byron SA, Gartside MG, Wellens CL, Mallon MA, Keenan JB, Powell MA, Goodfellow PJ, Pollock PM. Inhibition of activated fibroblast growth factor receptor 2 in endometrial cancer cells induces cell death despite PTEN abrogation. Cancer Res. 2008;68:6902–7.
171. Trudel S, Li ZH, Wei E, Wiesmann M, Chang H, Chen C, Reece D, Heise C, Stewart AK. CHIR-258, a novel, multitargeted tyrosine kinase inhibitor for the potential treatment of t(4;14) multiple myeloma. Blood. 2005;105:2941–8.
172. Fabbro D, Manley PW. Su-6668. SUGEN. Curr Opin Investig Drugs. 2001;2:1142–8.
173. Gavine PR, Mooney L, Kilgour E, Thomas AP, Al-Kadhimi K, Beck S, Rooney C, Coleman T, Baker D, Mellor MJ, Brooks AN, Klinowska T. AZD4547: an orally bioavailable, potent, and selective inhibitor of the fibroblast growth factor receptor tyrosine kinase family. Cancer Res. 2012;72:2045–56.
174. Celli G, Larochelle WJ, MacKem S, Sharp R, Merlino G. Soluble dominant-negative receptor uncovers essential roles for fibroblast growth-factors in multiorgan induction and patterning. EMBO J. 1998;17:1642–55.

175. Ciruna B, Rossant J. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell. 2001;1:37–49.
176. Ciruna BG, Schwartz L, Harpal K, Yamaguchi TP, Rossant J. Chimeric analysis of fibroblast growth-factor receptor-1 (FGFR1) function—a role for FGFR1 in morphogenetic movement through the primitive streak. Development. 1997;124:2829–41.
177. Deng CX, Wynshawboris A, Shen MM, Daugherty C, Ornitz DM, Leder P. Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev. 1994;8: 3045–57.
178. Freeman KW, Gangula RD, Welm BE, Ozen M, Foster BA, Rosen JM, Ittmann M, Greenberg NM, Spencer DM. Conditional activation of fibroblast growth factor receptor (FGFR) 1, but not FGFR2, in prostate cancer cells leads to increased osteopontin induction, extracellular signal-regulated kinase activation, and in vivo proliferation. Cancer Res. 2003;63:6237–43.
179. Freeman KW, Welm BE, Gangula RD, Rosen JM, Ittmann M, Greenberg NM, Spencer DM. Inducible prostate intraepithelial neoplasia with reversible hyperplasia in conditional FGFR1-expressing mice. Cancer Res. 2003;63:8256–63.
180. Partanen J, Schwartz L, Rossant J. Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. Genes Dev. 1998;12:2332–44.
181. Robinson ML, MacMillancrow LA, Thompson JA, Overbeek PA. Expression of a truncated FGF receptor results in defective lens development in transgenic mice. Development. 1995;121:3959–67.
182. Verheyden JM, Lewandoski M, Deng C, Harfe BD, Sun X. Conditional inactivation of Fgfr1 in mouse defines its role in limb bud establishment, outgrowth and digit patterning. Development. 2005;132:4235–45.
183. Welm BE, Freeman KW, Chen M, Contreras A, Spencer DM, Rosen JM. Inducible dimerization of FGFR1: development of a mouse model to analyze progressive transformation of the mammary gland. J Cell Biol. 2002;157:703–14.
184. Winter SF, Acevedo VD, Gangula RD, Freeman KW, Spencer DM, Greenberg NM. Conditional activation of FGFR1 in the prostate epithelium induces angiogenesis with concomitant differential regulation of Ang-1 and Ang-2. Oncogene. 2007;26:4897–907.
185. Xu X, Li C, Takahashi K, Slavkin HC, Shum L, Deng CX. Murine fibroblast growth factor receptor 1alpha isoforms mediate node regression and are essential for posterior mesoderm development. Dev Biol. 1999;208:293–306.
186. Yamaguchi TP, Harpal K, Henkemeyer M, Rossant J. FGFR-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes Dev. 1994;8:3032–44.
187. Yang J, Meyer M, Muller AK, Bohm F, Grose R, Dauwalder T, Verrey F, Kopf M, Partanen J, Bloch W, Ornitz DM, Werner S. Fibroblast growth factor receptors 1 and 2 in keratinocytes control the epidermal barrier and cutaneous homeostasis. J Cell Biol. 2010;188:935–52.
188. Zhang H, Dessimoz J, Beyer TA, Krampert M, Williams LT, Werner S, Grose R. Fibroblast growth factor receptor 1-IIIb is dispensable for skin morphogenesis and wound healing. Eur J Cell Biol. 2004;83:3–11.
189. Zhao H, Kegg H, Grady S, Truong HT, Robinson ML, Baum M, Bates CM. Role of fibroblast growth factor receptors 1 and 2 in the ureteric bud. Dev Biol. 2004;276:403–15.
190. De Moerlooze L, Spencer-Dene B, Revest J, Hajihosseini M, Rosewell I, Dickson C. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development. 2000;127:483–92.
191. Eswarakumar VP, Monsonego-Ornan E, Pines M, Antonopoulou I, Morriss-Kay GM, Lonai P. The IIIc alternative of Fgfr2 is a positive regulator of bone formation. Development. 2002;129:3783–93.
192. Jackson D, Bresnick J, Rosewell I, Crafton T, Poulsom R, Stamp G, Dickson C. Fibroblast growth factor receptor signalling has a role in lobuloalveolar development of the mammary gland. J Cell Sci. 1997;110(Pt 11):1261–8.

193. Lin Y, Liu G, Zhang Y, Hu YP, Yu K, Lin C, McKeehan K, Xuan JW, Ornitz DM, Shen MM, Greenberg N, McKeehan WL, Wang F. Fibroblast growth factor receptor 2 tyrosine kinase is required for prostatic morphogenesis and the acquisition of strict androgen dependency for adult tissue homeostasis. Development. 2007;134:723–34.
194. Mailleux AA, Spencer-Dene B, Dillon C, Ndiaye D, Savona-Baron C, Itoh N, Kato S, Dickson C, Thiery JP, Bellusci S. Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo. Development. 2002;129:53–60.
195. Parsa S, Kuremoto K, Seidel K, Tabatabai R, Mackenzie B, Yamaza T, Akiyama K, Branch J, Koh CJ, Al Alam D, Klein OD, Bellusci S. Signaling by FGFR2b controls the regenerative capacity of adult mouse incisors. Development. 2010;137:3743–52.
196. Peters K, Werner S, Liao X, Wert S, Whitsett J, Williams L. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. EMBO J. 1994;13:3296–301.
197. Revest JM, Spencer-Dene B, Kerr K, De Moerlooze L, Rosewell I, Dickson C. Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. Dev Biol. 2001;231:47–62.
198. Werner S, Smola H, Liao X, Longaker M, Krieg T, Hofschneider P, Williams L. The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. Science. 1994;266:819–22.
199. Werner S, Weinberg W, Liao X, Peters K, Blessing M, Yuspa S, Weiner R, Williams L. Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. EMBO J. 1993;12:2635–43.
200. Xu XL, Weinstein M, Li CL, Naski M, Cohen RI, Ornitz DM, Leder P, Deng CX. Fibroblast-growth-factor-receptor-2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. Development. 1998;125:753–65.
201. Yu K, Xu J, Liu Z, Sosic D, Shao J, Olson EN, Towler DA, Ornitz DM. Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development. 2003;130:3063–74.
202. Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat Genet. 1996;12:390–7.
203. Deng C, Wynshaw BA, Zhou F, Kuo A, Leder P. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell. 1996;84:911–21.
204. L'Hote CG, Knowles MA. Cell responses to FGFR3 signalling: growth, differentiation and apoptosis. Exp Cell Res. 2005;304:417–31.
205. Su N, Xu X, Li C, He Q, Zhao L, Chen S, Luo F, Yi L, Du X, Huang H, Deng C, Chen L. Generation of Fgfr3 conditional knockout mice. Int J Biol Sci. 2010;6:327–32.
206. Wang JM, Du XL, Li CL, Yin LJ, Chen B, Sun J, Su N, Zhao L, Song RH, Song WW, Chen L, Deng CX. Gly374Arg mutation in Fgfr3 causes achondroplasia in mice. Zhonghua Yi Xue Yi Chuan Xue Za Zhi. 2004;21:537–41.
207. Wang Y, Spatz MK, Kannan K, Hayk H, Avivi A, Gorivodsky M, Pines M, Yayon A, Lonai P, Givol D. A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. Proc Natl Acad Sci USA. 1999;96:4455–60.
208. Weinstein M, Xu XL, Ohyama K, Deng CX. FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. Development. 1998;125:3615–23.
209. Yu C, Wang F, Jin C, Wu X, Chan WK, McKeehan WL. Increased carbon tetrachloride-induced liver injury and fibrosis in FGFR4-deficient mice. Am J Pathol. 2002;161:2003–10.
210. Yu C, Wang F, Kan M, Jin C, Jones RB, Weinstein M, Deng CX, McKeehan WL. Elevated cholesterol metabolism and bile acid synthesis in mice lacking membrane tyrosine kinase receptor FGFR4. J Biol Chem. 2000;275:15482–9.

Chapter 7
The INSR/IGF1R Receptor Family

Rive Sarfstein and Haim Werner

7.1 Introduction to the INSR/IGF1R Tyrosine Kinase Family

The insulin/insulin-like growth factors (IGFs) constitute a network of ligands (insulin, IGF1, and IGF2), cell-surface receptors (insulin receptor [INSR], IGF1 receptor [IGF1R], and IGF2 receptor [IGF2R]), and six binding proteins (IGFBPs) involved in the regulation of multiple physiological processes, including metabolic, nutritional, endocrine, growth, and aging events (Fig. 7.1). In addition to the classical IGF-family members, a series of nonclassical members were identified. These members include the insulin receptor-related receptor (IRR), insulin–IGF1 hybrid receptors, an IGFBP-3 receptor, and a steadily growing number of IGFBP-related proteins (IGFBP-rP) as well as a group of IGFBP proteases [1]. The existence of the IGFs was postulated in the late 1950s, following the seminal observation by Salmon and Daughaday that growth hormone (GH) stimulated the incorporation of sulfate into cartilage in an indirect fashion, which involved activation of a specific serum factor. The factor that was originally termed "sulfation factor" and then "somatomedin" is now accepted as IGF1.

Insulin/IGFs play key developmental and metabolic roles at every stage of life, from early ontogeny until old age. Although the INSR and IGF1R share the majority of their downstream cytoplasmic mediators, most experimental and clinical evidence is consistent with the notion that INSR activation (mainly by insulin) leads primarily to metabolic activities, whereas IGF1R activation (mainly by IGF1 or IGF2) leads to proliferative and differentiative events. The IGF2R is identical to the mannose 6-phosphate receptor, a membrane protein involved in the recycling of lysosomal enzymes. This receptor lacks a tyrosine kinase domain and, therefore, is not involved in signaling events. It is clear, however, that there is cross talk between the various ligands and receptors of the IGF family. The literature has documented

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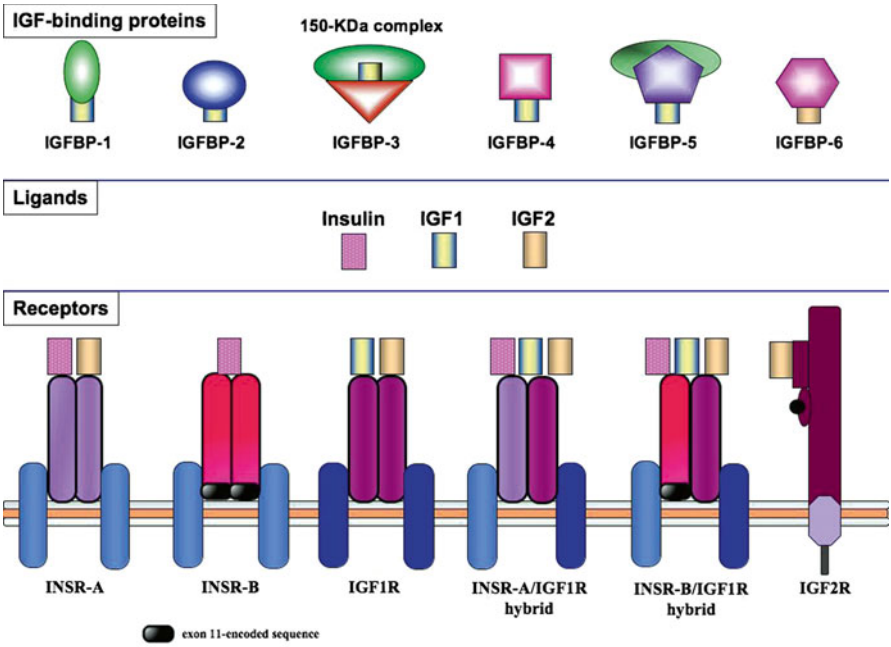

Fig. 7.1 Components of the insulin–IGF signaling pathway. The insulin–IGF axis comprises three ligands (insulin, IGF1, and IGF2), at least six IGF-binding proteins, and a family of cell-surface receptors. The INSR has two isoforms (INSR-A and INSR-B) that result from alternative splicing of exon 11 and, therefore, differ in the absence or presence of the exon-11-encoded sequence (12 amino acids). The hybrid INSR-IGF1R receptors include an INSR hemireceptor (either INSR-A or INSR-B) linked to an IGF1R hemireceptor. The IGF2R is identical to the mannose 6-phosphate receptor and appears to lack signaling activity. Hence, most biological activities of IGF2 are mediated by the IGF1R. INSR-A has been also shown to mediate IGF2 actions. IGFBP-3 is the main circulating binding protein. It forms a ternary complex with IGF and an acid-labile subunit. Additional members of the insulin–IGF network (not shown here) include IGFBP proteases, an insulin receptor-related receptor, and IGFBP-related proteins

the ability of insulin and IGF1 to activate the opposite receptor, showing that in some cases IGF1R can mediate metabolic activities [2], whereas INSR may exhibit growth, anti-apoptotic, and developmental functions [3]. Deregulation of IGF system expression and action is linked to diverse pathologies, ranging from growth deficits to cancer development. Targeting of the IGF axis emerged in recent years as a promising therapeutic approach in cancer and other medical conditions [4].

7.2 The Role of the INSR/IGF1R Tyrosine Kinase Family in Embryonic Development and Adult Physiology

INSR/IGF1R receptors display a remarkable similarity in genomic organization. Thus, 12 (out of 21) exons of the *IGF1R* gene are identical in size with the homologous exons of *INSR*, the main difference being that the *IGF1R* gene does not contain an

equivalent of the alternatively spliced *INSR* exon 11. This splicing event leads to the generation of two isoforms, INSR-A and INSR-B, which lack or contain, respectively, exon 11 [5]. These isoforms are differentially expressed during development, with INSR-A predominantly expressed in fetal tissues and INSR-B mainly expressed in adult organs, particularly liver, muscle, and adipocytes [2, 5]. The IGF1R displays an opposite pattern of expression, being absent in liver and present at low levels in adipose tissue and at high levels in brain [6].

Similarly, the *IGF1R* gene is developmentally regulated, with highest mRNA levels and IGF1 binding detected at embryonic stages. IGF1R levels markedly decrease in the adult animal. This developmental pattern reflects the key role of the IGF1R in cellular proliferation as well as its inherent anti-apoptotic, pro-survival action [7]. In addition, and in agreement with its potent neurotrophic activity, highest IGF1R mRNA levels in the adult rat are observed in the central nervous system. Intermediate levels of expression are found in kidney, stomach, testes, lung, and heart, with undetectable levels seen in liver.

7.3 The Role of the INSR/IGF1R Tyrosine Kinase Family in Human Disease

Many types of cancer have been shown to express INSR, particularly the fetal variant, INSR-A, which mediates proliferation and apoptosis protection in response to IGF2 [8]. It is conceivable that INSR-A and hybrid receptors (comprising an INSR hemireceptor linked to an IGF1R hemireceptor) may also be activated by high levels of insulin, as occur in patients with the "metabolic syndrome," characterized by obesity, type 2 diabetes, and insulin resistance [9]. Indeed, obesity and insulin resistance are linked to the risk of developing cancers of the esophagus, colon, kidney and endometrium, and with adverse prognosis in prostate and breast cancer [10, 11]. Consistent with this concept, diet-induced hyperinsulinemia was shown to accelerate the growth of prostate cancer xenografts [12]. Furthermore, an antibody that neutralizes both IGF1R and hybrid receptors showed a more potent antitumor activity than antibodies targeting only the IGF1R or receptor hybrids [13].

In addition, overexpression of the IGF1R constitutes a typical hallmark of most types of cancer [14]. The IGF1R exhibits a very potent anti-apoptotic activity in comparison with most other growth factor receptors described [15–17]. This activity confers upon IGF1R-expressing cells enhanced survivability, a key hallmark of cancer cells. Seminal studies from the laboratory of Renato Baserga provided evidence that cells derived from IGF1R "knockout" embryos (the total deficiency of IGF1R is a lethal condition), with a few exceptions, do not undergo malignant transformation when exposed to oncogenes [18, 19]. These early studies were already consistent with the notion that IGF1R expression and/or activation are fundamental prerequisites for cancer development. It is important to realize that IGF1R, per se, is neither genotoxic nor transforming. In other words, activation of the IGF1R by IGF1 is not an oncogenic event. IGF1, however, is an important progression factor

necessary for cell cycle progression after cell exposure to a competence factor (e.g., platelet-derived growth factor, fibroblast growth factor). Once an oncogenic event has occurred (i.e., a first hit), cell survival of already transformed cells is heavily dependent on IGF1 action. Unlike IGF1, overexpression of IGF2 has been linked to the etiology of a number of overgrowth syndromes (e.g., Beckwith–Wiedemann Syndrome) and cancers (e.g., Wilms' tumor, rhabdomyosarcoma) [20]. In this context, it was shown that the initial proliferative switch in oncogene-induced transformation was correlated with focal activation of IGF2 [21]. In agreement with its central role in neoplasia, the IGF1R emerged in recent years as a promising therapeutic target. Targeting modalities embrace the use of IGF1R monoclonal antibodies (as monotherapy or in combination with other antibodies and/or classical therapies) as well as small molecular weight tyrosine kinase inhibitors [22–24]. Given the structural similarity between IGF1R and INSR and in view of their overlapping pathways, the probability of "knocking down" the INSR (with ensuing metabolic impairment) when applying anti-IGF1R therapies became a matter of concern [25]. On the other hand, the recognition that the INSR (and, in particular, the INSR-A isoform) is an important player in breast cancer etiology might imply that dual (i.e., INSR *and* IGF1R) targeted therapy offers obvious advantages [26].

7.4 INSR

7.4.1 INSR *Gene*

7.4.1.1 Gene Structure

The human *INSR* gene is a single-copy gene located on chromosome 19. It extends over 130 kb DNA and contains 22 exons [27]. All introns interrupt protein-coding regions of the gene. Alternative splicing of exon 11 leads to generation of two species of mRNAs, encoding INSRs that differ in the presence or absence of a 12-amino acids insert [28]. The 11 exons encoding the α-subunit of the receptor are dispersed over greater than 90 kb, whereas the 11 exons encoding the β-subunit are located together in a region of approximately 30 kb (Table "Receptor at a glance: INSR").

7.4.1.2 Promoter Structure

Three transcriptional initiation sites have been identified, located 276, 282, and 283 bp upstream of the translation initiation site. In addition, a 247 bp fragment from the promoter region possessing 62.6 % of its maximal promoter activity has been identified. This promoter fragment is extremely GC rich and lacks typical TATA and CAAT boxes but contains multiple potential binding sites for zinc-finger proteins Sp1. Hence, the *INSR* promoter displays prototypical features of "housekeeping" genes [29].

7.4.1.3 Transcriptional Regulation

A number of transcription factors involved in *INSR* gene regulation have been identified, including nuclear proteins HT-FIR, IRNF-I, IRNF-II, Sp1, and HMGA1 [2].

7.4.1.4 mRNA Structure

There are at least five different INSR mRNA species ranging in length from approximately 5–10 kb [30–32]. This size heterogeneity arises from the utilization of alternate polyadenylation signals in exon 22.

7.4.2 INSR Protein

7.4.2.1 Amino Acids Sequence

Ullrich et al. [33] deduced the entire 1,370-amino acid sequence of the INSR from cDNA clones. The precursor protein starts with a 27-amino acid signal sequence, followed by the receptor α-subunit, a precursor processing enzyme cleavage site, and finally the β-subunit containing a single 23-amino acid transmembrane sequence.

7.4.2.2 Processing

As mentioned above, the precursor polypeptide includes a cleavage site that is specifically digested to generate α- and β-subunits. The formation of the mature, functionally active heterotetrameric protein is described below.

7.4.2.3 Domain Structure

The mature INSR is inserted into the plasma membrane of the cell as an α2 β2 heterotetramer [34]. The α-subunit (135,000 daltons) is highly glycosylated and contains a cysteine-rich domain. Hydropathy analysis of the amino acid sequence deduced from the INSR cDNA reveals no sequence of hydrophobic amino acids characteristic of a transmembrane domain. The α-subunit is, therefore, believed to be exposed exclusively on the extracellular surface of the plasma membrane. The β-subunit of the receptor does contain a sequence of 20 amino acids characteristic of a transmembrane domain. Based on this analysis, it has been inferred that, with a mass of 95,000 daltons, the carboxy-terminal one-third of the β-subunit is oriented on the extracellular side of the plasma membrane. The amino-terminal two-thirds of the β-subunit is oriented on the cytoplasmic face of the plasma membrane. In a series of elegant experiments, Finn et al. [35] investigated the nature of the disulfide bonds maintaining the

tetrameric structure of the INSR. In view of the finding that the INSR contains 47 cysteine residues in a αβ dimer, these investigators demonstrated that remarkably few disulfide bonds maintain the higher-order structure of the INSR. By selectively reducing the human placental INSR with tributylphosphine, followed by alkylation with N-ethylmaleimide, it has been found that upon reduction of only two disulfide bonds in the α-subunit and one disulfide bond in the β-subunit, two αβ dimers could be generated from the α2β2 tetrameric structure [35]. Further reduction and alkylation of two disulfide bonds in the β-subunit resulted in the generation of individual α- and β-subunits. Therefore, a total of five disulfide bonds account for the complex tetrameric structure of the INSR. The extracellular α-subunit of INSR contains the insulin-binding domain. It is the intracellular domain of the β-subunit that is autophosphorylated on tyrosine residues upon binding of insulin to the α-subunit. This autophosphorylation then activates the tyrosine kinase activity of INSR, rendering the receptor capable of phosphorylating other substrates. The INSR is therefore a multifunctional protein: it contains insulin binding activity, autotyrosine kinase activity, and tyrosine protein kinase activity [34].

7.4.2.4 Posttranslational Modification

Glycosylation

The INSR precursor, a single-chain high mannose polypeptide (210 kDa), enters the endoplasmic reticulum lumen where early maturation steps occur. Here, the polypeptide acquires dimeric structure and insulin binding capacity by rearrangement of disulfide bonds. Then, proteolytic cleavage and O-glycosylation of the insulin pro-receptor dimer (420 kDa) take place in the Golgi apparatus. Finally, the mature INSR α2β2 tetramer localizes on the cell surface as a transmembrane glycoprotein able to bind insulin and to transduce hormone signaling [36].

The INSR is heavily glycosylated and is estimated to contain 58–64 kDa of carbohydrate [37]. Oligosaccharides of both the high mannose and complex type are present, the latter containing additional fucose, N-acetylglucosamine, galactose, and sialic acid residues [38]. The INSR has 18 potential sites for N-linked glycosylation, 14 on the α chain and four on the β chain, of which 16 have been confirmed as glycosylated. O-linked glycosylation has been demonstrated only in the β-subunit. Other members of the INSR family have fewer potential N-linked glycosylation sites, suggesting that not all sites are required for correct folding, assembly, and function.

Phosphorylation and Desphosphorylation

For the INSR to autophosphorylate, a lysine at position 1030 is required to stabilize the γ-phosphate of ATP while the adenosine of ATP itself interacts with three glycines at residues 1003–1008. The first tyrosine residues to be autophosphorylated

are 1158, 1162, and 1163 in the tyrosine kinase domain. This is followed by Tyr-972 in the juxtamembrane domain and then tyrosines 1328 and 1330. These tyrosines fall into the three distinct tyrosine phosphorylation domains of the β-subunit. In total there are 13 potential tyrosines that may be phosphorylated. The receptor phosphorylates itself in a *trans* rather than *cis* manner. That is, one β-subunit of the receptor phosphorylates the other β-subunit rather than itself [39]. Phosphorylation of Tyr-999 is required for binding to IRS1, SHC1, and STAT5B. Dephosphorylation is catalyzed by protein tyrosine phosphatase receptor [PTPR (type Epsilon polypeptide)] at Tyr-999, Tyr-1185, Tyr-1189, and Tyr-1190 and by PTPRF (type F polypeptide) and PTPN1 (non-receptor type 1) [40–43].

SUMOylation

Recently, we have shown that the INSR can be modified by the small ubiquitin-like modifier (SUMO) protein, SUMO-1, with ensuing translocation to the nucleus [44]. SUMOylation is a posttranslational modification involved in various cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and cell cycle progression [45].

Ubiquitination

Although the INSR generally promotes the tyrosine phosphorylation of intermediate substrates, such as the insulin receptor substrate (IRS) family, to propagate its signal, it has also been reported that the INSR can directly recruit signaling adapters. The APS protein, which is mainly expressed in the target tissues of insulin (adipose tissue, skeletal muscle, and heart), is one of these adapters that directly interact with INSR [46]. APS can recruit c-Cbl to the INSR [47] in insulin-stimulated 3T3-L1 adipocytes. Then, c-Cbl protein acts as an ubiquitin-protein ligase (E3) that recognizes tyrosine-phosphorylated substrates by virtue of its SH2 domain and recruits an E2 ubiquitin-conjugating enzyme through its RING domain. RING domain-containing E3s (RING E3s) are thought to promote the ubiquitination of substrate lysines directly by E2 [48]. This succession of events leads to ubiquitination of the NSR and to the initiation of internalization [46].

Lipidation

The INSR also contains covalently linked fatty acids. In biosynthetic labeling studies, radiolabeled myristate and palmitate were found to be attached to the INSR. One of these fatty acids appears to be attached very early, in an amide linkage, as inhibition of protein synthesis prevents its occurrence [49].

7.4.2.5 Phosphorylation Sites and Known Functions

The major tyrosine autophosphorylation sites in INSR have been identified as Y960, Y1146, Y1150, Y1151, Y1316, and Y1322 [43, 50–52]. The tyrosine phosphorylation sites in the 1146–1151 region are necessary for the activation of the substrate kinase activity, whereas the 1316 and 1322 tyrosine sites are thought to be involved in the regulation of mitogenesis [53–56]. Interestingly, mutations of the INSR 960 tyrosine residue to phenylalanine do not alter INSR expression, assembly, binding, or autophosphorylation properties [57].

7.4.3 INS Ligands

7.4.3.1 Ligand Structure

Insulin is a peptide hormone composed of 51 amino acid residues normally secreted by the pancreatic islets of Langerhans in response to increasing levels of metabolic fuels in the blood. The mature, biologically active molecule is a dimer composed of an A-chain and a B-chain linked together by disulfide bonds.

7.4.3.2 Ligand Cleavage

There are two processes by which insulin can be degraded. The disulfide bonds can be reduced by the enzyme glutathione insulin transhydrogenase, resulting in the production of A and B chains [58]. These peptides, which are biologically inactive, are then susceptible to further degradation by nonspecific cellular hydrolases. This process (i.e., initial disulfide reduction and subsequent proteolysis) has been termed the sequential degradation of insulin [59] and, at one time, was felt to be the only mechanism of insulin degradation. The other insulin-degrading process is the direct proteolytic degradation of the insulin molecule by the insulin protease enzyme [60, 61]. Duckworth and coworkers [61] showed that the specific degradation of insulin by insulin protease alters the molecule so that it is susceptible to nonspecific endopeptidases, which do not attack the intact insulin molecule. The initial cleavage is at B16–B17, resulting in a molecule with three peptide chains held together by disulfide bonds. If, at this point, the disulfide bonds are reduced, intact A chain can be obtained along with the fragments of B chain. The B16 tyrosine residue is involved in the binding of insulin to its receptor; thus, a cleavage between B16 and B17 could alter greatly the binding properties of the molecule. Hydrolysis of the B16–B17 peptide bond by insulin protease followed by reduction of the A20–B19 disulfide bond (possibly by glutathione insulin transhydrogenase) would result in a peptide containing the residues B22–B26. These residues have been suggested to serve as the active portion of the insulin molecule since synthetic peptides with this sequence have insulin-like action.

7.4.4 INSR Activation and Signaling

7.4.4.1 Phosphorylation

As mentioned above, insulin binding to its receptor results in receptor autophosphorylation on tyrosine residues and the tyrosine phosphorylation of INSR substrates (e.g., IRS and Shc) by the INSR tyrosine kinase. This allows association of IRSs with downstream effectors such as PI3K via its Src homology-2 (SH2) domains, leading to end point events such as glucose transporter-4 (Glut-4) translocation to the cell membrane. Shc, when tyrosine phosphorylated, associates with Grb2 and can thus activate the Ras/MAPK pathway independent of the IRSs.

INSR-A upregulation is associated with increased IGF2 signaling, whereas INSR-B upregulation is predominantly associated with insulin-mediated metabolic effects [2]. Activation of INSR-A elicits different biological effects and intracellular signaling upon insulin or IGF2 binding. Thus, INSR-A-expressing cells undergo proliferation when stimulated by IGF2, whereas they preferentially activate glucose uptake when stimulated with insulin [5, 62]. These differential effects coincide with quantitative and temporal differences in the phosphorylation of intracellular substrates IRS1-4 and focal adhesion kinase (FAK) in response to either insulin or IGF2.

7.4.4.2 Pathway Activation and Major Genes Regulated

The activated IRS1 acts as a secondary messenger within the cell to stimulate the transcription of insulin-regulated genes. First, Grb2 binds the P-Tyr residue of IRS1 in its SH2 domain. Grb2 is then able to bind SOS that, in turn, catalyzes the replacement of bound GDP with GTP on Ras, a G protein. This protein then begins a phosphorylation cascade, culminating in the activation of mitogen-activated protein kinase (MAPK), which enters the nucleus and phosphorylates various nuclear transcription factors, including Elk1.

Glycogen synthesis is also stimulated by INSR via IRS1. In this case, it is the SH2 domain of PI3K that binds the P-Tyr of IRS1. Now activated, PI3K can convert the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). This indirectly activates protein kinase PKB (AKT). PKB then phosphorylates several target proteins, including glycogen synthase kinase-3 (GSK-3). GSK3 is responsible for phosphorylating (and thus deactivating) glycogen synthase. When GSK3 is phosphorylated, it is deactivated and prevented from deactivating glycogen synthase. In this roundabout manner, insulin increases glycogen synthesis.

7.4.4.3 Cross Talk with Other Receptors System

There is evidence for the existence of hybrid receptors (INSR-IGF1R), composed of INSR and IGF1R hemireceptors, in some tissues [63, 64]. A cross talk between insulin, IGFs, and their receptors appears to be a relatively common event in

multiple organs and systems. Hence, the recently described role of INSR in mitogenesis and cell motility may provide the foundation for its involvement in cancer development and progression [2, 65]. On the other hand, IGF1 exhibits important metabolic effects. For example, in vivo infusion of recombinant IGF1 was shown to lead to an acute decrease in circulating glucose values [66].

7.4.5 INSR Internalization, Processing, and Attenuation

The activated ligand–receptor complex, initially at the cell surface, is internalized into endosomes, a process that is dependent on tyrosine autophosphorylation. Endocytosis of activated receptors has the dual effect of concentrating receptors within endosomes and allowing the INSR tyrosine kinase to phosphorylate substrates that are spatially distinct from those accessible at the plasma membrane. Acidification of the endosomal lumen, due to the presence of proton pumps, results in dissociation of insulin from its receptor. The endosome constitutes the major site of insulin degradation, the endosomal acidic insulinase. Loss of the ligand–receptor complex attenuates any further insulin-driven receptor re-phosphorylation events and leads to receptor dephosphorylation by extra-lumenal endosomally associated protein tyrosine phosphatases [67].

7.4.6 Unique Features of the INSR

INSR signaling has a central role in mammalian biology, regulating cellular metabolism, growth, division, differentiation, and survival [68, 69]. Insulin resistance contributes to the pathogenesis of type 2 diabetes mellitus and may affect the onset of Alzheimer's disease [70]. Furthermore, aberrant signaling occurs in many cancers, exacerbated by cross talk with the homologous IGF1R [71].

A few patients with homozygous mutations in the *INSR* gene have been described, which causes Donohue syndrome or leprechaunism. This autosomal recessive disorder results in a totally nonfunctional INSR. These patients have low-set, often protuberant, ears, flared nostrils, thickened lips, and severe growth retardation. In most cases, the outlook for these patients is extremely poor, with death occurring within the first year of life. Other mutations of the same gene cause the less severe Rabson–Mendenhall syndrome, in which patients have characteristically abnormal teeth, hypertrophic gingiva (gums), and enlargement of the pineal gland. Both diseases present with fluctuations of the glucose level: after a meal the glucose is initially very high and then falls rapidly to abnormally low levels [72].

7.5 IGF1R

7.5.1 IGF1R *Gene*

7.5.1.1 Gene Structure

The human *IGF1R* gene spans more than 100 kb of genomic DNA at bands q25-26 at the distal end of chromosome 15. It contains 21 exons and its architecture is extremely similar to that of the *INSR* gene [73]. Exons 1–3 encode the ~1 kb long 5′ untranslated region (UTR), the signal peptide, and the N-terminal non-cysteine-rich and cysteine-rich domains of the α-subunit. The rest of the α-subunit is encoded by exons 4–10. Exon 11 encodes the cleavage site that generates mature α- and β-subunits from the proreceptor, and exons 12–21 encode the β-subunit, with exon 14 encoding the transmembrane domain and exons 16–20 encoding the tyrosine kinase domain. The size of the major human IGF1R mRNA transcript is ~11 kb, with an additional band at ~7 kb (that is missing in rodents) [74–76] (Table "Receptor at a glance: IGF1R").

7.5.1.2 Promoter Structure

The *IGF1R* promoter lacks canonical TATA and CAAT sequences, two promoter elements that are usually required for accurate transcription initiation, and that are absent in many cases of "housekeeping" genes [77]. Transcription of this gene, however, starts from a unique site contained within an "initiator" motif, a discrete promoter element able to direct initiation in the absence of a TATA box. Similar to other widely expressed genes, the *IGF1R* promoter is extremely GC rich (80 %) and contains several binding sites for members of the Sp1 family of zinc-finger nuclear proteins [6].

7.5.1.3 Transcriptional Regulation

Control of *IGF1R* gene expression is mainly attained at the level of transcription [2, 6, 14]. Comprehensive analyses have established that transcription of the *IGF1R* promoter is dependent on a number of stimulatory zinc-finger proteins, including Sp1 and Krüppel-like factors (e.g., KLF6) [78]. In addition, the *IGF1R* promoter was identified as a downstream target for tumor suppressor action, and multiple antioncogenes (e.g., p53, breast cancer gene-1 [BRCA1], and von-Hippel Lindau [VHL]) were shown to inhibit *IGF1R* transcription [79–81]. DNA affinity chromatography linked to mass spectroscopy analysis led recently to the identification of the entire collection of *IGF1R* promoter-binding proteins in estrogen receptor (ER)-positive and ER-negative breast cancer cells [78]. In addition, we showed that nuclear IGF1R acts as a transcriptional activator of its own promoter and a nuclear INSR functions as a negative regulator of *IGF1R* promoter activity [44]. Interactions

between stimulatory and inhibitory transcription factors play an important role in *IGF1R* regulation and, consequently, were postulated to have a major impact on the proliferative status of the cell [14, 82].

7.5.1.4 mRNA Structure

The size of the major human IGF1R mRNA transcript is ~11 kb, with an additional band at ~7 kb (that is missing in rodents) [74–76].

7.5.2 IGF1R Protein

7.5.2.1 Amino Acids Sequence

Ullrich and colleagues [75] determined the complete primary structure of the IGF1R from cloned cDNAs. The deduced sequence predicted a 1,367-amino acid receptor precursor, including a 30-residue signal peptide, which is removed during translocation of the nascent polypeptide chain. The 1,337-residue, unmodified proreceptor polypeptide had a predicted Mr of 151.869, which compares with the 180,000 Mr IGF1R precursor. In analogy with the 152,784 Mr INSR precursor, cleavage of the Arg-Lys-Arg-Arg sequence at position 707 of the IGF1R precursor generates α- (80,423 Mr) and β- (70,866 Mr) subunits, which compare with ~135.000 Mr (α) and 90,000 Mr (β) fully glycosylated subunits.

7.5.2.2 Processing

IGF1R is synthesized as a single chain pre-pro-peptide with a 30-amino acids signal peptide that is cleaved after translation. The pro-peptide is then glycosylated, dimerized, and transported to the Golgi where it is processed at a furin cleavage site to yield α- and β-subunits. A mature tetramer, β-α-α-β, is then formed through disulfide bridges, followed by transfer to the plasma membrane. It has been shown that N-linked glycosylation of IGF1R is necessary for its translocation to the cell surface.

7.5.2.3 Domain Structure

As mentioned above, the INSR and IGF1R are members of the superfamily of receptor tyrosine kinases (RTKs). However, unlike most RTKs that are single-chain monomeric transmembrane polypeptides, the INSR and IGF1R are covalent dimers composed of two extracellular α-subunits and two transmembrane β-subunits containing the tyrosine kinase domains. The β-subunits contain tyrosine kinase catalytic domains that are activated upon ligand binding. The α-subunits contain 706 amino acids and include two homologous domains, L1 and L2, separated by a cysteine-rich domain containing 25–27 cysteines in three repeating units. The cysteine domain

(residues 148–302) is also conserved in the INSR [83, 84]. The β-subunit contains 627 amino acid residues, spans the plasma membrane, and has three domains (i.e., extracellular, transmembrane, and intracellular domains). The extracellular domain of the β-subunit is 196 amino acids in length, while the transmembrane domain is 24 amino acids in length (located at position 906–929). The intracellular portion of the β-subunit can be divided into a juxtamembrane, a tyrosine kinase, and a C-terminal domain. The homology between the IGF1R and INSR at these different domains is clearly distinct. Thus, the tyrosine kinase domains exhibit the highest homology between the two receptors (84 %), the juxtamembrane domains share a 61 % homology, and the C-terminal domains share only 44 % similarity [75]. The C-terminal IGF1R domain, in conjunction with a nonapeptide at position 1073–1081, and the divergent membrane-proximal region between residues 933 and 955 are perhaps responsible for receptor-specific, ligand-induced, intracellular signal generation. Within the tyrosine kinase domain, a cluster of three tyrosine residues, located at position 1131, 1135, and 1136, is critical for receptor autophosphorylation [85]. In addition, the presence of the catalytic domain, containing an ATP binding motif (Gly-X-Gly-X-X-Gly), in which X can be any amino acid, at position 976–981, and a catalytic Lys at position 1003 are essential for ATP binding [86].

7.5.2.4 Posttranslational Modification

Glycosylation

The IGF1R has 16 potential N-linked glycosylation sites [38].

Phosphorylation and Desphosphorylation

Autophosphorylation of IGF1R, in response to ligand binding, occurs in a *trans* fashion, i.e., one subunit of the dimeric receptor phosphorylates tyrosine residues on the other subunit. Autophosphorylation occurs in a sequential manner: Tyr-1165 is predominantly phosphorylated first, followed by phosphorylation of Tyr-1161 and Tyr-1166. While every single phosphorylation increases kinase activity, all three tyrosine residues in the kinase activation loop (Tyr-1165, Tyr-1161, and Tyr-1166) have to be phosphorylated for optimal activity. In addition, IGF1R can be autophosphorylated in vitro at additional tyrosine residues. Autophosphorylation is followed by phosphorylation of juxtamembrane tyrosines and C-terminal serines. Phosphorylation of Tyr-980 is required for IRS1 and SHC1 binding. Dephosphorylation of IGF1R is catalyzed by PTPN1 [87–90].

SUMOylation

IGF1 stimulates the SUMOylation of IGF1R at three lysine residues (Lys1025, Lys1100, and Lys1120) located in its β-subunit [91]. Mutation of these residues blocked SUMOylation of the receptor and prevented its accumulation in the nucleus

and activation of transcription, but did not interfere with endocytosis of the receptor or its activation of the PI3K or MAPK pathways. SUMOylation is a prerequisite for the nuclear translocation of IGF1R; however, SUMOylated IGF1R is predominantly localized perinuclearly and at the nuclear membrane [44, 91].

Ubiquitination

Three E3 ligases, Nedd4, Mdm2, and c-Cbl, have been implicated in mediating IGF1R ubiquitination [92–94]. Polyubiquitination takes place at Lys-1138 and Lys-1141 through "Lys-48" and "Lys-29" linkages, promoting receptor endocytosis and subsequent degradation by the proteasome. Ubiquitination is facilitated by preexisting phosphorylation [95].

7.5.2.5 Phosphorylation Sites and Known Functions

Ligand binding induces conformational changes leading to IGF1R autophosphorylation of the β-subunit tyrosine kinase domain (spanning amino acids 973–1,229) and ubiquitination [92, 94]. As mentioned above, the activation loop of the IGF1R kinase domain contains three tyrosines residues (Tyr1131, Tyr1135, and Tyr1136) that serve as receptor autophosphorylation sites. Tyr1135 and Tyr1131 phosphorylation destabilizes the autoinhibitory conformation of the activation loop, whereas Tyr1136 phosphorylation stabilizes the catalytically optimized conformation [87], allowing substrate and ATP access. In addition, the C-terminal domain contains several additional tyrosines and serines, such as Tyrs 1250, 1251, and 1316 and Sers 1280–1283, whose phosphorylation plays a role in IGF1R signaling. Mutation of all [96] or some [97] of these residues dramatically reduces the kinase activity, as well as the mitogenic and transforming capabilities of IGF1R.

As mentioned before, the phosphorylated tyrosine residues serve as docking sites for other signaling molecules such as IRS1-4 and Shc, leading to the subsequent activation of the PI3K/MAPK and the 14-3-3 pathways [98–101]. Sehat and colleagues [91] reported that phosphorylation of the IGF1R is necessary for its ubiquitination.

7.5.3 IGF Ligands

7.5.3.1 Ligand Structure

IGF1 consists of 70 amino acids (7.65 kDa), whereas IGF2 consists of 67 amino acids (7.47 kDa). IGF1 and IGF2 have a 62 % homology in amino acid sequence and there is a 40 % homology between IGFs and proinsulin. Unlike insulin, where the connecting C-peptide is cleaved out during processing of the prohormone to the mature circulating hormone, the mature IGFs retain the C-domain, which links the A and B domains.

In addition, IGFs contain an extension to the A domain, the D domain, that is not found in insulin. Furthermore, both IGF prohormones contain C-terminal E peptides that are cleaved during processing of the precursors [102, 103].

7.5.3.2 Ligand Cleavage

IGF1 action is regulated by proteolytic processing by dipeptidyl peptidase IV (DPP-IV). DPP-IV is a membrane-bound serine protease that preferentially cleaves the peptide bond after the penultimate proline residue [104]. DPP-IV is ubiquitously expressed in various tissues. The attenuation of IGF1 signaling may be achieved by both a reduction in IGF1R activation and increased IGF1–IGFBP3 binding [105].

7.5.4 IGF1R Activation and Signaling

7.5.4.1 Phosphorylation

Binding of IGFs to the IGF1R α-subunits induces allosteric changes in the receptor molecule, which lead to autophosphorylation of the β-subunits. As is the case with INSR, the triple tyrosine cluster (located at positions 1131, 1134, 1135) is heavily phosphorylated, leading to activation of the intrinsic tyrosine kinase activity of the receptor [106]. Other β-subunit tyrosine residues (including tyrosines at positions 950, 1250, 1251, and 1316) are subsequently phosphorylated.

7.5.4.2 Pathway Activation and Major Genes Regulated

IGF1R and INSR are RTKs that most frequently signal through PI3K- and MAPK-dependent mechanisms [2]. IGF1 binding activates the IGF1R kinase, leading to receptor autophosphorylation followed by tyrosine phosphorylation of multiple substrates. These substrates function as signaling adapter proteins and include the INSR molecules (IRS1-4), Shc, and 14-3-3 proteins. Phosphorylation of IRSs proteins leads to the activation of two main signaling pathways: the PI3K-AKT/PKB pathway and the Ras–MAPK pathway [107–109]. The net result of activating the MAPK pathway is increased cellular proliferation whereas activation of the PI3K pathway inhibits apoptosis and stimulates protein synthesis. Phosphorylated IRS1 can activate the 85-kDa regulatory subunit of PI3K, leading to activation of several downstream substrates, including AKT/PKB. AKT phosphorylation, in turn, enhances protein synthesis through mTOR activation and triggers the anti-apoptotic effects of IGF1R through phosphorylation and inactivation of BAD. In parallel to PI3K-driven signaling, recruitment of Grb2/SOS by phosphorylated IRS1 or Shc leads to recruitment of Ras and activation of the Ras–MAPK pathway. In addition

to these two main signaling pathways, IGF1R signals also through the Janus kinase/signal transducer and activator of transcription pathway (JAK/STAT). Phosphorylation of JAK proteins can lead to phosphorylation/activation of signal transducers and activators of transcription (STAT) proteins. In particular, activation of STAT3 may be essential for the transforming activity of IGF1R. The JAK/STAT pathway activates gene transcription and may be responsible for its transforming activity. JNK kinases can also be activated by IGF1R. IGF1 exerts inhibiting activities on JNK activation via phosphorylation and inhibition of MAP3K5/ASK1, which is able to directly associate with the IGF1R [110, 111].

7.5.4.3 Cross Talk with Other Receptors System

In recent years, the literature has documented the ability of each ligand to activate the opposite receptor, showing that in some cases IGF1R can mediate metabolic activities and INSR growth, anti-apoptotic, and developmental activities [2, 66]. However, no prospective studies have directly assessed how activation of INSR or IGF1R leads to divergent biological events. In this respect, it is legitimate to question whether the distinct biological effects elicited by the activated receptors can be explained only by differential ligand affinity, divergent tissue distribution, differences in the internalization of the receptors or structural differences in the β-subunit, specifically in the C-terminus [112]. See also Sect. 7.4.4 on cross talk of INSR with other receptor system.

Recent studies have reported interactions between IGF1R and several cell-surface and nuclear receptors, including a cross talk between IGF1R and the ER pathway [113]. A cross talk between IGF1R and EGFR was detected in vitro and in vivo and it was shown that this interplay can lead to acquired resistance against EGFR-targeted drugs [114]. Finally, the androgen receptor (AR) was shown to activate IGF1R expression [115] while, on the other hand, IGF1R was demonstrated to stimulate AR activity in prostate cancer cells [116–118].

7.5.5 IGF1R Internalization, Processing, and Attenuation

The critical determinant of IGF1R signal termination is desensitization of receptors by the removal of activated receptors from the cell surface via endocytosis. For some membrane receptors the signal mediating receptor internalization/downregulation is constituted by ubiquitination. In vitro studies have implicated Nedd4 in IGF1R ubiquitination and processing. Nedd4 binds IGF1R trough Grb10. These studies have shown that the Grb10/Nedd4/IGF1R complex drives ligand-dependent ubiquitination of the internalized IGF1R. Moreover, ubiquitination was shown to occur at the plasma membrane, probably before the formation of endocytosis vesicles [119]. Another member of the E3 ligase family involved in IGF1R degradation is the MDM2 proto-oncoprotein, a RING finger ubiquitin ligase that is

transcriptionally regulated by IGF1 [120]. MDM2 binds to the IGF1R β-subunit through the adaptor β-arrestin, thereby recruiting ubiquitin to the IGF1R and initiating its degradation [121, 122].

7.5.6 *Unique Features of the IGF1R*

Under normal physiological conditions, the IGF1R plays an important role in protection from apoptosis, regulation of cell growth, and differentiation [38]. Anomalies of the IGF1R in mice lead to fetal growth retardation and malformations in the development of skin, muscle, bone, and the central nervous system [123]. High levels of IGF1R are detected in a diversity of human tumors, and interference with IGF1R function by antisense approaches, antibodies, or dominant-negative mutants reverses the transformed phenotype in multiple cancer models [98]. For these reasons, the IGF1R has emerged as a potential therapeutic target for the cure of human cancer.

Receptor at a glance: INSR

Chromosome location	19
Gene size (bp)	>130 kb
Intron/exon numbers	21/22. Two isoforms, INSR-A and –B, are generated by alternative splicing of exon 11
mRNA size (5′, ORF, 3′)	~5–10 kb
Amino acid number	1,370
kDa	Precursor, 200 kDa; mature receptor, 97 kDa
Posttranslational modifications	Glycosylation, phosphorylation and desphosphorylation, SUMOylation, ubiquitination, and lipidation
Domains	Ligand-binding domain in the extracellular α-subunit; tyrosine kinase domain in the cytoplasmic portion of the transmembrane β-subunit
Ligands	Insulin and IGF2 (mainly at INSR-A)
Known dimerizing partners	Preformed heterotetramer composed of two α and two β subunits
Pathways activated	PI3K/AKT and MAPK pathways
Tissues expressed	INSR-A predominantly expressed in fetal tissues and InsR-B predominately expressed in adult tissues, particularly liver, muscle, and adipocytes
Human diseases	Type 2 diabetes mellitus. May affect the onset of Alzheimer's disease, Donohue syndrome or leprechaunism, and Rabson–Mendenhall syndrome
Knockout mouse phenotype	Severe hyperglycemia, hyperketonemia, and growth retardation. KO mice die as a result of diabetic ketoacidosis within 48–72 h

Receptor at a glance: IGF1R

Chromosome location	15
Gene size (bp)	>100 kb
Intron/exon numbers	20/21
mRNA size (5′, ORF, 3′)	~11 kb with an additional band at ~7 kb
Amino acid number	1,337
kDa	Precursor, 200 kDa; mature receptor, 97 kDa
Posttranslational modifications	Glycosylation, phosphorylation and desphosphorylation, SUMOylation, and ubiquitination
Domains	Ligand-binding domain in the extracellular α-subunit; tyrosine kinase domain in the cytoplasmic portion of the transmembrane β-subunit
Ligands	IGF1, IGF2, and insulin (with low affinity)
Known dimerizing partners	Preformed heterotetramer composed of two α and two β subunits
Pathways activated	PI3K/AKT and MAPK pathways
Tissues expressed	Low levels in adipose tissue and high levels in brain. Highly expressed in most cancers
Human diseases	Mutations (very rare) of the IGF1R lead to intrauterine and postnatal growth failure, microcephaly, mental retardation, and deafness
Knockout mouse phenotype	Lethal condition with severe growth retardation. KO mice have anomalies in CNS, skin, and other organs

References

1. Hwa V, Tomasini-Sprenger C, Bermejo AL, Rosenfeld RG, Plymate SR. Characterization of insulin-like growth factor-binding protein-related protein-1 in prostate cells. J Clin Endocrinol Metab. 1998;83:4355–62.
2. Belfiore A, Frasca F, Pandini G, Sciacca L, Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr Rev. 2009;30:586–623.
3. Sandhu MS, Luben R, Day NE, Khaw KT. Self-reported birth weight and subsequent risk of colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2002;11:935–8.
4. Werner H, Bruchim I. Basic and clinical significance of IGF-I-induced signatures in cancer. BMC Med. 2010;8:2.
5. Frasca F, Pandini G, Scalia R, Sciacca L, Mineo R, Constantino A, et al. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol. 1999;19:3278–88.
6. Sarfstein R, Maor S, Reizner N, Abramovitch S, Werner H. Transcriptional regulation of the insulin-like growth factor-I receptor gene in breast cancer. Mol Cell Endocrinol. 2006; 252:241–6.
7. Werner H, Woloschak M, Adamo M, Shen-Orr Z, Roberts Jr CT, LeRoith D. Developmental regulation of the rat insulin-like growth factor I receptor gene. Proc Natl Acad Sci USA. 1989;86:7451–5.
8. Belfiore A. The role of insulin receptor isoforms and hybrid insulin/IGF-I receptors in human cancer. Curr Pharm Des. 2007;13:671–86.

9. Denley A, Wallace JC, Cosgrove LJ, Forbes BE. The insulin receptor isoform exon 11- (IR-A) in cancer and other diseases: a review. Horm Metab Res. 2003;35:778–85.
10. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat Rev Cancer. 2004;4:505–18.
11. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet. 2008;371:569–78.
12. Venkateswaran V, Haddad AQ, Fleshner NE, Fan R, Sugar LM, Nam R, et al. Association of diet-induced hyperinsulinemia with accelerated growth of prostate cancer (LNCaP) xenografts. J Natl Cancer Inst. 2007;99:1793–800.
13. Pandini G, Wurch T, Akla B, Corvaia N, Belfiore A, Goetsch L. Functional responses and in vivo anti-tumour activity of h7C10: a humanised monoclonal antibody with neutralising activity against the insulin-like growth factor-1 (IGF-1) receptor and insulin/IGF-1 hybrid receptors. Eur J Cancer. 2007;43:1318–27.
14. Werner H. Tumor suppressors govern insulin-like growth factor signaling pathways: implications in metabolism and cancer. Oncogene. 2012;31:2703–14.
15. Harrington EA, Bennett MR, Fanidi A, Evan GI. c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. EMBO J. 1994;13:3286–95.
16. O'Connor R, Kauffmann-Zeh A, Liu Y, Lehar S, Evan GI, Baserga R, et al. Identification of domains of the insulin-like growth factor I receptor that are required for protection from apoptosis. Mol Cell Biol. 1997;17:427–35.
17. Resnicoff M, Abraham D, Yutanawiboonchai W, Rotman HL, Kajstura J, Rubin R, et al. The insulin-like growth factor I receptor protects tumor cells from apoptosis in vivo. Cancer Res. 1995;55:2463–9.
18. Sell C, Rubini M, Rubin R, Liu JP, Efstratiadis A, Baserga R. Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor. Proc Natl Acad Sci USA. 1993;90:11217–21.
19. Sell C, Dumenil G, Deveaud C, Miura M, Coppola D, DeAngelis T, et al. Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts. Mol Cell Biol. 1994;14:3604–12.
20. Bentov I, Werner H. IGF, IGF receptor and overgrowth syndromes. Pediatr Endocrinol Rev. 2004;1:352–60.
21. Christofori G, Naik P, Hanahan D. A second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis. Nature. 1994;369:414–8.
22. Bruchim I, Attias Z, Werner H. Targeting the IGF1 axis in cancer proliferation. Expert Opin Ther Targets. 2009;13:1179–92.
23. Scotlandi K, Picci P. Targeting insulin-like growth factor 1 receptor in sarcomas. Curr Opin Oncol. 2008;20:419–27.
24. Yuen JSP, Macaulay VM. Targeting the type 1 insulin-like growth factor receptor as a treatment for cancer. Expert Opin Ther Targets. 2008;12:589–603.
25. Gualberto A, Pollak M. Emerging role of insulin-like growth factor receptor inhibitors in oncology: early clinical trial results and future directions. Oncogene. 2009; 28: 3009–21.
26. Belfiore A, Frasca F. IGF and insulin receptor signaling in breast cancer. J Mammary Gland Biol Neoplasia. 2008;13:381–406.
27. Araki E, Shichiri M. Characterization and expression of the insulin receptor gene and its promoter. Nihon Rinsho. 1994;52:2623–8.
28. Seino S, Seino M, Nishi S, Bell GI. Structure of the human insulin receptor gene and characterization of its promoter. Proc Natl Acad Sci USA. 1989;86:114–8.
29. Webster NJ, Resnik JL, Reichart DB, Strauss B, Haas M, Seely BL. Repression of the insulin receptor promoter by the tumor suppressor gene product p53: a possible mechanism for receptor overexpression in breast cancer. Cancer Res. 1996;56:2781–8.
30. Mamula PW, Wong KY, Maddux BA, McDonald AR, Goldfine ID. Sequence and analysis of promoter region of human insulin-receptor gene. Diabetes. 1988;37:1241–6.

31. McKeon C, Moncada V, Pham T, Salvatore P, Kadowaki T, Accili D, et al. Structural and functional analysis of the insulin receptor promoter. Mol Endocrinol. 1990;4:647–56.
32. Tewari DS, Cook DM, Taub R. Characterization of the promoter region and 3′ end of the human insulin receptor gene. J Biol Chem. 1989;264:16238–45.
33. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, et al. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature. 1985;313:756–61.
34. Zick Y. The insulin receptor: structure and function. Crit Rev Biochem Mol Biol. 1989; 24:217–69.
35. Finn FM, Ridge KD, Hofmann K. Labile disulfide bonds in human placental insulin receptor. Proc Natl Acad Sci USA. 1990;87:419–23.
36. Olson TS, Bamberger MJ, Lane MD. Post-translational changes in tertiary and quaternary structure of the insulin proreceptor. Correlation with acquisition of function. J Biol Chem. 1988;263:7342–51.
37. Cosgrove L, Lovrecz GO, Verkuylen A, Cavaleri L, Black LA, Bentley JD, et al. Purification and properties of insulin receptor ectodomain from large-scale mammalian cell culture. Protein Expr Purif. 1995;6:789–98.
38. Adams TE, Epa VC, Garrett TP, Ward CW. Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci. 2000;57:1050–93.
39. White MF, Kahn CR. The insulin signaling system. J Biol Chem. 1994;269:1–4.
40. Bandyopadhyay D, Kusari A, Kenner KA, Liu F, Chernoff J, Gustafson TA, et al. Protein-tyrosine phosphatase 1B complexes with the insulin receptor in vivo and is tyrosine-phosphorylated in the presence of insulin. J Biol Chem. 1997;272:1639–45.
41. Dickens M, Tavare JM. Analysis of the order of autophosphorylation of human insulin receptor tyrosines 1158, 1162 and 1163. Biochem Biophys Res Commun. 1992;186:244–50.
42. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A, McClain DA. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. EMBO J. 1990;9:2409–13.
43. Tavare JM, Denton RM. Studies on the autophosphorylation of the insulin receptor from human placenta. Analysis of the sites phosphorylated by two-dimensional peptide mapping. Biochem J. 1988;252:607–15.
44. Sarfstein R, Pasmanik-Chor M, Yeheskel A, Edry L, Shomron N, Warman N, et al. Insulin-like growth factor-I receptor (IGF-IR) translocates to nucleus and autoregulates IGF-IR gene expression in breast cancer cells. J Biol Chem. 2012;287:2766–76.
45. Hay RT. SUMO: a history of modification. Mol Cell. 2005;18:1–12.
46. Ahmed Z, Smith BJ, Kotani K, Wilden P, Pillay TS. APS, an adapter protein with a PH and SH2 domain, is a substrate for the insulin receptor kinase. Biochem J. 1999;341:665–8.
47. Ahmed Z, Smith B, Pillay TS. The APS adapter protein couples the insulin receptor to the phosphorylation of c-Cbl and facilitates ligand-stimulated ubiquitination of the insulin receptor. FEBS Lett. 2000;475:31–4.
48. Zheng N, Wang P, Jeffrey PD, Pavletich NP. Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell. 2000;102:533–9.
49. Hedo JA, Collier E, Watkinson A. Myristyl and palmityl acylation of the insulin receptor. J Biol Chem. 1987;262:954–7.
50. Feener EP, Backer JM, King GL, Wilden PA, Sun XJ, Kahn CR, et al. Insulin stimulates serine and tyrosine phosphorylation in the juxtamembrane region of the insulin receptor. J Biol Chem. 1993;268:11256–64.
51. Tavare JM, O'Brien RM, Siddle K, Denton RM. Analysis of insulin-receptor phosphorylation sites in intact cells by two-dimensional phosphopeptide mapping. Biochem J. 1988; 253:783–8.
52. Tornqvist HE, Pierce MW, Frackelton AR, Nemenoff RA, Avruch J. Identification of insulin receptor tyrosine residues autophosphorylated in vitro. J Biol Chem. 1987;262:10212–9.
53. Ando A, Momomura K, Tobe K, Yamamoto-Honda R, Sakura H, Tamori Y, et al. Enhanced insulin-induced mitogenesis and mitogen-activated protein kinase activities in mutant insulin receptors with substitution of two COOH-terminal tyrosine autophosphorylation sites by phenylalanine. J Biol Chem. 1992;267:12788–96.

54. Maegawa H, McClain DA, Freidenberg G, Olefsky JM, Napier M, Lipari T, et al. Properties of a human insulin receptor with a COOH-terminal truncation. II. Truncated receptors have normal kinase activity but are defective in signaling metabolic effects. J Biol Chem. 1988;263:8912–7.
55. McClain DA, Maegawa H, Levy J, Huecksteadt T, Dull TJ, Lee J, et al. Properties of a human insulin receptor with a COOH-terminal truncation. I. Insulin binding, autophosphorylation, and endocytosis. J Biol Chem. 1988;263:8904–11.
56. Myers MG, Backer JM, Siddle K, White MF. The insulin receptor functions normally in Chinese hamster ovary cells after truncation of the C terminus. J Biol Chem. 1991; 266:10616–23.
57. White MF, Livingston JN, Backer JM, Lauris V, Dull TJ, Ullrich A, et al. Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. Cell. 1988;54:641–9.
58. Katzen HM, Stetten Jr D. Hepatic glutathione-insulin transhydrogenase. Diabetes. 1962;11:271–80.
59. Varandani PT, Shroyer LA, Nafz MA. Sequential degradation of insulin by rat liver homogenates. Proc Natl Acad Sci USA. 1972;69:1681–4.
60. Brush JS. Purification and characterization of a protease with specificity for insulin from rat muscle. Diabetes. 1971;20:140–5.
61. Duckworth WC, Heinemann MA, Kitabchi AE. Purification of insulin-specific protease by affinity chromatography. Proc Natl Acad Sci USA. 1972;69:3698–702.
62. Morrione A, Valentinis B, Xu SQ, Yumet G, Louvi A, Efstratiadis A, et al. Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. Proc Natl Acad Sci USA. 1997;94:3777–82.
63. Bailyes EM, Nave BT, Soos MA, Orr SR, Hayward AC, Siddle K. Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptor species by selective immunoprecipitation and immunoblotting. Biochem J. 1997; 327:209–15.
64. Federici M, Porzio O, Zucaro L, Giovannone B, Borboni P, Marini MA, et al. Increased abundance of insulin/IGF-I hybrid receptors in adipose tissue from NIDDM patients. Mol Cell Endocrinol. 1997;135:41–7.
65. Baserga R. The decline and fall of the IGF-I receptor. J Cell Physiol. 2013;228:675–9.
66. Sandhu MS, Dunger DB, Giovannucci EL. Insulin, insulin-like growth factor-I, IGF binding proteins, their biologic interactions, and colorectal cancer. J Natl Cancer Inst. 2002; 94:972–80.
67. Bevan P. Insulin signalling. J Cell Sci. 2001;114:1429–30.
68. Cohen P. The twentieth century struggle to decipher insulin signalling. Nat Rev Mol Cell Biol. 2006;7:867–73.
69. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 2006;7:85–96.
70. Talbot K, Wang H-Y, Kazi H, Han L-Y, Bakshi KP, Stucky A, et al. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. J Clin Invest. 2012;122:1316–38.
71. Pollak M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. Nat Rev Cancer. 2012;12:159–69.
72. Longo N, Wang Y, Smith SA, Langley SD, DiMeglio LA, Giannella-Neto D. Genotype-phenotype correlation in inherited severe insulin resistance. Hum Mol Gen. 2002;11: 1465–75.
73. Abbott AM, Bueno R, Pedrini MT, Murray JM, Smith RJ. Insulin-like growth factor I receptor gene structure. J Biol Chem. 1992;267:10759–63.
74. Lowe Jr WL, Adamo M, Werner H, Roberts Jr CT, LeRoith D. Regulation by fasting of rat insulin-like growth factor I and its receptor. Effects on gene expression and binding. J Clin Invest. 1989;84:619–26.

75. Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, et al. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J. 1986;5:2503–12.
76. Werner H, Woloschak M, Stannard B, Shen-Orr Z, Roberts Jr CT, LeRoith D. Insulin-like growth factor receptor: molecular biology, heterogeneity, and regulation. In: LeRoith D, editor. Insulin-like growth factors: molecular and cellular aspects. Boca Raton, FL: CRC Press; 1991. p. 17–47.
77. Werner H, Bach MA, Stannard B, Roberts Jr CT, LeRoith D. Structural and functional analysis of the insulin-like growth factor I receptor gene promoter. Mol Endocrinol. 1992;6: 1545–58.
78. Sarfstein R, Belfiore A, Werner H. Identification of IGFR gene promoter binding proteins in estrogen receptor positive and ER-depleted breast cancer cells. Cancers. 2010;2:233–61.
79. Werner H, Bruchim I. IGF-1 and BRCA1 signalling pathways in familial cancer. Lancet Oncol. 2012;13:e537–44.
80. Werner H, Karnieli E, Rauscher FJ, LeRoith D. Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene. Proc Natl Acad Sci USA. 1996;93:8318–23.
81. Yuen JSP, Cockman ME, Sullivan M, Protheroe A, Turner GDH, Roberts IS, et al. The VHL tumor suppressor inhibits expression of the IGF1R and its loss induces IGF1R upregulation in human clear cell renal carcinoma. Oncogene. 2007;26:6499–508.
82. Boone DN, Lee AV. Targeting the insulin-like growth factor receptor: developing biomarkers from gene expression profiling. Crit Rev Oncog. 2012;17:161–73.
83. Andersen AS, Kjeldsen T, Wiberg FC, Christensen PM, Rasmussen JS, Norris K, et al. Changing the insulin receptor to possess insulin-like growth factor I ligand specificity. Biochemistry. 1990;29:7363–6.
84. Kjeldsen T, Andersen AS, Wiberg FC, Rasmussen JS, Schäffer L, Balschmidt P, et al. The ligand specificities of the insulin receptor and the insulin-like growth factor I receptor reside in different regions of a common binding site. Proc Natl Acad Sci USA. 1991;88:4404–8.
85. LeRoith D, Werner H, Beitner-Johnson D, Roberts Jr CT. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev. 1995;16:143–63.
86. Hanks SK, Quinn AM, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science. 1988;241:42–52.
87. Favelyukis S, Till JH, Hubbard SR, Miller WT. Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. Nat Struct Biol. 2001;8:1058–63.
88. Lopaczynski W, Terry C, Nissley P. Autophosphorylation of the insulin-like growth factor I receptor cytoplasmic domain. Biochem Biophys Res Commun. 2000;279:955–60.
89. Pautsch A, Zoephel A, Ahorn H, Spevak W, Hauptmann R, Nar H. Crystal structure of bisphosphorylated IGF-1 receptor kinase: insight into domain movements upon kinase activation. Structure. 2001;9:955–65.
90. Tollefsen SE, Stoszek RM, Thompson K. Interaction of the alpha beta dimers of the insulin-like growth factor I receptor is required for receptor autophosphorylation. Biochemistry. 1991;30:48–54.
91. Sehat B, Tofigh A, Lin Y, Trocme E, Liljedahl U, Lagergren J, et al. SUMOylation mediates the nuclear translocation and signaling of the IGF-1 receptor. Sci Signal. 2010;3:ra10.
92. Girnita L, Girnita A, Larsson O. Mdm2-dependent ubiquitination and degradation of the insulin-like growth factor 1 receptor. Proc Natl Acad Sci USA. 2003;100:8247–52.
93. Sehat B, Andersson S, Girnita L, Larsson O. Identification of c-Cbl as a new ligase for insulin-like growth factor-I receptor with distinct roles from Mdm2 in receptor ubiquitination and endocytosis. Cancer Res. 2008;68:5669–77.
94. Vecchione A, Marchese A, Henry P, Rotin D, Morrione A. The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor. Mol Cell Biol. 2003;23:3363–72.
95. Mao Y, Shang Y, Pham VC, Ernst JA, Lill JR, Scales SJ, et al. Polyubiquitination of insulin-like growth factor I receptor (IGF-IR) activation loop promotes antibody-induced receptor internalization and down-regulation. J Biol Chem. 2011;286:41852–61.

96. Gronborg M, Wulff BS, Rasmussen JS, Kjeldsen T, Gammeltoft S. Structure-function relationship of the insulin-like growth factor-I receptor tyrosine kinase. J Biol Chem. 1993;268:23435–40.
97. Hernandez-Sanchez C, Blakesley V, Kalebic T, Helman L, LeRoith D. The role of the tyrosine kinase domain of the insulin-like growth factor-I receptor in intracellular signaling, cellular proliferation, and tumorigenesis. J Biol Chem. 1995;270:29176–81.
98. Baserga R. The IGF-I receptor in cancer research. Exp Cell Res. 1999;253:1–6.
99. Baserga R. Insulin-like growth factor I receptor signalling in prostate cancer cells. Growth Horm IGF Res. 2000;10(Suppl A):S43–4.
100. LeRoith D, Werner H, Neuenschwander S, Kalebic T, Helman LJ. The role of the insulin-like growth factor-I receptor in cancer. Ann NY Acad Sci. 1995;766:402–8.
101. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst. 2000;92:1472–89.
102. Chan SJ, Nagamatsu S, Cao QP, Steiner DF. Structure and evolution of insulin and insulin-like growth factors in chordates. Prog Brain Res. 1992;92:15–24.
103. Werner H, LeRoith D. The role of the insulin-like growth factor system in human cancer. Adv Cancer Res. 1996;68:183–223.
104. Rosenblum JS, Kozarich JW. Prolyl peptidases: a serine protease subfamily with high potential for drug discovery. Curr Opin Chem Biol. 2003;7:496–504.
105. Lin C-T, Tang H-Y, Han Y-S, Liu H-P, Huang S-F, Chien C-H, et al. Downregulation of signaling-active IGF1 by dipeptidyl peptidase IV. Int J Biomed Sci. 2010;6:301–9.
106. Sepp-Lorenzino L. Structure and function of the insulin-like growth factor I receptor. Breast Cancer Res Treat. 1998;47:235–53.
107. Baserga R, Hongo A, Rubini M, Prisco M, Valentinis B. The IGF-I receptor in cell growth, transformation and apoptosis. Biochim Biophys Acta. 1997;1332:F105–26.
108. Dupont J, LeRoith D. Insulin and insulin-like growth factor-I receptors: similarities and differences in signal transduction. Horm Res. 2001;55 Suppl 2:22–6.
109. Myers Jr MG, Sun X-J, Cheatham B, Jachna BR, Glasheen EM, Backer JM, et al. IRS-1 is a common element in insulin and insulin-like growth factor-I signaling to the phosphatidylinositol 3′-kinase. Endocrinology. 1993;132:1421–30.
110. Galvan V, Logvinova A, Sperandio S, Ichijo H, Bredesen DE. Type 1 insulin-like growth factor receptor (IGF-IR) signaling inhibits apoptosis signal-regulating kinase 1 (ASK1). J Biol Chem. 2003;278:13325–32.
111. Zong CS, Chan J, Levy D, Horvath C, Sadowski HB, Wang LH. Mechanism of STAT3 activation by insulin-like growth factor I receptor. J Biol Chem. 2000;275:15099–105.
112. Werner H, Weinstein D, Bentov I. Similarities and differences between insulin and IGF-I: structures, receptors, and signaling pathways. Arch Physiol Biochem. 2008;114:17–22.
113. Klotz DM, Hewitt SC, Ciana P, Raviscioni M, Lindzey JK, Foley J, et al. Requirement of estrogen receptor-alpha in insulin-like growth factor-1 (IGF-1)-induced uterine responses and in vivo evidence for IGF-1/estrogen receptor cross-talk. J Biol Chem. 2002;277:8531–7.
114. van der Veeken J, Oliveira S, Schiffelers RM, Storm G, van Bergen En Henegouwen PM, Roovers RC. Crosstalk between epidermal growth factor receptor- and insulin-like growth factor-1 receptor signaling: implications for cancer therapy. Curr Cancer Drug Targets. 2009;9:748–60.
115. Pandini G, Mineo R, Frasca F, Roberts Jr CT, Marcelli M, Vigneri R, et al. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. Cancer Res. 2005;65:1849–57.
116. Plymate SR, Tennant MK, Culp SH, Woodke L, Marcelli M, Colman I, et al. Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF-I on proliferation and AR activity between localized and metastatic tumors. Prostate. 2004;61:276–90.
117. Sayeed A, Alam N, Trerotola M, Languino LR. Insulin-like growth factor 1 stimulation of androgen receptor activity requires beta(1A) integrins. J Cell Physiol. 2012;227:751–8.
118. Wu JD, Haugk K, Woodke L, Nelson P, Coleman I, Plymate SR. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. J Cell Biochem. 2006;99:392–401.

119. Charalambous M, Smith FM, Bennett WR, Crew TE, Mackenzie F, Ward A. Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. Proc Natl Acad Sci USA. 2003;100:8292–7.
120. Heron-Milhavet L, LeRoith D. Insulin-like growth factor I induces MDM2-dependent degradation of p53 via the p38 MAPK pathway in response to DNA damage. J Biol Chem. 2002;277:15600–6.
121. Girnita L, Shenoy SK, Sehat B, Vasilcanu R, Girnita A, Lefkowitz RJ, et al. β-Arrestin is crucial for ubiquitination and down-regulation of the insulin-like growth factor-1 receptor by acting as adaptor for the MDM2 E3 ligase. J Biol Chem. 2005;280:24412–9.
122. Girnita L, Shenoy SK, Sehat B, Vasilcanu R, Vasilcanu D, Girnita A, et al. β-arrestin and Mdm2 mediate IGF-1 receptor-stimulated ERK activation and cell cycle progression. J Biol Chem. 2007;282:11329–38.
123. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell. 1993;75:59–72.

Chapter 8
The MET Receptor Family

ChongFeng Gao and George F. Vande Woude

8.1 Introduction to the Met Receptor Tyrosine Kinase Family

8.1.1 MET

MET was isolated in 1984 as a transforming gene from a human osteosarcoma cell line which became more transformed after exposure to the carcinogen *N*-methyl-*N*′-nitroso-guanidine (MNNG-HOS). While the *MET* gene was named for *met*hyl [1], it is more appropriate for its function in tumor *met*astasis, as revealed in the study [2]. The first isolated *MET* gene was a chimeric gene (TPR-MET) containing sequences encoding the kinase domain and c-terminus of MET fused to TPR (translocated promoter region), which encodes a dimerization leucine zipper motif [3]. The two sequences are brought together through chromosomal rearrangement between chromosome 1 (TPR, 1q25) and chromosome 7 (MET, in 7q31). Subsequent studies indicated that the *MET* gene encoded a receptor tyrosine kinase (RTK), but the receptor's ligand was unknown at the time [4]. Molecular biological and biochemical experiments identified hepatocyte growth factor (HGF) as the MET ligand [5]. The ligand HGF was identified as a mitogen for hepatocytes [6–8], and independently identified as a fibroblast-derived cytokine that dissociated epithelial cells (scatter factor, SF) [9]. The two proteins were later found to be the same and were then referred to as HGF/SF [10].

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The binding of HGF/SF to MET elicits a diverse series of cellular responses including proliferation, scattering/motility, invasion into extracellular matrix, and branching morphogenesis. MET is primarily expressed in epithelial cells, while its ligand HGF/SF is produced by surrounding mesenchymal cells. An HGF/SF–MET-mediated interaction between epithelia and mesenchyme is required for cell migration and organ formation during embryonic development. MET signaling also participates in angiogenesis, wound healing, and organ regeneration in adults. However, aberrant activation of MET signaling has been found in a large number of different cancer types (www.vai.org/met). Numerous experimental studies and clinical investigations have demonstrated that aberrant MET signaling contributes to tumor development and malignant progression.

8.1.2 RON

In 1993, another member of the MET family, RON, was cloned by screening a cDNA library prepared from human foreskin keratinocytes [11]. RON encodes a receptor tyrosine kinase that is structurally similar to MET (Fig. 8.1); the proteins share a 63 overall sequence identity in their intracellular regions [11]. The ligand for RON is a serum-derived growth factor, MSP (macrophage stimulating protein) [12–15], which belongs to the HGF/SF family [16]. The RON receptor and its ligand are involved in embryonic development and are crucial in regulating certain physiological processes [17]. Aberrant activation of RON through overexpression or alternative splicing has been reported in various tumor types. Moreover, transgenic expression of RON in lung epithelial cells resulted in tumors with the pathological features of human bronchioloalveolar carcinoma. Thus, RON plays an important role in human cancers and may be a target for therapeutic intervention. Since RON and MET are structurally and functionally similar [18], we summarize the two receptors in table “Receptor at glance: comparison between Met and Ron.” This chapter will focus on MET.

8.2 The MET Receptor and Its Ligand HGF/SF

8.2.1 Genomic Organization, Transcription, and Synthesis of the MET Receptor

The *MET* gene is at 7q31 and consists of 21 exons. The 5′-regulatory region of the MET promoter lacks TATA or CAAT elements, but it has an extremely high G-C content and multiple Sp1-binding sites [19]. Besides Sp1, several other transcription factors including HIF-1 [20], Ets1 [21], Pax3 [22], and AP1 [23] were

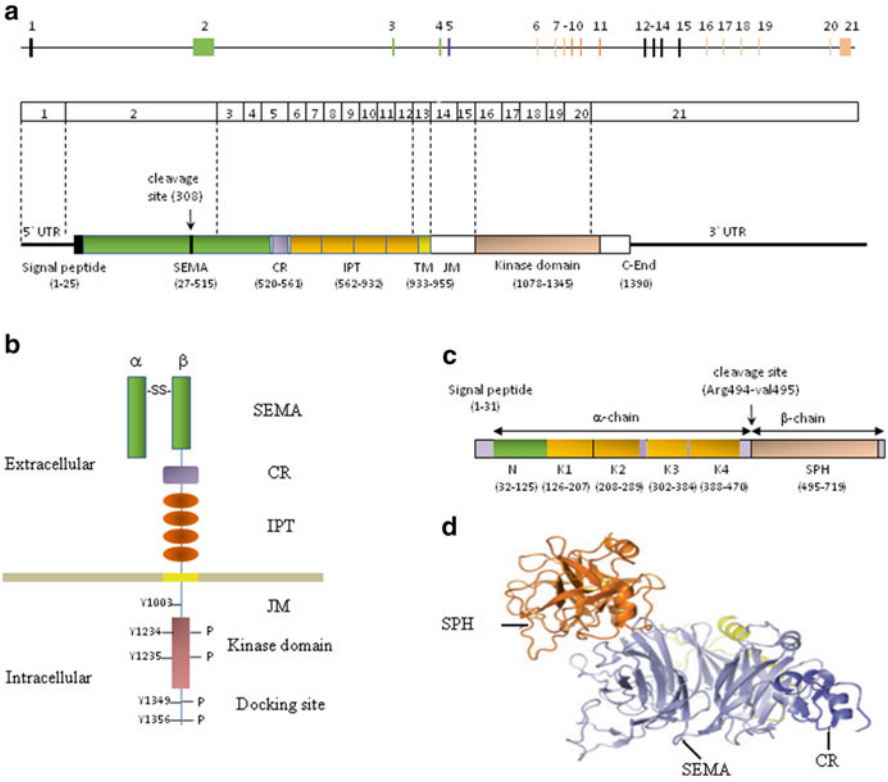

Fig. 8.1 Genomic structure and transcription of MET. (**a**) schematic representation of the *MET* gene locus. Exons are indicated by solid boxes and numbered above, while introns are indicated by the horizontal line. Numbered boxes indicate the exons of *MET*. MET protein is synthesized as a single-chain precursor and cleaved by furin during transit through the endoplasmic reticulum, thus yielding a smaller amino-terminal α-chain and a larger β-chain. (**b**) The MET ectodomain consists of a large N-terminal SEMA domain, which adopts a seven-bladed β-propeller fold and a stalk structure consisting of four immunoglobulin-like (Ig) domains. The SEMA domain and the stalk structure are separated by a small cystine-rich (CR) domain. The transmembrane (TM), the long juxtamembrane (JM) sequence, the kinase (K) domain, and a carboxy-terminal Docking site are also shown. (**c**) Hepatocyte growth factor/scatter factor (HGF/SF) is composed of six domains: an N-terminal (N) domain, four copies of the kringle domain (K1–4), and a C-terminal serine proteinase homology (SPH) domain that is structurally related to the catalytic domain of serine proteinases but that is enzymatically inactive. Mature, biologically active HGF/SF is a two-chain (α–β) protein that is produced by site-specific proteolysis in the extracellular space from single-chain pro-HGF/SF by the serine proteinases matriptase, hepsin, and HGF activator. HGF/SF contains two MET-binding sites: one in the NK1 fragment and one in the SPH domain. (**d**) The crystal structure of an SPH–MET complex is shown: the SPH domain of HGF/SF binds to an area of the SEMA domain within the MET α-chain (protein databank (PDB)

reported to be positive regulators of *MET* promoter. p53 was reported to be a transcription activator of MET, and a p53-responsive element was identified in *MET* promoter [24]. However, a later study showed that p53 could suppress MET expression at transcriptional and posttranslational levels in ovarian carcinoma cell lines, through inhibiting AP-1 and inducing miR-34, respectively [25]. The discrepancy may result from contextual difference of model systems used in the two studies.

Exon 1 of *MET* is noncoding and contains most of the 5′ UTR. Exon 2 is the largest internal coding exon (1,214 bp) in the *MET* gene and contains 14 bp of 5′ untranslated sequence followed by the initiating codon. Thus, the 4,170-bp open reading frame for the 1,390-amino-acid MET polypeptide precursor is distributed over 20 exons. After synthesis, the MET precursor undergoes proteolytic cleavage between Arg^{307} and Ser^{308}, forming an extracellular α chain and membrane-spanning β chain linked by disulfide bonds [26, 27]. Furin, a subtilisin-like mammalian endoprotease, has been identified as the processing endoprotease [28]. The 45 kDa α-chain is encoded by part of exon 2, whereas the 145 kDa β-chain is encoded by the rest of exon 2 together with exons 3–21 (Fig. 8.1).

The extracellular portion of the β subunit contains a semaphorin homology domain (SEMA), a cysteine-rich (CR) domain (also called the MET-related sequence, MRS), and four immunoglobulin-like (IPT, for IgG-like, plexins, transcription factors) domains. The intracellular portion of the β subunit contains a juxtamembrane domain (JM), a kinase domain, and a c-terminal docking site domain [29]. The HGF/SF-binding site is formed by the SEMA domain of the β-chain plus the α-chain [30]. The IPT domains 3 and 4 may be required for high-affinity binding between HGF/SF and MET [31]. The JM domain plays a key role in the binding of the CBL protein and in MET degradation. Phosphorylation of the JM domain at Y1003 is required for recruitment of CBL upon MET activation [32].

8.2.2 Genomic Organization, Transcription, and Synthesis of HGF/SF

The human HGF-encoding gene on chromosome 7q21.1 is composed of 18 exons and 17 introns. In the HGF/SF promoter region, an Sp1-binding site (at position –318 to –303 bp from the transcription start site) with a CTCCC motif has been identified [33]. Both Sp1 and Sp3 bind to this region and synergistically enhance HGF/SF gene expression. Other regulatory elements, CCAAT/enhancer-binding protein beta (C/EBP-β) and delta (C/EBP-δ), are located between –6 and +7 bp from the transcription start site. The core binding sequence for the inducible cis-acting factors was TTTGCAA (–4 to +3 bp). Partial hepatectomy increases C/EBP

binding activity to this region, providing a mechanistic explanation for the transcriptional induction of HGF/SF by extracellular signals (i.e., cytokines) that induce tissue regeneration [34]. A DNA element consisting of a mononucleotide repeat of 30 deoxyadenosines (deoxyadenosine tract element, DATE) is identified at 750 bp upstream from the transcription start site in the human *HGF* promoter. DATE acts as a transcriptional repressor, whose truncation leading to constitutive activation of the *HGF* promoter. DATE is a target of deletion in human breast cancer cells and tissues [35].

Expression of the *HGF* gene has been found to be upregulated by various cytokines and growth factors, including IL-1, TNF-α, EGF, FGF, and PDGF [36, 37], as well as by prostaglandins [38] and heparin [39]. In contrast, HGF expression is downregulated by dexamethasone and transforming growth factor β1 [40, 41]. *HGF* expression is restricted to non-epithelial cells, such as the fibroblasts of various tissues, Ito cells of the liver, macrophages, peripheral blood leukocytes, endothelial cells, and megakaryocytic cells [42].

HGF/SF is produced predominantly in mesenchymal cells as a precursor of 728 amino acid residues, which is mostly found in extracellular matrix [7]. While pro-HGF binds to MET with a high affinity, it is unable to activate MET [43]. Pro-HGF is proteolytically processed at Arg^{494}-Val^{495} to generate mature HGF/SF, a disulfide-linked heterodimer composed of a 69 kDa α subunit and a 34 kDa β subunit [44]. The α subunit contains a hairpin loop followed by four kringle (K1–K4) domains and is highly homologous to members of the plasminogen serine protease family. The β subunit resembles a serine protease homology domain (SPH), but lacks protease activity, partly due to mutations in residues forming the serine protease catalytic triad. The first kringle domain in the α-chain contains the high-affinity binding domain for MET [43, 45].

8.2.3 Activation of HGF/SF by Serine Proteinases

Three serine proteinases have been implicated in the activation of pro-HGF/SF: HGF activator (HGFA), matriptase (ST14), and hepsin. Matriptase and hepsin are type II transmembrane enzymes that efficiently activate pro-HGF at the cell surface [46]. In contrast, HGFA was originally isolated from bovine serum as a soluble proteinase capable of HGF/SF activation [47]. HGFA is present in human plasma as an inactive zymogen, which is processed by thrombin. The activated HGFA has a molecular mass of 34 kDa and consists of two chains held together by a disulfide bond [48]. The nucleotide sequence of the cDNA reveals that HGFA precursor protein contains 655 amino acid residues and consists of multiple putative domains homologous to those observed in blood coagulation factor XII [49]. Coagulation factor XIIa also has the ability to activate single-chain HGF, although

the specific activity is slightly lower than that of HGFA. The involvement of thrombin, a component of blood coagulation cascade, in HGFA activation suggests that HGFA is a key enzyme for HGF/SF activation during tissue regeneration [50, 51]. This idea is further supported by a study of HGFA-deficient mice, which exhibit decreased activation of HGF/SF and impaired regeneration of injured intestinal mucosa [52].

The activation of HGF/SF is finely tuned by two HGFA inhibitors: HGF activator inhibitor type 1 (HAI1; also known as SPINT1) and type 2 (HAI2; also known as SPINT2) [53, 54]. Both inhibitors are synthesized as integral membrane proteins containing two Kunitz domains and a transmembrane domain, and they are subsequently released by shedding from cell surface [55]. The inhibitors also inactivate matriptase, which is required for maintaining epithelial integrity [56], as well as for placental and neural development [57]. HGF/SF is known to associate with components of the extracellular matrix, including heparan sulfate proteoglycan, thrombospondin, fibronectin, and vitronectin [58, 59]. Matrix metalloprotease-mediated extracellular matrix degradation, which is triggered by the uPA/uPAR-plasmin system, facilitates the release and activation of sequestered pro-HGF from the extracellular matrix [60].

8.3 HGF/SF Activation of MET

8.3.1 Activation and Signaling

Binding of HGF/SF to MET receptor triggers dimerization and phosphorylation of the receptor. Phosphorylation at two tyrosine residues, Y1234 and Y1235, in the catalytic domain is crucial for activating MET as a tyrosine kinase [61], while phosphorylation at Y1349 and Y1356 in the C-terminal portion of the molecule is essential for its functioning as a docking site. Upon phosphorylation, the docking site recruits the Src homologous 2 (SH2)-domain-containing proteins, which in turn trigger specific signaling (Figs. 8.1 and 8.2). The adaptor proteins and signal transducers that physically bind to the phosphorylated MET receptor include Grb2 [29], Gab1 [62], SHC [63], Src [29], PI3K [64], and STAT3 [65].

Grb2 was isolated as a growth factor receptor-bound protein that contains a Src homology 2 (SH2) domain between two SH3 domains [66, 67]. The SH2 domain associates with the growth factor receptor, while the SH3 domains interact with the carboxyl-terminal domain of SOS (Son of Sevenless) to mediate RAS signaling [68–70]. The interaction between MET and Grb2 may be enhanced by SHC, which is recruited to the MET docking site and phosphorylated by activated MET. The phosphorylation of SHC produces a binding site ($pY^{317}VNV$) for Grb2 [63, 67]. Interestingly, the interaction between MET and SHC requires α6β4 integrin, which also physically interacts with MET [71]. Activated RAS triggers the activation of the MAPK pathway through RAS-RAF-MEK1, MEK2. This pathway is required

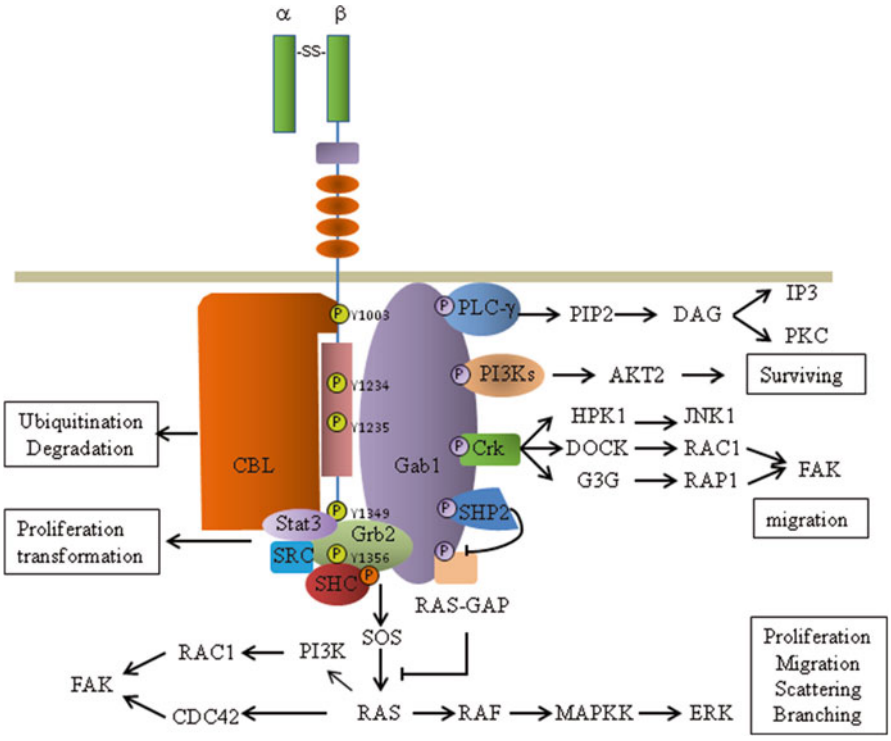

Fig. 8.2 Signaling pathways from HGF/SF activation of c-MET receptor. HGF/SF binding triggers MET dimerization and autophosphorylation activity. Phosphorylation at Tyr1234 and Tyr1235 of the kinase domain activates tyrosine kinase activity of MET. The phosphorylation of Tyr1349 and Tyr1356 at the docking site results in recruitment of various cytoplasmic effector molecules GRB2, GAB1, PLC, and SRC. Tyrosine-phosphorylated GAB1 that is bound to MET can attract further docking proteins, including SHP2, PI3K, and others. Phosphorylation and activation of these adaptors activate various downstream signaling cascades. Activation of MAPK results from sequential activation of several protein kinases including SOS, RAS, RAF, and MAPKK. PI3K is a lipid kinase catalyzing the formation of PIP3, which creates a docking site for Akt to the inner side of the plasma membrane. Activation of Akt leads to phosphorylation and activation of several substrates involved in cell proliferation and surviving. Phosphorylation of MET at Tyr1003 of JM domain results in the binding of CBL, an E3 ligase that triggers MET ubiquitination and degradation

for cell proliferation induced by growth factors, but it is also involved in other effects of MET signaling. For example, the activation of the MAPK pathway by MET induces the expression of urokinase, which plays an important role in cell invasion [72–74].

The role of Grb2 in MET-induced branching morphogenesis has been suggested from the use of mutant MET molecules that selectively disrupt the association: mutation at the consensus Grb2-binding site on MET, N1358H, disrupts the interaction between Grb2 and MET. Cells expressing this mutant receptor can scatter but

are unable to form branching tubules [75–78]. The role of Grb2 for the migration of muscle precursor cells in late myogenesis is also suggested by studies using this *MET* mutant as a mouse germline knock-in. These animals showed a striking reduction in limb muscle formation, while the development of placenta and liver was unaffected relative to animals nullizygous for *MET* [78].

Gab1 (Grb2-associated binder-1) was originally discovered as a Grb2 interacting protein that shares homology and structural features with IRS-1 (insulin-receptor substrate-1) [79]. Grb2 binds to Gab1 via its SH3 domain and to MET via its SH2 domain, thus coupling Gab1 to the MET receptor. Gab1 also directly binds to MET through its phosphotyrosine recognition domain (or MET-binding domain, MBD) [62]. Gab1 mutants deficient in Grb2 binding associate with MET but with a reduced strength, indicating that both direct and indirect binding are essential [80]. The N-terminal pleckstrin homology (PH) domain that binds phosphatidylinositol 3,4,5-triphosphate is critical for subcellular localization of Gab1. A Gab1 mutant lacking the PH domain is localized predominantly in the cytoplasm and loses the ability to induce branching morphogenesis [81]. Upon stimulation with HGF/SF, Gab1is recruited to the MET receptor and is phosphorylated at several tyrosine residues, which in turn recruit downstream adaptors and signaling molecules such as tyrosine phosphatase SHP2, PI3K, PLC-γ, and Crk/CRKL [80]. The specific tyrosine phosphorylation patterns on Gab1 specify the binding of different downstream molecules. For example, the phosphorylation of Y447, Y472, and Y589 is required for binding to the regulatory subunit p85 of PI3K [81, 82]; of Y627 for binding to SHP-2 [82, 83]; and of Y307, Y373, and Y407 for binding to PLC-γ [84].

The functions of SHP-2 and PLC-γ in MET signaling have been characterized by using Gab1 that is mutated at specific tyrosine residues required for its binding with distinct targets. The Gab1 C-terminal mutant Y637F fails to recruit SHP-2 and is unable to elicit sustained activation of ERK and epithelial morphogenesis in response to HGF/SF [80, 85]. As a tyrosine phosphatase, SHP2 may enhance RAS/ERK signaling by dephosphorylating the RAS-GAP-binding site on Gab1 and disengage RAS-GAP to sustain RAS activation [86]. A recent study showed that SHP2 deficiency compromises the mitotic checkpoint and results in chromosome instability and cancer predisposition. SHP2 is required for the optimal activation of the mitotic kinases PLK1 and Aurora B and thereby the proper kinetochore localization and phosphorylation of BubR1 [87]. Overexpression of the Gab1 mutant molecule Y307/373/407F, which is unable to bind PLC-γ, completely abolished HGF/SF-mediated tubulogenesis without altering scattering and only partially reduced cell growth [84].

Gab1 also contains multiple Tyr-*X*-*X*-Pro (Y*XX*P) motifs that bind to the adapter proteins c-Crk and Crk-like (CRKL) upon HGF/SF treatment [88]. c-Crk and CRKL are SH2- and SH3-domain-containing proteins, with the SH2 domain binding to Gab1 and the SH3 domain recruiting downstream adaptors including C3G, DOCK180, and HPK-1[89–91]. C3G is a guanine-nucleotide exchange factor that activates Rap1 [92], which in turn controls adherent junction positioning and cell adhesion [93]. DOCK180 is an activator of Rac1, which mediates MET-induced cell spreading and migration [94]. HPK-1 (hematopoietic progenitor

kinase1) is a well-established activator of JNK that is essential for MET-induced transformation [95–97].

PI3K is another Gab1-binding molecule that has been linked to HGF/SF-induced proliferation, scattering, and branching morphogenesis [98–100]. The PI3K/AKT pathway is a key to mediating cell survival in response to DNA damaging agents or serum starvation [101–103]. Survival signals emanating from HGF/SF–MET are enhanced by caspase-cleavage products of GAB1, a p35-GAB1 fragment that favors cell survival by maintaining HGF/SF-induced MET activation of AKT [104]. MET also mediates cell survival in PI3K/AKT-independent manner. For example, MET can prevent Fas-induced apoptosis by directly binding to Fas and blocking its self-aggregation and its ligand binding [105].

SHIP-1 (SH2-domain-containing inositol 5-phosphatase 1) was originally identified as a negative growth regulator in cytokine-stimulated hematopoietic cells [106]. In yeast two-hybrid screening, SHIP-1 was discovered to be a MET-binding protein [107]. MDCK cells that overexpress SHIP-1 branch early relative to wild-type cells in response to HGF/SF, while a mutant SHIP-1 molecule lacking catalytic activity impairs HGF/SF-mediated branching morphogenesis [107].

Upon HGF/SF activation of MET, Src binds to MDS domain of MET, which results in Src phosphorylation and activation. Activation of Src is required for HGF/SF-induced cell transformation [29]. HGF/SF also stimulates the recruitment of STAT-3 to MET receptor, which is followed by its tyrosine phosphorylation and nuclear translocation. STAT-3 is a transcription factor that activates the expression of genes required for HGF/SF-induced branching morphogenesis [65], or anchorage-independent growth and tumorigenic activity [108]. Also, Src and STAT-3 may cooperate to upregulate HGF expression [109].

8.3.2 Modulation of MET Activation by Other Signal Molecules

While HGF/SF is the only known ligand for MET, a number of signal molecules have been implicated in effective activation of MET. These proteins that augment MET activation include CD44, integrin, class B plexins, and other RTKs.

CD44 is a receptor for hyaluronic acid that is involved in cell–cell interactions, cell adhesion, and migration. CD44 exists in multiple isoforms that are generated through alternative splicing. CD44 isoforms containing the alternatively spliced exon v3 (CD44v3) carry heparan sulfate side chains that are able to bind HGF/SF. CD44v3 may enhance MET signaling by concentrating and presenting HGF/SF to MET [110]. Co-expression of CD44v3 and MET correlates with a poor prognosis of colon cancer, suggesting CD44v3 may promote HGF/SF-induced tumor progression [111]. Another isoform, CD44v6, forms a complex with HGF/SF–MET that enhances HGF-dependent MET phosphorylation [112] and activation of MAPK pathway in several tumor cell lines [112, 113].

The collaboration of CD44 and MET is required for development of the central and peripheral nervous systems; mice with MET (and HGF/SF and Gab1) heterozygous mutations on a $CD44^{-/-}$ background die at birth with defects in nervous system development. However, CD44-null animals or animals heterozygous for MET do not exhibit these defects, probably because ICAM-1 (intercellular adhesion molecule-1) can compensate for CD44 as a co-receptor for MET in CD44-null mice. In CD44 wild-type mice, MET activation and cell proliferation following partial hepatectomy were inhibited by CD44v6-specific antibodies, but ICAM-1-specific antibodies only interfered with liver cell proliferation and MET activation in CD44 knockout mice [114, 115]. These studies indicated that cross talk between CD44 and HGF/SF–MET signaling plays an important role in adult physiology and embryonic development.

Integrins are a group of membrane proteins that mediate the attachment of cells to the extracellular matrix. Certain integrins, such as α6β4, selectively associate with MET and potentiate HGF-triggered activation of the RAS and PI3K-dependent pathways [116]. Integrin-mediated cell–matrix adhesion may also activate MET in the absence of HGF/SF [117, 118]. The cross talk between integrin and MET may synergistically promote tumor invasion.

Plexins are single-pass transmembrane receptors for semaphorins, which modulate cytoskeletal remodeling and integrin-dependent adhesion [119]. Class B plexins and MET share homology in their extracellular domains: they both contain a Sema domain that forms a β-propeller structure, a cysteine-rich motif, and immunoglobulin-like domains [119]. The propeller domain mediates MET association with class B plexins [120–122]. The binding of Sema4D to plexinB1 increased MET signaling and enhanced cell invasion, while MET expression was also required for effective activation of plexinB1 by Sema4D [121].

The cross talk between HGF/SF–MET and other signaling molecules is also required for embryonic development. The absence of MET during renal development causes reduced branching of the ureteric bud and a decreased number of nephrons. Mice missing both MET and EGFR exhibit more serious defects in renal development [123], suggesting that cross talk between MET and EGFR family members is likely to be important [123–125]. Functional cross talk between MET and EGFR has been reported in several systems [126, 127]. Co-expression of MET and Her-2 is often detected in breast and gastric cancer cells [128, 129]. EGF stimulation of bladder, hepatocyte, epidermoid carcinoma, and non-small cell lung cancer cell lines activated both EGFR and MET [126, 130, 131]. In contrast, EGFR inhibition by Gefitinib significantly blocks HGF/SF activation of MET and the HGF/SF-induced proliferation and migration of mammary carcinoma cell lines [132], suggesting that the EGF/EGFR ligand/receptor pair is required for full activation of MET signaling. On the other hand, HGF/SF promotes transactivation of EGFR during retinal pigment epithelial wound healing, leading to an enhanced activation of downstream signaling pathways [133]. Activation of MET through amplification in lung cancer cells activates the ERBB3–PI3K pathway and promotes resistance to EGFR kinase inhibitors [134]. Therefore, the cross talk between MET and EGFR is an important mechanism for cancer progression and resistance to therapy.

Cross talk between MET and WNT–β-catenin occurs at several levels. First, MET can contribute to the transcriptional activation of WNT ligands such as WNT7B [135]. Second, MET can also stabilize β-catenin by inhibiting its degradation through AKT phosphorylation of glycogen synthase kinase-3β (GSK3β). Third, HGF/SF activation of MET promotes nuclear translocation of β-catenin and the transcription of their target genes in liver and bladder cancer cells [136]. On the other hand, MET is a direct transcriptional target of WNT–β-catenin in colon cancer cell lines [137].

8.3.3 MET Internalization, Processing, and Attenuation

The strength and duration of MET activation is tightly regulated to induce appropriate cellular responses [138]. Levels of MET expression at the cell surface are finely tuned by multiple mechanisms, including clathrin-mediated endocytosis, extracellular shedding, and intracellular cleavage. Downstream signaling is also restricted through negative feedback loops.

While HGF binding activates MET signaling, it also triggers the downregulation of MET through receptor-mediated endocytosis [139]. The proteasome activity seems to be necessary for MET internalization, although the detailed mechanism of how the proteasome participates is unknown [140, 141]. In this process, the MET-ligand complex is recruited to clathrin-coated pits, followed by internalization and endosomal trafficking, and ending with degradation in lysosomes or recycling to the plasma membrane [141]. Like other RTK receptors, the internalized receptor that is delivered to endosomal compartments remains capable of signaling during vesicle trafficking [142, 143] and is even required for certain signaling events such as the activation of ERK [144–146]. MET-activated ERK signaling within endosomal compartments is regulated by PKC-ε, which ensures the consequent accumulation of ERK in focal complexes [146]. In contrast, PKC-α is only required for the microtubule-based movement of MET from an early endosomal compartment to a perinuclear compartment. MET being delivered to a perinuclear endosomal compartment seems to be required to sustain phosphorylated STAT3 in the nucleus [147]. Thus, the route of trafficking can determine the nature of the signal output.

The proto-oncogene CBL plays a key role in MET ubiquitination and degradation. CBL is an E3 ubiquitin ligase that serves as a negative regulator for a number of receptor tyrosine kinases [148–150]. In addition to a RING finger domain that recruits E2 enzyme, CBL contains a tyrosine kinase binding domain, which recognizes the phosphorylated Tyr1003 residue in the juxtamembrane domain of MET, and a proline-rich domain that binds to Grb2. The site of ubiquitin binding is at the C-terminal ubiquitin-associated domain (UBA) [32, 151, 152].

Upon MET activation, CBL is recruited to MET through Y1003 and is subsequently phosphorylated by MET to activate its E3 ligase activity. MET is then ubiquitinated [153] and recruited to the endophilin-CIN85-Cbl complex in clathrin-coated pits [154]. Formation of the endophilin-CIN85-Cbl complex triggers invagination

and scission of the membrane to form early endosomes. After endocytosis, the ubiquitinated MET receptors are retained in endosomes through their interaction with the ubiquitin-interacting domain contained in the *h*epatocyte growth factor-*r*egulated tyrosine kinase *s*ubstrate (HRS) [155, 156]. HRS couples ubiquitinated MET to the endosomal sorting complex for transport (ESCRT) to initiate formation of the multivesicular body, which is then targeted to the lysosomes for degradation [157]. The endosomal sorting process also requires the signal-transducing adaptor molecule (STAM) that forms a heterodimeric complex with HRS [158]. The unubiquitinated MET interacts with GGA3 via the CRK adaptor and ARF6. The formation of a GGA3-MET complex promotes access of MET into a recycling pathway. GGA3-dependent entry of MET into the recycling pathway promotes sustained ERK1/2 activation [159].

Cbl-dependent ubiquitination is crucial to targeting the MET receptor to components of the lysosomal sorting machinery, but it appears to be dispensable for MET internalization [160]. Thus, MET with mutation or deletion of the CBL-binding site is still internalized upon ligand activation, but it escapes degradation owing to a change in endosomal sorting [32, 161]. Such receptor variants lead to sustained signaling and convert MET into a transforming protein [162]. Beyond mutations in the JM domain, MET mutations in the kinase domain (D1246N and M1268T) produce increased endocytosis/recycling activity and decreased degradation of MET, which leads to the accumulation of MET in endosomes [163]. Endosomal MET activates the GTPase Rac1, which is required for cell migration, tumorigenic activity, and experimental metastasis [163].

Another mechanism that leads to downregulation of MET involves the proteolysis and shedding of the extracellular domain. Shedding is mediated by members of the disintegrin and metalloproteinase (ADAM) family which generate a soluble MET ectodomain and a membrane-anchored cytoplasmic tail. The cytoplasmic tail undergoes proteolysis by γ-secretase and is rapidly cleared by proteasome-mediated degradation [164]. Unlike Cbl-mediated endosomal degradation, proteolysis of MET does not require the ligand-mediated activation of MET. The extracellular shedding of MET not only decreases the number of receptor molecules on the cell surface but also generates a decoy moiety that interacts with both HGF and full-length MET to further inhibit MET signaling [165]. In an immortal trophoblast cell line, B6Tert-1, HGF/SF–MET signaling induces ADAM10 and ADAM17, which in turn lead to proteolysis and MET shedding. Thus, HGF/SF could self-control its regulation on trophoblast cell invasion by enhancing proteolysis of its receptor. Interruption of this feedback loop may impede placentation during mammalian placental development [166].

MET signaling can be inhibited by downstream molecules of its signaling pathway. Spry2 was first identified as an inhibitor of the FGF and EGFR signaling pathways during *Drosophila* organogenesis [167, 168]. Subsequent study indicated that Spry2 is transcriptionally upregulated in cells treated with HGF/SF, and its expression inhibits MET signaling and HGF/SF-induced cellular responses [169]. MET activation also leads to transcriptional induction of the Notch ligand Delta and the Notch effector HES-1. The activation of Notch signaling downregulates the MET

receptor and suppresses RAS-ERK signaling [170]. Loss of Spry2 leads to activation of RAS-ERK signaling and contributes to tumorigenesis, indicating that the counter-regulatory mechanism is required for appropriate function of MET signaling [171–173].

8.4 Cellular Responses to HGF/SF

Activation of MET signaling induces various cellular responses including cell growth [6, 174], scattering/migration [9, 175], invasion [176], tubulogenesis/branching morphogenesis [124], and lumen formation [177, 178].

8.4.1 HGF/SF–MET Signaling in Cell Proliferation

Growth factor-induced cell proliferation is defined by its capacity to induce DNA synthesis in quiescent cells [179]. Indeed, HGF/SF was first identified based on its capacity to stimulate DNA synthesis [8]. The most sensitive method to measure HGF/SF-induced DNA synthesis is [^{3}H]-thymidine incorporation (Fig. 8.3a). Serum starvation before HGF/SF treatment may be required to measure the effect of HGF/SF on DNA synthesis in cultured tumor cells, since serum is a strong stimulator. The time to reach the peak of DNA synthesis after HGF/SF treatment may be cell type dependent. In the case of SK-LMS-1 cells, DNA synthesis peaks at 12 h of HGF/SF treatment.

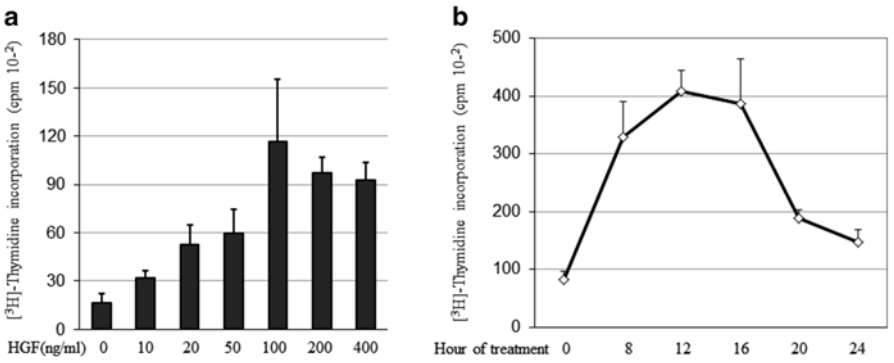

Fig. 8.3 HGF/SF stimulates proliferation on SK-LMS-1 cells as analyzed by [^{3}H]-thymidine incorporation assay. (**a**) Effects of HGF/SF on DNA synthesis. Cells were seeded in 96-well plate (2,000 cells/well) and cultured for 24 h. After serum starvation, the cells were treated with HGF/SF for 10 h. [^{3}H]-thymidine was added for 8 h before analysis. (**b**) DNA synthesis at various times after HGF/SF treatment. Cells were seeded in 96-well plate (5,000 cells/well) and cultured for 24 h. After serum starvation, the cells were treated with HGF/SF for various times. [^{3}H]-thymidine was added for 5 h before analysis

While the upregulation of cyclinD and downregulation of p27 through the RAS/MAPK and PI3K pathways are commonly involved in growth factor-induced cell growth [179], the activation of RAS/MAPK and PI3K is insufficient for HGF-induced growth, at least in some systems [180]. Activation of additional signaling, such p38 and NF-κB, may be also required [181, 182]. HGF/SF can induce proliferation through c-Myc in a proliferative subclone isolated from the DBTRG-05MG glioblastoma cell line [183]. The levels of phosphorylated ERK and AKT in the proliferative subclone were much lower than those of invasive subclones, which also exhibited low levels of c-Myc. This study suggests that high ERK and AKT activity is not required for c-Myc induction and proliferative response, although a basal level may be essential. Indeed, a high-intensity ERK signal mediates HGF/SF-induced proliferation inhibition in the human hepatocellular carcinoma cell line HepG2 [184, 185]. The role of Src in HGF/SF-induced proliferation was investigated using a Gab1 mutant having substitutions in the Src phosphorylation sites (Y242, Y259, Y317, and Y373). These Gab1 mutants failed to promote HGF-induced DNA synthesis but retained the ability to facilitate HGF-induced chemotaxis, indicating that Src is important for HGF-induced DNA synthesis [186].

8.4.2 HGF/SF–MET Signaling in Cell Scattering and EMT

The epithelial–mesenchymal transition (EMT) is a process characterized by loss of intercellular junctions and increased cell motility. In two-dimensional culture, EMT was reflected in cell spreading; a series of processes including disruption of cell–cell junctions; and subsequent cell scattering and migration [187]. HGF/SF was independently identified as scatter factor (SF), which causes a disruption of junctions, an increase in local motility, and a scattering of contiguous sheets of epithelial cells [9]. The role of HGF/SF in cell scattering is best manifested in MDCK cells (Fig. 8.4).

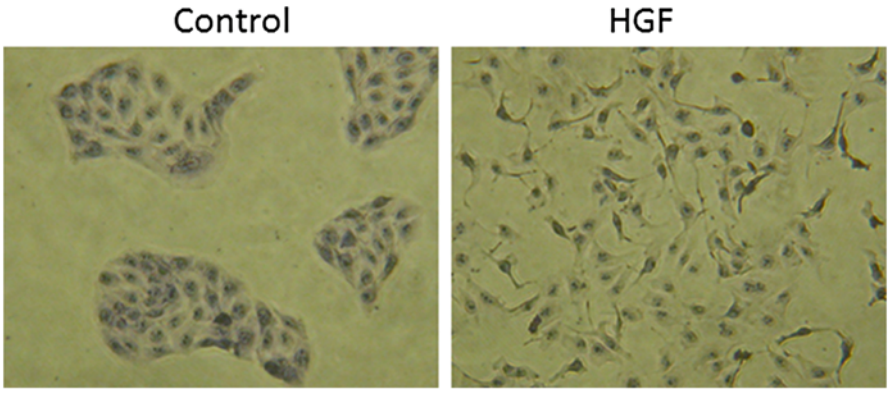

Fig. 8.4 HGF/SF-induced cell scattering in MDCK cells. Cells were treated with HGF/SF at 20 ng/ml for 24 h (HGF), or untreated (Control). Images were taken after cell staining with 0.005 % crystal violet

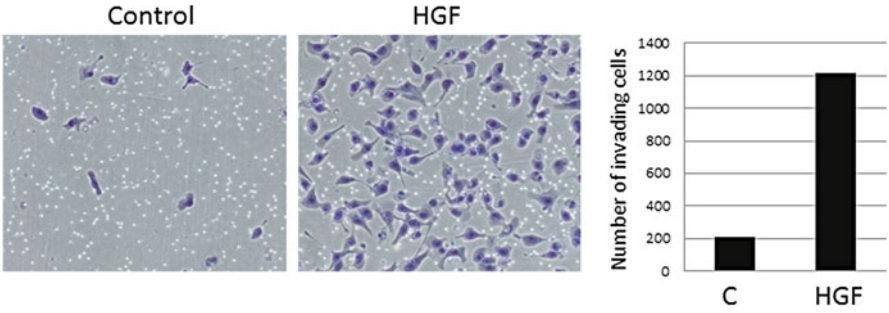

Fig. 8.5 HGF/SF-induced cell invasions through Matrigel. SK-LMS-1 cells (10,000 cells/chamber) were loaded into Boyden chamber and treated with HGF/SF at 100 ng/ml for 24 h (HGF), or were untreated (Control). Cells remaining inside the chamber were removed. Cells invading through Matrigel and attached to the bottom surface were stained. The number of invading cells in the whole insert was counted and presented in bar graph (unpublished data provided by Dr. Gao)

The RAS activation is sufficient for cell spreading and disruption of adherent junctions, while p42/p44 MAPK, PI3-kinase, and Rac are required for the downregulation of E-cadherin and the disruption of adherent junctions in MDCK cells [188]. The downregulation of E-cadherin may result from co-endo/exocytosis with MET [189]. HGF/SF-induced scattering may be a prerequisite for cell invasion; the process also needs the upregulation of uPA/uPAR [74] and members of the matrix metalloproteinase family [72, 73, 190–192] (Fig. 8.5). These studies suggest that HGF/SF-induced EMT may play a role in invasion and metastasis in human cancer. In fact, high level of circulating HGF/SF are associated with EMT in tumor tissue from small cell lung cancer and with poor outcome in patients [193].

EMT has been implicated in numerous developmental processes, including mesoderm formation and neural tube formation. HGF/SF–MET signaling is essential for the generation of myogenic precursor cells from the epithelial dermomyotome (i.e., EMT) as well as for the migration of myogenic precursor cells into the limbs, tongue, and other organs, where they differentiate to form a subset of the hypaxial muscles. The long-distance migration in the embryo is dependent on both MET and GAB1 [194, 195].

8.4.3 HGF/SF–MET Signaling in Tubulogenesis/Branching Morphogenesis

Tubulogenesis/branching morphogenesis refers to the organization of epithelial cells into branched tubular structures [196]. Branching morphogenesis is the structural basis for the formation of a variety of parenchymal organs, such as the kidney, liver, lung, and mammary gland during embryonic development.

Under physiological conditions, this is a highly complex process that involves the interaction of different cell types and is induced by various environmental cues. This process can be mimicked, in vitro, by culturing Madin-Darby canine kidney (MDCK) epithelial cells in three-dimensional (3D) collagen matrix in the presence of either fibroblasts or fibroblast-conditioned medium [197]. HGF/SF was subsequently identified as the sole growth factor responsible for branching morphogenesis [124]. None of the other known growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), transforming growth factor beta 1 (TGF-β1), insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), platelet-derived growth factor (PDGF), or keratinocyte growth factor (KGF) displayed the same activity in MDCK cells [197]. It was subsequently shown that HGF/SF induces branching morphogenesis in collagen matrix in a wide variety of epithelial cells from colon, pancreas, mammary gland, prostate, lung, and other organs [198]. The tubular structures formed in vitro culture system resemble the epithelial organization of the organ of origin, indicating that HGF/SF can induce morphogenesis in diverse epithelial cells, and the exact morphogenic events are determined by the intrinsic programs of the epithelia [198].

HGF/SF-induced branching morphogenesis includes a series of steps: starting from spheroid cysts of MDCK cells cultured in collagen matrix, HGF/SF stimulation induces membrane protrusions of individual MDCK cells in the cyst that extend into the extracellular matrix. The protrusions then develop chains of cells that are connected to the cyst. Next, the chains form cords that are two to three cells thick and develop discontinuous lumens. Finally, the discontinuous lumens grow and fuse to become continuous with the lumen of the cyst [199, 200]. These processes required a series of cellular responses including invasion, proliferation, migration, survival, and differentiation. Therefore, a sequential and coordinated activation of signaling is required for each of the cellular responses [196]. For example, activation of the RAS–MAPK pathway is required for HGF/SF-induced early steps of tubulogenesis when cells form protrusions, proliferate, migrate, and organize themselves into long chains, but is dispensable for the later dedifferentiation steps where polarity is reestablished and a fluid-filled lumen is formed [201]. The strength of MAPK activation is also critical for branching morphogenesis [127]. HGF/SF stimulates complete breakdown of cell–cell junctions to generate single cells in MDCK cells expressing constitutively activated ErbB2/Neu receptor (NeuNT). Those single cells do not form cell chains and cords, which are necessary steps for branching morphogenesis. HGF-induced cell dispersal of NeuNT-expressing cells is lessened by pretreatment with a pharmacological inhibitor of the mitogen-activated protein kinase kinase (MEK) pathway, which restores cell–cell junctions and branching morphogenesis [127]. This study suggest that moderate MAPK activity and partial EMT are required for generating cell chains and cords in early stage of branching morphogenesis.

MDCK cells forms tubes when cultured in Type I collagen gels, but not in basement membrane Matrigel [202], indicating that the interaction between cell membrane and the components within extracellular matrix (ECM) plays a key role in this

process. By adding back individual components Matrigel to MDCK cells grown in Type I collagen gels in the presence of HGF, it has been shown that certain ECM proteins, such as Type IV collagen, heparan sulfate proteoglycan, and vitronectin, caused marked inhibition of HGF-induced morphogenesis. However, other components in Matrigel, such as laminin, entactin, and fibronectin, actually facilitated the formation of branching tubular structures and increased their complexity [202]. It is worth noting that the stimulating or inhibitory effect of an ECM component on branching morphogenesis may be cell type dependent, since many tumor cell lines exhibit branching morphogenesis in 3D Matrigel [203–205] (Fig. 8.6).

HGF/SF-induced branching morphogenesis can be modulated by various signal molecules or microenvironmental factors. For example, EphA2 acts as a positive regulator for HGF/SF-induced mammary epithelial branching morphogenesis, since the HGF/SF-dependent morphogenesis was significantly reduced in EphA2-deficient cells relative to wild-type cells. The branching defects can be rescued by inhibition of Rho-Associated, Coiled-Coil-Containing Protein Kinase (ROCK) activity, suggesting that EphA2 mediates HGF/SF-induced branching morphogenesis through inhibition of RhoA–ROCK pathway [206]. HGF/SF-induced branching morphogenesis can also be antagonized by several morphogenic factors, such as TGF-β, which inhibit the formation of tubular structures in MDCK cells [202]. Hedgehog signaling in prostate stromal cells downregulates HGF/SF and thus inhibits branching morphogenesis in prostate cells. Such a signaling downregulates HGF/SF expression by inducing miR-26a and miR-26b, which in turn downregulate expression of HGF/SF [207].

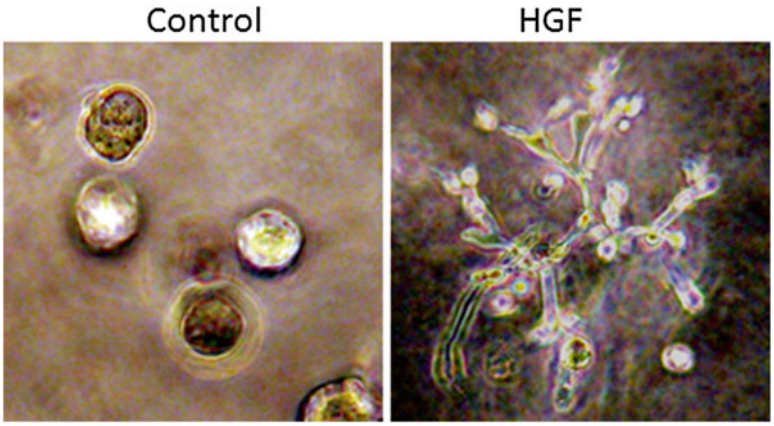

Fig. 8.6 HGF/SF induces branching morphogenesis in DU145 cells. 2,000 cells were suspended in 100 μl medium containing 50 % of Matrigel and loaded into 96-well plates. Cells were cultured in 37 °C for 30 min and fed with normal medium (Control) or medium supplemented with HGF/SF (100 ng/ml) for 10 days (HGF). Cells form acini in the absence of HGF/SF, while a portion of cells form branching structures in the presence of HGF/SF. Shown is a representative picture of branching structures formed when cells were cultured in the presence of HGF (unpublished data provided by Gao)

8.4.4 HGF/SF–MET Signaling and Stem Cell Properties

Stem cells are cells found in multicellular organisms that can divide and differentiate into diverse specialized cell types and can self-renew to produce more stem cells. Cancer stem cells, or cancer-initiating cells, are defined as a subpopulation of cancer cells that effectively reconstitutes the tumor heterogeneity after transplantation [208]. According to stem cell theory, the small fraction of cancer stem cells is the driving force for tumor growth and therefore should be the target of cancer therapy. Cancer stem cell theory has attracted a great interest, although the identity of these cells is still elusive [209].

HGF/SF–MET signaling has been implicated in the migration (but not the proliferation) of human mesenchymal stem cells isolated from bone marrow and cord blood [210, 211], as well as in cardiac stem cells after myocardial infarction [212]. MET signaling has also been implicated in the activation of the adult muscle stem cells [213, 214], hepatic stem cells [215], and pancreas stem or progenitor cells [216], and suggesting it is involved in the regeneration and repair of these organs.

HGF/SF–MET signaling has also been implicated in the stem cell properties of several types of cancers. In colon cancer, myofibroblast-secreted HGF/SF activates β-catenin-dependent transcription and CSC clonogenicity and even restores the CSC phenotype in more-differentiated tumor cells [217]. In human glioma, expression of the *MET* oncogene is associated with a mesenchymal and proneural subtype, but not the classical subtype of glioblastoma. The MET-expressing subpopulation in mesenchymal or proneural subtype neurospheres displays clonogenic potential and long-term self-renewal ability. These stem cell properties are further enhanced by HGF/SF treatment, suggesting that MET is a functional marker of glioblastoma stem cells [218]. A high level of MET is also associated with luminal progenitors in mouse models, and constitutive activation of MET in those progenitors generates stem cell properties, including clonogenic activity and the de novo ability to reconstitute mammary glands in repopulation assays. Activation of MET in luminal progenitors induces hyperplasic ductal morphogenesis and basal lineage commitment. These observations suggest a role for MET in promoting deregulated proliferation and generation of basal-like breast tumors [219].

8.5 HGF/SF–MET in Embryogenesis and Tissue Regeneration

One of the functions of HGF/SF–MET signaling in embryogenesis is in the generation of skeletal muscle that derives from long-range migrating precursor cells. Such precursor cells emigrate from the dermomyotome, an epithelial structure that develops from somites, and finally generate a subset of the hypaxial muscle groups. Loss of the *HGF/SF* or *MET* gene results in complete absence of the hypaxial muscle groups in the mouse embryo, whereas other muscle groups form normally [194, 195].

HGF/SF and MET are also involved in the development of epithelial organs. In *HGF/SF*- and *MET*-null mutant embryos, the liver is reduced in size, and the placental labyrinth layer formed by epithelial trophoblast is greatly reduced [194, 220, 221].

Regeneration is a fundamental part of liver response to injury. Among many growth factors and cytokines, HGF/SF plays important roles in this process [222]. Partial hepatectomy rapidly triggers HGF/SF mobilization from the extracellular matrix and the activation of MET in hepatocytes, which leads to proliferation. Mice with conditional knockout of *MET* in hepatocytes display impaired proliferation and incomplete liver regeneration after partial hepatectomy, providing genetic evidence for the crucial role of MET in liver regeneration [223, 224].

Upon injury to the skin, a set of complex biochemical events takes place in a closely orchestrated cascade to repair the damage. The basal keratinocytes at the wound edges play an important role in the epithelialization stage. HGF/SF and MET are co-expressed in keratinocytes during wound repair of the skin, implying that autocrine signaling is involved [225]. In mice with *MET* knockout in keratinocytes, only cells that had escaped recombination and that continued to express a functional MET could contribute to regeneration [225], suggesting that HGF/SF–MET signaling is essential for re-epithelialization in vivo.

8.6 Role of the MET Receptor Tyrosine Kinase in Human Disease

8.6.1 HGF/SF–MET Signaling in Cancer

MET was originally isolated as an activated oncogene, *Tpr-MET*, which possessed transforming activity [1]. The generation of an autocrine loop by co-expressing wild-type MET and HGF/SF molecules in NIH3T3 cells was also shown to be oncogenic, inducing tumor metastasis [2, 226]. The tumorigenicity of both *Tpr-MET* and autocrine HGF/SF–MET signaling was further proven in transgenic mouse models [227–229].

The involvement of *MET* in human tumorigenesis and metastasis was supported by the detection of *MET* amplification and overexpression in various tumors, especially in metastatic cancers. Most importantly, germline missense mutations in *MET* were discovered in both the sporadic and hereditary forms of human papillary renal carcinomas [230]. Most of these mutations are located in the kinase domain and are homologous to cancer-inducing mutations that occur in other RTKs. These mutants show increased levels of kinase activity, and NIH 3T3 cells expressing mutant MET forms in vitro are tumorigenic in nude mice [231, 232]. Mice carrying these mutations developed a variety of tumors including sarcomas, lymphomas, and carcinomas [233]. When expressed in the mammary gland, the mutant MET molecules induce basal-like breast carcinomas [234, 235]. Somatic MET mutations were detected in childhood hepatocellular carcinomas (HCCs) [236] and head and neck

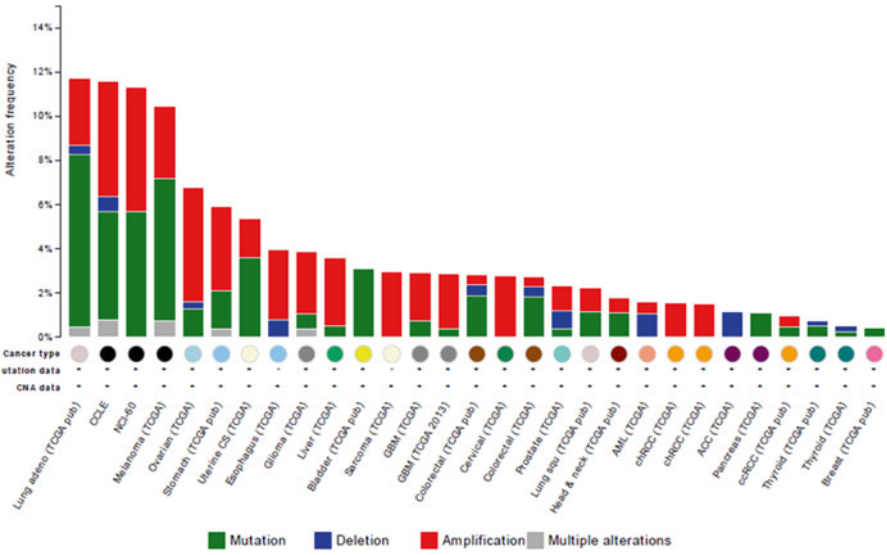

Fig. 8.7 *MET* gene alterations in major human cancer types. Mutations were identified in human cancers in The Cancer Genome Atlas (TCGA). *MET* mutations identified in cancer cell lines in Cancer Cell Line Encyclopedia (CCLE) and NCI-60 Cell Lines (NCI-60) are also included. The diagram is generated by the cBioPortal for Cancer Genomics

squamous cell carcinomas (HNSCC) [237]. The role of MET in tumor metastasis is supported by HNSCC, where the transcripts of the *MET* mutants are highly expressed in lymph node metastases but are barely detectable in the primary tumors, suggesting that the activating mutations of *MET* are clonally selected during the metastasis [236]. Recently, *MET* gene mutations, amplifications, and deletions have been inclusively studied in various types of human cancer in The Cancer Genome Atlas (TCGA) and other cancer genome projects (Fig. 8.7). The genetic alterations distribute across each domain of MET protein (Fig. 8.8).

MET/HGF can be dysregulated in human cancers through a number of other activating mechanisms, such as overexpression and alternative splicing, or HGF ligand-induced autocrine/paracrine loop signaling (refer to http://www.vai.org/met for comprehensive review of HGF/SF and MET in human cancers). For example, hypomethylation of a retrotransposon, LINE-1, was found to induce an alternate transcript of MET in bladder tumors and across the entire urothelium of tumor-bearing bladders [238]. In human breast cancers, deletion of a transcriptional repressor element (DATE, located 750 bp upstream from the transcription start) modulates chromatin structure and DNA–protein interactions, leading to constitutive activation of the *HGF* promoter [35]. Recently a new way for MET signaling to promote tumor metastasis was reported [239]. Highly metastatic melanoma cells produce MET containing exosomes that transfer MET protein to bone marrow progenitors and reprogram the bone marrow cells toward a pro-vasculogenic phenotype. Thus

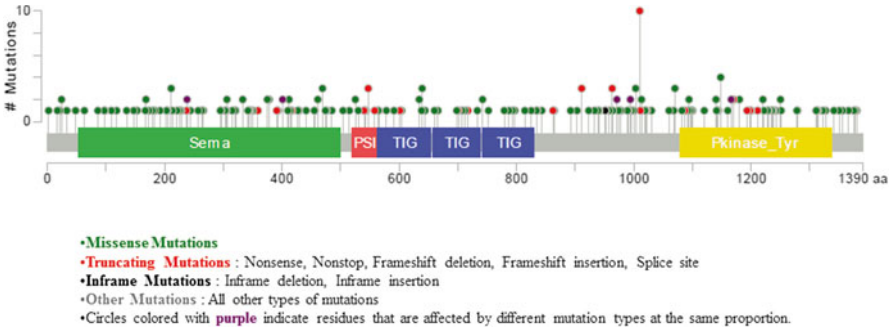

Fig. 8.8 MET mutations identified in human cancers in cancer genome projects. Circles representing mutations are colored according to the mutation type. Where different mutations are found at a single position, the color represents the most frequent mutation type. The diagram was generated by the cBioPortal for Cancer Genomics

melanoma cells increase the metastatic behavior through exosome production, transfer, and education of bone marrow cells to support angiogenesis [239].

It is well established that aberrant MET–HGF/SF signaling contributes to the development and progression of a variety of human cancers, so the interruption of HGF/SF–MET signaling has emerged as a useful intervention strategy. HGF/SF-neutralizing monoclonal antibody mixtures directed against epitopes that block HGF-induced MET signaling markedly inhibit tumor growth in animal models [240]. Subsequently, individual monoclonal antibodies that can block HGF/SF binding to MET have been isolated [241]. Beyond neutralizing antibodies, MET antagonists such as NK1, as well as various types of small molecules that inhibit the MET receptor tyrosine kinase, have been developed [242]. The availability of HGF/SF–MET inhibitors with a range of potencies and specificities has provided a strong basis for assessing their therapeutic value in human cancer, and the initial results from clinical studies have shown therapeutic benefits to patients with a variety of advanced or metastatic tumors, including NSCLC and breast, prostate, liver, and renal cancer. Several therapeutic studies have progressed to Phase III trials. Recently a durable, complete response was reported using an anti-MET receptor monoclonal antibody, MetMAb, in a patient population with chemotherapy-refractory, advanced gastric cancer [243, 244]. However, the cancer recurred after 2 years, and MetMAb therapy achieved a mixed response at recurrence. Larger studies and rigorous patient stratification procedures will clarify the therapeutic value and long-term safety of HGF/SF–MET inhibitors in cancer patients. The development of new intervention strategies that target HGF/SF–MET signaling will finally provide powerful weapon for fighting human cancers.

Drug resistance presents a challenge to target-based cancer therapy. Lung cancer with EGFR-activating mutations that responds initially to the EGFR inhibitors gefitinib and erlotinib invariably develops resistance to them. *MET* amplification has been detected in such lung cancer cell lines and lung cancer specimens.

MET amplification triggers gefitinib resistance through ERBB3-dependent activation of PI3K [134]. In addition to amplification, HGF/SF-mediated MET activation also contributes to the gefitinib resistance in lung cancer [245, 246]. However, in a Phase III lung cancer trial of the MET-specific antibody Onartuzumab in combination with EGFR inhibitor erlotinib did not provide any meaningful benefit over erlotinib alone [247]. The failure may partially due to the unselected population that includes patients with no MET alterations. In Phase II lung cancer trials, Onartuzumab plus erlotinib was associated with improved progression-free survival (PFS) and overall survival (OS) in a prespecified MET-positive population as determined by IHC [248, 249]. Other biomarkers, such as *MET* amplification measured by fluorescence in situ hybridization (FISH), may also be useful in selecting suitable patients [247].

Stromal cell secretion of HGF/SF has been identified as a major factor in the resistance to RAF inhibitors of *BRAF*-mutant melanoma, glioma, and colon cancer cells. In melanoma, the expression of HGF/SF in stromal cells significantly correlates to resistance to RAF inhibitor. Inhibiting HGF/SF or MET results in a reversal of the resistance to RAF inhibitors, suggesting that a combination therapy targeting both RAF and HGF/SF–MET is a therapeutic strategy for *BRAF*-mutant tumors [250, 251].

Vascular endothelial cell growth factor (VEGF) plays a key role in stimulating angiogenesis and driving tumor growth in many forms of cancer. The failure of antiangiogenic therapy with VEGF inhibitors has been partially ascribed to tumor invasion in response to treatment. In a mouse model of glioblastoma multiform (GBM), VEGF enhanced the recruitment of the protein tyrosine phosphatase 1B (PTP1B) to the MET/VEGFR2 complex and suppressed HGF/SF-dependent MET phosphorylation and tumor cell invasion. VEGF blockade with bevacizumab resulted in increases of MET activity and cell invasion. Dual inhibition of VEGF and MET blocked the cell invasion provoked by VEGF and resulted in a substantial survival benefit [252]. Indeed, endothelial cells express high levels of MET, which is activated by HGF/SF produced by tumor cells. The paracrine activation of endothelial MET contributes to tumor angiogenesis and confers resistance to antiangiogenic therapy with sunitinib. A combination of sunitinib and a selective MET inhibitor significantly inhibited tumor angiogenesis [253].

Beyond drug resistance, the activation of MET may also be involved in resistance to ionizing radiation therapy. Radiation induces overexpression and activation of the MET through the ATM-NF-κB signaling pathway in several human tumor cell lines. Activated MET, in turn, protects cells from apoptosis and promotes cell invasion, leading to radioresistance [254].

8.6.2 HGF/SF–MET Pathological Signaling in Diabetes, Autism, and Listeria *Infection*

HGF/SF is a pleiotropic growth factor involved in embryogenesis and in various adult physiological processes. Dysregulation of HGF/SF–MET signaling has been implicated in various diseases in addition to cancer.

The HGF/SF–MET axis regulates metabolism by stimulating hepatic glucose uptake and suppressing hepatic glucose output. MET receptor directly binds to INSR to form a hybrid complex, which is essential for an optimal hepatic insulin response. HGF/SF–MET restores insulin responsiveness in insulin-refractory mice, providing new insights into the molecular basis of hepatic insulin resistance [255]. HGF/SF–MET signaling is also critical for beta-cell survival. Pancreas-specific *MET*-null mice were more susceptible to multiple low-dose streptozotocin (MLDS)-induced diabetes, and they had higher blood glucose levels, marked hypoinsulinemia, and reduced beta-cell mass compared with wild-type littermates. In vitro, *MET*-null beta-cells were more sensitive to cytokine-induced cell death, an effect mediated by NF-κB activation and NO production. These results suggest that the activation of HGF/SF–MET signaling is a potential therapeutic strategy for diabetes [256].

Genetic studies of autism suggest that candidate genes may be located within the chromosome 7q31 region. HGF/SF–MET signaling participates in neocortical and cerebellar growth and maturation, immune function, and gastrointestinal repair, consistent with reported medical complications in some children with autism. A family-based study of autism including 1,231 cases showed a genetic association ($P = 0.0005$) of a common C allele in the promoter region of the *MET* gene in 204 families. Functional assays showed that the C allele results in a twofold decrease in MET promoter activity and in altered binding of specific transcription factor complexes. These data implicate reduced *MET* gene expression in autism susceptibility [257].

The bacterial pathogen *Listeria monocytogenes* uses its surface protein InlB to invade a variety of cell types. The interaction of InlB with MET is crucial for the occurrence of infection. Structural studies have indicated that InlB directly binds to MET to form a 2:2 complex with an InlB dimer at its center and one MET molecule bound peripherally to each InlB [258]. The InlB leucine-rich repeat region interacts with the first immunoglobulin-like domain of the MET stalk. A second contact, between InlB and the MET Sema domain, locks the otherwise flexible receptor in a rigid, signaling-competent conformation [259]. Upon binding of InlB to MET, the ubiquitin ligase Cbl is rapidly recruited to the complex. Purified InlB induces the Cbl-dependent monoubiquitination and endocytosis of MET, and the bacterium exploits the ubiquitin-dependent endocytosis machinery to invade mammalian cells [260, 261].

8.7 Conclusion

HGF/SF–MET signaling plays an important role in embryogenic development and adult physiological processes. Interruption or aberrant activation of HGF/SF–MET signaling has been implicated in several human diseases, especially cancers. Tumor cell addiction to MET and other RTKs is the basis for targeting cancer therapy. However, diverse pathways can be activated in a heterogeneous tumor. Resistant clones supported by signaling that are insensitive to the inhibitor will be selected.

Although combinations of different types of inhibitors may circumvent such resistance, genomic instability and the resulting clonal diversity of tumor cells may present may present a serious challenge for targeting therapy against human cancers. Targeting genomic instability could be the ultimate strategy for effective cancer therapy.

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Receptor at glance: comparison between MET and RON

	MET	RON
Other names	HGFR	MST1R; CD136; MSPR; PTK8
Chromosome location	7q31.2	3q21.31
Gene size (bp)	126,193	16,872
Intron/exon	20/21	19/20
mRNA size (5′, ORF, 3′)	6,695	4,785
Amino acids	1,390	1,400
Molecular weight	190	185
Subunit (α-chain/β chain)(kDa)	145/45	150/35
Posttranslational modifications	Proteolytic processing; phosphorylation; ubiquitination	Proteolytic processing; phosphorylation; ubiquitination
Domains	SEMA; CR; IPT; JM; kinase domain; docking site	SEMA; CR; IPT; JM; kinase domain; docking site
Phosphorylation sites	Tyr1234/1235 in kinase domain Tyr1349/1356 in docking site	Tyr1238/1239 in kinase domain Tyr1353/1360 in docking site
Pathways activated	PI3K/AKT2; RAS/MAPK; SRC; STAT3; PLCγ–PKC; Crk	PI3K/AKT2; RAS/MAPK; SRC; STAT3; PLCγ–PKC; Crk; NO
Tissues expressed	Mainly in epithelial cells; also found in endothelial cells, neurons, hepatocytes, hematopoietic cells, and melanocytes	Macrophages; epithelial and keratinocyte cells
Distribution in epithelial cells	Basal lateral membrane	Apical membrane
Transcriptional factor binds to promoter	AP1; SP1; Est1; Pax3; P53; HIF1α	NF-κB; Est-1 and estrogen receptor
Ligand for the receptor	HGF/SF	HGFL/MSP
Cell type that produces ligand	Mesenchymal cells	Hepatocyte
Interaction between ligand and receptor	Paracrine	Endocrine
Induction of cellular responses	Proliferation; scatting; migration/ invasion; surviving; branching morphogenesis; angiogenesis	Proliferation; scatting; migration/ invasion; surviving; branching morphogenesis; angiogenesis

(continued)

	MET	RON
Knockout mouse phenotype	Early embryonic lethality (e13.5)	Early embryonic lethality (e7.5)
Ligand knock out phenotype	Early embryonic lethality (e16.5)	No gross phenotype; fertile
Human diseases	Cancer; autism; diabetes;	Inflammation; cancer
Point mutation in cancers	Papillary renal carcinomas; HCC; lung cancer; brain tumors	Papillary renal carcinomas
Overexpression and aberrant activation	Most types of human cancer	Breast, lung, prostate, gastric, pancreatic, renal, bladder, ovarian, gastrointestinal, and colon cancers

The information about RON was obtained from the review by Wagh et al. [18].

References

1. Cooper CS, Park M, Blair DG, Tainsky MA, Huebner K, Croce CM, et al. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature. 1984;311(5981):29–33.
2. Rong S, Segal S, Anver M, Resau JH, Vande Woude GF. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc Natl Acad Sci USA. 1994;91(11):4731–5.
3. Park M, Dean M, Cooper CS, Schmidt M, O'Brien SJ, Blair DG, et al. Mechanism of met oncogene activation. Cell. 1986;45(6):895–904.
4. Dean M, Park M, Le Beau MM, Robins TS, Diaz MO, Rowley JD, et al. The human met oncogene is related to the tyrosine kinase oncogenes. Nature. 1985;318(6044):385–8.
5. Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, et al. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science. 1991;251(4995):802–4.
6. Nakamura T, Teramoto H, Ichihara A. Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. Proc Natl Acad Sci USA. 1986;83(17):6489–93.
7. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature. 1989;342(6248):440–3.
8. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun. 1984; 122(3):1450–9.
9. Stoker M, Gherardi E, Perryman M, Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature. 1987;327(6119):239–42.
10. Weidner KM, Arakaki N, Hartmann G, Vandekerckhove J, Weingart S, Rieder H, et al. Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proc Natl Acad Sci USA. 1991;88(16):7001–5.
11. Ronsin C, Muscatelli F, Mattei MG, Breathnach R. A novel putative receptor protein tyrosine kinase of the met family. Oncogene. 1993;8(5):1195–202.
12. Leonard EJ, Skeel AH. Isolation of macrophage stimulating protein (MSP) from human serum. Exp Cell Res. 1978;114(1):117–26.
13. Yoshimura T, Yuhki N, Wang MH, Skeel A, Leonard EJ. Cloning, sequencing, and expression of human macrophage stimulating protein (MSP, MST1) confirms MSP as a member of

the family of kringle proteins and locates the MSP gene on chromosome 3. J Biol Chem. 1993;268(21):15461–8.
14. Wang MH, Ronsin C, Gesnel MC, Coupey L, Skeel A, Leonard EJ, et al. Identification of the ron gene product as the receptor for the human macrophage stimulating protein. Science. 1994;266(5182):117–9.
15. Gaudino G, Follenzi A, Naldini L, Collesi C, Santoro M, Gallo KA, et al. RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. EMBO J. 1994; 13(15):3524–32.
16. Donate LE, Gherardi E, Srinivasan N, Sowdhamini R, Aparicio S, Blundell TL. Molecular evolution and domain structure of plasminogen-related growth factors (HGF/SF and HGF1/MSP). Protein Sci. 1994;3(12):2378–94.
17. Gaudino G, Avantaggiato V, Follenzi A, Acampora D, Simeone A, Comoglio PM. The proto-oncogene RON is involved in development of epithelial, bone and neuro-endocrine tissues. Oncogene. 1995;11(12):2627–37.
18. Wagh PK, Peace BE, Waltz SE. Met-related receptor tyrosine kinase Ron in tumor growth and metastasis. Adv Cancer Res. 2008;100:1–33.
19. Liu Y. The human hepatocyte growth factor receptor gene: complete structural organization and promoter characterization. Gene. 1998;215(1):159–69.
20. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell. 2003;3(4):347–61.
21. Gambarotta G, Boccaccio C, Giordano S, Ando M, Stella MC, Comoglio PM. Ets up-regulates MET transcription. Oncogene. 1996;13(9):1911–7.
22. Epstein JA, Shapiro DN, Cheng J, Lam PY, Maas RL. Pax3 modulates expression of the c-Met receptor during limb muscle development. Proc Natl Acad Sci USA. 1996;93(9): 4213–8.
23. Seol DW, Chen Q, Zarnegar R. Transcriptional activation of the hepatocyte growth factor receptor (c-met) gene by its ligand (hepatocyte growth factor) is mediated through AP-1. Oncogene. 2000;19(9):1132–7.
24. Seol DW, Chen Q, Smith ML, Zarnegar R. Regulation of the c-met proto-oncogene promoter by p53. J Biol Chem. 1999;274(6):3565–72.
25. Hwang CI, Matoso A, Corney DC, Flesken-Nikitin A, Korner S, Wang W, et al. Wild-type p53 controls cell motility and invasion by dual regulation of MET expression. Proc Natl Acad Sci USA. 2011;108(34):14240–5.
26. Tempest PR, Stratton MR, Cooper CS. Structure of the met protein and variation of met protein kinase activity among human tumour cell lines. Br J Cancer. 1988;58(1):3–7.
27. Giordano S, Di Renzo MF, Narsimhan RP, Cooper CS, Rosa C, Comoglio PM. Biosynthesis of the protein encoded by the c-met proto-oncogene. Oncogene. 1989;4(11):1383–8.
28. Komada M, Hatsuzawa K, Shibamoto S, Ito F, Nakayama K, Kitamura N. Proteolytic processing of the hepatocyte growth factor/scatter factor receptor by furin. FEBS Lett. 1993;328(1–2):25–9.
29. Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell. 1994;77(2):261–71.
30. Gherardi E, Youles ME, Miguel RN, Blundell TL, Iamele L, Gough J, et al. Functional map and domain structure of MET, the product of the c-met protooncogene and receptor for hepatocyte growth factor/scatter factor. Proc Natl Acad Sci USA. 2003;100(21):12039–44.
31. Basilico C, Arnesano A, Galluzzo M, Comoglio PM, Michieli P. A high affinity hepatocyte growth factor-binding site in the immunoglobulin-like region of Met. J Biol Chem. 2008;283(30):21267–77.
32. Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Langdon WY, et al. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell. 2001;8(5):995–1004.

33. Jiang JG, Chen Q, Bell A, Zarnegar R. Transcriptional regulation of the hepatocyte growth factor (HGF) gene by the Sp family of transcription factors. Oncogene. 1997;14(25): 3039–49.
34. Jiang JG, Zarnegar R. A novel transcriptional regulatory region within the core promoter of the hepatocyte growth factor gene is responsible for its inducibility by cytokines via the C/ EBP family of transcription factors. Mol Cell Biol. 1997;17(10):5758–70.
35. Ma J, DeFrances MC, Zou C, Johnson C, Ferrell R, Zarnegar R. Somatic mutation and functional polymorphism of a novel regulatory element in the HGF gene promoter causes its aberrant expression in human breast cancer. J Clin Invest. 2009;119(3):478–91.
36. Gohda E, Matsunaga T, Kataoka H, Takebe T, Yamamoto I. Induction of hepatocyte growth factor in human skin fibroblasts by epidermal growth factor, platelet-derived growth factor and fibroblast growth factor. Cytokine. 1994;6(6):633–40.
37. Matsumoto K, Okazaki H, Nakamura T. Up-regulation of hepatocyte growth factor gene expression by interleukin-1 in human skin fibroblasts. Biochem Biophys Res Commun. 1992;188(1):235–43.
38. Matsumoto K, Okazaki H, Nakamura T. Novel function of prostaglandins as inducers of gene expression of HGF and putative mediators of tissue regeneration. J Biochem. 1995;117(2): 458–64.
39. Matsumoto K, Tajima H, Okazaki H, Nakamura T. Heparin as an inducer of hepatocyte growth factor. J Biochem. 1993;114(6):820–6.
40. Gohda E, Matsunaga T, Kataoka H, Yamamoto I. TGF-beta is a potent inhibitor of hepatocyte growth factor secretion by human fibroblasts. Cell Biol Int Rep. 1992;16(9):917–26.
41. Matsumoto K, Tajima H, Okazaki H, Nakamura T. Negative regulation of hepatocyte growth factor gene expression in human lung fibroblasts and leukemic cells by transforming growth factor-beta 1 and glucocorticoids. J Biol Chem. 1992;267(35):24917–20.
42. Zarnegar R. Regulation of HGF and HGFR gene expression. EXS. 1995;74:33–49.
43. Lokker NA, Mark MR, Luis EA, Bennett GL, Robbins KA, Baker JB, et al. Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding. EMBO J. 1992;11(7):2503–10.
44. Naldini L, Tamagnone L, Vigna E, Sachs M, Hartmann G, Birchmeier W, et al. Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. EMBO J. 1992;11(13):4825–33.
45. Lokker NA, Presta LG, Godowski PJ. Mutational analysis and molecular modeling of the N-terminal kringle-containing domain of hepatocyte growth factor identifies amino acid side chains important for interaction with the c-Met receptor. Protein Eng. 1994;7(7):895–903.
46. Owen KA, Qiu D, Alves J, Schumacher AM, Kilpatrick LM, Li J, et al. Pericellular activation of hepatocyte growth factor by the transmembrane serine proteases matriptase and hepsin, but not by the membrane-associated protease uPA. Biochem J. 2010;426(2):219–28.
47. Shimomura T, Ochiai M, Kondo J, Morimoto Y. A novel protease obtained from FBS-containing culture supernatant, that processes single chain form hepatocyte growth factor to two chain form in serum-free culture. Cytotechnology. 1992;8(3):219–29.
48. Shimomura T, Kondo J, Ochiai M, Naka D, Miyazawa K, Morimoto Y, et al. Activation of the zymogen of hepatocyte growth factor activator by thrombin. J Biol Chem. 1993;268(30): 22927–32.
49. Miyazawa K, Shimomura T, Kitamura A, Kondo J, Morimoto Y, Kitamura N. Molecular cloning and sequence analysis of the cDNA for a human serine protease reponsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. J Biol Chem. 1993;268(14):10024–8.
50. Shimomura T, Miyazawa K, Komiyama Y, Hiraoka H, Naka D, Morimoto Y, et al. Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator. Eur J Biochem. 1995;229(1):257–61.
51. Miyazawa K, Shimomura T, Kitamura N. Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator. J Biol Chem. 1996;271(7): 3615–8.

52. Itoh H, Naganuma S, Takeda N, Miyata S, Uchinokura S, Fukushima T, et al. Regeneration of injured intestinal mucosa is impaired in hepatocyte growth factor activator-deficient mice. Gastroenterology. 2004;127(5):1423–35.
53. Shimomura T, Denda K, Kitamura A, Kawaguchi T, Kito M, Kondo J, et al. Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor. J Biol Chem. 1997;272(10):6370–6.
54. Kawaguchi T, Qin L, Shimomura T, Kondo J, Matsumoto K, Denda K, et al. Purification and cloning of hepatocyte growth factor activator inhibitor type 2, a Kunitz-type serine protease inhibitor. J Biol Chem. 1997;272(44):27558–64.
55. Kataoka H, Shimomura T, Kawaguchi T, Hamasuna R, Itoh H, Kitamura N, et al. Hepatocyte growth factor activator inhibitor type 1 is a specific cell surface binding protein of hepatocyte growth factor activator (HGFA) and regulates HGFA activity in the pericellular microenvironment. J Biol Chem. 2000;275(51):40453–62.
56. Carney TJ, von der Hardt S, Sonntag C, Amsterdam A, Topczewski J, Hopkins N, et al. Inactivation of serine protease Matriptase1a by its inhibitor Hai1 is required for epithelial integrity of the zebrafish epidermis. Development. 2007;134(19):3461–71.
57. Szabo R, Hobson JP, Christoph K, Kosa P, List K, Bugge TH. Regulation of cell surface protease matriptase by HAI2 is essential for placental development, neural tube closure and embryonic survival in mice. Development. 2009;136(15):2653–63.
58. Lamszus K, Joseph A, Jin L, Yao Y, Chowdhury S, Fuchs A, et al. Scatter factor binds to thrombospondin and other extracellular matrix components. Am J Pathol. 1996;149(3): 805–19.
59. Rahman S, Patel Y, Murray J, Patel KV, Sumathipala R, Sobel M, et al. Novel hepatocyte growth factor (HGF) binding domains on fibronectin and vitronectin coordinate a distinct and amplified Met-integrin induced signalling pathway in endothelial cells. BMC Cell Biol. 2005;6(1):8.
60. Matsuoka H, Sisson TH, Nishiuma T, Simon RH. Plasminogen-mediated activation and release of hepatocyte growth factor from extracellular matrix. Am J Respir Cell Mol Biol. 2006;35(6):705–13.
61. Ferracini R, Longati P, Naldini L, Vigna E, Comoglio PM. Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. J Biol Chem. 1991;266(29):19558–64.
62. Weidner KM, Di Cesare S, Sachs M, Brinkmann V, Behrens J, Birchmeier W. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature. 1996;384(6605):173–6.
63. Pelicci G, Giordano S, Zhen Z, Salcini AE, Lanfrancone L, Bardelli A, et al. The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. Oncogene. 1995;10(8):1631–8.
64. Graziani A, Gramaglia D, Cantley LC, Comoglio PM. The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. J Biol Chem. 1991;266(33):22087–90.
65. Boccaccio C, Ando M, Tamagnone L, Bardelli A, Michieli P, Battistini C, et al. Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature. 1998; 391(6664):285–8.
66. Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, et al. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell. 1992;70(3):431–42.
67. Rozakis-Adcock M, McGlade J, Mbamalu G, Pelicci G, Daly R, Li W, et al. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature. 1992;360(6405):689–92.
68. Chardin P, Camonis JH, Gale NW, van Aelst L, Schlessinger J, Wigler MH, et al. Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. Science. 1993; 260(5112):1338–43.

69. Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature. 1993; 363(6424):83–5.
70. Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, et al. Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature. 1993;363(6424):85–8.
71. Bertotti A, Comoglio PM, Trusolino L. Beta4 integrin activates a Shp2-Src signaling pathway that sustains HGF-induced anchorage-independent growth. J Cell Biol. 2006;175(6): 993–1003.
72. Ried S, Jager C, Jeffers M, Vande Woude GF, Graeff H, Schmitt M, et al. Activation mechanisms of the urokinase-type plasminogen activator promoter by hepatocyte growth factor/ scatter factor. J Biol Chem. 1999;274(23):16377–86.
73. Jeffers M, Rong S, Vande Woude GF. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol. 1996;16(3):1115–25.
74. Pepper MS, Matsumoto K, Nakamura T, Orci L, Montesano R. Hepatocyte growth factor increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in Madin-Darby canine kidney epithelial cells. J Biol Chem. 1992;267(28):20493–6.
75. Ponzetto C, Zhen Z, Audero E, Maina F, Bardelli A, Basile ML, et al. Specific uncoupling of GRB2 from the Met receptor. Differential effects on transformation and motility. J Biol Chem. 1996;271(24):14119–23.
76. Fournier TM, Kamikura D, Teng K, Park M. Branching tubulogenesis but not scatter of Madin-Darby canine kidney cells requires a functional Grb2 binding site in the Met receptor tyrosine kinase. J Biol Chem. 1996;271(36):22211–7.
77. Giordano S, Bardelli A, Zhen Z, Menard S, Ponzetto C, Comoglio PM. A point mutation in the MET oncogene abrogates metastasis without affecting transformation. Proc Natl Acad Sci USA. 1997;94(25):13868–72.
78. Maina F, Casagranda F, Audero E, Simeone A, Comoglio PM, Klein R, et al. Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. Cell. 1996;87(3):531–42.
79. Holgado-Madruga M, Emlet DR, Moscatello DK, Godwin AK, Wong AJ. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. Nature. 1996;379(6565):560–4.
80. Schaeper U, Gehring NH, Fuchs KP, Sachs M, Kempkes B, Birchmeier W. Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. J Cell Biol. 2000;149(7):1419–32.
81. Maroun CR, Holgado-Madruga M, Royal I, Naujokas MA, Fournier TM, Wong AJ, et al. The Gab1 PH domain is required for localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase. Mol Cell Biol. 1999; 19(3):1784–99.
82. Rocchi S, Tartare-Deckert S, Murdaca J, Holgado-Madruga M, Wong AJ, Van Obberghen E. Determination of Gab1 (Grb2-associated binder-1) interaction with insulin receptor-signaling molecules. Mol Endocrinol. 1998;12(7):914–23.
83. Lehr S, Kotzka J, Herkner A, Klein E, Siethoff C, Knebel B, et al. Identification of tyrosine phosphorylation sites in human Gab-1 protein by EGF receptor kinase in vitro. Biochemistry. 1999;38(1):151–9.
84. Gual P, Giordano S, Williams TA, Rocchi S, Van Obberghen E, Comoglio PM. Sustained recruitment of phospholipase C-gamma to Gab1 is required for HGF-induced branching tubulogenesis. Oncogene. 2000;19(12):1509–18.
85. Maroun CR, Naujokas MA, Holgado-Madruga M, Wong AJ, Park M. The tyrosine phosphatase SHP-2 is required for sustained activation of extracellular signal-regulated kinase and epithelial morphogenesis downstream from the met receptor tyrosine kinase. Mol Cell Biol. 2000;20(22):8513–25.
86. Montagner A, Yart A, Dance M, Perret B, Salles JP, Raynal P. A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation. J Biol Chem. 2005;280(7): 5350–60.

87. Liu X, Zheng H, Qu CK. Protein tyrosine phosphatase Shp2 (Ptpn11) plays an important role in maintenance of chromosome stability. Cancer Res. 2012;9.
88. Garcia-Guzman M, Dolfi F, Zeh K, Vuori K. Met-induced JNK activation is mediated by the adapter protein Crk and correlates with the Gab1 - Crk signaling complex formation. Oncogene. 1999;18(54):7775–86.
89. Knudsen BS, Feller SM, Hanafusa H. Four proline-rich sequences of the guanine-nucleotide exchange factor C3G bind with unique specificity to the first Src homology 3 domain of Crk. J Biol Chem. 1994;269(52):32781–7.
90. Ling P, Yao Z, Meyer CF, Wang XS, Oehrl W, Feller SM, et al. Interaction of hematopoietic progenitor kinase 1 with adapter proteins Crk and CrkL leads to synergistic activation of c-Jun N-terminal kinase. Mol Cell Biol. 1999;19(2):1359–68.
91. Hasegawa H, Kiyokawa E, Tanaka S, Nagashima K, Gotoh N, Shibuya M, et al. DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. Mol Cell Biol. 1996;16(4):1770–6.
92. Gotoh T, Hattori S, Nakamura S, Kitayama H, Noda M, Takai Y, et al. Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. Mol Cell Biol. 1995;15(12):6746–53.
93. Knox AL, Brown NH. Rap1 GTPase regulation of adherens junction positioning and cell adhesion. Science. 2002;295(5558):1285–8.
94. Kiyokawa E, Hashimoto Y, Kobayashi S, Sugimura H, Kurata T, Matsuda M. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. Genes Dev. 1998;12(21):3331–6.
95. Hu MC, Qiu WR, Wang X, Meyer CF, Tan TH. Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. Genes Dev. 1996; 10(18):2251–64.
96. Kiefer F, Tibbles LA, Anafi M, Janssen A, Zanke BW, Lassam N, et al. HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. EMBO J. 1996;15(24):7013–25.
97. Rodrigues GA, Park M, Schlessinger J. Activation of the JNK pathway is essential for transformation by the Met oncogene. EMBO J. 1997;16(10):2634–45.
98. Rahimi N, Tremblay E, Elliott B. Phosphatidylinositol 3-kinase activity is required for hepatocyte growth factor-induced mitogenic signals in epithelial cells. J Biol Chem. 1996; 271(40):24850–5.
99. Royal I, Park M. Hepatocyte growth factor-induced scatter of Madin-Darby canine kidney cells requires phosphatidylinositol 3-kinase. J Biol Chem. 1995;270(46):27780–7.
100. Royal I, Lamarche-Vane N, Lamorte L, Kaibuchi K, Park M. Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. Mol Biol Cell. 2000;11(5):1709–25.
101. Bowers DC, Fan S, Walter KA, Abounader R, Williams JA, Rosen EM, et al. Scatter factor/ hepatocyte growth factor protects against cytotoxic death in human glioblastoma via phosphatidylinositol 3-kinase- and AKT-dependent pathways. Cancer Res. 2000;60(15): 4277–83.
102. Xiao GH, Jeffers M, Bellacosa A, Mitsuuchi Y, Vande Woude GF, Testa JR. Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. Proc Natl Acad Sci USA. 2001;98(1):247–52.
103. Fan S, Ma YX, Gao M, Yuan RQ, Meng Q, Goldberg ID, et al. The multisubstrate adapter Gab1 regulates hepatocyte growth factor (scatter factor)-c-Met signaling for cell survival and DNA repair. Mol Cell Biol. 2001;21(15):4968–84.
104. Le Goff A, Ji Z, Leclercq B, Bourette RP, Mougel A, Guerardel C, et al. Anti-apoptotic role of caspase-cleaved GAB1 adaptor protein in hepatocyte growth factor/scatter factor-MET receptor protein signaling. J Biol Chem. 2012;287(42):35382–96.
105. Wang X, DeFrances MC, Dai Y, Pediaditakis P, Johnson C, Bell A, et al. A mechanism of cell survival: sequestration of Fas by the HGF receptor Met. Mol Cell. 2002;9(2):411–21.
106. Lioubin MN, Algate PA, Tsai S, Carlberg K, Aebersold A, Rohrschneider LR. p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. Genes Dev. 1996;10(9):1084–95.

107. Stefan M, Koch A, Mancini A, Mohr A, Weidner KM, Niemann H, et al. Src homology 2-containing inositol 5-phosphatase 1 binds to the multifunctional docking site of c-Met and potentiates hepatocyte growth factor-induced branching tubulogenesis. J Biol Chem. 2001;276(5):3017–23.
108. Zhang YW, Wang LM, Jove R, Vande Woude GF. Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. Oncogene. 2002;21(2):217–26.
109. Hung W, Elliott B. Co-operative effect of c-Src tyrosine kinase and Stat3 in activation of hepatocyte growth factor expression in mammary carcinoma cells. J Biol Chem. 2001; 276(15):12395–403.
110. van der Voort R, Taher TE, Wielenga VJ, Spaargaren M, Prevo R, Smit L, et al. Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. J Biol Chem. 1999;274(10): 6499–506.
111. Wielenga VJ, van der Voort R, Taher TE, Smit L, Beuling EA, van Krimpen C, et al. Expression of c-Met and heparan-sulfate proteoglycan forms of CD44 in colorectal cancer. Am J Pathol. 2000;157(5):1563–73.
112. Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H. CD44 is required for two consecutive steps in HGF/c-Met signaling. Genes Dev. 2002;16(23):3074–86.
113. Orian-Rousseau V, Morrison H, Matzke A, Kastilan T, Pace G, Herrlich P, et al. Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-actin. Mol Biol Cell. 2007;18(1):76–83.
114. Matzke A, Sargsyan V, Holtmann B, Aramuni G, Asan E, Sendtner M, et al. Haploinsufficiency of c-Met in cd44-/- mice identifies a collaboration of CD44 and c-Met in vivo. Mol Cell Biol. 2007;27(24):8797–806.
115. Olaku V, Matzke A, Mitchell C, Hasenauer S, Sakkaravarthi A, Pace G, et al. c-Met recruits ICAM-1 as a coreceptor to compensate for the loss of CD44 in Cd44 null mice. Mol Biol Cell. 2011;22(15):2777–86.
116. Trusolino L, Bertotti A, Comoglio PM. A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth. Cell. 2001;107(5):643–54.
117. Wang R, Kobayashi R, Bishop JM. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. Proc Natl Acad Sci USA. 1996;93(16):8425–30.
118. Nakamura Y, Matsubara D, Goto A, Ota S, Sachiko O, Ishikawa S, et al. Constitutive activation of c-Met is correlated with c-Met overexpression and dependent on cell-matrix adhesion in lung adenocarcinoma cell lines. Cancer Sci. 2008;99(1):14–22.
119. Gherardi E, Love CA, Esnouf RM, Jones EY. The sema domain. Curr Opin Struct Biol. 2004;14(6):669–78.
120. Conrotto P, Corso S, Gamberini S, Comoglio PM, Giordano S. Interplay between scatter factor receptors and B plexins controls invasive growth. Oncogene. 2004;23(30):5131–7.
121. Giordano S, Corso S, Conrotto P, Artigiani S, Gilestro G, Barberis D, et al. The semaphorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol. 2002;4(9): 720–4.
122. Swiercz JM, Worzfeld T, Offermanns S. ErbB-2 and met reciprocally regulate cellular signaling via plexin-B1. J Biol Chem. 2008;283(4):1893–901.
123. Ishibe S, Karihaloo A, Ma H, Zhang J, Marlier A, Mitobe M, et al. Met and the epidermal growth factor receptor act cooperatively to regulate final nephron number and maintain collecting duct morphology. Development. 2009;136(2):337–45.
124. Montesano R, Matsumoto K, Nakamura T, Orci L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell. 1991;67(5):901–8.
125. Kolatsi-Joannou M, Woolf AS, Hardman P, White SJ, Gordge M, Henderson RM. The hepatocyte growth factor/scatter factor (HGF/SF) receptor, met, transduces a morphogenetic signal in renal glomerular fibromuscular mesangial cells. J Cell Sci. 1995;108(Pt 12):3703–14.
126. Yamamoto N, Mammadova G, Song RX, Fukami Y, Sato K. Tyrosine phosphorylation of p145met mediated by EGFR and Src is required for serum-independent survival of human bladder carcinoma cells. J Cell Sci. 2006;119(Pt 22):4623–33.

127. Khoury H, Naujokas MA, Zuo D, Sangwan V, Frigault MM, Petkiewicz S, et al. HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion. Mol Biol Cell. 2005;16(2): 550–61.
128. Shattuck DL, Miller JK, Carraway 3rd KL, Sweeney C. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. Cancer Res. 2008;68(5): 1471–7.
129. Nakajima M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, et al. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer. 1999;85(9):1894–902.
130. Jo M, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. J Biol Chem. 2000;275(12):8806–11.
131. Dulak AM, Gubish CT, Stabile LP, Henry C, Siegfried JM. HGF-independent potentiation of EGFR action by c-Met. Oncogene. 2011;30(33):3625–35.
132. Bonine-Summers AR, Aakre ME, Brown KA, Arteaga CL, Pietenpol JA, Moses HL, et al. Epidermal growth factor receptor plays a significant role in hepatocyte growth factor mediated biological responses in mammary epithelial cells. Cancer Biol Ther. 2007;6(4):561–70.
133. Xu KP, Yu FS. Cross talk between c-Met and epidermal growth factor receptor during retinal pigment epithelial wound healing. Invest Ophthalmol Vis Sci. 2007;48(5):2242–8.
134. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007;316(5827):1039–43.
135. Liu Y, Chattopadhyay N, Qin S, Szekeres C, Vasylyeva T, Mahoney ZX, et al. Coordinate integrin and c-Met signaling regulate Wnt gene expression during epithelial morphogenesis. Development. 2009;136(5):843–53.
136. Monga SP, Mars WM, Pediaditakis P, Bell A, Mule K, Bowen WC, et al. Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. Cancer Res. 2002;62(7):2064–71.
137. Boon EM, van der Neut R, van de Wetering M, Clevers H, Pals ST. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. Cancer Res. 2002; 62(18):5126–8.
138. Lefebvre J, Ancot F, Leroy C, Muharram G, Lemiere A, Tulasne D. Met degradation: more than one stone to shoot a receptor down. FASEB J. 2012;26(4):1387–99.
139. Jeffers M, Taylor GA, Weidner KM, Omura S, Vande Woude GF. Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. Mol Cell Biol. 1997;17(2): 799–808.
140. Naka D, Shimomura T, Yoshiyama Y, Sato M, Ishii T, Hara H. Internalization and degradation of hepatocyte growth factor in hepatocytes with down-regulation of the receptor/c-Met. FEBS Lett. 1993;329(1–2):147–52.
141. Hammond DE, Urbe S, Vande Woude GF, Clague MJ. Down-regulation of MET, the receptor for hepatocyte growth factor. Oncogene. 2001;20(22):2761–70.
142. Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG, et al. Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J Biol Chem. 1998;273(2):685–8.
143. Burke P, Schooler K, Wiley HS. Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. Mol Biol Cell. 2001;12(6):1897–910.
144. Vieira AV, Lamaze C, Schmid SL. Control of EGF receptor signaling by clathrin-mediated endocytosis. Science. 1996;274(5295):2086–9.
145. Ceresa BP, Kao AW, Santeler SR, Pessin JE. Inhibition of clathrin-mediated endocytosis selectively attenuates specific insulin receptor signal transduction pathways. Mol Cell Biol. 1998;18(7):3862–70.
146. Kermorgant S, Zicha D, Parker PJ. PKC controls HGF-dependent c-Met traffic, signalling and cell migration. EMBO J. 2004;23(19):3721–34.

147. Kermorgant S, Parker PJ. Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. J Cell Biol. 2008;182(5):855–63.
148. Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, et al. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. 1998;12(23):3663–74.
149. Miyake S, Lupher Jr ML, Druker B, Band H. The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha. Proc Natl Acad Sci USA. 1998;95(14):7927–32.
150. Lee PS, Wang Y, Dominguez MG, Yeung YG, Murphy MA, Bowtell DD, et al. The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. EMBO J. 1999;18(13):3616–28.
151. Fixman ED, Holgado-Madruga M, Nguyen L, Kamikura DM, Fournier TM, Wong AJ, et al. Efficient cellular transformation by the Met oncoprotein requires a functional Grb2 binding site and correlates with phosphorylation of the Grb2-associated proteins, Cbl and Gab1. J Biol Chem. 1997;272(32):20167–72.
152. Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science. 1999;286(5438):309–12.
153. Carter S, Urbe S, Clague MJ. The met receptor degradation pathway: requirement for Lys48-linked polyubiquitin independent of proteasome activity. J Biol Chem. 2004;279(51): 52835–9.
154. Petrelli A, Gilestro GF, Lanzardo S, Comoglio PM, Migone N, Giordano S. The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. Nature. 2002; 416(6877):187–90.
155. Raiborg C, Bache KG, Gillooly DJ, Madshus IH, Stang E, Stenmark H. Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat Cell Biol. 2002;4(5):394–8.
156. Urbe S, Sachse M, Row PE, Preisinger C, Barr FA, Strous G, et al. The UIM domain of Hrs couples receptor sorting to vesicle formation. J Cell Sci. 2003;116(Pt 20):4169–79.
157. Katzmann DJ, Odorizzi G, Emr SD. Receptor downregulation and multivesicular-body sorting. Nat Rev Mol Cell Biol. 2002;3(12):893–905.
158. Row PE, Clague MJ, Urbe S. Growth factors induce differential phosphorylation profiles of the Hrs-STAM complex: a common node in signalling networks with signal-specific properties. Biochem J. 2005;389(Pt 3):629–36.
159. Parachoniak CA, Luo Y, Abella JV, Keen JH, Park M. GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. Dev Cell. 2011;20(6):751–63.
160. Abella JV, Peschard P, Naujokas MA, Lin T, Saucier C, Urbe S, et al. Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol Cell Biol. 2005;25(21):9632–45.
161. Lee JH, Han SU, Cho H, Jennings B, Gerrard B, Dean M, et al. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene. 2000;19(43):4947–53.
162. Mosesson Y, Mills GB, Yarden Y. Derailed endocytosis: an emerging feature of cancer. Nat Rev Cancer. 2008;8(11):835–50.
163. Joffre C, Barrow R, Menard L, Calleja V, Hart IR, Kermorgant S. A direct role for Met endocytosis in tumorigenesis. Nat Cell Biol. 2011;13(7):827–37.
164. Foveau B, Ancot F, Leroy C, Petrelli A, Reiss K, Vingtdeux V, et al. Down-regulation of the met receptor tyrosine kinase by presenilin-dependent regulated intramembrane proteolysis. Mol Biol Cell. 2009;20(9):2495–507.
165. Michieli P, Mazzone M, Basilico C, Cavassa S, Sottile A, Naldini L, et al. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. Cancer Cell. 2004; 6(1):61–73.

166. Yang Y, Wang Y, Zeng X, Ma XJ, Zhao Y, Qiao J, et al. Self-control of HGF regulation on human trophoblast cell invasion via enhancing c-Met receptor shedding by ADAM10 and ADAM17. J Clin Endocrinol Metab. 2012;97(8):E1390–401.
167. Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. Sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell. 1998;92(2):253–63.
168. Casci T, Vinos J, Freeman M. Sprouty, an intracellular inhibitor of Ras signaling. Cell. 1999;96(5):655–65.
169. Lee CC, Putnam AJ, Miranti CK, Gustafson M, Wang LM, Vande Woude GF, et al. Overexpression of sprouty 2 inhibits HGF/SF-mediated cell growth, invasion, migration, and cytokinesis. Oncogene. 2004;23(30):5193–202.
170. Stella MC, Trusolino L, Pennacchietti S, Comoglio PM. Negative feedback regulation of Met-dependent invasive growth by Notch. Mol Cell Biol. 2005;25(10):3982–96.
171. Shaw AT, Meissner A, Dowdle JA, Crowley D, Magendantz M, Ouyang C, et al. Sprouty-2 regulates oncogenic K-ras in lung development and tumorigenesis. Genes Dev. 2007; 21(6):694–707.
172. Gao M, Patel R, Ahmad I, Fleming J, Edwards J, McCracken S, et al. SPRY2 loss enhances ErbB trafficking and PI3K/AKT signalling to drive human and mouse prostate carcinogenesis. EMBO Mol Med. 2012;4(8):776–90.
173. Fong CW, Chua MS, McKie AB, Ling SH, Mason V, Li R, et al. Sprouty 2, an inhibitor of mitogen-activated protein kinase signaling, is down-regulated in hepatocellular carcinoma. Cancer Res. 2006;66(4):2048–58.
174. Kataoka H, Gohda E, Matsunaga T, Ishii T, Hara H, Yamamoto I. Stimulation of DNA synthesis in skin fibroblasts by human hepatocyte growth factor/scatter factor. Cell Biol Int. 1993;17(1):65–73.
175. Rosen EM, Meromsky L, Goldberg I, Bhargava M, Setter E. Studies on the mechanism of scatter factor. Effects of agents that modulate intracellular signal transduction, macromolecule synthesis and cytoskeleton assembly. J Cell Sci. 1990;96(Pt 4):639–49.
176. Weidner KM, Behrens J, Vandekerckhove J, Birchmeier W. Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. J Cell Biol. 1990;111(5 Pt 1): 2097–108.
177. Tsarfaty I, Resau JH, Rulong S, Keydar I, Faletto DL, Vande Woude GF. The met proto-oncogene receptor and lumen formation. Science. 1992;257(5074):1258–61.
178. Tsarfaty I, Rong S, Resau JH, Rulong S, da Silva PP, Vande Woude GF. The Met proto-oncogene mesenchymal to epithelial cell conversion. Science. 1994;263(5143):98–101.
179. Jones SM, Kazlauskas A. Connecting signaling and cell cycle progression in growth factor-stimulated cells. Oncogene. 2000;19(49):5558–67.
180. Day RM, Cioce V, Breckenridge D, Castagnino P, Bottaro DP. Differential signaling by alternative HGF isoforms through c-Met: activation of both MAP kinase and PI 3-kinase pathways is insufficient for mitogenesis. Oncogene. 1999;18(22):3399–406.
181. Recio JA, Merlino G. Hepatocyte growth factor/scatter factor activates proliferation in melanoma cells through p38 MAPK, ATF-2 and cyclin D1. Oncogene. 2002;21(7):1000–8.
182. Muller M, Morotti A, Ponzetto C. Activation of NF-kappaB is essential for hepatocyte growth factor-mediated proliferation and tubulogenesis. Mol Cell Biol. 2002;22(4): 1060–72.
183. Gao CF, Xie Q, Su YL, Koeman J, Khoo SK, Gustafson M, et al. Proliferation and invasion: plasticity in tumor cells. Proc Natl Acad Sci USA. 2005;102(30):10528–33.
184. Tsukada Y, Miyazawa K, Kitamura N. High intensity ERK signal mediates hepatocyte growth factor-induced proliferation inhibition of the human hepatocellular carcinoma cell line HepG2. J Biol Chem. 2001;276(44):40968–76.
185. Guegan JP, Ezan F, Gailhouste L, Langouet S, Baffet G. MEK1/2 overactivation can promote growth arrest by mediating ERK1/2-dependent phosphorylation of p70S6K. J Cell Physiol. 2014;229(7):903–15.

186. Chan PC, Sudhakar JN, Lai CC, Chen HC. Differential phosphorylation of the docking protein Gab1 by c-Src and the hepatocyte growth factor receptor regulates different aspects of cell functions. Oncogene. 2010;29(5):698–710.
187. Ridley AJ, Comoglio PM, Hall A. Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. Mol Cell Biol. 1995;15(2):1110–22.
188. Hordijk PL, ten Klooster JP, van der Kammen RA, Michiels F, Oomen LC, Collard JG. Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. Science. 1997;278(5342): 1464–6.
189. Kamei T, Matozaki T, Sakisaka T, Kodama A, Yokoyama S, Peng YF, et al. Coendocytosis of cadherin and c-Met coupled to disruption of cell-cell adhesion in MDCK cells–regulation by Rho, Rac and Rab small G proteins. Oncogene. 1999;18(48):6776–84.
190. Hanzawa M, Shindoh M, Higashino F, Yasuda M, Inoue N, Hida K, et al. Hepatocyte growth factor upregulates E1AF that induces oral squamous cell carcinoma cell invasion by activating matrix metalloproteinase genes. Carcinogenesis. 2000;21(6):1079–85.
191. Harvey P, Clark IM, Jaurand MC, Warn RM, Edwards DR. Hepatocyte growth factor/scatter factor enhances the invasion of mesothelioma cell lines and the expression of matrix metalloproteinases. Br J Cancer. 2000;83(9):1147–53.
192. Wang H, Keiser JA. Hepatocyte growth factor enhances MMP activity in human endothelial cells. Biochem Biophys Res Commun. 2000;272(3):900–5.
193. Canadas I, Taus A, Gonzalez I, Villanueva X, Gimeno J, Pijuan L, et al. High circulating hepatocyte growth factor levels associate with epithelial to mesenchymal transition and poor outcome in small cell lung cancer patients. Oncotarget. 2014;5(14):5246–56.
194. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature. 1995; 376(6543):768–71.
195. Dietrich S, Abou-Rebyeh F, Brohmann H, Bladt F, Sonnenberg-Riethmacher E, Yamaai T, et al. The role of SF/HGF and c-Met in the development of skeletal muscle. Development. 1999;126(8):1621–9.
196. Rosario M, Birchmeier W. How to make tubes: signaling by the Met receptor tyrosine kinase. Trends Cell Biol. 2003;13(6):328–35.
197. Montesano R, Schaller G, Orci L. Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. Cell. 1991;66(4):697–711.
198. Brinkmann V, Foroutan H, Sachs M, Weidner KM, Birchmeier W. Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J Cell Biol. 1995;131(6 Pt 1):1573–86.
199. Tsarfaty I, Ben-Jacob E. Secrets of tubule engineering by epithelial cells. Proc Natl Acad Sci USA. 2012;109(18):6790–1.
200. O'Brien LE, Zegers MM, Mostov KE. Opinion: building epithelial architecture: insights from three-dimensional culture models. Nat Rev Mol Cell Biol. 2002;3(7):531–7.
201. O'Brien LE, Tang K, Kats ES, Schutz-Geschwender A, Lipschutz JH, Mostov KE. ERK and MMPs sequentially regulate distinct stages of epithelial tubule development. Dev Cell. 2004;7(1):21–32.
202. Santos OF, Nigam SK. HGF-induced tubulogenesis and branching of epithelial cells is modulated by extracellular matrix and TGF-beta. Dev Biol. 1993;160(2):293–302.
203. Williams MJ, Clark P. Microscopic analysis of the cellular events during scatter factor/hepatocyte growth factor-induced epithelial tubulogenesis. J Anat. 2003;203(5):483–503.
204. Jeffers M, Rong S, Vande Woude GF. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol. 1996;16(3):1115–25.
205. Gao C, Furge K, Koeman J, Dykema K, Su Y, Cutler ML, et al. Chromosome instability, chromosome transcriptome, and clonal evolution of tumor cell populations. Proc Natl Acad Sci USA. 2007;104(21):8995–9000.
206. Vaught D, Chen J, Brantley-Sieders DM. Regulation of mammary gland branching morphogenesis by EphA2 receptor tyrosine kinase. Mol Biol Cell. 2009;20(10):2572–81.

207. Lim A, Shin K, Zhao C, Kawano S, Beachy PA. Spatially restricted Hedgehog signalling regulates HGF-induced branching of the adult prostate. Nat Cell Biol. 2014;2.
208. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730–7.
209. Kelly PN, Dakic A, Adams JM, Nutt SL, Strasser A. Tumor growth need not be driven by rare cancer stem cells. Science. 2007;317(5836):337.
210. Neuss S, Becher E, Woltje M, Tietze L, Jahnen-Dechent W. Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair, and wound healing. Stem Cells. 2004;22(3):405–14.
211. Son BR, Marquez-Curtis LA, Kucia M, Wysoczynski M, Turner AR, Ratajczak J, et al. Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells. 2006;24(5):1254–64.
212. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A, et al. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ Res. 2005;97(7): 663–73.
213. Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Dev Biol. 1998;194(1):114–28.
214. Yamada M, Tatsumi R, Yamanouchi K, Hosoyama T, Shiratsuchi S, Sato A, et al. High concentrations of HGF inhibit skeletal muscle satellite cell proliferation in vitro by inducing expression of myostatin: a possible mechanism for reestablishing satellite cell quiescence in vivo. Am J Physiol Cell Physiol. 2010;298(3):C465–76.
215. Kamiya A, Gonzalez FJ, Nakauchi H. Identification and differentiation of hepatic stem cells during liver development. Front Biosci. 2006;11:1302–10.
216. Suzuki A, Nakauchi H, Taniguchi H. Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting. Diabetes. 2004;53(8):2143–52.
217. Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol. 2010;12(5):468–76.
218. De Bacco F, Casanova E, Medico E, Pellegatta S, Orzan F, Albano R, et al. The MET oncogene is a functional marker of a glioblastoma stem cell subtype. Cancer Res. 2012;72(17): 4537–50.
219. Gastaldi S, Sassi F, Accornero P, Torti D, Galimi F, Migliardi G, et al. Met signaling regulates growth, repopulating potential and basal cell-fate commitment of mammary luminal progenitors: implications for basal-like breast cancer. Oncogene. 2012;7.
220. Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, et al. Scatter factor/hepatocyte growth factor is essential for liver development. Nature. 1995;373(6516): 699–702.
221. Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T, et al. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature. 1995;373(6516): 702–5.
222. Michalopoulos GK, DeFrances MC. Liver regeneration. Science. 1997;276(5309):60–6.
223. Borowiak M, Garratt AN, Wustefeld T, Strehle M, Trautwein C, Birchmeier C. Met provides essential signals for liver regeneration. Proc Natl Acad Sci USA. 2004;101(29):10608–13.
224. Huh CG, Factor VM, Sanchez A, Uchida K, Conner EA, Thorgeirsson SS. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci USA. 2004;101(13):4477–82.
225. Chmielowiec J, Borowiak M, Morkel M, Stradal T, Munz B, Werner S, et al. c-Met is essential for wound healing in the skin. J Cell Biol. 2007;177(1):151–62.
226. Rong S, Bodescot M, Blair D, Dunn J, Nakamura T, Mizuno K, et al. Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. Mol Cell Biol. 1992;12(11): 5152–8.

227. Liang TJ, Reid AE, Xavier R, Cardiff RD, Wang TC. Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. J Clin Invest. 1996;97(12):2872–7.
228. Takayama H, LaRochelle WJ, Sharp R, Otsuka T, Kriebel P, Anver M, et al. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc Natl Acad Sci USA. 1997;94(2):701–6.
229. Otsuka T, Takayama H, Sharp R, Celli G, LaRochelle WJ, Bottaro DP, et al. c-Met autocrine activation induces development of malignant melanoma and acquisition of the metastatic phenotype. Cancer Res. 1998;58(22):5157–67.
230. Schmidt L, Duh FM, Chen F, Kishida T, Glenn G, Choyke P, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet. 1997;16(1):68–73.
231. Jeffers M, Schmidt L, Nakaigawa N, Webb CP, Weirich G, Kishida T, et al. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci USA. 1997;94(21):11445–50.
232. Jeffers M, Fiscella M, Webb CP, Anver M, Koochekpour S, Vande Woude GF. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci USA. 1998;95(24):14417–22.
233. Graveel C, Su Y, Koeman J, Wang LM, Tessarollo L, Fiscella M, et al. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci USA. 2004;101(49):17198–203.
234. Ponzo MG, Lesurf R, Petkiewicz S, O'Malley FP, Pinnaduwage D, Andrulis IL, et al. Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. Proc Natl Acad Sci USA. 2009;106(31):12903–8.
235. Graveel CR, DeGroot JD, Su Y, Koeman J, Dykema K, Leung S, et al. Met induces diverse mammary carcinomas in mice and is associated with human basal breast cancer. Proc Natl Acad Sci USA. 2009;106(31):12909–14.
236. Park WS, Dong SM, Kim SY, Na EY, Shin MS, Pi JH, et al. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. Cancer Res. 1999;59(2):307–10.
237. Di Renzo MF, Olivero M, Martone T, Maffe A, Maggiora P, Stefani AD, et al. Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas. Oncogene. 2000;19(12):1547–55.
238. Wolff EM, Byun HM, Han HF, Sharma S, Nichols PW, Siegmund KD, et al. Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. PLoS Genet. 2010;6(4):e1000917.
239. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med. 2012;18(6):883–91.
240. Cao B, Su Y, Oskarsson M, Zhao P, Kort EJ, Fisher RJ, et al. Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models. Proc Natl Acad Sci USA. 2001;98(13):7443–8.
241. Jun HT, Sun J, Rex K, Radinsky R, Kendall R, Coxon A, et al. AMG 102, a fully human anti-hepatocyte growth factor/scatter factor neutralizing antibody, enhances the efficacy of temozolomide or docetaxel in U-87 MG cells and xenografts. Clin Cancer Res. 2007;13(22 Pt 1):6735–42.
242. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. Nat Rev Cancer. 2012;12(2):89–103.
243. Feng Y, Ma PC. Anti-MET targeted therapy has come of age: the first durable complete response with MetMAb in metastatic gastric cancer. Cancer Discov. 2011;1(7):550–4.
244. Catenacci DV, Henderson L, Xiao SY, Patel P, Yauch RL, Hegde P, et al. Durable complete response of metastatic gastric cancer with anti-Met therapy followed by resistance at recurrence. Cancer Discov. 2011;1(7):573–9.

245. Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. Cancer Res. 2008;68(22):9479–87.
246. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell. 2010; 17(1):77–88.
247. Garber K. MET inhibitors start on road to recovery. Nat Rev Drug Discov. 2014; 13(8):563–5.
248. Spigel DR, Ervin TJ, Ramlau RA, Daniel DB, Goldschmidt Jr JH, Blumenschein Jr GR, et al. Randomized phase II trial of onartuzumab in combination with erlotinib in patients with advanced non-small-cell lung cancer. J Clin Oncol. 2013;31(32):4105–14.
249. Koeppen H, Yu W, Zha J, Pandita A, Penuel E, Rangell L, et al. Biomarker analyses from a placebo-controlled phase II study evaluating erlotinib+/-onartuzumab in advanced non-small cell lung cancer: MET expression levels are predictive of patient benefit. Clin Cancer Res. 2014;20(17):4488–98.
250. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, et al. Tumour microenvironment elicits innate resistance to RAF inhibitors through HGF secretion. Nature. 2012;487(7408):500–4.
251. Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature. 2012;487(7408): 505–9.
252. Lu KV, Chang JP, Parachoniak CA, Pandika MM, Aghi MK, Meyronet D, et al. VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. Cancer Cell. 2012;22(1):21–35.
253. Shojaei F, Lee JH, Simmons BH, Wong A, Esparza CO, Plumlee PA, et al. HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. Cancer Res. 2010;70(24): 10090–100.
254. De Bacco F, Luraghi P, Medico E, Reato G, Girolami F, Perera T, et al. Induction of MET by ionizing radiation and its role in radioresistance and invasive growth of cancer. J Natl Cancer Inst. 2011;103(8):645–61.
255. Fafalios A, Ma J, Tan X, Stoops J, Luo J, Defrances MC, et al. A hepatocyte growth factor receptor (Met)-insulin receptor hybrid governs hepatic glucose metabolism. Nat Med. 2011;17(12):1577–84.
256. Mellado-Gil J, Rosa TC, Demirci C, Gonzalez-Pertusa JA, Velazquez-Garcia S, Ernst S, et al. Disruption of hepatocyte growth factor/c-Met signaling enhances pancreatic beta-cell death and accelerates the onset of diabetes. Diabetes. 2011;60(2):525–36.
257. Campbell DB, Sutcliffe JS, Ebert PJ, Militerni R, Bravaccio C, Trillo S, et al. A genetic variant that disrupts MET transcription is associated with autism. Proc Natl Acad Sci USA. 2006;103(45):16834–9.
258. Niemann HH. Structural basis of MET receptor dimerization by the bacterial invasion protein InlB and the HGF/SF splice variant NK1. Biochim Biophys Acta. 2012;30.
259. Niemann HH, Jager V, Butler PJ, van den Heuvel J, Schmidt S, Ferraris D, et al. Structure of the human receptor tyrosine kinase met in complex with the Listeria invasion protein InlB. Cell. 2007;130(2):235–46.
260. Veiga E, Cossart P. Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. Nat Cell Biol. 2005;7(9):894–900.
261. Shen Y, Naujokas M, Park M, Ireton K. InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. Cell. 2000;103(3):501–10.

Chapter 9
The MuSK Receptor Family

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Abbreviations

ACh	Acetylcholine
AChR	Acetylcholine receptor
ALS	Amyotrophic lateral sclerosis
CNS	Central nervous system
Lrp4	Low-density lipoprotein receptor-related protein 4
MG	Myasthenia gravis
MuSK	Muscle-specific kinase

9.1 Introduction

Muscle-specific kinase (MuSK) is a receptor tyrosine kinase that is expressed in skeletal muscle and has a crucial, master role in forming and maintaining neuromuscular synapses [1–4]. MuSK responds to Agrin, a signal provided by motor neurons, by stimulating differentiation of the muscle postsynaptic membrane and controlling production of retrograde signals for motor nerve terminal differentiation. In addition to skeletal muscle, MuSK is expressed in excitatory neurons in the mammalian central nervous system (CNS) [5, 6], as well as transiently in the liver

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in chick and rat and in additional tissues in *Xenopus* [7, 8]. Thus, MuSK is not a muscle-specific kinase, as the name implies. The roles for MuSK outside skeletal muscle are poorly understood, although one study reports a role for MuSK in memory consolidation (Garcia-Osta et al. 2006). Here, we describe how MuSK responds to neural Agrin and controls synaptic differentiation. Moreover, we discuss diseases of the neuromuscular synapse, caused either by mutations in genes within the MuSK signaling pathway or by autoantibodies to synaptic proteins, including MuSK.

9.2 MuSK Structure

MuSK is a single-pass, 120 kDa transmembrane protein, composed of an extracellular region containing three Ig-like domains and a Frizzled-like cysteine-rich domain (CRD) and an intracellular region containing a juxtamembrane region, a kinase domain, and a short cytoplasmic tail [1, 2] (Fig. 9.1, see Table "Receptor at a glance: MuSK").

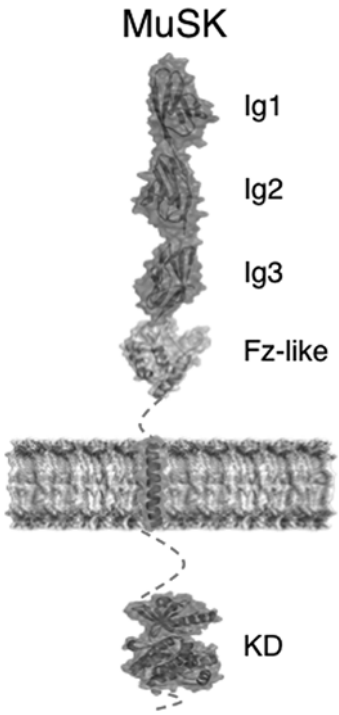

Fig. 9.1 MuSK contains three Ig-like domains and a Frizzled-like CRD in the extracellular region. The intracellular region of MuSK includes a juxtamembrane region, including a key site of tyrosine phosphorylation, the kinase domain, and a short cytoplasmic tail

In fish, amphibians, and avians, a kringle domain is additionally present in the extracellular region [1, 7, 8]. The first Ig-like domain is critical for MuSK function. A crystal structure of the first and second Ig-like domains reveals a dimer, mediated by a hydrophobic surface in the first Ig-like domain [9]. Mutation of this hydrophobic surface prevents Agrin from stimulating MuSK phosphorylation, suggesting that formation of a MuSK dimer is essential for transphosphorylation of MuSK. The opposite face of the first Ig-like domain has a separate but equally critical role in activating MuSK, as described below. The Frizzled-like CRD in MuSK is structurally distinct from the CRD of Frizzled 8 [10, 11] but is reported to bind Wnts [12–14] (see below). Mammals express two related kringle-containing kinases, Ror1 and Ror2, which are related to MuSK and homologous to CAM-1 in *C. elegans* and *Dnrk* and *Dror* in *Drosophila* [15].

9.3 MuSK Activation

The intracellular region of MuSK contains a typical tyrosine kinase domain that includes three tyrosine residues (Y750, Y754, Y755) in the activation loop. In the non-phosphorylated state, the Km for ATP is several mM, higher than typical for receptor tyrosine kinases [16]. The higher Km for ATP is likely due to greater stabilization of the non-phosphorylated activation loop in MuSK. Nonetheless, because the ATP concentration in resting skeletal muscle is 8 mM [17], greater than in most cell types, the high Km for ATP is unlikely to impede MuSK activation in muscle but may hinder MuSK phosphorylation in other cell types. Phosphorylation of Y754 is critical for activation of the kinase in vitro and in muscle cells, as expected for this pivotal activation-loop tyrosine. Tyrosine residues Y750 and Y755 are phosphorylated subsequently, and their phosphorylation potentiates MuSK signaling [19]. In addition to the activation-loop tyrosine residues, the juxtamembrane region contains a single tyrosine residue, Y553, which is phosphorylated during activation of the kinase [16, 18, 19]. Phosphorylation of Y553 is not essential for activation of the kinase in vitro but is surprisingly required for phosphorylation and activation of the kinase in muscle cells [19] (see below).

9.4 Dok-7 and Downstream from MuSK

Once phosphorylated, the pY553 region serves as a docking site for an adaptor protein, Dok-7 [20]. Dok-7 has amino-terminal PH and PTB domains and a carboxy-terminal region that becomes phosphorylated following recruitment to MuSK [20]. Dok-7 is an unusual adapter protein, possessing two critical functions [20, 21]. First, Dok-7 acts as an inside-out ligand that stimulates MuSK kinase activity. This

function underlies, in part, the absence of detectable MuSK phosphorylation in muscle cells lacking Dok-7 and in muscle cells expressing MuSK Y553F. Second, once recruited to MuSK, Dok-7 itself becomes tyrosine phosphorylated, leading to the recruitment of a signaling complex essential for synaptic differentiation.

These findings present an enigma, as phosphorylation of MuSK Y553 is required to recruit Dok-7, but in the absence of Dok-7, MuSK is apparently not tyrosine phosphorylated. The PH-PTB domains in Dok-7 mediate formation of a homodimer [21]. Therefore once recruited to MuSK, a dimer of Dok-7 stabilizes a MuSK dimer. Thus, MuSK phosphorylation may be labile, and the function of Dok-7, once recruited to MuSK pY553, may be to stabilize MuSK phosphorylation.

Because phosphorylation of Y553 is required for phosphorylation of the MuSK activation loop in muscle cells but not in vitro, muscle cells may express a protein that binds the non-phosphorylated Y553 region and inhibits adventitious MuSK phosphorylation in vivo [19]. If so, phosphorylation of Y553 may lead to displacement of this inhibitor in muscle and replacement with Dok-7, explaining why phosphorylation of Y553 is critical for activation of MuSK in muscle cells but not in vitro.

Mutations in Dok-7 are a major cause of congenital myasthenia [22] (see below). Although some mutations interfere with Dok-7 dimerization or recruitment of Dok-7 to phosphorylated MuSK, most mutations are within the carboxy-terminal region of Dok-7 [23, 24]. Agrin stimulates phosphorylation of two tyrosine residues in the carboxy-terminal region of Dok-7, which leads to recruitment of Crk/Crk-L, related adapter proteins composed of SH2 and SH3 domains [25]. Inactivation of Crk/Crk-L in mouse muscle leads to synaptic defects that are strikingly similar to the defects found in humans carrying mutations that truncate the carboxy-terminal region of Dok-7 [25]. The proteins that are recruited to Crk/Crk-L and function downstream of MuSK/Dok-7 have yet to be identified.

Ultimately, this signaling cascade impinges on Rapsyn, an intracellular peripheral membrane protein that binds directly to intracellular loops in acetylcholine receptor (AChR) subunits [26–29]. Rapsyn is essential for anchoring and clustering AChRs at synapses, although the mechanisms are poorly understood [30]. The stoichiometry of Rapsyn/AChR, isolated from mature *Torpedo* electric organ synapses, ranges from 1:1 to 3:1 [31], consistent with the idea that Rapsyn can bind to more than one of the subunits in an AChR pentamer (α_2, β, γ, δ). The association of Rapsyn with AChRs increases following Agrin stimulation and tyrosine phosphorylation of the AChR β subunit, which stabilizes anchoring of AChRs [29, 32–34]. Thus, the Agrin-stimulated recruitment of additional Rapsyn molecules to the AChR pentamer may facilitate cross-links with additional AChRs and further stabilize the anchoring of AChRs [31].

The proteins that function downstream of MuSK/Dok-7/Crk/Crk-L and control anchoring of Rapsyn have yet to be identified. Immunohistochemical studies have identified candidate proteins that are concentrated at synapses, and biochemical studies have identified proteins that interact with Rapsyn [35]. Moreover, dominant-negative forms of several signaling molecules interfere with AChR clustering in cultured muscle cells. Nonetheless, the combination of genetic analysis and biochemical studies, required to demonstrate a critical role for suspected

candidates, is currently lacking. Rac and Rho are essential for clustering AChRs, and together with actin and Tid1, they are likely components of this missing link [36–39]. Following the initial contact between motor nerve terminals and muscle, AChR clusters and nerve terminals undergo structural changes, transforming AChR clusters from a plaque-like shape into a pretzel-like shape. This transformation occurs concomitantly with the recruitment of additional proteins, such as dystrobrevin, LL5β, and podosomal proteins to the synapse, which may serve to maintain and stabilize synapses [40–43].

In addition to the role of MuSK/Dok-7 in building a protein complex to anchor AChRs/Rapsyn and other synaptic proteins, MuSK/Dok-7 signaling also leads to activation of a transcriptional pathway for stimulating expression of certain genes, including *AChR* genes, in nuclei near the site of MuSK/Dok-7 activation. This pathway involves JNK stimulation and one or more Ets-domain-containing proteins, but otherwise, this pathway is poorly understood [37, 38, 44–47].

9.5 Lrp4 Forms a Complex with MuSK and Confers Responsiveness to Agrin

Although Agrin stimulates MuSK phosphorylation, Agrin does not bind directly to MuSK. Instead, Agrin binds to Lrp4, a member of the LDLR family [48], causing an increase in association between Lrp4 and MuSK and stimulating MuSK kinase activity [49–51]. As such, Lrp4 acts as a cis-acting ligand for MuSK, and Agrin functions as an allosteric regulator that controls binding between the ligand and the kinase [51]. The association between Lrp4 and MuSK depends upon the first Ig-like domain in MuSK, revealing a second function, in addition to MuSK dimerization, for this Ig-like domain [51]. The first Ig-like domain in MuSK has an extra disulfide bond, not typically found in Ig-like domains, and this disulfide bond is on a solvent-exposed surface, opposite to the hydrophobic surface that mediates MuSK dimerization [9]. This solvent-exposed surface is essential for association between MuSK and Lrp4, as mutation of I96, adjacent to the extra disulfide bond, prevents association between MuSK and Lrp4 and precludes Agrin from activating MuSK in muscle cells [9, 51].

How association between Lrp4 and MuSK stimulates MuSK phosphorylation is poorly understood. A basal, Agrin-independent association between Lrp4 and MuSK confers low levels of MuSK phosphorylation [49], which is important for priming muscle prior to innervation (see below) [52]. In the absence of Agrin, Lrp4 is presumably in dynamic equilibrium, and one conformation is capable of binding and activating MuSK. Agrin binding to Lrp4 apparently alters the conformation of Lrp4, stabilizing a configuration that binds MuSK. Because Lrp4 forms homodimers and/or higher-order oligomers, recruitment of MuSK to Lrp4 may facilitate MuSK dimerization and transphosphorylation [51]. In addition, binding of MuSK to Lrp4 may reorient MuSK so that the hydrophobic surfaces in the first Ig-like domain are positioned in a manner that favors their association.

The extracellular region of Lrp4 is composed of eight LDLa repeats; two EGF-like domains; four β-propeller domains, each with an embedded EGF-like domain; and a juxtamembrane region. The intracellular region of Lrp4 is not essential for Agrin to activate MuSK or stimulate synaptic differentiation [53]. Indeed, a soluble form of Lrp4, containing the extracellular region, in the absence of transmembrane and intracellular regions, can restore Agrin-stimulated MuSK phosphorylation in *lrp4*-mutant muscle cells, indicating that a complex of Agrin, soluble Lrp4, and MuSK can be reconstituted on the cell surface [51, 54, 55]. A truncated Lrp4 protein, extending from the last few LDLa repeats through the first β-propeller domain, is necessary and sufficient for maximal binding of neural Agrin to Lrp4 [51]. Agrin-stimulated association of Lrp4 and MuSK additionally requires the third β-propeller domains in Lrp4 [51]. Finally, the fourth β-propeller domain in Lrp4 enhances MuSK activation [51]. Difficulties expressing the extracellular region of Lrp4 have hindered structural studies, but a crystal structure between a truncated form of Agrin, which is ~100-fold less effective than full-length Agrin in activating MuSK, reveals how neural Agrin binds to the first β-propeller domain in Lrp4 [56].

9.6 Synapse Formation

The formation of synapses requires a complex exchange of signals between motor neurons and skeletal muscle [57]. Fusion of myoblasts to form multinucleated myotubes begins prior to innervation and continues well after innervation is complete. In the mouse, muscle fusion begins at ~E10.5, whereas innervation occurs 2 to 3 days later. Over the following prenatal week and the first several weeks of postnatal life, synapses mature both morphologically and functionally. In mice, MuSK and Lrp4 are expressed in myotubes but not in myoblasts [49, 58], and their expression in muscle precedes innervation. Because myoblasts fuse to the ends of developing myotubes [59, 60], the nuclei that reside in the central region of developing myotubes are the first to express MuSK and Lrp4. Lrp4 stimulates MuSK phosphorylation, which leads to two positive feedback regulatory loops, one that clusters MuSK and Lrp4 and another that stimulates *MuSK* and *Lrp4* expression. Thus, the initial activation of MuSK in the central nuclei of developing myotubes leads to a sustained increase in MuSK activity and postsynaptic differentiation in the central region of developing muscle [61]. This Lrp4- and MuSK-dependent priming of muscle, prior to innervation, is termed muscle prepatterning, which regulates where motor axons will terminate and form synapses [52, 61–64] (Fig. 9.2).

Once motor axons contact muscle, Agrin, which is supplied by motor neurons and associated with the synaptic basal lamina, binds to Lrp4 and stimulates a dramatic increase in association between Lrp4 and MuSK and MuSK kinase activity, stabilizing nascent synapses [4, 51]. At the same time, postsynaptic differentiation is counteracted by a separate signaling pathway, activated by acetylcholine (ACh), also released by motor nerve terminals, which acts antagonistically to MuSK signaling and destabilizes AChR clusters [65, 66]. Because Agrin is associated with the synaptic basal lamina and acts focally, whereas ACh stimulates muscle

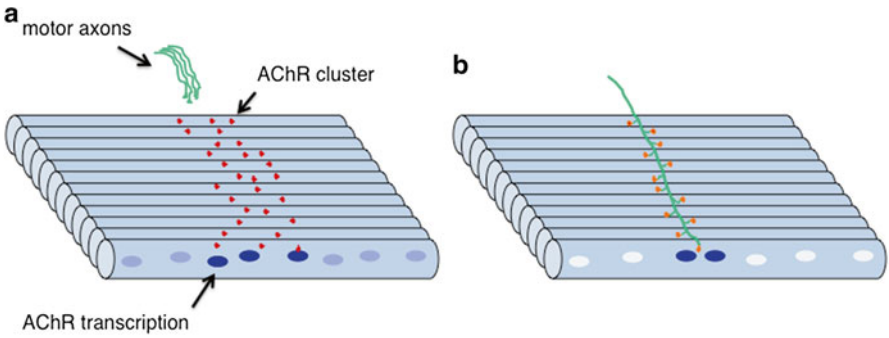

Fig. 9.2 (**a**) Motor axons approach muscles in which gene expression, including transcription of *MuSK*, *Lrp4*, and *AChR* subunit genes, and clustering of postsynaptic proteins are enhanced in the central region of muscle prior to and independent of innervation. (**b**) Motor axons form synapses in this prepatterned region and sharpen the pattern of gene expression

depolarization, which spreads from the site where ACh reacts with AChRs, these two signaling pathways insure that synaptic differentiation is maintained at sites where Agrin activates MuSK and extinguished at prepatterned sites that are not apposed by nerve terminals.

Because postsynaptic proteins, such as AChRs, are degraded and replaced at similar rates to maintain a steady-state number of AChRs in the postsynaptic membrane, the signaling pathways that are required to form synapses are required throughout life in order to maintain synaptic differentiation. As such, inactivation of MuSK, Rapsyn, or Lrp4, either by RNAi or by conditional gene inactivation, leads to disassembly of adult neuromuscular synapses [67–69].

Identification of the retrograde signals that are provided by muscle and control nerve terminal differentiation had proved elusive, but recent studies have identified Lrp4 itself as a critical retrograde signal that stimulates presynaptic differentiation of motor nerve terminals [70]. Thus, Lrp4 acts bidirectionally to coordinate synaptic differentiation. First, Lrp4 activates MuSK, stimulating muscle prepatterning; second, MuSK activation enhances expression and clustering of Lrp4, which acts as a retrograde signal to induce differentiation of motor nerve terminals; third, Agrin binds Lrp4, which increases MuSK phosphorylation and stabilizes synapses. How motor neurons recognize and respond to muscle-derived Lrp4 is not currently understood.

9.7 Neuromuscular Diseases Caused by Defects in the MuSK Signaling Pathway

Congenital myasthenia, a heterogeneous group of inherited neuromuscular disorders, has a prevalence of 1 per 500,000 and is caused by mutations in genes important for forming and maintaining neuromuscular synapses, including *Agrin*, *MuSK*, *Dok-7*, and *Rapsyn*. As described above, mutations in Dok-7 are a common cause of congenital myasthenia [22]. Mutations in Rapsyn are a less common cause of

congenital myasthenia [71], and mutations in Agrin and MuSK are far less frequent causes for congenital myasthenia [72–74]. In addition, mutations in genes critical for synaptic transmission, including *AChR* subunit genes, *acetylcholinesterase* and *choline acetyltransferase*, also cause congenital myasthenia [75]. Identification of the genes that cause congenital myasthenia has depended upon identification and analysis of these genes in model organisms, including *Torpedo*, a marine ray, and mice, and sequencing these candidate genes in patients diagnosed with congenital myasthenia. Currently, approximately half of the cases of congenital myasthenia can be attributed to mutations in one of the genes described above. It seems likely that further identification of genes that are critical for synapse formation as well as deep sequencing will reveal additional genes responsible for the remaining cases of congenital myasthenia.

Myasthenia gravis (MG) is an autoimmune disease with a prevalence of 1–2 per 10,000 [76]. Autoantibodies to the AChR are responsible for approximately 80 % of cases of MG. Like congenital myasthenia, MG causes muscle weakness and fatigue that is exacerbated by exercise. Approximately 15 % of cases of MG are caused by autoantibodies to MuSK, although the percentage varies among different ethnic groups. In anti-AChR MG, antibodies to the AChR deplete AChR from the cell surface by increasing AChR turnover and complement-mediated cellular disruption. Less frequently, the autoantibodies directly interfere with binding of ACh. In anti-MuSK MG, the disease-causing antibodies are IgG4, which are functionally monovalent and engage complement poorly if at all [77, 78]. In anti-MuSK MG, synapses appear to disassemble without evidence of cellular disruption, as expected if the antibodies directly interfere with MuSK function. Recent studies demonstrate that disease-causing autoantibodies to MuSK recognize the first Ig-like domain in MuSK and interfere with the ability of MuSK to associate with Lrp4, inhibiting Agrin-induced MuSK phosphorylation, providing a ready explanation for how the autoantibodies interfere with MuSK signaling and synaptic differentiation [79, 80].

Autoantibodies to Lrp4 are responsible for 0.5–50 % of the cases of MG that are not caused by autoantibodies to the AChR or MuSK [81–83]. The pathology and clinical symptoms of anti-Lrp4 MG have not been described in detail, but antibodies to Lrp4 can interfere with Agrin binding [81]. In addition, it remains possible that autoantibodies to Lrp4 obstruct binding of Lrp4 to MuSK and/or motor nerve terminals, thereby interfering with presynaptic differentiation. Since Lrp4 is expressed in tissues other than skeletal muscle and mutations in *lrp4* cause Cenani-Lenz syndrome, typified by bone malformations, autoantibodies to Lrp4 may cause clinical symptoms in addition to neuromuscular weakness [84, 85]. If so, autoantibodies to Lrp4 may be more common than currently believed but escape diagnosis as a neuromuscular disease. Likewise, deficits have not been reported for Cenani-Lenz syndrome, but the severe bone defects may disguise neuromuscular deficits and complicate a diagnosis [86].

Lrp4 mRNA is expressed in the brain, prominently in the hippocampus, olfactory bulb, cerebellum, and neocortex [87–90]. Moreover, Lrp4 protein co-fractionates with postsynaptic membranes [89], indicating a potential role for Lrp4 at synapse in the CNS. Lrp4 mutant mice die at birth, due to their neuromuscular deficits, prior to synapse formation in the CNS, but *lrp4* mutant mice that carry a transgene, which

restores Lrp4 expression selectively in skeletal muscle and rescues neuromuscular synapse formation, survive postnatally [53]. These rescued mice display striking defects in cognitive tasks that assess learning and memory. Moreover, the rescued mice lack hippocampal long-term potentiation, a form of synaptic plasticity that is associated with learning and memory [91]. These findings indicate that Lrp4 has a critical role in the CNS, although it remains unclear whether Lrp4 functions in the CNS by associating with Agrin and MuSK.

9.8 The Neuromuscular Synapse Deteriorates with Age

Not surprisingly, the neuromuscular synapse deteriorates with age. This deterioration is associated with structural and functional changes that lead to impaired synaptic transmission and contribute to a decrease in muscle size and function associated with aging, termed sarcopenia [92, 93]. Because synaptic and muscle activity increase muscle fiber size, the simplification of nerve terminals and the fragmentation of the postsynaptic membrane may trigger and surely exacerbate sarcopenia. The causes for the deterioration of the neuromuscular synapse during aging are poorly understood, but a decrease in expression or an altered distribution of proteins essential for forming and maintaining synapses, including Agrin, Lrp4, MuSK, Dok-7, or Rapsyn, may contribute to the progressive simplification and disassembly of the neuromuscular synapse. Because a similar simplification, followed by a loss of motor nerve terminals, is the first sign of disease in amyotrophic lateral sclerosis (ALS) [93], a decrease in signaling between motor neurons and muscle may contribute not only to sarcopenia but also to the progressive withdrawal of motor nerve terminals and lethal, respiratory paralysis in ALS.

Receptor at a glance: MuSK

Chromosome location	Mouse, chromosome 4, 31.87 cM
Gene size (bp)	Approx. 88–108 kb (depends on source)
Intron/ exon numbers	15/16
mRNA size (5′, ORF, 3′)	3422 (150 nt 5′; 2629 ORF; 593 3′)
Amino acid number	893
Kda	99.7
Posttranslational modifications	N-linked glycosylation
Domains	Ig-like domain, Frizzled-like domain, protein kinase domain
Ligands	Lrp4, Wnts (Wnt11, 4, 9a)
Known dimerizing partners	Lrp4, Dok-7, MuSK
Pathways activated	
Tissues expressed	Skeletal muscle, brain
Human diseases	Congenital myasthenia, myasthenia gravis
Knockout mouse phenotype	Failure to form neuromuscular synapses

References

1. Jennings CG, Dyer SM, Burden SJ. Muscle-specific trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. Proc Natl Acad Sci USA. 1993;90(7): 2895–9. Epub 1993/04/01.
2. Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K, Compton DL, et al. Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. Neuron. 1995;15(3):573–84. Epub 1995/09/01.
3. Ganju P, Walls E, Brennan J, Reith AD. Cloning and developmental expression of Nsk2, a novel receptor tyrosine kinase implicated in skeletal myogenesis. Oncogene. 1995;11(2): 281–90. Epub 1995/07/20.
4. DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, et al. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell. 1996;85(4):501–12. Epub 1996/05/17.
5. Garcia-Osta A, Tsokas P, Pollonini G, Landau EM, Blitzer R, Alberini CM. MuSK expressed in the brain mediates cholinergic responses, synaptic plasticity, and memory formation. J Neurosci. 2006;26(30):7919–32. Epub 2006/07/28.
6. Ksiazek I, Burkhardt C, Lin S, Seddik R, Maj M, Bezakova G, et al. Synapse loss in cortex of agrin-deficient mice after genetic rescue of perinatal death. J Neurosci. 2007;27(27):7183–95. Epub 2007/07/06.
7. Fu AK, Smith FD, Zhou H, Chu AH, Tsim KW, Peng BH, et al. Xenopus muscle-specific kinase: molecular cloning and prominent expression in neural tissues during early embryonic development. Eur J Neurosci. 1999;11(2):373–82. Epub 1999/03/03.
8. Ip FC, Glass DG, Gies DR, Cheung J, Lai KO, Fu AK, et al. Cloning and characterization of muscle-specific kinase in chicken. Mol Cell Neurosci. 2000;16(5):661–73. Epub 2000/11/21.
9. Stiegler AL, Burden SJ, Hubbard SR. Crystal structure of the agrin-responsive immunoglobulin-like domains 1 and 2 of the receptor tyrosine kinase MuSK. J Mol Biol. 2006;364(3):424–33. Epub 2006/10/03.
10. Stiegler AL, Burden SJ, Hubbard SR. Crystal structure of the frizzled-like cysteine-rich domain of the receptor tyrosine kinase MuSK. J Mol Biol. 2009;393(1):1–9. Epub 2009/08/12.
11. Janda CY, Waghray D, Levin AM, Thomas C, Garcia KC. Structural basis of Wnt recognition by Frizzled. Science. 2012;337(6090):59–64. Epub 2012/06/02.
12. Jing L, Lefebvre JL, Gordon LR, Granato M. Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/MuSK receptor. Neuron. 2009;61(5):721–33. Epub 2009/03/17.
13. Gordon LR, Gribble KD, Syrett CM, Granato M. Initiation of synapse formation by Wnt-induced MuSK endocytosis. Development. 2012;139(5):1023–33. Epub 2012/02/10.
14. Zhang B, Liang C, Bates R, Yin Y, Xiong WC, Mei L. Wnt proteins regulate acetylcholine receptor clustering in muscle cells. Mol Brain. 2012;5:7. Epub 2012/02/09.
15. Green JL, Kuntz SG, Sternberg PW. Ror receptor tyrosine kinases: orphans no more. Trends Cell Biol. 2008;18(11):536–44. Epub 2008/10/14.
16. Till JH, Becerra M, Watty A, Lu Y, Ma Y, Neubert TA, et al. Crystal structure of the MuSK tyrosine kinase: insights into receptor autoregulation. Structure. 2002;10(9):1187–96. Epub 2002/09/11.
17. Piras R, Staneloni R. In vivo regulation of rat muscle glycogen synthetase activity. Biochemistry. 1969;8(5):2153–60. Epub 1969/05/01.
18. Watty A, Neubauer G, Dreger M, Zimmer M, Wilm M, Burden SJ. The in vitro and in vivo phosphotyrosine map of activated MuSK. Proc Natl Acad Sci USA. 2000;97(9):4585–90. Epub 2000/04/26.
19. Herbst R, Burden SJ. The juxtamembrane region of MuSK has a critical role in agrin-mediated signaling. EMBO J. 2000;19(1):67–77. Epub 2000/01/05.
20. Okada K, Inoue A, Okada M, Murata Y, Kakuta S, Jigami T, et al. The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science. 2006;312(5781):1802–5. Epub 2006/06/24.

21. Bergamin E, Hallock PT, Burden SJ, Hubbard SR. The cytoplasmic adaptor protein Dok7 activates the receptor tyrosine kinase MuSK via dimerization. Mol Cell. 2010;39(1):100–9. Epub 2010/07/07.
22. Beeson D, Higuchi O, Palace J, Cossins J, Spearman H, Maxwell S, et al. Dok-7 mutations underlie a neuromuscular junction synaptopathy. Science. 2006;313(5795):1975–8. Epub 2006/08/19.
23. Muller JS, Herczegfalvi A, Vilchez JJ, Colomer J, Bachinski LL, Mihaylova V, et al. Phenotypical spectrum of DOK7 mutations in congenital myasthenic syndromes. Brain. 2007;130(Pt 6):1497–506. Epub 2007/04/19.
24. Selcen D, Milone M, Shen XM, Harper CM, Stans AA, Wieben ED, et al. Dok-7 myasthenia: phenotypic and molecular genetic studies in 16 patients. Ann Neurol. 2008;64(1):71–87. Epub 2008/07/16.
25. Hallock PT, Xu CF, Park TJ, Neubert TA, Curran T, Burden SJ. Dok-7 regulates neuromuscular synapse formation by recruiting Crk and Crk-L. Genes Dev. 2010;24(21):2451–61. Epub 2010/11/03.
26. Neubig RR, Krodel EK, Boyd ND, Cohen JB. Acetylcholine and local anesthetic binding to Torpedo nicotinic postsynaptic membranes after removal of nonreceptor peptides. Proc Natl Acad Sci USA. 1979;76(2):690–4. Epub 1979/02/01.
27. Burden SJ, DePalma RL, Gottesman GS. Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the beta-subunit and the 43 kd subsynaptic protein. Cell. 1983;35(3 Pt 2):687–92. Epub 1983/12/01.
28. Maimone MM, Merlie JP. Interaction of the 43 kd postsynaptic protein with all subunits of the muscle nicotinic acetylcholine receptor. Neuron. 1993;11(1):53–66. Epub 1993/07/01.
29. Lee Y, Rudell J, Ferns M. Rapsyn interacts with the muscle acetylcholine receptor via alpha-helical domains in the alpha, beta, and epsilon subunit intracellular loops. Neuroscience. 2009;163(1):222–32. Epub 2009/06/02.
30. Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, et al. Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. Nature. 1995;377(6546):232–6. Epub 1995/09/21.
31. Zuber B, Unwin N. Structure and superorganization of acetylcholine receptor-rapsyn complexes. Proc Natl Acad Sci USA. 2013;110(26):10622–7. Epub 2013/06/12.
32. Borges LS, Yechikhov S, Lee YI, Rudell JB, Friese MB, Burden SJ, et al. Identification of a motif in the acetylcholine receptor beta subunit whose phosphorylation regulates rapsyn association and postsynaptic receptor localization. J Neurosci. 2008;28(45):11468–76. Epub 2008/11/07.
33. Brockhausen J, Cole RN, Gervasio OL, Ngo ST, Noakes PG, Phillips WD. Neural agrin increases postsynaptic ACh receptor packing by elevating rapsyn protein at the mouse neuromuscular synapse. Dev Neurobiol. 2008;68(9):1153–69. Epub 2008/05/29.
34. Friese MB, Blagden CS, Burden SJ. Synaptic differentiation is defective in mice lacking acetylcholine receptor beta-subunit tyrosine phosphorylation. Development. 2007;134(23):4167–76. Epub 2007/10/26.
35. Burden SJ. SnapShot: neuromuscular junction. Cell. 2011;144(5):826–e1. Epub 2011/03/08.
36. Dai Z, Luo X, Xie H, Peng HB. The actin-driven movement and formation of acetylcholine receptor clusters. J Cell Biol. 2000;150(6):1321–34. Epub 2000/09/20.
37. Weston C, Gordon C, Teressa G, Hod E, Ren XD, Prives J. Cooperative regulation by Rac and Rho of agrin-induced acetylcholine receptor clustering in muscle cells. J Biol Chem. 2003;278(8):6450–5. Epub 2002/12/11.
38. Weston C, Yee B, Hod E, Prives J. Agrin-induced acetylcholine receptor clustering is mediated by the small guanosine triphosphatases Rac and Cdc42. J Cell Biol. 2000;150(1):205–12. Epub 2000/07/13.
39. Linnoila J, Wang Y, Yao Y, Wang ZZ. A mammalian homolog of Drosophila tumorous imaginal discs, Tid1, mediates agrin signaling at the neuromuscular junction. Neuron. 2008; 60(4):625–41. Epub 2008/11/29.

40. Grady RM, Zhou H, Cunningham JM, Henry MD, Campbell KP, Sanes JR. Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin–glycoprotein complex. Neuron. 2000;25(2):279–93. Epub 2000/03/17.
41. Grady RM, Akaaboune M, Cohen AL, Maimone MM, Lichtman JW, Sanes JR. Tyrosine-phosphorylated and nonphosphorylated isoforms of alpha-dystrobrevin: roles in skeletal muscle and its neuromuscular and myotendinous junctions. J Cell Biol. 2003;160(5):741–52. Epub 2003/02/27.
42. Kishi M, Kummer TT, Eglen SJ, Sanes JR. LL5beta: a regulator of postsynaptic differentiation identified in a screen for synaptically enriched transcripts at the neuromuscular junction. J Cell Biol. 2005;169(2):355–66. Epub 2005/04/27.
43. Proszynski TJ, Gingras J, Valdez G, Krzewski K, Sanes JR. Podosomes are present in a postsynaptic apparatus and participate in its maturation. Proc Natl Acad Sci USA. 2009; 106(43):18373–8. Epub 2009/10/14.
44. Schaeffer L, Duclert N, Huchet-Dymanus M, Changeux JP. Implication of a multisubunit Ets-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor. EMBO J. 1998;17(11):3078–90. Epub 1998/06/26.
45. Jaworski A, Smith CL, Burden SJ. GA-binding protein is dispensable for neuromuscular synapse formation and synapse-specific gene expression. Mol Cell Biol. 2007;27(13):5040–6. Epub 2007/05/09.
46. O'Leary DA, Noakes PG, Lavidis NA, Kola I, Hertzog PJ, Ristevski S. Targeting of the ETS factor GABPalpha disrupts neuromuscular junction synaptic function. Mol Cell Biol. 2007;27(9):3470–80. Epub 2007/02/28.
47. Hippenmeyer S, Huber RM, Ladle DR, Murphy K, Arber S. ETS transcription factor Erm controls subsynaptic gene expression in skeletal muscles. Neuron. 2007;55(5):726–40. Epub 2007/09/06.
48. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. J Clin Invest. 2001;108(6):779–84. Epub 2001/09/19.
49. Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, et al. Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell. 2008;135(2):334–42. Epub 2008/10/14.
50. Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. LRP4 serves as a coreceptor of agrin. Neuron. 2008;60(2):285–97. Epub 2008/10/30.
51. Zhang W, Coldefy AS, Hubbard SR, Burden SJ. Agrin binds to the N-terminal region of Lrp4 and stimulates association between Lrp4 and the first Ig-like domain in MuSK. J Biol Chem. 2011;286:40624–30. Epub 2011/10/05.
52. Arber S, Burden SJ, Harris AJ. Patterning of skeletal muscle. Curr Opin Neurobiol. 2002; 12(1):100–3. Epub 2002/02/28.
53. Gomez AM, Burden SJ. The extracellular region of Lrp4 is sufficient to mediate neuromuscular synapse formation. Dev Dyn. 2011;240(12):2626–33. Epub 2011/11/01.
54. Wu H, Lu Y, Shen C, Patel N, Gan L, Xiong WC, et al. Distinct roles of muscle and motoneuron LRP4 in neuromuscular junction formation. Neuron. 2012;75(1):94–107. Epub 2012/07/17.
55. Choi HY, Liu Y, Tennert C, Sugiura Y, Karakatsani A, Kroger S, et al. APP interacts with LRP4 and agrin to coordinate the development of the neuromuscular junction in mice. eLife. 2013;2:e00220. Epub 2013/08/30.
56. Zong Y, Zhang B, Gu S, Lee K, Zhou J, Yao G, et al. Structural basis of agrin-LRP4-MuSK signaling. Genes Dev. 2012;26(3):247–58. Epub 2012/02/04.
57. Burden SJ. The formation of neuromuscular synapses. Genes Dev. 1998;12(2):133–48. Epub 1998/03/07.
58. Glass DJ, Bowen DC, Stitt TN, Radziejewski C, Bruno J, Ryan TE, et al. Agrin acts via a MuSK receptor complex. Cell. 1996;85(4):513–23. Epub 1996/05/17.
59. Kitiyakara A, Angevine DM. A study of the pattern of postembryonic growth of M.Gracilis in Mice. Dev Biol. 1963;8:322–40. Epub 1963/12/01.
60. Zhang M, McLennan IS. During secondary myotube formation, primary myotubes preferentially absorb new nuclei at their ends. Dev Dyn. 1995;204(2):168–77. Epub 1995/10/01.

61. Kim N, Burden SJ. MuSK controls where motor axons grow and form synapses. Nat Neurosci. 2008;11(1):19–27. Epub 2007/12/18.
62. Yang X, Li W, Prescott ED, Burden SJ, Wang JC. DNA topoisomerase II beta and neural development. Science. 2000;287(5450):131–4. Epub 1999/12/30.
63. Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, et al. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron. 2001;30(2):399–410. Epub 2001/06/08.
64. Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature. 2001; 410(6832):1057–64. Epub 2001/04/27.
65. Misgeld T, Kummer TT, Lichtman JW, Sanes JR. Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. Proc Natl Acad Sci USA. 2005;102(31):11088–93. Epub 2005/07/27.
66. Lin W, Dominguez B, Yang J, Aryal P, Brandon EP, Gage FH, et al. Neurotransmitter acetylcholine negatively regulates neuromuscular synapse formation by a Cdk5-dependent mechanism. Neuron. 2005;46(4):569–79. Epub 2005/06/10.
67. Kong XC, Barzaghi P, Ruegg MA. Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference. EMBO Rep. 2004;5(2):183–8. Epub 2004/01/30.
68. Hesser BA, Henschel O, Witzemann V. Synapse disassembly and formation of new synapses in postnatal muscle upon conditional inactivation of MuSK. Mol Cell Neurosci. 2006; 31(3):470–80. Epub 2005/12/13.
69. Barik A, Lu Y, Sathyamurthy A, Bowman A, Shen C, Li L, et al. LRP4 is critical for neuromuscular junction maintenance. J Neurosci. 2014;34(42):13892–905. Epub 2014/10/17.
70. Yumoto N, Kim N, Burden SJ. Lrp4 is a retrograde signal for presynaptic differentiation at neuromuscular synapses. Nature. 2012;489:438–42. Epub 2012/08/03.
71. McConville J, Vincent A. Diseases of the neuromuscular junction. Curr Opin Pharmacol. 2002;2(3):296–301. Epub 2002/05/22.
72. Huze C, Bauche S, Richard P, Chevessier F, Goillot E, Gaudon K, et al. Identification of an agrin mutation that causes congenital myasthenia and affects synapse function. Am J Hum Genet. 2009;85(2):155–67. Epub 2009/07/28.
73. Chevessier F, Faraut B, Ravel-Chapuis A, Richard P, Gaudon K, Bauche S, et al. MUSK, a new target for mutations causing congenital myasthenic syndrome. Hum Mol Genet. 2004; 13(24):3229–40. Epub 2004/10/22.
74. Maselli RA, Fernandez JM, Arredondo J, Navarro C, Ngo M, Beeson D, et al. LG2 agrin mutation causing severe congenital myasthenic syndrome mimics functional characteristics of non-neural (z-) agrin. Hum Genet. 2012;131(7):1123–35. Epub 2011/12/30.
75. Engel AG, Ohno K, Sine SM. Sleuthing molecular targets for neurological diseases at the neuromuscular junction. Nat Rev Neurosci. 2003;4(5):339–52. Epub 2003/05/03.
76. Farrugia ME, Vincent A. Autoimmune mediated neuromuscular junction defects. Curr Opin Neurol. 2010;23(5):489–95. Epub 2010/07/24.
77. Niks EH, van Leeuwen Y, Leite MI, Dekker FW, Wintzen AR, Wirtz PW, et al. Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. J Neuroimmunol. 2008;195(1–2):151–6. Epub 2008/04/04.
78. Klooster R, Plomp JJ, Huijbers MG, Niks EH, Straasheijm KR, Detmers FJ, et al. Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice. Brain. 2012;135(Pt 4):1081–101. Epub 2012/03/08.
79. Huijbers MG, Zhang W, Klooster R, Niks EH, Friese MB, Straasheijm KR, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. Proc Natl Acad Sci USA. 2013;110(51):20783–8. Epub 2013/12/04.
80. Koneczny I, Cossins J, Waters P, Beeson D, Vincent A. MuSK myasthenia gravis IgG4 disrupts the interaction of LRP4 with MuSK but both IgG4 and IgG1-3 can disperse preformed agrin-independent AChR clusters. PLoS One. 2013;8(11):e80695. Epub 2013/11/19.

81. Higuchi O, Hamuro J, Motomura M, Yamanashi Y. Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. Ann Neurol. 2011;69(2):418–22. Epub 2011/03/10.
82. Zhang B, Tzartos JS, Belimezi M, Ragheb S, Bealmear B, Lewis RA, et al. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. Arch Neurol. 2012;69(4):445–51. Epub 2011/12/14.
83. Pevzner A, Schoser B, Peters K, Cosma NC, Karakatsani A, Schalke B, et al. Anti-LRP4 autoantibodies in AChR- and MuSK-antibody-negative myasthenia gravis. J Neurol. 2012; 259(3):427–35. Epub 2011/08/05.
84. Karner CM, Dietrich MF, Johnson EB, Kappesser N, Tennert C, Percin F, et al. Lrp4 regulates initiation of ureteric budding and is crucial for kidney formation–a mouse model for Cenani-Lenz syndrome. PLoS One. 2010;5(4):e10418. Epub 2010/05/11.
85. Li Y, Pawlik B, Elcioglu N, Aglan M, Kayserili H, Yigit G, et al. LRP4 mutations alter Wnt/beta-catenin signaling and cause limb and kidney malformations in Cenani-Lenz syndrome. Am J Hum Genet. 2010;86(5):696–706. Epub 2010/04/13.
86. Lindy AS, Bupp CP, McGee SJ, Steed E, Stevenson RE, Basehore MJ, et al. Truncating mutations in LRP4 lead to a prenatal lethal form of Cenani-Lenz syndrome. Am J Med Genet A. 2014;164A(9):2391–7. Epub 2014/06/14.
87. Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, et al. Genome-wide atlas of gene expression in the adult mouse brain. Nature. 2007;445(7124):168–76. Epub 2006/12/08.
88. Visel A, Thaller C, Eichele G. GenePaint.org: an atlas of gene expression patterns in the mouse embryo. Nucleic Acids Res. 2004;32(Database issue):D552–6.
89. Tian QB, Suzuki T, Yamauchi T, Sakagami H, Yoshimura Y, Miyazawa S, et al. Interaction of LDL receptor-related protein 4 (LRP4) with postsynaptic scaffold proteins via its C-terminal PDZ domain-binding motif, and its regulation by Ca/calmodulin-dependent protein kinase II. Eur J Neurosci. 2006;23(11):2864–76. Epub 2006/07/06.
90. Weatherbee SD, Anderson KV, Niswander LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development. 2006;133(24):4993–5000. Epub 2006/11/23.
91. Gomez AM. Synaptic plasticity and cognitive function are disrupted in the absence of Lrp4. eLife. 2014;3:e04287. doi:10.7554/eLife.04287.
92. Valdez G, Tapia JC, Kang H, Clemenson Jr GD, Gage FH, Lichtman JW, et al. Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise. Proc Natl Acad Sci USA. 2010;107(33):14863–8. Epub 2010/08/04.
93. Valdez G, Tapia JC, Lichtman JW, Fox MA, Sanes JR. Shared resistance to aging and ALS in neuromuscular junctions of specific muscles. PLoS One. 2012;7(4):e34640. Epub 2012/04/10.

Chapter 10
The PDGFR Receptor Family

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Abbreviations

17-AAG	17-allylamino-17demethoxygeldanamycin
a.a.	Amino acid
AGM	Aorta–gonad–mesonephros
AHCYL1	Adenosylhomocysteinase-like 1
Akt	v-Akt murine thymoma viral oncogene homologue
AL	Activation loop
ALL	Acute lymphoblastic leukemias

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ALSP	Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia
AML	Acute myeloid leukemia
AP1	Activator protein 1
ATP	Adenosine triphosphate
BARF1	Epstein–Barr virus lytic-cycle early protein 1
Bcl-2	B-cell lymphoma 2
Bcl-X(L)	B-cell lymphoma-extra large
BMM	Bone marrow-derived macrophages
bp	Base pairs
C/EBPα	CCAAT-enhancer-binding protein alpha
C1P	Ceramide-1 phosphate
cAMP	Cyclic adenosine monophosphate
Caspase	Cysteine-dependent aspartate-directed protease
Cbl	Casitas B-lineage lymphoma
CCL12	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDC	Lymphoid-tissue resident or classical DC
Cdc42	Cell division control protein 42 homologue
c-Fms	McDonough feline sarcoma virus oncogene (v-Fms) homologue
c-Fos	Finkel–Biskis–Jinkins murine osteogenic sarcoma virus oncogene (v-fos) homologue
CFU-GM	Colony-forming unit–granulocyte–macrophage
CFU-M	Colony-forming unit–macrophage
c-Kit	Hardy–Zuckerman 4 feline sarcoma viral oncogene (v-Kit) homologue
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor cells
CMT	Charcot–Marie–Tooth disease
c-Myc	(v-Myc) myelocytomatosis viral oncogene homologue
CNS	Central nervous system
CSF	Colony-stimulating factor
CSF-1	Colony-stimulating factor-1
CSF-1R	Colony-stimulating factor-1 receptor
CT	Cytoplasmic tail
CX3CR1	CX3C chemokine receptor 1
DAP12	DNAX-activating protein of 12 kDa
DC	Dendritic cells
DNMT1	DNA methyl transferase 1
Dok-1	Docking protein 1
DUSP	Dual-specificity phosphatase
DUSP5	Dual-specificity phosphatase 5
EBV	Epstein–Barr virus
ECD	Extracellular domain

Egr2	Early growth response 2
EM	Electron microscopy
EMT	Epithelial–mesenchymal transition
EPS8L3	Epidermal growth factor receptor pathway substrate 8 like 3
ERK1/2	Extracellular signal-regulated kinases 1 and 2
ERα	Estrogen receptor alpha
Ets	E26 transformation specific
EWS	Ewing sarcoma breakpoint region
FDMCs	Follicular dendritic cell-induced monocytes
FIMP	Fms-interacting protein
FIRE	Fms-intronic regulatory element
FL	FLT3 ligand
flk-2	Fetal liver kinase 2
Flt3	Fms-like tyrosine kinase 3
FUS/TLS	Fused in sarcoma/translocated in sarcoma
Fyn	Proto-oncogene tyrosine-protein kinase Fyn
Gab2	Grb2-associated-binding protein 2
GCL	Globoid cell leukodystrophy
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GM	Geldanamycin
GM-CSF	Granulocyte–macrophage CSF
GMP	Granulocyte–macrophage progenitor
GMP	Granulocyte–macrophage progenitors
GnRH	Gonadotropin-releasing hormone
Grb2	Growth factor receptor-bound protein 2
GSKβ	Glycogen synthase kinase 3 beta
HA	Herbimycin A
Hck	Tyrosine-protein kinase HCK
HDLS	Hereditary diffuse leukoencephalopathy with axonal spheroids
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HMGXB3	HMG-box domain containing 3
HSC	Hematopoietic stem cells
Ifi20	Interferon-inducible P204 protein
IFNγ	Interferon γ
Ig	Immunoglobulin
IL-3	Interleukin-3
IL-34	Interleukin-34
IL-4	Interleukin-4
IPC	Type 1 interferon-producing cells
IRSp53	Insulin receptor tyrosine kinase substrate p53
ISC	Intestinal stem cells
ITD	Internal tandem duplications

ITIM	Immunoreceptor tyrosine-based inhibitory motifs
JAK	Janus kinase
JDP2	Jun dimerization protein 2
JM	Juxtamembrane
JM-B	JM-binding motif
JMD	Juxtamembrane domain
JM-S	JM switch motif
JM-Z	Zipper segment
JNK	c-Jun N-terminal kinase
JunB	Transcription factor jun-B
KA	Kainic acid
KC	Keratinocyte chemoattractant
K_d	Dissociation constant
LC	Langerhans cells
Ldlr$^{-/-}$	Low-density lipoprotein receptor null
LEF	Lymphoid enhancer-binding factor
LH	Luteinizing hormone
LILRB	Leukocyte Ig-like receptor B
LIMK	LIM domain kinase
LMPP	Lymphoid primed multipotent progenitors
Lnk	Lnk adaptor protein
LPS	Lipopolysaccharide
LSK	lin-kit+sca-1+
LT-HSC	Long-term hematopoietic stem cells
Lyn	Tyrosine-protein kinase Lyn
MAP-kinase	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MDP	Macrophage–DC progenitors
MDS	Myelodysplastic syndrome
MEK	Mitogen-activated protein kinase kinase
MEP	Megakaryocyte/erythrocyte progenitors
MIP-2	Macrophage inflammatory protein-2
miR	Micro RNA
Mitf	Microphthalmia-associated transcription factor
MKP	MAP-kinase phosphatase
MKP-1	Mitogen-activated protein kinase phosphatase-1 (also known as DUSP-1)
Mo	Monocytes
Mona	Monocytic adaptor
MPP	Multipotent progenitor
mTOR	Mammalian target of rapamycin
MΦ	Macrophages
NBCn1	Na/HCO3 co-transporter 1
N-CoR	Nuclear receptor corepressor

NFAT	Nuclear factor of activated T cells
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPC	Neural progenitor cells
OC	Osteoclast
p38	p38 Mitogen-activated protein kinase
PA	Plasminogen activator
PAX5	Paired box protein 5
PC	Paneth cells
pDC	Plasmacytoid DC
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PIR-B	Paired Ig-like receptor B
PKCζ	Protein kinase Ca-dependent zeta
PLC	Phospholipase C
PLC	Phospholipase C
PLD2	Phospholipase D
POLD	Pigmented orthochromatic leukodystrophy
PP2A	Serine–threonine phosphatase 2A
Pro-B	Progenitors of B cells
Pro-NK	Progenitors of natural killer cells
Pro-T	Progenitors of T cells
PSTPIP2	Proline–serine–threonine (PEST)–phosphatase-interacting protein 2
PTB	Phosphotyrosine-binding domains
PTK	Protein-tyrosine kinase
PTPN12	Protein-tyrosine phosphatase nonreceptor type 12
PTPϕ	Nonreceptor protein-tyrosine phosphatase phi
PU.1	Transcription factor PU.1
Pyk2	Protein-tyrosine kinase 2 beta
Rac	Ras-related C3 botulinum toxin substrate
RANKL	Receptor activator of nuclear factor κB
Ras	Rat sarcoma small GTPase
RBM6	RNA-binding motif 6
RCC	Renal clear cell carcinoma
Rho	Rho small GTPase
Rho U/Wrch	Wnt-1-responsive Cdc42 homologue, Rho family GTPase
RIP	Regulated intramembrane proteolysis
RPTP-ζ	Receptor protein-tyrosine phosphatase-zeta
RSK2	Ribosomal S6 kinase 2
RTK	Receptor tyrosine kinase
RTK	Receptor tyrosine kinase
Runx1	Runt-related transcription factor 1

RV FV3	Ranavirus frog virus 3
S100A4	S100 family Ca2+-binding protein A4
SAXS	Small-angle X-ray scattering
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	Src family kinases
SH2	Src homology 2 domain
SH3BP2	SH3 domain-binding protein 2
Shc	Src homology 2 domain containing
SHIP	SH2-containing inositol phosphatase
Shp2	SH2 domain protein-tyrosine phosphatase 2
SIRPα	Signal regulatory protein alpha
SIV	Simian immunodeficiency virus
SKAP55R	Src kinase-associated phosphoprotein of 55 kDa (SKAP55)-related adaptor protein
SLAP	Src-like adaptor protein
SLAP2	Src-like adaptor protein 2
SLP-76	SH2 domain-containing leukocyte protein-76
SM-FeSV	Susan McDonough strain of feline sarcoma virus
Snord11B	Small nucleolar RNA CD box 11B
Socs1	Suppressor of cytokine signaling 1
Sos	Son of sevenless
Sp1/3	Transcription factors Sp1 and Sp3
Src	Rous sarcoma virus oncogenic tyrosine kinase homologue
STAP-2	Signal-transducing adaptor protein 2
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
T reg	T regulatory cells
TACE	TNFα-converting enzyme (also known as ADAM-17)
TAMs	Tumor-associated macrophages
Tbx3	T-box transcription factor 3
TCF	T-cell-specific, HMG-box transcription factor
Tcptp	T-cell protein-tyrosine phosphatase
TGFβ1	Transforming growth factor beta 1
TLR	Toll-like receptor
TRAF6	TNF receptor-associated factor 6
TRAP	Tartrate-resistant acid phosphatase
TREM2	Triggering receptor expressed on myeloid cells 2
Vac14	Vac14 homologue
Vav	Vav oncogene
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2.
WASP	Wiskott–Aldrich syndrome protein
WAVE2	WASP-family verprolin-homologous protein-2
Zp3-Cre	Zona pellucida 3 promoter-driven Cre recombinase

10.1 CSF-1R[1]

10.1.1 Introduction to the CSF-1 Receptor Tyrosine Kinase

The colony-stimulating factor-1 (CSF-1) receptor (CSF-1R) (also known as Fms, c-Fms, CD115, FIM2, or M-CSF receptor) is a class III receptor tyrosine kinase (RTK III) [1]. Similar to other RTK IIIs, the CSF-1R consists of seven modules: five extracellular Ig-like domains (D1–D5), a single transmembrane helix, and an intracellular split kinase domain [2, 3]. It is activated by two dimeric glycoprotein ligands, CSF-1 [4] and interleukin-34 (IL-34) [5]. IL-34 shares low primary sequence similarity with CSF-1, but they possess a similar three-dimensional structure [6–8]. In vitro, IL-34 and CSF-1 have comparable ability to support the proliferation and differentiation of myeloid cells and osteoclastogenesis to a degree proportional to their affinity for the CSF-1R [9]. However, they differ in their spatiotemporal expression patterns [9, 10], developmental roles [9–13], mechanism of interaction with CSF-1R [7, 8], signal activation kinetics, and strength [14].

The CSF-1R is the major regulator of tissue macrophage development and maintenance [15–17]. Macrophages are found in all tissues, where they represent 5–15 % of the cells [18]. They have roles in triggering immune responses, but also in enhancing and attenuating inflammation and promoting tissue repair. Through trophic and scavenger functions, they maintain tissue homeostasis and contribute to tissue remodeling during development [19, 20]. In combination with receptor activator of nuclear factor κB (RANK), the CSF-1R also regulates the differentiation of the bone-resorbing osteoclast and controls bone remodeling during embryonic and early postnatal development [21]. Studies in animal models suggest that CSF-1-dependent macrophages and/or osteoclasts play detrimental roles in cancer and inflammatory diseases (reviewed in [11, 22]). A chromosomal translocation involving the *Csf1r* gene [23], inappropriate overexpression of the CSF-1R [24–27], and/or inappropriate expression of CSF-1 [26–28] can contribute to the development of acute myeloid leukemias and Hodgkin's lymphoma. For these reasons, several small molecule inhibitors and antibodies targeting CSF-1R have been developed, and some are currently in clinical trials.

Outside the mononuclear phagocytic system, the CSF-1R directly regulates the development of intestinal Paneth cells [29], the innate immune functions of placental trophoblasts [30], prolactin secretion by pituitary cells [31], and neuronal survival and differentiation [10, 32]. CSF-1R deficiency in most mouse strains causes perinatal death [15, 21], and the surviving mice exhibit multiple developmental and functional deficits (Table 10.1). Dominant inactivating mutations in the CSF-1R in man lead to an adult-onset progressive dementia predominantly affecting the cerebral white matter [33, 34]. Homozygous inactivating mutations of the *Csf1r* or *Csf1* genes have not been reported in man.

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Table 10.1 Phenotypes of *Csf1r* $^{-/-}$, *Nes-Cre/+*, *Csf1r*$^{fl/fl}$, *Csf1*$^{op/op}$, and *IL-34* $^{-/-}$ mice compared to those of wild-type mice

Phenotype	Mouse mutant				References
	Csf1r$^{-/}$	*Nes-Cre/+; Csf1r*$^{fl/fl}$	*Csf1*$^{op/op}$	*IL-34* $^{-/-}$	
Gross phenotype					
Postnatal death (at 3 weeks)	60 %		20 %	Normal	[13, 15, 47, 544]
Body size	Reduced		Reduced	Normal	[13, 15, 47]
Body weight	Reduced		Reduced	Normal	[13, 15, 47]
Skeletal abnormalities (osteopetrosis, short limbs, domed skull)	Present		Present	Absent	[13, 15, 47]
Delayed tooth eruption	Present		Present	Absent	[13, 15, 47]
Low growth rate	Present		Present	Absent	[13, 15, 47]
Monocytic lineage					
Blood monocytes	Normal		Normal	Normal	[12, 13, 15, 55]
Tissue macrophages	Reduced		Reduced	Normal	[12, 15, 16, 54, 55]
Langerhans cells (LC)	Absent [a]		Reduced[b]	Severely reduced	[12, 13, 545]
Microglia	Absent	Normal	Reduced[b]	Reduced[c]	[10, 12, 13, 17, 544]
Osteoclasts	Reduced		Reduced[b]		[15, 21, 546]
Brain					[10]
Size	Reduced	Reduced	Reduced		
Mass	Increased	Normal	Increased		
Olfactory bulb atrophy	Present	Absent	Absent		
Ventricular size	Increased	Normal	Normal		
Corpus callosum crossing defect	Present (80%)	Absent	Present (22%)		
Cortical thickness	Reduced	Variable	Increased		
Cortical NPC proliferation	Increased	Increased	Increased		
Excitatory neuronal differentiation	Reduced	Variable	Reduced		
Sub-cortical OL differentiation	Reduced	Normal	Reduced		
Cortical cellular apoptsis	Increased	Increased	Normal		
Neurological deficits					
Hearing	Present		Present		[15, 94]
Vision			Present		[94]

(continued)

Table 10.1 (continued)

Phenotype	Mouse mutant				References
	Csf1r$^{-/}$	*Nes-Cre/+; Csf1r*$^{fl/fl}$	*Csf1*$^{op/op}$	*IL-34*$^{-/-}$	
Olfactory	Present				[95]
Abnormal GABAergic responses			Present		[94]
Intestine					
Paneth cells	Reduced[d]		Reduced		[29, 106]
Small intestine stem cells	Reduced[d]				[106]
Abnormal colon organization	Present		Present		[45]
Placenta					
Decreased response of trophoblasts to transplacental infection			Present		[30]
Reproductive defects					
Reduced fertility	Present		Present		[15, 96, 97, 110]
Delayed puberty	Present		Present		[15, 96, 97, 110]
Prolonged estrous cycle	Present		Present		[15, 96, 97, 110]
Failure to lactate	Present		Present		[15, 126]
Low testosterone	Present		Present		[15, 96, 97]
Low sperm viability	Present		Present		[15, 96, 97]
Reduced hypothalamic sex steroid hormone feedback response			Present		[96, 97]

[a]Also drastically reduced in mice with a LC-specific deletion of *Csf1r* achieved by crossing *Csf1r*$^{fl/fl}$ mice to mice expressing a Cre-transgene driven by an LC-specific promoter (*Langerin-Cre*) [13]
[b]Reduced postnatally, spontaneously recovered in the adult
[c]Only in areas of the brain with high IL-34 expression (i.e., cortex, hippocampus, corpus callosum, striatum, olfactory bulb) but not in the cerebellum or brain stem [12, 13]
[d]A reduction in Paneth cell number was also observed in a mice with gastrointestinal-specific deletion of *Csf1r* obtained using a tamoxifen inducible Cre-transgene driven by the villin promoter (*VillinCreERT2*) [106]
Nes-Cre/+; Csf1r$^{fl/fl}$, mice with neural progenitor-specific deletion of *Csf1r*, *OL* oligodendrocytes, *GABA* gamma amino butyric acid

10.1.2 The Role of the CSF-1 Receptor Tyrosine Kinase in Embryonic Development and Adult Physiology

The CSF-1R is expressed on all cells of the mononuclear phagocytic system, including monocytes, tissue macrophages, microglia, dendritic cells, Kupffer cells, and Langerhans cells (LC), as well as on the multinucleated bone-resorbing osteoclasts (reviewed in [11]). In addition, it is expressed at low levels on hematopoietic stem cells (HSC) [35, 36], and its expression increases as multipotent hematopoietic precursors become committed to the monocytic lineage and differentiate to macrophages [36–38]. CSF-1R mRNA is expressed in granulocytes but is not translated [39]. Outside the hematopoietic system, the CSF-1R is expressed on oocytes and preimplantation embryos [40, 41], as well as decidual and trophoblastic cells [42, 43], renal proximal tubule epithelial cells [44], Paneth cells [29], epithelial intestinal cells of the colon [45], neural progenitor cells [10], and in several subpopulations of neurons [10, 32, 46]. Consistent with its broad pattern of expression, the CSF-1R has pleiotropic actions in embryonic development and adult physiology (Table 10.1).

10.1.2.1 The Role of the CSF-1R in Embryonic and Early Postnatal Development

The viability of mice carrying a homozygous germline deletion of the CSF-1R (*Csf1r*$^{-/-}$ mice) varies from late embryonic lethality on the C3H/HeJ and C57/BL6 backgrounds to postnatal lethality by 3 weeks of age on the FVB/NJ background [15, 21]. On an outbred (mainly C3B6) background, 40 % of the mice survive beyond one month of age. CSF-1-deficient *osteopetrotic* (*Csf1*$^{op/op}$) mice [47–49] possess a phenotype similar to the phenotype of *Csf1r*$^{-/-}$ mice [15], but less severe, with 80 % of the mice surviving beyond one month of age on the outbred background (Table 10.1). In contrast, *Il34*$^{-/-}$ mice are grossly normal [12, 13]. The gross developmental defects of *Csf1r*$^{-/-}$ and *Csf1*$^{op/op}$ mice include skeletal abnormalities and delayed tooth eruption, both of which result from impaired bone remodeling in the absence of osteoclasts, a low growth rate, and lower adult body weight and body size (Table 10.1) [15, 21, 47, 50]. Administration of recombinant CSF-1 only partially rescues the phenotype of *Csf1*$^{op/op}$ mice [16, 51–53], indicating the importance of local regulation. Transgenic expression of full-length CSF-1 driven by the CSF-1 promoter and first intron fully corrects it [54], but there are differential effects of rescue by transgenic expression of the cell-surface and secreted CSF-1 isoforms on the bone phenotype and postnatal growth [55, 56]. Macrophages can produce insulin-like growth factor (IGF-1) in response to CSF-1, and in the CSF-1-deficient toothless (*tl/tl)* rat, there is gross deficiency of circulating IGF-1, which correlates with the defective postnatal growth. Thus, it has been suggested that CSF-1 might regulate postnatal growth by stimulating extrahepatic IGF-1 production by macrophages [57].

10.1.2.2 Regulation of Monocytes and Tissue Macrophages by the CSF-1R

The Role of the CSF-1R in Monocyte and Tissue Macrophage Development

Mouse tissue macrophages originate from the embryonic day (E) 7.0–7.5 extraembryonic yolk sac macrophages, the aorta–gonad–mesonephros (AGM) region of the embryo (E 8.5–12), fetal liver (E 9.5–21), or adult hematopoietic tissue-derived precursors [20, 58, 59]. Irrespective of their origin, they express the CSF-1R and respond by surviving or proliferating in response to CSF-1, the degree of proliferation depending on the tissue of origin [17, 37, 60]. Liver Kupffer cells, microglia, and most of the F4/80^{bright} CD11b^{low} tissue macrophages, including macrophages from the splenic red pulp, kidney, and the pancreatic islets, originate in CSF-1R^{+} yolk sac precursors that populate embryonic tissues between E9.5 and E10.5, after the development of the fetal circulation and before HSCs first emerge in the AGM region [17, 58, 61]. LCs have a mixed origin, the majority arising from fetal liver progenitors with a minor contribution (6–7 % in the adult) from yolk sac-derived macrophages [59]. Bone marrow HSCs give rise to blood monocytes and the F4/80^{low} CD11b^{high} macrophages, which represent a minor population of tissue macrophages (Fig. 10.1). In contrast to yolk sac and fetal liver-derived macrophages, which maintain themselves in situ throughout life, the F4/80^{low} CD11b^{high} macrophages are continuously replaced by bone marrow-derived progenitors [58, 62–64]. Studies in *Csf1r*$^{-/-}$ mice show that the CSF-1R is absolutely required for the development of embryonic precursor-derived LC and microglia [15, 17, 58, 59, 63]. Studies in IL-34-deficient mice show that the IL-34/CSF-1R complex controls LC development during embryogenesis and their homeostasis in adult skin [12, 13], as well as the maintenance of microglia in specific areas of the adult brain [13]. In addition, it has been shown that both under steady-state conditions and after infection- or inflammation-induced depletion, yolk sac precursor-derived tissue-resident macrophages self-renew independently of the bone marrow through local proliferation [62] and differentiation [65] in a CSF-1- and GM-CSF-dependent manner [62, 64, 65]. Furthermore, in adult mice, germline ablation of the *Csf1r, Csf1* deficiency, or inhibition of the CSF-1R decrease the macrophage numbers in most tissues, without affecting bone marrow or blood monocyte counts (Table 10.1) [15, 16, 22, 66, 67]. However, these studies also show that blockade of CSF-1R signaling leads to the selective depletion of the mature Ly6C^{-} blood monocytes, but not of their precursors, the Ly6C^{+} monocytes [62, 66, 67]. Taken together, they suggest that CSF-1R signaling is most critically required for the embryonic colonization of tissues by yolk sac- and fetal liver-derived progenitors and for the local maintenance and recovery of their progeny. In adults, CSF-1R is also necessary for the maturation of Ly6C^{+} blood monocytes into Ly6C^{-} monocytes, a late step in monocytopoiesis, which occurs only after Ly6C^{+} monocytes have been released into the circulation [62] (Fig. 10.1).

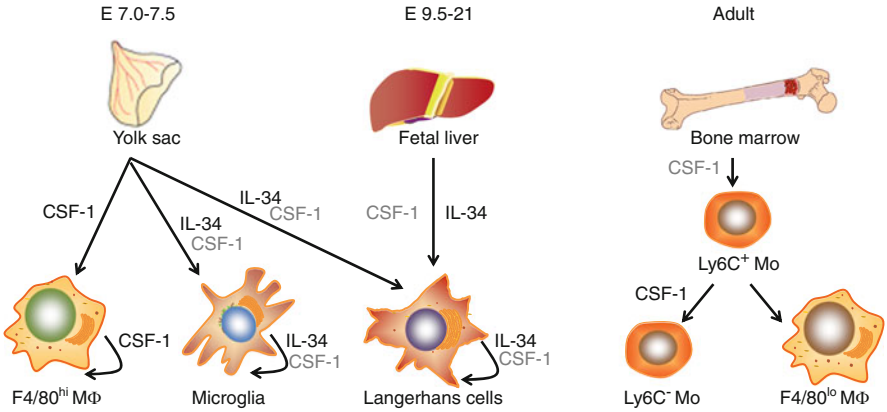

Fig. 10.1 Roles of the CSF-1R ligands in the development of adult monocyte, tissue macrophage, and Langerhans cell populations. In mouse, three different mononuclear phagocytic lineages arise from progenitors that populate tissues at different stages of development. Progenitors in the yolk sac at embryonic day (E) 7.0–7.5 give rise to microglia in the brain and to the F4/80^{hi} tissue-resident macrophages (F4/80^{hi} MΦ). By E11.5, the fetal liver, which is colonized by precursors from the yolk sac and the aorta–gonadal–mesonephros region of the embryo, becomes the source of monocytes during embryogenesis. Adult Langerhans cells are derived from both yolk sac and fetal liver precursors. In adult mice, at steady state, microglia, Langerhans cells, and F4/80^{hi} MΦ self-renew without contribution from circulating bone marrow-derived precursors. Postnatally, bone marrow precursors give rise to Ly6C^{+} blood monocytes that seed a few adult tissues (e.g., the kidneys, lungs, and the pregnant uterus) giving rise to F4/80^{lo} tissue-resident MΦ. F4/80^{lo} MΦ are continuously replaced by bone marrow progenitors. In circulation, Ly6C^{+} monocytes also give rise to Ly6C^{-} "patrolling" monocytes, which act to maintain vessel integrity and to detect pathogens. The CSF-1R is critical for the development of microglia, Langerhans cells, and F4/80^{hi} MΦ. CSF-1R and CSF-1 deficiencies have limited effects on Ly6C^{+} blood monocytes but contribute to their maturation into Ly6C^{-} monocytes. CSF-1 also contributes to the maintenance of F4/80^{hi} tissue MΦ. IL-34 is critical for the development of Langerhans cells and of microglia in specific areas of the brain, but has limited effects on other macrophage subsets and no effect on blood monocytes. Gray font indicates a lesser effect

Regulation of Emergency Monocytopoiesis by the CSF-1R

Emergency monocytopoiesis is the generation of large pools of monocytes from cells in the bone marrow in response to a sudden demand. Consistent with the involvement of CSF-1 in regulating this response, CSF-1 expression is increased by tissue stress (e.g., trauma or infection) and stimulates the production of monocytes and macrophages from their lineage-committed precursors [11, 36, 68–74]. In the adult hematopoietic system, CSF-1R is expressed at low levels on HSC [35]. CSF-1R expression increases ~10 fold at the earliest stage of commitment to the monocytic lineage (colony-forming unit–macrophage, CFU-M) and is further upregulated on their differentiated progeny (monoblasts, promonocytes, monocytes, and macrophages) [37, 38]. In vitro, CSF-1 stimulates the proliferation and differentiation of CFU-M and synergizes with other hematopoietic cytokines, such as

IL-1, IL-3, and stem cell factor (SCF), to increase the proliferation of more primitive multipotent, hematopoietic precursor cells [38, 75, 76]. Recent studies indicate that high systemic levels of CSF-1 induce HSC to adopt a myeloid lineage fate by activating the expression of the myeloid transcription factor PU.1 [36]. Similar instruction of macrophage differentiation by CSF-1 has been demonstrated at the level of the bipotent granulocyte–macrophage progenitor (GMP) [74] (Fig. 10.2).

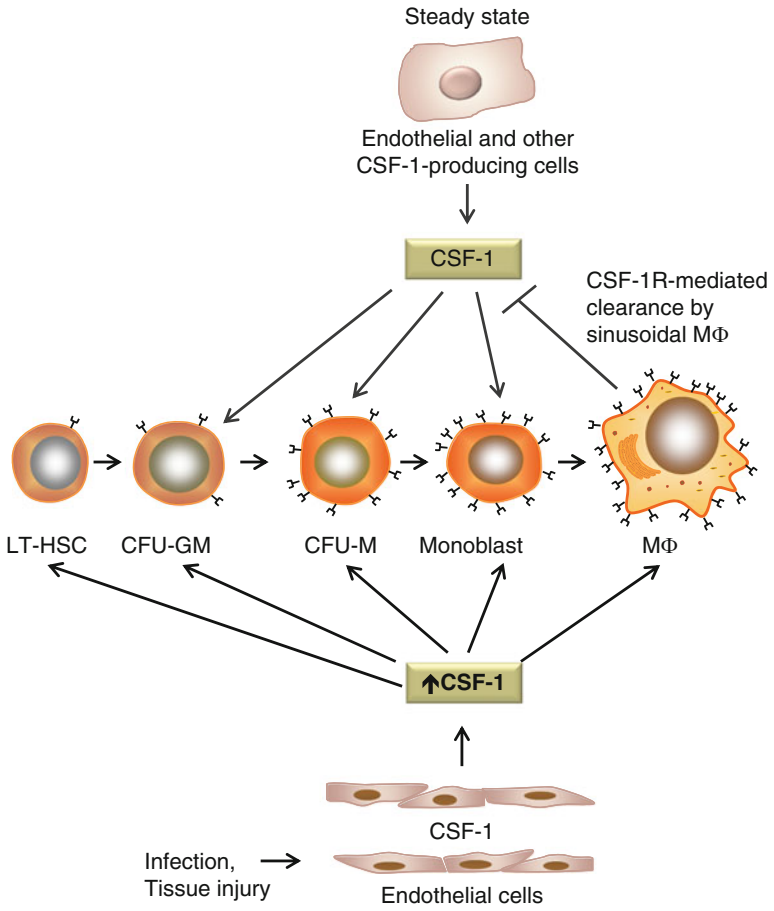

Fig. 10.2 Regulation of macrophage production by CSF-1 and the CSF-1R**.** At steady state, CSF-1 is cleared from circulation via CSF-1R-mediated internalization by tissue-resident, sinusoidally located macrophages, predominantly Kuppfer cells, which express high levels of the CSF-1R. This macrophage-mediated clearance of CSF-1 is a negative feedback control. Tissue damage or sepsis triggers the elevation of CSF-1 production by endothelial cells. Elevated CSF-1 instructs the commitment of HSC to a myeloid fate and of CFU-GM to a macrophage fate, thus triggering the rapid expansion of the monocytic pool. CSF-1 in the extracellular space also regulates proliferation of resident tissue macrophages. Relative CSF-1R density is indicated at each developmental stage

CSF-1R in Bone Development

The severe depletion of osteoclasts in *Csf1r*$^{-/-}$ mice leads to osteopetrotic phenotypes including toothlessness, increased radiopacity of long bones, and increased trabecular bone in histological sections [15]. These abnormalities are associated with severely decreased biomechanical strength of the long bones [21]. Studies examining bone formation and structural features during postnatal development showed that the postnatal development of lamellar bone is profoundly disturbed in these mice, witnessed by the disrupted cortical bone structure, disorganized collagen fibrils, and reduced bone mineralization [21]. While osteoblasts in *Csf1r*$^{-/-}$ mice had normal ultrastructure and matrix-depositing activity, their layered organization on the bone-forming surface and the direction of matrix deposition toward the bone surface were lost, resulting in their abnormal entrapment by matrix. Furthermore, the CSF-1R was not expressed in osteoblasts and the development of osteoclasts in normal mouse embryos preceded the time of appearance of these bone defects in *Csf1r*$^{-/-}$ mice. *Csf1r*$^{-/-}$ femoral anlagens transplanted into wt mice developed normal structured bone, indicating that the *Csf1r*$^{-/-}$ limb rudiment was fully capable of forming the normal bone structure in the presence of exogenously derived wt osteoclasts. These data suggest that by promoting osteoclastogenesis, CSF-1R plays an important role in regulating osteoblastic bone formation in development.

CSF-1R-dependent Tissue Repair

Tissue damage inflicted by infection, ischemia, or trauma triggers the CCR2-mediated recruitment of Ly6C^{high} monocytes from the circulation that differentiate into M1 macrophages which secrete inflammatory mediators that facilitate the clearance of the invading pathogen or digest the damaged tissue through their proteolytic and phagocytic activities (reviewed in [77]). Subsequently, anti-inflammatory macrophages become preponderant. It is not clear whether this is the result of a switch in the activation status of the M1 macrophages after exposure to factors produced by the M2 tissue-resident macrophages or other cell types [78], to the subsequent CX3CR1-dependent recruitment of Ly6C^{lo} blood monocytes which function as precursors of M2 macrophages [79], or to the proliferation of tissue-resident macrophages [80]. M2 macrophages produce growth factors such as TGFβ1 and PDGF that stimulate wound repair by promoting myofibroblast differentiation and expression of tissue inhibitors of matrix metalloproteinases that block the degradation of the extracellular matrix and by stimulating the synthesis of collagens [77, 79].

CSF-1R activation stimulates macrophage proliferation and drives macrophages toward a tolerogenic, M2-like phenotype [81–88]. Studies in mice have shown that acute kidney injury, triggered by ischemia/reperfusion or diphtheria toxin, induces the production of CSF-1 in the proximal tubule cells and the activation of CSF-1R signaling in resident macrophages that is essential for tissue repair [80]. CSF-1R

acted by promoting the proliferation of both M2 macrophages [80] and renal tubular epithelial cells in situ [89]. Genetic or pharmacologic inhibition of CSF-1R signaling blocked M2 polarization and macrophage proliferation and inhibited recovery [80], while the injection of CSF-1 hastened healing and improved renal function [89]. Studies in *Csf1*$^{op/op}$ mice have shown that CSF-1-dependent macrophages also contribute to the healing of gastric ulcers, by producing PGE2 and VEGF, which in turn stimulate angiogenesis in the ulcerated area [90]. A similar proangiogenic effect of CSF-1-dependent macrophages was found to contribute to both acute and chronic dermal wound healing in mice [91]. Increased local and circulating CSF-1 levels occur in patients with fractures [92]. Consistent with studies showing that CSF-1 promotes the formation of lamellar bone structures [21], administration of CSF-1 significantly increased the number of osteal macrophages at the injury site and promoted bone healing by enhancing matrix deposition and mineralization in a mouse tibial bone injury model [93].

10.1.2.3 Role of the CSF-1R in the Brain

The CSF-1R in Brain Development

CSF-1R and its ligands are highly expressed in the developing mouse embryonic brain as early as E 13.5 and the expression of CSF-1R and CSF-1 increases in late embryonic development and the early postnatal period concomitantly with the increase in parenchymal microglia [9, 10, 94, 95]. Furthermore, mice deficient in either CSF-1R or its ligands exhibit anatomic and functional defects in the brain (Table 10.1) that lead to phenotypes that are primarily neurological, such as altered GABAergic cortical circuitry [94], visual [94] and olfactory [95] deficits, and perturbations of hypothalamic function leading to decreased fertility [96, 97]. Since CSF-1R expression is highest in microglia and absolutely required for microglial development during embryogenesis [17], it was initially suggested that brain development was perturbed in CSF-1R deficiency due to the lack of microglia, which provide trophic factors and sculpt brain connectivity through their ability to prune synapses during neuronal development [95, 98]. However, recent studies show that CSF-1R is also expressed on neural progenitor cells (NPC), where it suppresses NPC self-renewal and promotes neuronal differentiation and the survival of NPC and neuronal lineage cells [10]. Thus, the CSF-1R regulates brain development by cell autonomously regulating both neuronal and microglial differentiation.

The CSF-1R in Brain Homeostasis

Protective Role Following Excitotoxic Injury Lineage-tracing experiments show that a small number of neurons in the adult mouse hippocampus and cortex express the CSF-1R under physiological conditions and that kainic acid (KA)-induced

excitotoxic injury results in an increase in CSF-1R neuronal expression [32]. Intraperitoneal administration of CSF-1 or IL-34 has neuroprotective effects against KA-induced neurodegeneration, activating the cAMP-responsive element-binding protein (CREB) pathway, which regulates survival of primary neurons. CSF-1 or IL-34 also suppresses KA-induced microgliosis in vivo. Opposite results are obtained when CSF-1R expression is selectively ablated in forebrain neurons indicating that the microgliosis is secondary to the neuronal cell death and that CSF-1R ligands suppress neurodegeneration primarily by promoting neuronal survival.

Control of Microglial Activation The expression of CSF-1 increases rapidly after brain injury or infection and triggers the upregulation of CSF-1R expression in microglia and microglial proliferation (reactive microgliosis). A transgenic mouse overexpressing CSF-1 in the glial fibrillary acidic protein (GFAP) compartment has been generated that permitted the examination of the effects of CSF-1 overexpression in vivo under basal conditions. These mice exhibited increased microglial proliferation and numbers. Gene expression analysis revealed that the microglia were not M1 or M2 polarized. However, they exhibited a decreased ability to respond to lipopolysaccharide administration in vivo [99]. Studies in mouse and human glial cultures have shown that CSF-1 increases DAP12; decreases the expression of antigen presentation proteins HLA-DP, HLA-DR, and HLA-DQ on microglia; and increases the phagocytic uptake of amyloid beta peptides [100, 101]. These data suggest that CSF-1 promotes a quiescent phenotype in microglia that may prevent their inappropriate activation and neurotoxicity.

CSF-1R in CNS Remyelination

Globoid cell leukodystrophy (GCL) is a neurodegenerative lysosomal storage disorder caused by the lack of ß-galactocerebrosidase which results in an accumulation of galactocerebroside, the primary lipid component of myelin. The galactocerebroside is converted to a cytotoxic metabolite which triggers oligodendrocyte apoptosis [102], leading to progressive demyelination [103] and the formation of highly phagocytic multinucleated globoid cells which are the hallmark of the disease [104]. In the absence of CSF-1, the symptoms are exacerbated in the *twitcher* mouse model of GCL. This is associated with a decrease in the number of microglia/macrophages, an increase in myelin debris, and a decrease in recruitment of oligodendrocyte precursor cells [103], suggesting that clearance of myelin debris by CSF-1-activated phagocytes is critical for remyelination indirectly. Also, as the CSF-1R stimulates oligodendrocyte precursor cell differentiation and survival [10], it is conceivable that it ensures the availability of oligodendrocytes for remyelination. In contrast, in a mouse model of the demyelinating peripheral neuropathy, Charcot–Marie–Tooth disease (CMT) type 1, CSF-1 supports the expansion of monocyte-derived macrophages and microglia that not only clear myelin debris but also cause further myelin damage [105]. These studies suggest that the activation status of the macrophage population expanded by CSF-1, not CSF-1-driven macrophage expansion per se, might be the essential factor deciding whether tissue repair or destruction will subsequently occur.

10.1.2.4 Regulation of Paneth Cells and the Intestinal Stem Cell Niche by the CSF-1R

CSF-1 deficiency in mice was initially found to cause depletion of the interstitial macrophages of the villi [16]. Subsequently, it was shown that the absence of CSF-1 in *Csf1*$^{op/op}$ mice, or of the CSF-1R in *Csf1r*$^{-/-}$ mice, also causes abnormal small intestine organization, with a dramatic reduction in epithelial cells, including Paneth cells (PC), and intestinal stem cells (ISC) [29, 106]. PCs express the CSF-1R and reside in close proximity to CSF-1-expressing cells [29]. In *Csf1*$^{op/op}$ mice, macrophage and PC deficiencies, as well as ISC activity, are rescued by transgenic expression of the cell-surface isoform of CSF-1, indicating that CSF-1 regulates PC development either directly, in a juxtacrine or paracrine manner, or indirectly via macrophages [29]. The possibility of indirect regulation was eliminated by subsequent studies utilizing intestine-specific deletion of *Csf1r* (*Csf1r*$^{fl/fl}$*; VillinCreERT2*), establishing that the CSF-1R cell autonomously supports PC replacement and maturation [106]. Furthermore, PCs were shown to fashion the intestinal stem cell niche to support ISC activity. As Paneth cells are a major component of innate immunity in the gut, as well as regulators of intestinal inflammation, digestion, detoxification, stem cell protection, and crypt development [107], these data suggest that CSF-1R is important for intestinal homeostasis. Consistent with this, both *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ mice were found to have abnormal colon organization, with defects in enterocytes and enteroendocrine cell fate, excessive goblet cell staining, and reduced cell proliferation [45].

10.1.2.5 Role of the CSF-1R in the Reproductive System

CSF-1R in the Regulation of Male Fertility

The CSF-1/CSF-1R axis is the major regulator of macrophages in male reproductive tissues [15, 108]. *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ male mice have reduced mating ability and low sperm numbers, and compared with wild-type (wt) mice, their levels of circulating testosterone and luteinizing hormone (LH) are reduced by 90 % [15, 97]. Administration of CSF-1 to *Csf1*$^{op/op}$ males throughout the postnatal period completely restores viable CSF-1 sperm numbers and significantly restores sexual behavior [109]. Testosterone deficiency in *Csf1*$^{op/op}$ mice results from reduced testicular Leydig cell steroidogenesis associated with ultrastructural abnormalities and lowered activity of steroidogenic enzymes [97]. Furthermore, the failure of castrated *Csf1*$^{op/op}$ males to increase LH secretion and to respond to exogenous testosterone indicates that the feedback responses of the hypothalamus are disrupted. In contrast, the release of LH by the pituitary in response to the gonadotropin releasing hormone (GnRH) analog histerilin is normal, suggesting that the primary defect is hypothalamic [97].

CSF-1R in the Regulation of Female Fertility and Pregnancy

Both *Csf1r*$^{-/-}$ and *Csf1*$^{op/op}$ female mice exhibit delayed puberty, extended estrus cycles, and poor ovulation rates leading to decreased fertility [15, 96, 110]. In ovaries, the CSF-1R is detected both in oocytes in the developing follicle and in macrophages that are recruited around the growing follicles [41, 110], and locally applied CSF-1 increases ovulation in vivo [110]. Compared to wt controls, *Csf1*$^{op/op}$ females exhibit a smaller increase in circulating LH following ovariectomy and fail to increase circulating LH levels following the administration of estrogen and progesterone, suggesting that the hypothalamic–pituitary feedback system to gonadal steroids is perturbed [96]. Administration of CSF-1 to *Csf1*$^{op/op}$ females over the first 2 weeks of life accelerates puberty and continuous CSF-1 treatment until puberty completely corrects the extended estrous cycles in adults, suggesting that the major role of CSF-1 is to regulate the establishment of the sex steroid hormone feedback regulatory system in the brain during the early postnatal period [96]. Interestingly, clinical studies indicate that the administration of CSF-1 increases ovulation in poor responders following controlled ovarian hyperstimulation [111] and that increased levels of circulating CSF-1 positively correlate with pregnancy rates following human in vitro fertilization [112].

During mouse pregnancy, CSF-1 is induced in high concentrations in the uterus [113–115]. Although uterine macrophages are absent in virgin *Csf1*$^{op/op}$ females, the implantation of fertilized oocytes is normal [110]. Furthermore, despite the absence of macrophages in *Csf1*$^{op/op}$ females after day 14 of gestation, embryo resorption rates are only modestly elevated, and the surviving litters have normal fetal and placental weights [116]. Thus, while required for ovulation, CSF-1 is dispensable for the normal progression of pregnancy.

In pregnant mice, local CSF-1 concentrations play a decisive role in determining tissue macrophage and DC density in the pregnant uterus [117]. CSF-1 drives the homeostatic expansion of macrophages in the growing myometrium, by stimulating the proliferation of myometrial-resident macrophages and CCR2 ligand (i.e., CCL2, CCL7, and CCL12) production by resident macrophages, leading to the recruitment of Ly6C^{hi} monocytes from the blood. In parallel, local CSF-1 also triggers pre-DC extravasation into the myometrium and promotes the expansion of CD11b^{hi} dendritic cells in a CCR2 ligand-independent manner. In contrast, decidual macrophages do not expand to match the growth of the tissue and decrease in density as pregnancy advances. Consistent with this, the E9.5–10.5 decidua contains low levels of CSF-1 mRNA compared to the myometrium, and CSF-1R blockade inhibits the high rate of macrophage proliferation in the myometrium, but not the low rate of macrophage proliferation seen in the decidua. High levels of serum and placental CSF-1 [118, 119], as well as increased decidual macrophage and DC densities [120–122], have been found in preeclamptic patients suggesting that inappropriate activation of CSF-1R negatively affects the outcome of pregnancy.

CSF-1R in the Regulation of Innate Immunity at the Maternal/Fetal Interface

The CSF-1R is highly expressed in the placental trophoblastic cells, fetally derived multinucleated epithelial cells that share several characteristics with macrophages. These shared characteristics include phagocytosis, invasiveness, expression of non-specific esterase, inflammatory cytokines and their receptors, and the Toll-like and nucleotide-binding oligomerization domain (NOD)-like innate immune receptors [123–125]. Studies in *Csf1*$^{op/op}$ mice have shown that CSF-1 is essential in the placental immune response to *Listeria monocytogenes,* a Gram-positive intracellular bacterium that has a preference for replication at the utero–placental interface [30]. CSF-1 induces the trophoblastic cells to synthesize the neutrophil chemoattractants, keratinocyte chemoattractant (KC), and macrophage inflammatory protein-2 (MIP-2), thereby stimulating the recruitment of neutrophils to the site of listerial infection. Thus, through its actions in trophoblasts, CSF-1R controls pregnancy-specific innate immune responses.

CSF-1R in the Regulation of Branching Morphogenesis

Branching morphogenesis is the remodeling of epithelial or endothelial sheaths leading to the formation of branched tubular structures such as those found in the mammary gland, lung, kidney, and the vasculature. At the beginning of puberty, *Csf1*$^{op/op}$ mice exhibit a defect in the outgrowth and branching of mammary ducts [126]. A similar defect occurs during pregnancy when further ductal outgrowth is impaired [127]. Both defects correlate with decreased macrophage recruitment. Transgenic expression of CSF-1 in the mammary epithelium of *Csf1*$^{op/op}$ mice rescues mammary macrophage populations corrects the branching morphogenesis defect, demonstrating that resident macrophages are important for mammary tissue remodeling [128]. Similar branching defects associated with macrophage paucity contribute to a decrease in insulin-producing pancreatic β cells, abnormal postnatal islet morphogenesis, and impaired pancreatic cell proliferation in late pregnancy in *Csf1*$^{op/op}$ mice [129].

10.1.3 The Role of the CSF-1 Receptor Tyrosine Kinase in Human Disease

Dominant inactivating mutations in the CSF-1R have been found in patients with neurodegenerative disease. Activating mutations and inappropriately increased expression of the CSF-1R have been shown to contribute to the development of leukemia and Hodgkin's lymphoma. In addition, autocrine and paracrine regulation by CSF-1 appear to contribute to the progression of a variety of human cancers. As discussed in detail below, studies showing that inhibitors of CSF-1 or CSF-1R

ameliorate inflammatory disease in mice, combined with observations of improvement of collateral arthritic symptoms in patients with cancer participating in clinical trials of CSF-1R inhibitors, suggest that therapeutic targeting of CSF-1R may be useful to treat both cancer and inflammatory disease.

10.1.3.1 Inactivating Mutations in CSF-1R Lead to Adult-Onset Leukoencephalopathy with Axonal Spheroids and Pigmented Glia (ALSP)

Dominant mutations in the *Csf1r* gene lead to ALSP, a term that encompasses two previously described microgliopathies involving CSF-1R mutations, hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS), and pigmented orthochromatic leukodystrophy (POLD) [33, 34]. Genetic studies of families with ALSP have identified multiple disease-associated mutations, including missense mutations affecting highly conserved residues and splice-site mutations leading to in-frame deletions [33, 34, 130–144]. Transfection experiments showed that both CSF-1 and IL-34-stimulated CSF-1R kinase activities were abolished in fifteen of the missense mutations (G589E, E633K, S688EfsX13, G765D, M766T, A770P, I775N, A781E, R782H, I794T, D837Y, F849S, L868P, M875T, and P878T) and the four aberrant splice variants (ASV1, ASV2, ASV3, and P824S) examined [33, 34, 145–147]. However, co-transfection experiments utilizing mutant and wild-type CSF-1R constructs that mimic the heterozygous mutant status of ALSP patients revealed that the expression of mutant chains does not suppress the phosphorylation of the wild-type chains [145]. Thus, in ALSP patients, it is expected that only 25 % of cell-surface CSF-1R dimers will be enzymatically inactive and the remainder will be active, 50 % containing one active chain and 25 % containing two. Furthermore, the discovery of a HDLS patient with a *CSF1R* frameshift mutation that abolished protein expression proved that *CSF1R* haploinsufficiency is sufficient to cause ALSP [145]. Further investigations are necessary to determine how changes in the composition of CSF-1R dimers (i.e., heterozygous kinase-dead mutation) or in cell-surface dimer density (expected from mutations leading to haploinsufficiency) affect the differentiation, survival, proliferation, and activity of CNS cells including neural precursors, mature neurons, and microglia.

ALSP is a central nervous system white-matter disease that usually starts with psychiatric symptoms (e.g., depression and anxiety) and progresses to epilepsy, dementia, ataxia, and gait impairment (reviewed in [148]). The brains of ALSP patients generally show extensive loss of myelin and axonal damage, numerous axonal spheroids, and hypertrophic astrocytes, as well as the distinctive feature of pigmented macrophages located predominantly in the frontal lobe [34]. The pigmentation is due to the accumulation of cytoplasmic autofluorescent granules containing lipofuscin, a product of oxidative damage of unsaturated fatty acids. The source of cellular lipofuscin is incompletely degraded products of mitochondria and endoplasmic reticulum autophagic processing [149], which accumulate in senescent microglia [150, 151]. It is possible that CSF-1R haploinsufficiency in ALSP

may result in premature microglial aging and/or microglial dysfunction. Indeed, studies in mice revealed that the development and maintenance of microglia depends on CSF-1R activation by CSF-1 and IL-34 [12, 13, 15, 17, 95]. Furthermore, as CSF-1 stimulates autophagy in macrophages, which in turn is necessary for CSF-1-induced macrophage differentiation and acquisition of phagocytic functions [152], a possible mechanism underlying ALSP pathology is that reduced CSF-1R signaling may lead to inefficient phagocytocis by microglia and consequent accumulation of cellular debris, triggering local inflammation. Indeed, microglial phagocytosis of axonal and myelin debris plays an essential role in brain homeostasis as demonstrated by the finding that loss-of-function mutations of either TREM2 or DAP12, microglial receptors that facilitate debris clearance in the absence of inflammation, leads to Nasu–Hakola neurodegenerative disease (NHD) that has striking similarities to ALSP [153–155]. Interestingly, other studies show that there is a close interplay between DAP12 and CSF-1R signaling in phagocytes. In human glial cultures, CSF-1 increases DAP12 expression in microglia [100], and the DAP12–TREM2 signaling complex mediates CSF-1R signaling for survival and proliferation in macrophages [156]. These data strengthen the assumption that ALSP is a primary microgliopathy that leads to secondary myelin and axonal damage [34, 148]. However, we have recently shown that CSF-1R expression on neural progenitor cells (NPC) is required for normal neuronal development in mice [10], raising the possibility that the effects of ALSP mutations in NPC may also contribute to disease development by impairing neurogenesis. Indeed, our recent studies in a *Csf1r+/-* mouse model of ALSP revealed that *Csf1r* haploinsufficiency causes anomalies in both neuronal development and microglial activation [157]. This model will permit further investigations of the basis of ALSP and evaluation of the efficacy of various therapeutic approaches.

10.1.3.2 CSF-1R in Human Cancers

The CSF-1R/CSF-1 axis has detrimental effects in human cancers. Increased circulating CSF-1 is found in various malignancies, including breast cancer, ovarian cancer, lung cancer, endometrial carcinoma, and leukemias, and its expression often correlates with poor prognosis [158–164]. The increase in circulating CSF-1 is likely to be due to production by CSF-1-expressing tumor cells [158, 165], but could be contributed to by other cells in response to the tumor. Studies in mice have suggested several mechanisms for the involvement of CSF-1 in human cancers. Inappropriate early expression of the CSF-1R [24] and autocrine regulation by CSF-1 are involved in the progression of acute leukemias in mice [28]. In addition, studies in mice have identified three mechanisms involved in the CSF-1-mediated progression of solid tumors: (1) CSF-1-dependent production of angiogenic and tumorigenic factors by M2-polarized tumor-associated macrophages (TAMs) [88, 166–168]; (2) a CSF-1/EGF paracrine loop in which tumor cells produce CSF-1 that activates CSF-1R-expressing TAMs to secrete EGF, which in turn promotes the

invasion of the tumor cells [169–171]; and (3) autocrine enhancement of tumor cell proliferation, invasion, and chemoresistance by CSF-1 in tumors expressing both ligand and receptor [[172], [163, 173–176]]. These and other mechanisms by which CSF-1 and the CSF-1R promote human neoplastic disease are discussed below.

Autocrine Regulation of Proliferation by CSF-1 in Human Cancers

Many types of neoplastic cells synthesize CSF-1. In several cases, the neoplastic cells also express the CSF-1R, and their proliferation is stimulated by CSF-1 in an autocrine manner [163, 175]. Studies in humans have shown that patients with acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) possess elevated levels of circulating CSF-1 [162, 177] that decrease in remission. Circulating CSF-1 is also elevated in patients with myelodysplastic syndrome (MDS) [162, 178], a disease that progresses to AML. AML cells [179, 180] express both CSF-1 and the CSF-1R [181], and CML cells express the CSF-1R [177]. Patients with Hodgkin's lymphoma also have elevated circulating CSF-1 that decreases in remission [162], and their cells express the CSF-1R in a lineage-inappropriate manner due to aberrant activation of an endogenous LTR through loss of expression of the corepressor CBFA2T3 [27]. Furthermore, a Hodgkin's lymphoma cell line was shown to co-express CSF-1 and the CSF-1R and to exhibit autocrine regulation by CSF-1 [26]. The CSF-1R is expressed in mammary epithelial cells of pregnant and lactating women [182], and elevated epithelial co-expression of CSF-1R and CSF-1 that correlates with invasiveness has been described for >50 % of mammary tumors (reviewed in [183]). In another study, both CSF-1 and CSF-1R were shown to be expressed in 16 out of 17 human breast cancer cell lines and those tested shown to exhibit autocrine regulated proliferation by CSF-1, via ERK1/2 signaling [184]. Autocrine CSF-1 regulation of cells of the human breast epithelial cell line MCF-10A induces hyperproliferation and progressive disruption of acinar structures formed in three-dimensional cultures. CSF-1R activation disrupts cell adhesion by uncoupling adherens junction complexes from the cytoskeleton and promoting cadherin internalization through a Src family kinase (SFK)-dependent mechanism [185]. In studies of another solid tumor type, analysis of biopsies obtained from patients with renal clear cell carcinoma (RCC) showed that both CSF-1 and CSF-1R are expressed in RCCs and adjacent tubular epithelial cells and that their expression is associated with enhanced proliferation and accelerated tumor progression [186]. Furthermore, the growth of xenogeneic transplanted RCC in mice is inhibited by treatment of the mice with CSF-1R kinase inhibitor.

CSF-1R Mutations and Dysregulation in Leukemias and Lymphomas

Apart from elevated expression of the CSF-1R in Hodgkin's lymphoma [27], translocation of the RNA-binding motif 6 (RBM6) gene to the CSF-1R gene in human acute megakaryoblastic leukemia leads to the expression of a fusion protein that

combines the amino terminal 36 amino acids of RBM6 with the carboxyterminal 399 amino acids of the CSF-1R, to generate a constitutively activated CSF-1R [23]. *RBM6-CSF1R* was shown to be essential for the growth and survival of cells of the patient-derived cell line, MKPL-1. Furthermore, retroviral transduction of *RBM6-CSF1R* transformed Ba/F3 cells to IL-3-independent growth and induced a myeloid proliferative disease with features of megakaryoblastic leukemia in a murine transplant model. Other studies have shown that elevation of CSF-1R expression in leukemic stem cells, secondary to a translocation not directly involving the CSF-1R, can also result in a CSF-1R-driven leukemia. The leukemia-associated monocytic leukemia zinc finger MOZ–TIF2 fusion protein induces AML in mice. MOZ–TIF2 interacts with the transcription factor PU.1 to stimulate the CSF-1R expression, and induction of AML by MOZ–TIF2 is suppressed by either PU.1 or CSF-1R deficiency [25]. Furthermore, the leukemia stem cell population is contained within a fraction of cells expressing high levels of CSF-1R, and deletion of these cells using a drug-induced suicide gene cures the AML. In addition, another translocation resulting in a *MEF2D/CSF1R* fusion encoding a constitutively active mitogenic kinase that is responsive to imatinib has been described in a lymphoid neoplasm [187].

The C-terminal tail of the CSF-1R has been shown to negatively regulate CSF-1R signal transduction, and the human CSF-1R Y969F mutation was shown to increase the transforming potential of the CSF-1R in mouse cells [188–191]. Tyr-969 mutations were found in 12.7 % of patients with myeloid malignancies [192]. These data suggest that therapeutic targeting of the CSF-1R may be useful in several types of human cancers.

10.1.3.3 CSF-1R in Human Immunodeficiency Virus Type 1 (HIV-1) Infection

Mononuclear phagocytes are a major target of HIV-1 infection and facilitate the dissemination of virus to various tissues. This is especially evident in the brain where HIV-1-infected monocytes cross the blood–brain barrier and mediate the infection of microglia by HIV-1 causing neurocognitive impairment [193]. CSF-1 enhances HIV-1 pathogenesis by upregulating the expression of viral receptors (i.e., CD4 and the chemokine receptor CCR5) and promoting viral replication in phagocytes [194]. Recent studies have shown that the level of CSF-1 in the cerebrospinal fluid directly correlates with the degree of cognitive impairment in patients [195]. Furthermore, studies in *Macacus rhesus* infected with simian immunodeficiency virus (SIV) revealed that perivascular macrophages express high levels of CSF-1 and are the major reservoir of productive SIV infection in the brain. In addition, both CSF-1 and IL-34 enhanced HIV-1 replication in primary human microglial cultures. In contrast, CSF-1R tyrosine kinase inhibitor GW-2580 inhibited viral replication [196]. Since the antiretroviral therapy does not target HIV in long-lived cells such as macrophages and microglia, these data suggest that inhibition of CSF-1R signaling may be a suitable adjunctive therapy for HIV-1 infection.

10.1.3.4 Pharmacologic Inhibitors of CSF-1R

Pharmacological inhibition of CSF-1R by small molecule inhibitors or blockade using neutralizing antibodies has proved beneficial in several inflammatory [22, 197, 198] and neoplastic [171, 186, 199–201] models. However, many of the CSF-1R small molecule inhibitors used in these studies (i.e., sunitinib (SU011248), imatinib, nilotinib, CYC10268, ABT-869) exhibit off-target effects, acting on many other unrelated RTKs [200, 202–208]. Others exhibit off-target effects for other RTK IIIs. These include PLX3397, which also inhibits c-Kit and oncogenic Flt3 [209] and JNJ-2312141, which also inhibits Flt3 [210]. Monospecific inhibitors such as GW2580 (also known as PLX6134) [211], ARRY-382, and PLX 5562 have also been developed. Studies in animal models using PLX3397 or neutralizing antibodies indicate that long-term treatment with CSF-1R inhibitors reduces the abundance of Ly6C^{low} monocytes, mononuclear phagocytes, and osteoclasts. However, apart from an increase in bone density, these inhibitors do not cause behavioral, cognitive, or physiological deficits [22, 65, 212, 213], suggesting that therapeutic blockade of CSF-1R signaling may not pose significant safety issues. However, metastasis of transplanted mouse mammary tumors, developed from two independent mouse cell lines, has recently been reported to be enhanced by blockade of CSF-1R, an effect that is reversed by G-CSF-R blockade [214], indicating a need for further investigation of the therapeutic blockade of CSF-1R signaling in cancer.

Several inhibitors are now in clinical trials for rheumatoid arthritis and cancer. PLX3397 is in phase 2 clinical trials for the treatment of castration-resistant prostate cancer (NCT01499043), recurrent glioblastoma (NCT01349036), refractory Hodgkin's lymphoma (NCT01217229), and AML (NCT01349049). ARRY-382 and PLX5622 are in phase I clinical trials for advanced/metastatic cancer (NCT01316822) and rheumatoid arthritis (NCT01329991), respectively. In addition, two neutralizing antibodies to the CSF-1R, AMG820, and IMC-CS4 have entered phase I clinical trials for the treatment of advanced solid tumors (NCT01444404 and NCT01346358) (http://clinicaltrials.gov).

10.1.4 CSF-1R

10.1.4.1 CSF-1R Gene

Discovery, Cloning, and Sequencing of the CSF-1R Gene

The CSF-1R was initially identified as a single class of high-affinity binding sites for ^{125}I-labeled CSF-1 on peritoneal exudate macrophages [215]. In an analysis of hematopoietic cells, binding was shown to be restricted to mononuclear phagocytic cells and their precursors [216]. However, recent studies have documented CSF-1R expression in other cell types (see Sect. 10.1.1–10.1.2). Detailed analysis of the

interaction of ^{125}I-CSF-1 with bone marrow-derived macrophages (BMM) provided valuable information concerning the kinetics of CSF-1 association with the CSF-1R as well as CSF-1 dissociation, internalization, and destruction [217]. The CSF-1R was purified and shown to possess intrinsic tyrosine kinase activity [218]. Independently, v-fms [219], the retroviral oncogene of the Susan McDonough strain of feline sarcoma virus (SM-FeSV) [220], derived from the cat c-*fms* proto-oncogene, had been shown to encode an integral transmembrane glycoprotein requiring expression at the cell surface for transforming activity [221, 222]. The human c-*fms* gene was cloned [223], and the c-*fms*-encoded protein shown to possess the topological properties of a growth factor receptor [224]. Then the two groups predominantly associated with this work demonstrated that mouse c-*fms* encodes the CSF-1R [1]. Following this, the human c-*fms* cDNA sequence was obtained [2].

Structure of the Genomic Locus Encoding the CSF-1R Receptor

The human *Csf1r* gene is located on chromosome 5, at position 5q32, on the minus strand, within a region that is syntenic to most mammals [225, 226], between the genes encoding PDGFR-B (upstream) and the transcription factor HMG-box domain containing 3 (HMGXB3) (downstream). The first intron of *Csf1r* gene contains a transcriptionally inactive processed ribosomal protein L7 pseudogene that is highly conserved across mammals [226]. The human *Csf1r* gene spans a region of 60 kb and is composed of 21 introns and 22 exons, of which the first exon is noncoding. The intron size varies from 26 kb for intron 1 to between 6.3 kb and less than 0.1 kb for the other introns (Fig 10.3). The mouse *Csf1r* gene is located on chromosome 18D [227] and is similarly organized, except that intron 1 is only 102 bp. In addition, the human exon 1 promoter, immediately distal to the PDGFR locus and driving trophoblast expression, is not conserved in mouse [228].

The *Csf1r* Gene Sequence

The human *Csf1r* coding sequence contains 60,077 nucleotides (Gene ID 1436) encoding a primary translational product of 972 amino acids. The mouse *Csf1r* coding sequence is 25,567 nucleotides in length (Gene ID 12978) encoding a 977-amino acid protein.

Csf1r-Null Mutations, Conditional Targeting of the *Csf1r* Gene, and *Csf1r* Promoter-Driven Transgenic Models

The mouse *Csf1r* gene was targeted by inserting the humanized green fluorescent protein (GFP) and neomycin resistance cassettes into the third exon of the *Csf1r* gene [15]. *Csf1r*$^{-/-}$ mice express the mRNA encoding GFP, but fail to express the

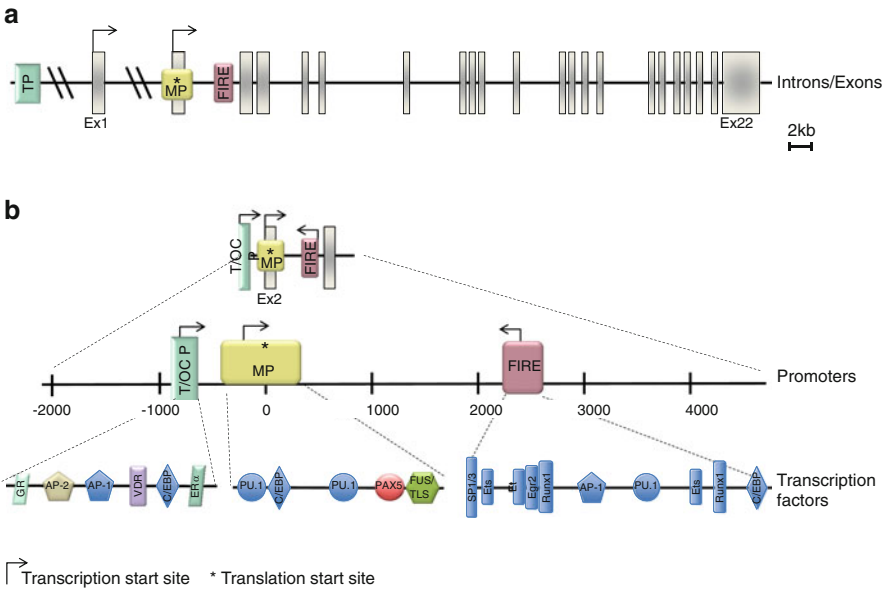

Fig. 10.3 Genomic structure and regulatory regions of the *Csf1r* locus. (**a**) Intron/exon organization and the locations of the trophoblast promoter (TP), macrophage promoter (MP), and Fms-intronic regulatory element (FIRE) enhancer regions of the human *Csf1r*. The mouse *Csf1r* exhibits a similar organization except that the exon 1 trophoblast promoter immediately distal to the *PDGFR* locus is not conserved (not shown). (**b**) Detailed structure of the mouse *Csf1r* regulatory regions. The expression of the *Csf1r* in mouse trophoblasts and osteoclasts is driven by a promoter (T/OC P) located ~200 bp upstream of the macrophage promoter (MP)

protein, indicative of a null allele. A floxed *Csf1r* allele (*Csf1rfl*) was developed by placing LoxP sites on either side of exon 5 and an additional Lox P site in intron 4. Removal of exon 5 results in the production of a 244-amino acid nonfunctional protein that lacks the transmembrane and tyrosine kinase domains. Excision of this floxed sequence in oocytes using a zona pellucida 3 promoter-driven Cre recombinase (*Zp3-Cre*) yielded a phenotype identical to the phenotype of the *Csf1r*$^{-/-}$ mouse [229]. In addition, mice expressing green fluorescent protein (MacGreen) or cyan fluorescent protein (MacBlue) under the control of the *Csf1r* promoter have been generated [213, 230]. These mice permit the visualization of monocyte and macrophage dynamics under various experimental conditions. Transgenic chicken lines expressing green or red fluorescent reporter proteins under the control of the Fms-intronic regulatory element (FIRE) (see 10.1.4.1.5, below) have also been developed [231].

In zebra fish, the *panther* mutant is a loss-of-function mutation of the CSF-1R that was initially shown to suppress the ventral migration and differentiation of the neural crest precursors of xanthophores [232]. *Panther* macrophage progenitors develop normally but fail to emigrate from the yolk sac and pericardial area to colonize embryonic tissues during the early embryonic development, a phenomenon

that is especially evident in brain [233]. It remains to be established whether delayed microglial invasion in the brain leads to cognitive and sensory deficits. Although *Panther* mutants are viable and fertile, they have reduced numbers of osteoclasts, leading to deformities in both the neural and hemal arches and abnormal development of the neural tube and blood vessels located inside these arches [234].

Csf1r Transcriptional Regulation

Most studies on CSF-1R transcriptional regulation have been performed in mouse; however, the human and mouse *Csf1r* genomic structures are highly conserved. In mouse macrophages, the transcription of *Csf1r* is under the control of two regulatory regions, one spanning the 3′ end of intron 1, exon 2, and the 5′ end of intron 2, referred to as the macrophage *Csf1r* promoter, and the other the Fms-intronic regulatory element (FIRE), a highly conserved 330-bp sequence enhancer element located in the 3′ end of intron 2 that is required to generate maximal CSF-1R expression in differentiated monocytes and macrophages [228, 235–237] (Fig. 10.3). Interestingly, the FIRE element also has reverse promoter activity. An antisense CSF-1R transcript starting at FIRE was detected in mouse B cells and macrophages [226, 235]. The antisense transcript is not involved in downregulating sense mRNA transcription. It is speculated that it contributes to the ability of the FIRE sequence to overcome repression by uncharacterized repressive elements within the remainder of intron 2 [235].

CSF-1R expression in placental trophoblasts is controlled by trophoblast-specific promoters. In humans, the trophoblast promoter lies within the 3′ UTR of the upstream *PDGFR-B*, approximately 20 kb upstream of the first exon [238, 239]. This promoter is not conserved in mice, where a 150-bp sequence approximately 200 bp upstream of the major macrophage promoter drives *Csf1r* expression in trophoblastic cells as well as in osteoclasts (referred to as the trophoblast/osteoclast (T/OC) promoter). The T/OC promoter also enhances *Csf1r* mRNA expression in macrophages and granulocytes [228, 240]. The T/OC promoter is conserved in mammals, although its activity has only been tested in the mouse [240].

Both macrophage and T/OC mouse promoters are unusual in the sense that they lack a TATA box and transcription initiates at multiple sites for each, generating several alternative 5′ noncoding exons [226, 228]. The T/OC promoter contains a number of candidate binding sites for transcription factors highly relevant to osteoclast biology, including AP1, which is implicated in RANKL-induced expression of osteoclast-specific genes, as well as the vitamin D receptor (VDR) and estrogen receptor alpha (ERα), both of which control osteoclast differentiation [240, 241] (Fig. 10.3). The macrophage promoter is AT rich but not GC rich, and there are no CpG islands in its vicinity [226]. It contains binding sites for transcription factors including a Runx1/CEBP binding site [242], PAX5 [243], EWS, FUS/TLS [244], and the myeloid master regulator PU.1 [245, 246] (Fig. 10.3). The FIRE enhancer element contains sites for Sp1/3, Egr-2, Ets, AP1, Runx1, PU.1, and C/EBP [235] (Fig. 10.3). Among these, PU.1, Egr-2, Ets, and Runx1 are activators of *Csf1r*

promoter [245, 247], while PAX5 acts as a repressor, silencing the *Csf1r* gene during B lymphocyte development by binding directly to a specific DNA sequence at the *Csf1r* promoter overlapping with the main transcriptional start sites recognized by EWS and Fus/TLS [226, 243].

Transcriptional Regulation of the *Csf1r* During Myeloid Development

During hematopoiesis, CSF-1R is expressed at low levels on hematopoietic stem cells (HSC) [35], and its expression increases ~10 fold at the earliest stage of commitment to the monocytic lineage (colony-forming unit–macrophage, CFU-M) [37]. It is gradually upregulated as CFU-M differentiate to macrophages (monoblast → promonocyte→ monocyte→macrophage) [38]. In agreement with this, low, equivalent levels of expression of *Csf1r* mRNA can be detected in hematopoietic stem cells (HSC), common myeloid progenitor (CMP) cells, and common lymphoid progenitor cells [248, 249]. As CMPs differentiate to macrophages, changes in the chromatin status of FIRE occur that are associated with its activation. Using a PU.1 $^{-/-}$ fetal liver cell line bearing an inducible form of the PU.1 protein to study the order of events during the activation of the *Csf1r* from the silent state, it was shown that transcription factor assembly (PU.1, Runx1 and C/EBP binding) and chromatin remodeling at the macrophage promoter is complete after 6 hours [245, 250]. In contrast, factor assembly and chromatin remodeling at FIRE is complete only after 48 h. It has been suggested that this biphasic activation occurs because PU.1 is required to induce the expression of secondary transcription factors [237], including the Egr-2 and JunB (a component of the AP-1 complex) [251], and that this two-step activation mechanism ensures that high levels of *Csf1r* mRNA and CSF-1R protein are only expressed in the more differentiated cells that respond to CSF-1 alone.

10.1.4.2 CSF-1R Protein

The CSF-1R gene encodes a type I transmembrane protein precursor of 972 amino acids (human) or 977 amino acids (mouse) (Fig. 10.4). Mouse and human CSF-1Rs are highly conserved, displaying 75 % amino acid identity and 82 % similarity. In the human CSF-1R, the first 19 amino acids representing the signal peptide are followed by an extracellular domain of 498 amino acids that can be further subdivided into five Ig-like domains designated D1–D5, bearing 9 N-linked glycosylation sites. D1–D3 and D5 contain conserved cysteine residues that are involved in intrachain disulfide bonding. The extracellular domain is followed by a 21-amino acid transmembrane helix and an intracellular domain comprised of a juxtamembrane regulatory region, a split kinase domain, and a C-terminal tail. The mouse CSF-1R has a similar domain organization with all the known covalent modification sites being conserved. Using SDS-PAGE and Western Blotting, two CSF-1R bands can be detected, a lower M_r species of ~130 kDa, representing the immature high

Fig. 10.4 The primary structure of human and mouse CSF-1R. The mouse (NCBI accession number P09581) and human (NCBI accession number P07333) sequences were aligned using ClustalW2. Maximal alignment of protein sequence was achieved by introducing 7 gaps in the human sequence and 2 gaps in the mouse sequence, each indicated by a dash. *Gray-boxed* regions indicate signal peptide and transmembrane domains. *Orange open boxes* indicate N-linked glycosylation sites. The cysteine residues involved in disulfide bridges are highlighted in *yellow*. The five Ig-like domains (D1–D5) are underlined with *green bars*. The N- and C-lobes of the tyrosine kinase domain are underlined with *blue* and *purple bars*, respectively. The *dashed boxes* delineate the juxtamembrane region (*black*), the catalytic loop (*purple*), and the activation loop (*red*). The phosphotyrosine sites detected by site-specific methods are boxed (*blue*)

mannose-containing glycoprotein, and a higher M_r form of ~165 kDa, corresponding to the mature N-glycosylated protein. In ligand-stimulated cells, additional higher molecular weight forms representing multi-ubiquitinated CSF-1Rs are also observed [252, 253]. In addition, a soluble form of CSF-1R that acts as a natural CSF-1R inhibitor has been reported in teleost fish [254].

10.1.4.3 CSF-1R Oncoproteins

The *Csf1r* gene is a proto-oncogene from which *v-fms* oncogenes encoded by SM-FeSV [220] and HZ5-FeSV [255] feline retroviruses have been derived. Both oncogenes are identical, except for C-terminal amino acid deletions of *c-fms* sequence that partially contribute to their transforming ability [189, 190]: HZ5-FeSV *v-fms* is missing the 24 C-terminal amino acids of the feline c-fms protein [255] and SM-FeSV *v-fms* a 40-amino acid C-terminal segment that is replaced by a sequence of 11 amino acids of unknown origin [188–190, 256]. While these C-terminal modifications present in *v-fms* are sufficient to generate partially transforming phenotypes, extracellular domain mutations Leu301Ser and Ala374Ser, located within a homodimerization interface [7, 257], are also required to generate a fully transforming *fms* gene [189]. CSF-1R oncoproteins involving deletions in CSF-1R extracellular domain that encompass the ligand-binding domain are also transforming, indicating that deletions in extracellular domain can result in constitutive activation in the absence of C-terminal truncations [258].

10.1.5 CSF-1R Ligands

CSF-1R interacts with two ligands, CSF-1 and the recently discovered interleukin-34 (IL-34) [5]. The discovery of a second ligand for CSF-1R was predicted, based on the more severe phenotype observed in CSF-1R-deficient mice compared to CSF-1-deficient *Csf1*$^{op/op}$ mice [15]. IL-34 shows only 10 % sequence similarity with CSF-1. However, they share a short-chain four alpha-helix bundle cytokine fold and interact in a similar manner with the CSF-1R. In vitro, CSF-1 and IL-34 functionally overlap in promoting macrophage differentiation, proliferation, and osteoclastogenesis [5, 9], but differ slightly in their signaling activation kinetics and strength [14]. When expressed under the control of the CSF-1 promoter, IL-34 rescues the CSF-1-deficient phenotype as effectively as the secreted glycoprotein isoform of CSF-1 [9]. However, in vivo the two CSF-1R ligands exhibit different spatiotemporal patterns of expression and play complementary roles in controlling the development, maintenance, and activity of specific macrophage populations, Langerhans cells, and neuronal progenitors [9, 10, 12, 13].

10.1.5.1 Colony-Stimulating Factor-1

CSF-1, also known as macrophage colony-stimulating factor (M-CSF), was the first colony-stimulating factor purified [4]. It was shown to stimulate the formation of macrophage colonies from single immature hematopoietic cells plated in semi-solid medium [60, 259]. CSF-1 is synthesized by a variety of cells, including endothelial cells, fibroblasts, myoblasts, thymic epithelial cells, keratinocytes, astrocytes, osteoblasts, mesothelial cells, liver parenchymal cells, ovarian granulosa cells, and the oviduct epithelium (reviewed in [51, 260–262]). It is also highly expressed by uterine epithelial cells during pregnancy [114, 115]. Consequently, CSF-1 mRNA is found in almost every tissue in the body and is particularly enriched in the heart and the skeletal muscle [9]. Also, as discussed above in detail (Section 10.1.3.2), many types of neoplastic cells synthesize CSF-1 that is involved in an autocrine or paracrine fashion in supporting tumor progression. Normal circulating levels of CSF-1 are 5–10 ng/ml for humans [162, 263] and 10–20 ng/ml for mice [15, 56]. Circulating CSF-1 levels increase during infection and sepsis and in autoimmune and inflammatory diseases, cancer, and pregnancy [44, 54, 114, 162, 264–272]. Bacterial lipopolysaccharide induces a rapid increase in circulating CSF-1, due to the increased synthesis and release of the growth factor by endothelial cells of several organs [68, 273]. Pharmacological studies utilizing biologically active, radiolabeled CSF-1 showed that liver and spleen are responsible for clearing most of the circulating CSF-1 (~88 % and ~6 %, respectively), the remainder being cleared by renal filtration [274]. The clearance is mediated by sinusoidal macrophages, mainly by the Kupffer cells of the liver, via CSF-1R-mediated internalization and intracellular degradation [274]. Consistent with the CSF-1R-dependence of clearance, levels of circulating CSF-1 are elevated 20-fold in CSF-1R-nullizygous mice [15]. As circulating CSF-1 is a major regulator of the development and maintenance of many macrophage subpopulations [16] (reviewed in [11]), this clearance mechanism provides a negative feedback control on macrophage numbers (Fig. 10.2). At physiological concentrations, circulating CSF-1 is cleared with a half-life of 10 min. At higher (pharmacologic) concentrations, this physiologic clearance mechanism is saturated, and CSF-1 is cleared by renal filtration with a half-life of at least 90 min [274–276].

Chromosomal Location of the CSF-1 Gene

The human CSF-1 gene is located on chromosome 1p13.3 between the epidermal growth factor receptor pathway substrate 8-like 3 (EPS8L3) and the adenosylhomocysteinase-like 1 (AHCYL1) loci [277, 278]. The mouse CSF-1 gene is situated in a syntenic region on chromosome 3 F3 between the predicted pseudogene Gm5075 and the gene encoding and Ahcyl1 [279, 280].

Structure of the CSF-1 Genomic Locus

The human CSF-1 gene contains 10 exons and 9 introns, which span 20 kb [281, 282]. The gene encoding mouse CSF-1 has a similar organization [54].

CSF-1 Isoforms

The human CSF-1 gene encodes a protein of 554 amino acids containing a signal peptide followed by a growth factor domain, a spacer region, a transmembrane domain, and a cytoplasmic tail [282–284] (Fig. 10.5). The full-length mouse gene encodes 552 amino acids [285]. Alternative splicing and differential proteolytic cleavage in the secretory vesicle generate three CSF-1 isoforms: a type I transmembrane glycoprotein, a secreted glycoprotein (N-terminal cleavage), and a secreted proteoglycan (C-terminal cleavage). The proteoglycan isoform contains a single ~18-kDa chondroitin sulfate glycosaminoglycan chain per monomeric subunit (Fig. 10.5) and is the predominant isoform secreted [286, 287]. The transmembrane cell-surface isoform of CSF-1 is biologically active and can interact with receptor-bearing target cells by direct cell–cell contact [288]. All three isoforms are biologically active disulfide-linked dimer containing the N-terminal 149 amino acids (amino acids 33–181 in the precursor form cytokine domain required for in vitro biological activity [281]. The cell-surface CSF-1 can be released via proteolytic cleavage by TNFα-converting enzyme (TACE) in the juxtamembrane region [289, 290] or by another protease near amino acid residue 158 (190 in the precursor) [6]. However, studies in mice exclusively expressing the cell-surface isoform revealed that the cleaved cell-surface CSF-1 does not significantly contribute to circulating CSF-1 levels [55].

The *Csf1* Osteopetrotic Mutations, Targeted Disruption of the *Csf1* Gene, and Other Experimental Models

Spontaneous null mutations in the *Csf1* gene include the mouse *osteopetrotic* ($Csf1_{op}$) and rat *toothless* (*tl*) mutations. In the $Csf1^{op/op}$ mouse, a thymidine insertion in exon 4 of the *Csf1* gene leads to a frameshift at base pair 262 from the ATG initiation codon and a stop codon 21 base pairs downstream, yielding a truncated CSF-1 protein of 63 amino acids, significantly shorter than the 149 required for biological activity [48]. In the *tl/tl* rat, a 10-bp insertion in exon 1 within the region encoding the signal peptide of the *Csf1* gene causes a frameshift mutation after codon 9, which creates a stop codon 96 base pairs downstream, yielding a greatly truncated, unrelated protein [291]. Mice harboring a *Csf1* floxed allele ($Csf1^{fl/fl}$), in which exons 4–6 are flanked by *loxP* sites, have been generated, and Meox2Cre-mediated (epiblast) deletion of *Csf1* in these mice during early embryogenesis recapitulates the skeletal and hematologic phenotypes of the $Csf1^{op/op}$ mice [292]. Other experimental models include mice individually expressing several *Csf1* promoter and first

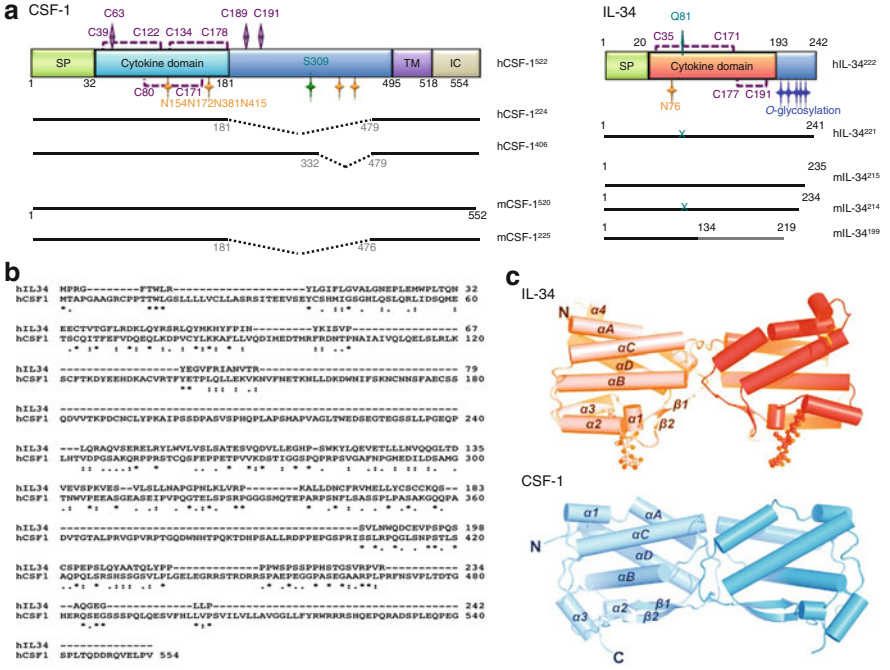

Fig. 10.5 Comparison of the major structural features of the CSF-1R ligands. (**a**) Domain structures of human (h) and mouse (m) CSF-1 and IL-34. *SP* signal peptide, *TM* transmembrane segment, *IC* intracellular domain. The cysteine residues involved in intrachain (*dashed lines*) and interchain (*purple diamonds*) disulfide bonds are shown. Sites of predicted N-linked glycosylation (*orange stars*), glycosaminoglycan addition (*green star*), and the putative O-linked glycosylation sites of IL-34 (*blue stars*) are also indicated (extensive O-linked glycosylation of CSF-1, not shown). Alternative splicing in exon 6 of CSF-1 mRNA leads to the expression of 3 CSF-1 precursors in man and two in mouse (*superscripted numbers* indicate length after signal peptide cleavage). hCSF-1^{224} and mCSF-1^{225} encode the membrane-spanning cell-surface isoform. N- or C-terminal proteolytic cleavage of hCSF-1^{406}, hCSF-1^{522}, or mCSF-1^{520} in the secretory vesicle, respectively, leads to secreted glycoprotein and chondroitin sulfate proteoglycan isoforms (reviewed in [350]). Both mIL34 and hIL34 pre-mRNAs exhibit alternative splicing of a CAG codon that leads to isoforms with and without a glutamine residue at position 81 (Q81, *green diamond*). An additional short isoform of mIL34 (mIL34^{199}) that, beyond amino acid 134, diverges in sequence from the other 2 isoforms (*gray line*) has been described in mouse and is biologically inactive. (**b**) ClustalW2 alignment of hCSF-1 and hIL-34 shows that they exhibit limited homology (10 % sequence identity and 26 % sequence similarity). (**c**) Ribbon representations of the IL-34 and CSF-1 dimeric structures show that both CSF-1R ligands share a four alpha-helix bundle cytokine architecture (panel reprinted with permission [7])

intron-driven transgenes, including the reporter gene β-galactosidase (*TgN(Csf1-Z)Ers*), the membrane-spanning cell-surface isoform of CSF-1 (*TgN(CSCsf1)Ers*), the precursor of the secreted proteoglycan (*TgN(SPPCsf1)Ers*), a precursor of the secreted CSF-1 isoforms that lacks the proteoglycan addition site (*TgN(SGPCsf1)Ers*), as well as the full-length CSF-1 encoding all three isoforms (*TgN(FLCsf1)*

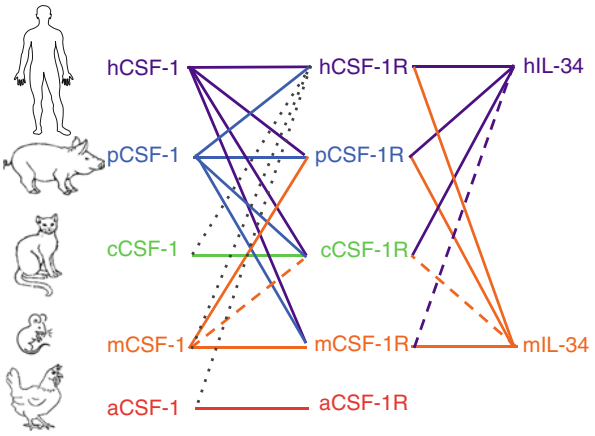

Fig. 10.6 CSF-1R ligands exhibit restricted cross-species reactivity. A one-letter prefix designates the CSF-1R or ligand species: human (h), pig (p), cat (c), murine (m), and avian (a). The *colored dashed lines* indicate lower activity than the human orthologue; the *gray dotted lines* indicate that the ligand fails to activate the receptor

Ers) [54–56]. These transgenic lines have been used to probe the functions of the various CSF-1 isoforms both developmentally [29, 54–56] and in disease models [293, 294].

Species Specificity of CSF-1

The primary sequences of the CSF-1R ligands, as well as of the CSF-1R, are more divergent among species than other genes that encode proteins not involved in immunity [73]. This phenomenon confers CSF-1R ligand cross-species specificity [9, 73, 295, 296] (Fig 10.6). While human CSF-1 can activate the CSF-1R in all species tested (human, mouse, feline, pig, sheep and dog), mouse CSF-1, which has an ~500-fold lower affinity for the human CSF-1R than for its cognate receptor, cannot activate the human CSF-1R [73, 257, 297] and is less potent than human CSF-1 in activating the cat receptor [73, 295, 296]. Furthermore, chicken and feline CSF-1 are unable to activate human and mouse CSF-1R and are restricted to activating their cognate receptors [73].

CSF-1 Interaction with BARF1

The Epstein–Barr virus (EBV) lytic-cycle early protein BARF1 (BamHI-A rightward frame-1) is a secreted hexameric, CSF-1-binding protein with limited homology to the extracellular domain of the CSF-1R [298]. BARF1 was shown to inhibit the secretion of the antiviral interferon-α [299] that is produced by human monocytes in response to CSF-1 [300] and which plays an important role with interferon-β in the primary immune response to viral infection [301, 302]. The high-affinity BARF1 binding renders CSF-1 incapable of binding the CSF-1R [302, 303] and is one mechanism by which EBV eludes the immune response. These findings provide opportunities for novel approaches to the therapeutic targeting of CSF-1 in inflammatory and neoplastic disease and of BARF1 in EBV infections.

10.1.5.2 Interleukin-34

In normal healthy human serum, IL-34 is either not detectable [304, 305] or present at low concentrations (0.15 ng/ml) [306]. In contrast to CSF-1, which is found in almost every tissue in the body [262], the expression of mouse IL-34 is tissue restricted [5, 9]. In adult mice, the messenger RNA (mRNA) for IL-34 is most abundant in skin followed by brain, salivary gland, adipose tissue, skeletal muscle, kidney, pancreas, and the mammary gland [9]. Notably, IL-34, but not CSF-1, was detected together with CSF-1R in E11.5 embryonic brain [9]. Consistent with this pattern of expression, studies in IL-34-deficient mice revealed that IL-34 produced by keratinocytes in the skin and neurons in the brain plays critical roles in the development and maintenance of resident Langerhans cells and of microglia, respectively [12, 13]. The development of both Langerhans cells and microglia is highly dependent on CSF-1R signaling [15, 17, 95]. The development and maintenance of Langerhans cells is primarily regulated by IL-34 [12, 13], whereas the regulation of microglia is shared between both ligands, which exhibit regional dominance [12, 13, 17]. Compared with CSF-1, IL-34 is strongly expressed in forebrain structures. It is the dominant CSF-1R ligand in the olfactory bulb, and consistent with this, atrophy of the olfactory bulb was reported to occur in *Csf1r*$^{-/-}$ mice, but not in *Csf-1*$^{op/op}$ mice [10]. In postnatal day 2 brain, CSF-1R is expressed predominantly in the meningeal microglia and neural progenitors and immature neurons located in the subventricular zone. Consistent with the dominant role of IL-34 in microglial development, IL-34, but not CSF-1, is co-expressed with CSF-1R in the meninges, while CSF-1 expression is restricted to neuronal layer VI, located in the vicinity of the subventricular zone [10]. In contrast, the finding that cultured osteoblasts express substantially more CSF-1 than IL-34 [9] suggested that IL-34 may be less important than CSF-1 in the regulation of osteoclastogenesis and hematopoiesis in the bone marrow. Indeed, later studies showed that in contrast to *Csf-1*$^{op/op}$ mice, IL-34$^{-/-}$ mice do not exhibit an osteopetrotic phenotype and have no apparent defects in blood monocytes, tissue macrophages, or DCs, with the exception of fewer CD11c^{+}CD11b^{+} DCs in the lung [12]. However, IL-34 is necessary for maintaining a splenic pool of osteoclast precursors in *Csf-1*$^{op/op}$ mice and contributes their age-dependent recovery of osteoclastogenesis [307]. Based on the relative mRNA expression levels of CSF-1R, CSF-1, and IL-34 [9] (www.biogps.org), it is expected that other tissues in which the CSF-1/CSF-1R complex may be preponderant include the female reproductive tract, heart, and retina. Thus, under normal conditions, the IL-34/CSF-1R complex is necessary for the development of mononuclear phagocytes in the epidermis and specific regions of the CNS, while CSF-1/CSF-1R signaling is sufficient for the development of the other tissue macrophages, monocytes, and osteoclasts.

Like CSF-1, IL-34 expression is induced by inflammation [308–310] and during stress-induced hematopoiesis [311]. However, in rainbow trout, IL-34, but not CSF-1, expression is induced in primary head kidney macrophages by pathogen-associated molecular patterns (PAMPs), inflammatory cytokines, and parasitic kidney infection [312]. Furthermore, TNF-α induces IL-34, rather than CSF-1 expression in

fibroblast-like synovial cells isolated from patients with rheumatoid arthritis [305]. These data suggest that IL-34 could play a nonredundant role with CSF-1 in the control of macrophage activation during inflammation. Indeed, studies in *Xenopus laevis* tadpoles infected with Ranavirus frog virus 3 (RV FV3) revealed that IL-34 and CSF-1 have divergent patterns of expression and opposite roles in antiviral immunity, with IL-34 promoting immunity, while CSF-1 increases the susceptibility to RV FV3 infection [313]. Furthermore, recent studies in mice have identified a new type of monocytic cell, the follicular dendritic cell-induced monocyte (FDMC) that arises through a CSF-1R-mediated differentiation process dependent on IL-34, but independent of CSF-1 [314]. FDMCs differ from monocytes by their ability to enhance IL-4-independent B-cell proliferation. Thus, IL-34 may have a unique role in the stimulation of antiviral and adaptive immune responses. Whether these unique functions of IL-34 are mediated via a newly identified IL-34 receptor, RPTP-ζ (Sect. 10.1.5.2.6) remains to be determined.

Chromosomal Location of the Il34 Gene

Human *Il34* is located on chromosome 16q22.1 between the Vac14 homologue (Vac14) and small nucleolar RNA CD box (Snord) 111B loci. Mouse *Il34* is located on chromosome 8E1, also between Vac14 and Snord 111.

Structure of the Il34 Genomic Locus

Human *Il34* is composed of 6 exons and 5 introns spanning ~81 kb. Mouse *Il34* has a similar structure consisting of 6 exons and 5 introns and extending over ~64 kb.

IL-34 Isoforms

IL-34 is a secreted homodimeric protein, comprising 242 amino acids in man and 235 amino acids in mouse, with an apparent monomeric molecular mass in SDS-PAGE of 39 kDa. The first 182 amino acids are sufficient to activate the CSF-1R [14]. This region also contains a predicted N-glycosylation site and six cysteine residues that are highly conserved across species. Four of these cysteine residues are involved in intramolecular disulfide bonding (Fig 10.5a, b). Both human and mouse *Il34* mRNAs exhibit alternative splicing of a CAG codon that leads to isoforms with and without a glutamine residue (Q) at position 81. Notably, the absence of Q81 is predicted to alter the nearby structure from coiled coil to alpha-helix, and isoforms lacking Q81 exhibit reduced biological activity [9]. IL-34 also carries N-linked glycans at Asn 76 and Asn 100 positions which are vital for its stability [8]. Other studies show that the C-terminal 50 amino acids of IL-34 are highly unstructured and also heavily O-glycosylated [315], but the physiological relevance of this finding is unclear as the last 40 amino acids are dispensable for CSF-1R binding and activation [7].

Although IL-34 lacks appreciable sequence similarity to CSF-1, structural studies reveal that they share a similar three-dimensional folding [7, 8] (Fig. 10.5c) characteristic of the short-chain four alpha-helix bundle cytokine family [316], which allows them to interact with CSF-1R.

Targeted Disruption of the Il34 Gene

A conditional floxed allele in which mouse *Il34* exons 3–5 are flanked by *loxP* sites as well as a *Il34*-deficient reporter allele in which exons 3–5 were replaced by *LacZ* have been reported [12, 13]. The IL-34-deficient mice are viable, and their phenotype is summarized in Table 10.1.

Species Specificity of IL-34

Similar to CSF-1, cross-species specificity was reported for IL-34, with human IL-34 activating human, cat, and pig CSF-1Rs, but, unlike CSF-1, displaying poor activation of the mouse CSF-1R. Conversely, murine IL-34 could activate human, mouse, and pig CSF-1 receptors, but was much less efficient in cats [9, 295] (Fig 10.6).

IL-34 Signaling Through Protein-Tyrosine Phosphatase-ζ (PTP-ζ)

The observations that IL-34 is often expressed in regions of the brain where there is minimal expression of the CSF-1R and that IL-34 is more active in suppressing neural progenitor cell self-renewal and stimulating their neuronal differentiation than CSF-1 [10] prompted the search for an additional receptor for IL-34. Proteomic studies identified PTP-ζ (also known as RPTP-β), a membrane-spanning, cell-surface chondroitin sulfate proteoglycan, as an additional IL-34 receptor [317]. In vitro, human IL-34 binds to the extracellular domain of PTP-ζ with significantly lower affinity than to the CSF-1R (K_d ~10^{-7} M vs. K_d ~ 10^{-12} M) [317]. However, IL-34 acts on cells at lower concentrations than expected from this high in vitro K_d, suggesting PTP-ζ clustering, or the involvement of a co-receptor. Ligand-induced dimerization of PTP-ζ leads to phosphatase inactivation and increased cellular tyrosine phosphorylation [318–320]. Consistent with this, IL-34 stimulation of the CSF-1R-negative human glioblastoma cell line U251 leads to a PTP-ζ phosphatase-dependent increase in tyrosine phosphorylation of focal adhesion kinase and paxillin, resulting in decreased cell motility [317]. In addition, IL-34 inhibits the viability and clonogenicity of U251 cells. Thus, IL-34 exerts CSF-1R-independent functions in PTP-ζ-expressing cells. Such functions may occur in the adult cerebral cortex, where CSF-1R expression is very low [10] and where PTP-ζ co-localizes with IL-34 in layers IV and V [317]. In the brain, PTP-ζ is expressed by neural progenitors, cerebral and cerebellar neurons, astrocytes, oligodendrocytes, radial glia, and

retinal Muller glia, suggesting that it plays an important role in the development and/or function of the nervous system [321–323]. Like *Il34*$^{-/-}$ mice, PTP-ζ$^{-/-}$ mice are viable and fertile and do not exhibit gross anatomical defects [324]. However, they exhibit early oligodendrocyte differentiation during central nervous system development, and adults are less susceptible to experimentally induced demyelinating disease, indicating that PTP-ζ negatively affects these processes [325].

Although PTP-ζ expression was initially thought to be restricted to the central nervous system [326], recent studies in mice showed that deletion of PTP-ζ increases both LT-HSC and ST-HSC pools in an HSC-autonomous manner, without altering the normal differentiation capacity of bone marrow HSC [327]. Furthermore, there is no evidence of splenomegaly, lymphadenopathy, or leukemia, in PTP-ζ$^{-/-}$ mice at 1 year, suggesting that deletion of PTP-ζ does not cause myeloproliferative or lymphoproliferative disease [327]. As discussed above (Sect. 10.1.2.2), the CSF-1R is also expressed in HSC and instructs their commitment to the myeloid lineage [35]. Because HSCs also express PTP-ζ, elevations in local concentrations of CSF-1 or IL-34 might trigger different HSC responses, by activating CSF-1R alone (CSF-1) or by simultaneously triggering both receptors (IL-34).

10.1.6 CSF-1R Activation and Signaling

10.1.6.1 Ligand Binding and CSF-1R Kinase Activation

Interaction of the CSF-1R with CSF-1 and IL-34

IL-34 and CSF-1 exhibit a similar three-dimensional architecture and both belong to the family of short-chain four alpha-helix bundle cytokines [6–8, 316]. Studies combining electron microscopy (EM) and small-angle X-ray scattering (SAXS) show that both CSF-1 and IL-34 dimers form structurally similar complexes with the CSF-1R [257, 315] (Fig. 10.7). Both cytokines interact with the CSF-1R with a stoichiometry of one ligand dimer to two receptors. The ligands bind to a concave surface formed by the D2 and D3 Ig-like domains of the CSF-1R with the D1 domain extending away from the core of the complex without making any interactions with the ligand or other receptor domains [257, 315] (Fig. 10.7b, c). Studies of mouse IL-34 interaction with its cognate receptor suggest that the interactions with D2 and D3 are not functionally equivalent. While CSF-1R D2 is essential for ligand binding, mutations in IL-34 designed to antagonize charge–charge interactions with D2 revealed that none of the individual hydrophilic residues of IL-34 interacting with CSF-1R D2 is essential for IL-34 biological activity. In contrast, disturbing the interactions with CSF-1R D3 abolished IL-34 activity. Based on these findings, it has been postulated that the strong charge attraction provided by CSF-1R D2 is first used to capture IL-34, while interactions with D3 are formed subsequently [8]. Interestingly, the engagement of CSF-1R by IL-34 triggers a rotation between D2 and D3 of the CSF-1R producing an elongated pose that is different from the kinked

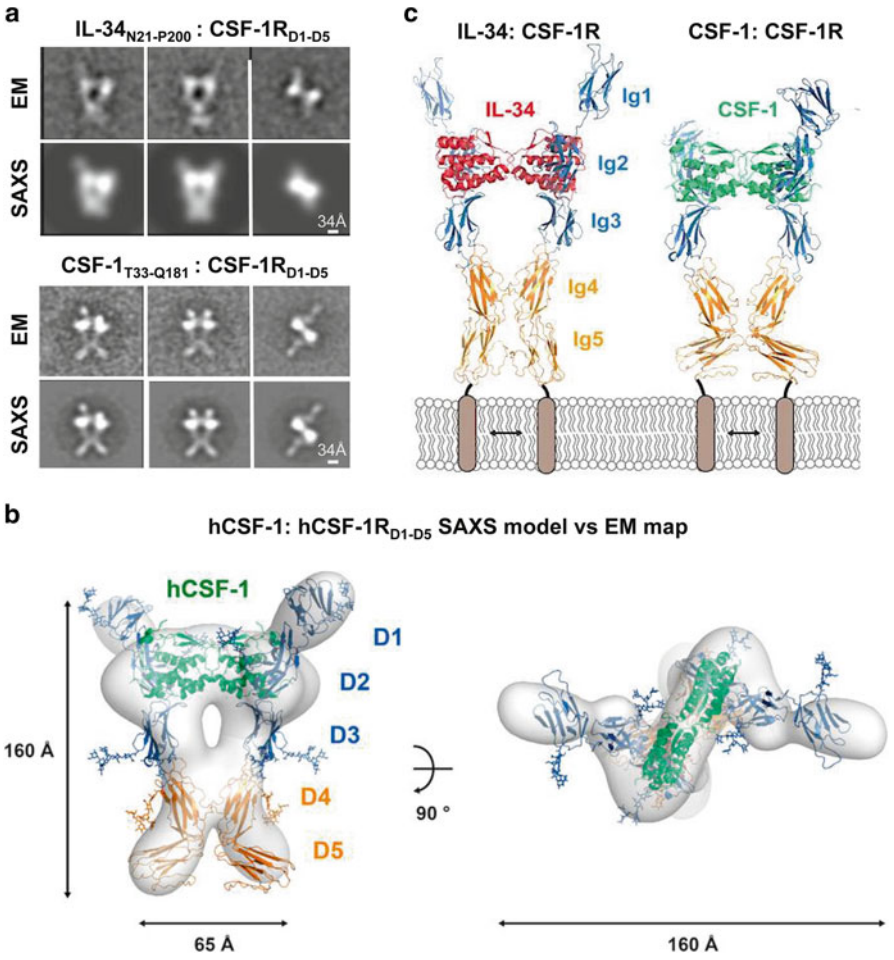

Fig. 10.7 Human IL-34 and CSF-1 form structurally similar complexes with the extracellular domains of the human CSF-1R. (**a**) Negative stain electron microscope images (EM) and SAXS model projections (SAXS) of the IL-$34_{N21-P200}$:CSF-$1R_{D1-D5}$ complexes (*top panel*, reproduced with permission [315]) and of the CSF-$1_{T33-Q181}$:CSF-$1R_{D1-D5}$ complexes (*bottom panel*, reproduced with permission [257]). (**b**) Superimposition of the SAXS model and EM density map for CSF-1T33-Q181:CSF-$1R_{D1-D5}$ (reproduced with permission [257]) (Electron Microscopy Data Bank accession code EMD-1977). (**c**) Ribbon representation of the CSF-$1_{T33-Q181}$:CSF-$1R_{D1-D5}$ and IL-$34_{N21-P200}$:CSF-$1R_{D1-D5}$ complexes (reproduced with permission [315])

configuration of the CSF-1R/CSF-1 complex [7] (Fig. 10.7c). Nevertheless, this reorientation of the hinge between D2 and D3 results in the D3–D4 junction being spaced equivalently in the two receptor complexes (distance between the two D3-D4 junctions is 60 Å in the CSF-1R/IL-34 complex and 62 Å in the CSF-1R/CSF-1 complex) and presumably triggers the homotypic interactions of D4 and the ensuing

signaling events [7]. Indeed, EM and SAXS studies of CSF-1/CSF-1R$_{D1–D5}$ and IL-34/CSF-1R$_{D1–D5}$ reveal that in receptor–ligand complexes, the D4 domains of the two CSF-1R molecules involved are in close proximity [257, 315] and CSF-1RD4 shares a dimerization sequence fingerprint with the other closely related RTK III receptors, Kit and PDGFR [328, 329]. Since both IL-34 and CSF-1 interact at higher affinity with CSF-1R D1–D5 than with CSF-1R D1–D3 [7], these studies suggest that ligand binding to the CSF-1R elicits homotypic CSF-1R$_{D4}$ interactions that dramatically increase the strength of interaction. The role of CSF-1R$_{D5}$ is unclear as in soluble receptor–ligand complexes, the D5 domains on the two CSF-1R molecules involved point away from each other [257, 315]. However, this orientation, described in a construct that only contains the extracellular domain of the CSF-1R, may not be identical to the conformation adopted in the full-length membrane-spanning CSF-1R and does not exclude a role for the membrane-proximal CSF-1R$_{D5}$ in stabilizing the ligand–receptor complex. Consistent with this, the K_d reported for the CSF-1/CSF-1R$_{D1–D5}$ interaction at 37 °C was 200-fold higher than the K_d for the binding of the human CSF-1 to the corresponding native receptor on cells [257, 330] and 50-fold higher than the K_d for the binding of the mouse CSF-1 to its cognate receptor on cells [217]. A similar phenomenon has been observed in the case of other RTK III [331, 332] and suggests a significant contribution of the spatial confinement of the membrane to affinity.

CSF-1R Kinase Activation

The structure of the inactive CSF-1R kinase domain closely resembles the two-lobed structures of the other RTK III family members, c-KIT and FLT3 [333, 334]. The smaller N-terminal lobe consists of a five-stranded, antiparallel β-sheet (β1–β5) and a single α-helix, αC. It is connected to the C-terminal lobe by the kinase insert domain and a hinge region. The C-lobe has seven α-helices (αD, αE, αEF, αF-αI) and two β strands (β6 and β7) (Fig. 10.8a). The N-lobe and hinge regions are mainly responsible for ATP binding (in a deep cleft between the N- and C-lobes) and provide some catalytic residues, while the C-lobe mediates substrate binding and catalysis. Similar to the inactive conformations of c-KIT and FLT3, the inactive conformation of the CSF-1R kinase domain possesses an activation loop (AL) that folds back onto the ATP-binding cleft preventing substrate binding. The conserved Asp-796 of the invariant DFG motif at the start of the AL that is required for Mg^{2+} coordination of ATP is displaced from the active site ("DFG-out" conformation), being tucked into the hydrophobic cleft between the N- and C-lobes and pointing away from the catalytic Asp-778. The sole AL tyrosine 809 acts as a pseudosubstrate with its hydroxyl group hydrogen bonding to the conserved catalytic residues Asp-778 and Arg-801 (Fig. 10.8b).

By analogy with other kinases, the activation of the CSF-1R would require flipping of the DFG motif ("DFG–in" conformation) and reorganization of the AL, involving the phosphorylation of Tyr-809. However, while studies of the mouse CSF-1R support the importance of this hydrogen bonding of Tyr-807 (the equivalent

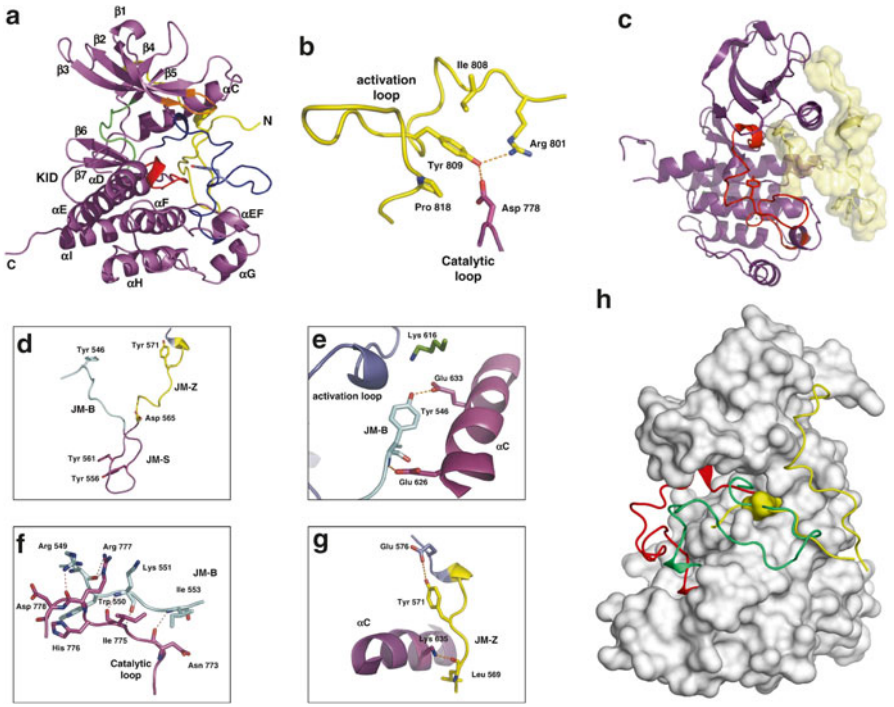

Fig. 10.8 Crystal structure of the autoinhibited human CSF-1R intracellular domain. (**a**) Overview of the structure of the kinase domain with the N-lobe above the C-lobe. The secondary structural elements and N and C termini are labeled. The juxtamembrane domain (JM, *yellow*), glycine loop (*orange*), hinge region (*green*), catalytic loop (CL, *red*), activation loop (AL) with Tyr809 (*blue*), and the position of the kinase insert domain (KID) are also shown. (**b**) Pseudosubstrate interaction of AL Tyr809 with CL residue Asp778 and AL residue Arg801. (**c**) Space-filling representation of the JMD (*yellow*) in relation to the kinase domain. The AL is colored in *red*. (**d**) CSF-1R JMD. JM-B (*cyan*), JM-Z (*magenta*), and JM-S (*yellow*), showing the positions of the JM tyrosines and of Asp-565, which facilitates the positioning of JM Tyr-571. (**e**) JM-B Tyr-546 interaction with αC residues Glu-633 and Glu-626 (*magenta*). (**f**) JM-B residues Arg-549, Trp-550, Lys-551, and Ile 553 interacting with CL residues (*magenta*). (**g**) Interactions of JM-S residues Leu-569 and Tyr-571 with αC residue Lys635 (*magenta*) and kinase domain residue Glu-576 (*light blue*), respectively. (**h**) Role of the JMD in stabilization of the inactive CSF-1R. Kinase domain is shown as a combination of white surface representation, AL (*red*) and JMD (*yellow*), with Trp-550 represented as a surface. The AL of active c-KIT (*green*) is superimposed. The JMD of the autoinhibited kinase inserts itself into the active site and prevents the AL from switching into an active conformation. Panels a–g reproduced with permission [333]. *Panel H* reproduced with permission [334]

tyrosine in mouse) in maintaining the structure of the active site, they do not confirm a role for Tyr-809 phosphorylation in the early activation of the CSF-1R [335]. In addition, the very late tyrosine phosphorylation of the corresponding AL Tyr-823 of the closely related c-KIT [336] suggests that the phosphorylation of the AL tyrosine in either receptor is not required for activation. Furthermore, the kinase activity of the mouse Y807F mutant CSF-1R was similar to the kinase activity of the wt

CSF-1R, suggesting that other tyrosine residues besides Tyr-807 are involved in activation [337]. Particularly important in this respect is Tyr-561 in the juxtamembrane domain (JMD) [253, 335, 338], for which the kinase activity of the corresponding mouse Y559F mutant CSF-1R is ~50 % of the kinase activity of the wt CSF-1R [337]. The JMD itself (Fig. 10.8c) mediates a critical autoinhibitory mechanism which is relieved by phosphorylation of Tyr-559, which acts as a switch, keeping kinase activity "off" in the absence of ligand, and as the first residue to be phosphorylated, turning it "on" by relieving autoinhibition, through its phosphorylation in response to ligand [335]. The JMD (Q542-K574) has been subdivided into three components: JM-B (binding or buried region, Y546-I553), JM-S (switch motif, E554-I564), and JM-Z (zipper region D565-K574) (Fig. 10.8d). The JM-B domain is largely buried, making direct contacts with the αC helix, the catalytic loop, and AL. The hydroxyl group of Tyr-546, whose equivalent, Tyr-544, in the mouse CSF-1R is critical for kinase activity [335, 337], forms hydrogen bonds with the conserved Glu633 in αC that in active kinase conformations forms a salt bridge with Lys616 that is important for ATP binding (Fig. 10.8e). Arg-549 forms hydrogen bonds with the catalytic loop Arg-777, and two other JM-B residues, Lys-551 and Ile-553, help to stabilize the buried JMD through interactions with Ile775 and Asn773, respectively (Fig. 10.8f). The JM-S domain forms a hairpin (Fig. 10.8d) that is located externally to the C-lobe (Fig. 10.8c) and contains the key Tyr561 switch residue, pointing toward the C-lobe that is involved in kinase activation. The JM-Z domain is positioned along the solvent-exposed face of the αC helix (Fig. 10.8c), with hydrogen bonding between Leu-569 and the conserved αC Lys-635 and a side-chain–side-chain interaction between the hydroxyl group of Tyr-571 and Glu-576 at the commencement of the kinase domain (Fig. 10.8g). Thus, in the inactive CSF-1R, the JM-B and JM-Z domains together block αC, preventing the AL from adopting an active conformation and restricting inter-lobe plasticity [333]. No structure of the activated CSF-1R kinase domain has been reported. However, based on the close structural similarities between c-KIT and the CSF-1R, how the CSF-1R AL is repositioned in the activated receptor can be envisaged from superimposition of the AL of activated c-KIT onto inactive CSF-1R [334] (Fig. 10.8h).

10.1.6.2 CSF-1R Signal Transduction in Macrophages and Myeloid Progenitor Cells

Systems Used to Study CSF-1R Signal Transduction

Studies of CSF-1R structure/function in macrophage survival, proliferation, and differentiation have utilized cells of the GM-CSF-responsive *Csf1r*$^{-/-}$ macrophage cell line, MacCsf1r$^{-/-}$, which, when transduced with the wt CSF-1R, mediates responses to CSF-1 that mimic those of primary bone marrow-derived macrophages [337]. Alternatively, BMM have been transduced with an erythropoietin receptor extracellular domain-CSF-1R fusion protein construct, in which erythropoietin elicits CSF-1R responses [339]. A differentiation response can be measured in the

MacCsf1r$^{-/-}$ cells in which CSF-1 signaling through the wt CSF-1R induces down-regulation of the dendritic cell marker CD11c and a 20-fold upregulation of the macrophage differentiation marker CD11b [337]. However, studies of CSF-1R structure/function in differentiation have predominantly utilized myeloid progenitor cells in which the CSF-1R is expressed by transfection. The multipotent myeloid progenitor cell line, FDC-P1, does not express the CSF-1R, but proliferates in response to IL-3. CSF-1 stimulation of wt CSF-1R-transfected FDC-P1 (FD-Fms) cells leads to macrophage differentiation [340]. A similar system utilizing CSF-1R-transfected myeloblasts of the growth factor-independent M1 cell line has also been used [341]. These systems have permitted elucidation of the CSF-1R signaling in the survival, proliferation, differentiation, and chemotaxis of myeloid cells as described below and illustrated elsewhere [342].

Short- and Long-Term CSF-1R Responses

Addition of CSF-1 to CSF-1-starved mouse macrophages results initially in CSF-1R tyrosine autophosphorylation, followed by CSF-1R ubiquitination and the membrane-proximal tyrosine phosphorylation of 0.02 % of the total cellular protein [343]. Downstream responses can be divided in short-term responses occurring within 30 min following CSF-1R activation and long-term responses that require changes in gene expression. The short-term responses include rapid cytoskeletal remodeling leading to increased membrane ruffling and membrane spreading followed by cell elongation and polarization, which permits effective cell migration [344–346]. The long-term responses include increased motility and chemotaxis [345–347] and changes in gene expression leading to the entry of the cells into the S phase (reviewed in [343, 348]) as well as to the increased expression of the macrophage differentiation marker Mac1 (CD11b) [337] and of cytokines, chemokines, and cell-surface markers characteristic of M2 polarization [82, 87].

Early Signaling by the Activated CSF-1R

Prior to CSF-1 addition, CSF-1Rs are clustered or are undergoing a rapid dimer-monomer transition [349] (Fig. 10.9, step 1). CSF-1 binding initially leads to the rapid dimerization of CSF-1R followed by phosphorylation of 8 of the 19 tyrosine residues in the intracellular domain (reviewed in [343, 350]) (Fig. 10.9, steps 2, 3, and 5). Activated CSF-1Rs form complexes with Grb2/Sos, Src family kinases (SFK), the ubiquitin ligase Cbl, the p85 regulatory subunit of PI-3 kinase (PI3K), Grb2, and other signaling molecules, many of which become tyrosine phosphorylated [349, 351–356] (Fig. 10.9, steps 3–5). The CSF-1R/Sos/Grb2 complexes dissociate rapidly (Fig. 10.9, step 4). Subsequently, Cbl-dependent ubiquitination leads to a second wave of CSF-1R tyrosine phosphorylation, increased CSF-1R serine phosphorylation [253, 349, 352], and CSF-1R endocytosis [252, 354] (Fig. 10.9, steps 5–7). After internalization, the CSF-1R/CSF-1 complex can continue to signal

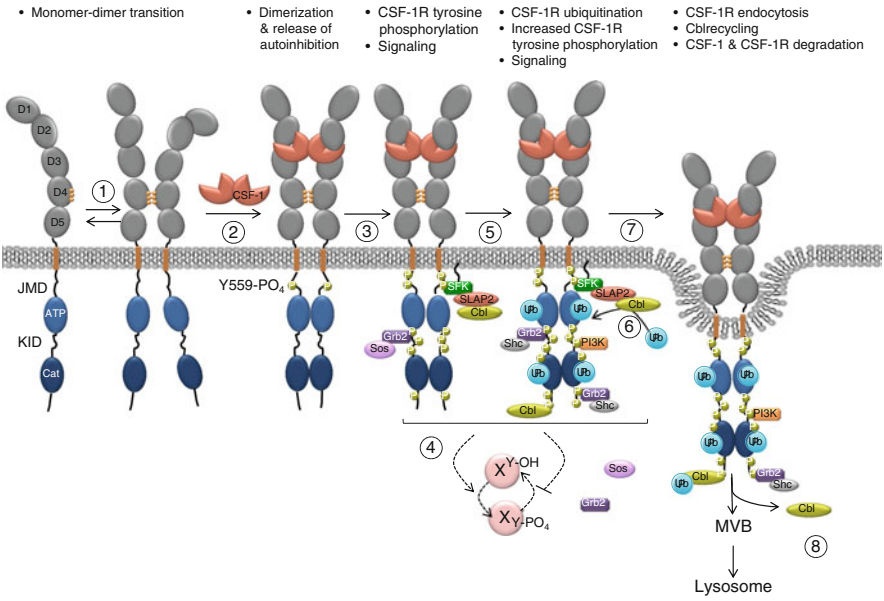

Fig. 10.9 Schematic representation of mouse CSF-1R activation and early signaling events. In the absence of ligand, the CSF-1R rapidly undergoes monomer–dimer transitions, probably mediated by the conserved dimerization motifs in D4 (*orange spheres*). The intracellular kinase domain is in an inactive conformation. Binding of CSF-1 stabilizes receptor dimerization and releases kinase autoinhibition through phosphorylation of the juxtamembrane domain (JMD) Tyr-559 (Y559-PO4). Activation of the kinase domain results in the phosphorylation of additional CSF-1R tyrosine residues (*yellow spheres*) that create binding sites for SH2- and PTB- domain-containing adaptors or enzymes, including SFK and Grb2, thus initiating downstream signaling events leading to the increased tyrosine phosphorylation of cytosolic proteins (X). The CSF-1R association with the Grb2/Sos complex is transient, compared with the more stable association of PI3K and Grb2-Shc. The CSF-1R-associated SFK recruits Cbl via SLAP2 and Cbl-mediated CSF-1R ubiquitination leads to enhanced CSF-1R tyrosine phosphorylation and the recruitment of additional downstream signaling molecules. The ubiquitinated CSF-1R signaling complexes are subsequently internalized. Both CSF-1 and the CSF-1R are lysosomally degraded, while Cbl is recycled. Steps 1–8 are described in detail in the text. *D1–D5* Ig-like domains, *KID* kinase insert domain, *Ub* ubiquitin, *MVB* multivesicular bodies

[357], prior to its subsequent targeting to the lysosomal system, where both CSF-1 [217] and the CSF-1R [252] are degraded (Fig. 10.9, step8). Simultaneously, Cbl also becomes ubiquitinated. However, in contrast to the CSF-1/CSF-1R complex, Cbl is not degraded, but rather deubiquitinated and translocated back to the cytosol 3–10 min after stimulation [252, 353, 354]. Tyrosine phosphorylation and ubiquitination of the cell-surface CSF-1R dimers are stoichiometric [349, 354] although ubiquitination may be asymmetric and restricted to only one monomer of the ubiquitinated CSF-1R dimer [253]. A previously reported covalent linkage of receptor dimers [349] was recently shown to be artifactual, but useful as a measure of receptor activation [253]. Similar behavior of the CSF-1R following ligand binding has been reported for myeloid progenitor cells [338, 358–360].

10.1.6.3 The Role of Individual CSF-1R Intracellular Domain Phosphotyrosines in Signal Transduction

CSF-1R Tyrosines Phosphorylated by Receptor Activation

Ligand-induced mouse CSF-1R dimerization triggers the phosphorylation of six intracellular tyrosine residues (Tyr-544, Tyr-559, Tyr-697, Tyr-706, Tyr-721, Tyr-807, Tyr-921, and Tyr-974) (reviewed in [350, 358, 359, 361]). The phosphorylation of two additional tyrosine residues, Tyr-544 and Tyr-921, has been demonstrated in the constitutively active *v-fms* oncoprotein [362, 363]. Phosphorylation of these tyrosines creates binding sites for effector and adaptor molecules containing phosphotyrosine-recognition domains (Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains); pTyr-559 for Src family kinases (SFKs); pTyr-697 for the Grb2, Mona, and Socs1 adaptor proteins; pTyr-721 for the p85 subunit of PI3K, PLCγ2, and Socs1; and pTyr-921 for Grb2 and pTyr-974 for Cbl (reviewed in [350, 358, 359, 361]). These downstream effector and adaptor proteins may also become tyrosine phosphorylated and/or activated to initiate a cascade of signaling events leading to cytoskeletal reorganization, cell proliferation, and differentiation (reviewed in [350, 361]). The necessity for the eight CSF-1R phosphotyrosines has been investigated in macrophages by their mutation to phenylalanine [337, 339] and their sufficiency for triggering specific responses examined by adding them back to a receptor backbone in which all 8 tyrosines were mutated to phenylalanine [253, 335]. Similar approaches have been used to study the role of CSF-1R tyrosine phosphorylation in the differentiation of myeloid progenitor cells to macrophages (reviewed in [342, 358, 359]) or to osteoclasts [364].

The CSF-1R Juxtamembrane Domain Tyrosine 559 Is Critically Required for CSF-1R Activation

Following CSF-1 binding to CSF-1R, the first phosphorylation event occurs at Tyr-559 of the CSF-1R *in trans* (step 2, Fig. 10.9) [253, 335, 337]. Phosphorylation of CSF-1R Tyr-559 triggers the binding of Src family kinases (SFKs) to this site and their activation [365] as well as the recruitment of the ubiquitin ligase Cbl to the CSF-1R pTyr-559/SFK complex, which is probably mediated by the Src-like adaptor protein 2 (SLAP 2) [366]. Cbl-mediated ubiquitination of CSF-1R is associated with a change in the cytoplasmic domain of the receptor that allows increased CSF-1R tyrosine phosphorylation [253]. The requirement for Cbl-mediated ubiquitination in CSF-1R activation was demonstrated in $Cbl^{-/-}$ macrophages and $Cbl^{-/-}$ macrophages expressing Cbl ubiquitin ligase inactive mutants. In both cases, addition of CSF-1 resulted in decreased CSF-1R ubiquitination and tyrosine phosphorylation. The initiating role of Tyr-559 in the initiation of the cascade of events leading to full CSF-1R activation is supported by several lines of evidence: (1) Maximum phosphorylation of Tyr-559 preceded the attainment of maximum phosphorylation of Tyr-807, Tyr-697, and Tyr-721, which was temporally correlated

with the onset of CSF-1R ubiquitination; (2) CSF-1R ubiquitination was dramatically compromised in the Tyr-559 F mutant and only slightly reduced in the Tyr-697 F and Tyr-807 F mutant receptors; and (3) reconstitution studies, adding back tyrosines to the a CSF-1R backbone in which all 8 tyrosines were mutated to phenylalanine (Y eight F, YEF), have demonstrated that phosphorylation of CSF-1R Tyr-559 was alone sufficient to trigger CSF-1R tyrosine phosphorylation and ubiquitination. In contrast, macrophages expressing receptor add-backs (AB) of tyrosines 697, 721, or 807 (Tyr-697AB, Tyr-721AB or Tyr-807AB CSF-1Rs) failed to undergo receptor tyrosine phosphorylation or exhibit significant receptor ubiquitination. Thus, phosphorylation of Tyr-559 is necessary and sufficient to induce the appropriate CSF-1R conformational change that leads to receptor activation [253].

CSF-1R Tyrosine Residues 544, 559, and 807 Are Necessary and Sufficient for CSF-1-Induced CSF-1R Activation and Macrophage Proliferation

Macrophages expressing a CSF-1R in which the juxtamembrane domain (JMD) Tyr-559 or the AL Tyr-807 were mutated to phenylalanine (CSF-1R Tyr-559F and Tyr-807F CSF-1R, respectively) exhibited severely impaired in vivo receptor tyrosine phosphorylation, consistent with the existence of cellular mechanisms inhibiting CSF-1R tyrosine phosphorylation that are relieved by phosphorylation of these two sites. These mutations also severely compromised macrophage proliferation and differentiation [337, 339]. When the Tyr-807 was the only conserved tyrosine phosphorylation site reconstituted in YEF (Y807 add-back, Y807AB), macrophages proliferated constitutively in the absence of CSF-1 in a CSF-1R kinase-dependent manner and in the absence of detectable Tyr-807 tyrosine phosphorylation. This proliferation was mediated by the phosphatidylinositol 3-kinase (PI3K) and ERK1/2 pathways. Addition of Tyr-559 alone (Y559AB) supported a low level of CSF-1-independent proliferation that was slightly enhanced by CSF-1, indicating that Tyr-559 has a positive Tyr-807-independent effect. However, the addition of Tyr-559 to the Y807AB background suppressed proliferation in the absence of CSF-1 and restored most of the CSF-1-stimulated proliferation, suggesting a switch function for Tyr-559. Full restoration of CSF-1R kinase activity and proliferation required the additional add-back of JMD Tyr-544 (YEF.Y544,559,807AB) [335] which contributes significantly to the in vitro kinase activity of the CSF-1R [337]. These studies [335] established that tyrosine residues 544, 559, and 807 are sufficient for CSF-1R kinase activation and trigger signaling events leading to normal proliferative responses.

CSF-1R Tyrosine Residues 706, 721, and 974 Mediate CSF-1R-Induced Morphological and Motility Responses

Granulocyte–macrophage CSF (GM-CSF) can substitute for CSF-1 to support macrophage proliferation in vitro; however, compared to CSF-1-derived macrophages, GM-CSF-derived macrophages are less elongated, and they express lower

cell-surface levels of a macrophage differentiation marker CD11b and higher levels of the dendritic cell marker CD11c [337]. These changes are reversible after 7 days of culture in CSF-1 [337]. Examination of the long-term morphological responses induced by CSF-1 in GM-CSF-derived macrophages revealed that mutations eliminating phosphorylation at residues Tyr-706, Tyr-721, and Tyr-974 altered CSF-1-induced morphological responses in a site-specific manner, while tyrosine to phenylalanine mutations in the remaining tyrosines did not cause major effects on macrophage morphology [337]. While the CSF-1R Tyr-721 and Tyr-974 were both necessary for macrophage elongation, Tyr-706 was inhibitory. Macrophages expressing the CSF-1R Y974F mutant spread poorly, suggesting that the Tyr-974 was required not only for cytoskeletal remodeling but also for the formation of membrane protrusions. As Tyr-974 is a binding site for Cbl [367], it is possible that, in addition to its actions in the early steps of CSF-1R activation discussed above (Sect. 10.1.6.2), Cbl mediates other events leading to cytoskeletal reorganization. In contrast, macrophages expressing the CSF-1R Y721F mutant spread more than those expressing the wt CSF-1R. Examination of the early biochemical events and morphological responses of macrophages to CSF-1 stimulation revealed that Tyr-721 was necessary and sufficient (on the YEF.Y544,559,807AB background) for mediating the association of CSF-1R with the p85 subunit of phosphoinositide 3-kinase (PI3K), polarized PtdIns(3,4,5)P_3 production at the putative leading edge of migrating cells, as well as CSF-1-induced chemotactic and chemokinetic responses, suggesting that the CSF-1R pTyr-721/PI3K pathway is a major regulator of CSF-1-induced macrophage migration [368].

CSF-1R Tyr-807 Is Necessary for Macrophage Differentiation Which Is Suppressed by Tyr-706

Studies of the role of the CSF-1R tyrosine autophosphorylation sites in FD-Fms differentiation to macrophages have shown that (1) Tyr-697, Tyr-706, Tyr-721, and Tyr-807 were not essential for CSF-1-dependent progenitor cell proliferation; (2) Tyr-697, Tyr-706, and Tyr-721, located in the kinase insert region of CSF-1R, were not necessary for differentiation, but their presence enhanced differentiation; and (3) Y807F FDC-P1 cells failed to differentiate and, conversely, exhibited an increased rate of proliferation, suggesting that Tyr-807 may control a switch between growth and differentiation [369]. The Y807F CSF-1R exhibited decreased binding and phosphorylation of PLC-γ2, which was also dependent on tyrosine phosphorylation of Tyr-721. Furthermore, a specific PLC-γ inhibitor abrogated the differentiation of the wt CSF-1R FDC-P1 cells [370]. These data suggest that a CSF-1R Tyr-807/PLC-γ2 pathway augmented by pTyr-721 signaling plays a major role in macrophage differentiation.

In MacCsf1r$^{-/-}$ macrophages expressing the wt CSF-1R (MacCsf1r$^{-/-}$.WT), CSF-1 signaling induces upregulation of the macrophage marker CD11b and downregulation of the dendritic cell marker CD11c [337]. When macrophages expressing various CSF-1R Tyr to Phe mutants were stimulated with CSF-1, the induction of

CD11b varied from ~70 % of the levels induced by the wt CSF-1R (for cells expressing the Y544F, Y697F, Y721F, or Y947F mutants) to 20–30 % in cells expressing CSF-1R YEF, Y559F, or Y807F mutant, suggesting that Tyr-559 and Tyr-807, but not the other tyrosine residues, play an important role in triggering macrophage differentiation in these more mature cells. In contrast, CSF-1R Y706F cells induced CD11b expression to levels above those obtained with the wt CSF-1R, suggesting that the phosphorylation of Tyr-706 negatively impacts signaling for macrophage differentiation [337].

10.1.6.4 Downstream Signaling Events Regulating Macrophage Differentiation, Survival, Proliferation, and Motility

CSF-1R Regulation of Macrophage Differentiation

CSF-1 directly induces the myeloid cell fate in mouse HSCs through upregulation of the myeloid transcription factor PU.1 and thus has an instructive role, rather than simply permitting the survival and/or expansion of determined progenitors [36]. It also instructs granulocyte–macrophage progenitors (GMP) to differentiate into macrophages [74].

Ca^{2+} signaling plays an important role in CSF-1R signaling for macrophage differentiation and both PLC-γ2 and PKC-δ [370–372] are critical downstream effectors. Stimulation of CSF-1R induces the rapid tyrosine phosphorylation, activation, and membrane translocation of PKC-δ which in turn upregulates the expression of PKA-related protein kinase (Pkare) [371]. This, together with the Ca^{2+}-dependent activation of nuclear factor of activated T-cell (NFAT) transcription factors, drives monocytic differentiation. Consistent with this, the CSF-1R Y807F mutant which failed to activate PLCγ2 and PKC-δ also fails to support differentiation [370] (see Sect. 10.1.6.3.5). In addition, proteomic studies in FDC-P1 multipotent progenitors transfected with CSF-1R revealed that the tyrosine phosphorylation of several proteins, including p46/52 Shc, depends on the phosphorylation of CSF-1R Tyr-807 [341]. Expression of a non-tyrosine-phosphorylatable form of p46/52 Shc prevented CSF-1-mediated macrophage differentiation, suggesting that p46/52(Shc) may play a role in CSF-1-induced macrophage differentiation [341].

Central Role of the ERK Pathway in CSF-1R-Regulated Myeloid Differentiation Studies in M1 myeloblasts revealed that CSF-1-induced differentiation was augmented by elevation of intracellular cAMP which also led to increased Erk1/2 activity, but paradoxically inhibited bulk protein-tyrosine phosphorylation [373], suggesting that the Erk1/2 pathway is central to CSF-1-induced differentiation. In FD-Fms myeloid progenitor cells, CSF-1 induces two temporally distinct phases of mitogen-activated protein kinase MEK/Erk1/2 phosphorylation characterized by an early and transient phase (reaching a peak at ~5 min of CSF-1 stimulation) and a late and persistent phase starting at 1 h of stimulation [374]. Studies utilizing FD-Fms cells treated with the MEK inhibitor, U0126, revealed that CSF-1R

signaling for differentiation was not dependent on the first wave of Erk1/2 phosphorylation but required MEK activity between 8 and 24 h of M-CSF stimulation. Furthermore, the differentiation signal was not dependent upon Grb2/Sos assembly or PI 3-kinase activity [374].

Myeloid Cell Mediators of CSF-1R Signaling Positively Regulating Erk1/2 Activation and Differentiation These mediators include the monocytic adaptor proteins, Mona, [375–377], Grb2-associated-binding protein (Gab)2 [378], and Gab3 [379]. The adaptor proteins Gab3 and Mona interact and are co-induced during monocytic differentiation [377]. Mona also interacts with CSF-1R pTyr-697, and FD-Fms Y697F cells exhibited reduced Gab3 tyrosine phosphorylation, a reduced ability to induce Mona expression and a reduced propensity to differentiate into macrophages [377]. Furthermore, CSF-1R Y807F also failed to induce Mona protein expression. While enforced expression of Mona in FD-Fms cells led to increased late-phase Erk phosphorylation, it did not alter CSF-1-induced differentiation, demonstrating that upregulation of Mona alone is not sufficient to enhance macrophage differentiation [376]. In contrast, overexpression of Gab3 in multipotent myeloid progenitor FD-Fms cells dramatically accelerated CSF-1-stimulated macrophage differentiation [379], suggesting that Gab3 has a significant role in CSF-1R-induced macrophage differentiation that could be partially mediated by the Gab3–Mona complex. However, as macrophage development is normal in Gab3-deficient mice [380], it appears that CSF-1R/Mona/Gab3/Erk1/2 pathway is not essential for steady-state macrophage development in vivo.

Gab 2 has also been implicated in CSF-1R-driven macrophage differentiation. Gab2 deficiency in mouse resulted in profoundly defective expansion of CSF-1R-dependent CMP and GMP in the bone marrow, through decreased proliferation and survival and accelerated differentiation to monocytes [378]. Structure–function studies, utilizing Gab2 mutants unable to interact with either PI3K or SH2 domain protein-tyrosine phosphatase 2 (Shp2), showed that Gab2 interaction with both enzymes was necessary to support the formation of CFU-M from bone marrow precursors. Interestingly, the absence of Gab2 disrupted CSF-1-induced signaling in a developmental stage-specific manner. The phosphorylation of Akt, Erk1/2, S6, and PI3K was decreased in Gab$^{-/-}$ CD31^{high} Ly6C^{-} myeloid progenitors compared to Gab$^{+/+}$ controls, while in the more differentiated CD31^{+} Ly6C^{+} progenitors, the activation of Akt was normal, and PI3K and Erk1/2 were much less affected. Gab2 deficiency also reduced CSF-1-induced proliferation of mature bone marrow-derived macrophages (BMM), but BMMs did not require Gab2 for full activation of Akt or S6 and, paradoxically, Gab2 deficiency caused increased phosphorylation of Erk1/2. Inhibitor and knockdown studies demonstrated that in mature BMM, Gab2 deficiency caused an increased phosphorylation of JNK, probably due to failure to upregulate the expression of inducible dual specificity phosphatases (DUSPs) [378]. These studies identify Gab2 as a node in the CSF-1R signaling network from which signals for macrophage and macrophage precursor differentiation, survival and proliferation emerge.

Negative Regulators of Erk1/2 Activation and Macrophage Differentiation These negative regulators include the following phosphatases: T-cell protein-tyrosine phosphatase (Tcptp)[381], which dephosphorylates the activated CSF-1R; the serine–threonine phosphatase 2A (PP2A) [382], which dephosphorylates, among other substrates, components of the Ras pathway acting upstream of Erk1/2 [383]; and the dual specificity phosphatase DUSP5 [384], which directly dephosphorylates and deactivates Erk1/2. Substrate-trapping experiments revealed that the tyrosine-phosphorylated CSF-1R is a substrate of Tcptp [381]. CSF-1 stimulation induced increased CSF-1R association with a Grb2/Gab2/Shp2 complex, and enhanced activation of Erk1/2 was observed in Tcptp$^{-/-}$ mouse macrophages. Furthermore, compared with wt mice, Tcptp$^{-/-}$ mice possessed increased numbers of bone marrow GMP and CFU-M and exhibited increased GMP differentiation into macrophages, suggesting that Tcptp is a significant regulator of CSF-1R signaling and mononuclear phagocyte development in hematopoiesis [381]. Studies in the M1 myeloblast cell line have shown that CSF-1R Tyr-559 initiates an SFK-dependent differentiation pathway leading both to the activation of STAT3 [360] and Erk1/2 and to the inactivation of PP2A through the tyrosine phosphorylation of its catalytic subunit [382]. Addition of the serine–threonine phosphatase inhibitor okadaic acid to M1 cells expressing CSF-1R Y559F partially restored Erk1/2 phosphorylation and rescued their ability to differentiate into macrophages in response to CSF-1, suggesting that inhibition of PP2A plays a significant role in enhancing Erk1/2-mediated macrophage differentiation. DUSP5 acts as a negative feedback regulator of Erk1/2, and its expression is induced by CSF-1 in various myeloid cells [384]. Overexpression of DUSP5 in two different multipotent progenitor cell lines (FD-Fms and the pro-T-cell line, EGER-Fms) increased CSF-1R-dependent proliferation, prevented macrophage differentiation, and favored granulocytic differentiation [384].

Cyclic Activation of the PI3K/Akt Pathway Is Required for the CSF-1-Induced Differentiation of Human Monocytes to Macrophages During CSF-1-induced differentiation of primary human monocytes to macrophages, the cells undergo a cyclic activation of the PI3K and Erk1/2 pathways that is correlated with successive rounds of phosphorylation and dephosphorylation of CSF-1R Tyr-723. Successive waves of Akt activation, increasing in amplitude and duration, were required for activation of caspase-8 and caspase-3 which contributed to macrophage differentiation through cleavage of nucleophosmin [385]. Nucleophosmin cleavage products inhibit macrophage migration and phagocytosis of bacteria, but not of apoptotic bodies, suggesting that they mediate differentiation of macrophages toward an M2-like phenotype [386]. Erk1/2 was activated with coordinated kinetics, but was not essential for nucleophosmin cleavage, and its role in human monocyte differentiation remains to be defined. In contrast, the SFKs, Hck, and, to a lesser extent, Lyn, but not Fyn or Src, mediated CSF-1R signals leading to the proteolysis of nucleophosmin [385].

Role of MicroRNAs in CSF-1-Induced Monocyte Differentiation Studies in human monocytes have shown that 45 microRNAs (miRs) were increased and 45 miRs were decreased during CSF-1-induced differentiation to macrophages [387].

Among these, miR132-3p, a hematopoietic-specific miR, was shown to suppress CSF-1R-induced monocyte differentiation by preventing the upregulation of early growth response 2 (Egr2), a transcription factor that suppresses granulocytic and promotes monocytic differentiation. CSF-1-induced elevation of Egr2 in turn suppressed miR132-3p expression and increased the expression of CSF-1R [387]. These data identify a novel molecular circuitry that regulates the monocyte to macrophage transition. In contrast, miR-mediated downregulation of the CSF-1R is a key event for the maturation of monocyte-derived dendritic cells [388].

CSF-1R Regulation of Myeloid Cell Proliferation

Early studies showed that CSF-1-stimulated BMM survival occurred at concentrations below those stimulating proliferation [37]. Cell survival was primarily associated with inhibition of total protein degradation, whereas the increase in total cellular protein, occurring at proliferation-inducing concentrations, was primarily associated with a CSF-1 dose-dependent increase in the rate of protein synthesis [389]. Following CSF-1 addition to CSF-1-starved cells, the growth factor was required for almost the entire lag period (~12 h) for the entry of macrophages into S phase [390], during which time the steady-state level of cell-surface CSF-1Rs was downregulated, but turning over rapidly and presumably signaling [217].

Positive Regulation of Myeloid Proliferation by the CSF-1R Studies in myeloid cells have shown that SFKs, ceramide-1P (C1P), MEK/Erk, PI3K, PLC, PKCζ, and β-catenin pathways contribute to the proliferative response in a CSF-1R pTyr-721-independent manner [335, 339, 391–395]. In mouse macrophages, C1P stimulates proliferation through activation of PI3K/Akt, JNK, and ERK1/2 pathways [395]. CSF-1R Tyr-559, required for CSF-1R ligand responsiveness, also contributes to the proliferative response through a SFK kinase-dependent pathway [335, 339], while CSF-1R Tyr-807 contributes by activating both the MEK and PI3K pathways [335].

In myeloid progenitors, Gab2 mediates the activation of the PI3K-Akt and Erk pathways that control progenitor cell survival and proliferation [378]. Multiple ERKs may be involved in the control of macrophage proliferation, as their contribution has been inferred from studies utilizing MEK inhibitors. A significant role has been assigned to ERK5, which is activated by the CSF-1R in a SFK-dependent manner [396]. ERK5 knockdown compromises the macrophage proliferative response to CSF-1 [396]. Interestingly, in actively proliferating *fes*-transformed macrophages, mitogenic concentrations of CSF-1 were shown to increase ERK1/2 activation, whereas low doses of CSF-1 reduced ERK1/2 phosphorylation, nuclear localization, and cell proliferation, indicating that low doses of CSF-1 can suppress cell proliferation by inhibiting ERK1/2 [393]. Concomitant with ERK1/2 activation, the CSF-1R also activates the membrane-associated PKCε, leading to the increased expression of mitogen-activated protein kinase phosphatase-1 (MKP-1, also known as DUSP-1) and thereby suppressing prolonged Erk1/2 activation and

preventing cell cycle arrest [397]. In myeloid progenitors, another member of the PKC family, PKCζ, was reported to mediate CSF-1-dependent proliferation and Erk1/2 activation [392]. PKCζ activity was increased by CSF-1 and overexpression of PKCζ increased the intensity and duration of Erk1/2 phosphorylation and rendered myeloid progenitors more responsive to CSF-1-induced proliferation. In contrast, in mature BMM, PKCζ inhibition had a modest effect on proliferation and induced a paradoxical increase in MEK and Erk phosphorylation, suggesting that PKCζ activates a negative regulatory step upstream of MEK [392].

In a MAPK- and Akt- independent manner, CSF-1R activation in macrophages induces the tyrosine phosphorylation of β-catenin, which requires the expression and tyrosine phosphorylation of the transmembrane adaptor protein, DAP12 [156]. DAP12-deficient humans and mice have defects in osteoclasts and microglia [398]. DAP12 deficiency in macrophages leads to impaired CSF-1R signaling for proliferation and survival in vitro, but does not affect differentiation [156]. The cytoplasmic tail of DAP12 contains immunoreceptor tyrosine-based activation motifs (ITAMs) which become phosphorylated in response to CSF-1R activation. Tyrosine-phosphorylated DAP12 ITAMs recruit and activate the cytosolic tyrosine kinase Syk which in turn activates another cytosolic tyrosine kinase, Pyk2. Pyk2 phosphorylates β-catenin, triggering its nuclear translocation. Nuclear β-catenin acts a coactivator of the transcription factors TCF and LEF, to induce the transcription of cell cycle genes, including cyclin D1 and c-Myc, thereby triggering cell proliferation. In a DAP12-independent manner, CSF-1R also inhibits the degradation of β-catenin by triggering the serine phosphorylation and inactivation of the serine–threonine kinase GSKβ.

Negative regulation of CSF-1R-induced myeloid proliferation Proteins that negatively regulate CSF-1R-mediated proliferative responses include the CSF-1R-interacting adaptor protein Lnk [399, 400], proline–serine–threonine (PEST)–phosphatase-interacting protein 2 (PSTPIP2) [401], suppressor of cytokine signaling 1 (Socs 1) [402], interferon-inducible P204 protein (Ifi204) [403], and Src kinase-associated phosphoprotein of 55 kDa (SKAP55)-related adaptor protein (SKAP55R).

Lnk is required for optimal Erk1/2 phosphorylation and inhibits the phosphorylation of Akt downstream of the CSF-1R. Lnk deficiency leads to the expansion of bone marrow CFU-M and of circulating monocytes [399, 400], but does not affect macrophage differentiation [399].

PSTPIP2 is a membrane-cytoskeletal adaptor expressed predominantly in the myeloid lineage [404]. PSTPIP2-deficient mice exhibit extramedullary hematopoiesis, increased tissue macrophages and osteoclasts, autoinflammatory disease, and osteopenia [22, 401, 405]. Absence of PSTPIP2 causes increased CSF-1-induced proliferation of myeloid precursors, while its overexpression in macrophages inhibited their growth. These phenotypes correlate with increased or decreased CSF-1-induced Erk1/2 phosphorylation [401]. PSTPIP2 interacts with PEST-family tyrosine phosphatases PTPN12 [22] and PTPN18 [406]. As recruitment of the PEST phosphatase PTPN12 to the T-cell receptor signalosome inhibits Erk1/2 activation [407],

it is possible that PSTPIP2 negatively regulates Erk1/2 by recruiting PTP-PEST to signaling complexes upstream of Erk1/2.

Socs1 is an adaptor protein that binds to all Janus kinase (JAK) family members and inhibits their tyrosine kinase activity, which is needed for the activation of signal transducers and activators of transcription (STATs). Socs1 is a negative regulator of Kit and Flt3 mitogenic signals [408]. Ectopic expression of Socs1 in hematopoietic cell lines decreased their growth rates in the presence of physiological, but not higher, concentrations of CSF-1 [402]. The molecular mechanism involved is unknown. It has been suggested that it may involve the interaction of Socs1 with signaling proteins other than JAK, e.g., Grb2 and Vav [402, 408].

Ifi204 is induced by the activated CSF-1R in FD-Fms cells and may act to tip the balance between CSF-1R-induced proliferation and differentiation toward differentiation [409].

SKAP55R is tyrosine phosphorylated following CSF-1R activation and associates with actin [410]. Overexpression of SKAP55R in FD-Fms cells decreased CSF-1R-induced proliferation but did not affect their differentiation into macrophages suggesting that SKAP55R is a negative regulator of macrophage progenitor cell growth.

Membrane and Cytoskeletal Reorganization and Chemotaxis

Addition of CSF-1 to macrophages triggers a rapid membrane ruffling response that peaks at 5 min and is followed by cell spreading and then by cell polarization, characterized by a leading edge formed by an extended lamellipod and a trailing edge that are characteristic of migratory cells [344, 346, 411]. All these processes involve dynamic reorganization of the actin cytoskeleton (reviewed in [347, 412]). CSF-1-stimulates a rapid actin polymerization response that peaks at 30 s of stimulation, followed by a longer lasting wave peaking at 5–6 min [368, 413]. After 5 min of CSF-1R stimulation, there is an increase in focal complex and point contact formation that coincides with the peak tyrosine phosphorylation of the focal adhesion kinases, Pyk2 and FAK, and is followed by peak focal complex formation at 15 min poststimulation, coincident with maximal phosphorylation of paxillin [345, 368].

As described above (Sect. 10.1.6.3.4), structure–function studies in macrophages have shown that the mechanism by which CSF-1 stimulates actin polymerization and adhesion critically requires the CSF-1R Tyr-721/PI3K pathway. PI3K p110δ is the major PI3K isoform recruited by the CSF-1R and the main regulator of actin polymerization, cytoskeletal remodeling, cell adhesion, and migration [414]. Indeed, specific inhibition of PI3K p110δ suppresses CSF-1-induced PIP3 production and Akt activation, as well as macrophage spreading and invasive capacity [415]. The exact pathway(s) by which induction of PIP3, the product of PI3K activation, mediates macrophage migration and chemotaxis remains to be elucidated. Loss of the p85α subunit of PI3K in BMM impairs their proliferation and chemotactic migration in response to CSF-1 and results in reduced activation of Akt and Rac but not Erk1/2 [416]. The essential role of Akt in CSF-1-induced

macrophage migration has been confirmed in several studies. Disruption of Akt2 expression inhibited the CSF-1-induced phosphorylation of PKCζ and downstream phosphorylation of LIMK/Cofilin, leading to defects in actin polymerization and chemotaxis [417]. In addition, two adaptor proteins that suppress CSF-1-induced activation of Akt, Lnk, and STAP-2 have also been shown to inhibit CSF-1-induced macrophage migration [399, 418].

CSF-1R-induced chemotaxis also requires the activation of the small GTPases, Rac, and Cdc42 and of their downstream effectors, members of the family of Wiskott–Aldrich syndrome protein (WASP), and WASP-family verprolin-homologous (WAVE)2 actin nucleators [413, 419–422]. Studies in SHIP-deficient macrophages, which exhibit increased and prolonged chemotactic responses to CSF-1, suggested that PI3K-induced elevation of PIP3 triggers the plasma membrane recruitment of Vav proteins, which become tyrosine phosphorylated in response to CSF-1 and activate the small GTPase Rac, thus triggering macrophage chemotaxis [423]. However, subsequent studies in single Vav-deficient BMM [424] as well as in triple-deficient Vav1/2/3 $^{-/-}$ BMM have shown that Vav proteins are not required for CSF-1-induced activation of Rac1, membrane ruffling, or cell spreading [425]. Furthermore, the CSF-1R Y721F mutation did not affect the ability of CSF-1 to activate Rac or Cdc42, suggesting that activation of the small GTPases is independent of the CSF-1RY721/PI3K pathway [368]. However, in primary BMM, neither Rac1 nor Rac2 were required for CSF-1-induced chemotaxis, and cells double deficient for Rac1 and 2 could also migrate to CSF-1 [426, 427]. In contrast, studies in macrophage cell lines suggest that Rac1 and 2 are important for CSF-1-induced membrane ruffling and chemotaxis [428, 429]. In RAW267.4 macrophages, CSF-1 stimulates the association of Grb2 with PLD2 which, in addition to its lipase activity, acts as a GEF for Rac2 [430, 431]. Grb2 association increases the lipase activity of PLD2 which cooperates with Rac2 to enhance CSF-1-stimulated membrane ruffling [430]. During chemotaxis, Rac2 has a dual effect on PLD, slightly enhancing its activity initially (positive feedback), but then inhibiting PLD by preventing its interaction with PIP2 at the plasma membrane during the late chemotactic response, thus leading to cell immobilization [432]. Other studies in RAW267.4 cells have shown that WAVE2 becomes activated downstream of Rac1 and IRSp53 and forms a complex with Abi1 that mediates the formation of F-actin-rich protrusions and macrophage migration in response to CSF-1 [420, 429]. Both CSF-1-induced activation of WASP and macrophage chemotaxis are fully dependent on the small GTPases Cdc42 and PI3K [421]. Cdc42-dependent activation of WASP is absolutely necessary for podosome formation, while combined CSF-1R-induced tyrosine phosphorylation and Cdc42-dependent activation of WASP are necessary for macrophage chemotaxis to CSF-1 [422].

Cell motility requires that the actin polymerization leading to the formation of adhesion structures be balanced by inhibitory proteins and actomyosin-dependent contractility for retracting the trailing edge. The membrane-cytoskeletal adaptor, PSTPIP2, is one of the earliest proteins to become tyrosine phosphorylated in macrophage response to CSF-1. PSTPIP2 phosphorylation peaks at 30 sec–1 min after CSF-1 stimulation, suggesting that it may be involved in the regulation of the early

responses involving plasma membrane and actin. Indeed, PSTPIP2 overexpression blunts the initial CSF-1-stimulated membrane ruffling, inhibits actin polymerization, and promotes migration and chemotaxis, while its reduced expression has opposite effects [346]. Modulation of the levels of expression of PSTPIP2 did not affect CSF-1R-induced activation of Rac, Rho, or Cdc42, indicating that PSTPIP2 acts either downstream or independently of small GTPases. The nonreceptor protein-tyrosine phosphatase ϕ (PTPϕ) increases motility and decreases adhesion in macrophages by dephosphorylating paxillin. In the presence of CSF-1, basal levels of paxillin phosphorylation are sufficient to sustain cell spreading and adhesion. In the absence of CSF-1, PTPϕ is upregulated leading to poor lamellipodial adhesion and increased dorsal ruffles [345].

S100A4 is a member of the S100 family of Ca^{2+}-binding proteins that interacts with and inhibits the assembly of non-muscle myosin II A [433]. S100A4$^{-/-}$ macrophages exhibit decreased chemotaxis to CSF-1, due to reduced persistence and size of membrane protrusions [434]. Associated with this phenotype are (1) persistent and enhanced actomyosin-IIA assembly that increases contractility and decreases actin polymerization in the lamellipodium; (2) altered CSF-1R signaling leading to the hyperphosphorylation of Pyk2, possibly triggered by enhanced myosin II assembly or by local disruption of Ca^{2+} homeostasis; and (3) hyperphosphorylation of paxillin, the downstream target of Pyk2, accompanied by paxillin mislocalization away from the leading edge.

The small GTPase, Rho, promotes tail retraction in migrating macrophages by controlling myosin activity [435] and is required for CSF-1-mediated macrophage chemotaxis [436]. In macrophages stimulated with CSF-1, Rho undergoes cycles of activation and deactivation. While the pathway mediating the activation of Rho by CSF-1R is unclear, RhoA deactivation is mediated by the PI3K p110δ/p190RhoGAP axis [437].

CSF-1R Regulation of Myeloid Cell Survival

Several antiapoptotic pathways involving lipid and protein mediators are activated by CSF-1R. CSF-1 withdrawal stimulates acid sphingomyelinase activity in macrophages leading to the accumulation of ceramides which, in turn, induce macrophage apoptosis [438]. In contrast, CSF-1 activates ceramide kinase, which phosphorylates ceramide generating C1P, which in turn inhibits the acid sphingomyelinase, enhances the activity of the PI3K/Akt pathway, and maintains the production of the antiapoptotic factor Bcl-X(L), thereby promoting cell survival [439, 440].

The PI3K/Akt pathway is a major mechanism by which CSF-1 promotes cell survival [441–444], while the Erk, p38, and JNK1 pathways are not involved in this process [445]. In macrophages, Akt can be activated directly through the CSF-1R pTyr721/PI3K pathway [368, 446] and indirectly, via Gab2, through CSF-1R pTyr559/SFK/PI3K signaling [335, 378, 446]. This last pathway is counteracted by a CSF-1R pTyr559/Lyn/SHIP-1 pathway in which SH2-containing inositol 5′-phosphatase 1 (SHIP-1) is recruited by Lyn to the plasma membrane, where it antagonizes the

increase in PI3K-induced phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) and the $PI(3,4,5)P_3$-dependent activation of Akt [447]. The transcription factor Ets2 is a downstream target of the PI3K/Akt/JNK pathway [448] that mediates increased expression of Bcl-x(L) and promotes macrophage survival [449, 450].

PI3K-independent pathways regulating CSF-1R survival signals involve phospholipase C (PLC) [394] and Fms-interacting protein (FIMP) [451]. PI3K and PLC independently enhance CSF-1-dependent macrophage survival by controlling glucose uptake [442]. Physiological stimuli such as hypoxia [452], or oxidized LDL [453], can prolong macrophage survival, either in the absence or presence of low CSF-1 concentrations, by enhancing the glycolytic activity which in turn is required to maintain Bcl-2 and Bcl-x(L) protein levels [453].

FIMP is transiently associated with and tyrosine phosphorylated by the activated CSF-1R. A substantial proportion of FIMP is nuclear localized, where it inhibits CSF-1R-mediated signaling for survival [451]. CSF-1R-regulated PKC-dependent serine phosphorylation translocates FIMP to the cytosol, enhancing macrophage survival and differentiation [451].

10.1.6.5 CSF-1R Signaling in Osteoclast Differentiation, Activation, and Survival

Role of the CSF-1R in Osteoclastogenesis

CSF-1 is essential for osteoclast (OC) development in vivo [15, 48, 49, 51, 454]. In combination with RANKL, CSF-1 drives OC differentiation from myeloid progenitors [455]. CSF-1 also sustains the survival of mature mouse OC [456, 457] and stimulates the spreading [458, 459] and migration [456, 460] of these cells.

Studies employing human umbilical cord-derived CFU-GM as OC precursors have shown that CSF-1 has similar developmental actions in human osteoclastogenesis [461]. As in mouse, CSF-1 appears to play a nonredundant role in human osteoclastogenesis, as it cannot be substituted by GM-CSF, FLT3 ligand, or VEGF [462]. IL-34 can substitute for CSF-1 in the stimulation of OC differentiation in vitro, or when expressed under control of the CSF-1 promoter in vivo [9]. However, OC differentiation is unaffected in *Il34*$^{-/-}$ mice [13]. In vitro, the effects of CSF-1 on OC differentiation are biphasic, with low concentrations (in the normal physiological range) enhancing OC precursor fusion and high concentrations inhibiting OC precursor fusion and promoting macrophage differentiation [461, 463]. Furthermore, addition of CSF-1 during the OC fusion phase caused increased cytoplasmic spreading and inhibited resorption, while blockade of CSF-1 had no effect on the number of OC formed, nor their size [461]. These data are consistent with previous studies in mouse bone marrow cultures showing that blockade of CSF-1 during the proliferative phase of osteoclast formation dramatically inhibited osteoclastogenesis, but had very little effect after the onset of osteoclast fusion [464]. They suggest that the major role of CSF-1 is to drive the expansion of osteoclast precursors and their maturation to a fusion-competent state, but that CSF-1 is not required for OC precursor fusion per se.

CSF-1R Signaling for OC Differentiation

The developmental signals of activated CSF-1R and RANK converge at the level of transcription factors essential for OC differentiation. The transcription factors TRAF6, NFkB, c-Fos, Mitf, NFATc, and C/EBPα play essential roles in OC differentiation, and their deficiency leads to the loss of multinucleated OC and severe osteopetrosis without impairing macrophage development [465–467]. CSF-1 induces RANK expression in OC precursors [468] in a c-Fos-dependent manner [469]. This process is inhibited by activated TLRs, which promote matrix metalloproteinase-mediated CSF-1R shedding, thus diverting OC precursors to a monocytic fate [470]. The combined actions of CSF-1 and RANKL induce high C/EBPα expression in murine bone marrow cells [466]. Based on the observation that the ectopic expression of C/EBPα induced RANK, c-Fos and NFATc1 expression, and reprogrammed monocytic cells to OC-like cells in the absence of RANKL, it has been suggested that C/EBPα is the key transcriptional regulator of OC lineage commitment [466]. RANKL induces the p38 MAPK-dependent phosphorylation of Mitf at Ser307, leading to increased tartrate-resistant acid phosphatase expression [471]. Furthermore, the CSF-1R-activated, Erk1/2-mediated phosphorylation of Mitf at Ser73 is required for the upregulation of TRAP activity and OC precursor fusion [472]. Another event critical for OC differentiation is the Tbx3-dependent induction of Jun dimerization protein 2 (JDP2) expression by the CSF-1/Erk1/2 pathway [473]. JDP2 mediates RANKL-induced upregulation of the TRAP and cathepsin K expression [474].

The small GTPase Cdc42 is another downstream target of both CSF-1R and RANKL signaling. Cdc42 is required for CSF-1 activation of p38 and the Akt/GSK3β pathway, but not of ERKs, and also mediates the RANKL-induced activation of p38, ERK, JNK, and Akt, but not NFkB. Cdc42 promotes CSF-1-driven proliferation in OC precursors and their osteoclastic differentiation by enhancing the expression of NFATc1 and RANKL-induced phosphorylation of Mitf [475]. In vivo, selective OC-specific ablation of Cdc42 using cathepsin K-Cre led to increased bone density and protection from ovariectomy-induced bone loss, while a Cdc42 gain-of-function mouse model exhibited a reciprocal phenotype [475]. The small GTPase RhoU/Wrch is the only Rho GTPase induced by RANKL during osteoclastogenesis [476]. Wrch inhibits CSF-1-induced OC precursor migration and integrin-mediated adhesion while simultaneously promoting their aggregation and fusion [477].

Although not critically required for basal bone homeostasis in vivo, the SFKs, Fyn and Lyn, have also been implicated in the fine-tuning of CSF-1R and RANK signaling for OC precursor proliferation, OC differentiation, and bone resorptive activity. In the monocytic/OC lineage, SFKs Fyn and Lyn are expressed in BMM and throughout osteoclastogenesis, while Src is absent from BMM and its expression is a marker of commitment toward the OC lineage [478–480]. Mice deficient in Fyn or Lyn exhibit normal basal osteoclastogenesis and bone density. However, their responses to RANKL-stimulated osteoclastogenesis in vivo were suppressed and increased, respectively. Lyn deficiency results in enhanced RANKL signaling

for OC differentiation but does not alter CSF-1-mediated activation of Erk1/2, OC precursor proliferation, or the bone-resorbing activity of mature osteoclasts [478]. In contrast, Fyn deficiency reduced CSF-1-stimulated proliferation of BMM and OC precursors, delayed differentiation, diminished osteoclastogenesis, and increased susceptibility of mature OC to apoptosis in vitro. While CSF-1 signaling to ERK1/2 and Akt was normal in $Fyn^{-/-}$ BMM, CSF-1-induced phosphorylation of Lyn was increased. In contrast, RANK-induced activation of Akt, IkB, and c-Jun was decreased [479]. Deficiency in the Hck or Src SFKs leads to cytoskeletal defects that cause either abnormal OC development or impaired bone resorption leading to osteopetrosis [480, 481]. OCs develop normally in $Src^{-/-}$ mice; however, they fail to form ruffled borders or resorb bone [481]. $Hck^{-/-}$ OC precursors exhibit defective podosome organization and migration through three-dimensional matrices, resulting in impaired bone homing of preosteoclasts and abnormal development of trabecular bone. In contrast, mature OCs exhibit normal cytoskeletal structures and enhanced bone resorption in vitro, probably due to a compensatory overexpression of Src [480].

Adaptor proteins such as SH3BP2, PSTPIP2, SLAP, and DAP12 play important roles in the control of OC development by CSF-1. Gain-of-function mutations in SH3BP2 lead to cherubism, an autoinflammatory disorder that is associated with enhanced CSF-1 and RANKL-mediated activation of Erk1/2 and Syk, leading to the formation of unusually large OCs, which resorbs the mandible and/or maxillar bone [482]. PSTPIP2 deficiency in mice also leads to an autoinflammatory disorder and osteopenia that is associated with increased numbers of OC and OC precursors. Multipotent myeloid precursors isolated from PSTPIP2-deficient mice have an increased propensity to form OC in the presence of CSF-1 and RANKL. In contrast, the ectopic expression of PSTPIP2 in OC precursors inhibits both TRAP expression and OC precursor fusion, while mutations that eliminated PSTPIP2 tyrosine phosphorylation in response to CSF-1R activation block the inhibitory activity, suggesting that PSTPIP2 is part of a negative feedback loop [22]. The adaptor protein, SLAP, regulates OC development and survival, but is not essential for bone homeostasis in vivo [483]. $SLAP^{-/-}$ mouse bone marrow cells compared with wt cells exhibit increased osteoclastogenic responses. In BMM, SLAP associates with the CSF-1R in lipid rafts and its deficiency leads to increased CSF-1-induced Erk1/2 activation and increased BMM proliferation without affecting RANKL signaling. In mature OC, SLAP is not necessary for bone resorption. However, its deficiency leads to increased caspase-3 activation and OC apoptosis following CSF-1 and RANKL withdrawal, suggesting that $SLAP^{-/-}$ mice counterbalance the effects of SLAP deficiency on OC differentiation and survival, to maintain normal OC numbers [483].

DAP12-deficient mice exhibit mild osteopetrosis and their cultured bone marrow cells differentiate into bone-resorbing TRAP+ mononuclear cells that fail to fuse into multinucleated OC in the presence of CSF-1 and RANKL suggesting that DAP12 regulates OC precursor fusion [484, 485]. In contrast, mouse BMM from $DAP12^{-/-}$ mice form multinucleated osteoclasts in vitro that exhibit cytoskeletal defects resulting in loss of bone resorptive activity [486]. Mice overexpressing

DAP12 have an osteopenic bone phenotype and an increased numbers of OC. The increase in OC numbers is associated with an increased rate of osteoclastogenesis from splenic, but not from the bone marrow cells, due to a greater splenic abundance of CFU-GM and CFU-M that hyperproliferate in the presence of CSF-1 and RANKL [487]. In contrast, DAP12$^{-/-}$-cultured splenocytes did not exhibit hyperresponsiveness to increasing concentrations of RANKL, indicating that RANK signaling was not affected. As DAP12 mediates the proliferative signals of the CSF-1R [156], it is possible that increased CSF-1R-driven amplification of OC progenitors explains the early OC differentiation of DAP12-overexpressing cells.

In osteoclast precursors, DAP12 associates with triggering receptor expressed in myeloid cells (TREM)-2 [488]. siRNA-mediated reduction of TREM2 expression in RAW 264.7 macrophages results in loss of OC formation in response to RANKL and CSF-1 [488], and DAP12- and TREM2- deficient human blood monocytes fail to fuse and form multinucleated OC in vitro [489, 490]. However, a recent study shows that TREM2$^{-/-}$ mice are osteopenic, and this phenotype is associated with decreased cell proliferation and accelerated differentiation during osteoclastogenesis and anomalies in CSF-1, but not RANK, signaling in bone marrow macrophages [491]. Compared to wt controls, CSF-1 stimulation of day 4 TREM2$^{-/-}$ BMM leads to enhanced phosphorylation of Erk1/2 and JNK and decreased β-catenin stabilization, all of which occur independently of DAP12 (see Sect. 10.1.6.4.2). In addition, DAP12-mediated CSF-1R signaling events including the phosphorylation of Syk, Pyk2, and β-catenin, as well as β-catenin nuclear translocation, were decreased [491]. Thus, signals emerging from TREM2 regulate CSF-1R signaling in osteoclast precursors at multiple levels.

CSF-1R also interacts functionally with integrins to promote osteoclastogenesis [364]. Bone marrow cells isolated from integrin $\beta_3^{-/-}$ mice exhibit defective upregulation of TRAP activity and fusion but normal proliferation and survival. The arrested differentiation was corrected by high-dose CSF-1 and required CSF-1R Tyr-697, but not CSF-1R Tyr-721, nor CSF-1R Tyr-921. In $\beta_3^{-/-}$ pre-OCs, CSF-1R Tyr-697 was specifically required for sustained CSF-1R-triggered Erk1/2 phosphorylation of ribosomal S6 kinase 2 (RSK2) and downstream activation of c-Fos. Like high-dose CSF-1, the overexpression of c-Fos rescued $\beta_3^{-/-}$ deficiency, showing that CSF-1R and $\beta_3^{-/-}$ collaborate via the shared activation of the Erk1/2 and c-Fos pathways.

Targeted disruption of either the inositol phosphatase SHIP or the SH2 domain-containing protein-tyrosine phosphatase-1 (SHP-1) resulted in increased osteoclastogenesis leading to osteoporosis, indicating that these nonreceptor phosphatases suppress osteoclastogenesis. *SHIP1*$^{-/-}$ mice have severe osteoporosis [492], and their BMM exhibit increased osteoclastogenic responses due to increased CSF-1-driven proliferation of OC precursors that is associated with increased PI3K/Akt activation, but unaltered MAPK pathway activation [493]. Viable motheaten (*mev*/*mev*) mice, in which tyrosine phosphatase activity of cytosolic enzyme SHP-1 is decreased by 80–90 %, show severe defects in hematopoiesis and osteopenia due to an increased number of osteoclasts and enhanced bone resorptive activity [494]. In macrophages, SHP-1 also interacts via its SH2 domain with the immunoreceptor

tyrosine-based inhibitory motif (ITIM)-containing receptors, SIRPα (also known as BIT) [495], the human leukocyte Ig-like receptor B (LILRB), and its mouse homologue, murine paired Ig-like receptor B (PIR-B) [496, 497]. Activation of LILRB receptors suppresses the differentiation of human $CD14^+$ monocytes into mononuclear osteoclast precursors, while PIR-B deficiency accelerates OC differentiation in bone marrow cells exposed to CSF-1 and RANKL [496]. Currently, it is unclear whether SHP-1/PIR-B complexes modulate CSF-1R and/or RANK activation. If present, the effect of SHP-1/PIR-B complex on CSF-1R signaling is likely to be indirect, as PIR-B does not interact with CSF-1R in macrophages [495, 497]. In contrast, SIRPα constitutively interacts with CSF-1R and mediates its association with SHP-1 [495]. SIRPα deficiency in mice leads to reduced cortical bone mass and increases OC actin ring formation while not affecting osteoclastogenesis [498]. SHP-1 also interacts constitutively with the adaptor protein, Dok-1, and negatively regulates CSF-1-induced tyrosine phosphorylation of Dok-1 [499]. Dok-1 deficiency does not affect bone structure, presumably because of redundancy with other Dok family members. However, double-deficient Dok-$1^{-/-}$/Dok-$2^{-/-}$ mice exhibit osteopenia, with increased numbers of osteoclasts. Their bone marrow cells exhibit increased osteoclastogenic responses at suboptimal concentrations of CSF-1, and their mature osteoclasts have increased bone resorptive capacity [500]. Thus, SHP-1 may play an important role in the negative regulation of CSF-1R signaling in OC precursors and mature OC.

CSF-1R Regulation of OC Migration and Bone Resorption

The CSF-1R regulates OC cytoskeletal organization, migration, chemotaxis, and resorption [501–503]. In mature human OC cultured on dentine, co-treatment with CSF-1 augmented RANKL-induced bone resorption by increasing the number of activated OCs [502]. CSF-1 facilitated RANKL-induced activation of c-Fos and increased Erk1/2 phosphorylation, but did not enhance NFκB nor NFATc1 activation. The MEK1 inhibitor, PD98059, partially blocked augmentation of resorption [502]. However, how the MEK/Erk/c-Fos pathway enhances bone resorption in mature human OC remains to be clarified.

Migration and resorption represent alternative states of osteoclast behavior. In vitro, nonresorbing osteoclasts move on the bone surface and do not show clear evidence of apical–basolateral polarity. Bone-resorbing osteoclasts are nonmotile and form an adhesion structure known as the sealing zone between the ruffled border and the rest of the cell membrane [504]. The sealing zone contains F-actin-rich podosomes and $\alpha_v\beta_3$ integrins [503, 505]. Inhibition of integrin-mediated adhesion using echistatin, a disintegrin containing RGD motifs, leads to the disruption of the sealing zone and inhibition of bone resorption [505]. A structure analogous to the sealing zone is the peripheral podosome belt formed after plating mature osteoclasts on glass. When stimulated with CSF-1, mature rodent osteoclasts exhibit a rapid spreading and chemotactic response concomitant with a decrease in their resorptive capacity [506, 507]. The spreading response is accompanied by the dissolution of

the podosome belt and the reorganization of F-actin in peripheral ruffles and perinuclear punctae [503, 508, 509]. At the same time, CSF-1 induces the tyrosine phosphorylation and activation of multiple proteins, including c-Src [503] and PI3K [507, 509, 510]. Early studies indicated that the CSF-1-induced spreading and chemotactic response are critically dependent on PI3K [507, 510]. However, disruption of the CSF-1R-p85 binding site (CSF-1R Y721F) does not suppress cytoskeletal remodeling or actin ring formation in osteoclasts [508]. Indeed, the simultaneous osteoclast-specific deletion of the mouse p85 α and β genes results in an osteopetrotic phenotype associated with decreased bone-resorbing activity, but normal sealing zone formation in osteoclasts cultured on mineralized matrix [509]. The bone resorptive defect is due to the inability of CSF-1 to activate Akt, resulting in the absence of a ruffled border and defective transport of cathepsin K-containing vesicles at the plasma membrane [509]. These defects are rescued by the expression of constitutively active Akt. Thus, CSF-1R signaling through PI3K/Akt is required for the ruffled border formation and vesicular transport, but not for formation of the sealing zone.

In mature OC, the Y559F mutation, which abrogates the SFK-binding site, blocks cytoskeletal reorganization [508], and several lines of evidence suggest that the SFK regulating the pathway mediating CSF-1R-induced actin remodeling in OC is c-Src: (1) deficiencies in Fyn or Lyn do not affect actin organization in OC[478, 479]; (2) Hck controls podosome formation in pre-OC before Src expression is upregulated, but is not essential for podosome formation or bone resorption by mature OC in which Src is highly expressed [480]; and (3) $Src^{-/-}$ mature OC plated on glass fails to form a podosome ring, and their F-actin reorganization in response to CSF-1 is abolished [503].

The small GTPase Wrch/RhoU was proposed as a mediator of cytoskeletal remodeling in OC downstream of the CSF-1R/Src pathway. The plasma membrane localization and activation of Wrch are suppressed by Src-mediated phosphorylation of Tyr-254 [511]. Studies in RAW264.7 cells have shown that Wrch specifically inhibits CSF-1-driven pre-OC migration and promotes OC fusion [477]. Overexpression of wt and constitutively active Wrch, but not of a GTPase inactive mutant, in mature OC leads to the reorganization of the podosomes from a peripheral belt configuration to clusters and rings distributed through the adherent surface of the cell. However, sealing zone formation and mineralized matrix resorption were normal, suggesting that Wrch is more relevant for OC development than for their activity.

In osteoclasts, the activated CSF-1R stimulated formation of a SFK/c-Cbl/PI3K complex in a CSF-1R pTyr-559-dependent manner leading to the downstream activation of the Rho/Rac guanine nucleotide exchange factors Vav3 and Rac [508]. Vav3 mediates CSF-1-induced activation of Rac1 in osteoclasts [512] and is a key mediator of CSF-1-induced OC spreading [458]. Rac1 and 2 mediate CSF-1-induced chemotactic migration in preosteoclasts and mature osteoclasts [508, 513, 514].

Another pathway through which CSF-1R pTyr-559-dependent activation of SFKs regulates OC cytoskeletal organization is mediated by DAP12 [486]. The phosphorylation of the ITAM motifs in DAP12 triggers the recruitment and

activation of the nonreceptor tyrosine kinase, Syk, via SH2 domain-mediated interactions [486]. Activated Syk phosphorylates the Src homology region 2 (SH2) domain-containing leukocyte protein-76 (SLP-76) adaptor which in turn recruits and mediates the tyrosine phosphorylation of Vav3 [515]. It is unclear whether the CSF-1R pTyr-559/Src/DAP12/Syk/SLP76 pathway induces OC migration, which would be expected to lead to a simultaneous decrease in bone resorptive capacity. Syk is not necessary for macrophage chemotaxis to CSF-1 [420], and deletion of DAP12, Syk, or SLP76 yields osteoclasts that fail to organize their cytoskeleton and exhibit defects in bone resorption [486, 515–517]. Furthermore, although both Syk and SLP-76 are necessary for CSF-1R- and adhesion-induced tyrosine phosphorylation of Vav3 in pre-OC, SLP76 deficiency does not impair the activation of Rac. Thus, it is possible that activation of the CSF-1R/Src/DAP12/Syk pathway in mature OC mediates cell spreading without promoting a migratory phenotype. While the requirement for the CSF-1R pTyr-559/Src pathway for CSF-1-triggered reorganization of the OC cytoskeleton has been clearly demonstrated, it should be noted that CSF-1R Tyr-559 is not sufficient to mediate CSF-1R-induced OC spreading, membrane ruffling, and migration, which requires cooperative signals triggered by Tyr-697 and Tyr-721 [508].

The CSF-1R also initiates a negative feedback pathway leading to phosphorylation of Syk at Tyr-317, which promotes Syk association with Cbl, the ubiquitination and degradation of Syk, and attenuation of OC function [516]. The Y317F mutation abolishes the association of Syk with Cbl resulting in CSF-1-induced hyperphosphorylation of the cytoskeleton-organizing molecules, SLP76, Vav3, and PLCγ2, and increased resorptive capacity of the osteoclasts [516].

CSF-1R Signaling for OC Survival

CSF-1 and RANKL withdrawal triggers rapid OC apoptosis by inducing the upregulation of the proapoptotic Bcl-2 family member Bim, a process which is suppressed in OC precursors by the tyrosine kinase Fyn [479] and in mature OC by the small GTPase Cdc42 [475]. In contrast, the activated CSF-1R was shown to induce Cbl-dependent ubiquitination and degradation of Bim in OC [518]. In OC precursors, CSF-1 exerts antiapoptotic effects through the upregulation of the antiapoptotic protein Bcl-X(L) which in turn inhibits caspase-9-mediated apoptosis [519]. CSF-1R promotes the survival of mature osteoclasts by activating mTOR/S6 kinase through multiple pathways including SLAP, PI3K/AKT, Erk, and geranylgeranylated proteins [483, 520]. In addition to its role in OC differentiation (Sect. 10.1.6.5.2), Mitf was shown to be necessary for the expression of the antiapoptotic protein Bcl-2 in the OC lineage. Since both Mitf- and Bcl2-deficient mice are osteopetrotic [521], it is possible that Mitf activation by CSF-1 and RANKL also contributes to OC survival. Another mechanism through which CSF-1 promotes OC survival is by stimulating the electroneutral Na^+/HCO_3^- co-transporter, NBCn1, leading to alkalinization of intracellular pH and inhibition of caspase-8–mediated apoptosis [457].

10.1.6.6 CSF-1R Signal Transduction Regulated by IL-34

Transgenic expression of IL-34 under the control of *Csf1* promoter rescues the developmental defects of *Csf-1*$^{op/op}$ mice to the same degree as the secreted glycoprotein isoform of CSF-1, demonstrating that the capacity of IL-34 to support CSF-1R signaling for monocyte and osteoclast differentiation is comparable to that of CSF-1 [9]. However, studies of the phenotype and function of the macrophages differentiated in the presence of IL-34 or CSF-1 reveal several differences in the biological activity of these ligands. In human peripheral blood mononuclear cells, cultured (PBMC) IL-34 cannot induce macrophages with elongated morphology to the same extent as CSF-1, and macrophages grown in IL-34 produced more eotaxin-2 and less MCP-1 than those grown in CSF-1 [14]. Human CD14^{+} monocytes exposed to IL-34 exhibit an immunosuppressive phenotype very similar to that induced by CSF-1 [82]. However, transcriptional profiling suggests that, although both IL-34 and CSF-1 regulate overlapping sets of genes, the effects of IL-34 on gene expression are dampened for about 25 % of the genes tested [522]. One difference, the decreased ability of IL-34 to repress CCR2, the receptor for the monocyte chemoattractant proteins (MCP) [523], raises the possibility of differential effects of IL-34 and CSF-1 in fine-tuning inflammatory responses, since the MCP-1/CCR2 axis is one of the key pathways that regulate migration and tissue infiltration of inflammatory monocytes in response to inflammation [524, 525].

The strength and duration of CSF-1R signaling elicited by the two ligands is also slightly different. IL-34 exhibits a reduced ability to activate the phosphorylation of p70 ribosomal S6 kinases and Stat proteins in human monocyte cultures [14]. Moreover, using a human pre-myeloid cell line stably transfected to express human CSF-1R (TF-1-fms), Chihara et al. have shown that compared to CSF-1, IL-34 induces a stronger but more transient tyrosine phosphorylation of several proteins including the adaptor protein p66Shc, focal adhesion kinase (FAK), and CSF-1R tyrosine residues 546 and 809 [14]. Thus, while signaling through the same receptor, CSF-1 and IL-34 elicit quantitatively different responses.

10.1.7 Regulation of Gene Expression by CSF-1R

Consistent with the roles of CSF-1R in adult physiology (Sect. 10.1.2), studies in mouse models have shown that among the genes upregulated by the CSF-1/CSF-R complex are regulators of cell survival, proliferation, and differentiation (Bcl-xL, c-Myc, Cyclin D1, c-Fos, JunB) [357, 526–529]; chemokines, chemokine receptors, and other genes involved in the control of inflammation (MCP-1, MCP-3, IP-10, IL-10 and urokinase plasminogen activator) [87, 205, 530]; as well as genes involved in cholesterol synthesis and efflux (DHCR24, HMGCR, MVD, IDI1, FDPS, SQLE, CYP51A1, EBP, NSDHL, DHCR7, and ABCG1)[205, 530]. Representative genes repressed by the CSF-1/CSF-R complex include Toll-like receptors TLR1, TLR2, TLR6, and TLR9 [531] and antiatherogenic proteins

(apolipoprotein E, CXCR4) [530]. Global analysis of gene expression using microarrays revealed that both IL-34 and CSF-1 induce in macrophages a gene expression signature that is similar to that of the anti-inflammatory M2 macrophages [82, 87]. Transcriptional profiling of human macrophages has shown that 9,436 genes exhibited a response to CSF-1 or IL-34 of a ≥1.7-fold upregulation or downregulation of mRNA after 7 days in culture with minor differences in magnitude or direction of change between the two ligands [522].

10.1.8 CSF-1R Internalization, Processing, and Attenuation

As described (Sect. 10.1.6.2.3), CSF-1 activates a CSF-1R pTyr-559/SFK/Cbl pathway that mediates CSF-1R multiubiquitination, permitting full CSF-1R tyrosine phosphorylation and kinase activation. CSF-1R ubiquitination also triggers internalization of the CSF-1/CSF-1R complex, which is followed by the degradation of both receptor and ligand [217, 252, 253]. CSF-1R pTyr-559 signaling is necessary and sufficient to trigger CSF-1-induced CSF-1R degradation as (1) CSF-1R degradation was significantly reduced in CSF-1R Y559F macrophages compared with CSF-1R WT- or CSF-1R Y807F- expressing cells and (2) the Y559AB CSF-1R displayed WT-like ubiquitination and degradation kinetics, whereas the Y807AB CSF-1R failed to undergo significant degradation [253]. Degradation of the internalized CSF-1 and CSF-1R is primarily intralysosomal [217, 252].

LPS and phorbol esters, which activate PKC, were shown to cause loss of cell-surface CSF-1Rs due to extracellular domain cleavage [532–535], and phorbol esters were also shown to generate a cleaved intracellular domain fragment [534]. Cytokines also downmodulate CSF-1Rs. These include IL-3 and GM-CSF [536] and the macrophage-activating cytokines, IL-2 [537] and IL-4 [538], the IL-2 and IL-4 effects exhibiting partial PKC and phospholipase C dependence. The macrophage activator, IFN-γ, does not downmodulate CSF-1Rs, but enhances the effects of LPS possibly through a rapid block of CSF-1R signaling [535, 538]. The phorbol ester- or LPS-induced shedding of the CSF-1R extracellular domain in response to macrophage activation is mediated by TNF-converting enzyme (TACE) which occurs between residues Gln^{503} and Ser^{504} [539, 540]. A second cleavage, by γ-secretase, occurs at two sites located in the CSF-1R transmembrane domain (main cleavage site between Leu^{532} and Leu^{533} and minor site between Leu^{535} and Tyr^{536}) [540], releasing a cleaved CSF-1R intracellular domain to the cytoplasm, where it is ubiquitinated and rapidly degraded by the proteasome [541, 542]. This two-step process, known as regulated intramembrane proteolysis or RIPping, also occurs when macrophages are stimulated by a variety of TLR agonists [543]. Induction of CSF-1R RIPping by macrophage activators could prevent both the expansion and prolonged survival of tissue-destructive macrophages as well as prevent their reversion to an alternatively activated (M2) state.

10.1.9 Perspective

The discovery of the new CSF-1R ligand, IL-34, provides additional mechanisms for CSF-1R regulation in development, immunity, and disease. As the prime regulator of tissue macrophage development and maintenance, the CSF-1R is important for innate immunity and for critical trophic and scavenger roles played by macrophages in the development of many tissues. This, together with the requirement of the CSF-1R for osteoclast development, also confers on this receptor a central role in the development and progression of many inflammatory and degenerative diseases. The importance of CSF-1R-regulated tumor-associated macrophages in tumor progression and metastasis, even in the case of tumors not expressing the CSF-1, renders the CSF-1R a novel target for multiple types of cancer. The demonstration that dominant inactivating mutations in the CSF-1R kinase domain lead to ALSP provides opportunities to further understand the role of the CSF-1R in brain and to develop new approaches to ALSP diagnosis and treatment. The recent description of cell-autonomous regulation by the CSF-1R in Paneth cells and in neural progenitor cells indicates that much remains to be learned about the fascinating biology of this RTK, how it signals, and its disease relevance.

10.2 FLT3[2]

10.2.1 Introduction to the FLT3 Receptor Tyrosine Kinase

Fms-like tyrosine kinase 3 (FLT3, also known as fetal liver kinase2 (Flk2), or CD135) is expressed in several hematopoietic lineages [547] and is activated by its cognate ligand, FLT3 ligand (FL) [548] which is expressed in soluble form or displayed as an active ligand on the cell surface. This class III receptor tyrosine kinase (RTK) is also detected in placenta, gonads, and brain [549, 550], where its roles remain to be established. In immature hematopoietic cells [550, 551], FLT3 controls the expansion, maintenance of clonogenic potential and self-renewal of early hematopoietic cells, and the proliferation and differentiation of dendritic cells (DC), B-cell progenitors, and myelomonocytic cells in a cell-autonomous fashion [550, 552–554]. Non-cell autonomously, it regulates the activation of natural killer (NK) and T regulatory (T reg) cells [555–557]. FLT3 signaling pathways include the phosphoinositide 3-kinase (PI3K)/Akt and the MAPK/ERK pathways [558, 559]. Constitutively activating mutations of FLT3 occur in hematological malignancies, most frequently in acute myeloid leukemia (AML), where they are predictive of poor outcome [556, 560]. Unlike the ligand-activated wild-type receptor, these mutant receptors phosphorylate STAT5 and upregulate STAT5 targets [556, 559], activities associated with their strong transforming potential, pronounced

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antiapoptotic effects, and suppression of myeloid cell differentiation [559, 561, 562]. The mutant FLT3 receptors exhibit impaired maturation, accumulating as incompletely glycosylated proteins in the endoplasmic reticulum [563, 564]. This information, together with the elucidation of the structure of the extracellular FLT3 receptor–FL ligand complex [565] and of the FLT3 kinase domain [566], has provided rationales for FLT3 inhibitor design. Small chemical inhibitors have been developed to target FLT3 folding, stabilization, activation, or biogenesis [567, 568].

10.2.2 The Role of the FLT3 Receptor Tyrosine Kinase in Embryonic Development and Adult Physiology

Despite effects on development of multipotent stem cells and lymphoid differentiation [569], *flt3*$^{-/-}$ mice survive to adulthood and remain healthy with a normal mature blood cell profile (Sect. 10.2.4.1). Thus, possible roles of FLT3 in embryonic development have not been studied. Instead, studies have focused on the role of FLT3 in the development of DC, B cells, and T cells from hematopoietic stem cells (HSC) as well as in NK cell activation. In hematopoietic organs, FLT3 expression is restricted to HSC and early committed progenitors, such as lin-kit+sca-1+ (LSK) bone marrow cells and lin-AA4.1+ fetal liver cells, and early B lymphocyte subsets. FLT3 is also expressed at low levels on monocytes [553, 570] (Fig. 10.10). FL binding is not detected on promyelocytes, myelocytes, promonocytes, metamyelocytes, polymorphonuclear cells, eosinophils, or nucleated erythroid cells [553].

Role of FLT3 in DC Development Dendritic cells (DC) are bone marrow-derived cells detected in lymphoid and most nonlymphoid tissues [571]. They are derived from common myeloid progenitor (CMP), from common lymphoid progenitors (CLP) [572], or from the recently described lymphoid-primed multipotent progenitors (LMPP) that are capable of forming DC, macrophages, and lymphoid cells, but lack erythroid, granulocyte, or megakaryocyte potential [573] (Fig. 10.10). DCs are classified into plasmacytoid DC that have the unique ability to produce type I interferon during infection (pDC, or type 1 interferon-producing cells (IPC)), lymphoid-tissue resident or classical DC (cDC), and nonlymphoid tissue or migratory DC such as epidermal DC (or Langerhans cells, LC) and dermal DC [572]. With respect to this classification, FLT3 is continuously expressed from progenitor cells to steady-state DC [574–577], and FL is considered a major, nonredundant cytokine required for DC development in mice [578–580], instructing the differentiation of progenitor cells into cDC and pDC [580–585]. Elevation of FL levels, either by injection or overexpression of FL, increases pDC and cDC numbers in all lymphoid and nonlymphoid organs [578, 586–588]. Conversely, mice with a targeted deletion of *flt3l* or *flt3* display dramatically reduced cDC, pDC, and interstitial dermal DC [578, 579], and treatment of mice with a FLT3 inhibitor dramatically reduces lymphoid-organ pDC and cDC [580, 589], demonstrating that mouse FL controls DC development in a FLT3-dependent manner. Constitutive depletion of DC in mice

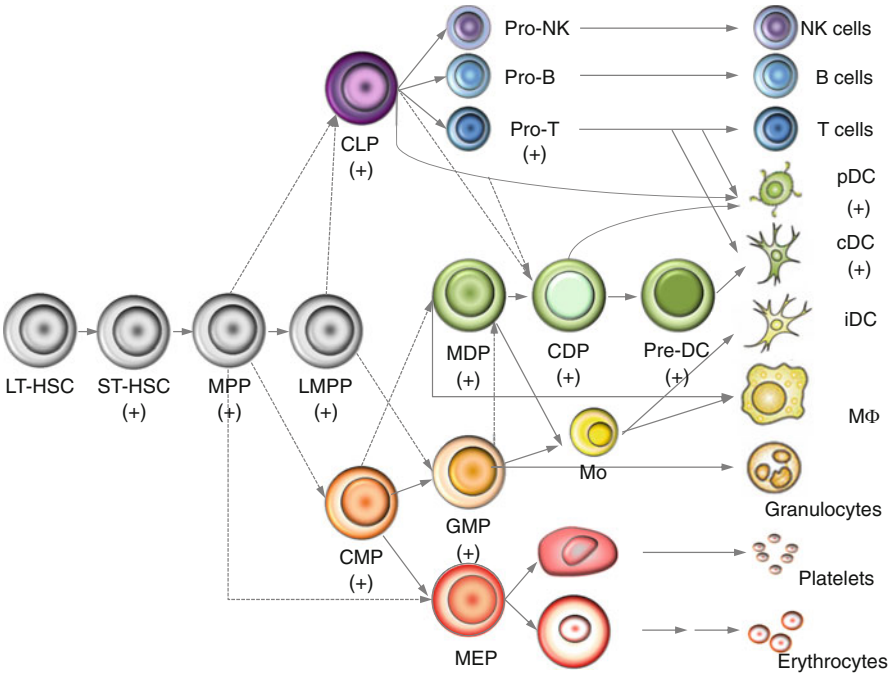

Fig. 10.10 Scheme showing FLT3 expression in normal hematopoiesis. Adult long-term hematopoietic stem cells (LT-HSCs) give rise to proliferating short-term HSCs, with limited self-renewal potential, that in turn generate multipotent progenitors (MPPs) committed to differentiating into mature cells. MPP gives rise to progenitors committed to myeloid (common myeloid progenitor, or CMP) or lymphoid (common lymphoid progenitor or CLP) fates. CMP further differentiates to megakaryocyte/erythrocyte progenitors (MEP) and granulocyte/macrophage progenitors (GMP). Alternatively, MPP develops into recently described lymphoid-primed multipotent progenitors (LMPP) that have lost megakaryocyte/erythrocyte developmental potential, but retain DC, myeloid, and lymphoid potential. CMP, CLP, and LMPP have DC potential that is restricted to progenitors expressing the FLT3 receptor ((+)). Macrophage–DC progenitors (MDP) give rise to monocytes (mo), macrophages (MΦ), classical DC (cDC), and plasmacytoid DC (pDC). MDP-derived monocytes can further differentiate into inflammatory DC (iDC). MDP generate common DC progenitors (CDP), which are DC restricted, giving rise to pDC and, via pre-DC, to cDC. Progenitors of T cells (pro-T), B cells (pro-B), and natural killer cells (pro-NK) are also shown. *Solid arrows* show confirmed pathways, whereas *dotted arrows* show suggested pathways that have not been formally proven. Figure adapted from [574]

using a CD11c-DTA transgene induced an increase in circulating FL, consistent with decreased receptor-mediated FL clearance, as well as a myeloproliferative syndrome, secondary to the elevated circulating FL [590].

FLT3 actions in both BM progenitors and peripheral DC precursors [579, 580, 589, 591] are mediated through the regulation of downstream transcription and differentiation factors including STAT3, IRF-8, PU.1, and FLT3 itself [592–594]. PI3K-AKT and the mammalian target of rapamycin (mTOR) act downstream of FLT3 to control DC development and expansion [595, 596]. Consistent with this

regulation, conditional deletion of *LAMTOR*2, an activator of mTOR and ERK, in CD11c$^+$ DC in mice resulted in plasma membrane accumulation of the FLT3 receptor, deficient ERK signaling from late endosomes, altered DC homeostasis (expansion of pDC and cDC), and a myeloproliferative syndrome in aging mice [597].

Role of FLT3 in Treg Cell Development The FL/FLT3 axis coordinates regulation of DC and Treg cells in vivo. In mice, DC are involved in Treg cell homeostasis. FL, by increasing DC, expands Treg cells [598], and loss of Treg cells increases DC division in a FL-dependent manner [599]. Similarly, an FL-mediated increase in DC, or DC depletion, was associated with increases or decreases in Treg cell frequency, respectively [590, 598]. These results suggest that FL-generated DC promotes Treg development and that low Treg levels feedback to enhance DC production [600].

Role of FLT3 in NK Cell Activation In mice, the presence of CD11c^{hi} DC is required for in vivo priming of NK cell responses to viral and bacterial pathogens [601]. Furthermore, maintenance of NK cells in the absence of infections requires the presence of a CD11c^{hi} DC population expressing IL-15 that mediates FL-induced NK cell expansion in vivo [602]. Thus, as in the regulation of Treg cells, the role of FL in NK cell activation is indirect, a consequence of DC support of NK effector function [603].

10.2.3 The Role of the FLT3 Receptor Tyrosine Kinase in Human Disease

Role of FLT3 in Acute Myeloid Leukemia (AML) Activating mutations in FLT3 are the most recurrent genetic modifications detected in AML, where they have prognostic value and determine the choice of therapy. Two main types of FLT3 mutations have been identified in approximately 40 % of AML patients. These are in-frame internal tandem duplications (ITD) within the juxtamembrane domain and point mutations within the activation loop of the kinase domain. Both types of mutation lead to constitutive (ligand-independent) activation of the receptor.

Both wild-type and mutant forms of FLT3 are highly expressed in certain hematopoietic malignancies, including 70–100 % of AML, acute lymphoblastic leukemias (ALL), and chronic myelogenous leukemias [560, 570, 604]. FLT3–ITDs were detected at low frequency in myelodysplastic syndrome (MDS or preleukemia), where a correlation between accumulation of FLT3 ITDs and disease progression toward leukemia was observed [605, 606]. Interestingly, the length of the ITD and the mutational load has been shown to vary among patients [607], and multiple studies have consistently associated higher levels of ITD mutational load with poorer outcomes [608–614]. However, the FLT3 receptor mutational status alone cannot fully describe the pathogenesis of AML, or predict the activity of the therapeutics targeting FLT3.

In addition, a recent genome-wide alternative splicing screen performed on 193 AML patients identified FLT3 as one of the most spliced transcripts in approximately 70 % of AML patients compared to normal donors, with significant correlations between the frequency of a particular splice variant and the stage of the disease progression [615]. Moreover, the spleen tyrosine kinase SYK transactivates FLT3 by direct binding, resulting in a protein complex, which activates MYC transcriptional programs. FLT3–ITD AML cells are more vulnerable to SYK inhibition than FLT3–WT cells. Furthermore, SYK is indispensable for the development of a myeloproliferative disease in a FLT3–ITD model in vivo. In this model, it promotes transformation to AML, suggesting that SYK regulates FLT3 enhancement of AML [616].

Role of FLT3 in Atherosclerosis Analysis of the non-lymphocyte population present in the adult mouse aorta revealed high expression of poorly phagocytic, immune stimulatory $CD11c^{+}MHC\ II^{hi}$ DC in the normal intima [617]. The intimal DC were of two types: classical FLT3–FL signaling-dependent $CD103^{+}CD11b^{-}$ DC and colony-stimulating factor-1 (CSF-1)-dependent $CD14^{+}CD11b^{+}DC\text{-}SIGN^{+}$ monocyte-derived DC. Both types expanded during atherosclerosis, and unlike macrophages, the $CD103^{+}$ cDC had an athero-protective function [617].

10.2.4 FLT3

10.2.4.1 *flt3* Gene

Discovery and Cloning of the flt3 Gene Two groups who employed different cloning strategies [547, 551, 618] simultaneously and independently identified the FLT3 receptor. Rosnet and colleagues screened a human testis cDNA library with a human genomic FMS probe from the CSF-1 receptor (CSF-1R) kinase domain [619] to isolate a cDNA clone encoding a fragment of a RTK that was named FLT3. The human *flt3* gene was mapped to chromosome 13q12.2 and further used to identify its mouse counterpart on chromosome 5 [551, 618]. In contrast, Matthews et al. identified conserved regions within the kinase domain of several tyrosine kinase receptors to define degenerate oligonucleotides and used a PCR-based strategy to screen a purified murine fetal liver stem cell cDNA library to isolate a cDNA clone encoding a full-length receptor that was named fetal liver kinase 2 (flk-2) [547].

Structure of the Genomic Locus Encoding the FLT3 Receptor The genomic loci encoding the *flt3*, *csf1r*, and *c-kit* receptors genes share overall conservation of exon size, number, sequence, and exon/intron boundary positions, suggesting that these genes have arisen from a common ancestral gene [620]. The two receptor-type tyrosine kinase genes *flt1* and *flt3* are arranged in a head-to-tail orientation, separated by ~150 kb containing three CpG islands, two of which are associated with *flt1* and *flt3* [621]. The third CpG island, in this intervening region, is part of the recently annotated PAB-dependent poly(A)-specific ribonuclease subunit 3 (PAN3) gene (Fig. 10.11a).

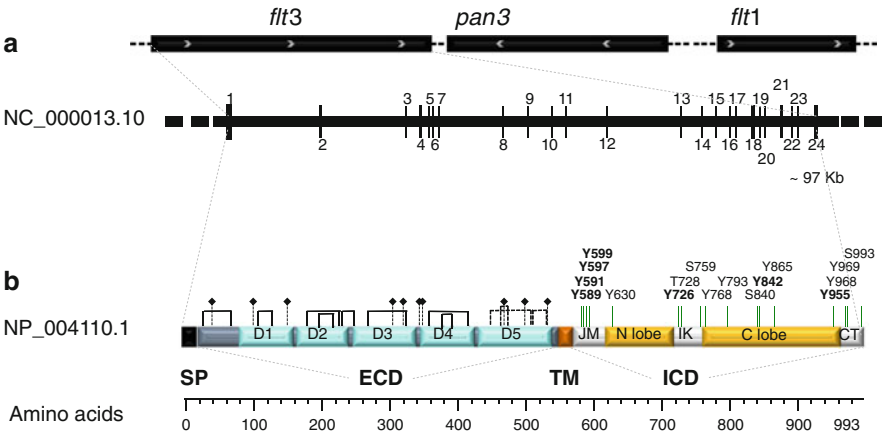

Fig. 10.11 Genetic organization of the *flt3* locus and domain organization of FLT3. (**a**) Human *flt3* is located on chromosome 13q12.2., adjacent to the *pan3* gene which separates it from the *flt1* gene, encoding a closely related *flt3* family member. The *flt3* gene consists of 24 exons (*vertical bars*) spanning ~97 kb, oriented at the minus strand, and encodes a full-length 3.7-kb transcript, with a 2,979-bp open reading frame (source: e!Ensembl. transcript ID: ENST00000241453). (**b**) Human FLT3 protein consists of a signal peptide (SP) (aa 1–26, *black*), an extracellular domain (ECD) (aa 27 to 543) containing five immunoglobulin-like domains (D1: aa 79–161, D2: aa 167–244, D3: aa 245–345, D4: aa 348–434, D5: aa 435:533, *blue*), a transmembrane domain (TM) (aa 544–563, *orange*), and an intracellular domain (ICD) (aa 597–993) comprised of a juxtamembrane domain (JM) (aa 591–597), an intracellular kinase domain (*yellow*) divided in two moieties, N-lobe (aa 618–718) and C-lobe (aa 762–962) by a hydrophilic interkinase domain (IK) (aa 719–761) and a short cytoplasmic tail (CT) (aa 962–993). The eight predicted N-linked glycosylation sites are indicated with *black diamonds* (source: UniProt). Established (*solid black lines*) and putative (*dashed black lines*, predicted by homology) disulfide bonds are shown [565]. *Green lines* indicate the positions of pTyr, pSer, or pThr sites detected by site-specific methods (*in bold*) or by MS analysis (*unbolded*) [561, 564, 658, 659, 667, 726, 727]

The flt3 Gene Sequence The human *flt3* coding sequence contains 2,979 nucleotides (NM_004119.2) encoding a primary translational product of 993 amino acids (NP_004110.2) (Fig. 10.11b) [622]. It possesses a polyadenylation signal 17 bp downstream of the STOP codon, but no poly(A) stretch. The mouse *flt3* coding sequence is 3,003 nucleotides in length (NM_010229.2) coding for a 1,000-amino acid protein (NP_034359) (Fig. 10.12).

Isoforms of the FLT3 Receptor Only one isoform of the FLT3 receptor has been reported, in mice [623]. This isoform lacks the extracellular D5 Ig-like domain as a result of the skipping of two exons during transcription. Although less abundant than the wild-type receptor, this alternative isoform, of unknown physiological significance, is able to bind FL and become phosphorylated, indicating that the Flt3 D5 domain is not necessary for either FL ligand binding or FLT3 receptor tyrosine kinase activation.

Targeted Disruption of the flt3 Gene In contrast to the severe phenotypes observed in mice with deletions in either *Csf1r* or *c-kit* [624], adult *flt3*$^{-/-}$ mice are healthy

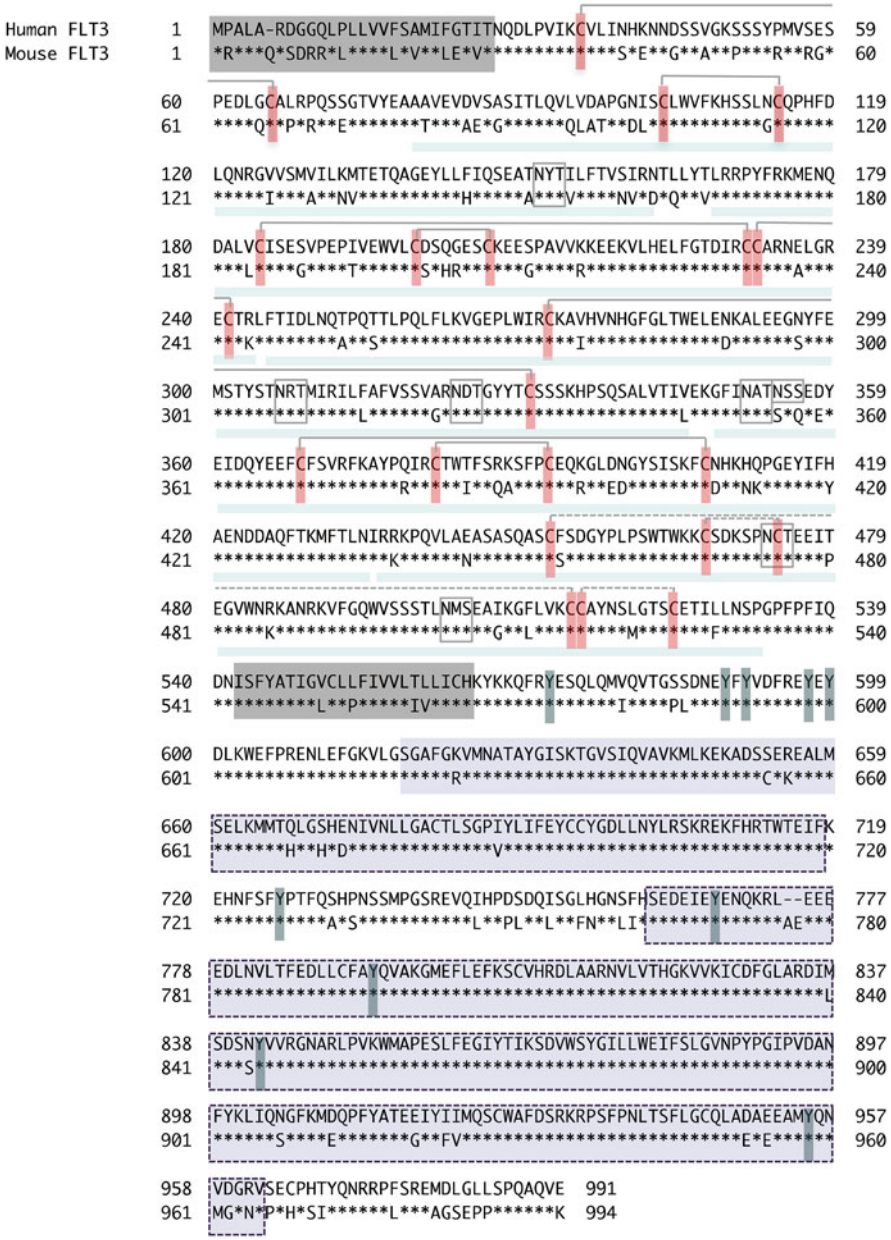

Fig. 10.12 The primary structure of human and mouse FLT3. Amino acid sequence identity of the mouse (NCBI accession number NP_034359) and human (NCBI accession number NP_004110) sequences is indicated by an asterisk. Maximal alignment of protein sequence was achieved by introducing three gaps in the human sequence, each indicated by a *dash*. Both sequences are numbered from the start of the signal sequence. The amino acid numbers referred to below are from the human sequence. *Gray-boxed* regions indicate signal peptide (aa: 1–26) and transmembrane (aa: 542–564) domains. Open *boxes* indicate N-linked glycosylation sites. The established (*solid lines*) and putative (*dashed lines*) disulfide bridges are shown in *red* [565]. The five Ig-like domains (D1–D5) are underlined with *blue bars*. The N- and C-lobes of the tyrosine kinase domain are within the *purple boxes*. The tyrosine phosphorylation sites, detected by site-specific methods, are indicated by vertical *green bars*

with normal mature hematopoietic populations [569]. However, they are deficient in primitive B-lymphoid progenitors, and bone marrow transplantation experiments have shown that *flt3*-deficient stem cells have a reduced capacity to reconstitute both T cells and myeloid cells [569]. Taken together, this data demonstrate an important role for FLT3 in the development of multipotent stem cells and lymphoid differentiation [569]. Choi and colleagues [617] found that genetic ablation of Flt3 in low-density lipoprotein receptor knockout ($Ldlr^{-/-}$) atherosclerosis prone mice correlated with lower numbers of classical DCs. These mice developed more severe atherosclerosis relative to control $Ldlr^{-/-}$ mice, and Flt3-deficient $Ldlr^{-/-}$ mice had less Treg cells and more inflammatory cytokines, IFNγ and TNFα, in the aorta. This identifies an athero-protective role of FLT-3-driven DCs.

flt3 Transcriptional Regulation Three transcription regulatory proteins, Pax5, N-CoR, and Hoxa9, have been shown to directly bind to the *flt3* promoter and to regulate *flt3* gene expression. Pax5, a transcription factor that is essential for the restriction of lymphoid progenitors to the B-cell fate, directly binds to the *flt*3 promoter within 200 bp upstream of the transcription start site and represses *flt*3 expression [625]. Thus, FLT3-mediated maintenance of multipotency of early lymphoid progenitors is suppressed, facilitating unilineage differentiation by Pax5 [625]. Similarly, the nuclear receptor corepressor (N-CoR) that is essential for the differentiation of erythroid cells binds the *flt3* promoter to repress *flt3* transcription, and posttranslational loss of N-CoR causes increased expression of FLT3 [626]. In contrast, Hoxa9 directly binds to the *flt*3 promoter to activate *flt3* transcription, consistent with the role of Hoxa9 in the positive regulation of lymphopoiesis and B-cell development [627].

10.2.4.2 FLT3 Protein

Amino Acid Sequence The FLT3 protein sequence is evolutionary conserved in higher vertebrates and displays all the features of a RTK [622] (Fig. 10.11b). It has a 26-amino acid signal peptide, followed by an extracellular region of 541 amino acids comprised of five Ig-like domains and bearing 9 potential asparagine-linked glycosylation sites [622] (Figs. 10.11b and 10.12). FLT3 also contains a 21-amino acid transmembrane domain, and an intracellular region comprised of a short juxtamembrane domain, a kinase domain split in two moieties by an interkinase insert, followed by a short cytoplasmic tail. The FLT3 protein has a calculated molecular mass of approximately 113 kDa. However, analysis by SDS-PAGE reveals a higher M_r form of 155–160 kDa, corresponding to the mature N-glycosylated protein, and a lower M_r species of ~130 KDa, representing the immature, high mannose-containing glycoprotein [549].

FLT3 Mutant Proteins Two types of FLT3-activating mutations, ITDs and activating point mutations, have been identified in approximately 40 % of AML patients. Up to 34 % of AML patient leukemic cells possess in-frame ITDs within the FLT3 juxtamembrane domain [609, 628]. However, their length (6–68 amino acids) and position vary from patient to patient [610]. It is believed that these repeat sequences

serve to disrupt the autoinhibitory role of the JM domain, causing constitutive activation of FLT3. The second class of FLT3-activating mutations comprises point mutations, the majority occurring at the key aspartate residue 835 (D835) within the activation loop of the FLT3 kinase domain [629, 630] (see Sect. 10.2.4.3). As observed for both c-kit D814 [631] and CSF-1R D802 [632], FLT3 D835 mutations led to constitutive activation of the receptor by stabilizing the active form of the FLT3 activation loop [630]. The most common substitution noticed in patients was D835Y, but other substitutions, including D835T, D835V, D835H, D835E, and D835N, have also been observed [630]. Additional activating point mutations have been identified in AML patients in which isoleucine 836 is either deleted or substituted with a threonine or with a methionine and an arginine [609]. Both FLT3-ITD and FLT3-point mutations triggered FLT3 autophosphorylation; phosphorylation of downstream ERK1/2, Akt, and STAT5; as well as upregulation and activation of Pim-2 kinase, PU.1, and C/EBPalpha [559, 562, 630, 633].

Recently, a G to A transition in the splice donor site of intron 9 of the mouse *flt3* gene [603] was shown to generate a translation product that lacked the exon 9-encoded amino acids Tyr 402 and Ser 403 within domain D4. This *flt3* mutant allele encodes a nonfunctional FLT3 protein [603].

10.2.4.3 FLT3 Crystal Structure and Structure–Function Studies

Extracellular Domain The structure of the extracellular FLT3 receptor–FL ligand complex has been elucidated [565]. Using the crystallographic data and thermodynamic analysis of complex formation, it has been shown that the FLT3–FL interaction involves high-affinity binding of the FL dimer to the Ig-like domain 3 (D3, a.a. 346–434) of the monomeric receptor, followed by receptor dimerization [565]. Unique characteristics of this class III RTK–ligand complex formation include a small receptor–ligand interface (Fig. 10.13a,b), ligand–receptor-binding preceding receptor dimerization (Fig. 10.13c), and a distinctive D1 domain plasticity (Fig. 10.13a,b).

At ~900 $Å^2$, the receptor–ligand interface is very compact and two times smaller than the measured interfaces of all other class III/V RTKs. Tight contacts are formed between the N-terminal domain of FL and the D3 domain of Flt3 (Fig. 10.13a) [565].

Unlike other members of the RTKIII/V family, Flt3 does not exhibit receptor homotypic interactions involving the membrane-proximal D4 and D5 domains (Fig. 10.13b), due to the absence of conserved amino acids located within these domains in the other RTKs. Analysis of the Flt3–FL complexes indicates that domains D4–D5 remain separated by a distance of ~20 Å in the dimerized receptor and do not contribute to FL dimer binding to FLT3 (Fig. 10.13b). Although this study does not eliminate the possibility of homotypic interactions involving the trans- or juxtamembrane domains of FLT3 [634, 635], nor addresses the homo- and heterodimers formed by FLT3-ITD and wild-type FLT3 that are constitutively

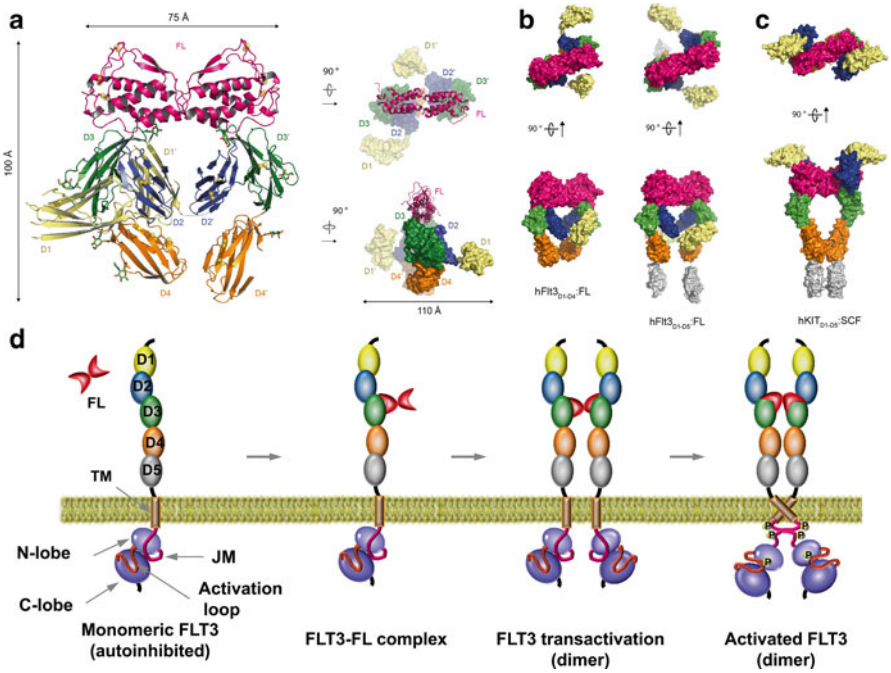

Fig. 10.13 Crystal structure of the human FLT3–FL complex and proposed mechanism of FL-dependent FLT3 activation. (**a**) The crystal structure of the $FLT3_{D1–D4}$–FL complex is shown in ribbon representation with the twofold symmetry axis of FL oriented along the vertical axis of the plane. As shown, a specific color is assigned to each of the four FLT3 domains. Disulfide bridges are shown as *yellow spheres* and N-linked glycans as *green sticks*. The structural panels to the right show 2 alternative views of the complex with FL in ribbon representation and the receptor in surface representation. (**b**) The architecture of FLT3–FL complex. Receptor ectodomains and ligand are colored as in **a**. (**c**) The architecture of human KIT–SCF complex. Receptor ectodomains and ligand are colored as in **a**. (**d**) Schematic of FL binding to FLT3. Activation of FLT3 is initiated by binding of dimeric FL to the D2–D3 interface of the extracellular FLT3 domain of a single receptor molecule, and this binary complex recruits a second FLT3 molecule, leading to receptor dimerization and juxtapositioning of FLT3 cytoplasmic domains for autophosphorylation. Panels A–C from [565], with permission. Panel D modified from [637]

active [636], it assists in prioritizing regions within FLT3 that could be targeted pharmacologically.

The analysis of the FLT3–FL complex also shows that FLT3 D1 (Fig. 10.13a,b) is the largest and the most atypical D1 domain in the entire RTK III/V family. As it does not make contact with any other complex components, its role remains unknown, possibly mediating intermolecular interactions or stabilization of the unbound receptor [565].

The structural analysis of the Flt3 receptor–FL ligand complex has provided valuable information on the extent of the FLT3–FL interaction epitope and on sites in the extracellular domain that can be exploited for pharmacological or antibody-based targeting of FLT3 activation [637] .

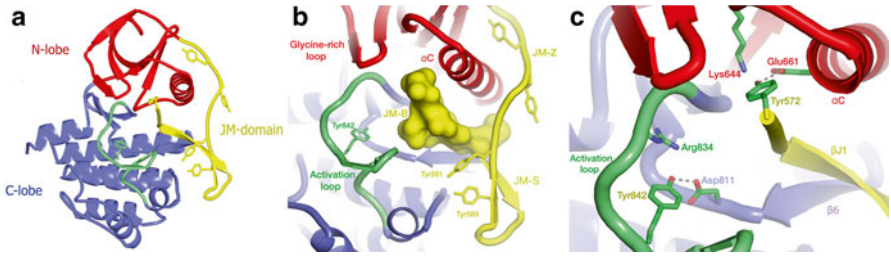

Fig. 10.14 Crystal structure of the autoinhibited FLT3 intracellular domain. (**a**) Structure of the intracellular domain. Ribbon diagram showing the spatial arrangement of N (*red*)- and C (*blue*)-lobes of the kinase domain, the activation loop (*green*) folded up between them, and the JM domain (*yellow*) that nearly spans the length of molecule. All tyrosines in the JM domain and the activation loop are displayed as "stick" representations. (**b**) Ribbon representation of the structure of the JM domain and its relation to other components of FLT3. Topologically, the FLT3 JM domain can be divided into three components: the JM-binding motif (JM-B), the JM switch motif (JM-S), and the zipper segment (JM-Z). JM-B (Tyr572-Met 578), in molecular surface representation, is a short fingerlike segment that makes contact with all structural components responsible for the activation/inactivation of the intracellular kinase domain, including the conserved glycine-rich loop located in the ATP-binding site, the activation loop, and the only α-helix of the N-lobe (αC) (see also *panel* C). As rotation of the N-lobe toward the C-lobe generates the activated kinase fold, the role of JM-B is to stabilize FLT3 in its inactive conformation by preventing this movement through tight interactions with the N-lobe of the kinase domain and the N-terminal hinge of the activation loop. JM-S (Val579–Val592) is located externally to the C-lobe and contains the key tyrosine residues Tyr-589 and Tyr-591, phosphorylation of which disrupts the interaction between JM-S and the C-lobe leading to kinase activation (shown schematically in Fig. 4d). JM-Z (Asp593–Trp603) is mainly associated with the N-lobe, forming contacts with αC. Since the role of JM-Z is to correctly align and maintain the JM-S during transitions between the activated and inactivated states of FLT3, its length is critical and known to be conserved between all members of the PDGFR family. (**c**) Active site of FLT3. Two sets of interactions involving hydrogen and salt bridges stabilize the FLT3 active site in its "loop-in conformation." In the first, Tyr–Lys–Glu, Tyr572 forms a hydrogen bond with a highly conserved Glu661, which together with Lys644 forms an ion pair known to be critical for ATP binding. In the second, Tyr–Arg–Asp, Tyr842, an anchoring point on the activation loop, is hydrogen bonded to Asp811, which forms a second ion pair with Arg834. Both tyrosines 572 and 842 are phosphorylated in activated receptor disrupting the loop-in conformation (shown schematically in Fig. 4d). Figure from [566], with permission

FLT3 Intracellular Domain Similar to the inactive conformations of ABL [638], c-KIT [639], and the insulin receptor tyrosine kinase [640], the inactive conformation of the FLT3 kinase domain (H564–V958) revealed an activation loop folded back onto the ATP-binding cleft (loop-in conformation) that prevented substrate binding [566] (Fig. 10.14a, b). The N-terminal region of the activation loop displayed the key Asp-Phe-Gly (DFG) motif in a DFG-out conformation in which the Asp side chain, required for Mg^{2+} coordination of ATP, was displaced from the active site. By analogy, activation of FLT3 would thus entail flipping of the DFG motif and reorganization of the activation loop [641]. This process exposes an additional hydrophobic binding site adjacent to the ATP-binding site that can be independently targeted therapeutically by type II (non-ATP-binding site) kinase inhibitors [641, 642].

In addition to providing a rationale for inhibitor design, the structure of the autoinhibited FLT3 has uncovered a crucial role of the FLT3 juxtamembrane (JM) domain in regulating FLT3 kinase activity. This mechanism is controlled by the phosphorylation state of Tyr589 and Tyr591 (Fig. 10.14b). When phosphorylated, these Tyr residues remove conformational restraints in the JM domain that are required for its binding to the C-lobe of the kinase domain. Similarly, the ITD insertions observed in AML patients offset the positioning of the JM domain relative to the C-lobe of the kinase domain [566], rendering FLT3 autoinhibition "leaky" [643] and FLT activation ligand independent [566].

Stabilization of the FLT3 active site in its "loop-in conformation" is realized through two sets of hydrogen bonds and salt bridges established between the amino acids of the two conserved peptide motifs Tyr-Lys/Arg-Glu/Asp involving Tyr572 (located in the JM domain) and Tyr842 (on the activation loop) (Fig 10.14c). Consequently, tyrosine phosphorylation at 572 and 842 contributes to activation by disrupting these interactions.

The study of the crystal structure of FLT3 kinase domain has demonstrated the ability of FLT3 to adopt distinctive "off" states, also identified for other kinases [566], and enabled the development of pharmacological inhibitors that selectively trap FLT3 in its inactive conformation [641].

10.2.5 FLT3 Ligand (FL)

10.2.5.1 Chromosomal Location of the *flt3l* Gene

The human *flt3l* gene maps to chromosome 19q13.3–13.4 (NC_000071.6) [555, 644], and the mouse *flt3l* gene is located on chromosome 17 (NC_000073.6).

10.2.5.2 Structure of the *flt3l* Genomic Locus

The similarities observed in the genomic organization of the c-kit ligand, CSF-1 and FL genes suggest that they are ancestrally related. Thus, they share the same number of exons with a high degree of conservation of exon size and all encode both membrane-spanning and secreted isoforms [644]. The human and mouse genomic loci encompassing the coding region of the FLT3 ligand differ in size, with the human gene (5.9 kb) being larger than the mouse (4.0 kb) due to the presence of repeated sequences of unknown significance within introns I, II, IV, V, and VI of the human locus [644].

10.2.5.3 FL Isoforms

Alternative splicing of the *flt3l* pre-mRNA yields mRNAs encoding different, active FL isoforms of undetermined function that fall into three main categories: cell-surface isoforms that are proteolytically processed, cell-surface isoforms that are

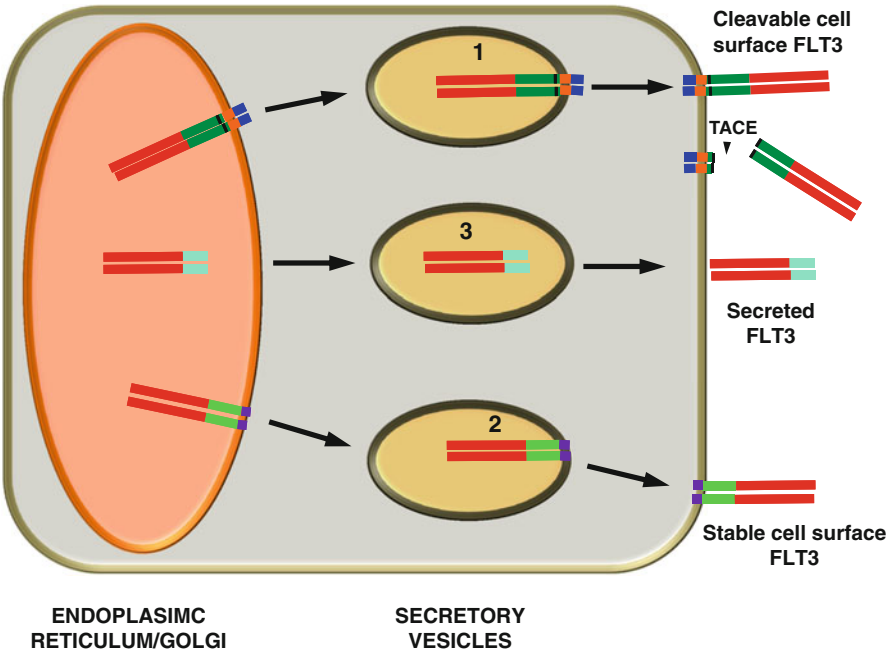

Fig. 10.15 Biosynthesis and expression of FLT3 ligand isoforms. The most abundant FL isoform is a type I membrane-spanning glycoprotein [547] that dimerizes during biosynthesis, is processed in the ER and the Golgi apparatus, and predominantly released by TACE proteolytic cleavage from the plasma membrane. As a result of alternative splicing, at least two other isoforms arise. One [548] is membrane associated, via a novel hydrophobic domain, and is abundantly expressed as a stable cell-surface protein. The other [549], in which synthesis terminates before the transmembrane domain, is secreted in low amounts. All isoforms are N- and O-glycosylated (not shown), noncovalent dimers. The location of the TACE proteolytic cleavage is indicated

stable, and secreted isoforms (Fig. 10.15). The most prevalent human isoform, also identified in mouse, is the full-length type I membrane-spanning molecule that readily undergoes extracellular domain proteolytic cleavage to generate a 158-amino acid biologically active glycoprotein [548, 645, 646]. Shedding of FL from the cell surface is metalloprotease-dependent and mainly mediated by TNFα-converting enzyme (TACE) [647]. A second FL isoform includes a 220-amino acid membrane-spanning, protease-resistant glycoprotein that arises from failure to splice an intron. This isoform lacks the TACE juxtamembrane proteolytic cleavage site and the transmembrane domain, which is replaced by a hydrophobic membrane-anchoring region [648]. A less prevalent third FL isoform, detected in lower amounts in both human [644] and mouse [648] tissues, contains an alternatively spliced sixth exon which introduces a stop codon near the C-terminus of the extracellular domain, resulting in a shorter, soluble, biologically active FL protein.

10.2.5.4 Targeted Disruption of the *flt3l* Gene

Similar to *flt3*$^{-/-}$ mice, *flt3l*$^{-/-}$ mice are viable, breed normally, have a healthy appearance, and show a defect in early B-cell development [580]. However, unlike *flt3*$^{-/-}$ mice, *flt3l*$^{-/-}$ mice display reduced cellularity in peripheral blood, spleen, and BM, a decrease in splenic DC, and a lack of NK cell activity in the spleens of either poly IC- or IL-15-treated mice [580]. These effects are reversible upon FL administration, confirming that they are specifically due to *flt3l* disruption. Although the *flt3*$^{-/-}$ mice were on a different genetic background from the *flt3l*$^{-/-}$ mice, the difference in the phenotypes between the ligand- and receptor-deficient mice, combined with the broader expression pattern of FL over FLT3 [547, 551, 646, 649], could point to the existence of a second FL receptor.

10.2.5.5 Species Specificity of FL

Human and mouse FLs are able to bind to and activate the FLT3 receptor expressed on both murine and human cells [650]. Moreover, the human FL is active on mouse, rabbit, nonhuman primate, and human cells [555], indicating a lack of species-related specificity.

10.2.5.6 Binding of FL to Its Receptor

FL was shown to bind the FLT3 receptor on human myeloid leukemia cells with high affinity ($K_d \sim 0.1$ nM) [554] comparable to the affinities of kit ligand (K_d 16–310 pM) [651] and CSF-1 ($K_d \sim 0.1$ nM) [217] for their cognate receptors on cells.

10.2.6 FLT3 Receptor Signal Transduction

10.2.6.1 Signaling by Wild-Type FLT3 Receptors

Upon binding of the FL dimer, the FLT3 receptor undergoes dimerization followed by RTK activation and receptor cytoplasmic domain transphosphorylation (Fig. 10.16). The phosphorylated tyrosine residues create binding sites that recruit signal transduction molecules containing phosphotyrosine-binding domains (i.e., Src homology 2 (SH2)- and phosphotyrosine-binding (PTB) domains) that mediate the activation of downstream signaling networks, with PI3K/Akt and RAS/MAPK as the main regulatory nodes (see below) [652–657]. Several intracellular tyrosine residues have been shown to be phosphorylated in the activated human (Tyr-572, Tyr-589, Tyr-591, Tyr-599) [658] and mouse (Tyr-572, Tyr-589, Tyr-591, Tyr-597, Tyr-599, Tyr-726, Tyr-768, Tyr-793, Tyr-842, and Tyr-955) [659] receptors. FLT3 signaling from the plasma membrane is negatively regulated by protein-tyrosine

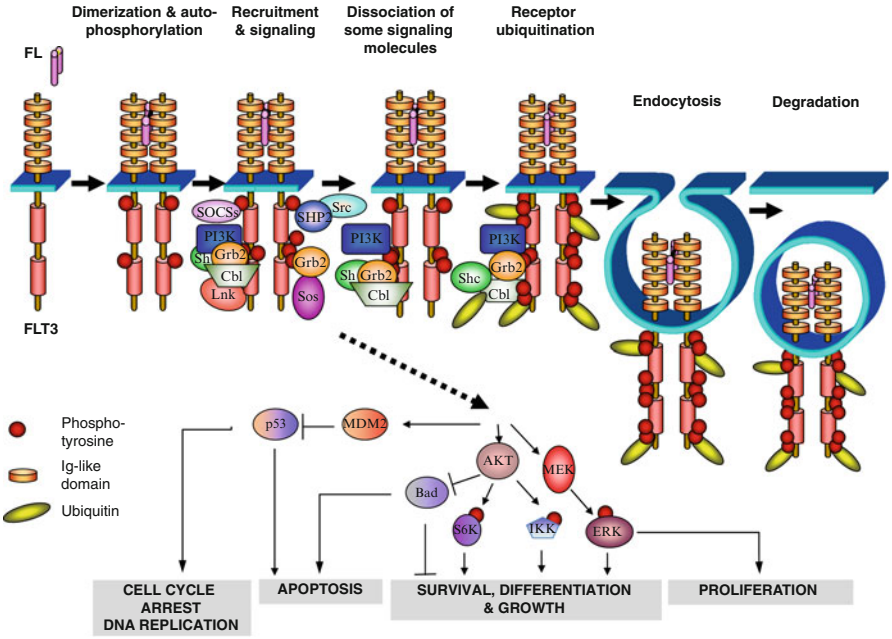

Fig. 10.16 Scheme depicting FLT3 signal transduction. Binding of FL ligand dimer induces receptor dimerization and activation of the intracellular kinase domain, resulting in the phosphorylation of tyrosine residues that create binding sites for phosphotyrosine-binding domain-containing adaptors or enzymes, including Grb2, SHP-2, SOCS, and Cbl. These FLT3 complexes initiate activation of components of downstream pathways, such as AKT, MAPKs, STATs, and MDM2, that control FL-regulated cellular functions of cell survival, growth, proliferation, differentiation, and apoptosis. Locations of tyrosine phosphorylation and of receptor-binding proteins do not reflect the actual sites. The connectors between pathway components do not necessarily indicate direct effects

phosphatases (PTPs) and dual specificity phosphatases (DUSPs) and terminated by adaptor-mediated receptor ubiquitination, internalization, and degradation [559, 633, 654–656, 660–662].

Apart from the role of the juxtamembrane (JM) residues Tyr-589 and Tyr-599 in regulating FLT3 kinase activity (Sect. 10.2.4.3), their phosphorylation leads to the recruitment of the Src homology 2-containing PTP2 (SHP2 or PTPN11) and Src family kinases (SFK) [658]. SHP2 interaction with FLT3 is dependent on Tyr-599 phosphorylation and enhances FL-mediated ERK1/2 activation and cell proliferation, whereas negative regulation of the mitogenic response by SFK is dependent on Tyr-589 phosphorylation [658].

Similar to its binding and negative regulation of other class III RTKs, the adaptor protein Lnk apparently binds to FLT3 in a Tyr-572, Tyr-591, and Tyr-919 phosphorylation-dependent manner and negatively regulates the receptor phosphorylation state and the downstream activation of PI3K and ERK1/2, suppressing FL-stimulated hematopoietic progenitor cell expansion [663]. The binding of *Src-like*

adaptor protein (SLAP) to Flt3 appears to be Tyr-572, Tyr-793, Tyr-919, and Tyr-955 phosphorylation dependent and results in downstream activation of Akt, ERK1/2, p38, and the ubiquitin ligase, Cbl, initiating Cbl-mediated receptor ubiquitination, internalization, and degradation [664]. Suppressors of cytokine signaling SOCS2 [665] and SOCS6 [666] also negatively regulate FLT3 signal transduction by directly binding FLT3 in a Tyr-589 and Tyr-919 phosphorylation-dependent manner, blocking activation of ERK1/2 and STAT5, but not Akt, and also by enhancing receptor ubiquitination, internalization, and degradation. The transmembrane PTP, density-enhanced phosphatase-1 (DEP-1 or PTPRJ), identified in a shRNA screen targeting PTPs expressed in myeloid cells [667], is a direct interactor of FLT3 that negatively regulates FLT3 phosphorylation and signaling [668]. DEP-1-deficient cells displayed increased site-specific phosphorylation of Tyr-589, Tyr-591, and Tyr-842 in response to FL. While DEP-1 depletion positively regulated ERK 1/2 and STAT5 activation and cell proliferation ex vivo, it did not allow the development of a 32D-FLT3 myeloproliferative disease in mice [650].

10.2.6.2 Signaling by FLT3 Mutant Receptors

FLT3–ITD mutant receptors activate aberrant signaling compared to the ligand-activated FLT3 receptor and exhibit stronger transforming potential [561, 576]. FL-independent activity of FLT3–ITD in AML cells leads to constitutive activation of ERK1/2, PI3K, p38, JAK2, STAT3, STAT5A, STAT5B, Cbl, VAV, and SHP2 [559, 633, 661, 669, 670], as well as decreased RTK ubiquitination, due to RTK mutations in ubiquitin ligase binding sites, or to inactivating mutations in the ubiquitin ligases [664]. Apart from these mechanisms, the altered signaling mediated by FLT3–ITD receptors can also be contributed to by retention of the receptor as a high-mannose precursor in an intracellular compartment [671, 672]. Indeed, genetic ablation of endoplasmic reticulum protein retention receptor 1 (KDELR1), a protein that is involved in the retention of proteins in the lumen of the endoplasmic reticulum, reduced FLT3–ITD-expressing 32D myeloblast-like cell proliferation, colony formation, and their ability to generate a leukemia-like disease in syngeneic C3H/HeJ mice [563], identifying KDELR1 as an additional potential target for FLT3–ITD-driven leukemias.

The consequences of abnormal FLT3–ITD signaling include oxidative inactivation of negative regulators of receptor signaling (e.g., DEP-1, [673]) and upregulation of STAT 5 gene targets such as the PIM1/2 kinases [556, 559, 633]. One example of altered signaling by FLT3 mutant receptors is constitutive STAT5 activation. Increased basal levels of pSTAT5 have been shown to predict the presence of FLT3–ITD mutations in AML patients' samples [558, 633, 674, 675], and FLT3–ITD binding of STAT5 was shown to be dependent on Tyr-589 and Tyr-591 [561], suggesting a role for these residues in altered FLT3–ITD signaling. Whereas FLT3–ITD activates STAT5 and STAT3 [558, 633, 674, 675], ligand-activated FLT3 or activating FLT3 activation loop point mutants are comparatively weak STAT5 activators [558, 661, 675–678]. Since FLT3–ITD is a more powerful inducer of

myeloproliferative disease in mice than the most common activated point mutant, FLT3 D835Y [661], STAT5 and STAT5 target gene activation may be particularly important in AML carcinogenesis.

Recently, a comprehensive phosphoproteomic analysis of FLT3–ITD-mediated signaling in human AML cell lines uncovered additional relevant downstream pathways, including Fc epsilon RI, BCR, and CD40 signaling [679]. FLT3–ITD was shown to regulate tyrosine phosphorylation of approximately 200 proteins, including cell-surface protein kinases, adaptor/scaffold proteins and phosphatases, as well as cytoskeletal or cytosolic proteins, including RNA processing proteins. On the basis of the results, the targeting of a DNA methyl transferase (DNMT1) that may specifically link FLT3–ITD activity with regulation of epigenetic events in human AML cells was suggested [667].

The diversity of cellular modifications associated with FLT3 oncogenic signaling offers a variety of molecules and processes that can be targeted pharmacologically.

10.2.6.3 FLT3 Signaling and Normal Homeostasis

Understanding how FLT3-associated networks regulate DC maturation and homeostasis is relevant to our understanding of autoimmune and inflammatory diseases, as well as to immune reprogramming and DC-based vaccine approaches. Relevant to the mechanisms connecting FL ligation and DC homeostasis, it was shown that FL acts on both bone marrow progenitors and peripheral DC precursors [579, 580, 589, 591] through regulation of the downstream transcription and differentiation factors STAT3, IRF-8, PU.1, ID2, and FLT3 itself [592–594]. However, lack of detailed knowledge of the transcriptional changes associated with deficiencies of these transcription factors hinders their manipulation for therapeutic purposes. Importantly, PI3K–mTOR signaling promotes maturation [595] and facilitates anti-inflammatory responses in cDC, through increased secretion of IL-10 [680, 681], whereas in pDC it is required for TLR-induced type I interferon production [682, 683]. Consistent with these observations, FL-induced mTOR signaling was shown to be necessary for FL-driven development of DC in vitro, whereas DC-intrinsic expression of PTEN restricted PI3K–mTOR signaling to maintain an optimal DC subset composition [596].

10.2.7 FLT3 Selective Inhibitors

The high frequency of FLT3–ITD [612, 684] and FLT3 activation loop mutations [630, 685], the overexpression of both FL and the FLT3 receptor in human leukemic blasts [604, 686], and the elucidation of the structure of the FLT3 [565, 566] have provided a rationale for designing small molecule inhibitors or antibody-based therapies targeting multiple forms of the FLT3 receptor. Small molecule inhibitors currently being developed are either indirect, targeting FLT3 folding or stabilization (e.g., heat shock protein (HSP)-90 inhibitors), or direct, targeting FLT3 activation (e.g., ATP mimetics) [567].

10.2.7.1 Indirect Inhibitors of FLT3

Unlike its WT counterpart, FLT3–ITD requires HSP-90 protein for proper folding and stabilization [687], and indirect inhibition of FLT3 has been achieved by targeting the chaperone function of HSP-90, which reduces the levels of several proteins involved in cell cycle and growth factor-mediated RTK signaling [688]. These inhibitors suppress mitogenesis by blocking Raf-1 signaling; by destabilizing and reducing the cellular levels of mutant oncoproteins; by inhibiting the activity of survival factors, such as Akt; by inducing cell cycle arrest; and by inhibiting angiogenesis, as well as tissue invasion and metastasis by decreasing the levels of metalloproteinases [689–691]. Three Hsp-90 inhibitors, the ansamycin antibiotics herbimycin A (HA), geldanamycin (GM), and 17-allylamino-17-demethoxygeldanamycin (17-AAG) were shown to exert anticancer effects by binding of the N-terminus pocket of HSP90 [692]. HA [693] and 17-AAG [687, 694–696] have been synthesized and successfully tested for in vitro activity against FLT3–ITD and in vivo efficacy against AML. 17-AAG displayed the lowest hepatotoxicity in vivo [697, 698] and is in phase I/II clinical trials for the treatment of young patients with recurrent or refractory leukemia or solid tumors [688, 699] and in two phase III studies for the treatment of refractory multiple myeloma [700].

10.2.7.2 Direct Inhibitors of FLT3

All the direct small molecule FLT3 inhibitors being developed are type I or type II ATP competitors [701–708]. No non-ATP-competitive, direct FLT3 inhibitors have been reported to date.

Type I direct FLT3 inhibitors bind in and around the region occupied by the adenine ring of ATP [701]. As such, their selectivity is significantly affected by changes in the tertiary structure of the binding pocket resulting from amino acid changes, such as those created by FLT3–ITD mutations [709, 710]. However, type 1 inhibitors may exhibit decreased selectivity due to the high sequence conservation between the ATP-binding domains of human TKs. In some cases, lack of selectivity may be an advantage. For instance, PKC412 (midostaurin, N-benzoyl-staurosporine; Novartis Pharma AG, Basel, Switzerland) is a multikinase inhibitor that targets FLT3, PKC alpha, vascular endothelial growth factor receptor 2 (VEGFR2), platelet-derived growth factor receptor b (PDGFRb), c-KIT, and c-FMS [708, 711]. It has potential antiangiogenic and antineoplastic activities and has been shown to disrupt cell cycle and inhibit proliferation, apoptosis, and angiogenesis in susceptible tumors (www.cancer.gov/clinicaltrials). PKC412 is currently in phase II clinical trials on AML patients with FLT3–ITD (2011-003168-63, 2011-002567-17, NCI-2013-00868) and in phase I clinical trials on relapsed or refractory leukemia in children (Clinical Trials.gov, ID: NCT00079404, ID: NCT00093821) and in patients older than 60 (NCI-2009-01285).

Type II direct FLT3 inhibitors, in addition to occupying the ATP-binding site, also engage an adjacent hydrophobic site that is accessible only when the kinase is in its inac-

tive configuration [701, 704, 707] (Sect. 10.2.4.3). As such, they are expected to be more selective in inhibiting mutant FLT3 kinase activity and to override resistance to other FLT3 inhibitors. For example, Weisberg and colleagues recently described first- and second-generation derivative analog type II FLT3 inhibitors that potently and selectively inhibit FLT3–IDT in vitro, with no apparent effect on cells harboring WT FLT3 [704, 707].

10.2.7.3 Future Prospects for FLT3 Inhibition

AML treatment with FLT3 inhibitors, tested as single agents or in combination with chemotherapy, indicated that only a fraction of FLT3–ITD patients showed a clinical response [712–714]. Unfortunately, most AML patients acquired resistance to these direct inhibitors, or relapsed with an increase in the mutant-to-wild-type FLT3 ratio [715–717]. Furthermore, due to incomplete suppression of downstream FLT3 signaling pathways, some FLT3–ITD patients did not benefit from FLT3 inhibition treatment, despite almost complete inhibition of FLT3 autophosphorylation [718–720]. Several suggestions have been made to address these and other problems associated with the use of FLT3 inhibitors in the treatment of AML. One approach is to use these inhibitors in combination with other small molecules that act synergistically in vitro to inhibit FLT3 and/or other downstream signaling molecules such as mTOR, PI3K, and MAPK/ERK 1/2 [702, 721, 722]. Also, based on the observation that only a subset of leukemic blasts display self-renewal and are able to initiate leukemia in irradiated recipient mice [568, 723], targeting this subset is appropriate [719]. In addition, since interactions with the stroma (possibly involving FL) may be important in maintaining these leukemia-initiating cells, disrupting these interactions provides a new therapeutic strategy [719, 724]. Finally, in view of the dramatic increase of circulating FL in patients post-chemotherapy, the combined use of inhibitors targeting both FL and FLT3 in AML could be considered [725]. Thus, it is likely that the treatment of AML patients whose leukemic cells express FLT3 mutations will have to be multifaceted, to target oncogenic signaling at several levels, including FLT3 phosphorylation, activation of downstream signaling molecules, FL expression, and the leukemia cell–stroma interaction.

10.3 The KIT Receptor Tyrosine Kinase[3]

10.3.1 Stem Cell Factor, SCF

The ligand for KIT is called stem cell factor (SCF), and it is produced by several cell types, including endothelial cells, keratinocytes, certain epithelial cells, fibroblasts, and some tumor cells [728]. Under normal condition, the concentration SCF in

[3] Johan Lennartsson and Lars Rönnstrand

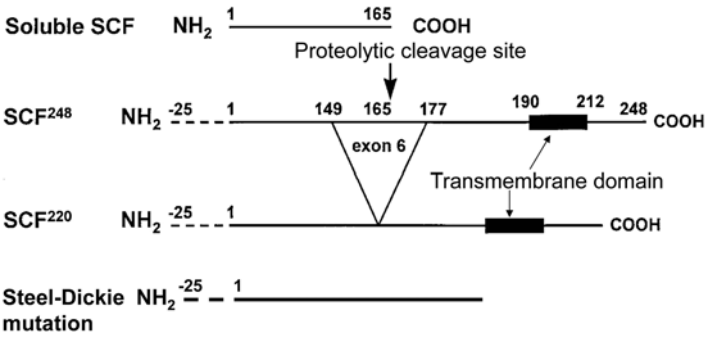

Fig. 10.17 Stem cell factor (also called KIT ligand) exists as a noncovalent constitutive dimer. Two alternative splice forms exist yielding a soluble form and a membrane-bound form

plasma is about 3 ng/ml [729]. However, it is likely that locally SCF concentrations may be significantly different. Alternative names for SCF are steel factor (SF), mast cell growth factor (MGF), and KIT ligand (KITLG). SCF is a 30-kDa glycoprotein, although the glycosylation has not been found to be important for its biological activity. The functionally active form is a noncovalent SCF homodimer held together by both polar and hydrophobic interactions, which allow simultaneous binding to two KIT monomers promoting their dimerization [730]. However, each SCF subunit contains disulfide bonds important for its structure. The gene encoding SCF is located on human chromosome 12 (mouse chromosome 10), and it contains nine exons [731]. The primary sequence of SCF consists of an extracellular domain, a transmembrane region, and a short intracellular tail [732]. Alternative splicing of exon 6 generates two forms of SCF; the translation product containing exon 6 generates a 248-amino acid protein that has a cleavage site in its extracellular region (Val–Ala–Ala–Ser) and can be converted into a soluble 165-amino acid form by proteolytic cleavage (Fig. 10.17). In contrast, the form of SCF lacking exon 6 is 220 amino acids long and resistant to cleavage and will remain membrane associated. There is a secondary cleavage site in exon 7 that may be used to release a soluble form of SCF [733]. Interestingly, the soluble form of SCF cannot fully compensate for loss of the membrane-associated form. This was illustrated in mice only expressing SCF with the Sl^d mutation (lack of transmembrane and intracellular domains) since these mice have macrocytic anemia and a reduced number of tissue mast cells, are sterile, and display pigmentation defects [734]. Conversely, the membrane-bound form of SCF cannot completely compensate for loss of soluble SCF. Mice expressing mutant SCF lacking the proteolytic cleavage site, and which thus remains membrane associated, showed reduced number of mast cells and increased sensitivity to sublethal irradiation [735]. Complete lack of SCF expression is embryonic lethal due to anemia.

10.3.2 KIT Gene Structure and Regulation of Its mRNA Expression

KIT is a transmembrane protein whose function is to sense the presence of SCF in the environment and transmit this information into the cell. The KIT gene was identified as the normal homologue to the previously identified viral oncogene v-Kit found in Hardy–Zuckerman 4 feline sarcoma virus [736]. Subsequent work showed the KIT gene to be at human chromosomal location 4q11 (chromosome 5 in mice) and span 34 kb of DNA encompassing 21 exons and that it generates a 5.5-kb transcript which is translated into a 145-kDa protein [737, 738]. The first exon encodes the initiation codon and a signal peptide, exon 2–9 the extracellular part, exon 10 the transmembrane domain, and exon 11–21 the intracellular domain containing the tyrosine kinase domain. Subsequent work showed that KIT is allelic to the white spotting locus (W) in mice [739, 740]. Mutations in the W locus result in anemia, reduced fertility, pigmentation defects, or lethality, depending on the identity and whether the mutation is hetero- or homozygous [734]. Complete loss of KIT expression leads to death in utero or perinatally, likely due to severe anemia. In addition, there is a testis-specific form of KIT that is truncated due to the use of an alternative promoter denoted tr-Kit [741].

KIT expression is dynamic during development and controlled by a range of growth factors, cytokines and even vitamin A (retinoic acid) [728, 742]. There are at least two regions in the promoter for KIT that have been demonstrated to be important in regulating its expression. In one region, located 58 bp upstream of the transcription initiation codon, there are binding sites for the transcription factors AP2, Sp1, Ets, and Myb [743]. The other region, located 139 bp upstream of the transcription initiation codon, contains binding sites for Myb and Ets2 [744]. Both Myb and Ets2 have been shown to be important for KIT expression in hematopoietic cells. The importance of AP2 for KIT expression is illustrated by the loss of KIT expression when AP2 is lost [745]. The impact of various transcription factors on KIT expression may also depend on cell type, for example, in melanocytes MITF has been demonstrated to regulate KIT expression [746]. In addition to transcription factor-mediated regulation, KIT expression is also influenced by microRNAs, which are noncoding RNAs that act as posttranscriptional regulators of protein expression, for example, miR-218, miR-221a, miR-222, and miR-193b [747–750]. Interestingly, MITF is a regulator of KIT, and KIT promotes miR-539 and miR-381 expression which in turn affects MITF expression [751], thus establishing an elaborate regulatory network.

KIT expression has been found in several cell types, including germ cells, hematopoietic stem cells, mast cells, melanocytes, epithelial cells, vascular smooth muscle cells, and umbilical vein endothelial cells [752–755]. Interestingly, KIT is expressed on primordial germ cells and SCF has been observed along the migratory route for these cells suggesting an important function guiding their migration during embryonic development [753, 756, 757]. Similarly, KIT is believed to be essential for migration of melanocytes [758].

10.3.3 KIT Protein

KIT is a 145-kDa protein that belongs to subclass III of receptor tyrosine kinases, which also includes the PDGFRα, PDGFRβ, M-CSF-R, and FLT3 [759]. Alternative names for KIT are CD117, stem cell factor receptor, and c-Kit. KIT is composed of an extracellular ligand-binding domain and a single hydrophobic transmembrane helix and an intracellular tyrosine kinase domain. The extracellular domain consists of five immunoglobulin (Ig)-like repeats, out of which the first three are involved in ligand binding and the fourth and fifth Ig-like repeats play an important role in forming a productive receptor dimer [328]. Furthermore, the extracellular domain is extensively glycosylated and is so in a variable manner. The transmembrane region is a hydrophobic α-helix. Since α-helices are rigid structures, this allows for a rotational coupling between the extracellular and intracellular regions, thus making ligand binding able to influence the relative positions of the kinase domains within a ligand-induced receptor dimer [760]. The intracellular part of KIT contains an inhibitory juxtamembrane region that plays a key role in suppressing the kinase activity in the monomeric receptor and a kinase domain divided into two parts in the primary structure by an about 80-amino acid-long kinase insert sequence. A consequence of ligand-induced dimerization is an extensive posttranslational modification of KIT, including autophosphorylation of tyrosine residues within the juxtamembrane (Tyr-568 and Tyr-570), kinase insert (Tyr-703, Tyr-721, and Tyr-730), kinase domain (Tyr-823), and C-terminal tail of the receptor (Tyr-936) [743]. In addition, Tyr-900 within the kinase domain of KIT is phosphorylated not directly by the receptor but by Src family kinases (SFK) activated by KIT [761]. KIT tyrosine phosphorylation serves two main purposes: First, it stabilizes the active conformation of the KIT kinase domain and, second, it allows intracellular signaling proteins containing domains, such as SH2 and PTB that have the ability to bind these tyrosine residues only when they are phosphorylated. This recruitment of signaling proteins initiates intracellular signaling pathways that influence the activity of existing proteins or change gene expression. In addition, it has been observed that KIT is extensively serine phosphorylated both in the absence or presence of ligand, and the reason for this is not clear, but it is possible that the negatively charged phosphate groups are important for stabilizing the KIT structure and/or in facilitating substrate selection. Furthermore, PKC can phosphorylate KIT on Ser-741 and Ser-746 resulting in decreased KIT kinase activity, thus serving as a negative feedback loop to limit signaling from the receptor [762]. Besides phosphorylation (and glycosylation during KIT maturation), a third prevalent modification is the addition of ubiquitin moieties on lysine residues. KIT ubiquitination is promoted by receptor activation and subsequent recruitment of ubiquitin ligases out of which c-Cbl is of particular importance [763, 764]. Receptor ubiquitination has been observed for most tyrosine kinase receptor and is linked to the downregulation process [765], probably by dictating the intracellular sorting pathways ultimately leading to receptor degradation or in some cases recycling back to the cell surface. The process of ubiquitination has at least two consequences: First, it removes the positive charge of

the lysine residues in KIT, and second, the ubiquitin is in itself a small protein that also can form different types of polyubiquitin chains that can be recognized by other proteins and hence facilitate sorting or degradation processes depending on the particular type of polyubiquitination that takes place.

Stem cell factor (SCF), the ligand for KIT, can exist in two versions: one soluble and one transmembrane [766]. Both the soluble and transmembrane forms of SCF can induce intracellular signaling by promoting KIT dimerization and activation. However, the transmembrane SCF can in addition also anchor cells together.

In humans, there are two KIT splice forms created by alternative splicing between exons 9 and 10 [767, 768]. This creates two versions of KIT that differs in the absence or presence of a tetrapeptide sequence (Gly–Asn–Asn–Lys) in the extracellular juxtamembrane region. Functional studies have shown that the signaling characteristics of these two receptor splice forms differ; KIT containing the Gly–Asn–Asn–Lys sequence is activated by ligand in a slower and less intense manner, but the receptor remains active for a longer time period, compared to the receptor lacking the Gly–Asn–Asn–Lys tetrapeptide [769, 770]. Both KIT splice forms have the same affinity for the ligand [770] and identical kinase domains, suggesting that these four amino acids affect the relative orientation of the kinase domains and this in turn influences the signaling potency. It was recently demonstrated that adding one amino acid at a time to the GNNK sequence gradually decreased kinase activity, irrespective of whether it was GNNK or AAAA sequence added [771], which suggests that the actual amino acids in the peptide insert is not critical, but the length of the insert.

Furthermore, in postmeiotic germ cells in the testis, there is a shorter approximately 3-kb transcript that starts within intron 15, which creates a truncated 202-amino acid-long version denoted tr-Kit [741]. Tr-Kit is not kinase active and cannot bind and be activated by ligand since it is lacking the extracellular domain and the first part of the kinase domain. Tr-Kit can be phosphorylated, and it probably functions as an adaptor protein and has been shown to promote metaphase to anaphase transition in oocytes [772].

Furthermore, through the use of alternative splice acceptor sites in humans (but not in mice), there exists an additional KIT variant with the absence or presence of a serine residue (Ser-715) in the kinase insert region [768]. The functional consequence of the absence or presence of this serine residue is not understood.

It has been observed that the extracellular part of KIT can be released from cells by the action of a protease that cleaves close to the plasma membrane within the 5th Ig-like domain of KIT [773]. The soluble extracellular fragment of KIT is called S-Kit. S-Kit binds ligand with the same affinity as KIT and can thus function as a natural ligand trap, thereby creating another level of control of KIT signal transduction.

Deregulated KIT activity has been found in different kind of tumors due to overproduction of SCF or by mutation affecting the amino acid sequence of the receptor. The majority of activating mutations occur in kinase domain (exon 13), in juxtamembrane region (exon 11), or in the extracellular domain (exon 9) [743]. These mutations act by promoting dimerization or by removing autoinhibitory functions.

10.3.4 KIT Activation

The mechanism of KIT dimerization and activation has been studied using both biochemical and structural approaches both supporting a common model. The ligand for KIT functions as an antiparallel homodimer where two KIT binding regions simultaneously are exposed. Binding of one SCF dimer to two monomeric KIT monomers has been suggested to be the driving force behind KIT dimerization [331]. It has been shown that it is the first three Ig-like domains of KIT that interacts with SCF by having a complementary binding surface [328]. The binding of SCF to KIT brings about a conformational change in the receptor allowing for additional interactions between Ig-like domain 4 and 5 in KIT [328]. These interactions bring the two transmembrane regions and the intracellular kinase domains close to each allowing for enzymatic activation. Mutation of key residues involved in these interactions showed that although this did not affect the formation of a KIT dimer, it did not allow for receptor activation, suggesting that receptor dimerization is just the first step in a process leading up to receptor activation. Antibodies directed against Ig-like domain 4 have been found to effectively inhibit KIT activation and SCF-driven proliferation, thus providing an alternative to kinase inhibitor strategies for KIT targeting in disease treatment [774]. Recently, by combining crystal structures with electron microscopy, it was suggested that the two KIT molecules within the dimer made close contact along the entire dimer, and the dimer could adopt several conformations [775]. Furthermore, in two prevalent KIT dimer configurations, the kinase domain interacted in an asymmetrical manner. In the related PDGFR, the transmembrane domains have been shown to have affinity for each other [635], and it is likely to be the case also in KIT. The association of two transmembrane domains which are predicted to have rigid α-helical structure may also serve to put the two intracellular kinase domains in a relative orientation suitable for enzymatic activation. Consistent with this hypothesis, experiments with the related PDGFR in which artificial dimerization motifs were introduced in the transmembrane region showed that only certain orientations were compatible with efficient kinase activation [760].

The structure of both the inactive and active KIT kinase domain has been solved (Mol et al. 2004; [776]). KIT has a classical kinase fold consisting of two lobes with the active site located in between. These studies revealed a central role for the intracellular juxtamembrane region in suppressing the kinase activity. This region folds into the cleft between the two kinase lobes and distorts the structure in a way that it does not support kinase activity [639]. In the active kinase, the juxtamembrane region is tyrosine phosphorylated and no longer binding to the kinase domain [776]. Thus, phosphorylation of the juxtamembrane region of KIT is a major regulatory event allowing for kinase activity. There are eight tyrosine residues in KIT that become phosphorylated in vivo: Tyr-568, Tyr-570, Tyr-703, Tyr-721, Tyr-730, Tyr-823, Tyr-900, and Tyr-936 [743]. It has been described that the order in which KIT autophosphorylation occurs is not random and that tyrosine

residues 568 and 570 were the first to be phosphorylated [336], consistent with their role in negatively regulating kinase activity. In contrast to many other kinases, autophosphorylation of the conserved activation loop tyrosine, Tyr-823 in KIT, is not necessary for kinase activation, but is linked to regulation of signals that promote proliferation and survival [777].

10.3.5 KIT Downregulation

In order for a cell to respond in a controlled manner to KIT activation, it is essential that the intensity as well as duration of signaling is appropriate. This is achieved by the initiation of several and in parallel acting feedback loops that serve to restrain signaling output from the receptor. There are three broad categories of feedback mechanisms acting on KIT. First, upon KIT activation, ubiquitin ligases are recruited to the receptor, for example, c-Cbl and SOCS6 [763, 764, 778, 779]. These can be recruited directly to the receptor by interacting with specific phosphorylated tyrosine residues or indirectly through adaptor proteins such as Grb2, p85, CrkL, and SLAP [763, 764, 780]. After ubiquitination of KIT, it is internalized through clathrin-coated pits and routed toward degradation in both proteasomes and lysosomes [778, 781]. Importantly, while the receptor is being sorted toward degradation, it is able to initiate certain signaling pathways. Second, a consequence of KIT stimulation is increased PKC activity which has diverse roles in signaling. In regard to negative regulation of KIT, it is has been found that PKC can phosphorylate two serine residues in the KIT kinase insert (Ser-741 and Ser-746) and this has been associated with decreased KIT kinase activity [762]. In addition, PKC activation also promotes shedding of the extracellular ligand-binding domain of KIT[782]. However, the involvement of PKC in KIT signaling is complex; in colon cancer cells, it has been observed that activation of PKCδ resulted in recycling of KIT, allowing for continuous signaling [783]. Third, KIT-induced signaling can also be restrained by dephosphorylation of tyrosine residues in KIT (or in downstream signaling components). The tyrosine phosphatase SHP1 has been shown to interact with the activated KIT and negatively regulate signaling ability [784, 785].

10.3.6 Signaling Downstream of KIT

Numerous signal transduction pathways are activated downstream of KIT (Fig. 10.18). Although they are listed here as individual pathways, in reality there is a high level of integration of signaling between the different pathways.

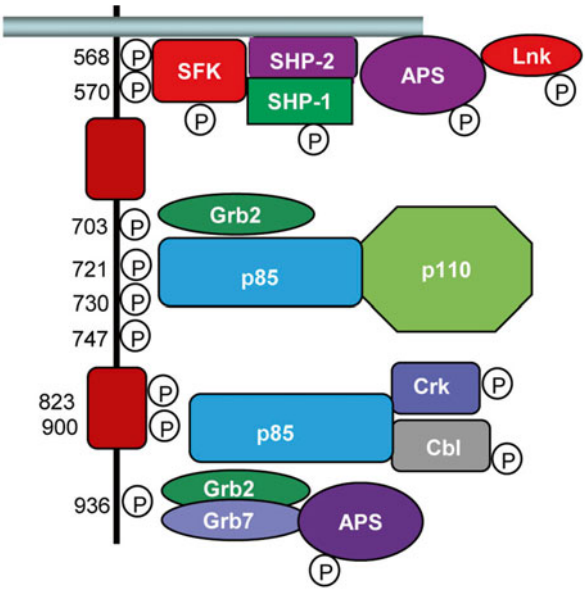

Fig. 10.18 Schematic illustration of KIT tyrosine phosphorylation sites and interaction with signaling proteins

10.3.6.1 Src Family Kinase Signaling

The Src family of tyrosine kinases (SFK) is a very central signal transduction component in signaling downstream of KIT, since it feeds into several other signal transduction pathways such as the Ras/Erk pathway, the PI3-kinase/Akt pathway, and the c-Cbl-mediated ubiquitination pathways (see above and below).

There are in total eight SFKs, of which three are ubiquitously expressed (Src, Yes, and Fyn) and others mainly expressed in the hematopoietic system (Lck, Hck, Lyn, Fgr, and Blk). The SFK has a domain structure consisting of membrane targeting domain, SH3, SH2, tyrosine kinase domain, and a carboxyterminal tail involved in negative regulation of SFK activity. In their inactive state, SFK is in a closed conformation where the kinase domain activity is inhibited. The closed conformation is stabilized through interaction of the SH2 domain with a carboxyterminal phosphorylation site, Tyr527, and further stabilized by interactions between the SH3 domain and proline-rich regions in the SFK. The SFKs are activated by molecular events that interfere with these interactions, e.g., binding of phosphotyrosine residue to the SH2 domain or interaction between a proline-rich sequence of a protein with the SH3 domain of SFK. Dephosphorylation Tyr527 in the carboxyterminal tail will also increase SFK activity.

Upon ligand binding, KIT becomes phosphorylated on two tyrosine residues in the juxtamembrane region (Tyr-568 and Tyr-570) [786–789]. Phosphorylated Tyr-568 docks to the phosphotyrosine-binding groove of the SH2 domain of SFK, while

phosphorylation of Tyr-570 strengthens the binding by providing an acidic determinant [788]. This leads to displacement of the C-terminal tail of SFK from the SH2 domain resulting in an opening of the structure leading to increased catalytic activity.

SFK has been demonstrated to regulate activation of several signal pathways downstream of KIT. Several groups have implicated SFK in SCF-induced ERK1/2 MAP-kinase activation [788, 790]. This has been reported to be mediated through phosphorylation of the Shc adaptor protein [788] as well as through phosphorylation of the scaffolding protein GAB2 [791, 792]. Activation of the JNK MAP-kinase pathway by KIT requires both PI3-kinase and SFK to act on Rac1 [793]. Several studies suggest that although SFKs are often viewed as one group of kinases, they have distinctive functions. In bone marrow-derived mast cells, which express KIT, Lyn was shown to be important for KIT-mediated activation of JNK and Stat3 and that it was a negative regulator of Akt [794]. In contrast, studies have shown that Fyn is important for KIT-mediated phosphorylation of the protein-tyrosine phosphatase SHP2 and p38, while it was dispensable for phosphorylation of either Stat3 or Akt [795].

An important aspect of SFK in KIT signaling is the fact that alternative splice forms of KIT display different capabilities to activate SFK. As described above, alternative splicing of the KIT mRNA generates two splice variants that differ by four amino acids in the extracellular juxtamembrane region. These two splice forms differ dramatically in their abilities to signal, where the form of KIT lacking the tetrapeptide sequence produces a stronger, more rapid, and also transient response [769, 770]. The reason for these differences was shown to depend to a large extent on their ability to recruit and activate SFK [769].

SFK are implicated in regulation of KIT-induced proliferation. In the megakaryocytic cell line Mo7e, it was demonstrated that Lyn promotes the G1/S phase transition and proliferation, which is inhibited by the SFK inhibitor PP1 [786, 796]. The same research team also demonstrated that mast cells lacking expression of Lyn displayed reduced proliferation, as well as migration, compared to control cells [797]. These data suggest that the SFK member Lyn is important for KIT-driven proliferation at least in hematopoietic cells. Furthermore, Lck was found to be important for KIT-mediated proliferation of the small-cell lung cancer cell line H526 [787]. Recent studies have demonstrated the importance of the transcription factor MITF in KIT-mediated proliferation in melanocytes and that SFK, among other signaling pathways downstream of KIT, was important for MITF activation [771]. In contrast, using endothelial cells transfected with KIT lacking the SFK-binding site Tyr-568, no obvious effect on proliferation could be noted [788]. It should be noted, however, that the splice form used in those early studies was the GNNK+ isoform which is a weaker activator of SFK.

Several studies have implicated SFK in mediating KIT-dependent cell migration, and this has been shown to occur through different routes. On one hand, SFK can interact with and phosphorylate FAK which is an important regulator of focal adhesions or directly phosphorylate the focal adhesion protein paxillin [798–800]. The SFK Lyn has been implicated downstream of KIT in promoting migration of mast cells [797].

To study the importance of the SFK-binding site in KIT in vivo, knock-in mouse was produced that expressed KIT^{Y567F} and $KIT^{Y567/569F}$ in place of wild-type KIT; corresponding to Tyr568 and Tyr570 in the human sequence, KIT^{Y567F} mice showed numbers of pro-T and pro-B cells in aged animals [801, 802]. In mice with both Tyr567 and Tyr569 mutated, there were also defects in mast cells and pigmentation and the mice displayed splenomegaly [802]. The difference in phenotype between mice carrying KIT^{Y567F} and $KIT^{Y567/569F}$ is interesting since in vitro it has been found that the single and double mutants are equally incompetent in activating SFK; however, only the double mutant was unable to support a proliferative response [788]. However, when interpreting these data, one should keep in mind that both Tyr568 and Tyr570 are the binding sites of many other proteins apart from SFK. Furthermore, the juxtamembrane region where Tyr568 and Tyr570 are located is of great importance in regulating the kinase activity of KIT. Finally, studies using low molecular weight inhibitors have to be interpreted with care since molecules targeting SFK kinase activity may also affect KIT, as has been demonstrated for the drug PP1 initially described as a SFK inhibitor but later shown to also effectively target KIT [803]. Additionally, SFK can contribute to the overall activity of KIT. Upon activation of KIT, there is a distinctive chronology of phosphorylation, in which the juxtamembrane sites, Tyr568 and Tyr570, are among the first ones to be phosphorylated [336]. Given the fact that Lyn has been shown to contribute to the overall phosphorylation of KIT [794], it is not unlikely, but not yet demonstrated, that SFK is involved in the regulation of KIT kinase activity.

As outlined above, it is clear that SFKs are integral parts of the signaling systems that mediate many KIT-driven responses such as proliferation, survival, and migration. The role of SFK in KIT-driven responses differs between cell types, which probably is a reflection of the expression profile of SFK members and substrate proteins in a given cell type. There are several mutant forms of KIT that can drive tumor progression of which the D816V mutant is the most common. It has been shown that KIT^{D816V}, in contrast to wild-type KIT, does not depend on activation of SFK for its ability to promote survival and proliferation [804]. This can be explained by the fact that the D816V mutant of KIT is not only constitutively active but also displays an altered substrate specificity resembling that of Abl and SFK, hence explaining the reduced need of SFK [804]. This alteration in substrate specificity of KIT has several consequences in that it enables the D816V mutant of KIT to phosphorylate additional substrates that wild-type KIT is not able to phosphorylate and hence alter the repertoire of activated signaling molecules [805, 806].

10.3.6.2 Phosphatidylinositol 3′-Kinase Signaling

Multiple classes of phosphatidylinositol 3′-kinases (PI3′-kinases) exist, but receptor tyrosine kinases predominantly activate class I_A. Receptors with a phosphorylated consensus PI3′-kinase binding site (YXXM) interact with the SH2 domains of the regulatory subunit (p85α, p50α, p55α, p85β, and p55γ) resulting in a conformation change of the associated catalytic p110 subunit (p110α, p110β, and p110δ).

This altered conformation leads to activation of its lipid kinase activity [807]. Furthermore, translocation of PI3′-kinase to the activated receptor at the plasma membrane positions PI3′-kinase in close proximity of its lipid substrates. One important substrate of PI3-kinase is phosphatidylinositol-(4,5)-bisphosphate (PIP_2) which by phosphorylation by PI3-kinase is converted to the second messenger phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3). One important function of PIP_3 is to recruit proteins containing pleckstrin homology (PH) domains to the membrane. One such molecule is the serine–threonine kinase Akt which is a key molecule downstream of PI3′-kinase that among other things promotes cell survival by interfering with the initiation of apoptosis [808]. Interestingly, p85 has been claimed to be expressed at a higher level than p110 in cells [809], suggesting that p85 might have additional functions apart from being a regulatory subunit of PI3-kinase. Indeed, p85 has been demonstrated to interact with the adaptor protein CrkL as well as the ubiquitin ligase c-Cbl [810, 811]. This suggests that p85 recruitment to phosphorylated tyrosine residues may have functions besides regulating p110 location and activity, although this remains to be firmly established. However, more recent studies claim that the expression levels of p85 match those of p110 in mammalian cell lines and tissues [812].

PI3′-kinase is activated by SCF both through direct binding to Tyr-721 in KIT [813, 814] and indirectly through binding to the tyrosine-phosphorylated scaffolding protein GAB2 [791, 815]. GAB2 becomes tyrosine phosphorylated through Grb2-dependent recruitment to the receptor followed by SFK-mediated phosphorylation [792, 815]. Activated Akt promotes cell survival through different mechanisms including phosphorylation of Bad, Foxo, and NF-κB. In the absence of survival signals, Bad heterodimerizes and thereby neutralizes the antiapoptotic proteins Bcl-X_L and Bcl-2 [816]. Downstream of PI3′-kinase, Akt is activated resulting in Bad phosphorylation on Ser-136 [817]. This phosphorylation leads to breakage of the interaction between Bad and Bcl-X_L, and Bad is sequestered by 14-3-3 proteins [818]. Bcl-X_L can then antagonize the proapoptotic Bax protein and thereby block apoptosis. In U2-OS cells transfected with a mutant KIT unable to interact with PI3′-kinase (Tyr721 to Phe mutation), SCF could only partially protect from starvation-induced apoptosis compared to the wild-type receptor [817]. The transcription factor NF-κB contains two subunits, p50 and p65, and is kept in the cytoplasm through interaction with IκB; phosphorylation of IκB results in its proteasomal degradation and thereby release of NF-κB which then enters the nucleus and regulates gene expression [819]. Consistent with NF-κB activation, Dhandapani et al. [820] observed SCF-induced and Akt-dependent IκBα phosphorylation [820]. Akt also promotes increased cell viability by phosphorylating and thereby negatively regulating forkhead transcription factors [821]. Phosphorylation of forkhead transcription factors (FoxO1, FoxO3, and FoxO4) leads to their retainment in the cytoplasm. In contrast, in the absence of phosphorylation, they bind to DNA and promote expression of proapoptotic genes. SCF-stimulated and Akt-mediated phosphorylation of FoxO3 is important to promote survival of hematopoietic progenitor cells [822]. Similarly, in mast cells, which are dependent of SCF as a survival factor, it was found that Akt-mediated FoxoO3a phosphorylation reduced the expression of

the proapoptotic Bcl-2 family member Bim [823]. Phosphorylation of Bim has been connected to its proteasomal degradation [824, 825].

SCF-induced production of reactive oxygen species (ROS) will cause a transient inactivation of protein-tyrosine phosphatases and allow for efficient signal transduction. Treatment of megakaryocytic cell line Mo7e cells with SCF results in an increased ROS production, which in these cells is associated with increased glucose uptake [826, 827]. The molecular mechanism by which KIT promotes ROS production is not clear, but for the closely related PDGF receptor, it has been shown to involve a PI3′-kinase-dependent translocation of cytosolic NAD(P)H oxidase subunits to the plasma membrane [828].

Activation of PI3-kinase by KIT also promotes activation of the Tec family members Btk and Tec. Btk and Tec contain a PH domain and can therefore be recruited to the plasma membrane by PIP_3 [829]. At the plasma membrane, Btk is activated through phosphorylation, presumably by SFKs.

10.3.6.3 Mitogen-Activated Protein Kinase Pathways

Mitogen-activated protein (MAP) kinases are activated downstream of numerous types of cell-surface receptors and play central roles in a multitude of biological processes. The MAP-kinase pathways are built up of three layers of distinctive kinase whose activity is induced in the plasma membrane and is transduced all the way to the nucleus where it regulates gene expression by phosphorylating transcription factors. Other processes occurring in the cytoplasm, for example, translation and cell migration, can be regulated by MAP-kinases. The biological responses of MAP-kinase activation are dependent on the magnitude as well as on the duration of MAP-kinase phosphorylation. All eukaryotic cells have at least one type of MAP-kinase, and human cells contain four major groups: ERK1 and 2 (ERK1/2), ERK5, p38, and JNK.

The ERK1/2 MAP-kinases have been studied in great detail. RTKs commonly engage ERK1/2 by recruiting the RAS guanine exchange factor Sos to plasma membrane. Sos is constitutively associated with the adaptor protein Grb2 that can interact either directly with phosphorylated receptors or indirectly through additional proteins such as Shc. Bringing Sos into the proximity of the small GTPase RAS provokes a nucleotide exchange from GDP to GTP, which induces a conformational change in RAS which in turn allows it to interact with downstream effectors. One of the most well-characterized downstream effector of active RAS is the serine–threonine kinase Raf, which through the interaction with active RAS translocates to the plasma membrane where Raf becomes activated through a process involving both phosphorylation and dephosphorylation [830]. Activated Raf phosphorylates and activates MEK1/2 which in turn phosphorylates and activates ERK1/2. Many of the proteins downstream of ERK1/2 are transcription factors, including c-Fos and Elk-1, but there are also cytoplasmic substrates such as the serine–threonine kinase Rsk. Furthermore, the presence of scaffolding proteins to

assemble the functional components of the MAP-kinase pathways, e.g., KSR, is of great importance [831].

Although stimulation of KIT has been shown to activate ERK1/2, p38, JNK, and ERK5, most studies have been focusing on the mechanisms of activation of ERK1/2. Using mutant receptors deficient in SFK activation, it has been demonstrated that SFK is important for SCF-induced ERK1/2 activation presumably by promoting Shc tyrosine phosphorylation [788]. It was suggested that the Grb2–Sos complex is recruited to KIT through tyrosine-phosphorylated Shc and not primarily through direct binding of Grb2 to the receptor. Interestingly, direct interaction sites for Grb2 exist in KIT, i.e., Tyr-703 and Tyr-936 [832], and the relative importance of these versus indirect Grb2 binding via Shc is not clear. Apart from acting to recruit Sos to the receptor, Grb2 can also act as an adapter recruiting the scaffolding protein GAB2 to the receptor which in turn is phosphorylated by SFKs. Thereby, binding sites for PI3-kinase and SHP-2 are created, contributing to activation of PI3-kinase/Akt pathway as well as the Ras/Erk pathway. The requirement of SFK in ERK1/2 activation appears, however, to be cell type specific since erythroblasts expressing a KIT mutant unable to bind SFK did not display defective ERK1/2 phosphorylation, but rather defective JNK activation [833]. Also studies in mast cells have implicated SFK in activation of JNK [793, 794]. Furthermore, the mechanism of ERK1/2 activation downstream of KIT appears to be different in different cell types or dependent on their state of differentiation; in primitive hematopoietic cells, ERK1/2 activation is dependent on PI3′-kinase signaling and not on RAS, whereas in mature mast cells, activation of ERK1/2 was shown to go through RAS [834]. It is possible that this is achieved through activation of p21-activated kinase 1 (PAK1). PAK1 is activated downstream of Rac which in turn is activated in a PI3′-kinase-dependent manner [835]. In mast cells lacking PAK1, it was seen that SCF-induced Mek1/2 and ERK1/2 phosphorylation was strongly suppressed, implicating a role of PAK1 in the activation process [836]. In a recent study using bone marrow-derived mast cells in which the KSR gene had been deleted, ERK1/2 phosphorylation was found to be reduced, but also p38 and PAK phosphorylation [837]. Several studies have implicated p38 in SCF-induced chemotaxis [836, 838, 839]. Ueda and coworkers found that SCF-induced p38 and PI3′-kinase activation was important for Ca^{2+} influx, which in turn activated ERK1/2 and promoted cell migration [840].

Mast cells deficient in the SFK member Lyn were defective in JNK activation [794], and furthermore, mast cells lacking the SFK member Fyn have a defective JNK as well as p38 activation [795]. Apart from SFKs, other tyrosine kinases are also involved in these responses. In mast cells lacking the Fes tyrosine kinase, SCF was unable to promote sustained p38 activation which correlated with impaired cell migration [841]. It was also demonstrated that SCF-induced Fes phosphorylation is dependent on Fyn.

It has been demonstrated that activation of KIT result in a SFK-dependent phosphorylation of GAB2 which then recruits SHP2, and this is critical for SCF-induced Rac/JNK but is also partially influencing RAS activation [815]. Consistent with the requirement of GAB2 for normal SCF-induced RAS activation [815], ERK1/2 activation has been found to be decreased in mast cells lacking GAB2 expression [791].

In response to KIT activation, ERK5 is activated in a manner dependent on PI3′-kinase activation, and the active ERK5 translocates to the nucleus [842]. In mast cells, it is was found that activation of KIT (as well as FcεRI) activates Mekk2, which in turn can promote JNK and ERK5 activations that are important for cytokine production [843].

10.3.6.4 Phospholipases C and D Signaling

Phospholipase C (PLC) enzymes can be found in all eukaryotic cells and function by hydrolyzing the polar head group from the membrane phospholipid PIP_2 generating the membrane-bound second messenger diacylglycerol (DAG) and soluble, negatively charged inositol-1,4,5-trisphosphate (IP_3). DAG interacts with effector proteins, e.g., certain PKC isoforms whereby it promotes their activation, whereas IP_3 leads to the release of Ca^{2+} from internal stores in the endoplasmic reticulum. The PLCγ family is activated by RTKs and contains two SH2 domains that allow them to interact with activated tyrosine kinase receptors. Another lipase that has been found to be important in KIT signaling is phospholipase D (PLD). PLD hydrolyzes the membrane lipid phosphatidylcholine into phosphatidic acid and soluble choline. Phosphatidic acid is then rapidly hydrolyzed to DAG by phosphatidic acid hydrolase, which can activate protein kinase C.

PLCγ has been found to interact with phosphorylated Tyr-730 in KIT, and interestingly, cell proliferation induced by membrane-bound SCF has been shown to depend on PLCγ association with and activation by KIT [844, 845]. Furthermore, soluble SCF was shown to be unable to active PLC. However, soluble SCF is able to activate phospholipase D (PLD) in a PI3-kinase-dependent manner [846, 847] and thereby indirectly activate PKC. This may be the dominating way for KIT to activate PKC in response to soluble SCF. Furthermore, it was found that DAG produced by PLD activation was necessary for the release of arachidonic acid from mast cells [846].

The role of PLCγ in KIT signaling was investigated by an add-back approach where intracellular tyrosine residues in KIT were mutated to phenylalanine and then added back one at a time. It was found that adding back the PLCγ binding site in KIT (Tyr-730) did not have a major impact on SCF-induced proliferation or migration [848]. However, PLCγ signaling may be more important for cells stimulated by membrane-bound SCF as some data suggests [844, 845].

Activation of KIT can induce a radioprotective effect and PLCγ has been found to be important for this. In cells expressing a mutant of KIT that is unable to bind and activate PLCγ or in cells treated with the PLC-γ inhibitor U73122, activation of KIT did not confer radioprotection [849]. It was shown that SCF-induced PLCγ activation blocked the production of ceramide, an inducer of apoptosis [849].

Alternative splicing in sperms generates a truncated cytoplasmic form of KIT (tr-Kit) that lacks kinase activity [850]. Furthermore, microinjection of tr-Kit in metaphase II-arrested mouse eggs leads their transition into anaphase, which can be blocked by either U73122 or a Ca^{2+} chelator [851]. Furthermore, it has been found

that the SFK Fyn is important in this process by causing phosphorylation of tr-Kit as well as of PLCγ [852]. A likely scenario is that the sperm brings the tr-Kit to the oocyte upon fertilization and that Fyn phosphorylates tr-Kit which in turn allows it to interact with and activate PLCγ. Through production of IP_3, PLCγ then triggers increased intracellular calcium which in turn is necessary for oocyte activation. In addition, tr-Kit also promotes interaction between the RNA-binding protein Sam68 and both Fyn and PLCγ [772].

10.3.6.5 The Grb7 Family of Adaptors

Adaptor proteins contain several domains that promote specific interactions but have no enzymatic function. Since they contain multiple interaction domains, they can interact with several other proteins or lipids simultaneously, thereby linking them together in functional complexes. p85 (the regulatory subunit of PI3-kinase), Grb2, GAB2, and Shc are examples of adaptor proteins that already have been described above in the context of their involvement in certain signaling pathways.

The adaptor protein Grb7 contains one SH2 domain, a PH domain, and a proline-rich sequence. It has been shown that Grb7 can interact through its SH2 domain with Tyr-936 in the carboxyterminus of KIT [832]. The function of Grb7 downstream of KIT is not known, but studies in other systems suggest a function of Grb7 in cell migration (reviewed in [853]). The related adaptor Grb10 also interacts with KIT through its SH2 domain [854] although the exact binding site has not been determined. Grb10 constitutively associates with Akt, suggesting that Akt is translocated to the plasma membrane when Grb10 interacts with KIT and thereby facilitates its subsequent activation [854].

10.3.6.6 The Lnk Family of Adaptors

The adaptor proteins APS, Lnk, and SH2B belong to the same family. They contain SH2 and PH domains and have in many systems been demonstrated to be negative regulators of signaling (for review, see [855]). Of these adapters, both APS and Lnk have been shown to interact with KIT; for Lnk, the binding site has been mapped to Tyr-568 [856], and for APS, this binding has been mapped to Tyr-568 and Tyr-936 in KIT [857]. SCF-mediated activation of ERK1/2, p38, and JNK was found to be negatively regulated by the adaptor protein Lnk [858]. Interestingly, since Lnk associates with Tyr-568, the major SFK-binding site in KIT, and since SFK have been implicated in ERK1/2 and JNK activation as well as regulation of protein stability through c-Cbl-mediated ubiquitination, the inhibitory effect of Lnk may at least partly be due to interference with SFK activation by KIT. Furthermore, Lnk has a conserved tyrosine phosphorylation site in its carboxyterminus that has been predicted to interact with c-Cbl [859]. By overexpressing Lnk in the MC9 mast cell line, it was proposed that Lnk serves to attenuate proliferative signaling from KIT [860]. The decreased proliferation correlated with reduced SCF-induced GAB2

phosphorylation and activation of the ERK1/2 pathway. However, expression of a mutant of Lnk lacking the proposed c-Cbl-binding site did not abrogate this effect. Thus, it is likely that other mechanisms of growth attenuation than binding of c-Cbl to the carboxyterminus of Lnk must exist. In contrast, bone marrow-derived mast cells from *lnk*$^{-/-}$, *APS*$^{-/-}$, and *SH2-B*$^{-/-}$ mice, respectively, did not display any impact in SCF-induced proliferation [861]. It is possible that the discrepancy in results arise from the fact that one study overexpressed the protein while the other eliminated the protein. Overexpression can lead to competition with other proteins binding to the same site, while knockout might lead to compensatory upregulation of other proteins. Consistent with a role of Lnk as a negative regulator of KIT signaling, *lnk*$^{-/-}$ mice had an enhanced hematopoiesis, and specifically the B-cell lineage was increased, which was suggested to be due to an increased sensitivity of KIT expressing precursor cells to SCF [860, 862].

10.3.6.7 The Crk Family of Adaptors

The Crk family of adaptor proteins, CrkI, CrkII, CrkL, and CrkIII, consists of one SH2 domain and one or two SH3 domains. Ligand stimulation of KIT induces phosphorylation of CrkL and its indirect association with KIT through the p85 adaptor protein [810]. CrkII is also phosphorylated by SCF and was shown to interact with Tyr-900 in KIT. This tyrosine residue is not an autophosphorylation site but rather a Src kinase phosphorylation site [761]. The functional consequence of Crk binding to KIT is not clear, but CrkL is known to interact with the ubiquitin E3 ligase c-Cbl, suggesting a possible role in KIT downregulation [810]. Binding of the nucleotide exchange protein C3G to Crk provides a possible link to JNK activation [863].

10.3.7 Signaling from Oncogenic KIT Mutants

Expression of KIT with oncogenic mutations in cells has been shown to lead to SCF-independent receptor phosphorylation to support both proliferation and survival. The majority of oncogenic KIT mutations are located in exon 11 encoding the juxtamembrane region (e.g., KIT^{V560G}) or within exon 17 encoding the kinase domain (e.g., KIT^{D816V}). The exact mechanism behind the constitutive activation is not fully understood. One possibility is that the mutation within the kinase domain results in a structural change that relieves autoinhibitory mechanisms. However, the crystal structure of the kinase domain of KIT with an activating mutation has not yet been elucidated. Recently, an in silico analysis was made on the structural consequences of D816V mutation. This modeling suggested that mutation of D816 caused a structural change in the activation loop, but also a weakened the binding of the juxtamembrane region to the kinase domain [864]. Consequently, the juxtamembrane region is no longer able to efficiently suppress the enzymatic activity of KIT.

Several groups have compared the ability of wild-type and oncogenic mutants of KIT to induce signal transduction and found that they differ qualitatively. This is potentially important since it suggests that there might be ways to selectively target oncogenic signaling with less impact on the normal situation. The reason for the different signaling abilities can stem from changes in intracellular localization of the mutant KIT, altered substrate specificity, or a combination of both.

It has been demonstrated that KIT with gain-of-function mutations in the kinase domain displays a reduced cell-surface expression, and inhibition of kinase activity in both KIT with juxtamembrane or kinase domain mutations restored cell-surface expression of receptor [865, 866]. Another study demonstrated that KIT^{D816V} was primarily localized to and could transmit oncogenic signals from the Golgi apparatus, whereas KIT trapped in the ER could not do this [867]. Although KIT^{D816V} has an increased intracellular localization, some is still surface expressed, and this has functional consequences. For instance, cells expressing KIT^{D816V} still migrate toward soluble SCF, indicating that signals emitted by KIT at the cell surface control chemotaxis [868]. Furthermore, it has been observed that even in cells expressing KIT^{D816V}, there is still a need for SCF to promote activation of Akt and ERK1/2 [804, 869, 870]. In contrast, other pathways such as JNK, c-Cbl, and Shc were constitutively activated in cells expressing KIT^{D816V}. The reason for the dependence of intracellular localization for signaling is not clear but may relate to which downstream substrates are available at different locations or that receptors are modified (e.g., phosphorylated or selectively dephosphorylated) by other enzymes which are located only in certain compartments.

There is also evidence that oncogenic mutations in KIT may alter its substrate specificity, which may lead to changes in signal transduction compared to that induced by the ligand-stimulated wild-type receptor. It has been seen that murine KIT^{D814Y} (corresponding to D816Y in the human sequence) displays altered autophosphorylation pattern and peptide substrate specificity compared to the wild-type receptor [806]. Consistently, another study also demonstrated that KIT^{D816V} displayed a substrate specificity that resembled that of Src and Abl tyrosine kinases [804]. Consequently, the mutant KIT could activate ERK1/2 and become ubiquitinated in manner that was independent on Src, whereas wild-type KIT required Src activity for these processes [763, 788]. Several proteins have been demonstrated to be selectively phosphorylated by KIT D816V but not by ligand-stimulated wild-type KIT. Recently, Sun and coworkers [805] demonstrated that the p110δ isoform of PI3-kinase is phosphorylated by KIT D816V, but not by wild-type KIT. This was also shown to be linked to the transforming capacity of the KIT D816V mutant which was shown to be dependent on tyrosine phosphorylation of p110δ but not its lipid kinase activity. The Src-like adapter protein (SLAP) was also shown to be selectively phosphorylated by KIT D816V [780], and this was linked to inactivation of SLAP-dependent recruitment of c-Cbl and the subsequent ubiquitination of KIT.

Several studies have tried to identify signaling pathways critical for the transforming abilities of mutant KIT. One study showed that the tyrosine kinase Fes was important for negative regulation of Stat and positive regulation of mTor phosphorylation downstream KIT^{D816V} [871]. Furthermore, silencing of Fes expression also

led to a partial reduction in KIT^{D816V}-driven proliferation. It has been demonstrated that PI3-kinase binding to KIT is necessary for the oncogenic properties of KIT^{D816V} or the corresponding mutation D814V in the murine receptor [869, 872]. The importance of PI3-kinase signaling was confirmed in a transplantation experiment where bone marrow cells expressing or lacking p85α were transduced by KIT^{D814V}; normal bone marrow cells were transformed, but not cells lacking p85α [873].

10.3.8 Conclusions

During the last couple of decades that has passed since the KIT/SCF system was discovered, our knowledge about the biological role of SCF and KIT has grown. Today, we have detailed information about the mechanisms of signal transduction, about the basis of cell type-specific signaling, and the role of alternative splice forms of both SCF and KIT. Most of the knowledge of signaling downstream of KIT is based on studies on cell lines. Transgenic mice with either the individual signaling molecules knocked out or with mutations of specific tyrosine residues in KIT have provided us with an information on the role of KIT in the physiological setting, but more work remains in order to provide the complete picture of the physiological role of these pathways. The role of membrane-bound versus soluble SCF in KIT signaling and the mechanisms of synergy between SCF and other cytokines deserve a deeper investigation. Signals of proliferation and survival transmitted through KIT are likely to at least partially contribute to the initiation and progression of many human malignancies. Thus, to summarize KIT is a suitable target for future drug development for the treatment of a multitude of human malignancies. The propensity of tumors to develop resistance to inhibition by KIT inhibitors emphasizes the importance of a deeper understanding of the signal transduction molecules that are selectively utilized by the oncogenic mutants of KIT but not by wild-type KIT.

10.4 Platelet-Derived Growth Factor Receptors[4]

10.4.1 Introduction

Platelet-derived growth factor (PDGF) α- and β-receptors (PDGFRα and PDGFRβ, respectively), together with the receptors for stem cell factor (SCF; Kit), colony-stimulating factor 1 (CSF-1; Fms), and Flt3 ligand (Flt3), form the type III class of protein-tyrosine kinase (PTK) receptors. Members of this family are characterized by five immunoglobulin (Ig)-like domains extracellularly, a single pass transmembrane domain, and a split tyrosine kinase domain intracellularly [759].

[4] Carl-Henrik Heldin

The PDGF receptor family is evolutionary related to the vascular endothelial growth factor (VEGF) receptor family; VEGFR1, 2, and 3, each has seven Ig-like domains in their extracellular parts [874]. Activation of receptors in these families occurs by ligand-induced receptor dimerization.

The aim of this chapter is to review the structural and functional properties of PDGFRα and PDGFRβ.

10.4.2 PDGF Receptor Ligands

There are four genes encoding PDGF polypeptide chains, and the corresponding products make up 5 dimeric isoforms, i.e., PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and the heterodimer PDGF-AB [875, 876]. The PDGF isoforms differ in their receptor-binding specificities; thus, the PDGFRα binds the A-, B-, and C-chains, whereas PDGFRβ binds B- and D-chains. This means that PDGFRα homodimers can be formed by PDGF-AA, PDGF-BB, PDGF-CC and PDGF-AB, PDGFRB homodimers by PDGF-BB and PDGF-DD, and PDGFRα/PDGFRβ heterodimers by PDGF-AB and PDGF-BB; receptor heterodimers have also been noted after stimulation with PDGF-CC and PDGF-DD (Fig. 10.19). Binding of the

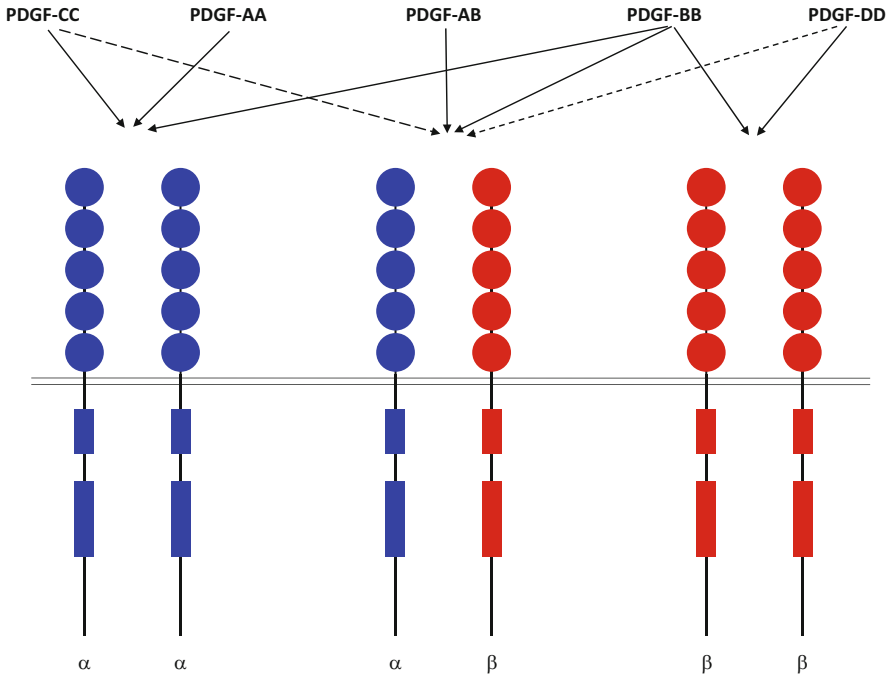

Fig. 10.19 Schematic illustration of the specificities of binding of the PDGF isoforms to αα, αβ, and ββ receptor dimers. *Broken lines* indicate interaction of low affinity

structurally related VEGF-A to PDGF receptors on bone marrow-derived mesenchymal cells has also been described [877], but the physiological significance of this finding remains to be elucidated.

PDGF-AA and PDGF-BB are synthesized as precursor molecules that are cleaved during synthesis by furin-like proteases and secreted as active molecules. In contrast, PDGF-CC and PDGF-DD are secreted as latent precursors containing, in addition to the growth factor domains, also CUB domains. The active growth factors are released after the CUB domains are cleaved off extracellularly by, e.g., plasmin; selective cleavage of PDGFR-CC also occurs by tissue-plasminogen activator (tPA) [878] and PDGF-DD by the closely related protease uPA [879].

The PDGF A-chain occurs as two different splice forms; the long form, but not the short form, has a C-terminal basic sequence that mediates interactions with molecules at the cell membrane and in the extracellular matrix [880]. The presence of the basic retention motif in the long PDGF-AA version restricts its diffusion and promotes autocrine stimulation and stimulation of cells in the close proximity of the producer cell. The PDGF-B chain does not occur as different splice forms, but also has C-terminal basic amino acid stretches that restrict its diffusion; this retention motif can be removed by proteolytic processing [881].

The PDGF isoforms have significant sequence homology with each other and with members of the VEGF family, with conservation of eight cysteine residues involved in inter- and intrachain disulfide bonds. Structurally, the two subunits of PDGF isoforms are arranged in an antiparallel manner and folded in a similar manner as nerve growth factor and transforming growth factor-β isoforms despite the lack of sequence homology between these molecules. The structures are characterized by the presence of three disulfide bridges forming a cystine knot-like structure, in which one of the disulfide bridges passes through the hole created by the other two and their neighboring amino acid sequences [882, 883].

10.4.3 PDGF Receptor Genes

The human PDGFRα gene is located on chromosome 4q12, close to the genes for the SCF receptor and VEGF receptor 2 [884]. It spans 65 kb and contains 23 exons [885] and encodes a protein of 1,089-amino acid residues, including a 23-amino acid residue long signal sequence [886, 887].

The human PDGFRβ gene is located on chromosome 5 [888], close to the CSF-1 receptor gene [889]. It spans 149.5 kb and contains 23 exons [890] and encodes a protein of 1,106-amino acid residues, including a 32-amino acid residue long signal sequence [888].

10.4.4 PDGF Receptor Proteins

The PDGFRα and PDGFRβ are single transmembrane-spanning membrane proteins, each with five immunoglobulin-like domains extracellularly and a tyrosine kinase domain intracellularly with a characteristic inserted sequence of about 100-amino acid residues without homology to kinases. Including glycosylation, the molecular masses of PDGFRα and PDGFRβ are about 170 and 180 kDa, respectively.

The sequence conservation between the two receptors is high in the kinase and juxtamembrane domain, but lower in the extracellular domain, the kinase insert, and the C-terminal tail. In response to ligand stimulation, the receptors undergo phosphorylation and ubiquitination (see further below).

10.4.5 PDGF Receptor Activation and Signaling

PDGF receptors are activated by ligand-induced dimerization. The dimeric ligands bind to Ig domains 2 and 3 and dimerize the receptors in a symmetric manner [891–894]. Whereas ligand binding initiates receptor dimerization, the receptor dimers need to be stabilized by direct interactions between Ig domains 4 and 5 in order for efficient activation to occur [329, 895].

After dimerization, the intracellular parts of the receptors are juxtaposed, allowing phosphorylation in trans between the receptors in the dimer. Thereby, certain tyrosine residues in the receptors are autophosphorylated. In the PDGFRα and PDGFRβ homodimers, 10 and 11 autophosphorylation sites have been identified (Fig. 10.20; [896]). Interestingly, the autophosphorylation sites in the PDGFRα/PDGFRβ heterodimer differ slightly from those of the homodimers, which is of functional importance (see below).

The autophosphorylation has two important functions. First, autophosphorylation occurs in certain regions of the receptors that are involved in keeping the kinase inactive, including the activation loop in the kinase domain [897], the juxtamembrane region [898], and the C-terminal tail [899]; autophosphorylation in these regions causes conformational changes that open up the catalytic site of the kinases allowing access of ATP and protein substrates. Second, autophosphorylation of specific tyrosine residues creates docking sites for SH2-domain-containing signaling molecules. SH2-domains can recognize phosphorylated tyrosines in certain environments; in particular, the 3–6-amino acid residue C-terminal of the phosphorylated tyrosine is important for binding specificity [900]. The PDGF receptors have been found to bind about ten different families of SH2-domain-containing molecules (Fig. 10.20; [896]).

Some SH2-domain-containing molecules that bind to the PDGF receptors have intrinsic enzymatic activities, e.g., members of the Src family of tyrosine kinases

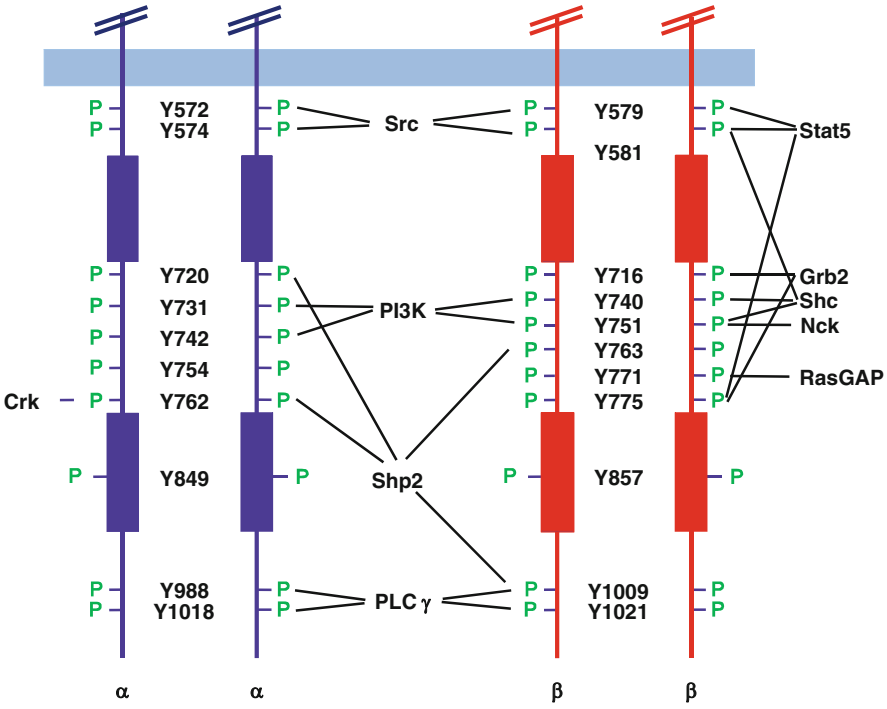

Fig. 10.20 Schematic illustration of autophosphorylation sites in αα and ββ receptor dimers and their specificities for binding of SH2 domain proteins

[901, 902], the tyrosine phosphatase SHP-2 [903, 904], and phospholipase C-γ [905]. In addition, PDGFRβ, but not PDGFRα, binds the GTPase protein (GAP) for Ras [906].

Other PDGF receptor binders are adaptor proteins without intrinsic enzymatic activity. Some of these form stable complexes with enzymes, e.g., Grb2, which occurs in a complex with the nucleotide exchange molecule SOS1 that activates Ras [907]. In addition, the α- and β-p85 regulatory subunits of the phosphatidylinositol 3′-kinase (PI3-kinase) occur in complexes with the catalytic α- or β-110 catalytic subunits [908–910]. Other adaptors, including Nck, Shc, Crk, Grb7, Grb10, Grb14, and GAB, mediate interactions with other downstream signaling molecules [896]. Certain members of the STAT family of transcription factors also bind to and are activated by the PDGF receptors [911].

There are also examples of molecules without SH2 domains that bind to PDGF receptors. Examples include the PDZ domain molecule NHERF, which binds to the C-terminal end of PDGFRβ and enhances receptor signaling [912–915]. Since the C-terminal sequences of PDGFRα and β are completely conserved, it is likely that NHERF also binds PDGFRα. Moreover, the adaptor molecule Alix has been shown to bind to PDGFRβ and to facilitate the binding of the ubiquitin ligase Cbl [916].

Attempts have been made to elucidate which of the signaling pathways activated by PDGF receptors that mediate the different responses observed, e.g., stimulation of cell proliferation, migration, and survival. This has turned out to be more difficult than anticipated, due to cell type differences and to an extensive cross-talk between different signaling pathways [917]. In general, activation of Ras and the downstream Erk MAP-kinase pathway, Src leading to activation of the transcription factor Myc, and PLCγ leading to activation of protein kinase C isoforms, have been shown to be important for the stimulation of cell proliferation. Activation of PI3-kinase has been found to be of particular importance for the antiapoptotic and motility responses of PDGF [875].

Signaling via PDGF receptors is carefully controlled. Thus, in the early phase of stimulation, various mechanisms assure that the signal rapidly reaches a high level. Examples include inhibition of tyrosine phosphatases which enhances tyrosine phosphorylation. This occurs by a PI3-kinase-dependent production of reactive oxygen species that react with a cysteine residue of the active sites of phosphatases and inactivate them [918, 919]. Another amplification mechanism is the ubiquitination and proteasomal degradation of MAP-kinase phosphatase-3 (MKP3), which dephosphorylates and deactivates Erk MAP-kinase; removal of this phosphatase, which occurs in an Erk MAP-kinase-dependent manner, is needed for an efficient Erk MAP-kinase activation [920].

There are also mechanisms that negatively modulate PDGF receptor signaling. Thus, at the same time as Grb2/SOS1 binds, directly or indirectly, to the PDGF receptors leading to activation of Ras and Erk MAP-kinase, RasGAP also binds to PDGFRβ and counteracts Ras activation [906]. The fact that PDGFRα does not bind RasGAP has as consequence that PDGF-AA activates Erk MAP-kinase more rapidly and efficiently compared to PDGF-BB [921]. Interestingly, Tyr771 in PDGFRβ which mediates binding of RasGAP is more efficiently phosphorylated in a PDGFRβ homodimer than in a PDGFRα/PDGFRβ heterodimer. Thus, the heterodimeric receptor induces a more efficient activation of Ras and the Erk MAP-kinase than the PDGFRβ homodimer; this may explain why PDGF-AB is a stronger mitogen than PDGF-BB for fibroblasts [922].

Another modulatory mechanism is exerted by the tyrosine phosphatase SHP2, which binds to PDGF receptors and dephosphorylates the receptors and their substrates [903], thus exerting a negatively modulatory effect on PDGF signaling. However, SHP2 also positively modulates signaling, e.g., by dephosphorylating the C-terminal inhibitory phosphorylation site in Src, thereby activating Src [923]; it can also act as an adaptor which after tyrosine phosphorylation binds Grb2/SOS1, thus promoting Ras activation [924].

Signaling via PDGF receptors is also modulated by interaction with other cell-surface receptors. Thus, PDGF receptors interact with integrins, which enhance signaling [925], and with the hyaluronan receptor CD44, which suppresses signaling [926].

10.4.6 PDGF Receptor Internalization, Processing, and Attenuation

After ligand binding, PDGF receptors are accumulated in coated pits at the cell membrane and are internalized in a clathrin- and dynamin-dependent manner; the internalization is partly dependent on the kinase activity of the receptors [927], and signaling continues in endosomes [928]. Receptor internalization is promoted by ubiquitination of the receptor by the ubiquitin ligase Cbl; further polyubiquitination of the receptors marks them for degradation in lysosomes [929]. Cbl itself is also subjected to ubiquitination and subsequent degradation in proteasomes, a process which is promoted by Alix [916].

The degradation of the PDGF receptors after ligand-induced activation is an important control mechanism to assure an appropriate level of stimulation. There are, however, sorting mechanisms which can promote recycling of the PDGF receptors to the cell surface. One such mechanism is exerted by activation of PLCγ via enhanced phosphorylation of Tyr1021 in PDGFRβ in cells deficient in the TC-PTP phosphatase [930]. Interestingly, recycling of PDGFRα was not observed in these cells. The mechanism involves activation of protein kinase C downstream of PLC-γ [931].

Another mechanism was found to involve overactivity of the PI3-kinase pathway. Thus, in cells transformed with Ras or stimulated by EGF, PDGF-induced autophosphorylation of PDGFRβ was enhanced; the enhanced activation of PI3-kinase promoted internalization of the receptor by macropinocytosis, which is accompanied by increased recycling [932]. In both cases, the increased receptor recycling was accompanied by increased amplitude and duration of receptor activation. Thus, modulation of receptor sorting mechanisms can affect signaling via PDGF receptors.

10.4.7 Role of PDGF Receptors in Embryonic Development

Studies on mice with the genes for PDGF ligands or receptors knocked out have unraveled important functions for PDGF signaling to promote proliferation, migration, and differentiation of specific cell types during embryonal development [876]. Often, PDGF isoforms are produced by epithelial or endothelial cells and act in a paracrine manner on nearby mesenchymal cells, such as fibroblasts, pericytes, and smooth muscle cells [876, 933]. PDGFRα and PDGFRβ have been found to have distinct roles during embryonal development. In order to explore whether these differences are due to different expression patterns or to different signaling capacities of the two receptors, Klinghoffer et al. [934] swapped the intracellular parts of the kinase domains of the receptors; whereas loss of the cytoplasmic part of PDGFRα could be rescued by the cytoplasmic part of PDGFRβ, the intracellular part of PDGFRα only partially rescued the loss of the PDGFRβ intracellular part. Thus, both the expression patterns and the signaling capacities account for the different roles of PDGFRα and PDGFRβ during embryonal development.

10.4.7.1 PDGFRα

Based on the use of dominant negative constructs and low molecular weight receptor kinase inhibitors, PDGF-AA and PDGFRα have been shown to have important roles during gastrulation in *Xenopus* [935] and sea urchins [936], as well as in the formation of mesendodermal cell protrusions and cell polarization in zebra fish embryos [937].

During mouse development, PDGF-A and PDGFRα are co-expressed in the blastocyst inner cell mass [938]. Knockout of PDGF-A or PDGFRα genes leads to severe impairment of early mesenchymal derivatives in both embryo and extraembryonic tissues [939]; depending on genetic background, a portion of these animals die before or at E10.5. PDGFRα knockout mice show defects in neural crest mesenchyme derivatives, including the cardiac outflow tract and the thymus, as well as skeletal components in the facial and other regions affecting the development of the palate and teeth [940–943].

Those PDGF-A knockout mice that survive birth were found to develop lung emphysema; PDGFRα-positive alveolar myofibroblast precursors were found not to migrate to the alveolar saccules [944, 945]. In addition, knockout of PDGF-A or PDGFRα was found to perturb the interaction between the PDGFRα expressed by mesenchymal cells of different kinds and the ligand expressed by neighboring epithelial cells. The defects seen include abnormal development of gastrointestinal villi [946], skin blistering [940], reduced hair development [947], and abnormal development of Leydig cells of the testis [948, 949].

10.4.7.2 PDGFRβ

Knockout of PDGF-B or PDGFRβ genes unraveled important roles of signaling via PDGFRβ for the recruitment of pericytes and smooth muscle cells to blood vessels [950, 951]. Thus, PDGF-BB is produced by endothelial cells and stimulates PDGFRβ-expressing pericytes and smooth muscle cells [952–954]. In particular, PDGF-BB is secreted by the tip cells that lead the angiogenic sprout [955]. PDGF-B or PDGFRβ knockout mice die at E16-E19, due to impaired glomeruli function in the kidneys because of developmental defect of mesangial cells [950, 951, 956], capillary aneurysm [954], cardiac defects [957], and placental defects [958]. A role for PDGFRβ in the early development of hematopoietic/endothelial precursors has also been demonstrated; activation of PDGFRβ in these cells drives differentiation toward endothelial cells [959].

10.4.8 Role of PDGF Receptors in Adult Physiology

PDGF-BB has been shown to stimulate wound healing [960, 961]. Presumably, both PDGFRα and PDGFRβ are involved in the response, since these receptors are expressed by several cell types involved in wound healing, including fibroblasts,

smooth muscle cells, neutrophils, and macrophages [962]. PDGF isoforms released from blood platelets or other cells recruit these cell types to the wounded area. PDGF also contributes to wound healing by stimulating the production of matrix molecules (reviewed by 3).

10.4.8.1 PDGFRα

A potentially important function for PDGFRα was recently reported, i.e., to promote proliferation of insulin-producing β-cells in juvenile pancreatic islets [963].

10.4.8.2 PDGFRβ

PDGFRβ controls the interstitial fluid pressure of tissues and thus counteracts edema formation [964]. A possible mechanism is that stromal fibroblasts and myofibroblasts make contacts with collagen fibers via their integrins and that PDGFRβ activation induces contraction of the cells, which controls the interstitial fluid pressure [965].

10.4.9 Roles of PDGF Receptors in Human Disease

Overactivity of PDGF receptors has been found to contribute to tumor progression, as well as to the progression of other diseases characterized by increased cell proliferation, such as atherosclerosis and fibrosis.

Whereas certain diseases can be ascribed to overactivity of either PDGFRA or PDGFRβ (see below), overactivity of both receptors probably contribute to the development of fibrotic diseases, such as lung fibrosis, liver cirrhosis, glomerulonephritis, and myelofibrosis [966]. During chronic inflammation, macrophages and other cell types secrete growth factors and cytokines which act on mesenchymal cells and upregulate PDGF receptors. In response to PDGF stimulation, these cells then proliferate and produce matrix molecules, thus contributing to fibrosis. Knock-in of constitutively active PDGFRα mutants was found to promote a progressive fibrotic phenotype of many organs [967], and knock-in of similar PDGFRβ mutants was found to lead to an enhanced wound-healing response in the skin and the liver [968]. These findings support the notion that enhanced signaling via PDGF receptors promotes fibrosis.

10.4.9.1 PDGFRα

The PDGFRα gene is mutated in certain malignancies. About 5 % of gastrointestinal stromal tumors (GIST) show point mutations in the PDGFRα gene. The mutations affect the control mechanisms that keep the kinase activity inhibited, leading

to a constitutively active kinase [969]. Similar activating point mutations in the PDGFRα gene have been observed in hypereosinophilic syndrome [970]. In this syndrome, and in systemic mastocytosis, the PDGFα gene is also often fused to the FIP1L gene [971–973]. The resulting fusion proteins have constitutively active kinases as a result of the juxtaposition of the receptor kinases, as well as by the loss of regulatory sequences in the juxtamembrane [974] and transmembrane [975] sequences. Moreover, the PDGFRα gene is amplified in a subset of glioblastomas [976–978], anaplastic oligodendrogliomas [979], esophageal squamous carcinoma [980], and pulmonary artery intimal sarcoma [981]. The resulting increased number of PDGFRα on the surface of the cells makes them very sensitive to stimulation by PDGF, and, at very high receptor levels, receptors may be activated in a ligand-independent manner. An activated deletion mutant of PDGFRα has also been found in a human glioblastoma [982].

Increased expression of PDGFRα has been associated with invasiveness and metastasis. Thus, epithelial tumors can undergo epithelial–mesenchymal transition (EMT), e.g., in response to certain cytokines and growth factors, such as transforming growth factor-β [983]. During EMT, tumor cells lose their epithelial characteristics, such as cell–cell junctions, and acquire mesenchymal characteristics, including synthesis of fibronectin and expression of PDGFRα. Whereas epithelial tumor cells generally do not respond to PDGF, they can do so after having gone through EMT [984]. EMT correlates with increased invasiveness and ability to form metastases; interestingly, inhibition of PDGFRα was found to lead to inhibition of metastasis in mouse models of breast cancer [985], hepatocellular carcinoma [986], and prostate cancer [987, 988].

10.4.9.2 PDGFRβ

In chronic myelomonocytic leukemia, the sequences encoding the intracellular part of PDGFRβ were found to be fused to the gene coding for the transcription factor TEL [989] or other genes encoding proteins that can dimerize or oligomerize [990]. Activation of PDGFRβ was found to drive the development of the rare skin tumor dermatofibrosarcoma protuberans since in this tumor the PDGF-B gene is fused to the collagen 1A1 gene [991, 992]; this results in the production of large amounts of a fusion protein which after processing becomes very similar to mature PDGF-BB that activates the PDGF receptors in an autocrine manner [993].

In addition to mediating direct effects on certain tumor cells, PDGFRβ also has a more general effect in solid tumors by stimulating cells in the stroma. Thus, PDGF-B and PDGFRβ are overexpressed in the stroma of glioblastoma cells [994] and in other tumor types [995]. PDGFRβ expressed on pericytes and smooth muscle cells mediates proangiogenic signals, and activation of PDGFRβ expressed on myofibroblasts contributes to the increased interstitial fluid pressure seen in many solid tumors; the latter is an obstacle in tumor treatment since it decreases transcapillary transport and thereby lowers the uptake of chemotherapeutical drugs [996].

Overactivity of PDGF receptors, particularly PDGFRβ, has also been linked to atherosclerosis and restenosis. PDGF isoforms released by platelets, inflammatory cells, and, possibly, endothelial cells act on smooth muscle cells in the media layer of the vessel wall and stimulate them to migrate into the intima layer; subsequent cell proliferation, lipid deposition, and inflammation lead to narrowing of the vessel lumen [997, 998].

10.4.10 PDGF Receptor Inhibitors

Signaling via PDGF receptors can be inhibited by sequestration of PDGF isoforms by, for example, monoclonal antibodies, soluble extracellular parts of the receptors, and DNA aptamers. Moreover, inhibitory antibodies against the extracellular parts of the receptors have been developed. Finally, selective low molecular weight inhibitors of the receptor kinases are available, e.g., imatinib, sunitinib, and sorafenib [999].

PDGF signal antagonists are used clinically for the treatment of patients with the rare malignancies driven by overactivity of PDGF signaling (see above), with some reported beneficial effects [1000]. Moreover, there are indications that PDGF receptor antagonists can be of more general applicability to inhibit metastasis [1001] and to lower the interstitial fluid pressure and thus increase the uptake of chemotherapeutical drugs [996].

The observed side effects after treatment with PDGF receptor inhibitors reflect the known in vivo function of PDGF. They include a tendency for edema, reflecting an important role for PDGFRβ in controlling the interstitial fluid pressure of tissues [964], and heart failure, reflecting an important role of PDGF in stress-induced cardiac angiogenesis [1002]. In patients treated with the inhibitory Fab fragment CDP860 for advanced ovarian cancer, significant ascites was developed [1003]; the exact mechanism behind this effect remains to be elucidated.

In conclusion, it seems as if inhibitors of PDGF receptor signaling will be valuable tools for the treatment of certain diseases.

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Receptor at a glance: CSF-1R (human)

Chromosome location	5q32
Gene size (bp)	60,077
Intron/exon numbers	21/22
Amino acid number	972
kDa	M_r 130 (the immature, high mannose-containing form) M_r 165 (the mature, N-glycosylated form)
Posttranslational modifications	Glycosylation, phosphorylation, ubiquitination
Domains	ECD (D1–D5), TM, ICD (JM, KD, IK, CT)
Ligands	CSF-1, IL-34
Known dimerizing partners	CSF-1R
Pathways activated	MAPK, PI3K, Rac, Rho, JAK/STAT
Tissues expressed	Hematopoietic stem cells and myeloid progenitors (e.g., CFU-GM, CFU-M), monocytes, tissue macrophages, microglia, osteoclasts, dendritic, Kupffer and Langerhans cells, neuronal subsets, neural progenitors, mammary epithelial cells, Paneth cells, renal proximal tubule epithelial cells, trophoblasts, oocytes
Human diseases	Activating CSF-1R mutations in myeloid malignancies, including acute megakaryoblastic leukemia, AML, CML Inactivating mutations in ALSP
Knockout mouse phenotype	Late embryonic/early postnatal lethality. Osteopetrosis, toothlessness, growth impairment, neurological and reproductive defects

Receptor at a glance: FLT3 (human)

Chromosome location	13q12.2 (minus strand)
Gene size (bp)	96,982
Intron/exon numbers	23/24
mRNA size (5′, ORF, 3′)	3.7 kb (5′ 700 bp; ORF 2979 bp, 3′ 21 bp)
Amino acid number	993
KDa	M_r 130 (the immature, high mannose-containing form) M_r 155/160 (the mature, N-glycosylated form)
Posttranslational modifications	Glycosylation, phosphorylation, ubiquitination
Domains	ECD (D1-D5), TM, ICD (JM, KD, IK, CT)
Ligands	FL
Known dimerizing partners	FLT3
Pathways activated	RAS/RAF/MAPK, PI3K/AKT/mTOR
Tissues expressed	Hematopoietic stem cells and early committed progenitors, such as lin-kit+sca-1+ (LSK) bone marrow cells and lin-AA4.1+ fetal liver cells, some B lymphocyte subsets, with lower expression on monocytes; placenta, gonads, and brain; blast cells from most ANLL and B-ALL

(continued)

Human diseases	Activating FLT3 mutations in ALL, CML
Knockout mouse phenotype	Normal mature hematopoietic populations, deficient in primitive B-lymphoid progenitors. *flt3*$^{-/-}$ HSCs have a reduced capacity to reconstitute T cells and myeloid cells

Receptor at a glancve: KIT

Chromosome location	4
Gene size (bp)	82,797 bp
Intron/exon numbers	20 introns, 21 exons
mRNA size (5′, ORF, 3′)	5 kbp
Amino acid number	976 amino acids
kDa	109,685 unglycosylated, 145 kDa as the mature, glycosylated form
Posttranslational modifications	glycosylation, phosphorylation, ubiquitylation
Domains	extracellular domain, transmembrane domain, juxtamembrane domain, kinase domain with kinase insert, carboxyterminal tail
Ligands	KITLG
Known dimerizing partners	
Pathways activated	Ras/Erk pathway, PI3-kinase/Akt pathway, p38, Src family kinases, Cbl, Grb10, Gab10, Grb7, Crk, CrkL, SLAP, APS, Lnk, SHP1, SHP2
Tissues expressed	Mast cells, interstitial cells of Cajal, bone marrow stem cells, melanocytes, spermatogonia, oocytes
Human diseases	Mastocytosis, acute myeloid leukemia (in particular core factor binding leukemia), testicular seminoma, ovarian dysgerminoma, teratoma, small-cell lung cancer, gastrointestinal stroma tumors, malignant melanoma, allergy, asthma
Knockout mouse phenotype	Loss of pigment cells in skin, anemia, sterility, loss of intestinal cells of Cajal with resulting constipation, sometimes loss of hearing

References

1. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell. 1985;41(3):665–76.
2. Coussens L, Van Beveren C, Smith D, Chen E, Mitchell RL, Isacke CM, et al. Structural alteration of viral homologue of receptor proto-oncogene fms at carboxy-terminus. Nature. 1986;32(6059):277–80.
3. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010;141(7): 1117–34.
4. Stanley ER, Heard PM. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. J Biol Chem. 1977;252(12):4305–12.
5. Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E, et al. Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. Science. 2008;320(5877): 07–11.

6. Pandit J, Bohm A, Jancarik J, Halenbeck R, Koths K, Kim SH. Three-dimensional structure of dimeric human recombinant macrophage colony-stimulating factor. Science. 1992; 258(5086):1358–62.
7. Ma X, Lin WY, Chen Y, Stawicki S, Mukhyala K, Wu Y, et al. Structural basis for the dual recognition of helical cytokines IL-34 and CSF-1 by CSF-1R. Structure. 2012;20(4): 676–87.
8. Liu H, Leo C, Chen X, Wong BR, Williams LT, Lin H, et al. The mechanism of shared but distinct CSF-1R signaling by the non-homologous cytokines IL-34 and CSF-1. Biochem Biophys Acta. 2012;1824(7):93–45.
9. Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, et al. Functional overlap, but differential expression of CSF-1 and IL-34, in their regulation of macrophages and osteoclasts via the CSF-1 receptor. J Leukoc Biol. 2010;88(3):495–505.
10. Nandi S, Gokhan S, Dai XM, Wei S, Enikolopov G, Lin H, et al. The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation. Dev Biol. 2012;367(2):100–13.
11. Chitu V, Stanley ER. Colony-stimulating factor-1 in immunity and inflammation. Curr Opin Immunol. 2006;18(1):39–48.
12. Wang Y, Szretter KJ, Vermi W, Gilfillan S, Rossini C, Cella M, et al. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. Nat Immunol. 2012;13(8):753–60.
13. Greter M, Lelios I, Pelczar P, Hoeffel G, Price J, Leboeuf M, et al. Stroma-derived interleukin-34 controls the development and maintenance of langerhans cells and the maintenance of microglia. Immunity. 2012;37(6):1050–60.
14. Chihara T, Suzu S, Hassan R, Chutiwitoonchai N, Hiyoshi M, Motoyoshi K, et al. IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. Cell Death Differ. 2010;17(12):1917–27.
15. Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, et al. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood. 2002;99(1):111–20.
16. Cecchini MG, Dominguez MG, Mocci S, Wetterwald A, Felix R, Fleisch H, et al. Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. Development. 1994;120(6):1357–72.
17. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 2010; 330(6005):841–5.
18. Hume DA. Macrophages as APC and the dendritic cell myth. J Immunol. 2008;181(9): 5829–35.
19. Pollard JW. Trophic macrophages in development and disease. Nat Rev Immunol. 2009;9(4): 259–70.
20. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496(7446):445–55.
21. Dai XM, Zong XH, Akhter MP, Stanley ER. Osteoclast deficiency results in disorganized matrix, reduced mineralization, and abnormal osteoblast behavior in developing bone. J Bone Miner Res. 2004;19(9):1441–51.
22. Chitu V, Nacu V, Charles JF, Henne MW, McMahon HT, Nandi S, et al. PSTPIP2 deficiency in mice causes osteopenia and increased differentiation of multipotent myeloid precursors into osteoclasts. Blood. 2012;120(15):3126–35.
23. Gu TL, Mercher T, Tyner JW, Goss VL, Walters DK, Cornejo MG, et al. A novel fusion of RBM6 to CSF1R in acute megakaryoblastic leukemia. Blood. 2007;110(1):323–33.
24. Gisselbrecht S, Fichelson S, Sola B, Bordereaux D, Hampe A, Andre C, et al. Frequent c-fms activation by proviral insertion in mouse myeloblastic leukaemias. Nature. 1987;329(6136):259–61.

25. Aikawa Y, Katsumoto T, Zhang P, Shima H, Shino M, Terui K, et al. PU.1-mediated upregulation of CSF1R is crucial for leukemia stem cell potential induced by MOZ-TIF2. Nat Med. 2010;16(5):580–5.
26. Paietta E, Racevskis J, Stanley ER, Andreeff M, Papenhausen P, Wiernik PH. Expression of the macrophage growth factor, CSF-1 and its receptor c-fms by a Hodgkin's disease-derived cell line and its variants. Cancer Res. 1990;50(7):2049–55.
27. Lamprecht B, Walter K, Kreher S, Kumar R, Hummel M, Lenze D, et al. Derepression of an endogenous long terminal repeat activates the CSF1R proto-oncogene in human lymphoma. Nat Med. 2010;16(5):571–9. 1p following 9.
28. Haran-Ghera N, Krautghamer R, Lapidot T, Peled A, Dominguez MG, Stanley ER. Increased circulating colony-stimulating factor-1 (CSF-1) in SJL/J mice with radiation-induced acute myeloid leukemia (AML) is associated with autocrine regulation of AML cells by CSF-1. Blood. 1997;89(7):2537–45.
29. Huynh D, Dai XM, Nandi S, Lightowler S, Trivett M, Chan CK, et al. Colony stimulating factor-1 dependence of paneth cell development in the mouse small intestine. Gastroenterology. 2009;137(1):136–44. 44 e1–3.
30. Guleria I, Pollard JW. The trophoblast is a component of the innate immune system during pregnancy. Nat Med. 2000;6(5):589–93.
31. Hoshino S, Kurotani R, Miyano Y, Sakahara S, Koike K, Maruyama M, et al. Macrophage colony-stimulating factor induces prolactin expression in rat pituitary gland. Zoolog Sci. 2014;31(6):390–7.
32. Luo J, Elwood F, Britschgi M, Villeda S, Zhang H, Ding Z, et al. Colony-stimulating factor 1 receptor (CSF1R) signaling in injured neurons facilitates protection and survival. J Exp Med. 2013;210(1):157–72.
33. Rademakers R, Baker M, Nicholson AM, Rutherford NJ, Finch N, Soto-Ortolaza A, et al. Mutations in the colony stimulating factor 1 receptor (CSF1R) gene cause hereditary diffuse leukoencephalopathy with spheroids. Nat Genet. 2011;44(2):200–5.
34. Nicholson AM, Baker MC, Finch NA, Rutherford NJ, Wider C, Graff-Radford NR, et al. CSF1R mutations link POLD and HDLS as a single disease entity. Neurology. 2013; 80(11):1033–40.
35. Sarrazin S, Mossadegh-Keller N, Fukao T, Aziz A, Mourcin F, Vanhille L, et al. MafB restricts M-CSF-dependent myeloid commitment divisions of hematopoietic stem cells. Cell. 2009;138(2):300–13.
36. Mossadegh-Keller N, Sarrazin S, Prashanth KK, Espinoza L, Stanley ER, Nutt SL, et al. M-CSF instructs myeloid lineage fate in single hematopoietic stem cells. Nature. 2013; 497(7448):239–43.
37. Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER. Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell. 1982;28(1):71–81.
38. Bartelmez SH, Bradley TR, Bertoncello I, Mochizuki DY, Tushinski RJ, Stanley ER, et al. Interleukin 1 plus interleukin 3 plus colony-stimulating factor 1 are essential for clonal proliferation of primitive myeloid bone marrow cells. Exp Hematol. 1989;17(3):240–5.
39. Sasmono RT, Ehrnsperger A, Cronau SL, Ravasi T, Kandane R, Hickey MJ, et al. Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages in vitro in response to CSF-1. J Leukoc Biol. 2007; 82(1):111–23.
40. Arceci RJ, Pampfer S, Pollard JW. Expression of CSF-1/c-fms and SF/c-kit mRNA during preimplantation mouse development. Dev Biol. 1992;151(1):1–8.
41. Arceci RJ, Pampfer S, Pollard JW. Role and expression of colony stimulating factor-1 and steel factor receptors and their ligands during pregnancy in the mouse. Reprod Fertil Dev. 1992;4(6):619–32.
42. Arceci RJ, Shanahan F, Stanley ER, Pollard JW. Temporal expression and location of colony-stimulating factor 1 (CSF-1) and its receptor in the female reproductive tract are

consistent with CSF-1-regulated placental development. Proc Natl Acad Sci USA. 1989;86(22):8818–22.
43. Regenstreif LJ, Rossant J. Expression of the c-fms proto-oncogene and of the cytokine, CSF-1, during mouse embryogenesis. Dev Biol. 1989;133(1):284–94.
44. Menke J, Rabacal WA, Bryne KT, Iwata Y, Schwartz MM, Stanley ER, et al. Circulating CSF-1 promotes monocyte and macrophage phenotypes that enhance lupus nephritis. J Am Soc Nephrol. 2009;20(12):2581–92.
45. Huynh D, Akçora D, Malaterre J, Chan CK, Dai XM, Bertoncello I, et al. CSF-1 receptor-dependent colon development, homeostasis and inflammatory stress response. PLoS One. 2013;8(2):e56951.
46. Wang Y, Berezovska O, Fedoroff S. Expression of colony stimulating factor-1 receptor (CSF-1R) by CNS neurons in mice. J Neurosci Res. 1999;57(5):616–32.
47. Marks Jr SC, Lane PW. Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. J Hered. 1976;67(1):11–8.
48. Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, et al. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature. 1990;345(6274):442–4.
49. Wiktor-Jedrzejczak W, Bartocci A, Ferrante Jr AW, Ahmed-Ansari A, Sell KW, Pollard JW, et al. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic ($^{op/op}$) mouse. Proc Natl Acad Sci USA. 1990;87(12):4828–32.
50. Wise GE. Cellular and molecular basis of tooth eruption. Orthod Craniofac Res. 2009;12(2): 67–73.
51. Pollard JW, Stanley ER. Pleiotropic roles for CSF-1 in development defined by the mouse mutation osteopetrotic. Adv Dev Biochem. 1996;4:153–93.
52. Wiktor-Jedrzejczak W, Urbanowska E, Aukerman SL, Pollard JW, Stanley ER, Ralph P, et al. Correction by CSF-1 of defects in the osteopetrotic $^{op/op}$ mouse suggests local, developmental, and humoral requirements for this growth factor. Exp Hematol. 1991;19(10): 1049–54.
53. Sundquist KT, Cecchini MG, Marks Jr SC. Colony-stimulating factor-1 injections improve but do not cure skeletal sclerosis in osteopetrotic (op) mice. Bone. 1995;16(1):39.
54. Ryan GR, Dai XM, Dominguez MG, Tong W, Chuan F, Chisholm O, et al. Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1(op)/Csf1(op)) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. Blood. 2001;98(1):74–84.
55. Dai XM, Zong XH, Sylvestre V, Stanley ER. Incomplete restoration of colony-stimulating factor 1 (CSF-1) function in CSF-1-deficient Csf1op/Csf1op mice by transgenic expression of cell surface CSF-1. Blood. 2004;103(3):1114–23.
56. Nandi S, Akhter MP, Seifert MF, Dai XM, Stanley ER. Developmental and functional significance of the CSF-1 proteoglycan chondroitin sulfate chain. Blood. 2006;107(2): 786–95.
57. Gow DJ, Sester DP, Hume DA. CSF-1, IGF-1, and the control of postnatal growth and development. J Leukoc Biol. 2010;88(3):475–81.
58. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science. 2012;336(6077):86–90.
59. Hoeffel G, Wang Y, Greter M, See P, Teo P, Malleret B, et al. Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. J Exp Med. 2012;209(6):1167–81.
60. Stanley E, Chen D, Lin H. Induction of macrophage production and proliferation by a purified colony stimulating factor. Nature. 1978;274(5667):168–70.
61. Kierdorf K, Erny D, Goldmann T, Sander V, Schulz C, Perdiguero EG, et al. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nat Neurosci. 2013;16(3):273–80.

62. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity. 2013; 38(1):79–91.
63. Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol. 2002;3(12): 1135–41.
64. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity. 2013;18(4):792–804.
65. Elmore MR, Najafi AR, Koike MA, Dagher NN, Spangenberg EE, Rice RA, et al. Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. Neuron. 2014;82(2):380–97.
66. MacDonald KPA, Palmer JS, Cronau S, Seppanen E, Olver S, Raffelt NC, et al. An antibody against the colony-stimulating factor 1 receptor (CSF1R) depletes the resident subset of monocytes and tissue and tumor-associated macrophages but does not inhibit inflammation. Blood. 2010;116(19):3955–63.
67. Lenzo JC, Turner AL, Cook AD, Vlahos R, Anderson GP, Reynolds EC, et al. Control of macrophage lineage populations by CSF-1 receptor and GM-CSF in homeostasis and inflammation. Immunol Cell Biol. 2012;90(4):429–40.
68. Roth P, Bartocci A, Stanley ER. Lipopolysaccharide induces synthesis of mouse colony-stimulating factor-1 in vivo. J Immunol. 1997;158:3874–80.
69. Cheers C, Stanley ER. Macrophage production during murine listeriosis: colony- stimulating factor 1 (CSF-1) and CSF-1-binding cells in genetically resistant and susceptible mice. Infect Immun. 1988;56(11):2972–8.
70. Santangelo S, Gamelli RL, Shankar R. Myeloid commitment shifts toward monocytopoiesis after thermal injury and sepsis. Ann Surg. 2001;233(1):97–106.
71. Howell K, Posluszny J, He LK, Szilagyi A, Halerz J, Gamelli RL, et al. High MafB expression following burn augments monocyte commitment and inhibits DC differentiation in hemopoietic progenitors. J Leukoc Biol. 2012;91(1):69–81.
72. François B, Trimoreau F, Vignon P, Fixe P, Praloran V, Gastinne H. Thrombocytopenia in the sepsis syndrome: role of hemophagocytosis and macrophage colony-stimulating factor. Am J Med. 1997;103(2):114–20.
73. Garceau V, Smith J, Paton IR, Davey M, Fares MA, Sester DP, et al. Pivotal Advance: Avian colony-stimulating factor 1 (CSF-1), interleukin-34 (IL-34), and CSF-1 receptor genes and gene products. J Leukoc Biol. 2010;87(5):753–64.
74. Rieger MA, Hoppe PS, Smejkal BM, Eitelhuber AC, Schroeder T. Hematopoietic cytokines can instruct lineage choice. Science. 2009;325(5937):217–8.
75. Stanley ER, Bartocci A, Patinkin D, Rosendaal M, Bradley TR. Regulation of very primitive multipotent hematopoietic cells by hemopoietin-1. Cell. 1986;45(5):667–74.
76. Williams N, Bertoncello I, Kavnoudias H, Zsebo K, McNiece I. Recombinant rat stem cell factor stimulates the amplification and differentiation of fractionated mouse stem cell populations. Blood. 1992;79(1):58–64.
77. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol. 2011;11(11):723–37.
78. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci. 2013;16(9):1211–8.
79. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med. 2007;204(12):3037–47.
80. Zhang MZ, Yao B, Yang S, Jiang L, Wang S, Fan X, et al. CSF-1 signaling mediates recovery from acute kidney injury. J Clin Invest. 2012;122(12):4519–32.

81. Li G, Kim YJ, Broxmeyer HE. Macrophage colony-stimulating factor drives cord blood monocyte differentiation into IL-10(high)IL-12absent dendritic cells with tolerogenic potential. J Immunol. 2005;174(8):4706–7.
82. Foucher ED, Blanchard S, Preisser L, Garo E, Ifrah N, Guardiola P, et al. IL-34 induces the differentiation of human monocytes into immunosuppressive macrophages. antagonistic effects of GM-CSF and IFNγ. PLoS One. 2013;8(2):e56045.
83. Puig-Kröger A, Sierra-Filardi E, Domínguez-Soto A, Samaniego R, Corcuera MT, Gómez-Aguado F, et al. Folate receptor beta is expressed by tumor-associated macrophages and constitutes a marker for M2 anti-inflammatory/regulatory macrophages. Cancer Res. 2009;69(24):9395–403.
84. Sierra-Filardi E, Vega MA, Sánchez-Mateos P, Corbí AL, Puig-Kröger A. Heme Oxygenase-1 expression in M-CSF-polarized M2 macrophages contributes to LPS-induced IL-10 release. Immnobiology. 2010;215(9–10):788–95.
85. Svensson J, Jenmalm MC, Matussek A, Geffers R, Berg G, Ernerudh J. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. J Immunol. 2011;187(7):3671–82.
86. Zhang W, Wang X, Xia X, Liu X, Suo S, Guo J, et al. Klf10 inhibits IL-12p40 production in macrophage colony-stimulating factor-induced mouse bone marrow-derived macrophages. Eur J Immunol. 2013;43(1):258–69.
87. Fleetwood AJ, Lawrence T, Hamilton JA, Cook AD. Granulocyte-macrophage colony stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. J Immunol. 2007;178(8):5245–52.
88. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. Nat Med. 2013;19(10):1264–72.
89. Menke J, Iwata Y, Rabacal WA, Basu R, Yeung YG, Humphreys BD, et al. CSF-1 signals directly to renal tubular epithelial cells to mediate repair in mice. J Clin Invest. 2009; 119(8):2330–42.
90. Kawahara Y, Nakase Y, Isomoto Y, Matsuda N, Amagase K, Kato S, et al. Role of macrophage colony-stimulating factor (M-CSF)-dependent macrophages in gastric ulcer healing in mice. J Physiol Pharmacol. 2011;62(4):441–8.
91. Okuno Y, Nakamura-Ishizu A, Kishi K, Suda T, Kubota Y. Bone marrow-derived cells serve as proangiogenic macrophages but not endothelial cells in wound healing. Blood. 2011;117(19):5264–72.
92. Sarahrudi K, Mousavi M, Thomas A, Eipeldauer S, Vécsei V, Pietschmann P, et al. Elevated levels of macrophage colony-stimulating factor in human fracture healing. J Orthop Res. 2010;28(5):671–6.
93. Alexander KA, Chang MK, Maylin ER, Kohler T, Müller R, Wu AC, et al. Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model. J Bone Miner Res. 2011;26(7):1517–32.
94. Michaelson MD, Bieri PL, Mehler MF, Xu H, Arezzo JC, Pollard JW, et al. CSF-1 deficiency in mice results in abnormal brain development. Development. 1996;122:2661–72.
95. Erblich B, Zhu L, Etgen AM, Dobrenis K, Pollard JW. Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits. PLoS One. 2011;6(10):e26317.
96. Cohen PE, Zhu L, Nishimura K, Pollard JW. Colony-stimulating factor 1 regulation of neuroendocrine pathways that control gonadal function in mice. Endocrinology. 2002;143(4): 1413–22.
97. Cohen PE, Hardy MP, Pollard JW. Colony-stimulating factor-1 plays a major role in the development of reproductive function in male mice. Mol Endocrinol. 1997;11(11): 1636–50.

98. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, et al. Synaptic pruning by microglia is necessary for normal brain development. Science. 2011;333(6048): 1456–8.
99. De I, Nikodemova M, Steffen MD, Sokn E, Maklakova VI, Watters JJ, et al. CSF1 overexpression has pleiotropic effects on microglia in vivo. Glia. 2014;62:1955–67.
100. Smith AM, Gibbons HM, Oldfield RL, Bergin PM, Mee EW, Curtis MA, et al. M-CSF increases proliferation and phagocytosis while modulating receptor and transcription factor expression in adult human microglia. J Neuroinflammation. 2013;10(1):85.
101. Fischer HG, Bielinsky AK, Nitzgen B, Daubener W, Hadding U. Functional dichotomy of mouse microglia developed in vitro: differential effects of macrophage and granulocyte/macrophage colony-stimulating factor on cytokine secretion and antitoxoplasmic activity. J Neuroimmunol. 1993;45(1–2):193–201.
102. Taniike M, Mohri I, Eguchi N, Irikura D, Urade Y, Okada S, et al. An apoptotic depletion of oligodendrocytes in the twitcher, a murine model of globoid cell leukodystrophy. Exp Neurol. 1999;58(6):644–53.
103. Kondo Y, Adams JM, Vanier MT, Duncan ID. Macrophages counteract demyelination in a mouse model of globoid cell leukodystrophy. J Neurosci. 2011;31(10):3610–24.
104. Ijichi K, Brown GD, Moore CS, Lee JP, Winokur PN, Pagarigan R, et al. MMP-3 mediates psychosine-induced globoid cell formation: implications for leukodystrophy pathology. Glia. 2013;61(5):765–77.
105. Groh J, Weis J, Zieger H, Stanley ER, Heuer H, Martini R. Colony-stimulating factor-1 mediates macrophage-related neural damage in a model for Charcot-Marie-Tooth disease type 1X. Brain. 2012;135(Pt 1):88–104.
106. Akcora D, Huynh D, Lightowler S, Germann M, Robine S, de May JR, et al. The CSF-1 receptor fashions the intestinal stem cell niche. Stem Cell Res. 2013;10(2):203–12.
107. Porter EM, Bevins CL, Ghosh D, Ganz T. The multifaceted Paneth cell. Cell Mol Life Sci. 2002;59(1):156–70.
108. Pollard JW, Dominguez MG, Mocci S, Cohen PE, Stanley ER. Effect of the colony-stimulating factor-1 null mutation, osteopetrotic ($csfm^{op}$), on the distribution of macrophages in the male mouse reproductive tract. Biol Reprod. 1997;56(5):1290–300.
109. Cohen PE, Chisholm O, Arceci RJ, Stanley ER, Pollard JW. Absence of colony-stimulating factor-1 in osteopetrotic (csfmop/csfmop) mice results in male fertility defects. Biol Reprod. 1996;55(2):310–7.
110. Cohen PE, Zhu L, Pollard JW. Absence of colony stimulating factor-1 in osteopetrotic (csfmop/csfmop) mice disrupts estrous cycles and ovulation. Biol Reprod. 1997;56(1):110–8.
111. Takasaki A, Ohba T, Okamura Y, Honda R, Seki M, Tanaka N, et al. Clinical use of colony-stimulating factor-1 in ovulation induction for poor responders. Fertil Steril. 2008;90(6): 2287–90.
112. Salmassi A, Mettler L, Jonat W, Buck S, Koch K, Schmutzler AG. Circulating level of macrophage colony-stimulating factor can be predictive for human in vitro fertilization outcome. Fertil Steril. 2010;93(1):116–23.
113. Bradley TR, Stanley ER, Sumner MA. Factors from mouse tissues stimulating colony growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci. 1971;49(6): 595–603.
114. Bartocci A, Pollard JW, Stanley ER. Regulation of colony-stimulating factor 1 during pregnancy. J Exp Med. 1986;164(3):956–61.
115. Pollard JW, Bartocci A, Arceci R, Orlofsky A, Ladner MB, Stanley ER. Apparent role of the macrophage growth factor, CSF-1, in placental development. Nature. 1987;330:484–6.
116. Pollard JW, Hunt JS, Wiktor-Jedrzejczak W, Stanley ER. A pregnancy defect in the osteopetrotic ($^{op/op}$) mouse demonstrates the requirement for CSF-1 in female fertility. Dev Biol. 1991;148(1):273–83.
117. Tagliani E, Shi C, Nancy P, Tay CS, Pamer EG, Erlebacher A. Coordinate regulation of tissue macrophage and dendritic cell population dynamics by CSF-1. J Exp Med. 2011;208(9): 1901–6.

118. Hayashi M, Hoshimoto K, Ohkura T, Inaba N. Increased levels of macrophage colony-stimulating factor in the placenta and blood in preeclampsia. Am J Reprod Immunol. 2002;47(1):19–24.
119. Hayashi M, Numaguchi M, Watabe H, Yaoi Y. High blood levels of macrophage colony-stimulating factor in preeclampsia. Blood. 1996;88(12):4426–8.
120. Schonkeren D, van der Hoorn ML, Khedoe P, Swings G, van Beelen E, Claas F, et al. Differential distribution and phenotype of decidual macrophages in preeclamptic versus control pregnancies. Am J Pathol. 2011;178(2):709–17.
121. Lockwood CJ, Matta P, Krikun G, Koopman LA, Masch R, Toti P, et al. Regulation of monocyte chemoattractant protein-1 expression by tumor necrosis factor-alpha and interleukin-1beta in first trimester human decidual cells: implications for preeclampsia. Am J Pathol. 2006;168(2):445–52.
122. Huang SJ, Chen CP, Schatz F, Rahman M, Abrahams VM, Lockwood CJ. Pre-eclampsia is associated with dendritic cell recruitment into the uterine decidua. J Pathol. 2008;214(3): 328–36.
123. Guilbert L, Robertson SA, Wegmann TG. The trophoblast as an integral component of a macrophage-cytokine network. Immunol Cell Biol. 1993;71(Pt 1):49–57.
124. Abrahams VM, Mor G. Toll-like receptors and their role in the trophoblast. Placenta. 2005;26(7):540–7.
125. Abrahams VM. The role of the Nod-like receptor family in trophoblast innate immune responses. J Reprod Immunol. 2011;88(2):112–7.
126. Gouon-Evans V, Rothenberg ME, Pollard JW. Postnatal mammary gland development requires macrophages and eosinophils. Development. 2000;127(11):2269–82.
127. Pollard JW, Hennighausen L. Colony stimulating factor 1 is required for mammary gland development during pregnancy. Proc Natl Acad Sci USA. 1994;91(20):9312–6.
128. Van Nguyen A, Pollard JW. 1;247(1):11-25. DBJ. Colony stimulating factor-1 is required to recruit macrophages into the mammary gland to facilitate mammary ductal outgrowth. Dev Biol. 2002;247(1):11–25.
129. Banaei-Bouchareb L, Gouon-Evans V, Samara-Boustani D, Castellotti MC, Czernichow P, Pollard JW, et al. Insulin cell mass is altered in Csf1op/Csf1op macrophage-deficient mice. J Leukoc Biol. 2004;76(2):359–67.
130. Saitoh BY, Yamasaki R, Hayashi S, Yoshimura S, Tateishi T, Ohyagi Y, et al. A case of hereditary diffuse leukoencephalopathy with axonal spheroids caused by a de novo mutation in CSF1R masquerading as primary progressive multiple sclerosis. Mult Scler. 2013;19(10):1367–70.
131. Inui T, Kawarai T, Fujita K, Kawamura K, Mitsui T, Orlacchio A, et al. A new CSF1R mutation presenting with an extensive white matter lesion mimicking primary progressive multiple sclerosis. J Neurol Sci. 2013;334(1–2):192–5.
132. Fujioka S, Broderick DF, Sundal C, Baker MC, Rademakers R, Wszolek ZK. An adult-onset leukoencephalopathy with axonal spheroids and pigmented glia accompanied by brain calcifications: a case report and a literature review of brain calcifications disorders. J Neurol. 2013;260(10):2665–8.
133. Terasawa Y, Osaki Y, Kawarai T, Sugimoto T, Orlacchio A, Abe T, et al. Increasing and persistent DWI changes in a patient with hereditary diffuse leukoencephalopathy with spheroids. J Neurol Sci. 2013;335(1–2):213–5.
134. Battisti C, Di Donato I, Bianchi S, Monti L, Formichi P, Rufa A, et al. Hereditary diffuse leukoencephalopathy with axonal spheroids: three patients with stroke-like presentation carrying new mutations in the CSF1R gene. J Neurol. 2014;261(4):768–72.
135. La Piana R, Webber A, Guiot MC, Del Pilar Cortes M, Brais B. A novel mutation in the CSF1R gene causes a variable leukoencephalopathy with spheroids. Neurogenetics. 2014; 15:289–94.
136. Guerreiro R, Kara E, Le Ber I, Bras J, Rohrer JD, Taipa R, et al. Genetic analysis of inherited leukodystrophies: genotype-phenotype correlations in the CSF1R gene. JAMA Neurol. 2013;70(7):875–82.

137. Sundal C, Lash J, Aasly J, Oygarden S, Roeber S, Kretzschman H, et al. Hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS): a misdiagnosed disease entity. J Neurol Sci. 2012;314(1–2):130–7.
138. Hoffmann S, Murrell J, Harms L, Miller K, Meisel A, Brosch T, et al. Enlarging the nosological spectrum of hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS). Brain Pathol. 2014;24:452–8.
139. Karle KN, Biskup S, Schule R, Schweitzer KJ, Kruger R, Bauer P, et al. De novo mutations in hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS). Neurology. 2013;81(23):2039–44.
140. Kinoshita M, Kondo Y, Yoshida K, Fukushima K, Hoshi K, Ishizawa K, et al. Corpus callosum atrophy in patients with hereditary diffuse leukoencephalopathy with neuroaxonal spheroids: an MRI-based study. Intern Med. 2014;53(1):21–7.
141. Kleinfeld K, Mobley B, Hedera P, Wegner A, Sriram S, Pawate S. Adult-onset leukoencephalopathy with neuroaxonal spheroids and pigmented glia: report of five cases and a new mutation. J Neurol. 2013;260(2):558–71.
142. Mitsui J, Matsukawa T, Ishiura H, Higasa K, Yoshimura J, Saito TL, et al. CSF1R mutations identified in three families with autosomal dominantly inherited leukoencephalopathy. Am J Med Genet B Neuropsychiatr Genet. 2012;159B(8):951–7.
143. Ahmed R, Guerreiro R, Rohrer JD, Guven G, Rossor MN, Hardy J, et al. A novel A781V mutation in the CSF1R gene causes hereditary diffuse leucoencephalopathy with axonal spheroids. J Neurol Sci. 2013;332(1–2):141–4.
144. Sundal C, Baker M, Karrenbauer V, Gustavsen M, Bedri S, Glaser A, et al. Hereditary diffuse leukoencephalopathy with spheroids with phenotype of primary progressive multiple sclerosis. Eur J Neurol. 2014;22:329–33.
145. Konno T, Tada M, Tada M, Koyama A, Nozaki H, Harigaya Y, et al. Haploinsufficiency of CSF-1R and clinicopathologic characterization in patients with HDLS. Neurology. 2014; 82(2):139–48.
146. Hiyoshi M, Hashimoto M, Yukihara M, Bhuyan F, Suzu S. M-CSF receptor mutations in hereditary diffuse leukoencephalopathy with spheroids impair not only kinase activity but also surface expression. Biochem Biophys Res Commun. 2013;440(4):589–93.
147. Pridans C, Sauter KA, Baer K, Kissel H, Hume DA. CSF1R mutations in hereditary diffuse leukoencephalopathy with spheroids are loss of function. Sci Rep. 2013;3:3013.
148. Wider C, Van Gerpen JA, DeArmond S, Shuster EA, Dickson DW, Wszolek ZK. Leukoencephalopathy with spheroids (HDLS) and pigmentary leukodystrophy (POLD): a single entity? Neurology. 2009;72(22):1953–9.
149. Brunk UT. On the origin of lipofuscin; the iron content of residual bodies, and the relation of these organelles to the lysosomal vacuome. A study on cultured human glial cells. Adv Exp Med Biol. 1989;266:313–20.
150. Streit WJ, Xue QS. The brain's aging immune system. Aging Dis. 2010;1(3):254–61.
151. Sierra A, Gottfried-Blackmore AC, McEwen BS, Bulloch K. Microglia derived from aging mice exhibit an altered inflammatory profile. Glia. 2007;55(4):412–24.
152. Jacquel A, Obba S, Boyer L, Dufies M, Robert G, Gounon P, et al. Autophagy is required for CSF-1-induced macrophagic differentiation and acquisition of phagocytic functions. Blood. 2012;119(19):4527–31.
153. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J Exp Med. 2005; 201(4):647–57.
154. Neumann H, Takahashi K. Essential role of the microglial triggering receptor expressed on myeloid cells-2 (TREM2) for central nervous tissue immune homeostasis. J Neuroimmunol. 2007;184(1–2):92–6.
155. Paloneva J, Kestila M, Wu J, Salminen A, Bohling T, Ruotsalainen V, et al. Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts. Nat Genet. 2000;25(3):357–61.

156. Otero K, Turnbull IR, Poliani PL, Vermi W, Cerutti E, Aoshi T, et al. Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and beta-catenin. Nat Immunol. 2009;10(7):734–43.
157. Chitu V, Gokhan S, Gulinello M, Branch CA, Patil M, Basu R, et al. Phenotypic characterization of a Csf1r haploinsufficient mouse model of adult-onset leukodystrophy with axonal spheroids and pigmented glia (ALSP). Neurobiol Dis. 2015;74:219–28.
158. Kacinski BM. CSF-1 and its receptor in breast carcinomas and neoplasms of the female reproductive tract. Mol Reprod Dev. 1997;46(1):71–4.
159. Chambers SK, Kacinski BM, Ivins CM, Carcangiu ML. Overexpression of epithelial macrophage colony-stimulating factor (CSF-1) and CSF-1 receptor: a poor prognostic factor in epithelial ovarian cancer, contrasted with a protective effect of stromal CSF-1. Clin Cancer Res. 1997;3(6):999–1007.
160. Kluger HM, Dolled-Filhart M, Rodov S, Kacinski BM, Camp RL, Rimm DL. Macrophage colony-stimulating factor-1 receptor expression is associated with poor outcome in breast cancer by large cohort tissue microarray analysis. Clin Cancer Res. 2004;10(11):173–7.
161. Smith HO, Anderson PS, Kuo DY, Goldberg GL, DeVictoria CL, Boocock CA, et al. The role of colony-stimulating factor 1 and its receptor in the etiopathogenesis of endometrial adenocarcinoma. Clin Cancer Res. 1995;1(3):313–25.
162. Janowska-Wieczorek A, Belch AR, Jacobs A, Bowen D, Padua RA, Paietta E, et al. Increased circulating colony-stimulating factor-1 in patients with preleukemia, leukemia, and lymphoid malignancies. Blood. 1991;77(8):1796–803.
163. Hung JY, Horn D, Woodruff K, Prihoda T, LeSaux C, Peters J, et al. Colony-stimulating factor 1 potentiates lung cancer bone metastasis. Lab Invest. 2014;94(4):371–81.
164. Aharinejad S, Salama M, Paulus P, Zins K, Berger A, Singer CF. Elevated CSF-1 serum concentration predicts poor overall survival in women with early breast cancer. Endocr Relat Cancer. 2013;20:777–83.
165. Qin L, Wu YL, Toneff MJ, Li D, Liao L, Gao X, et al. NCOA1 directly targets M-CSF1 expression to promote breast cancer metastasis. Cancer Res. 2014;74(13):3477–88.
166. Aharinejad S, Abraham D, Paulus P, Abri H, Hofmann M, Grossschmidt K, et al. Colony stimulating factor-1 antisense treatment suppresses growth of human tumor xenografts in mice. Cancer Res. 2002;62(18):5317–24.
167. Aharinejad S, Paulus P, Sioud M, Hofmann M, Zins K, Schafer R, et al. Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice. Cancer Res. 2004;64(15):5378–84.
168. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res. 2006;66(23):11238–46.
169. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res. 2004;64(19):7022–9.
170. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, et al. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Res. 2005;65(12):5278–83.
171. Coniglio SJ, Eugenin E, Dobrenis K, Stanley ER, West BL, Symons MH, et al. Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. Mol Med. 2012;18(1):519–27.
172. Patsialou A, Wyckoff J, Wang Y, Goswami S, Stanley ER, Condeelis JS. Invasion of human breast cancer cells in vivo requires both paracrine and autocrine loops involving the colony-stimulating factor-1 receptor. Cancer Res. 2009;69(24):9498–506.
173. Patsialou A, Wang Y, Pignatelli J, Chen X, Entenberg D, Oktay M, et al. Autocrine CSF1R signaling mediates switching between invasion and proliferation downstream of TGFbeta in claudin-low breast tumor cells. Oncogene. 2014. doi:10.1038/onc.2014.226.
174. Cioce M, Canino C, Goparaju C, Yang H, Carbone M, Pass HI. Autocrine CSF-1R signaling drives mesothelioma chemoresistance via AKT activation. Cell Death Dis. 2014;5:e1167.

175. Barbetti V, Morandi A, Tusa I, Digiacomo G, Riverso M, Marzi I, et al. Chromatin-associated CSF-1R binds to the promoter of proliferation-related genes in breast cancer cells. Oncogene. 2014;33(34):4359–64.
176. Azzam G, Wang X, Bell D, Murphy ME. CSF1 is a novel p53 target gene whose protein product functions in a feed-forward manner to suppress apoptosis and enhance p53-mediated growth arrest. PLoS One. 2013;8(9):e74297.
177. Specchia G, Liso V, Capalbo S, Fazioli F, Bettoni S, Bassan R, et al. Constitutive expression of IL-1 beta, M-CSF and c-fms during the myeloid blastic phase of chronic myelogenous leukaemia. Br J Haematol. 1992;80(3):310–6.
178. Gilbert HS, Praloran V, Stanley ER. Increased circulating CSF-1 (M-CSF) in myeloproliferative disease: association with myeloid metaplasia and peripheral bone marrow extension. Blood. 1989;74(4):1231–4.
179. Dubreuil P, Torres H, Courcoul M-A, Birg F, Mannoni P. c-fms expression is a molecular marker of human acute myeloid leukemias. Blood. 1988;72(3):1081–5.
180. Ashmun RA, Look AT, Roberts WM, Roussel MF, Seremetis S, Ohtsuka M, et al. Monoclonal antibodies to the human CSF-1 receptor (c-fms proto-oncogene product) detect epitopes on normal mononuclear phagocytes and on human myeloid leukemic blast cells. Blood. 1989;73(3):827–37.
181. Rambaldi A, Wakamiya N, Vellenga E, Horiguchi J, Warren MK, Kufe D, et al. Expression of the macrophage colony-stimulating factor and c-fms genes in human acute myeloblastic leukemia cells. J Clin Invest. 1988;81(4):1030–5.
182. Sapi E, Flick MB, Rodov S, Carter D, Kacinski BM. Expression of CSF-I and CSF-I receptor by normal lactating mammary epithelial cells. J Soc Gynecol. 1998;5(2):94–101.
183. Kacinski BM, Scata KA, Carter D, Yee LD, Sapi E, King BL, et al. FMS (CSF-1 receptor) and CSF-1 transcripts and protein are expressed by human breast carcinomas in vivo and in vitro. Oncogene. 1991;6(6):941–52.
184. Morandi A, Barbetti V, Riverso M, Dello Sbarba P, Rovida E. The colony-stimulating factor-1 (CSF-1) receptor sustains ERK1/2 activation and proliferation in breast cancer cell lines. PLoS One. 2011;6(11):e27450.
185. Wrobel CN, Debnath J, Lin E, Beausoleil S, Roussel MF, Brugge JS. Autocrine CSF-1R activation promotes Src-dependent disruption of mammary epithelial architecture. J Cell Biol. 2004;165(2):263–73.
186. Menke J, Kriegsmann J, Schimanski CC, Schwartz MM, Schwarting A, Kelley VR. Autocrine CSF-1 and CSF-1 receptor coexpression promotes renal cell carcinoma growth. Cancer Res. 2012;72(1):187–200.
187. Lilljebjorn H, Agerstam H, Orsmark-Pietras C, Rissler M, Ehrencrona H, Nilsson L, et al. RNA-seq identifies clinically relevant fusion genes in leukemia including a novel MEF2D/CSF1R fusion responsive to imatinib. Leukemia. 2014;28(4):977–9.
188. Browning PJ, Bunn HF, Cline A, Shuman M, Nienhuis AW. "Replacement" of COOH-terminal truncation of v-fms with c-fms sequences markedly reduces transformation potential. Proc Natl Acad Sci USA. 1986;83(20):7800–4.
189. Woolford J, McAuliffe A, Rohrschneider LR. Activation of the feline c-fms proto-oncogene: multiple alterations are required to generate a fully transformed phenotype. Cell. 1988;55(6):965–77.
190. Roussel MF, Dull TJ, Rettenmier CW, Ralph P, Ullrich A, Sherr CJ. Transforming potential of the c-fms proto-oncogene (CSF-1 receptor). Nature. 1987;325(6104):549–52.
191. McGlynn H, Baker AH, Padua RA. Biological consequences of a point mutation at codon 969 of the FMS gene. Leuk Res. 1998;22(4):365–72.
192. Ridge SA, Worwood M, Oscier D, Jacobs A, Padua RA. FMS mutations in myelodyplastic, leukemic, and normal subjects. Proc Natl Acad Sci USA. 1990;87(4):1377–80.
193. Strazza M, Pirrone V, Wigdahl B, Nonnemacher MR. Breaking down the barrier: the effects of HIV-1 on the blood-brain barrier. Brain Res. 2011;1399:96–115.

194. Haine V, Fischer-Smith T, Rappaport J. Macrophage colony-stimulating factor in the pathogenesis of HIV infection: potential target for therapeutic intervention. J Neuroimmune Pharmacol. 2006;1(1):32–40.
195. Lentz MR, Degaonkar M, Mohamed MA, Kim H, Conant K, Halpern EF, et al. Exploring the relationship of macrophage colony-stimulating factor levels on neuroaxonal metabolism and cognition during chronic human immunodeficiency virus infection. J Neurovirol. 2010; 16(5):368–76.
196. Gerngross L, Fischer T. Evidence for cFMS signaling in HIV production by brain macrophages and microglia. J Neurovirol. 2015;21(3):249–56.
197. Akashi N, Matsumoto I, Tanaka Y, Inoue A, Yamamoto K, Umeda N, et al. Comparative suppressive effects of tyrosine kinase inhibitors imatinib and nilotinib in models of autoimmune arthritis. Mod Rheumatol. 2010;21(3):267–75.
198. Lenda DM, Kikawada E, Stanley ER, Kelley VR. Reduced macrophage recruitment, proliferation, and activation in colony-stimulating factor-1-deficient mice results in decreased tubular apoptosis during renal inflammation. J Immunol. 2003;170(6):3254–62.
199. Denardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov. 2011;1(1):54–67.
200. Murray LJ, Abrams TJ, Long KR, Ngai TJ, Olson LM, Hong W, et al. SU11248 inhibits tumor growth and CSF-1R-dependent osteolysis in an experimental breast cancer bone metastasis model. Clin Exp Metastasis. 2003;20(8):757–66.
201. Mitchem JB, Brennan DJ, Knolhoff BL, Belt BA, Zhu Y, Sanford DE, et al. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. Cancer Res. 2013;73(3):1128–41.
202. Fratto ME, Imperatori M, Vincenzi B, Tomao F, Santini D, Tonini G. New perspectives: role of sunitinib in breast cancer. Clin Ter. 2010;161(5):475–82.
203. Blay JY, von Mehren M. Nilotinib: a novel, selective tyrosine kinase inhibitor. Semin Oncol. 2011;38 Suppl 1:S3–9.
204. Manley PW, Drueckes P, Fendrich G, Furet P, Liebetanz J, Martiny-Baron G, et al. Extended kinase profile and properties of the protein kinase inhibitor nilotinib. Biochim Biophys Acta. 2010;104(3):445–53.
205. Irvine KM, Burns CJ, Wilks AF, Su S, Hume DA, Sweet MJ. A CSF-1 receptor kinase inhibitor targets effector functions and inhibits pro-inflammatory cytokine production from murine macrophage populations. FASEB J. 2006;20(11):1921–3.
206. Guo J, Marcotte PA, McCall JO, Dai Y, Pease LJ, Michaelides MR, et al. Inhibition of phosphorylation of the colony-stimulating factor-1 receptor (c-Fms) tyrosine kinase in transfected cells by ABT-869 and other tyrosine kinase inhibitors. Mol Cancer Ther. 2006;5(4): 1007–13.
207. Abrams TJ, Lee LB, Murray LJ, Pryer NK, Cherrington JM. SU11248 inhibits KIT and platelet-derived growth factor receptor beta in preclinical models of human small cell lung cancer. Mol Cancer Ther. 2003;2(5):471–8.
208. O'Farrell AM, Abrams TJ, Yuen HA, Ngai TJ, Louie SG, Yee KW, et al. SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. Blood. 2003; 101(9):3597–605.
209. Burton E, Wong B, Zhang J, West B, Bollag G, Habets G, et al. The novel inhibitor PLX3397 effectively inhibits FLT3-mutant AML. Blood. 2011;118(21):3632.
210. Manthey CL, Johnson DL, Illig CR, Tuman RW, Zhou Z, Baker JF, et al. JNJ-28312141, a novel orally active colony-stimulating factor-1 receptor/FMS-related receptor tyrosine kinase-3 receptor tyrosine kinase inhibitor with potential utility in solid tumors, bone metastases, and acute myeloid leukemia. Mol Cancer Ther. 2009;8(11):3151–61.
211. Ryder M, Gild M, Hohl TM, Pamer E, Knauf J, Ghossein R, et al. Genetic and pharmacological targeting of CSF-1/CSF-1R inhibits tumor-associated macrophages and impairs BRAF-induced thyroid cancer progression. PLoS One. 2013;1:e54302.

212. Wei S, Lightwood D, Ladyman H, Cross S, Neale H, Griffiths M, et al. Modulation of CSF-1-regulated post-natal development with anti-CSF-1 antibody. Immunobiology. 2005; 210(2–4):109–19.
213. Sauter KA, Pridans C, Sehgal A, Tsai YT, Bradford BM, Raza S, et al. Pleiotropic effects of extended blockade of CSF1R signaling in adult mice. J Leukoc Biol. 2014;96:265–74.
214. Swierczak A, Cook AD, Lenzo JC, Restall CM, Doherty JP, Anderson RL, et al. The promotion of breast cancer metastasis caused by inhibition of CSF-1R/CSF-1 signaling is blocked by targeting the G-CSF receptor. Cancer Immunol Res. 2014;2:765–76.
215. Guilbert LJ, Stanley ER. Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J Cell Biol. 1980;85(1):153–9.
216. Byrne PV, Guilbert LJ, Stanley ER. Distribution of cells bearing receptors for a colony-stimulating factor (CSF) in murine tissues. J Cell Biol. 1981;91:848–53.
217. Guilbert LJ, Stanley ER. The interaction of ^{125}I-colony stimulating factor-1 with bone marrow-derived macrophages. J Biol Chem. 1986;261:4024–32.
218. Yeung Y-G, Jubinsky PT, Sengupta A, Yeung DC-Y, Stanley ER. Purification of the colony-stimulating factor 1 receptor and demonstration of its tyrosine kinase activity. Proc Natl Acad Sci USA. 1987;84(5):1268–71.
219. Donner L, Fedele LA, Garon CF, Anderson SJ, Sherr CJ. McDonough feline sarcoma virus: characterization of the molecularly cloned provirus and its feline oncogene (v-fms). J Virol. 1982;41(2):489–500.
220. McDonough SK, Larsen S, Brodey RS, Stock ND, Hardy Jr WD. A transmissible feline fibrosarcoma of viral origin. Cancer Res. 1971;31(7):353–6.
221. Anderson SJ, Gonda MA, Rettenmier CW, Sherr CJ. Subcellular localization of glycoproteins encoded by the viral oncogene v-fms. J Virol. 1984;51(3):730–41.
222. Roussel MF, Rettenmier CW, Look AT, Sherr CJ. Cell surface expression of v-fms-coded glycoproteins is required for transformation. Mol Cell Biol. 1984;4(10):1999–2009.
223. Roussel MF, Sherr CJ, Barker PE, Ruddle FH. Molecular cloning of the c-fms locus and its assignment to human chromosome 5. J Virol. 1983;48(3):770–3.
224. Rettenmier CW, Chen JH, Roussel MF, Sherr CJ. The product of the c-fms proto-oncogene: a glycoprotein with associated tyrosine kinase activity. Science. 1985;228(4697):320–2.
225. Le Beau MM, Westbrook CA, Diaz MO, Larson RA, Rowley JD, Gasson JC, et al. Evidence for the involvement of GM-CSF and FMS in the deletion (5q) in myeloid disorders. Science. 1986;231(4741):984–7.
226. Bonifer C, Hume DA. The transcriptional regulation of the colony-stimulating factor 1 receptor (csf1r) gene during hematopoiesis. Front Biosci. 2008;13:549–60.
227. Hoggan MD, Halden NF, Buckler CE, Kozak CA. Genetic mapping of the mouse c-fms proto-oncogene to chromosome 18. J Virol. 1988;62(3):1055–6.
228. Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ, et al. A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. Blood. 2003;101(3):1155–63.
229. Li J, Chen K, Zhu L, Pollard JW. Conditional deletion of the colony stimulating factor-1 receptor (c-fms proto-oncogene) in mice. Genesis. 2006;44(7):328–35.
230. Sasmono RT, Williams E. Generation and characterization of MacGreen mice, the Cfs1r-EGFP transgenic mice. Methods Mol Biol. 2012;844:157–76.
231. Balic A, Garcia-Morales C, Vervelde L, Gilhooley H, Sherman A, Garceau V, et al. Visualisation of chicken macrophages using transgenic reporter genes: insights into the development of the avian macrophage lineage. Development. 2014;141(16):3255–65.
232. Parichy DM, Ransom DG, Paw B, Zon LI, Johnson SL. An orthologue of the kit-related gene fms is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio. Development. 2000;127(14): 3031–44.
233. Herbomel P, Thisse B, Thisse C. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. Dev Biol. 2001;238(2):274–88.

234. Chatani M, Takano Y, Kudo A. Osteoclasts in bone modeling, as revealed by in vivo imaging, are essential for organogenesis in fish. Dev Biol. 2011;360(1):96–109.
235. Sauter KA, Bouhlel MA, O'Neal J, Sester DP, Tagoh H, Ingram RM, et al. The function of the conserved regulatory element within the second intron of the mammalian Csf1r locus. PLoS One. 2013;8(1):e54935.
236. Himes SR, Tagoh H, Goonetilleke N, Sasmono T, Oceandy D, Clark R, et al. A highly conserved c-fms gene intronic element controls macrophage-specific and regulated expression. J Leukoc Biol. 2001;70(5):812–20.
237. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, et al. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. Cell. 2006; 126(4):755–66.
238. Visvader J, Verma IM. Differential transcription of exon 1 of the human c-fms gene in placental trophoblasts and monocytes. Mol Cell Biol. 1989;9(3):1336–41.
239. Roberts WM, Shapiro LH, Ashmun RA, Look AT. Transcription of the human colony-stimulating factor-1 receptor gene is regulated by separate tissue-specific promoters. Blood. 1992;79(3):586–93.
240. Ovchinnikov DA, DeBats CE, Sester DP, Sweet MJ, Hume DA. A conserved distal segment of the mouse CSF-1 receptor promoter is required for maximal expression of a reporter gene in macrophages and osteoclasts of transgenic mice. J Leukoc Biol. 2010;87(5):815–22.
241. Pang M, Martinez AF, Fernandez I, Balkan W, Troen BR. AP-1 stimulated the cathepsin K promoter in RAW264.7 cells. Gene. 2007;403(1–2):151–8.
242. Zhang DE, Hetherington CJ, Chen H-M, Tenen DG. The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. Mol Cell Biol. 1994;14(1):373–81.
243. Tagoh H, Ingram R, Wilson N, Salvagiotto G, Warren AJ, Clarke D, et al. The mechanism of repression of the myeloid-specific c-fms gene by Pax5 during B lineage restriction. EMBO J. 2006;25(5):1070–80.
244. Hume DA, Sasmono T, Himes SR, Sharma SM, Bronisz A, Constantin M, et al. The Ewing sarcoma protein (EWS) binds directly to the proximal elements of the macrophage-specific promoter of the CSF-1 receptor (csf1r) gene. J Immunol. 2008;180(10):6733–42.
245. Krysinska H, Hoogenkamp M, Ingram R, Wilson N, Tagoh H, Laslo P, et al. A two-step, PU.1-dependent mechanism for developmentally regulated chromatin remodeling and transcription of the c-fms gene. Mol Cell Biol. 2007;27(3):878–87.
246. Ross IL, Yue X, Ostrowski MC, Hume DA. Interaction between PU.1 and another Ets family transcription factor promotes macrophage-specific basal transcription initiation. J Biol Chem. 1998;273(12):6662–9.
247. Li X, Vradii D, Gutierrez S, Lian JB, van Wijnen AJ, Stein JL, et al. Subnuclear targeting of Runx1 is required for synergistic activation of the myeloid specific M-CSF receptor promoter by PU.1. J Cell Biochem. 2005;96(4):795–809.
248. Tagoh H, Himes R, Clarke D, Leenen PJ, Riggs AD, Hume D, et al. Transcription factor complex formation and chromatin fine structure alterations at the murine c-fms (CSF-1 receptor) locus during maturation of myeloid precursor cells. Genes Dev. 2002;16(13): 1721–37.
249. Tagoh H, Schebesta A, Lefevre P, Wilson N, Hume D, Busslinger M, et al. Epigenetic silencing of the c-fms locus during B-lymphopoiesis occurs in discrete steps and is reversible. EMBO J. 2004;23(21):4275–85.
250. Walsh JC, DeKoter RP, Lee HJ, Smith ED, Lancki DW, Gurish MF, et al. Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. Immunity. 2002;17(5):665–76.
251. Steidl U, Rosenbauer F, Verhaak RG, Gu X, Ebralidze A, Otu HH, et al. Essential role of Jun family transcription factors in PU.1 knockdown-induced leukemic stem cells. Nat Genet. 2006;38(11):1269–77.

252. Lee PS, Wang Y, Dominguez MG, Yeung YG, Murphy MA, Bowtell DD, et al. The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. EMBO J. 1999;18(13):3616–28.
253. Xiong Y, Song D, Cai Y, Yu W, Yeung YG, Stanley ER. A CSF-1 receptor phosphotyrosine 559 signaling pathway regulates receptor ubiquitination and tyrosine phosphorylation. J Biol Chem. 2011;286(2):952–60.
254. Rieger AM, Hanington PC, Belosevic M, Barreda DR. Control of CSF-1 induced inflammation in teleost fish by a soluble form of the CSF-1 receptor. Fish Shellfish Immunol. 2014;41:45–51.
255. Besmer P, Lader E, George PC, Bergold PJ, Qiu FH, Zuckerman EE, et al. A new acute transforming feline retrovirus with fms homology specifies a C-terminally truncated version of the c-fms protein that is different from SM-feline sarcoma virus v-fms protein. J Virol. 1986;60(1):194–203.
256. Tamura T, Hadwiger-Fangmeier A, Simon E, Smola U, Geschwill H, Schutz B, et al. Transforming mechanism of the feline sarcoma virus encoded v-fms oncogene product. Behring Inst Mitt. 1991;89:93–9.
257. Elegheert J, Desfosses A, Shkumatov AV, Wu X, Bracke N, Verstraete K, et al. Extracellular complexes of the hematopoietic human and mouse CSF-1 receptor are driven by common assembly principles. Structure. 2011;19(12):1762–72.
258. de Parseval N, Bordereaux D, Varlet P, Gisselbrecht S, Sola B. Isolation of new oncogenic forms of the murine c-fms gene. J Virol. 1995;69(6):3597–604.
259. Stanley ER. Colony-stimulating factor (CSF) radioimmunoassay: detection of a CSF subclass stimulating macrophage production. Proc Natl Acad Sci USA. 1979;76(6):2969–73.
260. Stanley ER. Colony stimulating factor-1 (Macrophage colony stimulating factor). In: Thomson AW, editor. The cytokine handbook. San Diego: Academic; 1994. p. 387–418.
261. Cohen PE, Nishimura K, Zhu L, Pollard JW. Macrophages: important accessory cells for reproductive function. J Leuk Biol. 1999;66(5):765–72.
262. Stanley ER. CSF-1. In: Oppenheim JJ, Feldmann M, editors. Cytokine reference: a compendium of cytokines and other mediators of host defense. London, UK: Academic; 2000. p. 911–34.
263. Cebon J, Layton J. Measurement and clinical significance of circulating hematopoietic growth factor levels. Curr Opin Hematol. 1994;1(3):228–34.
264. Cheers C, Haigh AM, Kelso A, Metcalf D, Stanley ER, Young AM. Production of colony-stimulating factors (CSFs) during infection: separate determinations of macrophage-, granulocyte-, granulocyte-macrophage-, and multi-CSFs. Infect Immun. 1988;56(1):247–51.
265. Cebon J, Layton JE, Maher D, Morstyn G. Endogenous haemopoietic growth factors in neutropenia and infection. Br J Haematol. 1994;86(2):265–74.
266. Oren H, Duman N, Abacioglu H, Ozkan H, Irken G. Association between serum macrophage colony-stimulating factor levels and monocyte and thrombocyte counts in healthy, hypoxic, and septic term neonates. Pediatrics. 2001;108(2):329–32.
267. Das Roy L, Pathangey LB, Tinder TL, Schettini JL, Gruber HE, Mukherjee P. Breast-cancer-associated metastasis is significantly increased in a model of autoimmune arthritis. Breast Cancer Res. 2009;11(4):R56.
268. Morishita E, Sekiya A, Hayashi T, Kadohira Y, Maekawa M, Yamazaki M, et al. Increased macrophage colony-stimulating factor levels in patients with Graves' disease. Int J Hematol. 2008;88(3):272–7.
269. Mroczko B, Groblewska M, Wereszczyńska-Siemiatkowska U, Okulczyk B, Kedra B, Łaszewicz W, Dabrowski A, Szmitkowski M. Serum macrophage-colony stimulating factor levels in colorectal cancer patients correlate with lymph node metastasis and poor prognosis. Clin Chim Acta. 2007;380(1–2):208–12.
270. Scholl SM, Bascou CH, Mosseri V, Olivares R, Magdelenat H, Dorval T, et al. Circulating levels of colony-stimulating factor 1 as a prognostic indicator in 82 patients with epithelial ovarian cancer. Br J Cancer. 1994;69(2):342–6.

271. Suzu S, Yanai N, Sato-Somoto Y, Yamada M, Kawashima T, Hanamura T, et al. Characterization of macrophage colony-stimulating factor in body fluids by immunoblot analysis. Blood. 1991;77(10):2160–5.
272. Stanley ER, Berg KL, Einstein DB, Lee PSW, Pixley FJ, Wang Y, et al. Biology and action of CSF-1. Mol Reprod Dev. 1997;46(1):4–10.
273. Roth P, Stanley ER. The biology of CSF-1 and its receptor. Curr Top Microbiol Immunol. 1992;181:141–67.
274. Bartocci A, Mastrogiannis DS, Migliorati G, Stockert RJ, Wolkoff AW, Stanley ER. Macrophages specifically regulate the concentration of their own growth factor in the circulation. Proc Natl Acad Sci USA. 1987;84(17):6179–83.
275. Cole DJ, Sanda MG, Yang JC, Schwartzentruber DJ, Weber J, Ettinghausen SE, et al. Phase I trial of recombinant human macrophage colony-stimulating factor administered by continuous intravenous infusion in patients with metastatic cancer. J Natl Cancer Inst. 1994; 86(1):39–45.
276. Garnick MB, Stoudemire JB. Preclinical and clinical evaluation of recombinant human macrophage colony-stimulating factor (rhM-CsF). Int J Cell Cloning. 1990;8 Suppl 1:356–73.
277. Morris SW, Valentine MB, Shapiro DN, Sublett JE, Deaven LL, Foust JT, et al. Reassignment of the human CSF1 gene to chromosome 1p13-p21. Blood. 1991;78(8):2013–20.
278. Saltman DL, Dolganov GM, Hinton LM, Lovett M. Reassignment of the human macrophage colony stimulating factor gene to chromosome 1p13-21. Biochem Biophys Res Commun. 1992;182(3):1139–43.
279. Gisselbrecht S, Sola B, Fichelson S, Bordereaux D, Tambourin P, Mattei MG, et al. The murine M-CSF gene is localized on chromosome 3. Blood. 1989;73(6):1742–5.
280. Buchberg AM, Jenkins NA, Copeland NG. Localization of the murine macrophage colony-stimulating factor gene to chromosome 3 using interspecific backcross analysis. Genomics. 1989;5(2):363–7.
281. Kawasaki ES, Ladner MB. Molecular biology of macrophage colony-stimulating factor. In: Dexter TM, Garland JM, Testa NG, editors. Colony-stimulating factors molecular and cellular biology. New York and Basel: Marcel Dekker; 1990. p. 155–76.
282. Ladner MB, Martin GA, Noble JA, Nikoloff DM, Tal R, Kawasaki ES, et al. Human CSF-1: gene structure and alternative splicing of mRNA precursors. EMBO J. 1987;6(9):2693–8.
283. Kawasaki ES, Ladner MB, Wang AM, Van Arsdell J, Warren MK, Coyne MY, et al. Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). Science. 1985;230(4723):291–6.
284. Wong GG, Temple PA, Leary AC, Witek-Giannotti JS, Yang YC, Ciarletta AB, et al. Human CSF-1: molecular cloning and expression of 4-kb cDNA encoding the human urinary protein. Science. 1987;235(4795):1504–8.
285. Ladner MB, Martin GA, Noble JA, Wittman VP, Warren MK, McGrogan M, et al. cDNA cloning and expression of murine macrophage colony-stimulating factor from L929 cells. Proc Natl Acad Sci USA. 1988;85(18):6706–10.
286. Price LKH, Choi HU, Rosenberg L, Stanley ER. The predominant form of secreted colony stimulating factor-1 is a proteoglycan. J Biol Chem. 1992;267(4):2190–9.
287. Suzu S, Ohtsuki T, Yanai N, Takatsu Z, Kawashima T, Takaku F, et al. Identification of a high molecular weight macrophage colony-stimulating factor as a glycosaminoglycan-containing species. J Biol Chem. 1992;267:4345–8.
288. Stein J, Borzillo GV, Rettenmier CW. Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. Blood. 1990;76(7):1308–14.
289. Horiuchi K, Miyamoto T, Takaishi H, Hakozaki A, Kosaki N, Miyauchi Y, et al. Cell surface colony-stimulating factor 1 can be cleaved by TNF-alpha converting enzyme or endocytosed in a clathrin-dependent manner. J Immunol. 2007;179(10):6715–24.
290. Douglass TG, Driggers L, Zhang JG, Hoa N, Delgado C, Williams CC, et al. Macrophage colony stimulating factor: not just for macrophages anymore! A gateway into complex biologies. Int Immunopharmacol. 2008;8(10):1354–76.

291. Van Wesenbeeck L, Odgren PR, MacKay CA, D'Angelo M, Safadi FF, Popoff SN, et al. The osteopetrotic mutation toothless (tl) is a loss-of-function frameshift mutation in the rat Csf1 gene: Evidence of a crucial role for CSF-1 in osteoclastogenesis and endochondral ossification. Proc Natl Acad Sci USA. 2002;99(22):14303–8.
292. Harris SE, MacDougall M, Horn D, Woodruff K, Zimmer SN, Rebel VI, et al. Meox2Cre-mediated disruption of CSF-1 leads to osteopetrosis and osteocyte defects. Bone. 2012; 50(1):42–53.
293. Menke J, Iwata Y, Rabacal WA, Basu R, Stanley ER, Kelley VR. Distinct roles of CSF-1 isoforms in lupus nephritis. J Am Soc Nephrol. 2011;22(10):1821–33.
294. Hiroyasu S, Chinnasamy P, Hou R, Hotchkiss K, Casimiro I, Dai XM, et al. Donor and recipient cell surface colony stimulating factor-1 promote neointimal formation in transplant-associated arteriosclerosis. Arterioscler Thromb Vasc Biol. 2013;33(1):87–95.
295. Gow DJ, Garceau V, Pridans C, Gow AG, Simpson KE, Gunn-Moore D, et al. Cloning and expression of feline colony stimulating factor receptor (CSF-1R) and analysis of the species specificity of stimulation by colony stimulating factor-1 (CSF-1) and interleukin-34 (IL-34). Cytokine. 2013;61(2):630–8.
296. Gow DJ, Garceau V, Kapetanovic R, Sester DP, Fici GJ, Shelly JA, et al. Cloning and expression of porcine Colony Stimulating Factor-1 (CSF-1) and Colony Stimulating Factor-1 Receptor (CSF-1R) and analysis of the species specificity of stimulation by CSF-1 and Interleukin 34. Cytokine. 2012;60(3):793–805.
297. Koths K. Structure-function studies on human macrophage colony-stimulating factor (M-CSF). Mol Reprod Dev. 1997;46(1):31–7.
298. Strockbine LD, Cohen JI, Farrah T, Lyman SD, Wagener F, DuBose RF, et al. The Epstein-Barr virus BARF1 gene encodes a novel, soluble colony- stimulating factor-1 receptor. J Virol. 1998;72(5):4015–21.
299. Cohen JI, Lekstrom K. Epstein-Barr virus BARF1 protein is dispensable for B-cell transformation and inhibits alpha interferon secretion from mononuclear cells. J Virol. 1999;73(9): 7627–32.
300. Warren MK, Ralph P. Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor, and colony stimulating activity. J Immunol. 1986;137:2281–5.
301. Lin JC, Zhang ZX, Chou TC, Sim I, Pagano JS. Synergistic inhibition of Epstein-Barr virus: transformation of B lymphocytes by alpha and gamma interferon and by 3′-azido-3′-deoxythymidine. J Infect Dis. 1989;159(2):248–54.
302. Shim AH, Chang RA, Chen X, Longnecker R, He X. Multipronged attenuation of macrophage-colony stimulating factor signaling by Epstein-Barr virus BARF1. Proc Natl Acad Sci USA. 2012;109(32):12962–7.
303. Elegheert J, Bracke N, Pouliot P, Gutsche I, Shkumatov AV, Tarbouriech N, et al. Allosteric competitive inactivation of hematopoietic CSF-1 signaling by the viral decoy receptor BARF1. Nat Struct Mol Biol. 2012;19(9):938–47.
304. Tian Y, Shen H, Xia L, Lu J. Elevated serum and synovial fluid levels of interleukin-34 in rheumatoid arthritis: possible association with disease progression via interleukin-17 production. J Interferon Cytokine Res. 2013;33(7):398–401.
305. Hwang SJ, Choi B, Kang SS, Chang JH, Kim YG, Chung YH, et al. Interleukin-34 produced by human fibroblast-like synovial cells in rheumatoid arthritis supports osteoclastogenesis. Arthritis Res Ther. 2012;14(1):R14.
306. Chang EJ, Lee SK, Song YS, Jang YJ, Park HS, Hong JP, et al. IL-34 is associated with obesity, chronic inflammation, and insulin resistance. J Clin Endocrinol Metab. 2014;99: 1263–71.
307. Nakamichi Y, Mizoguchi T, Arai A, Kobayashi Y, Sato M, Penninger JM, et al. Spleen serves as a reservoir of osteoclast precursors through vitamin D-induced IL-34 expression in osteopetrotic $^{op/op}$ mice. Proc Natl Acad Sci USA. 2012;109(25):10006–11.

308. Yu Y, Yang D, Qiu L, Okamura H, Guo J, Haneji T. Tumor necrosis factor-alpha induces interleukin-34 expression through nuclear factorkappaB activation in MC3T3-E1 osteoblastic cells. Mol Med Rep. 2014;10:1371–6.
309. Bostrom EA, Lundberg P. The newly discovered cytokine IL-34 is expressed in gingival fibroblasts, shows enhanced expression by pro-inflammatory cytokines, and stimulates osteoclast differentiation. PLoS One. 2013;8(12):e81665.
310. Hong S, Li R, Xu Q, Secombes CJ, Wang T. Two types of TNF-alpha exist in teleost fish: phylogeny, expression, and bioactivity analysis of type-II TNF-alpha3 in rainbow trout Oncorhynchus mykiss. J Immunol. 2013;191(12):5959–72.
311. Kuzmac S, Grcevic D, Sucur A, Ivcevic S, Katavic V. Acute hematopoietic stress in mice is followed by enhanced osteoclast maturation in the bone marrow microenvironment. Exp Hematol. 2014;42:966–75.
312. Wang T, Kono T, Monte MM, Kuse H, Costa MM, Korenaga H, et al. Identification of IL-34 in teleost fish: differential expression of rainbow trout IL-34, MCSF1 and MCSF2, ligands of the MCSF receptor. Mol Immunol. 2013;53(4):39–409.
313. Grayfer L, Robert J. Divergent antiviral roles of amphibian (Xenopus laevis) macrophages elicited by colony-stimulating factor-1 and interleukin-34. J Leukoc Biol. 2014;96: 1143–53.
314. Yamane F, Nishikawa Y, Matsui K, Asakura M, Iwasaki E, Watanabe K, et al. CSF-1 receptor-mediated differentiation of a new type of monocytic cell with B cell-stimulating activity: its selective dependence on IL-34. J Leukoc Biol. 2014;95(1):19–31.
315. Felix J, Elegheert J, Gutsche I, Shkumatov AV, Wen Y, Bracke N, et al. Human IL-34 and CSF-1 establish structurally similar extracellular assemblies with their common hematopoietic receptor. Structure. 2013;21(4):52–39.
316. Rozwarski DA, Gronenborn AM, Clore GM, Bazan JF, Bohm A, Wlodawer A, et al. Structural comparisons among the short-chain helical cytokines. Structure. 1994;2(3): 159–73.
317. Nandi S, Cioce M, Yeung YG, Nieves E, Tesfa L, Lin H, et al. Receptor-type protein tyrosine phosphatase zeta is a functional receptor for interleukin-34. J Biol Chem. 2013;288(30):21972–86.
318. Majeti R, Bilwes AM, Noel JP, Hunter T, Weiss A. Dimerization-induced inhibition of receptor protein tyrosine phosphatase function through an inhibitory wedge. Science. 1998; 279(5347):88–91.
319. Meng K, Rodriguez-Pena A, Dimitrov T, Chen W, Yamin M, Noda M, et al. Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. Proc Natl Acad Sci USA. 2000;97(6):2603–8.
320. Barr AJ, Ugochukwu E, Lee WH, King ON, Filippakopoulos P, Alfano I, et al. Large-scale structural analysis of the classical human protein tyrosine phosphatome. Cell. 2009;136(2): 352–63.
321. Shock LP, Bare DJ, Klinz SG, Maness PF. Protein tyrosine phosphatases expressed in developing brain and retinal Müller glia. Brain Res Mol Brain Res. 1995;28(1):110–6.
322. Ranjan M, Hudson LD. Regulation of tyrosine phosphorylation and protein tyrosine phosphatases during oligodendrocyte differentiation. Mol Cell Neurosci. 1996;7(5):404–18.
323. Shintani T, Watanabe E, Maeda N, Noda M. Neurons as well as astrocytes express proteoglycan-type protein tyrosine phosphatase zeta RPTPb: analysis of mice in which the PTPzeta/RPTPb gene was replaced with the LacZ gene. Neurosci Lett. 1998;247(2–3): 135–8.
324. Harroch S, Palmeri M, Rosenbluth J, Custer A, Okigaki M, Shrager P, et al. No obvious abnormality in mice deficient in receptor protein tyrosine phosphatase beta. Mol Cell Biol. 2000;20(20):7706–15.
325. Kuboyama K, Fujikawa A, Masumura M, Suzuki R, Matsumoto M, Noda M. Protein tyrosine phosphatase receptor type z negatively regulates oligodendrocyte differentiation and myelination. PLoS One. 2012;7(11):e48797.

326. Levy JB, Canoll PD, Silvennoinen O, Barnea G, Morse B, Honegger AM, et al. The cloning of a receptor-type protein tyrosine phosphatase expressed in the central nervous system. J Biol Chem. 1993;268(14):10573–81.
327. Himburg HA, Harris JR, Ito T, Daher P, Russell JL, Quarmyne M, et al. Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche. Cell Rep. 2012;2(4):964–75.
328. Yuzawa S, Opatowsky Y, Zhang Z, Mandiyan V, Lax I, Schlessinger J. Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. Cell. 2007;130(2): 323–34.
329. Yang Y, Yuzawa S, Schlessinger J. Contacts between membrane proximal regions of the PDGF receptor ectodomain are required for receptor activation but not for receptor dimerization. Proc Natl Acad Sci USA. 2008;105(22):7681–6.
330. Roussel MF, Rettenmier CW, Sherr CJ. Introduction of a human colony stimulating factor-1 gene into a mouse macrophage cell line induces CSF-1 independence but not tumorigenicity. Blood. 1988;71(5):1218–25.
331. Lemmon MA, Pinchasi D, Zhou M, Lax I, Schlessinger J. Kit receptor dimerization is driven by bivalent binding of stem cell factor. J Biol Chem. 1997;272(10):6311–7.
332. Graddis TJ, Brasel K, Friend D, Srinivasan S, Wee S, Lyman SD, et al. Structure-function analysis of FLT3 ligand-FLT3 receptor interactions using a rapid functional screen. J Biol Chem. 1998;273(28):17626–33.
333. Walter M, Lucet IS, Patel O, Broughton SE, Bamert R, Williams NK, et al. The 2.7 A crystal structure of the autoinhibited human c-Fms kinase domain. J Mol Biol. 2007;367(3): 839–47.
334. Schubert C, Schalk-Hihi C, Struble GT, Ma HC, Petrounia IP, Brandt B, et al. Crystal structure of the tyrosine kinase domain of colony-stimulating factor-1 receptor (cFMS) in complex with two inhibitors. J Biol Chem. 2007;282(6):4094–101.
335. Yu W, Chen J, Xiong Y, Pixley FJ, Yeung YG, Stanley ER. Macrophage proliferation is regulated through CSF-1 receptor tyrosines 544, 559, and 807. J Biol Chem. 2012;287(17): 13694–704.
336. DiNitto JP, Deshmukh GD, Zhang Y, Jacques SL, Coli R, Worrall JW, et al. Function of activation loop tyrosine phosphorylation in the mechanism of c-Kit auto-activation and its implication in sunitinib resistance. J Biochem. 2010;147(4):601–9.
337. Yu W, Chen J, Xiong Y, Pixley F, Dai X, Yeung Y, et al. CSF-1 receptor structure/function in MacCsf1r$^{-/-}$ macrophages: regulation of proliferation, differentiation, and morphology. J Leukoc Biol. 2008;84(3):852–63.
338. Rohde CM, Schrum J, Lee AW. A juxtamembrane tyrosine in the colony stimulating factor-1 receptor regulates ligand-induced Src association, receptor kinase function, and downregulation. J Biol Chem. 2004;279(42):43448–61.
339. Takeshita S, Faccio R, Chappel J, Zheng L, Feng X, Weber JD, et al. c-Fms tyrosine 559 is a major mediator of M-CSF-induced proliferation of primary macrophages. J Biol Chem. 2007;282(26):18980–90.
340. Bourette RP, Myles GM, Carlberg K, Chen AR, Rohrschneider LR. Uncoupling of the proliferation and differentiation signals mediated by the murine macrophage colony-stimulating factor receptor expressed in myeloid FDC-P1 cells. Cell Growth Differ. 1995;6(6): 631–45.
341. Csar XF, Wilson NJ, McMahon KA, Marks DC, Beecroft TL, Ward AC, et al. Proteomic analysis of macrophage differentiation. p46/52(Shc) Tyrosine phosphorylation is required for CSF-1-mediated macrophage differentiation. J Biol Chem. 2001;276(28):26211–7.
342. Stanley ER, Chitu V. CSF-1 receptor signaling in myeloid cells. Cold Spring Harb Perspect Biol. 2014;6(6):a021857.
343. Yeung YG, Stanley ER. Proteomic approaches to the analysis of early events in colony-stimulating factor-1 signal transduction. Mol Cell Proteomics. 2003;2(11):1143–55.

344. Boocock CA, Jones GE, Stanley ER, Pollard JW. Colony-stimulating factor-1 induces rapid behavioural responses in the mouse macrophage cell line, BAC1.2 F5. J Cell Sci. 1989;93(Pt 3):447–56.
345. Pixley FJ, Lee PS, Condeelis JS, Stanley ER. Protein tyrosine phosphatase phi regulates paxillin tyrosine phosphorylation and mediates colony-stimulating factor 1-induced morphological changes in macrophages. Mol Cell Biol. 2001;21(5):1795–809.
346. Chitu V, Pixley FJ, Macaluso F, Larson DR, Condeelis J, Yeung YG, et al. The PCH family member MAYP/PSTPIP2 directly regulates F-actin bundling and enhances filopodia formation and motility in macrophages. Mol Biol Cell. 2005;16(6):2947–59.
347. Pixley FJ. Macrophage migration and its regulation by CSF-1. Int J Cell Biol. 2012; 2012:501962.
348. Sherr CJ. Mitogenic response to colony-stimulating factor 1. TIG. 1991;7:398–402.
349. Li W, Stanley ER. Role of dimerization and modification of the CSF-1 receptor in its activation and internalization during the CSF-1 response. EMBO J. 1991;10(2):277–88.
350. Pixley FJ, Stanley ER. CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol. 2004;14(11):628–38.
351. Li W, Yeung YG, Stanley ER. Tyrosine phosphorylation of a common 57-kDa protein in growth factor- stimulated and -transformed cells. J Biol Chem. 1991;266(11):6808–14.
352. Baccarini M, Li W, Dello Sbarba P, Stanley ER. Increased phosphorylation of the colony stimulating factor-1 receptor following transmembrane signaling. Receptor. 1991;1(4): 243–59.
353. Wang Y, Yeung YG, Langdon WY, Stanley ER. c-Cbl is transiently tyrosine-phosphorylated, ubiquitinated, and membrane-targeted following CSF-1 stimulation of macrophages. J Biol Chem. 1996;271(1):17–20.
354. Wang Y, Yeung YG, Stanley ER. CSF-1 stimulated multiubiquitination of the CSF-1 receptor and of Cbl follows their tyrosine phosphorylation and association with other signaling proteins. J Cell Biochem. 1999;72(1):119–34.
355. Kanagasundaram V, Jaworowski A, Hamilton JA. Association between phosphatidylinositol-3 kinase, Cbl and other tyrosine phosphorylated proteins in colony-stimulating factor-1-stimulated macrophages. Biochem J. 1996;320(Pt 1):69–77.
356. Husson H, Mograbi B, Schmid-Antomarchi H, Fischer S, Rossi B. CSF-1 stimulation induces the formation of a multiprotein complex including CSF-1 receptor, c-Cbl, PI 3-kinase, Crk-II and Grb2. Oncogene. 1997;14(19):2331–8.
357. Huynh J, Kwa MQ, Cook AD, Hamilton JA, Scholz GM. CSF-1 receptor signalling from endosomes mediates the sustained activation of Erk1/2 and Akt in macrophages. Cell Signal. 2012;24(9):1753–61.
358. Bourette RP, Rohrschneider LR. Early events in M-CSF receptor signaling. Growth Factors. 2000;17(3):155–66.
359. Hamilton JA. CSF-1 signal transduction. J Leukoc Biol. 1997;62(2):145–55.
360. Marks DC, Csar XF, Wilson NJ, Novak U, Ward AC, Kanagasundaram V, et al. Expression of a Y559F mutant of the CSF-1 receptor in M1 myeloid cells: a role for the Src kinases in CSF-1 receptor mediated differentiation. Mol Cell Biol Res Commun. 1999;1(2):144–52.
361. Mouchemore KA, Pixley FJ. CSF-1 signaling in macrophages: pleiotrophy through phosphotyrosine-based signaling pathways. Crit Rev Clin Lab Sci. 2012;49(2):49–61.
362. Joos H, Trouliaris S, Helftenbein G, Niemann H, Tamura T. Tyrosine phosphorylation of the juxtamembrane domain of the v-Fms oncogene product is required for its association with a 55-kDa protein. J Biol Chem. 1996;271(40):24476–81.
363. Mancini A, Niedenthal R, Joos H, Koch A, Trouliaris S, Niemann H, et al. Identification of a second Grb2 binding site in the v-Fms tyrosine kinase. Oncogene. 1997;15(13):1565–72.
364. Faccio R, Takeshita S, Zallone A, Ross FP, Teitelbaum SL. c-Fms and the alphavbeta3 integrin collaborate during osteoclast differentiation. J Clin Invest. 2003;111(5):749–58.
365. Alonso G, Koegl M, Mazurenko N, Courtneidge SA. Sequence requirements for binding of Src family tyrosine kinases to activated growth factor receptors. J Biol Chem. 1995; 270(17):9840–8.

366. Pakuts B, Debonneville C, Liontos LM, Loreto MP, McGlade CJ. The Src-like adaptor protein 2 regulates colony-stimulating factor-1 receptor signaling and down-regulation. J Biol Chem. 2007;282(25):17953–63.
367. Wilhelmsen K, Burkhalter S, van der Geer P. C-Cbl binds the CSF-1 receptor at tyrosine 973, a novel phosphorylation site in the receptor's carboxy-terminus. Oncogene. 2002;21(7): 1079–89.
368. Sampaio N, Yu W, Cox D, Wyckoff J, Condeelis J, Stanley ER, et al. Phosphorylation of Y721 of the CSF-1R mediates PI3K association to regulate macrophage motility and enhancement of tumor cell invasion. J Cell Sci. 2011;124(Pt 12):2021–31.
369. Rohrschneider LR, Bourette RP, Lioubin MN, Algate PA, Myles GM, Carlberg K. Growth and differentiation signals regulated by the M-CSF receptor. Mol Reprod Dev. 1997;46(1): 96–103.
370. Bourette RP, Myles GM, Choi JL, Rohrschneider LR. Sequential activation of phosphatidylinositol 3-kinase and phospholipase C-g2 by the M-CSF receptor is necessary for differentiation signaling. EMBO J. 1997;16(19):5880–93.
371. Junttila I, Bourette RP, Rohrschneider LR, Silvennoinen O. M-CSF induced differentiation of myeloid precursor cells involves activation of PKC-delta and expression of Pkare. J Leukoc Biol. 2003;73(2):281–8.
372. Barbosa CM, Bincoletto C, Barros CC, Ferreira AT, Paredes-Gamero EJ. PLCgamma2 and PKC are important to myeloid lineage commitment triggered by M-SCF and G-CSF. J Cell Biochem. 2014;115(1):42–51.
373. Wilson NJ, Cross M, Nguyen T, Hamilton JA. cAMP inhibits CSF-1-stimulated tyrosine phosphorylation but augments CSF-1R-mediated macrophage differentiation and ERK activation. FEBS J. 2005;272(16):4141–52.
374. Gobert Gosse S, Bourgin C, Liu WQ, Garbay C, Mouchiroud G. M-CSF stimulated differentiation requires persistent MEK activity and MAPK phosphorylation independent of Grb2-Sos association and phosphatidylinositol 3-kinase activity. Cell Signal. 2005;17(11): 1352–62.
375. Bourette RP, Arnaud S, Myles GM, Blanchet JP, Rohrschneider LR, Mouchiroud G. Mona, a novel hematopoietic-specific adaptor interacting with the macrophage colony-stimulating factor receptor, is implicated in monocyte/macrophage development. EMBO J. 1998;17(24): 7273–81.
376. Bourgin C, Bourette R, Mouchiroud G, Arnaud S. Expression of Mona (monocytic adapter) in myeloid progenitor cells results in increased and prolonged MAP kinase activation upon macrophage colony-stimulating factor stimulation. FEBS Lett. 2000;480(2–3):113–7.
377. Bourgin C, Bourette RP, Arnaud S, Liu Y, Rohrschneider LR, Mouchiroud G. Induced expression and association of the Mona/Gads adapter and Gab3 scaffolding protein during monocyte/macrophage differentiation. Mol Cell Biol. 2002;22(11):3744–56.
378. Lee AW, Mao Y, Penninger JM, Yu S. Gab2 promotes colony-stimulating factor 1-regulated macrophage expansion via alternate effectors at different stages of development. Mol Cell Biol. 2011;31(22):4563–81.
379. Wolf I, Jenkins BJ, Liu Y, Seiffert M, Custodio JM, Young P, et al. Gab3, a new DOS/Gab family member, facilitates macrophage differentiation. Mol Cell Biol. 2002;22(1):231–44.
380. Seiffert M, Custodio JM, Wolf I, Harkey M, Liu Y, Blattman JN, et al. Gab3-deficient mice exhibit normal development and hematopoiesis and are immunocompetent. Mol Cell Biol. 2003;23(7):2415–24.
381. Simoncic PD, Bourdeau A, Lee-Loy A, Rohrschneider LR, Tremblay ML, Stanley ER, et al. T-cell protein tyrosine phosphatase (Tcptp) is a negative regulator of colony-stimulating factor 1 signaling and macrophage differentiation. Mol Cell Biol. 2006;26(11):4149–60.
382. McMahon KA, Wilson NJ, Marks DC, Beecroft TL, Whitty GA, Hamilton JA, et al. Colony-stimulating factor-1 (CSF-1) receptor-mediated macrophage differentiation in myeloid cells: a role for tyrosine 559-dependent protein phosphatase 2A (PP2A) activity. Biochem J. 2001;358(Pt 2):431–6.

383. Raabe T, Rapp UR. Ras signaling: PP2A puts Ksr and Raf in the right place. Curr Biol. 2003;13(16):R635–7.
384. Grasset MF, Gobert-Gosse S, Mouchiroud G, Bourette RP. Macrophage differentiation of myeloid progenitor cells in response to M-CSF is regulated by the dual-specificity phosphatase DUSP5. J Leukoc Biol. 2010;87(1):127–35.
385. Jacquel A, Benikhlef N, Paggetti J, Lalaoui N, Guery L, Dufour EK, et al. Colony-stimulating factor-1-induced oscillations in phosphatidylinositol-3 kinase/AKT are required for caspase activation in monocytes undergoing differentiation into macrophages. Blood. 2009;114(17):3633–41.
386. Guery L, Benikhlef N, Gautier T, Paul C, Jego G, Dufour E, et al. Fine-tuning nucleophosmin in macrophage differentiation and activation. Blood. 2011;118(17):4694–704.
387. Lagrange B, Martin RZ, Droin N, Aucagne R, Paggetti J, Largeot A, et al. A role for miR-142-3p in colony-stimulating factor 1-induced monocyte differentiation into macrophages. Biochim Biophys Acta. 2013;1833(8):1936–46.
388. Riepsaame J, van Oudenaren A, den Broeder BJ, van Ijcken WF, Pothof J, Leenen PJ. MicroRNA-mediated down-regulation of M-CSF receptor contributes to maturation of mouse monocyte-derived dendritic cells. Front Immunol. 2013;4:353.
389. Tushinski RJ, Stanley ER. The regulation of macrophage protein turnover by a colony stimulating factor (CSF-1). J Cell Physiol. 1983;116(1):67–75.
390. Tushinski RJ, Stanley ER. The regulation of mononuclear phagocyte entry into S phase by the colony stimulating factor CSF-1. J Cell Physiol. 1985;122(2):221–8.
391. Lee A, States D. Colony-stimulating factor-1 requires PI3-kinase-mediated metabolism for proliferation and survival in myeloid cells. Cell Death Differ. 2006;13(11):1900–14.
392. Lee AW. The role of atypical protein kinase C in CSF-1-dependent Erk activation and proliferation in myeloid progenitors and macrophages. PLoS One. 2011;6(10):e25580.
393. Rovida E, Baccarini M, Olivotto M, Sbarba PD. Opposite effects of different doses of MCSF on ERK phosphorylation and cell proliferation in macrophages. Oncogene. 2002;21(23):3670–6.
394. Xu XX, Tessner TG, Rock CO, Jackowski S. Phosphatidylcholine hydrolysis and c-myc expression are in collaborating mitogenic pathways activated by colony-stimulating factor 1. Mol Cell Biol. 1993;13(3):1522–33.
395. Gangoiti P, Granado MH, Wang SW, Kong JY, Steinbrecher UP, Gómez-Muñoz A. Ceramide 1-phosphate stimulates macrophage proliferation through activation of the PI3-kinase/PKB, JNK and ERK1/2 pathways. Cell Signal. 2008;20(4):726–36.
396. Rovida E, Spinelli E, Sdelci S, Barbetti V, Morandi A, Giuntoli S, et al. ERK5/BMK1 is indispensable for optimal colony-stimulating factor 1 (CSF-1)-induced proliferation in macrophages in a Src-dependent fashion. J Immunol. 2008;180(6):4166–72.
397. Valledor AF, Xaus J, Marques L, Celada A. Macrophage colony-stimulating factor induces the expression of mitogen- activated protein kinase phosphatase-1 through a protein kinase C- dependent pathway. J Immunol. 1999;163(5):2452–62.
398. Nataf S, Anginot A, Vuaillat C, Malaval L, Fodil N, Chereul E, et al. Brain and bone damage in KARAP/DAP12 loss-of-function mice correlate with alterations in microglia and osteoclast lineages. Am J Pathol. 2005;166(1):275–86.
399. Gueller S, Goodridge HS, Niebuhr B, Xing H, Koren-Michowitz M, Serve H, et al. Adaptor protein Lnk inhibits c-Fms-mediated macrophage function. J Leukoc Biol. 2010;88(4): 699–706.
400. Velazquez L, Cheng AM, Fleming HE, Furlonger C, Vesely S, Bernstein A, et al. Cytokine signaling and hematopoietic homeostasis are disrupted in Lnk-deficient mice. J Exp Med. 2002;195(12):1599–611.
401. Chitu V, Ferguson P, de Bruijn R, Schlueter A, Ochoa L, Waldschmidt T, et al. Primed innate immunity leads to autoinflammatory disease in PSTPIP2-defcient cmo mice. Blood. 2009; 114(12):2497–24505.

402. Bourette RP, De Sepulveda P, Arnaud S, Dubreuil P, Rottapel R, Mouchiroud G. Suppressor of cytokine signaling 1 interacts with the macrophage colony-stimulating factor receptor and negatively regulates its proliferation signal. J Biol Chem. 2001;276(25):22133–9.
403. Bourette RP, Mouchiroud G. The biological role of interferon-inducible P204 protein in the development of the mononuclear phagocyte system. Front Biosci. 2008;13:879–86.
404. Chitu V, Stanley ER. PSTPIP1 and PSTPIP2. In: Aspenström P, editor. Pombe Cdc15 homology proteins. Landes Bioscience: Austin, TX; 2008. p. 49–61.
405. Grosse J, Chitu V, Marquardt A, Hanke P, Schmittwolf C, Zeitlmann L, et al. Mutation of mouse MAYP/PSTPIP2 causes a macrophage autoinflammatory disease. Blood. 2006; 107(8):3350–8.
406. Wu Y, Dowbenko D, Lasky LA. PSTPIP 2, a second tyrosine phosphorylated, cytoskeletal-associated protein that binds a PEST-type protein-tyrosine phosphatase. J Biol Chem. 1988;273(46):30487–96.
407. Yang H, Reinherz E. CD2BP1 modulates CD2-dependent T cell activation via linkage to protein tyrosine phosphatase (PTP)-PEST. J Immunol. 2006;176(10):5898–907.
408. De Sepulveda P, Okkenhaug K, Rose JL, Hawley RG, Dubreuil P, Rottapel R. Socs1 binds to multiple signalling proteins and suppresses steel factor-dependent proliferation. EMBO J. 1999;18(4):904–15.
409. Dauffy J, Mouchiroud G, Bourette RP. The interferon-inducible gene, Ifi204, is transcriptionally activated in response to M-CSF, and its expression favors macrophage differentiation in myeloid progenitor cells. J Leukoc Biol. 2006;79(1):173–83.
410. Bourette RP, Therier J, Mouchiroud G. Macrophage colony-stimulating factor receptor induces tyrosine phosphorylation of SKAP55R adaptor and its association with actin. Cell Signal. 2005;17(8):941–9.
411. Webb SE, Pollard JW, Jones GE. Direct observation and quantification of macrophage chemoattraction to the growth factor CSF-1. J Cell Sci. 1996;110(Pt 4):707–20.
412. Park H, Ishihara D, Cox D. Regulation of tyrosine phosphorylation in macrophage phagocytosis and chemotaxis. Arch Biochem Biophys. 2011;510(2):101–11.
413. Ishihara D, Dovas A, Park H, Isaac BM, Cox D. The chemotactic defect in wiskott-Aldrich syndrome macrophages is due to the reduced persistence of directional protrusions. PLoS One. 2012;7(1):e30033.
414. Papakonstanti EA, Zwaenepoel O, Bilancio A, Burns E, Nock GE, Houseman B, et al. Distinct roles of class IA PI3K isoforms in primary and immortalised macrophages. J Cell Sci. 2008;121(Pt24):4124–33.
415. Mouchemore KA, Sampaio NG, Murrey MW, Stanley ER, Lannutti BJ, Pixley FJ. Specific inhibition of PI3K p110delta inhibits CSF-1-induced macrophage spreading and invasive capacity. FEBS J. 2013;280:5228–36.
416. Munugalavadla V, Borneo J, Ingram DA, Kapur R. p85alpha subunit of class IA PI-3 kinase is crucial for macrophage growth and migration. Blood. 2005;106(1):103–9.
417. Zhang B, Ma Y, Guo H, Sun B, Niu R, Ying G, et al. Akt2 is required for macrophage chemotaxis. Eur J Immunol. 2009;39(3):894–901.
418. Ikeda O, Sekine Y, Kakisaka M, Tsuji S, Muromoto R, Ohbayashi N, et al. STAP-2 regulates c-Fms/M-CSF receptor signaling in murine macrophage Raw 264.7 cells. Biochem Biophys Res Commun. 2007;358(3):931–7.
419. Ridley AJ. Regulation of macrophage adhesion and migration by Rho GTP-binding proteins. J Microsc. 2008;231(3):518–23.
420. Kheir WA, Gevrey JC, Yamaguchi H, Isaac B, Cox D. A WAVE2-Abi1 complex mediates CSF-1-induced F-actin-rich membrane protrusions and migration in macrophages. J Cell Sci. 2005;118(Pt22):5369–79.
421. Cammer M, Gevrey JC, Lorenz M, Dovas A, Condeelis J, Cox D. The mechanism of CSF-1-induced Wiskott-Aldrich syndrome protein activation in vivo: a role for phosphatidylinositol 3-kinase and Cdc42. J Biol Chem. 2009;284(35):23302–11.

422. Dovas A, Gevrey JC, Grossi A, Park H, Abou-Kheir W, Cox D. Regulation of podosome dynamics by WASp phosphorylation: implication in matrix degradation and chemotaxis in macrophages. J Cell Sci. 2009;122(Pt21):3872–82.
423. Vedham V, Phee H, Coggeshall KM. Vav activation and function as a rac guanine nucleotide exchange factor in macrophage colony-stimulating factor-induced macrophage chemotaxis. Mol Cell Biol. 2005;25(10):4211–20.
424. Wells CM, Bhavsar PJ, Evans IR, Vigorito E, Turner M, Tybulewicz V, et al. Vav1 and Vav2 play different roles in macrophage migration and cytoskeletal organization. Exp Cell Res. 2005;310(2):303–10.
425. Bhavsar PJ, Vigorito E, Turner M, Ridley AJ. Vav GEFs regulate macrophage morphology and adhesion-induced Rac and Rho activation. Exp Cell Res. 2009;315(19):3345–58.
426. Wheeler AP, Wells CM, Smith SD, Vega FM, Henderson RB, Tybulewicz VL, et al. Rac1 and Rac2 regulate macrophage morphology but are not essential for migration. J Cell Sci. 2006;119(Pt 13):2749–57.
427. Wells CM, Walmsley M, Ooi S, Tybulewicz V, Ridley AJ. Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. J Cell Sci. 2004;117(Pt 7):1259–68.
428. Allen WE, Zicha D, Ridley AJ, Jones GE. A role for Cdc42 in macrophage chemotaxis. J Cell Biol. 1998;141(5):1147–57.
429. Abou-Kheir W, Isaac B, Yamaguchi H, Cox D. Membrane targeting of WAVE2 is not sufficient for WAVE2-dependent actin polymerization: a role for IRSp53 in mediating the interaction between Rac and WAVE2. J Cell Sci. 2008;121(Pt3):379–90.
430. Mahankali M, Peng HJ, Cox D, Gomez-Cambronero J. The mechanism of cell membrane ruffling relies on a phospholipase D2 (PLD2), Grb2 and Rac2 association. Cell Signal. 2011;23(8):1291–8.
431. Mahankali M, Peng HJ, Henkels KM, Dinauer MC, Gomez-Cambronero J. Phospholipase D2 (PLD2) is a guanine nucleotide exchange factor (GEF) for the GTPase Rac2. Proc Natl Acad Sci USA. 2011;108(49):19617–22.
432. Peng HJ, Henkels KM, Mahankali M, Marchal C, Bubulya P, Dinauer MC, et al. The dual effect of Rac2 on phospholipase D2 regulation that explains both the onset and termination of chemotaxis. Mol Cell Biol. 2011;31(11):2227–40.
433. Li ZH, Spektor A, Varlamova O, Bresnick AR. Mts1 regulates the assembly of nonmuscle myosin-IIA. Biochemistry. 2003;42(48):14258–66.
434. Li ZH, Dulyaninova NG, House RP, Almo SC, Bresnick AR. S100A4 regulates macrophage chemotaxis. Mol Biol Cell. 2010;21(15):2598–610.
435. Hanley PJ, Xu Y, Kronlage M, Grobe K, Schon P, Song J, et al. Motorized RhoGAP myosin IXb (Myo9b) controls cell shape and motility. Proc Natl Acad Sci USA. 2010;107(27):12145–50.
436. Jones GE, Allen WE, Ridley AJ. The Rho GTPases in macrophage motility and chemotaxis. Cell Adhes Commun. 1998;6(2–3):237–45.
437. Papakonstanti EA, Ridley AJ, Vanhaesebroeck B. The p110delta isoform of PI 3-kinase negatively controls RhoA and PTEN. EMBO J. 2007;26(13):3050–61.
438. Gómez-Muñoz A, Kong JY, Salh B, Steinbrecher UP. Ceramide-1-phosphate blocks apoptosis through inhibition of acid sphingomyelinase in macrophages. J Lipid Res. 2004;45(1):99–105.
439. Gómez-Muñoz A, Gangoiti P, Granado MH, Arana L, Ouro A. Ceramide-1-phosphate in cell survival and inflammatory signaling. Adv Exp Med Biol. 2010;688:118–30.
440. Gómez-Muñoz A, Kong JY, Parhar K, Wang SW, Gangoiti P, González M, et al. Ceramide-1-phosphate promotes cell survival through activation of the phosphatidylinositol 3-kinase/protein kinase B pathway. FEBS Lett. 2005;579(17):3744–50.
441. Kelley TW, Graham MM, Doseff AI, Pomerantz RW, Lau SM, Ostrowski MC, et al. Macrophage colony-stimulating factor promotes cell survival through Akt/protein kinase B. J Biol Chem. 1999;274(37):26393–8.

442. Chang M, Hamilton JA, Scholz GM, Masendycz P, Macaulay SL, Elsegood CL. Phosphatidylinostitol-3 kinase and phospholipase C enhance CSF-1-dependent macrophage survival by controlling glucose uptake. Cell Signal. 2009;21(9):1361–9.
443. Golden LH, Insogna KL. The expanding role of PI3-kinase in bone. Bone. 2004;34(1): 3–12.
444. Murray JT, Craggs G, Wilson L, Kellie S. Mechanism of phosphatidylinositol 3-kinase-dependent increases in BAC1.2 F5 macrophage-like cell density in response to M-CSF: phosphatidylinositol 3-kinase inhibitors increase the rate of apoptosis rather than inhibit DNA synthesis. Inflamm Res. 2000;49(11):610–8.
445. Jaworowski A, Wilson NJ, Christy E, Byrne R, Hamilton JA. Roles of the mitogen-activated protein kinase family in macrophage responses to colony stimulating factor-1 addition and withdrawal. J Biol Chem. 1999;274(21):15127–33.
446. Lee AW, States DJ. Both src-dependent and -independent mechanisms mediate phosphatidylinositol 3-kinase regulation of colony-stimulating factor 1-activated mitogen-activated protein kinases in myeloid progenitors. Mol Cell Biol. 2000;20(18):6779–98.
447. Baran CP, Tridandapani S, Helgason CD, Humphries RK, Krystal G, Marsh CB. The inositol 5′-phosphatase SHIP-1 and the Src kinase Lyn negatively regulate macrophage colony-stimulating factor-induced Akt activity. J Biol Chem. 2003;278(40):38628–36.
448. Smith JL, Schaffner AE, Hofmeister JK, Hartman M, Wei G, Forsthoefel D, et al. Ets-2 is a target for an akt (protein kinase B)/Jun N-terminal kinase signaling pathway in macrophages of motheaten-viable mutant mice. Mol Cell Biol. 2000;20(21):8026–34.
449. Sevilla L, Aperlo C, Dulic V, Chambard JC, Boutonnet C, Pasquier O, et al. The Ets2 transcription factor inhibits apoptosis induced by colony- stimulating factor 1 deprivation of macrophages through a Bcl-xL- dependent mechanism. Mol Cell Biol. 1999;19(4): 2624–34.
450. Sevilla L, Zaldumbide A, Carlotti F, Dayem MA, Pognonec P, Boulukos KE. Bcl-XL expression correlates with primary macrophage differentiation, activation of functional competence, and survival and results from synergistic transcriptional activation by Ets2 and PU.1. J Biol Chem. 2001;276(21):17800–7.
451. Mancini A, Koch A, Whetton AD, Tamura T. The M-CSF receptor substrate and interacting protein FMIP is governed in its subcellular localization by protein kinase C-mediated phosphorylation, and thereby potentiates M-CSF-mediated differentiation. Oncogene. 2004; 23(39):6581–9.
452. Roiniotis J, Dinh H, Masendycz P, Turner A, Elsegood CL, Scholz GM, et al. Hypoxia prolongs monocyte/macrophage survival and enhanced glycolysis is associated with their maturation under aerobic conditions. J Immunol. 2009;182(12):7974–81.
453. Elsegood CL, Chang M, Jessup W, Scholz GM, Hamilton JA. Glucose metabolism is required for oxidized LDL-induced macrophage survival: role of PI3K and Bcl-2 family proteins. Arterioscler Thromb Vasc Biol. 2009;29(9):1283–9.
454. Norgard M, Marks Jr SC, Reinholt FP, Andersson G. The effects of colony-stimulating factor-1 (CSF-1) on the development of osteoclasts and their expression of tartrate-resistant acid phosphatase (TRAP) in toothless (tl-osteopetrotic) rats. Crit Rev Eukaryot Gene Expr. 2003;13(2–4):117–32.
455. Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, et al. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophys Res Commun. 1998;253(2):395–400.
456. Fuller K, Owens JM, Jagger CJ, Wilson A, Moss R, Chambers TJ. Macrophage colony-stimulating factor stimulates survival and chemotactic behavior in isolated osteoclasts. J Exp Med. 1993;178(5):1733–44.
457. Bouyer P, Sakai H, Itokawa T, Kawano T, Fulton CM, Boron WF, et al. Colony-stimulating factor-1 increases osteoclast intracellular pH and promotes survival via the electroneutral Na/HCO3 cotransporter NBCn1. Endocrinology. 2007;148(2):831–40.

458. Sakai H, Chen Y, Itokawa T, Yu KP, Zhu ML, Insogna K. Activated c-Fms recruits Vav and Rac during CSF-1-induced cytoskeletal remodeling and spreading in osteoclasts. Bone. 2006;39(6):1290–301.
459. Palacio S, Felix R. The role of phosphoinositide 3-kinase in spreading osteoclasts induced by colony-stimulating factor-1. Eur J Endocrinol. 2001;144(4):431–40.
460. Owens J, Chambers TJ. Macrophage colony-stimulating factor (M-CSF) induces migration in osteoclasts in vitro. Biochem Biophys Res Commun. 1993;195(3):1401–7.
461. Hodge JM, Kirkland MA, Nicholson GC. Multiple roles of M-CSF in human osteoclastogenesis. J Cell Biochem. 2007;102(13):759–68.
462. Hodge JM, Kirkland MA, Nicholson GC. GM-CSF cannot substitute for M-CSF in human osteoclastogenesis. Biochem Biophys Res Commun. 2004;321(1):7–12.
463. Perkins SL, Kling SJ. Local concentrations of macrophage colony-stimulating factor mediate osteoclastic differentiation. Am J Physiol. 1995;269(6):E1024–30.
464. Biskobing DM, Fan X, Rubin J. Characterization of MCSF-induced proliferation and subsequent osteoclast formation in murine marrow culture. J Bone Miner Res. 1995;10(7): 1025–32.
465. Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. Bone. 2007;40(2):251–64.
466. Chen W, Zhu G, Hao L, Wu M, Ci H, Li YP. C/EBPα regulates osteoclast lineage commitment. Proc Natl Acad Sci USA. 2013;110(18):7294–9.
467. Murphy HM. The osteopetrotic syndrome in the microphthalmic mutant mouse. Calcif Tissue Res. 1973;13(1):19–26.
468. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, et al. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med. 1999;190(12):1741–54.
469. Arai A, Mizoguchi T, Harada S, Kobayashi Y, Nakamichi Y, Yasuda H, et al. Fos plays an essential role in the upregulation of RANK expression in osteoclast precursors within the bone microenvironment. J Cell Sci. 2012;125(Pt 12):2910–7.
470. Ji JD, Park-Min KH, Shen Z, Fajardo RJ, Goldring SR, McHugh KP, et al. Inhibition of RANK expression and osteoclastogenesis by TLRs and IFN-gamma in human osteoclast precursors. J Immunol. 2009;183(11):7223–33.
471. Mansky KC, Sankar U, Han J, Ostrowski MC. Microphthalmia transcription factor is a target of the p38 MAPK pathway in response to receptor activator of NF-kappa B ligand signaling. J Biol Chem. 2002;277(13):11077–83.
472. Weilbaecher KN, Motyckova G, Huber WE, Takemoto CM, Hemesath TJ, Xu Y, et al. Linkage of M-CSF signaling to Mitf, TFE3, and the osteoclast defect in Mitf(mi/mi) mice. Mol Cell. 2001;8(4):749–58.
473. Yao C, Yao GQ, Sun BH, Zhang C, Tommasini SM, Insogna K. The transcription factor T-box3 regulates colony stimulating factor 1-dependent Jun dimerization protein 2 expression and plays an important role in osteoclastogenesis. J Biol Chem. 2014;289:6775–90.
474. Kawaida R, Ohtsuka T, Okutsu J, Takahashi T, Kadono Y, Oda H, et al. Jun dimerization protein 2 (JDP2), a member of the AP-1 family of transcription factor, mediates osteoclast differentiation induced by RANKL. J Exp Med. 2003;197(8):1029–35.
475. Ito Y, Teitelbaum SL, Zou W, Zheng Y, Johnson JF, Chappel J, et al. Cdc42 regulates bone modeling and remodeling in mice by modulating RANKL/M-CSF signaling and osteoclast polarization. J Clin Invest. 2010;120(6):1981–93.
476. Brazier H, Stephens S, Ory S, Fort P, Morrison N, Blangy A. Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis: identification of essential genes in osteoclasts. J Bone Miner Res. 2006;21(9):1387–98.
477. Brazier H, Pawlak G, Vives V, Blangy A. The Rho GTPase Wrch1 regulates osteoclast precursor adhesion and migration. Int J Biochem Cell Biol. 2009;41(6):1391–40.
478. Kim HJ, Zhang K, Zhang L, Ross FP, Teitelbaum SL, Faccio R. The Src family kinase, Lyn, suppresses osteoclastogenesis in vitro and in vivo. Proc Natl Acad Sci USA. 2009;106(7): 2325–30.

479. Kim HJ, Warren JT, Kim SY, Chappel JC, DeSelm CJ, Ross FP, et al. Fyn promotes proliferation, differentiation, survival and function of osteoclast lineage cells. J Cell Biochem. 2010;111(5):1107–13.
480. Vérollet C, Gallois A, Dacquin R, Lastrucci C, Pandruvada SN, Ortega N, et al. Hck contributes to bone homeostasis by controlling the recruitment of osteoclast precursors. FASEB J. 2013;27:3608–18.
481. Lowe C, Yoneda T, Boyce BF, Chen H, Mundy GR, Soriano P. Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. Proc Natl Acad Sci USA. 1993; 90(10):4485–9.
482. Ueki Y, Lin CY, Senoo M, Ebihara T, Agata N, Onji M, et al. Increased myeloid cell responses to M-CSF and RANKL cause bone loss and inflammation in SH3BP2 "cherubism" mice. Cell. 2007;12(1):71–83.
483. Kim HJ, Zou W, Ito Y, Kim SY, Chappel J, Ross FP, et al. Src-like adaptor protein regulates osteoclast generation and survival. J Cell Biochem. 2010;110(1):201–9.
484. Humphrey MB, Ogasawara K, Yao W, Spusta SC, Daws MR, Lane NE, et al. The signaling adapter protein DAP12 regulates multinucleation during osteoclast development. J Bone Miner Res. 2004;19(2):224–34.
485. Kaifu T, Nakahara J, Inui M, Mishima K, Momiyama T, Kaji M, et al. Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice. J Clin Invest. 2003;111(3):323–32.
486. Zou W, Reeve JL, Liu Y, Teitelbaum SL, Ross FP. DAP12 couples c-Fms activation to the osteoclast cytoskeleton by recruitment of Syk. Mol Cell. 2008;31(3):422–31.
487. Despars G, Pandruvada SN, Anginot A, Domenget C, Jurdic P, Mazzorana M. DAP12 overexpression induces osteopenia and impaired early hematopoiesis. PLoS One. 2013;8(6): e65297.
488. Humphrey MB, Daws MR, Spusta SC, Niemi EC, Torchia JA, Lanier LL, et al. TREM2, a DAP12-associated receptor, regulates osteoclast differentiation and function. J Bone Miner Res. 2006;21(2):237–45.
489. Paloneva J, Mandelin J, Kiialainen A, Bohling T, Prudlo J, Hakola P, et al. DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. J Exp Med. 2003;198(4):669–75.
490. Cella M, Buonsanti C, Strader C, Kondo T, Salmaggi A, Colonna M. Impaired differentiation of osteoclasts in TREM-2-deficient individuals. J Exp Med. 2003;198(4):645–51.
491. Otero K, Shinohara M, Zhao H, Cella M, Gilfillan S, Colucci A, et al. TREM2 and β-catenin regulate bone homeostasis by controlling the rate of osteoclastogenesis. J Immunol. 2012;188(6):2612–21.
492. Takeshita S, Namba N, Zhao JJ, Jiang Y, Genant HK, Silva MJ, et al. SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. Nat Med. 2002;8(9):943–9.
493. Zhou P, Kitaura H, Teitelbaum SL, Krystal G, Ross FP, Takeshita S. SHIP1 negatively regulates proliferation of osteoclast precursors via Akt-dependent alterations in D-type cyclins and p27. J Immunol. 2006;177(12):8777–84.
494. Umeda S, Beamer WG, Takagi K, Naito M, Hayashi S, Yonemitsu H, et al. Deficiency of SHP-1 protein-tyrosine phosphatase activity results in heightened osteoclast function and decreased bone density. Am J Pathol. 1999;155(1):223–33.
495. Timms JF, Carlberg K, Gu H, Chen H, Kamatkar S, Nadler MJ, et al. Identification of major binding proteins and substrates for the SH2-containing protein tyrosine phosphatase SHP-1 in macrophages. Mol Cell Biol. 1998;18(7):3838–50.
496. Mori Y, Tsuji S, Inui M, Sakamoto Y, Endo S, Ito Y, et al. Inhibitory immunoglobulin-like receptors LILRB and PIR-B negatively regulate osteoclast development. J Immunol. 2008;181(7):4742–51.
497. Berg KL, Carlberg K, Rohrschneider LR, Siminovitch KA, Stanley ER. The major SHP-1-binding, tyrosine-phosphorylated protein in macrophages is a member of the KIR/LIR family and an SHP-1 substrate. Oncogene. 1998;17(19):2535–41.

498. van Beek EM, de Vries TJ, Mulder L, Schoenmaker T, Hoeben KA, Matozaki T, et al. Inhibitory regulation of osteoclast bone resorption by signal regulatory protein alpha. FASEB J. 2009;23(12):4081–90.
499. Berg KL, Siminovitch KA, Stanley ER. SHP-1 regulation of p62(DOK) tyrosine phosphorylation in macrophages. J Biol Chem. 1999;274(50):35855–65.
500. Kawamata A, Inoue A, Miyajima D, Hemmi H, Mashima R, Hayata T, et al. Dok-1 and Dok-2 deficiency induces osteopenia via activation of osteoclasts. J Cell Physiol. 2011; 226(12):3087–93.
501. Ross F, Teitelbaum S. alphavbeta3 and macrophage colony-stimulating factor: partners in osteoclast biology. Immunol Rev. 2005;208:88–105.
502. Hodge JM, Collier FM, Pavlos NJ, Kirkland MA, Nicholson GC. M-CSF potently augments RANKL-induced resorption activation in mature human osteoclasts. PLoS One. 2011;6(6): e21462.
503. Insogna KL, Sahni M, Grey AB, Tanaka S, Horne WC, Neff L, et al. Colony-stimulating factor-1 induces cytoskeletal reorganization and c-src-dependent tyrosine phosphorylation of selected cellular proteins in rodent osteoclasts. J Clin Invest. 1997;100(10):2476–85.
504. Lakkakorpi PT, Vaananen HK. Cytoskeletal changes in osteoclasts during the resorption cycle. Microsc Res Tech. 1996;33(2):171–81.
505. Nakamura I, Pilkington MF, Lakkakorpi PT, Lipfert L, Sims SM, Dixon SJ, et al. Role of alpha(v)beta(3) integrin in osteoclast migration and formation of the sealing zone. J Cell Sci. 1999;112(Pt22):3985–93.
506. Teti A, Taranta A, Migliaccio S, Degiorgi A, Santandrea E, Villanova I, et al. Colony stimulating factor-1-induced osteoclast spreading depends on substrate and requires the vitronectin receptor and the c-src proto-oncogene. J Bone Miner Res. 1998;13(1):50–8.
507. Pilkington MF, Sims SM, Dixon SJ. Wortmannin inhibits spreading and chemotaxis of rat osteoclasts in vitro. J Bone Miner Res. 1998;13(4):688–94.
508. Faccio R, Takeshita S, Colaianni G, Chappel J, Zallone A, Teitelbaum SL, et al. M-CSF regulates the cytoskeleton via recruitment of a multimeric signaling complex to c-Fms Tyr-559/697/721. J Biol Chem. 2007;282(26):18991–9.
509. Shinohara M, Nakamura M, Masuda H, Hirose J, Kadono Y, Iwasawa M, et al. Class IA phosphatidylinositol 3-kinase regulates osteoclastic bone resorption through protein kinase B-mediated vesicle transport. J Bone Miner Res. 2012;27(12):2464–75.
510. Grey A, Chen Y, Paliwal I, Carlberg K, Insogna K. Evidence for a functional association between phosphatidylinositol 3-kinase and c-src in the spreading response of osteoclasts to colony-stimulating factor-1. Endocrinology. 2000;141(6):2129–38.
511. Alan JK, Berzat AC, Dewar BJ, Graves LM, Cox AD. Regulation of the Rho family small GTPase Wrch-1/RhoU by C-terminal tyrosine phosphorylation requires Src. Mol Cell Biol. 2010;30(17):4324–38.
512. Faccio R, Teitelbaum SL, Fujikawa K, Chappel J, Zallone A, Tybulewicz VL, et al. Vav3 regulates osteoclast function and bone mass. Nat Med. 2005;11(3):284–90.
513. Itokowa T, Zhu ML, Troiano N, Bian J, Kawano T, Insogna K. Osteoclasts lacking Rac2 have defective chemotaxis and resorptive activity. Calcif Tissue Int. 2011;88(1):75–86.
514. Wang Y, Lebowitz D, Sun C, Thang H, Grynpas MD, Glogauer M. Identifying the relative contributions of Rac1 and Rac2 to osteoclastogenesis. J Bone Miner Res. 2008;23(2): 260–70.
515. Reeve JL, Zou W, Liu Y, Maltzman JS, Ross FP, Teitelbaum SL. SLP-76 couples Syk to the osteoclast cytoskeleton. J Immunol. 2009;183(3):1804–12.
516. Zou W, Reeve JL, Zhao H, Ross FP, Teitelbaum SL. Syk tyrosine 317 negatively regulates osteoclast function via the ubiquitin-protein isopeptide ligase activity of Cbl. J Biol Chem. 2009;284(28):18833–9.
517. Mócsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, Spusta SC, et al. The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase. Proc Natl Acad Sci USA. 2004;101(16):6158–63.

518. Akiyama T, Bouillet P, Miyazaki T, Kadono Y, Chikuda H, Chung UI, et al. Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim. EMBO J. 2003;22(24):6653–64.
519. Woo KM, Kim HM, Ko JS. Macrophage colony-stimulating factor promotes the survival of osteoclast precursors by up-regulating Bcl-X(L). Exp Mol Med. 2002;34(5):340–6.
520. Glantschnig H, Fisher JE, Wesolowski G, Rodan GA, Reszka AA. M-CSF, TNFalpha and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. Cell Death Differ. 2003;10(10):1165–77.
521. McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, et al. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell. 2002;109(6):707–18.
522. Barve RA, Zack MD, Weiss D, Song RH, Beidler D, Head RD. Transcriptional profiling and pathway analysis of CSF-1 and IL-34 effects on human monocyte differentiation. Cytokine. 2013;63(1):10–7.
523. Proudfoot AEI. Chemokine receptors: multifaceted therapeutic targets. Nat Rev Immunol. 2002;2:106–15.
524. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003;19(1):71–82.
525. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29(6):313–26.
526. Cheng M, Wang D, Roussel MF. Expression of c-Myc in response to colony-stimulating factor-1 requires mitogen-activated protein kinase kinase-1. J Biol Chem. 1999;274(10): 6553–8.
527. Aziz N, Cherwinski H, McMahon M. Complementation of defective colony-stimulating factor 1 receptor signaling and mitogenesis by Raf and v-Src. Mol Cell Biol. 1999;19(2): 1101–15.
528. Roussel MF. Regulation of cell cycle entry and G1 progression by CSF-1. Mol Reprod Dev. 1997;46(1):11–8.
529. Roussel MF, Cleveland JL, Shurtleff SA, Sherr CJ. Myc rescue of a mutant CSF-1 receptor impaired in mitogenic signalling. Nature. 1991;353(6342):361–3.
530. Irvine KM, Andrews MR, Fernandez-Rojo MA, Schroder K, Burns CJ, Su S, et al. Colony-stimulating factor-1 (CSF-1) delivers a proatherogenic signal to human macrophages. J Leukoc Biol. 2009;85(2):278–88.
531. Sweet MJ, Campbell CC, Sester DP, Xu D, McDonald RC, Stacey KJ, et al. Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages. J Immunol. 2002;168(1):392–9.
532. Chen BD, Lin HS, Hsu S. Tumor-promoting phorbol esters inhibit the binding of colony-stimulating factor (CSF-1) to murine peritoneal exudate macrophages. J Cell Physiol. 1983;116(2):207–12.
533. Guilbert LJ, Stanley ER. Modulation of receptors for the colony-stimulating factor, CSF-1, by bacterial lipopolysaccharide and CSF-1. J Immunol Methods. 1984;73(1):17–28.
534. Downing JR, Roussel MF, Sherr CJ. Ligand and protein kinase C downmodulate the colony-stimulating factor 1 receptor by independent mechanisms. Mol Cell Biol. 1989;9(7): 2890–6.
535. Baccarini M, Dello Sbarba P, Buscher D, Bartocci A, Stanley ER. IFN-gamma/lipopolysaccharide activation of macrophages is associated with protein kinase C-dependent down-modulation of the colony-stimulating factor-1 receptor. J Immunol. 1992;149(8):2656–61.
536. Walker F, Nicola NA, Metcalf D, Burgess AW. Hierarchical down-modulation of hemopoietic growth factor receptors. Cell. 1985;43(1):269–76.
537. Dello Sbarba P, Nencioni L, Labardi D, Rovida E, Caciagli B, Cipolleschi MG. Interleukin 2 down-modulates the macrophage colony-stimulating factor receptor in murine macrophages. Cytokine. 1996;8(6):488–94.

538. Dello Sbarba P, Rovida E, Caciagli B, Nencioni L, Labardi D, Paccagnini A, et al. Interleukin-4 rapidly down-modulates the macrophage colony-stimulating factor receptor in murine macrophages. J Leukoc Biol. 1996;60(5):644–50.
539. Rovida E, Paccagnini A, Del Rosso M, Peschon J, Dello SP. TNF-alpha-converting enzyme cleaves the macrophage colony-stimulating factor receptor in macrophages undergoing activation. J Immunol. 2001;166(3):1583–9.
540. Vahidi A, Glenn G, van der Geer P. Identification and mutagenesis of the TACE and gamma-secretase cleavage sites in the colony-stimulating factor 1 receptor. Biochem Biophys Res Commun. 2014;450(1):782–7.
541. Wilhelmsen K, van der Geer P. Phorbol 12-myristate 13-acetate-induced release of the colony-stimulating factor 1 receptor cytoplasmic domain into the cytosol involves two separate cleavage events. Mol Cell Biol. 2004;24(1):454–64.
542. Glenn G, van der Geer P. CSF-1 and TPA stimulate independent pathways leading to lysosomal degradation or regulated intramembrane proteolysis of the CSF-1 receptor. FEBS Lett. 2007;581(28):5377–81.
543. Glenn G, van der Geer P. Toll-like receptors stimulate regulated intramembrane proteolysis of the CSF-1 receptor through Erk activation. FEBS Lett. 2008;582(6):911–5.
544. Kondo Y, Duncan ID. Selective reduction in microglia density and function in the white matter of colony-stimulating factor-1-deficient mice. J Neurosci Res. 2009;87(12): 2686–95.
545. Ginhoux F, Tacke F, Angeli V, Bogunovic M, Loubeau M, Dai XM, et al. Langerhans cells arise from monocytes in vivo. Nat Immunol. 2006;7(3):265–73.
546. Begg SK, Radley JM, Pollard JW, Chisholm OT, Stanley ER, Bertoncello I. Delayed hematopoietic development in osteopetrotic ($^{op/op}$) mice. J Exp Med. 1993;177(1):237–42.
547. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell. 1991; 65(7):1143–52.
548. Lyman SD, James L, Vanden Bos T, de Vries P, Brasel K, Gliniak B, Hollingsworth LT, Picha KS, McKenna HJ, Splett RR, et al. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. Cell. 1993; 17(756):1157–67.
549. Maroc N, Rottapel R, Rosnet O, Marchetto S, Lavezzi C, Mannoni P, Birnbaum D, Dubreuil P. Biochemical characterization and analysis of the transforming potential of the FLT3/FLK2 receptor tyrosine kinase. Oncogene. 1993;8(4):909–18.
550. de Lapeyrière O, Naguet P, Planche J, Marchetto S, Rottapel R, Gambarelli D, Rosnet O, Birnbaum D. Expression of Flt3 tyrosine kinase receptor gene in mouse hematopoietic and nervous tissues. Differentiation. 1995;58(5):351–9.
551. Rosnet O, Marchetto S, deLapeyriere O, Birnbaum D. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. Oncogene. 1991;6(9):1641–50.
552. Brasel K, Escobar S, Anderberg R, de Vries P, Gruss HJ, Lyman SD. Expression of the flt3 receptor and its ligand on hematopoietic cells. Leukemia. 1995;9(7):1212–8.
553. Rasko JE, Metcalf D, Rossner MT, Begley CG, Nicola NA. The flt3/flk-2 ligand: receptor distribution and action on murine haemopoietic cell survival and proliferation. Leukemia. 1995;9(12):2058–66.
554. Turner AM, Lin N, Issarachai S, Lyman SD, Broudy VC. FLT3 receptor expression on the surface of normal and malignant human hematopoietic cells. Blood. 1996;88(9):3383–90.
555. Lyman SD, Jacobsen SE. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood. 1998;91(4):1101–34.
556. Stirewalt DL, Radich J. The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer. 2003;3(9):650–65.
557. Schmidt-Arras D, Schwable J, Böhmer FD, Serve H. Flt3 receptor tyrosine kinase as a drug target in leukemia. Curr Pharm Des. 2004;10(16):1867–83.
558. Choudhary C, Müller-Tidow C, Berdel WE, Serve H. Signal transduction of oncogenic Flt3. Int J Hematol. 2005;82(2):93–9.

559. Hayakawa F, Towatari M, Kiyoi H, Tanimoto M, Kitamura T, Saito H, Naoe T. Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. Oncogene. 2000;19(5):624–31.
560. Gilliland DG, Griffin J. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002; 100(5):1532–42.
561. Rocnik JL, Okabe R, Yu JC, Lee BH, Giese N, Schenkein DP, Gilliland DG. Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD. Blood. 2006;108(4):1339–45.
562. Mizuki M, Schwable J, Steur C, Choudhary C, Agrawal S, Sargin B, Steffen B, Matsumura I, Kanakura Y, Böhmer FD, Müller-Tidow C, Berdel WE, Serve H. Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations. Blood. 2003;101(8):3164–73.
563. Caldarelli A, Müller JP, Paskowski-Rogacz M, Herrmann K, Bauer R, Koch S, Heninger AK, Krastev D, Ding L, Kasper S, Fischer T, Brodhun M, Böhmer FD, Buchholz F. A genome-wide RNAi screen identifies proteins modulating aberrant FLT3-ITD signaling. Leukemia. 2013;27:2301–10. doi:10.1038/leu.2013.83.
564. Schmidt-Arras DE, Böhmer A, Markova B, Choudhary C, Serve H, Böhmer FD. Tyrosine phosphorylation regulates maturation of receptor tyrosine kinases. Mol Cell Biol. 2005;25(9):3690–703.
565. Verstraete K, Vandriessche G, Januar M, Elegheert J, Shkumatov AV, Desfosses A, Van Craenenbroeck K, Svergun DI, Gutsche I, Vergauwen B, Savvides SN. Structural insights into the extracellular assembly of the hematopoietic Flt3 signaling complex. Blood. 2011;118(1):60–8.
566. Griffith J, Black J, Faerman C, Swenson L, Wynn M, Lu F, Lippke J, Saxena K. The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Mol Cell. 2004;13(2): 169–78.
567. Knapper S. FLT3 inhibition in acute myeloid leukaemia. Br J Haematol. 2007;138(6): 687–99.
568. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730–7.
569. Mackarehtschian K, Hardin JD, Moore KA, Boast S, Goff SP, Lemischka IR. Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoietic progenitors. Immunity. 1995;3(1):147–61.
570. Rosnet O, Bühring HJ, Marchetto S, Rappold I, Lavagna C, Sainty D, Arnoulet C, Chabannon C, Kanz L, Hannum C, Birnbaum D. Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. Leukemia. 1996;10(2):238–48.
571. Bigley V, Haniffa M, Doulatov S, Wang XN, Dickinson R, McGovern N, Jardine L, Pagan S, Dimmick I, Chua I, Wallis J, Lordan J, Morgan C, Kumararatne DS, Doffinger R, van der Burg M, van Dongen J, Cant A, Dick JE, Hambleton S, Collin M. The human syndrome of dendritic cell, monocyte, B and NK lymphoid deficiency. J Exp Med. 2011;208(2):227–34.
572. Merad M, Manz MG. Dendritic cell homeostasis. Blood. 2009;113(15):3418–27.
573. Doulatov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, Dick JE. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nat Immunol. 2010;11(7):585–93.
574. Schmid MA, Kingston D, Boddupalli S, Manz MG. Instructive cytokine signals in dendritic cell lineage commitment. Immunol Rev. 2010;234(1):32–44.
575. Mende I, Karsunky H, Weissman IL, Engleman EG, Merad M. Flk2+ myeloid progenitors are the main source of Langerhans cells. Blood. 2006;107(4):1383–90.
576. Karsunky H, Merad M, Cozzio A, Weissman IL, Manz MG. Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. J Exp Med. 2003;198(2):305–13.

577. D'Amico A, Wu L. The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. J Exp Med. 2003;198(2):293–303.
578. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. J Immunol. 2007;179(11):7577–84.
579. Waskow C, Liu K, Darrasse-Jèze G, Guermonprez P, Ginhoux F, Merad M, Shengelia T, Yao K, Nussenzweig M. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. Nat Immunol. 2008;9(6):676–83.
580. McKenna HJ, Stocking KL, Miller RE, Brasel K, De Smedt T, Maraskovsky E, Maliszewski CR, Lynch DH, Smith J, Pulendran B, Roux ER, Teepe M, Lyman SD, Peschon JJ. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood. 2000;95(11):3489–97.
581. Gilliet M, Boonstra A, Paturel C, Antonenko S, Xu XL, Trinchieri G, O'Garra A, Liu YJ. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. J Exp Med. 2002;195(7):953–8.
582. Onai N, Manz MG. The STATs on dendritic cell development. Immunity. 2008;28(4): 490–2.
583. Naik SH, Metcalf D, van Nieuwenhuijze A, Wicks I, Wu L, O'Keeffe M, Shortman K. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. Nat Immunol. 2006;7(6):663–71.
584. Kingston D, Schmid MA, Onai N, Obata-Onai A, Baumjohann D, Manz MG. The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. Blood. 2009;114(4):835–43.
585. Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, Fehling HJ, Hardt WD, Shakhar G, Jung S. Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity. 2009;31(3):502–12.
586. Vempati S, Reindl C, Kaza SK, Kern R, Malamoussi T, Dugas M, Mellert G, Schnittger S, Hiddemann W, Spiekermann K. Arginine 595 is duplicated in patients with acute leukemias carrying internal tandem duplications of FLT3 and modulates its transforming potential. Blood. 2007;110(2):686–94.
587. Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R, Kalchenko V, Geissmann F, Jung S. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. J Exp Med. 2007;204(1):171–80.
588. Maraskovsky E, Daro E, Roux E, Teepe M, Maliszewski CR, Hoek J, Caron D, Lebsack ME, McKenna HJ. In vivo generation of human dendritic cell subsets by Flt3 ligand. Blood. 2000;96(3):878–84.
589. Tussiwand R, Onai N, Mazzucchelli L, Manz MG. Inhibition of natural type I IFN-producing and dendritic cell development by a small molecule receptor tyrosine kinase inhibitor with Flt3 affinity. J Immunol. 2005;175(6):3674–80.
590. Birnberg T, Bar-On L, Sapoznikov A, Caton ML, Cervantes-Barragán L, Makia D, Krauthgamer R, Brenner O, Ludewig B, Brockschnieder D, Riethmacher D, Reizis B, Jung S. Lack of conventional dendritic cells is compatible with normal development and T cell homeostasis, but causes myeloid proliferative syndrome. Immunity. 2008;29(6):986–97.
591. Hochweller K, Miloud T, Striegler J, Naik S, Hämmerling GJ, Garbi N. Homeostasis of dendritic cells in lymphoid organs is controlled by regulation of their precursors via a feedback loop. Blood. 2009;114(20):4411–21.
592. Schiavoni G, Mattei F, Sestili P, Borghi P, Venditti M, Morse 3rd HC, Belardelli F, Gabriele L. ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. J Exp Med. 2002;196(11): 1415–25.
593. Laouar Y, Welte T, Fu XY, Flavell RA. STAT3 is required for Flt3L-dependent dendritic cell differentiation. Immunity. 2003;19(6):903–12.

594. Carotta S, Dakic A, D'Amico A, Pang SH, Greig KT, Nutt SL, Wu L. The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. Immunity. 2010;32(5):628–41.
595. Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, Gessner A, Thomson AW. Rapamycin inhibits IL-4-induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. Blood. 2003;101(11):4457–63.
596. Sathaliyawala T, O'Gorman WE, Greter M, Bogunovic M, Konjufca V, Hou ZE, Nolan GP, Miller MJ, Merad M, Reizis B. Mammalian target of rapamycin controls dendritic cell development downstream of Flt3 ligand signaling. Immunity. 2010;33(4):597–606.
597. Scheffler JM, Sparber F, Tripp CH, Herrmann C, Humenberger A, Blitz J, et al. LAMTOR2 regulates dendritic cell homeostasis through FLT3-dependent mTOR signalling. Nat Commun. 2014;5:5138.
598. Swee LK, Bosco N, Malissen B, Ceredig R, Rolink A. Expansion of peripheral naturally occurring T regulatory cells by Fms-like tyrosine kinase 3 ligand treatment. Blood. 2009;113(25):6277–87.
599. Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, Yao K, Chu FF, Randolph GJ, Rudensky AY, Nussenzweig M. In vivo analysis of dendritic cell development and homeostasis. Science. 2009;324(5925):392–7.
600. Darrasse-Jèze G, Deroubaix S, Mouquet H, Victora GD, Eisenreich T, Yao KH, Masilamani RF, Dustin ML, Rudensky A, Liu K, Nussenzweig MC. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. J Exp Med. 2009;206(9):1853–62.
601. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity. 2007;26(4):503–17.
602. Guimond M, Freud AG, Mao HC, Yu J, Blaser BW, Leong JW, Vandeusen JB, Dorrance A, Zhang J, Mackall CL, Caligiuri MA. In vivo role of Flt3 ligand and dendritic cells in NK cell homeostasis. J Immunol. 2010;184(6):2769–75.
603. Eidenschenk C, Crozat K, Krebs P, Arens R, Popkin D, Arnold CN, Blasius AL, Benedict CA, Moresco EM, Xia Y, Beutler B. Flt3 permits survival during infection by rendering dendritic cells competent to activate NK cells. Proc Natl Acad Sci USA. 2010;107(21): 9759–64.
604. Drexler. Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. Leukemia. 1996;10(4):588–99.
605. Horiike S, Yokota S, Nakao M, Iwai T, Sasai Y, Kaneko H, Taniwaki M, Kashima K, Fujii H, Abe T, Misawa S. Tandem duplications of the FLT3 receptor gene are associated with leukemic transformation of myelodysplasia. Leukemia. 1997;11(9):1442–6.
606. Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, Sonoda Y, Abe T, Kahsima K, Matsuo Y, Naoe T. Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. Leukemia. 1997; 11(10):1605–9.
607. Rosen DB, Minden MD, Kornblau SM, Cohen A, Gayko U, Putta S, Woronicz J, Evensen E, Fantl WJ, Cesano A. Functional characterization of FLT3 receptor signaling deregulation in acute myeloid leukemia by single cell network profiling (SCNP). PLoS One. 2010;5(10): e13543.
608. Whitman SP, Archer KJ, Feng L, Baldus C, Becknell B, Carlson BD, Carroll AJ, Mrózek K, Vardiman JW, George SL, Kolitz JE, Larson RA, Bloomfield CD, Caligiuri MA. Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a cancer and leukemia group B study. Cancer Res. 2001;61(19):7233–9.
609. Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, Wermke M, Bornhäuser M, Ritter M, Neubauer A, Ehninger G, Illmer T. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99(12):4326–35.

610. Kottaridis PD, Gale RE, Linch DC. Prognostic implications of the presence of FLT3 mutations in patients with acute myeloid leukemia. Leuk Lymphoma. 2003;44(6):905–13.
611. Baldus CD, Thiede C, Soucek S, Bloomfield CD, Thiel E, Ehninger G. BAALC expression and FLT3 internal tandem duplication mutations in acute myeloid leukemia patients with normal cytogenetics: prognostic implications. J Clin Oncol. 2006;24(5):790–7.
612. Stirewalt DL, Kopecky KJ, Meshinchi S, Engel JH, Pogosova-Agadjanyan EL, Linsley J, Slovak ML, Willman CL, Radich JP. Size of FLT3 internal tandem duplication has prognostic significance in patients with acute myeloid leukemia. Blood. 2006;107(9):3724–6.
613. Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, Linch DC. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood. 2008;111(5):2776–84.
614. Stirewalt DLMS, Meshinchi S, Kopecky KJ, Fan W, Pogosova-Agadjanyan EL, Engel JH, Cronk MR, Dorcy KS, McQuary AR, Hockenbery D, Wood B, Heimfeld S, Radich JP. Identification of genes with abnormal expression changes in acute myeloid leukemia. Genes Chromosomes Cancer. 2008;47(1):8–20.
615. Adamia S, Bar-Natan M, Haibe-Kains B, Pilarski PM, Bach C, Pevzner S, et al. NOTCH2 and FLT3 gene mis-splicings are common events in patients with acute myeloid leukemia (AML): new potential targets in AML. Blood. 2014;123(18):2816–25.
616. Puissant A, Fenouille N, Alexe G, Pikman Y, Bassil CF, Mehta S, et al. SYK is a critical regulator of FLT3 in acute myeloid leukemia. Cancer Cell. 2014;25(2):226–42.
617. Choi JH, Cheong C, Dandamudi DB, Park CG, Rodriguez A, Mehandru S, Velinzon K, Jung IH, Yoo JY, Oh GT, Steinman RM. Flt3 signaling-dependent dendritic cells protect against atherosclerosis. Immunity. 2011;35(5):819–31.
618. Rosnet O, Mattei MG, Marchetto S, Birnbaum D. Isolation and chromosomal localization of a novel FMS-like tyrosine kinase gene. Genomics. 1991;9(2):380–5.
619. Dubreuil P, Courcoul M, Birnbaum D, Planche J, Pebusque M-J, Mannoni P, et al. Cloning and functional analysis of the human c-fms gene. In: Dornand J, Mani J-C, editors. Lymphocyte activation and differentiation. Berlin: de Gruyter; 1988. p. 351–4.
620. Agnès F, Shamoon B, Dina C, Rosnet O, Birnbaum D, Galibert F. Genomic structure of the downstream part of the human FLT3 gene: exon/intron structure conservation among genes encoding receptor tyrosine kinases (RTK) of subclass III. Gene. 1994;145(2):283–8.
621. Imbert A, Rosnet O, Marchetto S, Ollendorff V, Birnbaum D, Pébusque MJ. Characterization of a yeast artificial chromosome from human chromosome band 13q12 containing the FLT1 and FLT3 receptor-type tyrosine kinase genes. Cytogenet Cell Genet. 1994;67(3):175–7.
622. Rosnet O, Schiff C, Pebusque MJ, Marchetto S, Tonnelle C, Toiron Y, Birg F, Birnbaum D. Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. Blood. 1993;82(4):1110–9.
623. Lavagna C, Marchetto S, Birnbaum D, Rosnet O. Identification and characterization of a functional murine FLT3 isoform produced by exon skipping. J Biol Chem. 1995;270(7):3165–71.
624. Fleischman RA, Saltman DL, Stastny V, Zneimer S. Deletion of the c-kit protooncogene in the human developmental defect piebald trait. Proc Natl Acad Sci USA. 1991;88(23):10885–9.
625. Holmes ML, Carotta S, Corcoran LM, Nutt SL. Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment. Genes Dev. 2006;20(8):933–8.
626. Nin DS, Kok WK, Li F, Takahashi S, Chng WJ, Khan M. Role of misfolded N-CoR mediated transcriptional deregulation of Flt3 in acute monocytic leukemia (AML)-M5 subtype. PLoS One. 2012;7(4):e34501.
627. Gwin K, Frank E, Bossou A, Medina KL. Hoxa9 regulates Flt3 in lymphohematopoietic progenitors. J Immunol. 2010;185(11):6572–83.
628. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, Sonoda Y, Fujimoto T, Misawa S. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996;10(12):1911–8.

629. Abu-Duhier FM, Goodeve AC, Wilson GA, Gari MA, Peake IR, Rees DC, Vandenberghe EA, Winship PR, Reilly JT. FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. Br J Haematol. 2000;111(1):190–5.
630. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Saito H, Ueda R, Ohno R, Naoe T. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001;97(8):2434–9.
631. Moriyama Y, Tsujimura T, Hashimoto K, Morimoto M, Kitayama H. Role of aspartic acid 814 in the function and expression of c-kit receptor tyrosine kinase. J Biol Chem. 1996; 271(7):3347–50.
632. Morley GM, Uden M, Gullick WJ, Dibb NJ. Cell specific transformation by c-fms activating loop mutations is attributable to constitutive receptor degradation. Oncogene. 1999;18(20):3076–84.
633. Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, Müller C, Grüning W, Kratz-Albers K, Serve S, Steur C, Büchner T, Kienast J, Kanakura Y, Berdel WE, Serve H. Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. Blood. 2000;96(12):3907–14.
634. Finger C, Escher C, Schneider D. The single transmembrane domains of human receptor tyrosine kinases encode self-interactions. Sci Signal. 2009;2(89):ra56.
635. Oates J, King G, Dixon AM. Strong oligomerization behavior of PDGFbeta receptor transmembrane domain and its regulation by the juxtamembrane regions. Biochim Biophys Acta. 2010;1798(3):605–15.
636. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999;93(9):3074–80.
637. Verstraete K, Savvides SN. Extracellular assembly and activation principles of oncogenic class III receptor tyrosine kinases. Nat Rev Cancer. 2012;12(11):753–66.
638. Levinson NM, Kuchment O, Shen K, Young MA, Koldobskiy M, Karplus M, Cole PA, Kuriyan J. A Src-like inactive conformation in the abl tyrosine kinase domain. PLoS Biol. 2006;4(5):e144.
639. Mol CD, Dougan DR, Schneider TR, Skene RJ, Kraus ML, Scheibe DN, et al. Structural basis for the autoinhibition and STI-571 inhibition of c-Kit tyrosine kinase. J Biol Chem. 2004;279(30):31655–63.
640. Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, McKenna HJ. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med. 1996;184(5): 1953–62.
641. Smith CC, Wang Q, Chin CS, Salerno S, Damon LE, Levis MJ, Perl AE, Travers KJ, Wang S, Hunt JP, Zarrinkar PP, Schadt EE, Kasarskis A, Kuriyan J, Shah NP. Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. Nature. 2012;485(7397):260–3.
642. Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. Nat Rev Cancer. 2009;9(1):28–39.
643. Schlessinger J. Signal transduction. Autoinhibition control. Science. 2003;300(5620): 750–2.
644. Lyman SD, Stocking K, Davison B, Fletcher F, Johnson L, Escobar S. Structural analysis of human and murine flt3 ligand genomic loci. Oncogene. 1995;11(6):1165–72.
645. Hannum C, Culpepper J, Campbell D, McClanahan T, Zurawski S, Bazan JF, Kastelein R, Hudak S, Wagner J, Mattson J. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. Nature. 1994; 368(6472):643–8.
646. Lyman SD, James L, Johnson L, Brasel K, de Vries P, Escobar SS, Downey H, Splett RR, Beckmann MP, McKenna HJ. Cloning of the human homologue of the murine flt3 ligand: a growth factor for early hematopoietic progenitor cells. Blood. 1994;83(10):2795–801.

647. Horiuchi K, Morioka H, Takaishi H, Akiyama H, Blobel CP, Toyama Y. Ectodomain shedding of FLT3 ligand is mediated by TNF-alpha converting enzyme. J Immunol. 2009;182(12):7408–14.
648. Lyman SD, James L, Escobar S, Downey H, de Vries P, Brasel K, Stocking K, Beckmann MP, Copeland NG, Cleveland LS, et al. Identification of soluble and membrane-bound isoforms of the murine flt3 ligand generated by alternative splicing of mRNAs. Oncogene. 1995;10(1):149–57.
649. McClanahan T, Culpepper J, Campbell D, Wagner J, Franz-Bacon K, Mattson J, Tsai S, Luh J, Guimaraes MJ, Mattei MG, Rosnet O, Birnbaum D, Hannum CH. Biochemical and genetic characterization of multiple splice variants of the Flt3 ligand. Blood. 1996; 88(9):3371–82.
650. Lyman SD, Brasel K, Rousseau AM, Williams DE. The flt3 ligand: a hematopoietic stem cell factor whose activities are distinct from steel factor. Stem Cells. 1994;12 Suppl 1:99–107.
651. Broudy VC, Kovach NL, Bennett LG, Lin N, Jacobsen FW, Kidd PG. Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of the c-kit receptor. Blood. 1994;83(8):2145–52.
652. Lavagna-Sévenier C, Marchetto S, Birnbaum D, Rosnet O. FLT3 signaling in hematopoietic cells involves CBL, SHC and an unknown P115 as prominent tyrosine-phosphorylated substrates. Leukemia. 1998;12(3):301–10.
653. Zhang S, Broxmeyer HE. Flt3 ligand induces tyrosine phosphorylation of gab1 and gab2 and their association with shp-2, grb2, and PI3 kinase. Biochem Biophys Res Commun. 2000;277(1):195–9.
654. Zhang S, Mantel C, Broxmeyer HE. Flt3 signaling involves tyrosyl-phosphorylation of SHP-2 and SHIP and their association with Grb2 and Shc in Baf3/Flt3 cells. J Leukoc Biol. 1999;65(3):372–80.
655. Zhang S, Broxmeyer HE. p85 subunit of PI3 kinase does not bind to human Flt3 receptor, but associates with SHP2, SHIP, and a tyrosine-phosphorylated 100-kDa protein in Flt3 ligand-stimulated hematopoietic cells. Biochem Biophys Res Commun. 1999;254(2): 440–5.
656. Rosnet O, Bühring HJ. Expression and signal transduction of the FLT3 tyrosine kinase receptor. Acta Haematol. 1996;95(3–4):218–23.
657. Zhang S, Fukuda S, Lee Y, Hangoc G, Cooper S, Spolski R, Leonard WJ, Broxmeyer HE. Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling. J Exp Med. 2000;192(5):719–28.
658. Heiss E, Masson K, Sundberg C, Pedersen M, Sun J, Bengtsson S, Rönnstrand L. Identification of Y589 and Y599 in the juxtamembrane domain of Flt3 as ligand-induced autophosphorylation sites involved in binding of Src family kinases and the protein tyrosine phosphatase SHP2. Blood. 2006;108(5):1542–50.
659. Razumovskaya E, Masson K, Khan R, Bengtsson S, Rönnstrand L. Oncogenic Flt3 receptors display different specificity and kinetics of autophosphorylation. Exp Hematol. 2009;37(8):979–89.
660. Lavagna-Sévenier C, Marchetto S, Birnbaum D, Rosnet O. The CBL-related protein CBLB participates in FLT3 and interleukin-7 receptor signal transduction in pro-B cells. J Biol Chem. 1998;273(24):14962–7.
661. Grundler R, Miething C, Thiede C, Peschel C, Duyster J. FLT3-ITD and tyrosine kinase domain mutants induce 2 distinct phenotypes in a murine bone marrow transplantation model. Blood. 2005;105(12):4792–9.
662. Srinivasa SP, Doshi PD. Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways cooperate in mediating cytokine-induced proliferation of a leukemic cell line. Leukemia. 2002;16(2):244–53.
663. Lin DC, Yin T, Koren-Michowitz M, Ding LW, Gueller S, Gery S, Tabayashi T, Bergholz U, Kazi JU, Rönnstrand L, Stocking C, Koeffler HP. Adaptor protein Lnk binds to and inhibits normal and leukemic FLT3. Blood. 2012;120(16):3310–7.

664. Kazi JU, Rönnstrand L. Src-Like adaptor protein (SLAP) binds to the receptor tyrosine kinase Flt3 and modulates receptor stability and downstream signaling. PLoS One. 2012; 7(12):e53509.
665. Kazi JU, Rönnstrand L. Suppressor of cytokine signaling 2 (SOCS2) associates with FLT3 and negatively regulates downstream signaling. Mol Oncol. 2013;7(3):693–703.
666. Kazi JU, Sun J, Phung B, Zadjali F, Flores-Morales A, Rönnstrand L. Suppressor of cytokine signaling 6 (SOCS6) negatively regulates Flt3 signal transduction through direct binding to phosphorylated tyrosines 591 and 919 of Flt3. J Biol Chem. 2012;287(43): 36509–17.
667. Arora D, Stopp S, Böhmer SA, Schons J, Godfrey R, Masson K, Razumovskaya E, Rönnstrand L, Tänzer S, Bauer R, Böhmer FD, Müller JP. Protein-tyrosine phosphatase DEP-1 controls receptor tyrosine kinase FLT3 signaling. J Biol Chem. 2011;286(13): 10918–29.
668. Böhmer SA, Weibrecht I, Söderberg O, Böhmer FD. Association of the Protein-Tyrosine Phosphatase DEP-1 with Its Substrate FLT3 Visualized by In Situ Proximity Ligation Assay. PLoS One. 2013;8:5.
669. Tse KF, Mukherjee G, Small D. Constitutive activation of FLT3 stimulates multiple intracellular signal transducers and results in transformation. Leukemia. 2000;14(10):1766–76.
670. Tse KF, Allebach J, Levis M, Smith BD, Bohmer FD, Small D. Inhibition of the transforming activity of FLT3 internal tandem duplication mutants from AML patients by a tyrosine kinase inhibitor. Leukemia. 2002;16(10):2027–36.
671. Schmidt-Arras D, Böhmer SA, Koch S, Müller JP, Blei L, Cornils H, Bauer R, Korasikha S, Thiede C, Böhmer FD. Anchoring of FLT3 in the endoplasmic reticulum alters signaling quality. Blood. 2009;113(15):3568–76.
672. Choudhary C, Olsen JV, Brandts C, Cox J, Reddy PN, Böhmer FD, Gerke V, Schmidt-Arras DE, Berdel WE, Müller-Tidow C, Mann M, Serve H. Mislocalized activation of oncogenic RTKs switches downstream signaling outcomes. Mol Cell. 2009;36(2):326–39.
673. Godfrey R, Arora D, Bauer R, Stopp S, Müller JP, Heinrich T, Böhmer SA, Dagnell M, Schnetzke U, Scholl S, Östman A, Böhmer FD. Cell transformation by FLT3 ITD in acute myeloid leukemia involves oxidative inactivation of the tumor suppressor protein-tyrosine phosphatase DEP-1/ PTPRJ. Blood. 2012;119(19):4499–511.
674. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med. 2005;353(2):172–87.
675. Choudhary C, Brandts C, Schwable J, Tickenbrock L, Sargin B, Ueker A, Böhmer FD, Berdel WE, Müller-Tidow C, Serve H. Activation mechanisms of STAT5 by oncogenic Flt3-ITD. Blood. 2007;110(1):370–4.
676. Kelly LM, Yu JC, Boulton CL, Apatira M, Li J, Sullivan CM, Williams I, Amaral SM, Curley DP, Duclos N, Neuberg D, Scarborough RM, Pandey A, Hollenbach S, Abe K, Lokker NA, Gilliland DG, Giese NA. CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML). Cancer Cell. 2002;1(5):421–32.
677. Müller-Tidow C, Steur C, Mizuki M, Schwäble J, Brandts C, Berdel WE, Serve H. Mutations of growth factor receptor Flt3 in acute myeloid leukemia: transformation of myeloid cells by Ras-dependent and Ras-independent mechanisms. Dtsch Med Wochenschr. 2002;127(42): 2195–200.
678. Murata K, Kumagai H, Kawashima T, Tamitsu K, Irie M, Nakajima H, Suzu S, Shibuya M, Kamihira S, Nosaka T, Asano S, Kitamura T. Selective cytotoxic mechanism of GTP-14564, a novel tyrosine kinase inhibitor in leukemia cells expressing a constitutively active Fms-like tyrosine kinase 3 (FLT3). J Biol Chem. 2003;278(35):32892–8.
679. Gu TL, Nardone J, Wang Y, Loriaux M, Villén J, Beausoleil S, Tucker M, Kornhauser J, Ren J, MacNeill J, Gygi SP, Druker BJ, Heinrich MC, Rush J, Polakiewicz RD. Survey of activated FLT3 signaling in leukemia. PLoS One. 2011;6(4):e19169.
680. Ohtani M, Nagai S, Kondo S, Mizuno S, Nakamura K, Tanabe M, Takeuchi T, Matsuda S, Koyasu S. Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells. Blood. 2008;112(3):635–43.

681. Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, Stuhlmeier KM, Kolbe T, Stulnig TM, Hörl WH, Hengstschläger M, Müller M, Säemann MD. The TSC-mTOR signaling pathway regulates the innate inflammatory response. Immunity. 2008;29(4):565–77.
682. Cao W, Manicassamy S, Tang H, Kasturi SP, Pirani A, Murthy N, Pulendran B. Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. Nat Immunol. 2008;9(10):1157–64.
683. Guiducci C, Ghirelli C, Marloie-Provost MA, Matray T, Coffman RL, Liu YJ, Barrat FJ, Soumelis V. PI3K is critical for the nuclear translocation of IRF-7 and type I IFN production by human plasmacytoid predendritic cells in response to TLR activation. J Exp Med. 2008;205(2):315–22.
684. Levis M, Small D. FLT3: ITDoes matter in leukemia. Leukemia. 2003;17(9):1738–52.
685. Mead AJ, Linch DC, Hills RK, Wheatley K, Burnett AK, Gale RE. FLT3 tyrosine kinase domain mutations are biologically distinct from and have a significantly more favorable prognosis than FLT3 internal tandem duplications in patients with acute myeloid leukemia. Blood. 2007;110(4):1262–70.
686. Zheng R, Levis M, Piloto O, Brown P, Baldwin BR, Gorin NC, Beran M, Zhu Z, Ludwig D, Hicklin D, Witte L, Li Y, Small D. FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. Blood. 2004;103(1):267–74.
687. Yao Q, Nishiuchi R, Kitamura T, Kersey JH. Human leukemias with mutated FLT3 kinase are synergistically sensitive to FLT3 and Hsp90 inhibitors: the key role of the STAT5 signal transduction pathway. Leukemia. 2005;19(9):1605–12.
688. Weigel BJ, Blaney S, Reid JM, Safgren SL, Bagatell R, Kersey J, Neglia JP, Ivy SP, Ingle AM, Whitesell L, Gilbertson RJ, Krailo M, Ames M, Adamson PC. A phase I study of 17-allylaminogeldanamycin in relapsed/refractory pediatric patients with solid tumors: a Children's Oncology Group study. Clin Cancer Res. 2007;13(6):1789–93.
689. Maloney A, Workman P. HSP90 as a new therapeutic target for cancer therapy: the story unfolds. Expert Opin Biol Ther. 2002;2(1):3–24.
690. Bagatell R, Whitesell L. Altered Hsp90 function in cancer: a unique therapeutic opportunity. Mol Cancer Ther. 2004;3:1021–30.
691. Whitesell L, Lindquist SL. Hsp90 and the chaperoning of cancer. Nat Rev Cancer. 2005;10:761–2.
692. Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU, Pavletich NP. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. Cell. 1997;98(2):239–50.
693. Minami Y, Kiyoi H, Yamamoto Y, Yamamoto K, Ueda R, Saito H, Naoe T. Selective apoptosis of tandemly duplicated FLT3-transformed leukemia cells by Hsp90 inhibitors. Leukemia. 2002;16(8):1535–40.
694. Yao Q, Nishiuchi R, Li Q, Kumar AR, Hudson WA, Kersey JH. FLT3 expressing leukemias are selectively sensitive to inhibitors of the molecular chaperone heat shock protein 90 through destabilization of signal transduction-associated kinases. Clin Cancer Res. 2003; 9(12):4483–93.
695. George P, Bali P, Annavarapu S, Scuto A, Fiskus W, Guo F, Sigua C, Sondarva G, Moscinski L, Atadja P, Bhalla K. Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and AML cells with activating mutation of FLT-3. Blood. 2005;105(4):1768–76.
696. Yao Q, Weigel B, Kersey J. Synergism between etoposide and 17-AAG in leukemia cells: critical roles for Hsp90, FLT3, topoisomerase II, Chk1, and Rad51. Clin Cancer Res. 2007;13(5):1591–600.
697. Schulte TW, Neckers L. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. Cancer Chemother Pharmacol. 1998;42(4):273–9.
698. Schnur RC, Corman ML, Gallaschun RJ, Cooper BA, Dee MF, Doty JL, Muzzi ML, Moyer JD, DiOrio CI, Barbacci EG, et al. Inhibition of the oncogene product p185erbB-2 in vitro and in vivo by geldanamycin and dihydrogeldanamycin derivatives. J Med Chem. 1995;38(19):3806–12.

699. Bagatell R, Gore L, Egorin MJ, Ho R, Heller G, Boucher N, Zuhowski EG, Whitlock JA, Hunger SP, Narendran A, Katzenstein HM, Arceci RJ, Boklan J, Herzog CE, Whitesell L, Ivy SP, Trippett TM. Phase I pharmacokinetic and pharmacodynamic study of 17-N-allylamino-17-demethoxygeldanamycin in pediatric patients with recurrent or refractory solid tumors: a pediatric oncology experimental therapeutics investigators consortium study. Clin Cancer Res. 2007;13(6):1783–8.
700. Usmani SZ, Bona R, Li Z. 17 AAG for HSP90 inhibition in cancer—from bench to bedside. Curr Mol Med. 2009;9(5):654–64.
701. Liu Y, Gray NS. Rational design of inhibitors that bind to inactive kinase conformations. Nat Chem Biol. 2006;2(7):358–64.
702. Weisberg E, Banerji L, Wright RD, Barrett R, Ray A, Moreno D, Catley L, Jiang J, Hall-Meyers E, Sauveur-Michel M, Stone R, Galinsky I, Fox E, Kung AL, Griffin JD. Potentiation of antileukemic therapies by the dual PI3K/PDK-1 inhibitor, BAG956: effects on BCR-ABL- and mutant FLT3-expressing cells. Blood. 2008;111(7):3723–34.
703. Weisberg E, Barrett R, Liu Q, Stone R, Gray N, Griffin JD. FLT3 inhibition and mechanisms of drug resistance in mutant FLT3-positive AML. Drug Resist Updat. 2009;12(3):81–9.
704. Weisberg E, Choi HG, Barrett R, Zhou W, Zhang J, Ray A, Nelson EA, Jiang J, Moreno D, Stone R, Galinsky I, Fox E, Adamia S, Kung AL, Gray NS, Griffin JD. Discovery and characterization of novel mutant FLT3 kinase inhibitors. Mol Cancer Ther. 2010;9(9):2468–77.
705. Weisberg E, Ray A, Nelson E, Adamia S, Barrett R, Sattler M, Zhang C, Daley JF, Frank D, Fox E, Griffin JD. Reversible resistance induced by FLT3 inhibition: a novel resistance mechanism in mutant FLT3-expressing cells. PLoS One. 2011;6(9):e25351.
706. Weisberg E, Roesel J, Bold G, Furet P, Jiang J, Cools J, Wright RD, Nelson E, Barrett R, Ray A, Moreno D, Hall-Meyers E, Stone R, Galinsky I, Fox E, Gilliland G, Daley JF, Lazo-Kallanian S, Kung AL, Griffin JD. Antileukemic effects of the novel, mutant FLT3 inhibitor NVP-AST487: effects on PKC412-sensitive and -resistant FLT3-expressing cells. Blood. 2008;112(13):5161–70.
707. Weisberg E, Roesel J, Furet P, Bold G, Imbach P, Flörsheimer A, Caravatti G, Jiang J, Manley P, Ray A, Griffin JD. Antileukemic effects of novel first- and second-generation FLT3 inhibitors: Structure-affinity comparison. Genes Cancer. 2010;1(10):1021–32.
708. Weisberg E, Sattler M, Ray A, Griffin JD. Drug resistance in mutant FLT3-positive AML. Oncogene. 2010;29(37):5120–34.
709. Grundler R, Thiede C, Miething C, Steudel C, Peschel C, Duyster J. Source. Sensitivity toward tyrosine kinase inhibitors varies between different activating mutations of the FLT3 receptor. Blood. 2003;102(2):646–51.
710. Clark JJ, Cools J, Curley DP, Yu JC, Lokker NA, Giese NA, Gilliland DG. Variable sensitivity of FLT3 activation loop mutations to the small molecule tyrosine kinase inhibitor MLN518. Blood. 2004;104(9):2867–72.
711. Weisberg E, Boulton C, Kelly LM, Manley P, Fabbro D, Meyer T, Gilliland DG, Griffin JD. Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. Cancer Cell. 2002;1(5):433–43.
712. DeAngelo DJ, Stone RM, Heaney ML, Nimer SD, Paquette RL, Klisovic RB, Caligiuri MA, Cooper MR, Lecerf JM, Karol MD, Sheng S, Holford N, Curtin PT, Druker BJ, Heinrich MC. Phase 1 clinical results with tandutinib (MLN518), a novel FLT3 antagonist, in patients with acute myelogenous leukemia or high-risk myelodysplastic syndrome: safety, pharmacokinetics, and pharmacodynamics. Blood. 2006;108(12):3674–81.
713. Ravandi F, Cortes JE, Jones D, Faderl S, Garcia-Manero G, Konopleva MY, O'Brien S, Estrov Z, Borthakur G, Thomas D, Pierce SR, Brandt M, Byrd A, Bekele BN, Pratz K, Luthra R, Levis M, Andreeff M, Kantarjian HM. Phase I/II study of combination therapy with sorafenib, idarubicin, and cytarabine in younger patients with acute myeloid leukemia. J Clin Oncol. 2010;28(11):1856–62.
714. Levis M, Ravandi F, Wang ES, Baer MR, Perl A, Coutre S, Erba H, Stuart RK, Baccarani M, Cripe LD, Tallman MS, Meloni G, Godley LA, Langston AA, Amadori S, Lewis ID, Nagler A, Stone R, Yee K, Advani A, Douer D, Wiktor-Jedrzejczak W, Juliusson G, Litzow

MR, Petersdorf S, Sanz M, Kantarjian HM, Sato T, Tremmel L, Bensen-Kennedy DM, Small D, Smith BD. Results from a randomized trial of salvage chemotherapy followed by lestaurtinib for patients with FLT3 mutant AML in first relapse. Blood. 2011; 117(12):3294–301.
715. Schnittger S, Bacher U, Haferlach C, Kern W, Alpermann T, Haferlach T. Clinical impact of FLT3 mutation load in acute promyelocytic leukemia with t(15;17)/PML-RARA. Haematologica. 2011;96(12):1799–807.
716. Huang Y, Ratajczak MZ, Reca R, Xu H, Tanner M, Rezzoug F, Hussain LR, Fugier-Vivier I, Bolli R, Ildstad ST. Fms-related tyrosine kinase 3 expression discriminates hematopoietic stem cells subpopulations with differing engraftment-potential: identifying the most potent combination. Transplantation. 2008;85(8):1175–84.
717. Pratz KW, Sato T, Murphy KM, Stine A, Rajkhowa T, Levis M. Source. FLT3-mutant allelic burden and clinical status are predictive of response to FLT3 inhibitors in AML. Blood. 2010;115(7):1425–32.
718. Knapper S, Mills KI, Gilkes AF, Austin SJ, Walsh V, Burnett AK. The effects of lestaurtinib (CEP701) and PKC412 on primary AML blasts: the induction of cytotoxicity varies with dependence on FLT3 signaling in both FLT3-mutated and wild-type cases. Blood. 2006;108(10):3494–503.
719. Kindler T, Lipka DB, Fischer T. FLT3 as a therapeutic target in AML: still challenging after all these years. Blood. 2010;116(24):5089–102.
720. Eriksson A, Hermanson M, Wickström M, Lindhagen E, Ekholm C, Jenmalm Jensen A, Löthgren A, Lehmann F, Larsson R, Parrow V, Höglund M. The novel tyrosine kinase inhibitor AKN-028 has significant antileukemic activity in cell lines and primary cultures of acute myeloid leukemia. Blood Cancer J. 2012;2:e81.
721. Mohi MG, Boulton C, Gu TL, Sternberg DW, Neuberg D, Griffin JD, Gilliland DG, Neel BG. Combination of rapamycin and protein tyrosine kinase (PTK) inhibitors for the treatment of leukemias caused by oncogenic PTKs. Proc Natl Acad Sci USA. 2004;101(9): 3130–5.
722. Nishioka C, Ikezoe T, Yang J, Takeshita A, Taniguchi A, Komatsu N, Togitani K, Koeffler HP, Yokoyama A. Blockade of MEK/ERK signaling enhances sunitinib-induced growth inhibition and apoptosis of leukemia cells possessing activating mutations of the FLT3 gene. Leuk Res. 2008;32(6):865–72.
723. Dick JE. Acute myeloid leukemia stem cells. Ann N Y Acad Sci. 2005;1044:1–5.
724. Konopleva M, Konoplev S, Hu W, Zaritskey AY, Afanasiev BV, Andreeff M. Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. Leukemia. 2002;16(9):1713–24.
725. Sato T, Yang X, Knapper S, White P, Smith BD, Galkin S, Small D, Burnett A, Levis M. FLT3 ligand impedes the efficacy of FLT3 inhibitors in vitro and in vivo. Blood. 2011; 117(12):3283–93.
726. Kiyoi H, Towatari M, Yokota S, Hamaguchi M, Ohno R, Saito H, Naoe T. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia. 1998;12(9):1333–7.
727. Oppermann FS, Gnad F, Olsen JV, Hornberger R, Greff Z, Kéri G, Mann M, Daub H. Large-scale proteomics analysis of the human kinome. Mol Cell Proteomics. 2009;8(7):1751–64.
728. Ashman L. The biology of stem cell factor and its receptor c-kit. Int J Biochem Cell Biol. 1999;31(10):1037–51.
729. Langley KE, Bennett LG, Wypych J, Yancik SA, Liu XD, Westcott KR, et al. Soluble stem cell factor in human serum. Blood. 1993;81(3):656–60. Epub 1993/02/01. eng.
730. Zhang Z, Zhang R, Joachimiak A, Schlessinger J, Kong XP. Crystal structure of human stem cell factor: implication for stem cell factor receptor dimerization and activation. Proc Natl Acad Sci USA. 2000;97(14):7732–7.
731. Ropers HH, Craig IW. Report of the committee on the genetic constitution of chromosomes 12 and 13. Cytogenet Cell Genet. 1989;51(1–4):259–79. Epub 1989/01/01. eng.

732. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, et al. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell. 1990;63:213–24.
733. Majumdar MK, Feng L, Medlock E, Toksoz D, Williams DA. Identification and mutation of primary and secondary proteolytic cleavage sites in murine stem cell factor cDNA yields biologically active, cell-associated protein. J Biol Chem. 1994;269(2):1237–42. Epub 1994/01/14. eng.
734. Lev S, Blechman JM, Givol D, Yarden Y. Steel factor and c-kit protooncogene: genetic lessons in signal transduction. Crit Rev Oncog. 1994;5(2–3):141–68.
735. Tajima Y, Moore MA, Soares V, Ono M, Kissel H, Besmer P. Consequences of exclusive expression in vivo of Kit-ligand lacking the major proteolytic cleavage site. Proc Natl Acad Sci USA. 1998;95(20):11903–8.
736. Besmer P, Murphy JE, George PC, Qiu FH, Bergold PJ, Lederman L, et al. A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. Nature. 1986;320(6061):415–21.
737. D'Auriol L, Mattei MG, Andre C, Galibert F. Localization of the human c-kit protooncogene on the q11-q12 region of chromosome 4. Hum Genet. 1988;78(4):374–6. Epub 1988/04/01. eng.
738. Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, et al. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. 1987;6(11):3341–51.
739. Chabot B, Stephenson DA, Chapman VM, Besmer P, Bernstein A. The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature. 1988;335(6185):88–9.
740. Geissler EN, Ryan MA, Housman DE. The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. Cell. 1988;55(1):185–92.
741. Rossi P, Marziali G, Albanesi C, Charlesworth A, Geremia R, Sorrentino V. A novel c-kit transcript, potentially encoding a truncated receptor, originates within a kit gene intron in mouse spermatids. Dev Biol. 1992;152(1):203–7.
742. Katayama I, Otoyama K, Yokozeki H, Nishioka K. Retinoic acid upregulates c-kit ligand production by murine keratinocyte in vitro and increases cutaneous mast cell in vivo. J Dermatol Sci. 1995;9(1):27–35. Epub 1995/01/01. eng.
743. Lennartsson J, Rönnstrand L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. Physiol Rev. 2012;92(4):1619–49. Epub 2012/10/18. eng.
744. Ratajczak MZ, Perrotti D, Melotti P, Powzaniuk M, Calabretta B, Onodera K, et al. Myb and ets proteins are candidate regulators of c-kit expression in human hematopoietic cells. Blood. 1998;91(6):1934–46. Epub 1998/04/16. eng.
745. Huang S, Jean D, Luca M, Tainsky MA, Bar-Eli M. Loss of AP-2 results in downregulation of c-KIT and enhancement of melanoma tumorigenicity and metastasis. EMBO J. 1998;17(15):4358–69.
746. Opdecamp K, Nakayama A, Nguyen MT, Hodgkinson CA, Pavan WJ, Arnheiter H. Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. Development. 1997; 124(12):2377–86. Epub 1997/06/01. eng.
747. Gao XN, Lin J, Gao L, Li YH, Wang LL, Yu L. MicroRNA-193b regulates c-Kit proto-oncogene and represses cell proliferation in acute myeloid leukemia. Leuk Res. 2011;35(9):1226–32. Epub 2011/07/05. eng.
748. Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, et al. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. Proc Natl Acad Sci USA. 2005;102(50):18081–6. Epub 2005/12/07. eng.
749. Fan R, Zhong J, Zheng S, Wang Z, Xu Y, Li S, et al. MicroRNA-218 inhibits gastrointestinal stromal tumor cell and invasion by targeting KIT. Tumour Biol. 2014;35(5):4209–17.

750. Igoucheva O, Alexeev V. MicroRNA-dependent regulation of cKit in cutaneous melanoma. Biochem Biophys Res Commun. 2009;379(3):790–4. Epub 2009/01/08. eng.
751. Lee YN, Brandal S, Noel P, Wentzel E, Mendell JT, McDevitt MA, et al. KIT signaling regulates MITF expression through miRNAs in normal and malignant mast cell proliferation. Blood. 2011;117(13):3629–40. Epub 2011/01/29. eng.
752. Keshet E, Lyman SD, Williams DE, Anderson DM, Jenkins NA, Copeland NG, et al. Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. EMBO J. 1991;10(9):2425–35.
753. Matsui Y, Zsebo KM, Hogan BL. Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. Nature. 1990;347(6294):667–9. Epub 1990/10/18. eng.
754. Orr-Urtreger A, Avivi A, Zimmer Y, Givol D, Yarden Y, Lonai P. Developmental expression of c-kit, a proto-oncogene encoded by the W locus. Development. 1990;109(4):911–23. Epub 1990/08/01. eng.
755. Nocka K, Majumder S, Chabot B, Ray P, Cervone M, Bernstein A, et al. Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice–evidence for an impaired c-kit kinase in mutant mice. Genes Dev. 1989;3(6):816–26.
756. De Felici M, Di Carlo A, Pesce M. Role of stem cell factor in somatic-germ cell interactions during prenatal oogenesis. Zygote. 1996;4(4):349–51. Epub 1996/11/01. eng.
757. Runyan C, Schaible K, Molyneaux K, Wang Z, Levin L, Wylie C. Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration. Development. 2006;133(24):4861–9. Epub 2006/11/17. eng.
758. Yoshida H, Kunisada T, Grimm T, Nishimura EK, Nishioka E, Nishikawa SI. Review: melanocyte migration and survival controlled by SCF/c-kit expression. J Investig Dermatol Symp Proc. 2001;6(1):1–5. Epub 2002/01/05. eng.
759. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature. 2001;411(6835):355–65.
760. Bell CA, Tynan JA, Hart KC, Meyer AN, Robertson SC, Donoghue DJ. Rotational coupling of the transmembrane and kinase domains of the Neu receptor tyrosine kinase. Mol Biol Cell. 2000;11(10):3589–99. Epub 2000/10/12. eng.
761. Lennartsson J, Wernstedt C, Engström U, Hellman U, Rönnstrand L. Identification of Tyr900 in the kinase domain of c-Kit as a Src-dependent phosphorylation site mediating interaction with c-Crk. Exp Cell Res. 2003;288(1):110–8.
762. Blume-Jensen P, Wernstedt C, Heldin CH, Rönnstrand L. Identification of the major phosphorylation sites for protein kinase C in kit/stem cell factor receptor in vitro and in intact cells. J Biol Chem. 1995;270(23):14192–200.
763. Masson K, Heiss E, Band H, Rönnstrand L. Direct binding of Cbl to Tyr568 and Tyr936 of the stem cell factor receptor/c-Kit is required for ligand-induced ubiquitination, internalization and degradation. Biochem J. 2006;399(1):59–67. Epub 2006/06/20. eng.
764. Sun J, Pedersen M, Bengtsson S, Rönnstrand L. Grb2 mediates negative regulation of stem cell factor receptor/c-Kit signaling by recruitment of Cbl. Exp Cell Res. 2007;313(18):3935–42. Epub 2007/10/02. eng.
765. Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol. 2003;5(5):461–6.
766. Anderson DM, Williams DE, Tushinski R, Gimpel S, Eisenman J, Cannizzaro LA, et al. Alternate splicing of mRNAs encoding human mast cell growth factor and localization of the gene to chromosome 12q22-q24. Cell Growth Differ. 1991;2(8):373–8. Epub 1991/08/01. eng.
767. Zhu WM, Dong WF, Minden M. Alternate splicing creates two forms of the human kit protein. Leuk Lymphoma. 1994;12(5–6):441–7.
768. Crosier PS, Ricciardi ST, Hall LR, Vitas MR, Clark SC, Crosier KE. Expression of isoforms of the human receptor tyrosine kinase c-kit in leukemic cell lines and acute myeloid leukemia. Blood. 1993;82(4):1151–8.

769. Voytyuk O, Lennartsson J, Mogi A, Caruana G, Courtneidge S, Ashman LK, et al. Src family kinases are involved in the differential signaling from two splice forms of c-Kit. J Biol Chem. 2003;278(11):9159–66.
770. Caruana G, Cambareri AC, Ashman LK. Isoforms of c-KIT differ in activation of signalling pathways and transformation of NIH3T3 fibroblasts. Oncogene. 1999;18(40):5573–81.
771. Phung B, Steingrímsson E, Rönnstrand L. Differential activity of c-KIT splice forms is controlled by extracellular peptide insert length. Cell Signal. 2013;25(11):2231–8.
772. Paronetto MP, Venables JP, Elliott DJ, Geremia R, Rossi P, Sette C. Tr-kit promotes the formation of a multimolecular complex composed by Fyn, PLCgamma1 and Sam68. Oncogene. 2003;22(54):8707–15.
773. Broudy VC, Lin NL, Sabath DF. The fifth immunoglobulin-like domain of the Kit receptor is required for proteolytic cleavage from the cell surface. Cytokine. 2001;15(4):188–95.
774. Reshetnyak AV, Nelson B, Shi X, Boggon TJ, Pavlenco A, Mandel-Bausch EM, et al. Structural basis for KIT receptor tyrosine kinase inhibition by antibodies targeting the D4 membrane-proximal region. Proc Natl Acad Sci USA. 2013;110(44):17832–7.
775. Opatowsky Y, Lax I, Tome F, Bleichert F, Unger VM, Schlessinger J. Structure, domain organization, and different conformational states of stem cell factor-induced intact KIT dimers. Proc Natl Acad Sci USA. 2014;111(5):1772–7.
776. Mol CD, Lim KB, Sridhar V, Zou H, Chien EY, Sang BC, et al. Structure of a c-kit product complex reveals the basis for kinase transactivation. J Biol Chem. 2003;278(34):31461–4.
777. Agarwal S, Kazi JU, Rönnstrand L. Phosphorylation of the activation loop tyrosine 823 in c-Kit is crucial for cell survival and proliferation. J Biol Chem. 2013;288(31):22460–8.
778. Zeng S, Xu Z, Lipkowitz S, Longley JB. Regulation of stem cell factor receptor signaling by CBL family proteins (CBL-B/c-CBL). Blood. 2004;17.
779. Zadjali F, Pike AC, Vesterlund M, Sun J, Wu C, Li SS, et al. Structural basis for c-KIT inhibition by the suppressor of cytokine signaling 6 (SOCS6) ubiquitin ligase. J Biol Chem. 2011;286(1):480–90. Epub 2010/10/30. eng.
780. Kazi JU, Agarwal S, Sun J, Bracco E, Rönnstrand L. Src-like adaptor protein (SLAP) differentially regulates normal and oncogenic c-Kit signaling. J Cell Sci. 2014;127:653–62.
781. Miyazawa K, Toyama K, Gotoh A, Hendrie PC, Mantel C, Broxmeyer HE. Ligand-dependent polyubiquitination of c-kit gene product: a possible mechanism of receptor down modulation in M07e cells. Blood. 1994;83(1):137–45.
782. Yee NS, Hsiau CW, Serve H, Vosseller K, Besmer P. Mechanism of down-regulation of c-kit receptor. Roles of receptor tyrosine kinase, phosphatidylinositol 3′-kinase, and protein kinase C. J Biol Chem. 1994;269(50):31991–8.
783. Park M, Kim WK, Song M, Park M, Kim H, Nam HJ, et al. Protein kinase C-delta-mediated recycling of active KIT in colon cancer. Clin Cancer Res. 2013;19(18):4961–71.
784. Paulson RF, Vesely S, Siminovitch KA, Bernstein A. Signalling by the W/Kit receptor tyrosine kinase is negatively regulated in vivo by the protein tyrosine phosphatase Shp1. Nat Genet. 1996;13(3):309–15.
785. Kozlowski M, Larose L, Lee F, Le DM, Rottapel R, Siminovitch KA. SHP-1 binds and negatively modulates the c-Kit receptor by interaction with tyrosine 569 in the c-Kit juxtamembrane domain. Mol Cell Biol. 1998;18(4):2089–99.
786. Linnekin D, DeBerry CS, Mou S. Lyn associates with the juxtamembrane region of c-Kit and is activated by stem cell factor in hematopoietic cell lines and normal progenitor cells. J Biol Chem. 1997;272(43):27450–5.
787. Krystal GW, DeBerry CS, Linnekin D, Litz J. Lck associates with and is activated by Kit in a small cell lung cancer cell line: inhibition of SCF-mediated growth by the Src family kinase inhibitor PP1. Cancer Res. 1998;58(20):4660–6.
788. Lennartsson J, Blume-Jensen P, Hermanson M, Pontén E, Carlberg M, Rönnstrand L. Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. Oncogene. 1999;18(40):5546–53.

789. Price DJ, Rivnay B, Fu Y, Jiang S, Avraham S, Avraham H. Direct association of Csk homologous kinase (CHK) with the diphosphorylated site Tyr568/570 of the activated c-KIT in megakaryocytes. J Biol Chem. 1997;272(9):5915–20.
790. Bondzi C, Litz J, Dent P, Krystal GW. Src family kinase activity is required for Kit-mediated mitogen- activated protein (MAP) kinase activation, however loss of functional retinoblastoma protein makes MAP kinase activation unnecessary for growth of small cell lung cancer cells. Cell Growth Differ. 2000;11(6):305–14.
791. Nishida K, Wang L, Morii E, Park SJ, Narimatsu M, Itoh S, et al. Requirement of Gab2 for mast cell development and KitL/c-Kit signaling. Blood. 2002;99(5):1866–9.
792. Sun J, Pedersen M, Rönnstrand L. Gab2 is involved in differential phosphoinositide 3-kinase signaling by two splice forms of c-Kit. J Biol Chem. 2008;283(41):27444–51.
793. Timokhina I, Kissel H, Stella G, Besmer P. Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. EMBO J. 1998;17(21):6250–62.
794. Shivakrupa R, Linnekin D. Lyn contributes to regulation of multiple Kit-dependent signaling pathways in murine bone marrow mast cells. Cell Signal. 2005;17(1):103–9.
795. Samayawardhena LA, Hu J, Stein PL, Craig AW. Fyn kinase acts upstream of Shp2 and p38 mitogen-activated protein kinase to promote chemotaxis of mast cells towards stem cell factor. Cell Signal. 2006;18(9):1447–54.
796. Mou S, Linnekin D. Lyn is activated during late G1 of stem-cell-factor-induced cell cycle progression in haemopoietic cells. Biochem J. 1999;342:163–70.
797. O'Laughlin-Bunner B, Radosevic N, Taylor ML, Shivakrupa R, DeBerry C, Metcalfe DD, et al. Lyn is required for normal stem cell factor-induced proliferation and chemotaxis of primary hematopoietic cells. Blood. 2001;98(2):343–50.
798. Glenney Jr JR, Zokas L. Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. J Cell Biol. 1989;108(6):2401–8.
799. Thomas SM, Soriano P, Imamoto A. Specific and redundant roles of Src and Fyn in organizing the cytoskeleton. Nature. 1995;376(6537):267–71.
800. Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, et al. FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol. 2000;2(5): 249–56.
801. Agosti V, Corbacioglu S, Ehlers I, Waskow C, Sommer G, Berrozpe G, et al. Critical role for Kit-mediated Src kinase but Not PI 3-kinase signaling in Pro T and Pro B cell development. J Exp Med. 2004;199(6):867–78.
802. Kimura Y, Jones N, Kluppel M, Hirashima M, Tachibana K, Cohn J, et al. Targeted mutations of the juxtamembrane tyrosines in the Kit receptor tyrosine kinase selectively affect multiple cell lineages. Proc Natl Acad Sci USA. 2004;101(16):6015–20.
803. Tatton L, Morley GM, Chopra R, Khwaja A. The Src-selective kinase inhibitor PP1 also inhibits Kit and Bcr-Abl tyrosine kinases. J Biol Chem. 2003;278(7):4847–53.
804. Sun J, Pedersen M, Rönnstrand L. The D816V mutation of c-Kit circumvents a requirement for Src family kinases in c-Kit signal transduction. J Biol Chem. 2009;284(17):11039–47.
805. Sun J, Mohlin S, Lundby A, Kazi JU, Hellman U, Påhlman S, et al. The PI3-kinase isoform p110δ is essential for cell transformation induced by the D816V mutant of c-Kit in a lipid-kinase independent manner. Oncogene. 2014;33(46):5360–9.
806. Piao X, Paulson R, Van Der Geer P, Pawson T, Bernstein A. Oncogenic mutation in the Kit receptor tyrosine kinase alters substrate specificity and induces degradation of the protein tyrosine phosphatase SHP-1. Proc Natl Acad Sci USA. 1996;93:14665–9.
807. Klippel A, Escobedo JA, Hirano M, Williams LT. The interaction of small domains between the subunits of phosphatidylinositol 3-kinase determines enzyme activity. Mol Cell Biol. 1994;14(4):2675–85.
808. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell- intrinsic death machinery. Cell. 1997;91(2):231–41.

809. Ueki K, Fruman DA, Brachmann SM, Tseng YH, Cantley LC, Kahn CR. Molecular balance between the regulatory and catalytic subunits of phosphoinositide 3-kinase regulates cell signaling and survival. Mol Cell Biol. 2002;22(3):965–77.
810. Sattler M, Salgia R, Shrikhande G, Verma S, Pisick E, Prasad KV, et al. Steel factor induces tyrosine phosphorylation of CRKL and binding of CRKL to a complex containing c-kit, phosphatidylinositol 3-kinase, and p120(CBL). J Biol Chem. 1997;272(15):10248–53.
811. Hartley D, Meisner H, Corvera S. Specific association of the beta isoform of the p85 subunit of phosphatidylinositol-3 kinase with the proto-oncogene c-cbl. J Biol Chem. 1995; 270(31):18260–3.
812. Geering B, Cutillas PR, Nock G, Gharbi SI, Vanhaesebroeck B. Class IA phosphoinositide 3-kinases are obligate p85-p110 heterodimers. Proc Natl Acad Sci USA. 2007;104(19): 7809–14.
813. Lev S, Givol D, Yarden Y. Interkinase domain of kit contains the binding site for phosphatidylinositol 3′ kinase. Proc Natl Acad Sci USA. 1992;89(2):678–82.
814. Serve H, Hsu Y, Besmer P. Tyrosine residue 719 of the c-kit receptor is essential for binding of the P85 subunit of phosphatidylinositol (PI) 3-kinase and for c-kit-associated PI 3-kinase activity in cos-1 cells. J Biol Chem. 1994;269:6026–30.
815. Yu M, Luo J, Yang W, Wang Y, Mizuki M, Kanakura Y, et al. The scaffolding adapter Gab2, via Shp-2, regulates kit-evoked mast cell proliferation by activating the Rac/JNK pathway. J Biol Chem. 2006;281(39):28615–26.
816. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell. 1995;80(2): 285–91.
817. Blume-Jensen P, Janknecht R, Hunter T. The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. Curr Biol. 1998;8(13):779–82.
818. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell. 1996;87(4):619–28.
819. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol. 2009;27:693–733.
820. Dhandapani KM, Wade FM, Wakade C, Mahesh VB, Brann DW. Neuroprotection by stem cell factor in rat cortical neurons involves AKT and NFkappaB. J Neurochem. 2005;95(1): 9–19.
821. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999;96(6): 857–68.
822. Engström M, Karlsson R, Jönsson JI. Inactivation of the forkhead transcription factor FoxO3 is essential for PKB-mediated survival of hematopoietic progenitor cells by kit ligand. Exp Hematol. 2003;31(4):316–23.
823. Möller C, Alfredsson J, Engström M, Wootz H, Xiang Z, Lennartsson J, et al. Stem cell factor promotes mast cell survival via inactivation of FOXO3a-mediated transcriptional induction and MEK-regulated phosphorylation of the proapoptotic protein Bim. Blood. 2005; 106(4):1330–6.
824. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein. Bim. J Biol Chem. 2003;278(21):18811–6.
825. Luciano F, Jacquel A, Colosetti P, Herrant M, Cagnol S, Pages G, et al. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. Oncogene. 2003;22(43):6785–93.
826. Sattler M, Winkler T, Verma S, Byrne CH, Shrikhande G, Salgia R, et al. Hematopoietic growth factors signal through the formation of reactive oxygen species. Blood. 1999; 93(9):2928–35.

827. Fiorentini D, Prata C, Maraldi T, Zambonin L, Bonsi L, Hakim G, et al. Contribution of reactive oxygen species to the regulation of Glut1 in two hemopoietic cell lines differing in cytokine sensitivity. Free Radic Biol Med. 2004;37(9):1402–11.
828. Baumer AT, Ten Freyhaus H, Sauer H, Wartenberg M, Kappert K, Schnabel P, et al. Phosphatidylinositol 3-kinase-dependent membrane recruitment of Rac-1 and p47phox is critical for alpha-platelet-derived growth factor receptor-induced production of reactive oxygen species. J Biol Chem. 2008;283(12):7864–76.
829. Ortutay C, Nore BF, Vihinen M, Smith CI. Phylogeny of Tec family kinases identification of a premetazoan origin of Btk, Bmx, Itk, Tec, Txk, and the Btk regulator SH3BP5. Adv Genet. 2008;64:51–80.
830. Chong H, Guan KL. Regulation of Raf through phosphorylation and N terminus-C terminus interaction. J Biol Chem. 2003;278(38):36269–76.
831. Brown MD, Sacks DB. Protein scaffolds in MAP kinase signalling. Cell Signal. 2009; 21(4):462–9.
832. Thömmes K, Lennartsson J, Carlberg M, Rönnstrand L. Identification of Tyr-703 and Tyr-936 as the primary association sites for Grb2 and Grb7 in the c-Kit/stem cell factor receptor. Biochem J. 1999;341(Pt 1):211–6.
833. Agosti V, Karur V, Sathyanarayana P, Besmer P, Wojchowski DM. A KIT juxtamembrane PY567-directed pathway provides nonredundant signals for erythroid progenitor cell development and stress erythropoiesis. Exp Hematol. 2009;37(2):159–71.
834. Wandzioch E, Edling CE, Palmer RH, Carlsson L, Hallberg B. Activation of the MAP kinase pathway by c-Kit is PI-3 kinase dependent in hematopoietic progenitor/stem cell lines. Blood. 2004;104(1):51–7.
835. Bokoch GM. Biology of the p21-activated kinases. Annu Rev Biochem. 2003;72:743–81.
836. McDaniel AS, Allen JD, Park SJ, Jaffer ZM, Michels EG, Burgin SJ, et al. Pak1 regulates multiple c-Kit mediated Ras-MAPK gain-in-function phenotypes in Nf1+/− mast cells. Blood. 2008;112(12):4646–54.
837. Chen M, Burgin S, Staser K, He Y, Li X, Robinson M, et al. Kinase suppressor of Ras (KSR1) modulates multiple kit-ligand-dependent mast cell functions. Exp Hematol. 2011;39(10):969–76.
838. Sundström M, Alfredsson J, Olsson N, Nilsson G. Stem cell factor-induced migration of mast cells requires p38 mitogen-activated protein kinase activity. Exp Cell Res. 2001;267(1): 144–51.
839. Kuang D, Zhao X, Xiao G, Ni J, Feng Y, Wu R, et al. Stem cell factor/c-kit signaling mediated cardiac stem cell migration via activation of p38 MAPK. Basic Res Cardiol. 2008; 103(3):265–73.
840. Ueda S, Mizuki M, Ikeda H, Tsujimura T, Matsumura I, Nakano K, et al. Critical roles of c-kit tyrosine residues 567 and 719 in stem cell factor-induced chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration. Blood. 2002;99:3342–9.
841. Smith JA, Samayawardhena LA, Craig AW. Fps/Fes protein-tyrosine kinase regulates mast cell adhesion and migration downstream of Kit and beta1 integrin receptors. Cell Signal. 2010;22(3):427–36.
842. Lee SJ, Yoon JH, Song KS. Chrysin inhibited stem cell factor (SCF)/c-Kit complex-induced cell proliferation in human myeloid leukemia cells. Biochem Pharmacol. 2007;74(2): 215–25.
843. Garrington TP, Ishizuka T, Papst PJ, Chayama K, Webb S, Yujiri T, et al. MEKK2 gene disruption causes loss of cytokine production in response to IgE and c-Kit ligand stimulation of ES cell-derived mast cells. EMBO J. 2000;19(20):5387–95.
844. Gommerman JL, Sittaro D, Klebasz NZ, Williams DA, Berger SA. Differential stimulation of c-Kit mutants by membrane-bound and soluble Steel Factor correlates with leukemic potential. Blood. 2000;96(12):3734–42.

845. Trieselmann NZ, Soboloff J, Berger SA. Mast cells stimulated by membrane-bound, but not soluble, steel factor are dependent on phospholipase C activation. Cell Mol Life Sci. 2003;60(4):759–66.
846. Koike T, Hirai K, Morita Y, Nozawa Y. Stem cell factor-induced signal transduction in rat mast cells. Activation of phospholipase D but not phosphoinositide-specific phospholipase C in c-kit receptor stimulation. J Immunol. 1993;151(1):359–66.
847. Kozawa O, Blume-Jensen P, Heldin CH, Rönnstrand L. Involvement of phosphatidylinositol 3′-kinase in stem-cell-factor- induced phospholipase D activation and arachidonic acid release. Eur J Biochem. 1997;248(1):149–55.
848. Hong L, Munugalavadla V, Kapur R. c-Kit-mediated overlapping and unique functional and biochemical outcomes via diverse signaling pathways. Mol Cell Biol. 2004;24(3): 1401–10.
849. Maddens S, Charruyer A, Plo I, Dubreuil P, Berger S, Salles B, et al. Kit signaling inhibits the sphingomyelin-ceramide pathway through PLC gamma 1: implication in stem cell factor radioprotective effect. Blood. 2002;100(4):1294–301.
850. Albanesi C, Geremia R, Giorgio M, Dolci S, Sette C, Rossi P. A cell- and developmental stage-specific promoter drives the expression of a truncated c-kit protein during mouse spermatid elongation. Development. 1996;122(4):1291–302.
851. Sette C, Bevilacqua A, Bianchini A, Mangia F, Geremia R, Rossi P. Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. Development. 1997;124(11):2267–74.
852. Sette C, Paronetto MP, Barchi M, Bevilacqua A, Geremia R, Rossi P. Tr-kit-induced resumption of the cell cycle in mouse eggs requires activation of a Src-like kinase. EMBO J. 2002;21(20):5386–95.
853. Han DC, Shen TL, Guan JL. The Grb7 family proteins: structure, interactions with other signaling molecules and potential cellular functions. Oncogene. 2001;20(44):6315–21.
854. Jahn T, Seipel P, Urschel S, Peschel C, Duyster J. Role for the adaptor protein Grb10 in the activation of Akt. Mol Cell Biol. 2002;22(4):979–91.
855. Gery S, Koeffler HP. Role of the adaptor protein LNK in normal and malignant hematopoiesis. Oncogene. 2013;32(26):3111–8.
856. Gueller S, Gery S, Nowak V, Liu L, Serve H, Koeffler HP. Adaptor protein Lnk associates with Tyr(568) in c-Kit. Biochem J. 2008;415(2):241–5.
857. Wollberg P, Lennartsson J, Gottfridsson E, Yoshimura A, Rönnstrand L. The adapter protein APS associates to the multifunctional docking sites Tyr568 and Tyr936 in c-Kit: possible role in v-Kit transformation. Biochem J. 2003;370:1033–8.
858. Simon C, Dondi E, Chaix A, de Sepulveda P, Kubiseski TJ, Varin-Blank N, et al. Lnk adaptor protein down-regulates specific Kit-induced signaling pathways in primary mast cells. Blood. 2008;112(10):4039–47.
859. Hu J, Hubbard SR. Structural characterization of a novel Cbl phosphotyrosine recognition motif in the APS family of adapter proteins. J Biol Chem. 2005;280(19):18943–9.
860. Takaki S, Morita H, Tezuka Y, Takatsu K. Enhanced hematopoiesis by hematopoietic progenitor cells lacking intracellular adaptor protein, Lnk. J Exp Med. 2002;195(2):151–60.
861. Kubo-Akashi C, Iseki M, Kwon SM, Takizawa H, Takatsu K, Takaki S. Roles of a conserved family of adaptor proteins, Lnk, SH2-B, and APS, for mast cell development, growth, and functions: APS-deficiency causes augmented degranulation and reduced actin assembly. Biochem Biophys Res Commun. 2004;315(2):356–62.
862. Takaki S, Sauer K, Iritani BM, Chien S, Ebihara Y, Tsuji K, et al. Control of B cell production by the adaptor protein lnk. Definition of a conserved family of signal-modulating proteins. Immunity. 2000;13(5):599–609.
863. Tanaka S, Ouchi T, Hanafusa H. Downstream of Crk adaptor signaling pathway: activation of Jun kinase by v-Crk through the guanine nucleotide exchange protein C3G. Proc Natl Acad Sci USA. 1997;94(6):2356–61.

864. Laine E. Chauvot de Beauchene I, Perahia D, Auclair C, Tchertanov L. Mutation D816V alters the internal structure and dynamics of c-KIT receptor cytoplasmic region: implications for dimerization and activation mechanisms. PLoS Comput Biol. 2011;7(6):e1002068.
865. Bougherara H, Subra F, Crepin R, Tauc P, Auclair C, Poul MA. The aberrant localization of oncogenic kit tyrosine kinase receptor mutants is reversed on specific inhibitory treatment. Mol Cancer Res. 2009;7(9):1525–33.
866. Tabone-Eglinger S, Subra F, El Sayadi H, Alberti L, Tabone E, Michot JP, et al. KIT mutations induce intracellular retention and activation of an immature form of the KIT protein in gastrointestinal stromal tumors. Clin Cancer Res. 2008;14(8):2285–94.
867. Xiang Z, Kreisel F, Cain J, Colson A, Tomasson MH. Neoplasia driven by mutant c-KIT is mediated by intracellular, not plasma membrane, receptor signaling. Mol Cell Biol. 2007; 27(1):267–82.
868. Taylor ML, Dastych J, Sehgal D, Sundström M, Nilsson G, Akin C, et al. The Kit-activating mutation D816V enhances stem cell factor-dependent chemotaxis. Blood. 2001;98: 1195–9.
869. Chian R, Young S, Danilkovitch-Miagkova A, Rönnstrand L, Leonard E, Ferrao P, et al. Phosphatidylinositol 3 kinase contributes to the transformation of hematopoietic cells by the D816V c-Kit mutant. Blood. 2001;98(5):1365–73.
870. Burke P, Schooler K, Wiley HS. Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. Mol Biol Cell. 2001;12(6):1897–910.
871. Voisset E, Lopez S, Dubreuil P, De Sepulveda P. The tyrosine kinase FES is an essential effector of KITD816V proliferation signal. Blood. 2007;110(7):2593–9.
872. Hashimoto K, Matsumura I, Tsujimura T, Kim D, Ogihara H, Ikeda H, et al. Necessity of tyrosine 719 and phosphatidylinositol 3′-kinase-mediated signal pathway in constitutive activation and oncogenic potential of c-Kit receptor tyrosine kinase with the Asp814Val mutation. Blood. 2003;101(3):1094–102.
873. Munugalavadla V, Sims EC, Chan RJ, Lenz SD, Kapur R. Requirement for p85alpha regulatory subunit of class IA PI3K in myeloproliferative disease driven by an activation loop mutant of KIT. Exp Hematol. 2008;36(3):301–8.
874. Tarsitano M, De Falco S, Colonna V, McGhee JD, Persico MG. The C. elegans pvf-1 gene encodes a PDGF/VEGF-like factor able to bind mammalian VEGF receptors and to induce angiogenesis. FASEB J. 2006;20(2):227–33. Epub 2006/02/02.
875. Heldin C-H, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev. 1999;79(4):1283–316. Epub 1999/10/03.
876. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev. 2008;22(10):1276–312. Epub 2008/05/17.
877. Ball SG, Shuttleworth CA, Kielty CM. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. J Cell Biol. 2007;177(3):489–500. Epub 2007/05/02.
878. Fredriksson L, Li H, Fieber C, Li X, Eriksson U. Tissue plasminogen activator is a potent activator of PDGF-CC. EMBO J. 2004;23(19):3793–802.
879. Ustach CV, Kim HR. Platelet-derived growth factor D is activated by urokinase plasminogen activator in prostate carcinoma cells. Mol Cell Biol. 2005;25(14):6279–88. Epub 2005/07/01.
880. Östman A, Andersson M, Betsholtz C, Westermark B, Heldin C-H. Identification of a cell retention signal in the B-chain of platelet-derived growth factor and in the long splice version of the A-chain. Cell Regul. 1991;2:503–12.
881. Kelly JL, Sánchez A, Brown GS, Chesterman CN, Sleigh MJ. Accumulation of PDGF B and cell-binding forms of PDGF A in the extracellular matrix. J Cell Biol. 1993;121(5): 1153–63.
882. Murray-Rust J, McDonald NQ, Blundell TL, Hosang M, Oefner C, Winkler F, et al. Topological similarities in TGF-β2, PDGF-BB and NGF define a superfamily of polypeptide growth factors. Structure. 1993;1:153–9.

883. McDonald NQ, Hendrickson WA. A structural superfamily of growth factors containing a cystine knot motif. Cell. 1993;73:421–4.
884. Spritz RA, Strunk KM, Lee ST, Lu-Kuo JM, Ward DC, Le Paslier D, et al. A YAC contig spanning a cluster of human type III receptor protein tyrosine kinase genes (PDGFRA-KIT-KDR) in chromosome segment 4q12. Genomics. 1994;22(2):431–6. Epub 1994/07/15.
885. Kawagishi J, Kumabe T, Yoshimoto T, Yamamoto T. Structure, organization, and transcription units of the human α-platelet-derived growth factor receptor gene, PDGFRA. Genomics. 1995;30(2):224–32.
886. Claesson-Welsh L, Eriksson A, Westermark B, Heldin C-H. cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. Proc Natl Acad Sci USA. 1989;86:4917–21.
887. Matsui T, Heidaran M, Miki T, Toru M, Popescu N, La Rochelle W, et al. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science. 1989; 243:800–3.
888. Yarden Y, Escobedo JA, Kuang W-J, Yang-Feng TL, Daniel TO, Tremble PM, et al. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature. 1986;323:226–32.
889. Roberts WM, Look AT, Roussel MF, Scherr CJ. Tandem linkage of human CSF-1 receptor (c-*fms*) and PDGF receptor genes. Cell. 1988;55:655–61.
890. Vizmanos JL. PDGFRB (platelet-derived growth factor receptor, beta polypeptide) (5q31-q32). Atlas Genet Cytogenet Oncol Haematol. 2005;9(4):587–97.
891. Heidaran MA, Pierce JH, Jensen RA, Matsui T, Aaronson SA. Chimeric α- and β-platelet-derived growth factor (PDGF) receptors define three immunoglobulin-like domains of the α-PDGF receptor that determine PDGF-AA binding specificity. J Biol Chem. 1990;265(31): 18741–4.
892. Lokker NA, O'Hare JP, Barsoumian A, Tomlinson JE, Ramakrishnan V, Fretto LJ, et al. Functional importance of platelet-derived growth factor (PDGF) receptor extracellular immunoglobulin-like domains. Identification of PDGF binding site and neutralizing monoclonal antibodies. J Biol Chem. 1997;272(52):33037–44.
893. Miyazawa K, Bäckström G, Leppänen O, Persson C, Wernstedt C, Hellman U, et al. Role of immunoglobulin-like domains 2–4 of the platelet-derived growth factor α-receptor in ligand-receptor complex assembly. J Biol Chem. 1998;273(39):25495–502.
894. Shim AH, Liu H, Focia PJ, Chen X, Lin PC, He X. Structures of a platelet-derived growth factor/propeptide complex and a platelet-derived growth factor/receptor complex. Proc Natl Acad Sci USA. 2010;107(25):11307–12. Epub 2010/06/11.
895. Omura T, Heldin C-H, Östman A. Immunoglobulin-like domain 4-mediated receptor-receptor interactions contribute to platelet-derived growth factor-induced receptor dimerization. J Biol Chem. 1997;272(19):12676–82.
896. Heldin C-H, Östman A, Rönnstrand L. Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta. 1998;1378(1):F79–F113.
897. Baxter RM, Secrist JP, Vaillancourt RR, Kazlauskas A. Full activation of the platelet-derived growth factor beta-receptor kinase involves multiple events. J Biol Chem. 1998;273(27):17050–5.
898. Irusta PM, Luo Y, Bakht O, Lai CC, Smith SO, DiMaio D. Definition of an inhibitory juxtamembrane WW-like domain in the platelet-derived growth factor beta receptor. J Biol Chem. 2002;277(41):38627–34.
899. Chiara F, Bishayee S, Heldin C-H, Demoulin J-B. Autoinhibition of the platelet-derived growth factor β receptor tyrosine kinase by its C-terminal tail. J Biol Chem. 2004;279(19): 19732–8.
900. Pawson T. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. Cell. 2004;116(2):191–203.
901. Kypta RM, Goldberg Y, Ulug ET, Courtneidge SA. Association between the PDGF receptor and members of the *src* family of tyrosine kinases. Cell. 1990;62:481–92.

902. Mori S, Rönnstrand L, Yokote K, Engström Å, Courtneidge SA, Claesson-Welsh L, et al. Identification of two juxtamembrane autophosphorylation sites in the PDGF β-receptor; involvement in the interaction with Src family tyrosine kinases. EMBO J. 1993;12(6): 2257–64.
903. Lechleider RJ, Sugimoto S, Bennett AM, Kashishian AS, Cooper JA, Shoelson SE, et al. Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human platelet-derived growth factor receptor β. J Biol Chem. 1993;268(29):21478–81.
904. Kazlauskas A, Feng G-S, Pawson T, Valius M. The 64-kDa protein that associates with the platelet-derived growth factor receptor β subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Syp. Proc Natl Acad Sci USA. 1993;90(15):6939–43.
905. Rönnstrand L, Mori S, Arvidsson A-K, Eriksson A, Wernstedt C, Hellman U, et al. Identification of two C-terminal autophosphorylation sites in the PDGF β-receptor: involvement in the interaction with phospholipase C-γ. EMBO J. 1992;11(11):3911–9.
906. Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M, McCormick F, et al. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell. 1992;69:413–23.
907. Arvidsson A-K, Rupp E, Nånberg E, Downward J, Rönnstrand L, Wennström S, et al. Tyr-716 in the platelet-derived growth factor β-receptor kinase insert is involved in GRB2 binding and Ras activation. Mol Cell Biol. 1994;14(10):6715–26.
908. Kazlauskas A, Cooper JA. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. Cell. 1989;58(6):1121–33.
909. Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell. 1989;57:167–75.
910. Coughlin SR, Escobedo JA, Williams LT. Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science. 1989;243:1191–4.
911. Valgeirsdóttir S, Paukku K, Silvennoinen O, Heldin C-H, Claesson-Welsh L. Activation of Stat5 by platelet-derived growth factor (PDGF) is dependent on phosphorylation sites in PDGF β-receptor juxtamembrane and kinase insert domains. Oncogene. 1998;16(4): 505–15.
912. Demoulin J-B, Seo JK, Ekman S, Grapengiesser E, Hellman U, Rönnstrand L, et al. Ligand-induced recruitment of Na^+/H^+ exchanger regulatory factor to the PDGF (platelet-derived growth factor) receptor regulates actin cytoskeleton reorganization by PDGF. Biochem J. 2003;376(Pt 2):505–10.
913. Maudsley S, Zamah AM, Rahman N, Blitzer JT, Luttrell LM, Lefkowitz RJ, et al. Platelet-derived growth factor receptor association with Na(+)/H(+) exchanger regulatory factor potentiates receptor activity. Mol Cell Biol. 2000;20(22):8352–63.
914. Takahashi Y, Morales FC, Kreimann EL, Georgescu MM. PTEN tumor suppressor associates with NHERF proteins to attenuate PDGF receptor signaling. EMBO J. 2006;25(4): 910–20.
915. Theisen CS, Wahl 3rd JK, Johnson KR, Wheelock MJ. NHERF links the N-cadherin/catenin complex to the platelet-derived growth factor receptor to modulate the actin cytoskeleton and regulate cell motility. Mol Biol Cell. 2007;18(4):1220–32. Epub 2007/01/19.
916. Lennartsson J, Wardega P, Engström U, Hellman U, Heldin C-H. Alix facilitates the interaction between c-Cbl and platelet-derived growth factor β-receptor and thereby modulates receptor downregulation. J Biol Chem. 2006;281(51):39152–8.
917. Fambrough D, McClure K, Kazlauskas A, Lander ES. Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. Cell. 1999;97(6):727–41.
918. Bae YS, Sung J-Y, Kim O-S, Kim YJ, Hur KC, Kazlauskas A, et al. Platelet-derived growth factor-induced H_2O_2 production requires the activation of phosphatidylinositol 3-kinase. J Biol Chem. 2000;275(14):10527–31.

919. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science. 1995;270(5234):296–9.
920. Jurek A, Amagasaki K, Gembarska A, Heldin C-H, Lennartsson J. Negative and positive regulation of MAPK phosphatase 3 controls platelet-derived growth factor-induced Erk activation. J Biol Chem. 2009;284(7):4626–34.
921. Jurek A, Heldin C-H, Lennartsson J. Platelet-derived growth factor-induced signaling pathways interconnect to regulate the temporal pattern of Erk1/2 phosphorylation. Cell Signal. 2011;23(1):280–7.
922. Ekman S, Rupp Thuresson E, Heldin C-H, Rönnstrand L. Increased mitogenicity of an αβ heterodimeric PDGF receptor complex correlates with lack of RasGAP binding. Oncogene. 1999;18(15):2481–8.
923. Peng Z-Y, Cartwright CA. Regulation of the Src tyrosine kinase and Syp tyrosine phosphatase by their cellular association. Oncogene. 1995;11(10):1955–62.
924. Dance M, Montagner A, Salles JP, Yart A, Raynal P. The molecular functions of Shp2 in the Ras/Mitogen-activated protein kinase (ERK1/2) pathway. Cell Signal. 2008;20(3):453–9. Epub 2007/11/13.
925. Sundberg C, Rubin K. Stimulation of β_1 integrins on fibroblasts induces PDGF independent tyrosine phosphorylation of PDGF β-receptors. J Cell Biol. 1996;132(4):741–52.
926. Li L, Heldin C-H, Heldin P. Inhibition of platelet-derived growth factor-BB-induced receptor activation and fibroblast migration by hyaluronan activation of CD44. J Biol Chem. 2006;281(36):26512–9.
927. Sorkin A, Westermark B, Heldin C-H, Claesson-Welsh L. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF β-receptor. J Cell Biol. 1991;112(3):469–78.
928. Wang Y, Pennock SD, Chen X, Kazlauskas A, Wang Z. Platelet-derived growth factor receptor-mediated signal transduction from endosomes. J Biol Chem. 2004;279(9):8038–46. Epub 2003/12/09.
929. Miyake S, Mullane-Robinson KP, Lill NL, Douillard P, Band H. Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation: a critical role for Cbl tyrosine kinase-binding domain. J Biol Chem. 1999;274(23):16619–28.
930. Karlsson S, Kowanetz K, Sandin Å, Persson C, Östman A, Heldin C-H, et al. Loss of T-cell protein tyrosine phosphatase induces recycling of the platelet-derived growth factor (PDGF) β-receptor but not the PDGF α-receptor. Mol Biol Cell. 2006;17(11):4846–55.
931. Hellberg C, Schmees C, Karlsson S, Åhgren A, Heldin C-H. Activation of protein kinase C α is necessary for sorting the PDGF β-receptor to Rab4a-dependent recycling. Mol Biol Cell. 2009;20(12):2856–63.
932. Schmees C, Villaseñor R, Zheng W, Ma H, Zerial M, Heldin C-H, et al. Macropinocytosis of the PDGF β-receptor promotes fibroblast transformation by H-RasG12V. Mol Biol Cell. 2012;23(13):2571–82.
933. Hoch RV, Soriano P. Roles of PDGF in animal development. Development. 2003;130(20):4769–84. Epub 2003/09/04.
934. Klinghoffer RA, Mueting-Nelsen PF, Faerman A, Shani M, Soriano P. The two PDGF receptors maintain conserved signaling in vivo despite divergent embryological functions. Mol Cell. 2001;7(2):343–54.
935. Ataliotis P, Symes K, Chou MM, Ho L, Mercola M. PDGF signalling is required for gastrulation of Xenopus laevis. Development. 1995;121(9):3099–110.
936. Ramachandran RK, Wikramanayake AH, Uzman JA, Govindarajan V, Tomlinson CR. Disruption of gastrulation and oral-aboral ectoderm differentiation in the Lytechinus pictus embryo by a dominant/negative PDGF receptor. Development. 1997;124(12):2355–64.
937. Montero JA, Heisenberg CP. Gastrulation dynamics: cells move into focus. Trends Cell Biol. 2004;14(11):620–7. Epub 2004/11/03.
938. Palmieri SL, Payne J, Stiles CD, Biggers JD, Mercola M. Expression of mouse PDGF-A and PDGF α-receptor genes during pre- and post-implantation development: evidence for a

developmental shift from an autocrine to a paracrine mode of action. Mech Dev. 1992;39(3):181–91.
939. Hamilton TG, Klinghoffer RA, Corrin PD, Soriano P. Evolutionary divergence of platelet-derived growth factor alpha receptor signaling mechanisms. Mol Cell Biol. 2003;23(11):4013–25. Epub 2003/05/16.
940. Soriano P. The PDGFα receptor is required for neural crest cell development and for normal patterning of the somites. Development. 1997;124(14):2691–700.
941. Tallquist MD, Soriano P. Cell autonomous requirement for PDGFRα in populations of cranial and cardiac neural crest cells. Development. 2003;130(3):507–18. Epub 2002/12/20.
942. Tallquist MD, Weismann KE, Hellstrom M, Soriano P. Early myotome specification regulates PDGFA expression and axial skeleton development. Development. 2000;127(23):5059–70. Epub 2000/11/04.
943. Xu X, Bringas Jr P, Soriano P, Chai Y. PDGFR-α signaling is critical for tooth cusp and palate morphogenesis. Dev Dyn. 2005;232(1):75–84. Epub 2004/11/16.
944. Boström H, Willetts K, Pekny M, Levéen P, Lindahl P, Hedstrand H, et al. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. Cell. 1996;85(6):863–73.
945. Lindahl P, Karlsson L, Hellström M, Gebre-Medhin S, Willetts K, Heath JK, et al. Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. Development. 1997; 124(20):3943–53.
946. Karlsson L, Lindahl P, Heath JK, Betsholtz C. Abnormal gastrointestinal development in PDGF-A and PDGFR-α deficient mice implicates a novel mesenchymal structure with putative instructive properties in villus morphogenesis. Development. 2000;127(16):3457–66.
947. Karlsson L, Bondjers C, Betsholtz C. Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle. Development. 1999;126(12): 2611–21.
948. Brennan J, Tilmann C, Capel B. PDGFR-α mediates testis cord organization and fetal Leydig cell development in the XY gonad. Genes Dev. 2003;17(6):800–10. Epub 2003/03/26.
949. Gnessi L, Basciani S, Mariani S, Arizzi M, Spera G, Wang C, et al. Leydig cell loss and spermatogenic arrest in platelet-derived growth factor (PDGF)-A-deficient mice. J Cell Biol. 2000;149(5):1019–26.
950. Levéen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev. 1994; 8(16):1875–87.
951. Soriano P. Abnormal kidney development and hematological disorders in PDGF β-receptor mutant mice. Genes Dev. 1994;8(16):1888–96.
952. Bjarnegård M, Enge M, Norlin J, Gustafsdottir S, Fredriksson S, Abramsson A, et al. Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities. Development. 2004;131(8):1847–57. Epub 2004/04/16.
953. Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR-β in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development. 1999;126(14):3047–55.
954. Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science. 1997;277(5323):242–5.
955. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol. 2003;161(6): 1163–77.
956. Lindahl P, Hellström M, Kalén M, Karlsson L, Pekny M, Pekna M, et al. Paracrine PDGF-B/PDGF-Rbeta signaling controls mesangial cell development in kidney glomeruli. Development. 1998;125(17):3313–22.
957. Van den Akker NM, Winkel LC, Nisancioglu MH, Maas S, Wisse LJ, Armulik A, et al. PDGF-B signaling is important for murine cardiac development: its role in developing atrio-

ventricular valves, coronaries, and cardiac innervation. Dev Dyn. 2008;237(2):494–503. Epub 2008/01/24.
958. Ohlsson R, Falck P, Hellström M, Lindahl P, Boström H, Franklin G, et al. PDGFB regulates the development of the labyrinthine layer of the mouse fetal placenta. Dev Biol. 1999;212(1): 124–36.
959. Rolny C, Nilsson I, Magnusson P, Armulik A, Jakobsson L, Wentzel P, et al. Platelet-derived growth factor receptor-beta promotes early endothelial cell differentiation. Blood. 2006;108(6):1877–86.
960. Robson MC, Phillips LG, Thomason A, Robson LE, Pierce GF. Platelet-derived growth factor BB for the treatment of chronic pressure ulcers. Lancet. 1992;339:23–5.
961. Steed DL. Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity ulcers. Plast Reconstr Surg. 2006;117(7 Suppl):143S–9S. discussion 50S-51S. Epub 2006/06/27.
962. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev. 2003;83(3):835–70. Epub 2003/07/05.
963. Chen H, Gu X, Liu Y, Wang J, Wirt SE, Bottino R, et al. PDGF signalling controls age-dependent proliferation in pancreatic β-cells. Nature. 2011;478(7369):349–55. Epub 2011/10/14.
964. Rodt SÅ, Åhlén K, Berg A, Rubin K, Reed RK. A novel physiological function for platelet-derived growth factor-BB in rat dermis. J Physiol. 1996;495(Pt 1):193–200.
965. Lidén A, Berg A, Nedrebø T, Reed RK, Rubin K. Platelet-derived growth factor BB-mediated normalization of dermal interstitial fluid pressure after mast cell degranulation depends on beta3 but not beta1 integrins. Circ Res. 2006;98(5):635–41. Epub 2006/02/04.
966. Bonner JC. Regulation of PDGF and its receptors in fibrotic diseases. Cytokine Growth Factor Rev. 2004;15(4):255–73. Epub 2004/06/23.
967. Olson LE, Soriano P. Increased PDGFRα activation disrupts connective tissue development and drives systemic fibrosis. Dev Cell. 2009;16(2):303–13. Epub 2009/02/17.
968. Krampert M, Heldin C-H, Heuchel R. A gain-of-function mutation in the PDGFR-β alters the kinetics of injury response in liver and skin. Lab Invest. 2008;88(11):1204–14.
969. Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, et al. PDGFRA activating mutations in gastrointestinal stromal tumors. Science. 2003;299(5607):708–10.
970. Elling C, Erben P, Walz C, Frickenhaus M, Schemionek M, Stehling M, et al. Novel imatinib-sensitive PDGFRA-activating point mutations in hypereosinophilic syndrome induce growth factor independence and leukemia-like disease. Blood. 2011;117(10):2935–43. Epub 2011/01/13.
971. Cools J, DeAngelo DJ, Gotlib J, Stover EH, Legare RD, Cortes J, et al. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. N Engl J Med. 2003;348(13):1201–14.
972. Griffin JH, Leung J, Bruner RJ, Caligiuri MA, Briesewitz R. Discovery of a fusion kinase in EOL-1 cells and idiopathic hypereosinophilic syndrome. Proc Natl Acad Sci USA. 2003;100(13):7830–5.
973. Pardanani A, Ketterling RP, Brockman SR, Flynn HC, Paternoster SF, Shearer BM, et al. CHIC2 deletion, a surrogate for FIP1L1-PDGFRA fusion, occurs in systemic mastocytosis associated with eosinophilia and predicts response to imatinib mesylate therapy. Blood. 2003;102(9):3093–6. Epub 2003/07/05.
974. Stover EH, Chen J, Folens C, Lee BH, Mentens N, Marynen P, et al. Activation of FIP1L1-PDGFRα requires disruption of the juxtamembrane domain of PDGFRα and is FIP1L1-independent. Proc Natl Acad Sci USA. 2006;103(21):8078–83. Epub 2006/05/13.
975. Toffalini F, Hellberg C, Demoulin J-B. Critical role of the platelet-derived growth factor receptor (PDGFR) β transmembrane domain in the TEL-PDGFRβ cytosolic oncoprotein. J Biol Chem. 2010;285(16):12268–78.
976. Fleming TP, Saxena A, Clark WC, Robertson JT, Oldfield EH, Aaronson SA, et al. Amplification and/or overexpression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. Cancer Res. 1992;52:4550–3.

977. Kumabe T, Sohma Y, Kayama T, Yoshimoto T, Yamamoto T. Amplification of α-platelet-derived growth factor receptor gene lacking an exon coding for a portion of the extracellular region in a primary brain tumor of glial origin. Oncogene. 1992;7:627–33.
978. Puputti M, Tynninen O, Sihto H, Blom T, Maenpaa H, Isola J, et al. Amplification of KIT, PDGFRA, VEGFR2, and EGFR in gliomas. Mol Cancer Res. 2006;4(12):927–34.
979. Smith JS, Wang XY, Qian J, Hosek SM, Scheithauer BW, Jenkins RB, et al. Amplification of the platelet-derived growth factor receptor-A (PDGFRA) gene occurs in oligodendrogliomas with grade IV anaplastic features. J Neuropathol Exp Neurol. 2000;59(6):495–503. Epub 2000/06/13.
980. Arai H, Ueno T, Tangoku A, Yoshino S, Abe T, Kawauchi S, et al. Detection of amplified oncogenes by genome DNA microarrays in human primary esophageal squamous cell carcinoma: comparison with conventional comparative genomic hybridization analysis. Cancer Genet Cytogenet. 2003;146(1):16–21. Epub 2003/09/23.
981. Zhao J, Roth J, Bode-Lesniewska B, Pfaltz M, Heitz PU, Komminoth P. Combined comparative genomic hybridization and genomic microarray for detection of gene amplifications in pulmonary artery intimal sarcomas and adrenocortical tumors. Genes Chromosomes Cancer. 2002;34(1):48–57. Epub 2002/03/29.
982. Clarke ID, Dirks PB. A human brain tumor-derived PDGFR-α deletion mutant is transforming. Oncogene. 2003;22(5):722–33.
983. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139(5):871–90. Epub 2009/12/01.
984. Jechlinger M, Grunert S, Tamir IH, Janda E, Ludemann S, Waerner T, et al. Expression profiling of epithelial plasticity in tumor progression. Oncogene. 2003;22(46):7155–69. Epub 2003/10/17.
985. Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodiecci P, et al. Autocrine PDGFR signaling promotes mammary cancer metastasis. J Clin Invest. 2006;116(6): 1561–70.
986. Gotzmann J, Fischer AN, Zojer M, Mikula M, Proell V, Huber H, et al. A crucial function of PDGF in TGF-β-mediated cancer progression of hepatocytes. Oncogene. 2006;25(22): 3170–85.
987. Dolloff NG, Shulby SS, Nelson AV, Stearns ME, Johannes GJ, Thomas JD, et al. Bone-metastatic potential of human prostate cancer cells correlates with Akt/PKB activation by alpha platelet-derived growth factor receptor. Oncogene. 2005;24(45):6848–54.
988. Russell MR, Jamieson WL, Dolloff NG, Fatatis A. The α-receptor for platelet-derived growth factor as a target for antibody-mediated inhibition of skeletal metastases from prostate cancer cells. Oncogene. 2009;28(3):412–21. Epub 2008/10/14.
989. Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor β to a novel *ets*-like gene, *tel*, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. Cell. 1994;77(2):307–16.
990. Magnusson MK, Meade KE, Brown KE, Arthur DC, Krueger LA, Barrett AJ, et al. Rabaptin-5 is a novel fusion partner to platelet-derived growth factor β receptor in chronic myelomonocytic leukemia. Blood. 2001;98(8):2518–25.
991. O'Brien KP, Seroussi E, Dal Cin P, Sciot R, Mandahl N, Fletcher JA, et al. Various regions within the α-helical domain of the COL1A1 gene are fused to the second exon of the PDGFB gene in dermatofibrosarcomas and giant-cell fibroblastomas. Gene Chrom Cancer. 1998;23(2):187–93.
992. Simon M-P, Pedeutour F, Sirvent N, Grosgeorge J, Minoletti F, Coindre J-M, et al. Deregulation of the platelet-derived growth factor B-chain gene via fusion with collagen gene *COL1A1* in dermatofibrosarcoma protuberans and giant-cell fibroblastoma. Nat Genet. 1997;15(1):95–8.
993. Shimizu A, O'Brien KP, Sjöblom T, Pietras K, Buchdunger E, Collins VP, et al. The dermatofibrosarcoma protuberans-associated collagen type Iα1/platelet-derived growth factor (PDGF) B-chain fusion gene generates a transforming protein that is processed to functional PDGF-BB. Cancer Res. 1999;59(15):3719–23.

994. Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin C-H, Westermark B, et al. Platelet-derived growth factor and its receptors in human glioma tissue: Expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res. 1992;52:3213–9.
995. Pietras K, Pahler J, Bergers G, Hanahan D. Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. PLoS Med. 2008; 5(1):e19.
996. Heldin C-H, Rubin K, Pietras K, Östman A. High interstitial fluid pressure: an obstacle in cancer therapy. Nat Rev Cancer. 2004;4(10):806–13.
997. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature. 1993; 362:801–9.
998. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. 2005;352(16):1685–95. Epub 2005/04/22.
999. Demetri GD. Differential properties of current tyrosine kinase inhibitors in gastrointestinal stromal tumors. Semin Oncol. 2011;38 Suppl 1:S10–S9. Epub 2011/04/01.
1000. Pietras K, Sjöblom T, Rubin K, Heldin C-H, Östman A. PDGF receptors as cancer drug targets. Cancer Cell. 2003;3:439–43.
1001. Catena R, Luis-Ravelo D, Anton I, Zandueta C, Salazar-Colocho P, Larzabal L, et al. PDGFR signaling blockade in marrow stroma impairs lung cancer bone metastasis. Cancer Res. 2010;71(1):164–74. Epub 2010/11/26.
1002. Chintalgattu V, Ai D, Langley RR, Zhang J, Bankson JA, Shih TL, et al. Cardiomyocyte PDGFR-β signaling is an essential component of the mouse cardiac response to load-induced stress. J Clin Invest. 2010;120(2):472–84. Epub 2010/01/15.
1003. Jayson GC, Parker GJ, Mullamitha S, Valle JW, Saunders M, Broughton L, et al. Blockade of platelet-derived growth factor receptor-beta by CDP860, a humanized, PEGylated di-Fab', leads to fluid accumulation and is associated with increased tumor vascularized volume. J Clin Oncol. 2005;23(5):973–81. Epub 2004/10/07.

Chapter 11
The PTK7 Receptor Family

Anne-Catherine Lhoumeau, Sébastien Martinez, Thomas Prébet, and Jean-Paul Borg

11.1 Introduction to the PTK7 Receptor Tyrosine Kinase Family

This receptor tyrosine kinase was identified in the early 1990s by S.T. Lee and Axel Ullrich's groups, who named the receptor PTK7 (Protein Tyrosine Kinase 7) and CCK4 (Colon Carcinoma Kinase 4), respectively [1–4]. Other acronyms were given to this conserved receptor in *Drosophila* in reference to its function (OTK for Off Track Kinase), or its similarity to TRK receptors (DTRK for Drosophila TRK). Other names were given in chicken (KLG for Kinase-Like Gene), and in Hydra (Lemon). PTK7 is now the commonly used name of this receptor that represents the only member of this receptor tyrosine kinase family.

PTK7 is a RTK presenting a classical organization with an extracellular region, a single transmembrane region, and a tyrosine kinase domain without insert. It belongs to the group of pseudokinases as important residues required for catalytic activity are missing within its kinase domain. Results obtained in *Xenopus* and in mammals have nevertheless emphasized the critical role of the tyrosine kinase domain that acts as a protein interaction domain. PTK7 is an atypical RTK implicated in Wnt pathways and processed by the metalloproteinase MT1-MMP. Identity of its ligand(s) is presently unknown, but accumulated data from groups working principally in Vertebrates pictured PTK7 as a partner for receptors (VEGFR, Frizzled, PlexinA1) inserted into the plasma membrane. Besides a role of

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co-receptor, phylogenetic studies also suggest that PTK7 is potentially endowed with cell adhesion functions through its extracellular immunoglobulin loops.

PTK7 has indisputable important functions in embryonic development by playing a crucial and conserved role in embryonic development in Metazoans due to its role in planar cell polarity (PCP). PCP serves to structure many epithelial tissues and organs polarized within the plane of the epithelium. Impaired vertebrate PCP gene function provokes an array of phenotypes ranging from classical PCP defects to defects in convergent extension (a polarized morphogenetic movement of mesenchymal cells) during gastrulation [5]. Although no PTK7 mutation has been yet found in human diseases, frequent overexpression of the receptor is observed in solid and hematological cancers. As PTK7 overexpression leads to increased cell invasion and is correlated to poor prognosis, it is anticipated that PTK7 inhibitors may have therapeutic values in certain pathological contexts.

11.2 The Role of the PTK7 Receptor Tyrosine Kinase Family in Embryonic Development and Adult Physiology

Ptk7 null mice die perinatally, making impossible the study of PTK7 in adult life [6]. Death is due to severe embryonic developmental defects originated from abnormal PCP and convergent extension [6]. In multicellular organisms, organization of tissues, especially epithelial tissues, is represented by two types of polarity: apico-basal and planar cell polarity. Apico-basal is established along an *x*–*y* axis of epithelial cells and is supported by an evolutionarily conversed set of proteins that participate to the formation and maintenance of cell–cell junctions (i.e., the Crumbs, Scrib, and Par complexes) [7]. PCP defines the organized orientation of single cells, or group of cells within the plane of an epithelial sheet. For instance, actin-rich hairs that decorate the surface of epithelial cells of *Drosophila* wings are uniformly oriented in the same direction according to PCP. Core PCP genes originally discovered in the fly (*frizzled*, *van gogh, dishevelled*,…) have one or more homologues in mammals that play a role in tissue organization. Impaired vertebrate PCP gene function leads to phenotypes ranging from classical PCP defects such as misorientation of hair bundle in inner ear sensory cells, and of hair follicles in the skin, to defects in convergent extension (a polarized morphogenetic movement) during gastrulation, neural tube closure, eyelid closure, primary cilium formation, and asymmetric division [5]. Interestingly, studies in vertebrate model systems have revealed a distinct class of PCP genes that includes *ptk7*, which are not implicated in *Drosophila* PCP. *Ptk7* null mice have misorientated stereociliary bundles of sensory hair cells in the inner ear, defective neural tube closure, smaller kidney, eyelid closure defects, polydactyly, and an impaired gastrulation, due to impaired convergent extension and related defects of polarized cell movements (Fig. 11.1) [6, 8]. These defects are linked to a PTK7-Src signaling pathway along cell–cell contacts which controls the spatial regulation of ROCK activity and actomyosin contractility [9]. PTK7 is genetically linked to *vangl2*, a core PCP gene. Double heterozygous $vangl2^{+/-}$/$ptk7^{+/-}$ mice recapitulate the strong PCP defects of homozygous $vangl2^{-/-}$ or

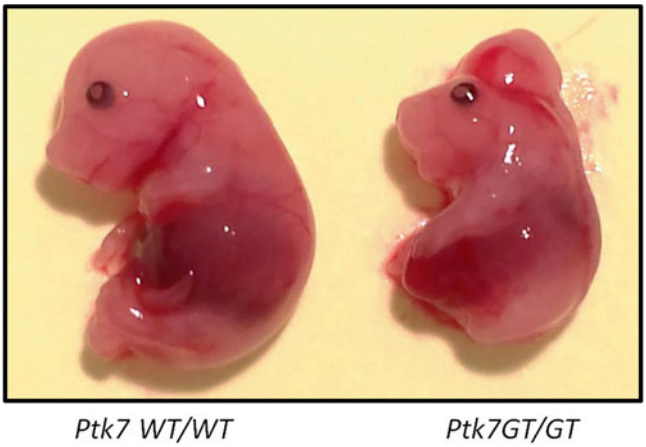


Fig. 11.1 Phenotype of *Ptk7* null embryos. *Ptk7* deficient mice display a severe planar cell polarity defect with a major neural tube closure defect (craniorachischisis) and an abdomen closure defect

ptk7$^{-/-}$ deficient mice [6]. No biochemical evidence has yet supported the idea that Vangl2 and PTK7 belong to a common protein complex, but a cooperation between two proteins was revealed in the mouse neural plate [10].

Knockdown of *ptk7* in *Xenopus* also impairs PCP and convergence extension, indicating a conservation of PTK7 PCP functions along evolution in Vertebrates [6, 11]. In *Xenopus* embryos, targeting of *ptk7* with morpholino-oligonucleotides (MOs) not only provokes PCP defects (neural tube closure defects at tailbud stage) but also often leads to incomplete blastopore closure, and reduced nervous system in the brain region, consistent with impaired Spemann's organizer activity [6, 11]. This structure is required for axis formation during embryogenesis and is under the control of Wnt/β catenin pathway. Likewise, this phenotype is recapitulated by injection of β-catenin MOs [12]. Thus, PTK7 is implicated in canonical and non-canonical (PCP) Wnt signaling pathway.

Despite the lack of obvious implication of Ptk7 in *Drosophila* PCP, the receptor has nevertheless an important role in axon guidance in the fly central nervous system and in the projections of the motor nerves. Defects depicted in *ptk7* deficient flies are similar to abnormalities reported for *plexA* and *sema1a* (encoding a ligand for the Plexin A receptor) loss-of-function mutants [13].

11.3 The Role of the PTK7 Receptor Tyrosine Kinase Family in Human Diseases

The multiplicity of developmental processes that employ PCP genes predicts that a broad range of diseases may stem from deregulations of PCP functions. Neural tube defects (NTDs) are common human birth defects that can be recapitulated in

vertebrate models, including the mouse, by disrupting PCP genes such as *vangl2*, *scrib*, or *ptk7*. Mutations of *VANGL1* and *VANGL2* were recently discovered in patients affected by severe NTDs [14, 15]. However, no *PTK7* mutation has been described yet in human NTDs.

In humans, a deregulated expression of *PTK7* mRNA has been documented at the transcriptomic level in solid tumors including colon cancers [1], pulmonary adenocarcinoma [16], gastric cancers [17], and metastatic melanoma [18], breast cancer [19–21], esophageal squamous cell carcinoma [22], prostate cancer [23], intrahepatic cholangiocarcinoma [24], and glioma cells [25]. *PTK7* is overexpressed in all these cancers, except in advanced stage melanoma, where it is downregulated as compared to localized tumors [18]. In advanced lung squamous cell carcinoma, *PTK7* is also downregulated and seems to play a tumor suppressing role by inhibiting ERK and AKT activity [26, 27]. A few mutations of *PTK7* have been described, two in ovarian cancer and one in colon cancer, but no functional consequences have been reported yet.

Our group was the first to demonstrate that PTK7 is not only expressed in neural and epithelial tissues but also on human hematopoietic stem cells and myeloid progenitors [28]. No PTK7 expression is found on mature blood cells. PTK7 was recognized as a specific marker of a small subset of T CD4+ cells called Recent Thymic Emigrant (RTE) cells [29]. These cells are naïve CD4+ T lymphocytes found in peripheral blood after thymic education whose functions are not yet well established.

In acute myeloid leukemia, PTK7 is overexpressed in leukemic cells as compared to normal hematopoietic stem cells and progenitors. This overexpression is correlated to poor prognosis and to increased resistance to chemotherapeutic agents. Resistance to apoptosis correlated to PTK7 overexpression is recapitulated in vitro and can be partially decreased by a recombinant soluble PTK7-Fc protein [28]. Role of PTK7 overexpression in resistance to apoptosis induced by anthracycline-based chemotherapy has also been described in breast cancer [20]. A PTK7-dependent resistance to apoptosis has been also suggested in colon cancer cells. The underlying mechanism is not yet elucidated, but involvement of PTK7 in a caspase 10-dependent mitochondrial apoptosis pathway has been suggested [30]. These results suggest that PTK7 may be a good prognostic and predictive marker associated with resistance to anthracycline-based chemotherapy.

PTK7 was found overexpressed in a subset of poor-prognosis metastatic breast cancers with high propensity to metastase in bones [31, 32, 21], and in metastatic colorectal cancers [33]. PTK7 plays a role in cell migration. Both pro- and anti-migratory properties of PTK7 have been described in different cell contexts. In leukemia cells, overexpressed PTK7 increases chemotaxis toward growth factors (stem cell factor or SCF) and serum [28]. In colon cancer cells, expression of a PTK7 mutant resistant to MT1-MMP cleavage represses cell migration while soluble PTK7 has an inverse property [34]. Two reports also implicate PTK7 in endothelial cell migration and angiogenesis. PTK7 is expressed in vascular endothelial cells and has a promigratory role in human umbilical vein endothelial cells (HUVECs). Cell migration of HUVECs stimulated by VEGF is inhibited by

repression of PTK7 expression with small interference RNAs or by a soluble recombinant PTK7 protein [35]. Moreover, PTK7 forms a protein complex with VEGFR1 and is required for VEGF signaling in this model system [36].

11.4 PTK7

11.4.1 PTK7 Gene

11.4.1.1 Promoter Structure

The human *PTK7* gene structure has been described in 2002 by S.T. Lee's team [4]. The gene is located on chromosome 6 (6p21.1–p12.2) [3, 4, 37] and spans approximately 85 kb. The 883-bp 5′-flanking sequence from the ATG start codon is functional as a promoter and has high CG content with 420-bp-long CpG islands but does not contain TATA or CATT box. The *PTK7* gene shares some features with housekeeping genes. Two GC boxes with Sp1 binding motifs and several potential transcription factor binding motifs (NFAT, dEF1, LMO2COM, v-MYB, TCF11, NF1, IK-2, AP4) might be involved in the regulation of PTK7 expression [4].

The *PTK7* gene is organized into 20 exons in mammals. In humans, exon 1 encodes the translation initiation codon and the signal peptide. Exons 2–13 encode the extracellular domain. The 5′-half of exon 14 encodes the transmembrane domain, and the rest of exon 14 and 5′-half of exon 15 encode the juxtamembrane domain. The 3′-half of exon 15 and exons 16–20 encode the tyrosine kinase domain [4].

Organization and nucleotide sequences are highly conserved between mouse and human *PTK7* genes, although the mouse gene is shorter than the human gene and spans approximately 65 kb. The mouse *Ptk7* gene, located on chromosome 17, consists of 20 exons and has exactly the same exon–intron structure as the human gene. Moreover, the human–mouse homology map shows synteny around the human and mouse loci of *PTK7* (mouse chromosome 17B3 and human chromosome 6p21), suggesting that genomic organization and chromosomal localization of the *PTK7* family are conserved in mammalian evolution [38].

11.4.1.2 mRNA Structure

In humans, five alternative transcripts have been identified in testis (PTK7-1 to PTK7-5). The encoded protein isoforms result in loss of one immunoglobulin loop by alternative splicing. The PTK7-5 variant contains an in-frame termination codon, generated by a frameshift in exon 16 and theoretically producing a PTK7 isoform lacking the tyrosine kinase domain (Fig. 11.2) [4].

Alternative transcripts	mRNA transcript	Missing exons	Number of amino acids	Predicted Molecular weights (kDa)	Structure
Isoform 1	3213 bp	_	1070	118.5	Full lenght protein 
Isoform 2	3093 bp	exon 10	1030	114	Loss of half of the 6th Ig loop 
Isoform 3	3045 bp	exons 8,9,10	1014	103.7	Loss of the 5th and a part of 6th Ig loops 
Isoform 4	2823 bp	exons 12,13	940	112.4	Loss of the 7th Ig loop 
Isoform 5	2451 bp	exon 16	816	90	Loss of the tyrosine kinase domain 

Fig. 11.2 Overview of *Ptk7* transcripts. Five isoforms are produced by alternative splicing

11.4.1.3 Transcriptional Regulation

PTK7 has a ubiquitous expression and is expressed at low level in normal mouse and human tissues. The 5′-flanking sequence of the human *PTK7* gene has a promoter activity and contains several canonical binding sites for transcription factors, such as NFAT, SP1, dEF1, LMO2COM, v-MYB, TCF11, NF1, IK-2, and AP4, that might be important for PTK7 gene expression[4]. Most of these binding sites are conserved in the mouse gene. More recently, a report showed that the *Cdx* family genes could be involved in the regulation of *Ptk7* expression. The vertebrate *Cdx* genes encode homeodomain transcription factors with well-established role in anteroposterior patterning. *Cdx1–Cdx2* double mutants exhibit a severe phenotype of neural tube defects, similar to those found when PCP genes such as *Ptk7* are mutated. The authors found that *Ptk7* expression is downregulated in *Cdx1/2* double KO mutants at E7.5, preceding initiation of neural tube closure. Several Cdx response elements in the 5′-flanking sequence of *Ptk7* have been identified by chromatin immunoprecipitation analysis and activity of these sites has been demonstrated in cultured cells. These promoter sequences are conserved between mouse and human and respond to Cdx1 and Cdx2, suggesting that *Ptk7* is a direct Cdx target [39].

In a recent study, gene expression analysis in glioma cells shows that PTK7 could regulate the inhibitor of DNA binding site (*Id1*) gene by modulating the

TGFβ-SMAD signaling pathway [25]. Downregulation of PTK7 reduced *Id1* expression, suppressed tumor growth, and induced apoptosis in an orthotopic glioma tumor mouse model.

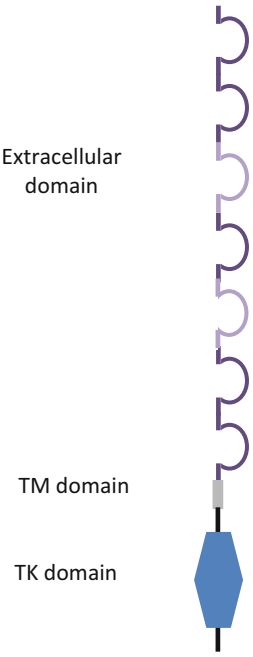
11.4.2 PTK7 Protein

11.4.2.1 Amino Acid Sequence

The human and mouse PTK7 proteins comprise 1,070 and 1,062 amino acids (AA), respectively, and share 92.6 % identity. PTK7 has a classical RTK organization as it is composed, from N-terminal to C-terminal, of seven extracellular immunoglobulin-like loops followed by a transmembrane domain and an intracellular tyrosine kinase domain. The tyrosine kinase domain is evolutionarily conserved during evolution (for example, 71.6 % identity between mouse and chicken PTK7). In all species, this domain lacks important amino acids required for catalytic activity. Indeed, among the three major sites required for enzymatic activity, the GXGXXG ATP binding motif and the HDRL motif required for the catalytic proton transfer are changed in GXSXXG and HKDL motifs, respectively. The aspartate residue of the DFG motif coordinating Mg^{2+}-ATP binding is changed to an uncharged alanine residue (ALG motif). Until now, no convincing experimental data support a catalytic function of the PTK7 tyrosine kinase domain (Fig. 11.3) [3, 4, 38].

11.4.2.2 Processing

The mouse PTK7 polypeptide sequence contains an AXA motif (residues 20–22), which defines a cleavage site of the signal peptide by the endoplasmic reticulum signal peptidase complex. This is confirmed by the N-terminal sequencing of the protein [38]. Furthermore, the PTK7 extracellular domain is processed by membrane proteases. Recent work has indeed shown that PTK7 is cleaved by the membrane type-1 matrix metalloproteinase (MT1-MMP), a prototypic member of a membrane-anchored MMP subfamily [34]. MT1-MMP can switch on or off the activity of a broad range of cell surface receptors including RTKs and adhesion molecules [40]. Cleavage of PTK7 between Ig loops 6 and 7 ($L^{621}I^{622}$ sequence; see Fig. 11.3) by MT1-MMP releases a promigratory soluble PTK7 form whose production is abolished by the introduction of a L622D mutation in the PTK7 sequence [41]. Moreover, another cleavage site for ADAMs proteins has been identified after the 7th loop (AA 722). These two processed fragments (cleaved by MT1-MPP or by ADAMs) can be sequentially processed in the transmembrane by γ-secretase, leading to an intracellular fragment of PTK7 which is able to translocate into the nucleus [42]. From in vivo and in vitro studies, it appears that a fine-tuned balance between full-length PTK7 and soluble PTK7 is required for normal embryonic development and directional cell migration [34, 41]. Indeed, in colon cancer cells, levels of PTK7



Domains	Residues and features
Loop1: IgC2	44-108
Loop2: IgC2	141-207
Loop3: Ig	231-320
Loop4: IgC2	334-398
Loop5: Ig	418-499
Loop6: IgC2	515-577
Loop7: IgC2	604-671. $PKP^{621}LI$: cleavage site of MT1-MMP
Transmembrane	704-726
Kinase domain:	796-1061 GXGXXG changed in GXSXXG HDRL changed in HKDL DFG changed in ALG

Fig. 11.3 Structure of the PTK7 receptor. The extracellular domain encompasses 7 Immunoglobulin-like loops (in *purple*), a transmembrane region (in *gray*), and an inactive tyrosine kinase domain (in *blue*). Important PTK7 amino acids are listed in the *right* column

expression and proteolysis were directly linked to the formation of cell protrusions, including lamellipodia and invadopodia. Authors showed, using an in vivo model, that PTK7 expression and proteolysis, rather than levels of full-length PTK7, contributed to efficient directional cell motility and metastatic development [43, 44].

11.4.2.3 Domain Structure

PTK7 comprises an extracellular domain of seven immunoglobulin-like loops similar to the overall organization of the VEGFR family, although no obvious identity exists at the sequence level. Instead, the PTK7 Ig-like loops domains have a strong homology to those of cell adhesion molecules of the immunoglobulin superfamily. Studies on the evolution of protein families involved in cellular recognition, in particular within the hematopoietic system, have emphasized the central role played by

immunoglobulin superfamily (Ig Sf) molecules. Ig and Ig-like domains are grouped into four subtypes: V-set (variable), C1-set (constant-1), C2-set (constant-2), and I-set (intermediate) [45]. Subtype domains V and C are typically found in antigen receptors (TcR and BcR) or Major Histocompatibility Complexes (MHC). Interestingly, like the *PTK7* gene, the *MHC* genes are located on human chromosome 6. The majority of the seven Ig-like loops domains of PTK7 are IgC2 subtype while loop 3 and loop 5 are not classified (Fig. 11.3). The IgC2 subtype domains are considered as the most primitive domains because they are present in many adhesion molecules including in Invertebrates and are probably required for basic functions such as cell–cell or cell–matrix adhesion.

Besides the extracellular domain, PTK7 has a juxtamembrane region that shares no significant homology with other RTKs and a tyrosine kinase domain with highest identity with MUSK (42 %). In a phylogenetic study, Grassot et al. reported that, among the RTKs of the immunoglobulin superfamily, the *PTK7* and *MUSK* genes have derived very early during evolution of this subfamily and have created an independent branch probably endowed with particular functions [46].

11.4.2.4 Posttranslational Modifications

Ten total putatives sites can be predicted from the polypeptide sequence analysis. Four sites of N-glycosylation have been identified until now by mass spectrometry at residues 116, 175, 268, and 283 in human PTK7 [47].

11.4.2.5 Phosphorylation Sites and Known Functions

Despite a poor tyrosine kinase activity, *Drosophila* PTK7 was found tyrosine phosphorylated in in vitro kinase assays and during cell adhesion. Functional importance of PTK7 phosphorylation in cell adhesion was not demonstrated [48]. In the absence of known ligand, Mossie and colleagues fused the extracellular domain of EGFR to the transmembrane and intracellular domains of human PTK7. No tyrosine phosphorylation of the EGFR-PTK7 chimera was evidenced following transfection and stimulation with EGF [1].

11.4.3 PTK7 Ligands

11.4.3.1 Ligand Structure

No ligand has been yet identified for the extracellular domain of PTK7. Nevertheless, PTK7 can indirectly interact with ligands thanks to its interaction with other receptors. *Drosophila* Ptk7 is able to coimmunoprecipitate with Plexin A1, a receptor for Sema1a, a ligand of the Semaphorin family [13]. This protein complex

is implicated in axon guidance in the central nervous system and in the projections of the motor nerves. In *Xenopus*, the canonical Wnt8 and Wnt3a ligands coprecipitate with Ptk7 only when the Fz7 receptor is coexpressed. This interaction is specific as non-canonical Wnt ligands, Wnt5a and Wnt11, do not interact with the Ptk7/Fz7 complex (see below) (Table 11.1).

Table 11.1 Interactors of PTK7 and their functions. Known interactors of PTK7, their identified functions, and their implication in Wnt signaling pathway are summarized in this table

	Species	PTK7 interactors	Function of PTK7 interactors	Mapping of the interaction	Signaling pathway	ref
OTK	Drosophila	Wnt4	Ligand	coIP between proteins	Antagonizes canonical Wnt signaling	[52]
		Dsh	Adapter		Canonical and non-canonical Wnt pathway	[11]
		Plexin A	Ternary complex with Plexin A and Sema1a	coIP between proteins	Signaling of semaphorin ligands	[13]
PTK7	Xenopus	Wnt3a/Fz7 complex	Ligand/ Receptor	coIP between proteins	Inhibition of canonical Wnt signaling	[52]
		Wnt8 (not Wnt5a or Wnt11)	Ligand	coIP between proteins	Inhibition of canonical Wnt signaling	[52]
		Dsh	Adapter protein in complex with RACK1,Fz7	coIP between proteins	Required for canonical Wnt signaling	[11]
		RACK1	Adapter protein In complex with PKCδ1	Tyrosine kinase domain of PTK7	Required for membrane localization of Dsh	[50]
		β-catenin	Transcriptional activity	Tyrosine kinase domain of PTK7	Activation of canonical pathway upstream of GSK3	[12]
		LRP6	Adapter protein	CoIP between proteins Transmembrane domains	Activation of canonical pathway by stabilization of LRP6	[53]
PTK7	Human	Wnt3a	Ligand	coIP between proteins	Inhibition of canonical Wnt signaling	[52]

(continued)

Table 11.1 (continued)

	Species	PTK7 interactors	Function of PTK7 interactors	Mapping of the interaction	Signaling pathway	ref
		Fz7	Receptor	coIP between proteins	Required for both canonical and non-canonical Wnt signaling	[52]
		Dsh	Adapter protein	coIP between proteins	Activation of non-canonical PCP pathway	[11]
		β-catenin	Transcriptional activity	Tyrosine kinase domain of PTK7	Activation of canonical pathway upstream GSK3	[12]
		Src	Adapter protein	Tyrosine kinase domain of PTK7	Localization of Src signaling and dowstream actomyosin regulation at cell–cell contact	[9]

11.4.3.2 Ligand Cleavage

N/A

11.4.4 PTK7 Activation and Signaling

11.4.4.1 Dimerization

PTK7 has been described to heterodimerize with VEGFR1 (Flt-1) but not with VEGFR2 (KDR/Flk-1) or VEGFR3 (Flt-4). This interaction takes place in human endothelial cells and seems to be improved by addition of VEGF-A, as measured in vitro by Surface Plasmon Resonance assays. VEGFR1 signaling (AKT, FAK) and function (angiogenesis) are dependent on PTK7 expression in HUVECs [36].

11.4.4.2 Phosphorylation

Although phosphorylation of PTK7 was described in *Drosophila* using an in vitro kinase assay and in cell adhesion [48], no phosphorylation site of PTK7 has been yet identified, nor any substrate for its hypothetical tyrosine kinase activity.

11.4.4.3 Pathway Activation

Despite the absence of catalytic activity, PTK7 appears to have a role in signal transduction and emerges as an important regulator of VEGFR1 and Wnt pathways.

In endothelial cells, VEGFA triggers VEGFR1 phosphorylation and association with PTK7. This interaction is required for optimal phosphorylation and activation of downstream components of VEGFR1 signaling, including AKT and FAK that are required for the angiogenic process (Fig. 11.4) [36]. Accordingly, downregulation of PTK7 expression in vitro and in vivo leads to a decreased angiogenesis [35]. Interestingly, an anti-angiogenic effect can also be obtained using a soluble recombinant form of PTK7, suggesting a dominant-negative effect on the extracellular domain and the capture of a putative ligand.

Wnt pathways are subdivided into canonical (β-catenin dependent) and non-canonical (β-catenin independent) pathways. Wnt ligands and their Frizzled (Fz) receptors represent core components of Wnt pathways, the output of a Wnt–Fz complex depending on the specific combination of Wnt ligands, Fz receptors, and co-receptors. While canonical Wnts such as Wnt3a stabilize β-catenin and promote its transcriptional functions, non-canonical Wnts (Wnt5a, Wnt11) use Fz receptors together with PCP proteins, including PTK7, to promote the so-called PCP pathway inducing a Rho/Rac/JNK signaling cascade that controls actin cytoskeleton remodeling [49] (Fig. 11.5). Cross talks exist between canonical and non-canonical Wnt pathways that shared some molecular components such as Dishevelled (Dsh). *Xenopus* Ptk7 is part of a Fz7-Dsh complex that is required for PCP [11]. The tyrosine kinase domain of PTK7 is mandatory for membrane recruitment of Dishevelled. Furthermore, PTK7 associates with RACK1 (Receptor of ACtivated

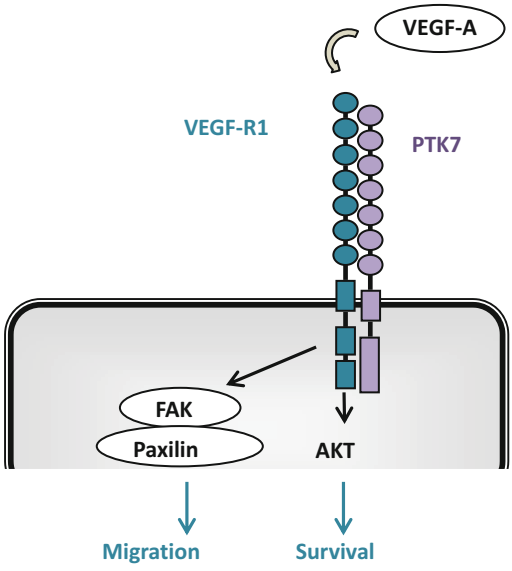


Fig. 11.4 Dimerization of PTK7 with the VEGFR1 receptor and downstream signaling. The interaction between VEGFR1 and PTK7 is intensified by VEGF-A binding. Downregulation of PTK7 leads to decreased phosphorylation of focal adhesion kinase (FAK) and AKT, and decreased cell migration and survival

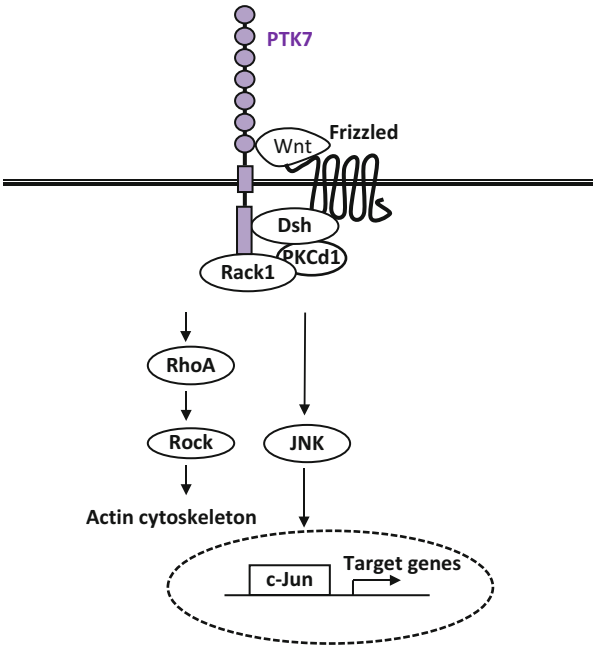


Fig. 11.5 Role of PTK7 in non-canonical Wnt pathway or PCP. PTK7 is member of a protein complex comprising Fz7, PKCδ1, and RACK1 that promotes the membrane recruitment of Dsh. It is likely that this signaling complex affects JNK phosphorylation and transcriptional activity of Jun

protein Kinase C1) and PKCδ1 to recruit Dsh [50]. Since RACK1 is known to antagonize canonical Wnt pathways, the PTK7/RACK1 complex can potentially repress the canonical Wnt signaling [51]. In mammalian cells, we recently found that PTK7 can directly interact with β-catenin through its tyrosine kinase domain. PTK7 deficient cells exhibit weakened β-catenin/T-cell factor transcriptional activity upon Wnt3a stimulation. These data are consistent with a role for PTK7 in the Wnt canonical signaling pathway. We decided to investigate the role of PTK7 in Wnt canonical signaling using the *Xenopus* embryo, which is ideally suited for this question. Using the combination of MOs shown to abolish Ptk7 function in PCP, we observed anterior truncations in a significant number of injected embryos. This phenotype is compatible with reduced β-catenin activity. This was supported by the reduction of dorsal organizer genes' expression (*siamois*, *chordin*, *noggin*, *goosecoid*), which are known to depend on β-catenin activity, in Ptk7 morphants [12]. Importantly, these defects were observed prior to overt gastrulation movements and could not be secondary to deficient PCP due to the lack of Ptk7. Finally, organizer gene induction caused by ectopic Wnt8 expression was dramatically reduced upon Ptk7 knockdown. Taken together, these data indicate that Ptk7 is required for β-catenin activity (Fig. 11.6). Studies by Peradziryi et al. showed that Ptk7 inhibits the canonical Wnt pathway in *Xenopus* and *Drosophila*. The proposed mechanism is that Ptk7

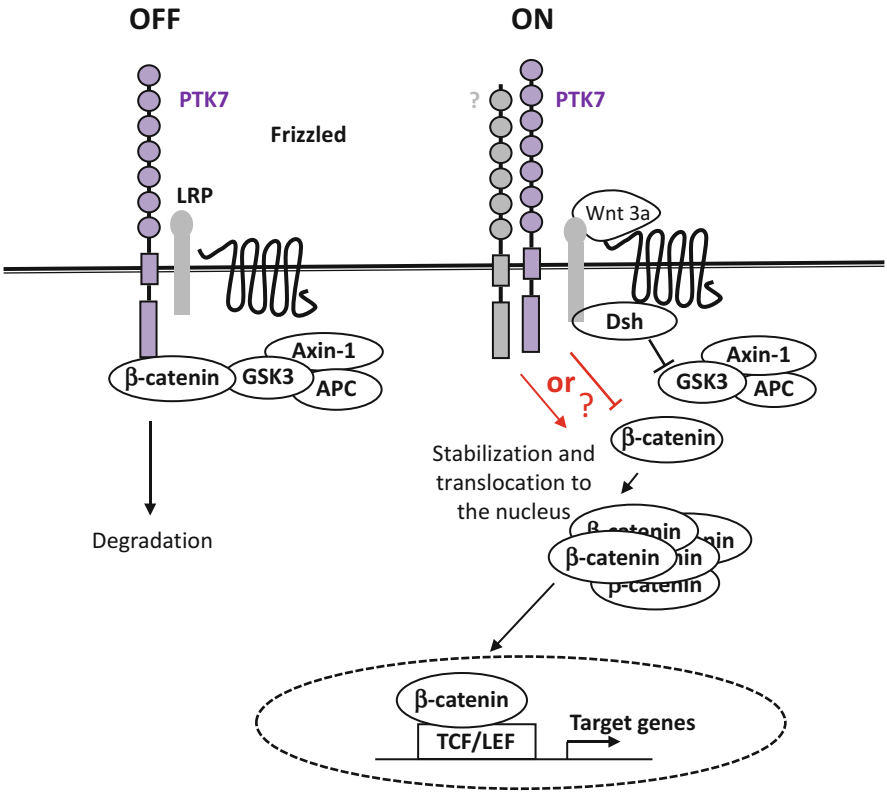


Fig. 11.6 Controversial roles of PTK7 in canonical Wnt pathway. In the absence of Wnt ligands (OFF), cytoplasmic β-catenin is complexed to Adenomatous polyposis coli (APC), Axin-1, and Glycogen synthase kinase 3β (GSK3β) and gets phosphorylated and targeted for ubiquitin-mediated degradation. Upon Wnt stimulation (ON), the complex is disrupted in a Dsh-dependent manner and stabilized β-catenin translocates to the nucleus, where it binds to LEF/TCF transcription factors and activates target gene expression. The positive or negative role of PTK7 on the canonical Wnt pathway is probably cell context dependent and could be directed by the presence or absence of additional receptor or ligand

interacts with canonical Wnt ligands (Wnt3a and Wnt8 in *Xenopus*, Wnt4 in *Drosophila*) and represses canonical Wnt signaling in embryonic patterning [52]. These controversial results could be explained by different cell contexts, and presence or absence of ligand or receptor at the plasma membrane (Fig. 11.6). A recent interaction described between PTK7 and LRP6 confirmed the role of PTK7 in canonical Wnt pathway. PTK7 knockdown inhibits embryonic Wnt/β-catenin signaling by strongly reducing LRP6 protein levels. Maintenance of high LRP6 protein levels by PTK7 triggers PCP inhibition. PTK7 could thus be a modulator of Wnt/PCP activity via LRP6 [53].

11.4.4.4 Major Genes Regulated

Being at the crossroads between canonical and non-canonical Wnt pathways, PTK7 expression positively or negatively impacts on the regulation of genes lying downstream of these signaling networks. In the non-canonical PCP pathway, PTK7 potentially triggers JNK activation that may ultimately lead to phosphorylation of the Jun transcription factor. To date, there is no report relating the direct implication of PTK7 in expression of PCP target genes.

As for the implication of PTK7 in the Wnt canonical pathways, its role in β-catenin/T-cell factor transcriptional activity is controversial. In HCT116 colon cancer cells, repression of PTK7 using small hairpin RNA decreases the transcriptional activity of β-catenin monitored by a classical TOP/FOP luciferase assay [12]. On the other hand, data obtained with a similar readout in another cellular system (HEK293 cells) showed an inhibition of canonical Wnt signaling [52]. In *Xenopus*, we showed that depletion of Ptk7 leads to reduced β-catenin activity correlated to reduced expression of β-catenin target genes (*siamois*, *chordin*, *noggin*, *goosecoid*) [12]. Contradictory results were obtained by others showing the capacity of Ptk7 to bind canonical Wnt ligands (Wnt3a and Wnt8) and repress the canonical Wnt pathway [52]. In this case, downregulation of Ptk7 leads to higher canonical Wnt pathway (Fig. 11.6).

11.4.4.5 Cross Talk with Other Receptor Systems

In addition to its implication in VEGFR1 (Fig. 11.4) and Wnt signaling (Figs. 11.5 and 11.6), PTK7 can also form a protein complex with members of the Plexin protein family. Plexins are transmembrane receptors that transduce signals upon binding of ligands of the Semaphorin family, leading to cytoskeleton remodeling thereby affecting cell shape, migration, and cell–cell interactions. The Plexin–Semaphorin pathway is important during embryogenesis as well as during adult life in playing a role in homeostasis of many tissues. Deregulation of the Plexin–Semaphorin pathway is also observed in many tumoral situations. In *Drosophila*, Ptk7 forms a complex with Plexin A1 [13]. This interaction is conserved in Vertebrates. Ptk7 indeed genetically cooperates with Plexin A1 and this pathway is required for migration of cranial neural crest cell in *Xenopus* [54]. Furthermore, in chicken, the Ptk7/Plexin A1 complex is involved in heart development in response to Sema6D [55].

11.4.5 PTK7 Internalization, Processing, and Attenuation

As mentioned early on, the extracellular domain of PTK7 is processed by MT1-MMP. Indeed, a soluble PTK7 form is generated by this membrane protease leading to the release of the cleaved receptor in the supernatants of cultured cell lines.

This cleavage is functionally important as shown by in vivo data in *zebrafish* and mice. Injection of MT1-MMP MOs increases Ptk7 levels in *zebrafish* embryos. Furthermore, genetic interaction between Ptk7 and MT1-MMP was demonstrated in these animals using combined injection of MOs that knockdown expression of the proteins. PCP and convergent extension defects are indeed clearly observed in *zebrafish* embryos treated by a simultaneous injection of suboptimal doses of Ptk7 and MT1-MMP MOs [34]. A N-ethyl-N-nitrosourea (ENU)-induced mouse PCP mutant, *chuzhoi*, develops strong PCP defects likely due to a mutation introducing an additional MT1-MPP cleavage site (Ala-Asn-Pro motif) in between the Ig loops 5 and 6 of Ptk7. This mutation leads to an increased production of soluble Ptk7 and, in parallel, to decreased amounts of full-length Ptk7 found at the plasma membrane. PCP defects observed in the *chuzhoi* mutant are probably the combined result of reduced membrane localization of Ptk7 and hyperproduction of soluble Ptk7 [41].

11.4.6 Unique Features of PTK7

PTK7 is an atypical RTK highly conserved through evolution. Accumulated data from several labs place PTK7 as a crucial regulator of Wnt pathways in both Invertebrates and Vertebrates. Its suspected role in cell adhesion in *Drosophila* remains to be investigated in Vertebrates. As previously mentioned, many issues (ligands, signaling pathway, target genes) have now to be addressed to precise PTK7 functions. PTK7 has without doubt an important role in physiology as demonstrated by the spectacular PCP phenotype observed during embryonic development of null mutants. One important issue is now to address the role of PTK7 in adult normal tissues.

From a clinical standpoint, PTK7 has been described as overexpressed in a large number of cancers. A potential role of PTK7 in cell migration and/or apoptosis was observed in colon cancer and acute myeloid leukemia, and this suggests that PTK7 may represent a novel therapeutic target. In addition, positive PTK7 implication in VEGF signaling may be thought as a new way to develop anti-angiogenic therapies. Being overexpressed in many cancers, PTK7 could be also considered as a suitable receptor for targeted drug delivery. Recent studies described the use of PTK7-specific aptamers able to deliver chemotherapy in leukemia cell lines [56, 57]. As Wnt pathways are implicated in many diseases, targeting of PTK7 may also offer some opportunities in the future for treatments other than cancers (cardiac diseases, skeletal diseases,…).

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Receptor at a glance: human PTK7

Chromosome location	Chromosome 6p21.1 → 12.2
Gene size (bp)	4,272 bp
Intron/exon numbers	20 exons
mRNA Size (5′ORF3′)	3,213 bp
Amino acid number	1,070
kDa	118.5
Posttranslational modifications	• Four characterized N-glycosylation sites (N116, N175, N268, and N283) • Cleavage of the extracellular domain by MT1-MMP ($L^{621}I^{622}$)
Domains	Extracellular domain: seven immunoglobulin loops Transmembrane region Intracellular domain: Tyrosine kinase domain with poor activity
Ligands	Not yet identified
Known dimerizing partners	VEGFR1
Pathways activated	Non-canonical Wnt pathway (RhoA, JNK) Canonical Wnt pathway (β-catenin)
Tissues expressed	Ubiquitous expression at low level in epithelial, endothelial, and hematopoietic tissues
Human diseases	High expression in tumoral tissues: colon cancer, melanoma, breast cancer, gastric cancer, prostate cancer, glioma cells, acute myeloid leukemia, acute lymphoid leukemia
Knockout mouse phenotype	PCP and convergent extension defects: major neural tube closure defect (craniorachischisis), abdomen closure defect, polydactyly, smaller kidney, open eyes

References

1. Mossie K, Jallal B, Alves F, Sures I, Plowman GD, Ullrich A. Colon carcinoma kinase-4 defines a new subclass of the receptor tyrosine kinase family. Oncogene. 1995;11(10): 2179–84.
2. Lee ST, Strunk KM, Spritz RA. A survey of protein tyrosine kinase mRNAs expressed in normal human melanocytes. Oncogene. 1993;8(12):3403–10.
3. Park SK, Lee HS, Lee ST. Characterization of the human full-length PTK7 cDNA encoding a receptor protein tyrosine kinase-like molecule closely related to chick KLG. J Biochem. 1996;119(2):235–9.
4. Jung JW, Ji AR, Lee J, Kim UJ, Lee ST. Organization of the human PTK7 gene encoding a receptor protein tyrosine kinase-like molecule and alternative splicing of its mRNA. Biochim Biophys Acta. 2002;1579(2–3):153–63.
5. Simons M, Mlodzik M. Planar cell polarity signaling: from fly development to human disease. Annu Rev Genet. 2008;42:517–40.
6. Lu X, Borchers AGM, Jolicoeur C, Rayburn H, Baker JC, Tessier-Lavigne M. PTK7/CCK-4 is a novel regulator of planar cell polarity in vertebrates. Nature. 2004;430(6995):93–8.
7. Margolis B, Borg J-P. Apicobasal polarity complexes. J Cell Sci. 2005;118(Pt 22):5157–9.
8. Yen WW, Williams M, Periasamy A, Conaway M, Burdsal C, Keller R, et al. PTK7 is essential for polarized cell motility and convergent extension during mouse gastrulation. Development. 2009;136(12):2039–48.

9. Andreeva A, Lee J, Lohia M, Wu X, Macara IG, Lu X. PTK7-Src signaling at epithelial cell contacts mediates spatial organization of actomyosin and planar cell polarity. Dev Cell. 2014;29(1):20–33.
10. Williams M, Yen W, Lu X, Sutherland A. Distinct apical and basolateral mechanisms drive planar cell polarity-dependent convergent extension of the mouse neural plate. Dev Cell. 2014;29(1):34–46.
11. Shnitsar I, Borchers A. PTK7 recruits dsh to regulate neural crest migration. Development. 2008;135(24):4015–24.
12. Puppo F, Thomé V, Lhoumeau A-C, Cibois M, Gangar A, Lembo F, et al. Protein tyrosine kinase 7 has a conserved role in Wnt/β-catenin canonical signalling. EMBO Rep. 2011; 12(1):43–9.
13. Winberg ML, Tamagnone L, Bai J, Comoglio PM, Montell D, Goodman CS. The transmembrane protein Off-track associates with Plexins and functions downstream of Semaphorin signaling during axon guidance. Neuron. 2001;32(1):53–62.
14. Iliescu A, Gravel M, Horth C, Kibar Z, Gros P. Loss of membrane targeting of Vangl proteins causes neural tube defects. Biochemistry (Mosc). 2011;50(5):795–804.
15. Kibar Z, Torban E, McDearmid JR, Reynolds A, Berghout J, Mathieu M, et al. Mutations in VANGL1 associated with neural-tube defects. N Engl J Med. 2007;356(14):1432–7.
16. Endoh H, Tomida S, Yatabe Y, Konishi H, Osada H, Tajima K, et al. Prognostic model of pulmonary adenocarcinoma by expression profiling of eight genes as determined by quantitative real-time reverse transcriptase polymerase chain reaction. J Clin Oncol. 2004;22(5):811–9.
17. Gorringe KL, Boussioutas A, Bowtell DDL, Melbourne Gastric Cancer Group, Peter Mac Micro Array Facility. Novel regions of chromosomal amplification at 6p21, 5p13, and 12q14 in gastric cancer identified by array comparative genomic hybridization. Genes Chromosomes Cancer. 2005;42(3):247–59.
18. Easty DJ, Mitchell PJ, Patel K, Flørenes VA, Spritz RA, Bennett DC. Loss of expression of receptor tyrosine kinase family genes PTK7 and SEK in metastatic melanoma. Int J Cancer. 1997;71(6):1061–5.
19. Ataseven B, Angerer R, Kates R, Gunesch A, Knyazev P, Högel B, et al. PTK7 expression in triple-negative breast cancer. Anticancer Res. 2013;33(9):3759–63.
20. Ataseven B, Gunesch A, Eiermann W, Kates RE, Högel B, Knyazev P, et al. PTK7 as a potential prognostic and predictive marker of response to adjuvant chemotherapy in breast cancer patients, and resistance to anthracycline drugs. Onco Targets Ther. 2014;7:1723–31.
21. Gärtner S, Gunesch A, Knyazeva T, Wolf P, Högel B, Eiermann W, et al. PTK 7 is a transforming gene and prognostic marker for breast cancer and nodal metastasis involvement. PLoS One. 2014;9(1):e84472.
22. Shin W-S, Kwon J, Lee HW, Kang MC, Na H-W, Lee S-T, et al. Oncogenic role of protein tyrosine kinase 7 in esophageal squamous cell carcinoma. Cancer Sci. 2013;104(8):1120–6.
23. Zhang H, Wang A, Qi S, Cheng S, Yao B, Xu Y. Protein tyrosine kinase 7 (PTK7) as a predictor of lymph node metastases and a novel prognostic biomarker in patients with prostate cancer. Int J Mol Sci. 2014;15(7):11665–77.
24. Jin J, Ryu HS, Lee KB, Jang J-J. High expression of protein tyrosine kinase 7 significantly associates with invasiveness and poor prognosis in intrahepatic cholangiocarcinoma. PLoS One. 2014;9(2):e90247.
25. Liu Q, Zhang C, Yuan J, Fu J, Wu M, Su J, et al. PTK7 regulates Id1 expression in CD44-high glioma cells. Neuro-Oncol. 2014;17:505–15.
26. Kim J-H, Kwon J, Lee HW, Kang MC, Yoon H-J, Lee S-T, et al. Protein tyrosine kinase 7 plays a tumor suppressor role by inhibiting ERK and AKT phosphorylation in lung cancer. Oncol Rep. 2014;31(6):2708–12.
27. Chen R, Khatri P, Mazur PK, Polin M, Zheng Y, Vaka D, et al. A meta-analysis of lung cancer gene expression identifies PTK7 as a survival gene in lung adenocarcinoma. Cancer Res. 2014;74(10):2892–902.

28. Prebet T, Lhoumeau A-C, Arnoulet C, Aulas A, Marchetto S, Audebert S, et al. The cell polarity PTK7 receptor acts as a modulator of the chemotherapeutic response in acute myeloid leukemia and impairs clinical outcome. Blood. 2010;116(13):2315–23.
29. Haines CJ, Giffon TD, Lu L-S, Lu X, Tessier-Lavigne M, Ross DT, et al. Human CD4+ T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function. J Exp Med. 2009;206(2):275–85.
30. Meng L, Sefah K, O'Donoghue MB, Zhu G, Shangguan D, Noorali A, et al. Silencing of PTK7 in colon cancer cells: caspase-10-dependent apoptosis via mitochondrial pathway. PLoS One. 2010;5(11):e14018.
31. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell. 2003;3(6):537–49.
32. Speers C, Tsimelzon A, Sexton K, Herrick AM, Gutierrez C, Culhane A, et al. Identification of novel kinase targets for the treatment of estrogen receptor-negative breast cancer. Clin Cancer Res. 2009;15(20):6327–40.
33. Saha S, Bardelli A, Buckhaults P, Velculescu VE, Rago C, St Croix B, et al. A phosphatase associated with metastasis of colorectal cancer. Science. 2001;294(5545):1343–6.
34. Golubkov VS, Chekanov AV, Cieplak P, Aleshin AE, Chernov AV, Zhu W, et al. The Wnt/planar cell polarity protein-tyrosine kinase-7 (PTK7) is a highly efficient proteolytic target of membrane type-1 matrix metalloproteinase: implications in cancer and embryogenesis. J Biol Chem. 2010;285(46):35740–9.
35. Shin W-S, Maeng Y-S, Jung J-W, Min J-K, Kwon Y-G, Lee S-T. Soluble PTK7 inhibits tube formation, migration, and invasion of endothelial cells and angiogenesis. Biochem Biophys Res Commun. 2008;371(4):793–8.
36. Lee HK, Chauhan SK, Kay E, Dana R. Flt-1 regulates vascular endothelial cell migration via a protein tyrosine kinase-7-dependent pathway. Blood. 2011;117(21):5762–71.
37. Banga SS, Ozer HL, Park SK, Lee ST. Assignment of PTK7 encoding a receptor protein tyrosine kinase-like molecule to human chromosome 6p21.1–>p12.2 by fluorescence in situ hybridization. Cytogenet Cell Genet. 1997;76(1–2):43–4.
38. Jung J-W, Shin W-S, Song J, Lee S-T. Cloning and characterization of the full-length mouse Ptk7 cDNA encoding a defective receptor protein tyrosine kinase. Gene. 2004;328:75–84.
39. Savory JGA, Mansfield M, Rijli FM, Lohnes D. Cdx mediates neural tube closure through transcriptional regulation of the planar cell polarity gene Ptk7. Development. 2011;138(7):1361–70.
40. López-Otín C, Hunter T. The regulatory crosstalk between kinases and proteases in cancer. Nat Rev Cancer. 2010;10(4):278–92.
41. Golubkov VS, Aleshin AE, Strongin AY. Potential relation of aberrant proteolysis of human protein tyrosine kinase 7 (PTK7) chuzhoi by membrane type 1 matrix metalloproteinase (MT1-MMP) to congenital defects. J Biol Chem. 2011;286(23):20970–6.
42. Golubkov VS, Strongin AY. Insights into ectodomain shedding and processing of protein-tyrosine pseudokinase 7 (PTK7). J Biol Chem. 2012;287(50):42009–18.
43. Golubkov VS, Prigozhina NL, Zhang Y, Stoletov K, Lewis JD, Schwartz PE, et al. Protein-tyrosine pseudokinase 7 (PTK7) directs cancer cell motility and metastasis. J Biol Chem. 2014;289(35):24238–49.
44. Golubkov VS, Strongin AY. Downstream signaling and genome-wide regulatory effects of PTK7 pseudokinase and its proteolytic fragments in cancer cells. Cell Commun Signal. 2014;12:15.
45. Smith DK, Xue H. Sequence profiles of immunoglobulin and immunoglobulin-like domains. J Mol Biol. 1997;274(4):530–45.
46. Grassot J, Gouy M, Perrière G, Mouchiroud G. Origin and molecular evolution of receptor tyrosine kinases with immunoglobulin-like domains. Mol Biol Evol. 2006;23(6):1232–41.
47. Wollscheid B, Bausch-Fluck D, Henderson C, O'Brien R, Bibel M, Schiess R, et al. Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. Nat Biotechnol. 2009;27(4):378–86.

48. Pulido D, Campuzano S, Koda T, Modolell J, Barbacid M. Dtrk, a Drosophila gene related to the trk family of neurotrophin receptors, encodes a novel class of neural cell adhesion molecule. EMBO J. 1992;11(2):391–404.
49. Montcouquiol M, Crenshaw EB, Kelley MW. Noncanonical Wnt signaling and neural polarity. Annu Rev Neurosci. 2006;29:363–86.
50. Wehner P, Shnitsar I, Urlaub H, Borchers A. RACK1 is a novel interaction partner of PTK7 that is required for neural tube closure. Development. 2011;138(7):1321–7.
51. Peradziryi H, Tolwinski NS, Borchers A. The many roles of PTK7: a versatile regulator of cell-cell communication. Arch Biochem Biophys. 2012;524(1):71–6.
52. Peradziryi H, Kaplan NA, Podleschny M, Liu X, Wehner P, Borchers A, et al. PTK7/Otk interacts with Wnts and inhibits canonical Wnt signalling. EMBO J. 2011;30(18):3729–40.
53. Bin-Nun N, Lichtig H, Malyarova A, Levy M, Elias S, Frank D. PTK7 modulates Wnt signaling activity via LRP6. Development. 2014;141(2):410–21.
54. Wagner G, Peradziryi H, Wehner P, Borchers A. PlexinA1 interacts with PTK7 and is required for neural crest migration. Biochem Biophys Res Commun. 2010;402(2):402–7.
55. Toyofuku T, Zhang H, Kumanogoh A, Takegahara N, Suto F, Kamei J, et al. Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2. Genes Dev. 2004;18(4):435–47.
56. Taghdisi SM, Abnous K, Mosaffa F, Behravan J. Targeted delivery of daunorubicin to T-cell acute lymphoblastic leukemia by aptamer. J Drug Target. 2010;18(4):277–81.
57. Xiao Z, Shangguan D, Cao Z, Fang X, Tan W. Cell-specific internalization study of an aptamer from whole cell selection. Chemistry. 2008;14(6):1769–75.

Chapter 12
The RET Receptor Family

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Abbreviations

aa	Amino acid
ARTN	Artemin
BCR	Breakpoint cluster region
CAKUT	Congenital anomalies of the kidney or lower urinary tract
CCHS	Congenital central hypoventilation syndrome
Chip	Chromatin immunoprecipitation
CLD	Cadherin-like domain
CMML	Chronic myelomonocytic leukemia
CNS	Central nervous system
CRC	Colorectal cancer
CRD	Cysteine-rich domain
CREB	Cyclic-AMP-response element binding protein
DA	Dopaminergic
DNM2	Dynamin 2 GTPase
ENS	Enteric nervous system
ERE	Estrogen-responsive elements
ET	Endothelin
FDA	Food and Drug Administration
FGFR1OP	Fibroblast Growth Factor Receptor 1 Oncogenic Partner
GDNF	Glial cell line-derived neurotrophic factor
GFL	Glial cell line-derived neurotrophic factor family
GFRα	GDNF receptor-α family

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GPI	Glycosylphosphatidylinositol
HMG	High-mobility group
HSC	Hematopoietic stem cell
HSCR	Hirschsprung's disease
JNK	c-Jun N-terminal kinase
kbp	Kilobase pair
KIF5B	Kinesin family member 5B
LAR	Leukocyte antigen related
LTβ	Lymphotoxin β
LTβR	LTβ receptor
MAPK	Mitogen-activated protein kinases
MEN	Multiple endocrine neoplasia
MEN2A	Multiple endocrine neoplasia type 2A
MEN2B	Multiple endocrine neoplasia type 2B
MTC	Medullary thyroid carcinoma
NGF	Nerve growth factor
NRTN	Neurturin
NSCLC	Non small cell lung cancer
OMIM	Online Mendelian Inheritance in Men
PD	Parkinson's disease
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PLCγ	Phospholipase Cγ
PNI	Perineural invasion
PP	Peyer's patches
PSPN	Persephin
PTB	Phosphotyrosine-binding domains
PTC	Papillary thyroid carcinoma
PTP	Protein tyrosine phosphatase(s)
RA	Retinoic acid
RET	REarranged during Transfection
RTK	Receptor tyrosine kinase
SAXS	Solution low-angle X-ray scattering
SCG	Superior cervical ganglion
SCLC	Small cell lung adenocarcinoma
SERM	Selective estrogen receptor modulator
TGF-β	Transforming growth factor β
TK	Tyrosine kinase
TRPA1	Transient receptor potential family of cation channels
TSS	Transcription start site
UTR	Untranslated regions

12.1 Introduction to the RET Receptor Tyrosine Kinase

RET (REarranged during Transfection) is a unique receptor tyrosine kinase (RTK) characterized by the presence of cadherin-like domains in its extracellular region. *RET* gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, and fruit fly. *RET* plays a critical role in oncogenesis and organogenesis [1–4].

As far as oncogenesis is concerned, *RET* was originally identified as an oncogene activated by a gene rearrangement occurred in vitro during transfection of NIH3T3 fibroblasts with DNA from a human T-cell lymphoma [5]. Subsequently, by searching for activated oncogenes in human papillary thyroid carcinomas (PTC), a transforming oncogene (*RET*/PTC) was isolated, which turned out to be a rearranged version of *RET* [6, 7]. More recently, *RET* gene rearrangements have been also found in lung adenocarcinoma, chronic myeloproliferative disorders, and Spitz tumors [8, 9]. *RET* germline point mutations cause multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B) and, at somatic level, are associated with sporadic medullary thyroid carcinoma (MTC) [8–11].

As far as organogenesis is concerned, *RET* loss-of-function mutations are found in patients with Hirschsprung's disease (HSCR, aganglionic megacolon) and in patients with congenital anomalies of the kidney or lower urinary tract (CAKUT) [12–14].

The human *RET* gene is located on chromosome 10q11.2. RET extracellular portion contains 4 cadherin-like domains (CLD1-CLD4) followed by a juxtamembrane cysteine-rich domain (CRD). Its intracytosolic tyrosine kinase domain (TKD) is splitted into two subdomains by a short kinase insert. Alternative splicing generates three different RET forms differing at the carboxyl-terminal tail (Fig. 12.1) [1–4].

RET ligands belong to glial cell line-derived neurotrophic factor (GDNF) family (GFL). GFLs are members of the cystine-knot superfamily, which also includes transforming growth factor (TGF)-β. GFLs include GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN) [1–4]. Binding to GFLs occurs with a 2:2:2 stoichiometry within a ternary receptor complex, in which ligand-binding components are represented by glycosylphosphatidylinositol (GPI)-linked co-receptors (GDNF receptor alpha: GFRα) and the signaling unit is represented by RET. Four different GFRαs (GFRα 1–4) feature preferential binding specificity for GDNF, NRTN, ARTN, and PSPN, respectively (Fig. 12.1) [1–4].

RET expression is typically found in neuroendocrine tumors, such as neuroblastoma, pheochromocytoma, and MTC. As far as normal tissues are concerned, *RET* is expressed in neural tissues, including peripheral enteric, sympathetic, and sensory neurons. *RET* expression is also detected in motor, dopaminergic, and noradrenergic neurons, in the developing excretory system (mesonephric duct and branching ureteric bud) and in spermatogonia [1–4]. Consistent with this expression pattern, targeted inactivation of the murine *RET* locus results in a complex phenotype, including defective development of enteric neurons and kidney [14].

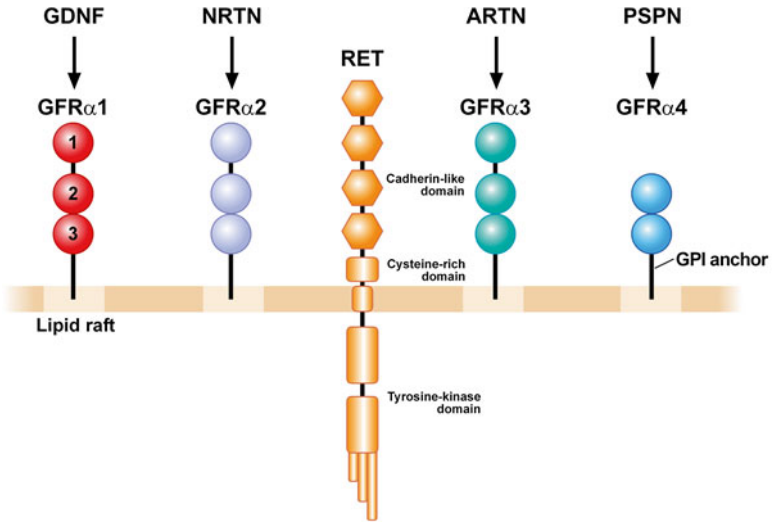

Fig. 12.1 RET, ligands, and co-receptors. RET protein with the four extracellular cadherin-like, cysteine-rich, transmembrane, and intracellular tyrosine kinase (splitted into two portions) domains are represented. Alternative splicing generates three different RET forms differing at the COOH-tail. Also represented are the RET functional ligands of the GFL (GDNF family ligands) family: glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), and persephin (PSPN). GDNF family receptor-α (GFRαs) are glycosylphosphatidylinositol (GPI)-anchored proteins and feature three (GFRα4 has only two) globular cysteine-rich domains

12.2 The Role of RET in Embryonic Development and Adult Physiology

Mouse *RET* transcripts are detected beginning at day 8.5 p.c. in neural crest cells migrating from rhombomere 4 and forming the anlage of the facioacoustic ganglion, and in closely associated domains of surface ectoderm and pharyngeal endoderm [15, 16]. *RET* expression is detected in spinal and encephalic ganglia, and in sensory organs, such as ganglial layer of the retina and olfactory epithelium. *RET* expression is also found in the wall of the gut (enteric nervous system, ENS) and in the nephrogenic zone of the developing kidney cortex (metanephrogenic vesicles) [16]. A similar pattern of expression is found in rat [17] and fruit fly [18]. *RET* mRNA is also present in distinct areas of the mouse central nervous system (CNS), such as developing spinal cord and brain stem. In spinal cord, *RET* expression is found in somatic and visceral motor columns. In the brain stem, *RET* is expressed in cranial motor and sensory nuclei [1–4].

Consistent with its expression pattern, targeted inactivation of *RET* in mice causes defective development of ENS and agenesis or dysgenesis of the kidney [12–14]. *RET* inactivation impairs colonization of the gut by the enteric neuroblasts also in zebrafish [19, 20]. Mechanistically, *RET* inactivation causes defective

migration, decreased proliferation, and apoptotic death of ENS progenitors into the gastrointestinal mesenchyme [21, 22]. Accordingly, about 50 % of patients with HSCR display *RET* mutations, indicating a critical role played by *RET* in the innervation of the human gut (see Sect. 12.3.2) [23]. *RET* inactivation in the mouse also causes the loss or size reduction of superior cervical ganglion (SCG). The defect in SCG is likely due to combined effects of impaired migration, proliferation, survival, and delayed differentiation [24]. *RET* is also required for the development of mouse cholinergic sympathetic neurons [25]. *RET* is expressed in dorsal root ganglia (DRG) [26]. Although *RET* knockout does not affect the number of DRG neurons, the expression of a member of the transient receptor potential family of cation channels (TRPA1) is completely lost in DRG at postnatal day 14 in *RET-null* mice. TRPA1 has been proposed to function in diverse sensory processes, including thermosensation and pain. Consistently, GDNF and ARTN regulate responsiveness of DRG sensory neurons to mechanical, thermal, and chemical challenges, and mice overexpressing these ligands feature increased levels of TRPA1 in DRG neurons [27, 28].

RET-knockout mice present kidney agenesis or dysgenesis (see Sect. 12.3.2) [12–14]. The development of kidney is a highly regulated process induced by the reciprocal interaction between the nephrogenic mesenchyme and the ureteric bud [12, 13]. Mesenchyme-derived GDNF stimulates *RET*-positive cells on the tips of branching ureteric bud [21, 29–31]. In turn, RET stimulation induces a feedback signal through the upregulation of the *Spry1* gene; this, in turn, downregulates GDNF-mediated signaling [32].

Disruption of the GDNF/RET/GFRα1 signaling in testis results in disturbed spermatogenesis. Testis-specific overexpression of GDNF results in disturbed spermatogenesis and, in older animals, testicular tumors [33]. In seminiferous tubules, GDNF is expressed by Sertoli cells, the somatic cells that regulate gametogenesis. *RET* and *GFRa1*, instead, are expressed by a subset of spermatogonial cells, probably representing stem cells. Accordingly, mice expressing a dominant-negative *RET* allele, which displayed reduced kinase activity, show alterations of early spermatogenesis [34]. Thus, during spermatogenesis, reduced levels of GDNF lead to increased differentiation and depletion of the stem cell pool. In contrast, excessive RET/GFRα1 signaling promotes stem cell renewal and inhibits differentiation [33, 34].

RET is expressed in several adult neuronal populations, including peripheral (enteric, sympathetic, and sensory) and central (motor, dopaminergic, and noradrenergic) neurons. The neonatal mortality of *RET*-null mice precludes the evaluation of postnatal role of *RET*. Nevertheless, selective *RET* deletion in dopaminergic neurons shows that *RET* is dispensable for their maintenance. However, nigrostriatal abnormality, with loss of dopaminergic neurons, alteration in dopamine release, degeneration of striatum, and glial activation, is observed in aged mice following conditional *RET* ablation. These features are suggestive of presymptomatic Parkinson's Disease (PD) [35]. Accordingly, RET was able to rescue the phenotype, in particular mitochondrial function, of *Drosophila Pink1* mutant, a model for autosomal recessive PD [36]. *RET*, *GFRα*s, and *GFL*s are expressed in the retina

[37, 38], and GFLs' administration inhibits photoreceptor cell death in animal models of retinal degeneration [39]. *RET*-hypomorphic mice, able to survive for several weeks after birth, show altered electro-retinograms and selective *RET* ablation causes abnormal retinal function [40]. Finally, selective *RET* ablation decreases number and size of nociceptive neurons. Accordingly, mutant mice display abnormal response to cold and pain [41].

As far as its role outside nervous system is considered, *RET* contributes to the formation of gut-associated secondary lymphoid tissue (Peyer's patches: PP) and in *RET*-knockout mice intestine lacks PPs [42]. PP formation is driven by myeloid inducer and stromal organizer cells. The first secrete lymphotoxin β (LTβ) and the second express the LTβ receptor (LTβR). A particular myeloid cell population, which expresses both RET and LTβ, is recruited in PPs by organizer cells, which secrete ARTN. In turn, RET-positive cells, by producing LTβ, induce organizer cells to express adhesion molecules and several lymphoid chemoattractants. These interactions lead to the recruitment of lymphocytes that finally form PPs [42].

In mice, *RET* and its co-receptors are expressed in hematopoietic stem cells (HSCs) resident in fetal liver and in bone marrow. HSCs' survival depends on signals deriving from microenvironment, including GFLs. In *RET-null* mice, HSCs maintain their differentiation potential while losing their ability to replace bone marrow upon irradiation. This is caused by lack of survival signals secondary to impaired RET-p38MAPK-CREB (cyclic-AMP-response element binding protein) cascade, which is necessary for the transcription of anti-apoptotic Bcl2/Bcl2l1 factors [43].

Finally, in normal human and rodent pituitary somatotroph cells and GH-secreting somatotroph pituitary adenomas (somatotropinomas), RET is expressed together with GDNF and GFRα1 and functions as a dependence receptor, e.g., able to trigger cell death when unbound to the cognate ligand (see Sect. 12.4.2.1) [44–46]. In fact, when somatotroph cells are deprived by ligand, RET protein is processed by caspases, releasing an intracellular fragment that induces the expression of Pit1 transcription factor, which in turn activates a p19Arf/p53-dependent apoptotic pathway. *RET-null* mice display pituitary hyperplasia secondary to the lack of such a mechanism [44, 45].

Finally, additional roles in developing olfactory system, preventing keratoconjunctivitis sicca, limiting consequences of cerebral ischemia, and sustaining thyroid calcitonin levels were unveiled through genetic manipulations of GFLs or their co-receptors [1, 8, 47–49].

12.3 The Role of RET in Human Disease

12.3.1 RET in Cancer

Different human cancers, including thyroid carcinoma, display gain-of-function structural alterations in *RET* which convert it into a dominantly transforming oncogene. Thus, RET kinase inhibitory compounds can be used to treat these neoplastic diseases.

12.3.1.1 RET Mutations in Multiple Endocrine Neoplasia type 2 (MEN2) and Medullary Thyroid Carcinoma

Multiple Endocrine Neoplasia type 2 (MEN2) syndromes (Online Mendelian Inheritance in Men, OMIM: #171400) comprise two related dominantly inherited Mendelian disorders: MEN2A and MEN2B [9–11]. Medullary Thyroid Carcinoma (MTC) is always present in MEN2 patients. MTC arises from neural crest-derived calcitonin-producing thyroid parafollicular C cells. It represents less than 5 % of all thyroid cancers and it is familial or sporadic in about 25 % and 75 % of cases, respectively [9–11]. MEN2A is characterized by MTC associated with pheochromocytoma (a benign tumor of adrenal medulla) in 50 % of cases and parathyroid hyperplasia or adenoma in 10–30 % of cases. Several authors suggest that isolated Familial Medullary Thyroid Carcinoma (FMTC) should no longer be considered as a separate entity but as a variant of MEN2A [10]. MEN2B is characterized by MTC, pheochromocytoma, ganglioneuromatosis of the intestine, thickening of corneal nerves, and marfanoid habitus [9–11].

Germline *RET* gene mutations are responsible for virtually all MEN2 cases. Most common mutations target exons 10 and 11 in the extracellular domain or exons 13–16 in the intracellular domain of *RET* [9–11]. These mutations are annotated at www.sanger.ac.uk/genetics/CGP/cosmic and www.arup.utah.edu/database/MEN2 [50]. Most common mutations are represented in Fig. 12.2. MEN2A is associated most frequently with mutations of cysteine 634 (85 %) (exon 11), particularly C634R (single letter code), in the CRD. Other mutations are evenly distributed among the various cysteines of the CRD (C609, C611, C618, C620 in exon 10 or C630 in exon 11) and are associated with isolated form of familial MTC [9–11]. Mutations also target non-cysteine residues in *RET* ectodomain and *RET* TKD (including E768D, V804L, V804M). Most common MEN2B mutation is M918T in *RET* TKD (exon 16); more rarely, MEN2B patients harbor the A883F substitution or complex double mutations clustered on the same or two different *RET* allele [9–11] (Fig. 12.2).

MEN2-associated *RET* mutations have been classified into different disease risk levels, with M918T and A883F bearing the highest risk. This notion is important for planning the optimal timing of prophylactic thyroidectomy to prevent MTC [11].

RET mutations, mainly M918T, occur at the somatic level in about 50 % of sporadic MTC and their presence correlates with an aggressive disease phenotype [51].

MEN2/MTC-associated mutations activate *RET* oncogenic potential. In the case of CRD mutations, cysteine removal is believed to prevent the formation of intramolecular disulfide bonds, thus allowing free cysteines to form covalent intermolecular RET dimers [52]. Crystallographic analysis has shown that wild-type RET kinase domain adopts a tethered autoinhibited state that may be destabilized by the M918T mutation (see Sect. 12.4.2.3) [53, 54]. Accordingly, this mutation accelerates RET autophosphorylation and alters RET substrate specificity [54, 55].

Based on RET role in MTC formation, vandetanib and cabozantinib, 2 tyrosine kinase inhibitors with activity against RET and other tyrosine kinases, have been approved by the FDA for the treatment of medullary thyroid carcinoma [56–58].

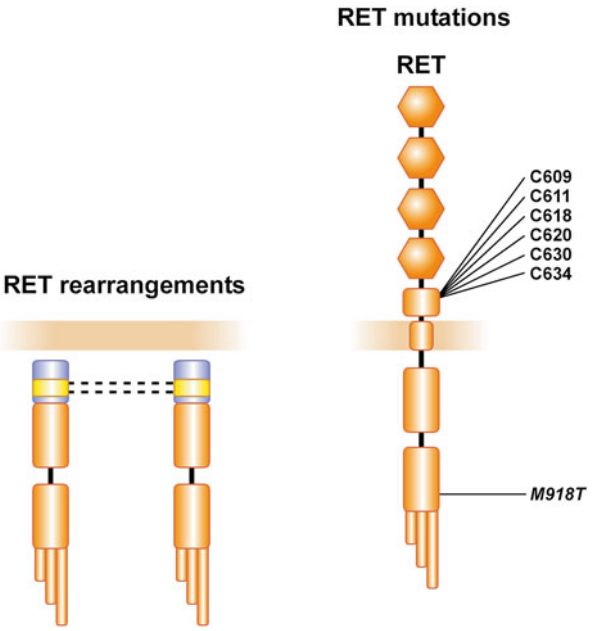

Fig. 12.2 RET oncogenic mutations. RET protein structure is represented. On the *left*, structure of RET gene rearrangements associated with papillary thyroid carcinoma (RET/PTC), lung adenocarcinoma, chronic myelomonocytic leukemia, and Spitz tumor is depicted. Dimerization mediated by coiled-coil domains in the RET fusion partners is shown. On the *right*, residues most commonly targeted by point mutations associated with sporadic MTC (frequently, M918T), MEN2A, and MEN2B are shown

12.3.1.2 RET Gene Rearrangements in Papillary Thyroid Carcinoma

Papillary thyroid carcinoma (PTC) originates from endodermal-derived thyroid follicular cells and it is the most frequent thyroid cancer type [59]. In PTC, with a frequency that varies in different patient series, chromosomal rearrangements, most commonly paracentric inversions of chromosome 10, cause the generation of chimeric oncogenes named RET/PTC [59]. These oncogenes are composed by the TKD (starting from residue 713) and COOH-tail encoding sequence of *RET* (from exon 12 to the 3′-end) fused, at the 5′-end, to the promoter sequence and 5′-terminal exons of different heterologous genes [59] (Fig. 12.2). Most common (90 % of the cases) rearrangements are RET/PTC1 (*CCDC6-RET*) and RET/PTC3 (*NCOA4-RET*) [7, 8, 59]. RET/PTC oncoproteins transform thyroid cells in culture [60] and thyroid-directed RET/PTC expression induces thyroid hyperplasia or neoplasia in transgenic mice [61].

CCDC6, *NCOA4*, and *RET* loci display close proximity in the chromatin of thyroid cells, this facilitating their illegitimate recombination in PTC [62, 63]. *RET* gene rearrangements, as well as rearrangements of other RTKs, are common in PTC

patients with a history of exposure to ionizing radiation, as those exposed to the Chernobyl power plant explosion, thereby suggesting that these rearrangements may be caused by ionizing radiation [64–66].

The RET fusion protein partners feature protein–protein interaction domains, such as coiled-coil motifs (Fig. 12.2). Thus, fusion results in constitutive dimerization of RET kinase and ligand-independent activation [67]. In addition, secondary to gene fusion, *RET* TKD is placed under the transcriptional control of promoter elements of fusion gene partners that are ubiquitously expressed and able to drive *RET* expression in thyroid follicular cells [59].

12.3.1.3 RET Gene Rearrangements in Lung Adenocarcinoma

Lung adenocarcinoma is a common type of non-small cell lung cancer (NSCLC) and it is commonly associated with mutations targeting RTKs, such as *EGFR*, *ROS1, NTRK1*, and *ALK* [68]. *RET* gene is rearranged in a fraction (about 2 %) of lung adenocarcinoma [69–73]. In these cases, chromosomal inversions cause the fusion of the *RET*-encoded TKD (from exon 12 to the 3′-end, as in RET/PTC rearrangements) to different 5′-terminal exons of *KIF5B* (kinesin family member 5B) gene (Fig. 12.2). Less commonly, the *RET*-encoded TKD is fused to *CCDC6* (as in RET/PTC1), *NCOA4* (as in RET/PTC3), or *TRIM33* (as in RET/PTC7) genes [74]. *RET*-rearranged lung adenocarcinomas tend to be poorly differentiated, to occur in young and never-smokers patients, and to have solid morphology [74]. Similar to RET/PTCs, KIF5B-RET fusion proteins form homodimers. Consistently, they display ligand-independent activation of RET kinase and transform fibroblasts in vitro [69]. An activating *RET* M918T mutation has been found in one case of small cell lung adenocarcinoma (SCLC) displaying metastatic behavior, and the introduction of the mutated *RET* allele in SCLC cell lines resulted in increased proliferation [75].

12.3.1.4 RET Gene Rearrangements in Chronic Myeloproliferative Disorder

RET gene rearrangements have been described in chronic myelomonocytic leukemia (CMML) [76] (Fig. 12.2). In one case, the *RET*-encoding TKD (from exon 12 to the 3′-end as in RET/PTC and in KIF5B-RET) was fused to the 5′-terminal four exons of *BCR* (breakpoint cluster region). In a second case, *RET* TKD was fused to the 5′-terminal twelve exons of *FGFR1OP* (Fibroblast Growth Factor Receptor 1 Oncogenic Partner). BCR-RET and FGFR1OP-RET fusion proteins transform hematopoietic cells in vitro [76]. A FGFR1OP-RET fusion event has been also described in one patient affected by primary myelofibrosis with secondary acute myeloid leukemia [77].

12.3.1.5 RET Rearrangements in Spitz Tumor

Spitz tumors include benign and malignant neoplasms, formed by spindle or epithelioid melanocytes that, differently from melanomas, generally lack activation of *BRAF*. Recently, a fraction (3 %) of Spitz tumors were found to carry *RET* gene rearrangements involving *KIF5B* and *GOLGA5* genes [78]. The resulting chimeric oncoproteins displayed increased phosphorylation and activation of downstream signaling, which could be obstructed by RET kinase inhibitors [78].

12.3.1.6 RET in Other Cancer Types

An increasing number of human cancer types display *RET* aberrant expression levels. *RET* is overexpressed in breast cancer, particularly in ERα (estrogen receptor α)-positive cases [79, 80] and *RET* levels associate with decreased metastasis-free and overall survival [81]. In breast cancer cells, *RET* expression is induced by estrogens and GDNF expression is induced by inflammatory cytokines [80–83]. RET stimulation activates breast cancer cell proliferation, survival, and motility [80–83]. In turn, RET stimulation induces mTOR-dependent phosphorylation and hormone-independent activation of ERα [83]. RET stimulation also triggers breast cancer cell resistance to aromatase inhibitors [84]. RET and IL-6 are linked in a feed-forward transcriptional loop, whereby IL-6-induced cell migration requires RET and both RET and IL-6 signaling impinge on FAK signaling for the induction of cell migration and metastatic ability [81]. Finally, the cholesterol metabolite 27-hydroxycholesterol, an endogenous selective estrogen receptor modulator (SERM), stimulates ER- and GDNF-RET-dependent breast cancer cell proliferation [85]. All together, these findings suggest that RET targeting could be exploited for the treatment of breast cancer.

In pancreatic ductal carcinomas, *RET* expression was found in about half of cases, correlating with tumor grade and metastasis. In particular, RET function is involved in perineural invasion (PNI) of pancreatic carcinomas, an event that is associated with poor prognosis. Secretion of RET ligands, GDNF or ARTN, and of soluble GFRα1 by nerves, enhances PNI [86, 87].

Despite its well-established role as an oncogene in several malignancies, *RET* has been recently proposed as a *bona fide* tumor suppressor in some colorectal cancers (CRC). Accordingly, CpG islands' promoter methylation silences *RET* expression in CRC with respect to normal tissue and this has been associated with poor prognosis [88, 89]. Moreover, rare somatic mutations target *RET* extracellular domain in CRC [90]. RET is able to trigger apoptosis of colon epithelial cells and loss of RET-mediated apoptosis, secondary to *RET* silencing or CRC-associated mutations, may foster CRC formation [88, 91].

12.3.2 RET in Developmental Disorders

12.3.2.1 RET in Hirschsprung's Disease

Hirschsprung's disease (HSCR, aganglionic megacolon: OMIM #142623) (1/5,000 live births) is a genetic neurocristopathy (disease of neural crest) characterized by absence of the parasympathetic ganglia of the myenteric and submucosal plexuses of ENS, leading to impaired peristalsis, functional obstruction, and chronic enlargement of the colon [92, 93]. HSCR can be familial or sporadic and occurs isolated or combined with other malformations in syndromes such as Shah-Waardenburg, Down, Bardet–Biedl, and congenital central hypoventilation (Ondine's curse) syndromes. At least ten different genes have been involved in HSCR [92, 93].

RET is expressed in ENS and *RET-null* mice display impaired development of ENS (see Sect. 12.2). Accordingly, familial and sporadic HSCR diseases are associated with germline mutations of *RET* in about 50 % and 15 % of the cases, respectively [23, 92, 93]. Over 100 different *RET* mutations have been described in HSCR patients; databases of these mutations can be found at UniProt (www.uniprot.org/) and Human Gene Mutation Database (www.hgmd.cf.ac.uk/ac/index.php) [94, 95].

Differently from cancer-associated mutations, HSCR-associated *RET* mutations do not cluster in hot-spots, are heterogeneous (deletions, nonsense and missense point mutations), and in most of the cases cause a loss of function of RET. Mutations in the RET extracellular domain impair cell surface expression, probably secondary to misfolding of the protein, while mutations in the RET TKD reduce enzymatic activity [96–98]. Finally, a few mutations in the RET carboxyl-terminal tail, close to RET tyrosine 1062 (Y1062), impair binding to signaling adaptors, such as SHC and others (see Sect. 12.4.2.3) [99]. Paradoxically, in some cases, HSCR cosegregates with MEN2A/MTC that, as described above, is, instead, associated with RET gain of function. These promiscuous HSCR-MEN2A/FMTC cases typically display mutations in RET CRD cysteines other than C634. Possibly, these particular mutations not only cause constitutive kinase activity but also a decreased cell surface expression of the RET protein that may cause HSCR [100, 101].

Finally, besides coding sequence mutations, *RET* haplotypes have been associated with HSCR [93, 102–104]. In particular, a nucleotide variant in the transcriptional enhancer in *RET* intron 1, which reduces *RET* expression, is a common low penetrance variant predisposing (>20-fold greater risk) to HSCR [105].

12.3.2.2 RET in Congenital Anomalies of the Kidney or Lower Urinary Tract

Congenital anomalies of the kidney or lower urinary tract (CAKUT) are a common (1/250 live births) cause of kidney failure and believed to be secondary to gene defects acquired early during development [12, 13]. *RET* is involved in kidney and lower urinary tract development and *RET-null* mice feature renal agenesis or aplasia (see Sect. 12.2). About 5–35 % of CAKUT patients harbor various *RET* and *RET*

signaling complex mutations [106–108]. Studies with genetically modified mice suggest that signals originated at the level of RET Y1015 and Y1062 docking sites are particularly important for proper kidney development, and loss of these RET-mediated signals may cause CAKUT (see Sect. 12.4.2.3).

12.4 RET

12.4.1 RET *Gene*

RET gene maps to chromosome 10q11.2, in a 5′ centromeric/3′ telomeric orientation [109, 110]. *RET* gene includes 21 coding exons and its size is approximately 55 kbp. Exon 1 and 2 are separated by a 24 kb intron, while exons 2–20 are included in a 31 kbp genomic region. A highly polymorphic CA repeat sequence is present within intron 5. Such a gene structure, i.e., a large first intron between exons 1 and 2 and other exons (2–20) clustered at the 3′-half of the gene, is reminiscent of other RTKs (*PDGFRB* and *KIT*) gene structures [109, 110].

12.4.1.1 RET Promoter Structure

A short genomic fragment of 453 bp located at the 5′-terminus of the human *RET* gene displays transcriptional promoter activity. In particular, a sequence including nucleotide −167 to +98 with respect to transcription start site (tss) is able to drive transcription [111]. This region is characterized by several transcription factor binding sites, such as Sp1, AP-2, and ETF. This region also contains four tandemly repeated GC boxes. High GC content and lack of canonical TATA and CAAT boxes are typical of Sp1 factor-regulated promoters. Accordingly, *RET* expression in the TT cell line, derived from a human MTC, depends on the binding of Sp1 and Sp3 to GC boxes distant 70 bp from the tss [112]. Through analysis of sequences conserved among species coupled with chromatin immunoprecipitation (Chip), many potential *RET* regulatory elements have been identified able to bind several transcription factors [113]. Furthermore, an analysis of the sequence 5 kbp upstream from *RET* tss revealed putative recognition sites for different constitutive, inducible, or developmentally regulated transcription factors [114].

As its human counterpart, the murine *RET* promoter lacks TATA and CAAT boxes and shows a high GC content. The major tss has been mapped 254 bp upstream the predicted translation initiating (ATG) site, and putative Sp1-binding sites have been identified in its most conserved regions. A region of 12 kb containing the regulatory sequences of the murine *RET* gene was fused to the β-galactosidase gene. Progressive deletions of this construct have been used to generate transgenic mice. These studies have shown that the 12 kb *RET* flanking sequence contains separable mesodermal and neuronal regulatory regions. However, β-galactosidase expression was not detected in developing motor neurons, in enteric and autonomic ganglia,

and in developing urogenital system, which normally express *RET,* thus suggesting that still unidentified additional *cis*-regulatory elements may be responsible for tissue-specific *RET* expression [115]. A small fragment of 380 bp upstream of the mouse ATG start codon was able to drive *RET* expression in neuronal cells [116]. Consistently, transgenic mouse lines carrying this fragment partially recapitulated the pattern of endogenous *RET* expression [117].

12.4.1.2 RET mRNA Structure

The *RET* gene codes for multiple transcripts thanks to alternative splicing mechanisms [118–122]. Moreover, multiple alternatively spliced *RET* transcripts have been detected in human neoplasms [122–124].

Alternative splicing of 3′ terminal exons results in transcripts encoding three RET protein isoforms bearing different C-terminal amino acid (aa) sequences: RET9 (1072 aa), RET51 (1114 aa), and RET43 (1106 aa) (Fig. 12.1) [118–122]. Such alternative splicing of *RET* 3′ sequences is highly conserved in human, murine, and other vertebrate species [125, 126]. RET9 and RET51 are the most abundant forms. *RET* exon 19 is present in all transcripts, and splicing at the 3′-end of exon 19 results in transcripts where exon 19 is unspliced (RET9), spliced to exon 20 (RET51), or spliced to exon 21 (RET43). Multiple polyadenylation sites and 3′ UTRs (untranslated regions) are associated with these three variants. The amino acid sequences of the alternative RET products diverge at codon 1063 which encodes a glycine in RET9 and RET51 and an aspartic acid in RET43. Thus, the last common amino acid for RET9, RET43, and RET51 is tyrosine 1062 (Y1062), a site which is essential for RET signaling (see Sect. 12.4.2.3). Alternative splicing places Y1062 in different amino acid contexts, thus conferring different binding properties. Moreover, RET51 contains an extra Grb2 docking site (Y1096) that preferentially activates phosphatidylinositol-3-kinase (PI3K)/AKT pathway (see Sect. 12.4.4.1) [127, 128].

To address functional significance of the alternatively spliced RET products, mice that express exclusively either the RET9 or RET51 isoform have been generated. In one study, a chimeric RET51 form consisting of mouse extracellular and human cytoplasmic RET domains was used. The resulting mice featured defects in kidney and ENS development, thus suggesting that RET51 was insufficient to drive normal RET functions [129]. Another transgenic model, exclusively expressing human RET9 or RET51 isoforms, suggested that the two forms are redundant since either one was fully competent for proper kidney development [130].

12.4.1.3 RET Transcriptional Regulation

Several transcription factors involved in embryonic development mediate regulation of *RET* gene expression. Besides already mentioned Sp1 factors (see Sect. 12.4.1.1), PAX proteins are also involved in *RET* regulation. PAX are members of the

paired-box containing transcription factor family and control the development of a variety of structures in which RET is involved, including neural crest and kidney (see Sect. 12.2). PAX3, in particular, is necessary for enteric ganglia formation and it is required for the regulation of *RET* gene expression during the development of ENS. PAX3 is capable of physically interacting and functionally synergizing with SOX10 to activate an enhancer element in the *RET* gene [131]. SOX10, in turn, belongs to the high-mobility group (HMG) of nuclear factors and regulates ENS differentiation [132–135]. Both *PAX3* and *SOX10* genes harbor mutations in patients with genetic syndromes characterized by defects in neural crest-derived structures including HSCR (see Sect. 12.3.2.1).

Mice and humans with renal hypoplasia display heterozygous mutations in *PAX2,* another *PAX* family member [136]. *PAX2,* together with *PAX8,* is expressed early in the mammalian metanephric duct and precedes *RET* expression. In vitro, both PAX2 and PAX8 can bind to *RET* promoter and activate its transcription [136].

Homeobox gene *HOXB5* is expressed in neural crest and its ablation in the mouse causes defective ENS development. HOXB5 binds *RET* promoter at −875 bp upstream tss [137]. HOXB5 synergizes with another transcription factor, NKX2-1/TTF1, to stimulate *RET* gene transcription [137]. NKX2-1/TTF1 is important for thyroid and lung development and it is also strongly expressed in ENS. A NKX2-1/TTF1 binding sequence is present in *RET* promoter [103].

Other factors are involved in the regulation of *RET* expression during development. The orphan nuclear receptor *Nurr1* is critical for the generation of dopaminergic (DA) neurons of the substantia nigra [138–140]. Noteworthy, also *RET* is involved in the maintenance of DA neurons (see Sect. 12.2). Knockdown of *Nurr1* in DA neurons results in a significant decrease in *RET* expression, and Nurr1 is capable of transactivating *RET* gene promoter. The fragment of *RET* gene that mediates Nurr1 activity is located in the proximal promoter, which however does not contain canonical Nurr1 binding elements, indicating that Nurr1 effect may be indirect [141].

PHOX2B is a neuronal type-specific paired-homeodomain transcription factor that is expressed throughout the developing sympathetic, parasympathetic, and enteric ganglia [142]. Autonomic ganglia fail to form properly in mice lacking PHOX2B, and PHOX2B is needed for the expression of *RET* [142]. Mutations in *PHOX2B* gene cause congenital central hypoventilation syndrome (CCHS), characterized by idiopathic failure of autonomic breathing and HSCR, also known as Ondine's curse (see Sect. 12.3.2.1) [143].

RET expression can be induced by differentiating agents, such as retinoic acid (RA) [144–146], and estrogens (E2) [82]. Consistently, different potential retinoic acid responsive elements (RARE) have been identified in *RET* regulatory region [114]. Moreover, estrogen-responsive elements (ERE) have been identified in the *RET* promoter [82]. Epigenetic modifications, such as chromatin and DNA methylation, influence *RET* promoter activity and are associated with RA-mediated *RET* transcriptional activation [147, 148]. Chromatin acetylation at the *RET* promoter has been detected that may modulate *RET* transcription rate [113].

12.4.2 RET Protein

12.4.2.1 RET Amino Acid Sequence and Processing

Different RET protein products of 1072 (RET9), 1106 (RET43), and 1114 (RET51) amino acids are generated secondary to alternative splicing of *RET* 3'mRNA (see Sect. 12.4.1.2). RET signal peptide spans amino acids 1–28. The transmembrane segment is composed of 22 amino acids (residues 636–657), among which S649 and S653 mediate self-association and dimerization of RET [149]. The intracellular portion of RET contains the TKD (residues 732–1112) splitted into two subdomains (Fig. 12.1).

RET is synthesized as a 120 kDa precursor, representing the unglycosylated core protein. Secondary to glycosylation, RET glycoproteins of 150 and 170 kDa are produced [15]. Cell fractionation experiments showed that only the 170 kDa isoform is sorted to the plasma membrane, while, as suggested by its sensitivity to endoglycosidase H, the 150 kDa isoform is an incompletely glycosylated immature precursor that resides in the endoplasmic reticulum [150–152].

RET belongs to the family of so-called "dependence" receptors. In the absence of cognate ligands, dependence receptors exert a pro-apoptotic activity that is blocked upon ligand stimulation [91, 153]. Such a pro-apoptotic activity is mediated by cleavage of RET cytosolic portion (between residues 707 and 708) by caspase-3, which, in turn, releases a C-terminal RET peptide that is able to induce cell death [46]. It is feasible that such activity is important for RET developmental function, because it may control migration of RET expressing cells by limiting survival of cells that move beyond ligand availability.

12.4.2.2 RET Domain Structure

Distinct domains can be identified in RET protein: (1) four cadherin-like domains (CLD) and one cysteine-rich region (CRD) in the extracellular region; (2) one transmembrane domain; and (3) one intracellular domain which includes the tyrosine kinase (TKD) domain split into two subdomains (Fig. 12.1). The structural domains of the extracellular RET are essential for binding to ligands and co-receptors (see Sect. 12.4.3).

CLD domains are stretches of 110 amino acids showing homology with the cadherin family of Ca^{2+}-dependent cell adhesion molecules. They share a common sequence which includes a Ca^{2+}-binding site. Accordingly, RET ectodomain binds Ca^{2+}, and this is required for RET binding to its ligands [154].

RET CRD contains 16 of the 28 cysteine residues of the RET ectodomain. Interestingly, most MEN2A or FMTC-causing mutations affect six of these extracellular cysteines (see Sect. 12.3.1.1).

RET TKD consists of a small N-terminal lobe and a large C-terminal lobe connected by a short linker [53].

12.4.2.3 RET Phosphorylation Sites

By phosphopeptide mapping [155] and mass spectrometry [156], several autophosphorylation sites were identified in RET intracellular domain: Y687, Y826, Y900, Y905, Y981, Y1015, Y1029, Y1062, and Y1096. Y687 maps in the juxtamembrane region, Y806 and Y809 in the N-terminal lobe of the TKD, Y826 in the kinase insert, Y900 and Y905 in the kinase activation loop between the DFG and APE (single letter amino acid code) motifs, Y981 in the C-terminal lobe of the TKD, and Y1015, Y1029, and Y1062 in the carboxyl-terminal tail. Y1090 and Y1096 are present only in RET51 isoform (see Sect. 12.4.1.2). Studies with phosphospecific antibodies confirmed phosphorylation of some of these sites (Y905, Y981, Y1015, Y1062, and Y1096) in vivo in intact cells [54, 157, 158].

The temporal sequence of RET phosphorylation events in physiological and oncogenic settings has been defined [54]. Upon ligand triggering, RET autophosphorylation occurs in *trans* and involves, initially, tyrosine residues flanking the TKD (Y687, Y1062), and, at a later time point, those located within the activation loop (Y900, Y905) and in other TKD flanking regions (Y1015, Y1029). Thus, at a variance from other RTKs for which phosphorylation events involving tyrosines within the activation loop are activating, in the case of RET Y900 and Y905 are not bona fide activating tyrosines [54]. Importantly, such a phosphorylation trajectory was perturbed by oncogenic RET point mutations (M918T and V804M) (see Sect. 12.3.1.1) that, indeed, cause a faster phosphorylation of activation loop residues. This is explained by the observation that the M918T mutation destabilizes the "in" conformation of the activation loop and exposes it to a faster phosphorylation in *trans*. However, such an oncogenic mutation does not simply enhance substrate presentation but also potentiates RET enzymatic activity by augmenting ATP occupancy of the kinase [54].

Autophosphorylated tyrosines serve as docking sites for signaling proteins (see Sect. 12.4.4.1) (Fig. 12.3). Y905 functions as a binding site for Grb7/10 adaptors [159]. Y981 is a docking site for SRC [160]. Y905 and Y981 are also involved in the binding of SH2B1β, a member of the SH2B family of adapters [161]. Y1015 binds phospholipase Cγ (PLCγ), this mediating activation of protein kinase C (PKC) [162]. Y1062 is a multidocking site for several phosphotyrosine-binding domains (PTB) containing adapters such as SHC, SHC-C, IRS1/2, FRS2, and DOK1/4/5. This residue is embedded in a NXXY sequence, which recognizes PTB-containing substrates, and is essential for the transforming ability of RET-derived oncogenes in cell cultures [163–165] and in transgenic mice [166]. Binding to SHC and FRS2 mediates recruitment of Grb2-SOS complexes leading to RAS/BRAF/MAPK stimulation, and of Grb2-GAB1/2 complexes leading to stimulation of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway (see Sect. 12.4.4.1). SHC-C and IRS1/2 are required to trigger PI3K/AKT activation. Y1096, specific for RET51, recruits Grb2/GAB complex, and is important for the activation of the PI3K/AKT pathway (see Sect. 12.4.1.2). Binding to DOK4 and DOK5 is implicated in MAPK stimulation, whereas binding to DOK1 is involved in c-Jun N-terminal kinase (JNK) stimulation [167] (Fig. 12.3). Other RET phosphotyrosines have been implicated in

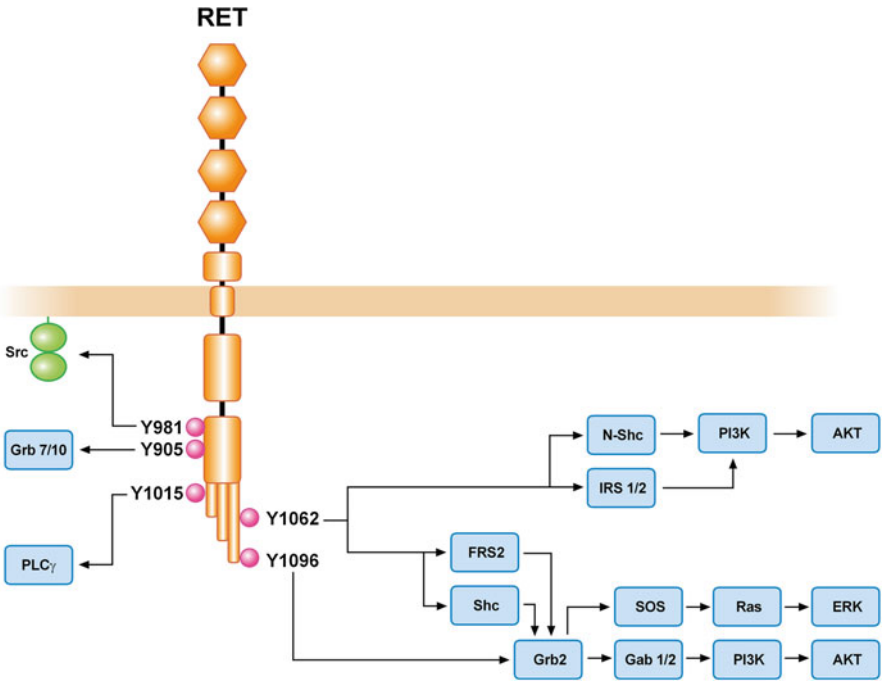

Fig. 12.3 RET signaling. Major RET autophosphorylated tyrosines with their binding partners are represented. Signaling pathways downstream Y1062 and Y1096 are also represented

docking to signal transducer and activator of transcription 3 (STAT3) [168, 169]. Phosphorylated Y687 in the RET juxtamembrane is a binding site for SHP2 phosphatase and influenced by the phosphorylation of the nearby S696 residue by protein kinase A (PKA) [170, 171]. Phosphorylation-independent RET interactors have been also identified, such as SHANK3 that associates selectively with the C-terminal tail of RET9 isoform and sustains ERK and PI3K signaling and Enigma that selectively associates with Y1062 in the context of RET9 and prevents CBL-3-mediated RET ubiquitylation and degradation [172–174]. The role of Enigma as a positive regulator of RET is supported by data showing that, among breast cancers with high RET expression, those that concurrently display high Enigma levels showed reduced survival [174].

To address the functional role of RET autophosphorylation sites, transgenic mice that express Tyr to Phe mutations at Y981, Y1015, or Y1062 have been generated. Abrogation of the PLCγ-binding site (Y1015F) significantly impaired kidney development (see Sect. 12.3.2.2). Moreover, loss of multidocking site (Y1062), in the absence of the additional Grb2-binding site (Y1096) that is specific to RET51 isoform, phenocopied *RET* knockout and caused defective development of kidney and ENS as well as spermatogonial deficiency and hearing loss [175–178].

12.4.3 RET Ligands

RET ligands include four GFL members (Fig. 12.1). The first member of this family, GDNF, was identified from a glioma cell line conditioned medium as a trophic factor for different neuronal populations [179] and then identified as a functional RET ligand [180–182]. GFL family includes also neurturin (NRTN), artemin (ARTN), and persephin (PSPN). These factors are characterized by the presence of a cystine knot, a structural motif that includes seven cysteine residues, six of which form disulfide bonds [2–4]. Accordingly, GFLs function as homodimers. They are produced by different tissues as precursors and undergo removal of the signal peptide before secretion. Once secreted, they bind to heparan sulfate, a glycosaminoglycan of the extracellular matrix.

In order to activate RET, GFLs first bind to co-receptors, named GDNF family receptor-α (GFRα). GFRαs are glycosylphosphatidylinositol (GPI)-anchored proteins, secondary to a posttranslational modification that allows attachment to the outer leaflet of the plasma membrane. Soluble forms of GFRαs can be generated by the action of phospholipases or proteases. Thus, RET stimulation by its ligands can occur in *cis* or in *trans* by GFLs complexed with GPI-anchored or soluble GFRα, respectively [2–4]. Four distinct GFRαs have been identified (GFRα1–4). They share a similar structure, with three globular cysteine-rich domains (GFRα4 has only two cysteine-rich domains) joined by linker sequences (Fig. 12.1) [183–188]. Each ligand preferentially binds to a specific GFRα: GDNF binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3, and PSPN to GFRα4 (Fig. 12.1). Despite this specificity, a certain degree of cross talk between ligands and co-receptors has been reported. RET activating complex has a stoichiometry GFL(2)–GFRα(2)–RET(2), with one GFL dimer binding to 2 GFRα and 2 RET monomers, finally inducing RET dimerization and activation. The GDNF–GFRα1–RET binding has been recently elucidated by solution low-angle X-ray scattering (SAXS) and revealed to occur within a flower-shaped structure, whereby RET CLD/CRD domains make multiple contacts with a bipartite GDNF-GFRα1, this, in turn, driving a homotypic interaction between the membrane proximal RET CRD regions [189]. Few contacts occur between RET and GDNF and involve the CRD, while major contacts are between RET extracellular domain and GFRα1. Importantly, RET binding to the GDNF–GFRα1 fosters self-association of two RET monomers via their CRD, an ability that is exploited by the CRD mutations associated with MEN2A [189].

GFLs may activate intracellular signaling pathways also in the absence of RET. GDNF is capable of high-affinity binding and signaling through the cell adhesion molecule NCAM [190–192].

Genetic ablation in mice has defined the specific role of each GFL. While *RET*, *GDNF*, or *GFRα1* genetic ablation causes lethality at birth, with kidney agenesis and defects in ENS, mice lacking the other GFLs or GFRαs are viable and fertile (see Sect. 12.2). In general, cells and tissues that are affected by the knockout of

each ligand constantly co-express RET, pointing to RET as the major signaling receptor in vivo; moreover, ligand and co-receptor ablation in mice induced an overlapping phenotype, confirming preferential binding of each ligand to cognate co-receptor. NRTN- or ARTN-knockout mice display a common phenotype characterized by ptosis (drooping eyelids), thought to result from damage to the eyelid muscle, the superior cervical sympathetic ganglion, or the oculomotor nerve [193, 194]. ARTN is important for the formation of the superior cervical ganglion [195, 196]. GFRα4-knockout mice display defective development of thyroid parafollicular C cells [197]; intriguingly, C cells are the cell of origin of MTC, a tumor that is associated with RET mutations (see Sect. 12.3.1.1).

12.4.4 RET Activation and Signaling

12.4.4.1 RET Pathway Activation

As discussed above (see Sect. 12.4.2.3), RET activates a number of intracellular signaling cascades, with major autophosphorylation sites, Y981, Y1015, Y1062, and Y1096, involved in the activation of SRC (Y981), PLCγ-PKC (Y1015), RAS/BRAF/MAPK (Y1062), and PI3K/AKT (Y1062 and Y1096), respectively.

Defects of these signaling mechanisms are involved in defective development (see Sect. 12.3.2). Furthermore, RET signaling pathway includes activation of BRAF and RAS proteins. In turn, *BRAF* is the oncogene most commonly activated in RET/PTC-negative PTC cases, and *RAS* genes are commonly mutated in sporadic PTC and MTC negative for *RET* mutations [11, 59]. This strongly indicates that both follicular cell and parafollicular C cell-derived thyroid tumors are caused by aberrant activation of the RET-RAS-MAPK epistatic signaling cascade (see Sect. 12.3.1).

12.4.4.2 Cross talk with Other Receptor Systems

Several ligand–receptor systems have been shown to interact with GFL–RET axis. *EDNRB*, which encodes the seven-pass G-coupled endothelin (ET-1, -2, and -3) receptor, is implicated in HSCR (about 5 % of cases). Accordingly, ET-3 is essential for formation of the ENS. Genome-wide association studies and mouse models revealed genetic interactions between *RET* and *EDNRB* genes [198, 199].

Nerve growth factor (NGF)-triggered activation of the TRKA kinase receptor promotes RET phosphorylation through GFL-independent inter-receptor kinase signaling [200]. This TRKA–RET interaction selectively involves RET51. Of note, both RET and TRKA are also involved in the pathogenesis of PTC (see Sect. 12.3.1) [201, 202].

12.4.5 RET Internalization, Processing, and Attenuation

Duration of RTK signaling is governed by receptor dephosphorylation, through protein tyrosine phosphatases (PTP), internalization, and degradation. PTPs, such as SHP1 and Leukocyte antigen-related (LAR) phosphatase, counteract RET autophosphorylation and biological activity [203, 204].

Following GDNF binding, RET is internalized through the clathrin-coated pit pathway. Ligand stimulation induces maximal RET phosphorylation in 15 min. Then, between 15 and 120 min, RET protein is internalized, colocalizes with markers of clathrin-coated vescicles and early endosomes such as Rab5a, and degraded. RET internalization depends on dynamin 2 GTPase (DNM2) and requires RET kinase activity. Interestingly, RET-mediated signaling is also affected by internalization, as inhibition of this process, through a DNM2 dominant-negative mutant, blunts MAPK, but not AKT activation [205]. Surface labeling experiments have shown that RET can also be partially recycled back to the plasma membrane [206].

RET internalization requires ubiquitylation, which is carried out by the ubiquitin ligase CBL. CBL associates with tyrosine phosphorylated RET through SHC and Grb2 adapters. By binding RET Y1062, SHC recruits the Grb2/CBL complex. Grb2 recruits CBL also by binding directly to RET Y1096 (see Sect. 12.4.2.3) [207]. Despite CBL docking sites are also present in the oncogenic RET/PTC oncoproteins (see Sect. 12.3.1.2), both RET/PTC1 and RET/PTC3 fail to interact with CBL, possibly because of their aberrant intracellular localization. Failure to bind CBL results in decreased ubiquitylation, and higher protein stability of RET/PTC oncoproteins with respect to membrane-bound RET [208].

At least a part of GDNF-induced RET degradation is mediated by the proteasome in sympathetic neurons. In these cells, GDNF-stimulated RET moves to lipid rafts where it is very stable. Instead, RET proteins located outside rafts undergo ubiquitylation and proteasome-dependent degradation [209].

Two novel RET interactors, CD2-associated protein (C2AP) and CBL-3, one of the three members of the CBL family, bind RET directly. C2AP and CBL-3 interact with RET in resting conditions; then, upon GDNF stimulation, C2AP and CBL-3 dissociate from RET, this inducing RET ubiquitylation and proteasomal degradation [210]. As mentioned above, binding to Enigma interferes with CBL-3 binding to RET, thus enhancing RET stability and downstream signaling (see Sect. 12.4.2.3).

Receptor at a glance: *RET*

Chromosome location	10q11.2
Gene Size (bp)	55 kb
Intron/exon numbers	20/21
mRNA size (5`, ORF, 3`)	7.0 kb; 6.0 kb; 4.5 kb; 3.9 kb
Amino acid number	1114 aa (RET51); 1106 aa (RET43); 1072 aa (RET9)
kDa	150 and 170 kDa

Posttranslational modifications	N- and O-glycosylation
Domains	4 cadherin-like domains (CLD) and 1 cysteine-rich region (CRD) in the extracellular region; 1 transmembrane domain and 1 tyrosine kinase domain (TKD) in the intracellular region split into two subdomains
Ligands	GDNF (glial cell line-derived neurotrophic factor); NRTN (neurturin); ARTN (artemin); PSPN (persephin)
Known dimerizing partners	TRKA; EDNRB
Pathways activated	RAS/RAF/MAP kinase; PI3K/AKT; PLCγ; SRC; STAT3
Tissues expressed	Peripheral and central nervous system; thyroid C-cells; adrenal medulla; developing ureteric bud; spermatogonia
Human diseases	Multiple Endocrine Neoplasia type 2 syndromes (MEN 2A, MEN 2B); sporadic medullary thyroid carcinoma; papillary thyroid carcinoma; lung adenocarcinoma; chronic myelomonocytic leukemia; Spitz tumor; breast and other carcinomas; Hirschsprung's disease (colonic aganglionosis); congenital anomalies of the kidney or lower urinary tract (CAKUT)
Knockout mouse phenotype	Defective development of enteric nervous system; agenesis or dysgenesis of the kidney; defective spermatogenesis; defective Peyer's patches; abnormal retinal function

References

1. Ibáñez CF. Structure and physiology of the RET receptor tyrosine kinase. Cold Spring Harb Perspect Biol. 2013;5(2).
2. Airaksinen MS, Holm L, Hätinen T. Evolution of the GDNF family ligands and receptors. Brain Behav Evol. 2006;68(3):181–90.
3. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci. 2002;3(5):383–94.
4. Baloh RH, Enomoto H, Johnson Jr EM, Milbrandt J. The GDNF family ligands and receptors implications for neural development. Curr Opin Neurobiol. 2000;10(1):103–10.
5. Takahashi M, Ritz J, Cooper GM. Activation of a novel human transforming gene, ret, by DNA rearrangement. Cell. 1985;42(2):581–8.
6. Fusco A, Grieco M, Santoro M, Berlingieri MT, Pilotti S, Pierotti MA, Della Porta G, Vecchio G. A new oncogene in human thyroid papillary carcinomas and their lymph-nodal metastases. Nature. 1987;328(6126):170–2.
7. Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, Pierotti MA, Della Porta G, Fusco A, Vecchio G. PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell. 1990;60(4):557–63.
8. Plaza-Menacho I, Mologni L, McDonald NQ. Mechanisms of RET signaling in cancer: current and future implications for targeted therapy. Cell Signal. 2014;26(8):1743–52.
9. Mulligan LM. RET revisited: expanding the oncogenic portfolio. Nat Rev Cancer. 2014;14(3):173–86.
10. de Groot JW, Links TP, Plukker JT, Lips CJ, Hofstra RM. RET as a diagnostic and therapeutic target in sporadic and hereditary endocrine tumors. Endocr Rev. 2006;27(5):535–60.

11. Wells Jr SA, Pacini F, Robinson BG, Santoro M. Multiple endocrine neoplasia type 2 and familial medullary thyroid carcinoma: an update. J Clin Endocrinol Metab. 2013;9(8): 3149–64.
12. Costantini F, Shakya R. GDNF/Ret signaling and the development of the kidney. Bioessays. 2006;28(2):117–27.
13. Jain S. The many faces of RET dysfunction in kidney. Organogenesis. 2009;5(4):177–90.
14. Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature. 1994;367(6461):380–3.
15. Iwamoto T, Taniguchi M, Asai N, Ohkusu K, Nakashima I, Takahashi M. cDNA cloning of mouse ret proto-oncogene and its sequence similarity to the cadherin superfamily. Oncogene. 1993;8(4):1087–91.
16. Pachnis V, Mankoo B, Costantini F. Expression of the c-ret proto-oncogene during mouse embryogenesis. Development. 1993;119(4):1005–17.
17. Tsuzuki T, Takahashi M, Asai N, Iwashita T, Matsuyama M, Asai J. Spatial and temporal expression of the ret proto-oncogene product in embryonic, infant and adult rat tissues. Oncogene. 1995;10(1):191–8.
18. Sugaya R, Ishimaru S, Hosoya T, Saigo K, Emori Y. A Drosophila homolog of human proto-oncogene ret transiently expressed in embryonic neuronal precursor cells including neuroblasts and CNS cells. Mech Dev. 1994;45(2):139–45.
19. Shepherd IT, Beattie CE, Raible DW. Functional analysis of zebrafish GDNF. Dev Biol. 2001;231(2):420–35.
20. Shepherd IT, Pietsch J, Elworthy S, Kelsh RN, Raible DW. Roles for GFRalpha1 receptors in zebrafish enteric nervous system development. Development. 2004;131(1):241–9.
21. Moore MW, Klein RD, Fariñas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A. Renal and neuronal abnormalities in mice lacking GDNF. Nature. 1996;382(6586):76–9.
22. Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E. Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development. 2002;129(22):5151–60.
23. Amiel J, Sproat-Emison E, Garcia-Barcelo M, Lantieri F, Burzynski G, Borrego S, Pelet A, Arnold S, Miao X, Griseri P, Brooks AS, Antinolo G, de Pontual L, Clement-Ziza M, Munnich A, Kashuk C, West K, Wong KK, Lyonnet S, Chakravarti A, Tam PK, Ceccherini I, Hofstra RM, Fernandez R, Hirschsprung Disease Consortium. Hirschsprung disease, associated syndromes and genetics: a review. J Med Genet. 2008;45(1):1–14.
24. Enomoto H, Crawford PA, Gorodinsky A, Heuckeroth RO, Johnson Jr EM, Milbrandt J. RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development. 2001;128(20):3963–74.
25. Burau K, Stenull I, Huber K, Misawa H, Berse B, Unsicker K, Ernsberger U. c-ret regulates cholinergic properties in mouse sympathetic neurons: evidence from mutant mice. Eur J Neurosci. 2004;20(2):353–62.
26. Ernsberger U. The role of GDNF family ligand signalling in the differentiation of sympathetic and dorsal root ganglion neurons. Cell Tissue Res. 2008;333(3):353–71.
27. Elitt CM, McIlwrath SL, Lawson JJ, Malin SA, Molliver DC, Cornuet PK, Koerber HR, Davis BM, Albers KM. Artemin overexpression in skin enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and leads to behavioral sensitivity to heat and cold. J Neurosci. 2006;26(33):8578–87.
28. Marcos C, Pachnis V. The effect of the ret- mutation on the normal development of the central and parasympathetic nervous systems. Int J Dev Biol. 1996; Suppl 1:137S–138S.
29. Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H. Defects in enteric innervation and kidney development in mice lacking GDNF. Nature. 1996;382(6586):73–6.
30. Sánchez MP, Silos-Santiago I, Frisén J, He B, Lira SA, Barbacid M. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature. 1996;382(6586):70–3.

31. Shakya R, Jho EH, Kotka P, Wu Z, Kholodilov N, Burke R, D'Agati V, Costantini F. The role of GDNF in patterning the excretory system. Dev Biol. 2005;283(1):70–84.
32. Basson MA, Akbulut S, Watson-Johnson J, Simon R, Carroll TJ, Shakya R, Gross I, Martin GR, Lufkin T, McMahon AP, Wilson PD, Costantini FD, Mason IJ, Licht JD. Sprouty1 is a critical regulator of GDNF/RET-mediated kidney induction. Dev Cell. 2005;8(2):229–39.
33. Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science. 2000;287(5457):1489–93.
34. Jain S, Naughton CK, Yang M, Strickland A, Vij K, Encinas M, Golden J, Gupta A, Heuckeroth R, Johnson Jr EM, Milbrandt J. Mice expressing a dominant-negative Ret mutation phenocopy human Hirschsprung disease and delineate a direct role of Ret in spermatogenesis. Development. 2004;131(21):5503–13.
35. Kramer ER, Aron L, Ramakers GM, Seitz S, Zhuang X, Beyer K, Smidt MP, Klein R. Absence of Ret signaling in mice causes progressive and late degeneration of the nigrostriatal system. PLoS Biol. 2007;5(3):e39.
36. Klein P, Müller-Rischart AK, Motori E, Schönbauer C, Schnorrer F, Winklhofer KF, Klein R. Ret rescues mitochondrial morphology and muscle degeneration of Drosophila Pink1 mutants. EMBO J. 2014;33(4):341–55.
37. Jomary C, Thomas M, Grist J, Milbrandt J, Neal MJ, Jones SE. Expression patterns of neurturin and its receptor components in developing and degenerative mouse retina. Invest Ophthalmol Vis Sci. 1999;40(3):568–74.
38. Jomary C, Darrow RM, Wong P, Organisciak DT, Jones SE. Expression of neurturin, glial cell line-derived neurotrophic factor, and their receptor components in light-induced retinal degeneration. Invest Ophthalmol Vis Sci. 2004;45(4):1240–6.
39. Carwile ME, Culbert RB, Sturdivant RL, Kraft TW. Rod outer segment maintenance is enhanced in the presence of bFGF, CNTF and GDNF. Exp Eye Res. 1998;66(6):791–805.
40. Brantley Jr MA, Jain S, Barr EE, Johnson Jr EM, Milbrandt J. Neurturin-mediated ret activation is required for retinal function. J Neurosci. 2008;28(16):4123–35.
41. Golden JP, Hoshi M, Nassar MA, Enomoto H, Wood JN, Milbrandt J, Gereau 4th RW, Johnson Jr EM, Jain S. RET signaling is required for survival and normal function of nonpeptidergic nociceptors. J Neurosci. 2010;30(11):3983–94.
42. Veiga-Fernandes H, Coles MC, Foster KE, Patel A, Williams A, Natarajan D, Barlow A, Pachnis V, Kioussis D. Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. Nature. 2007;446(7135):547–51.
43. Fonseca-Pereira D, Arroz-Madeira S, Rodrigues-Campos M, Barbosa IA, Domingues RG, Bento T, Almeida AR, Ribeiro H, Potocnik AJ, Enomoto H, Veiga-Fernandes H. The neurotrophic factor receptor RET drives haematopoietic stem cell survival and function. Nature. 2014;514(7520):98–101.
44. Diaz-Rodriguez E, Garcia-Rendueles AR, Ibáñez-Costa A, Gutierrez-Pascual E, Garcia-Lavandeira M, Leal A, Japon MA, Soto A, Venegas E, Tinahones FJ, Garcia-Arnes JA, Benito P, Angeles Galvez M, Jimenez-Reina L, Bernabeu I, Dieguez C, Luque RM, Castaño JP, Alvarez CV. Somatotropinomas, but not nonfunctioning pituitary adenomas, maintain a functional apoptotic RET/Pit1/ARF/p53 pathway that is blocked by excess GDNF. Endocrinology. 2014;155(11):4329–40.
45. Cañibano C, Rodriguez NL, Saez C, Tovar S, Garcia-Lavandeira M, Borrello MG, Vidal A, Costantini F, Japon M, Dieguez C, Alvarez CV. The dependence receptor Ret induces apoptosis in somatotrophs through a Pit-1/p53 pathway, preventing tumor growth. EMBO J. 2007;26(8):2015–28.
46. Bordeaux MC, Forcet C, Granger L, Corset V, Bidaud C, Billaud M, Bredesen DE, Edery P, Mehlen P. The RET proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. EMBO J. 2000;19(15):4056–63.
47. Marks C, Belluscio L, Ibáñez CF. Critical role of GFRα1 in the development and function of the main olfactory system. J Neurosci. 2012;32(48):17306–20.

48. Song XJ, Li DQ, Farley W, Luo LH, Heuckeroth RO, Milbrandt J, Pflugfelder SC. Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. Invest Ophthalmol Vis Sci. 2003;44(10):4223–9.
49. Tomac AC, Agulnick AD, Haughey N, Chang CF, Zhang Y, Bäckman C, Morales M, Mattson MP, Wang Y, Westphal H, Hoffer BJ. Effects of cerebral ischemia in mice deficient in Persephin. Proc Natl Acad Sci USA. 2002;99(14):9521–6.
50. Margraf RL, Crockett DK, Krautscheid PM, Seamons R, Calderon FR, Wittwer CT, Mao R. Multiple endocrine neoplasia type 2 RET protooncogene database: repository of MEN2-associated RET sequence variation and reference for genotype/phenotype correlations. Hum Mutat. 2009;30(4):548–56.
51. Romei C, Elisei R, Pinchera A, Ceccherini I, Molinaro E, Mancusi F, Martino E, Romeo G, Pacini F. Somatic mutations of the ret protooncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. J Clin Endocrinol Metab. 1996;81(4):1619–22.
52. Santoro M, Carlomagno F, Romano A, Bottaro DP, Dathan NA, Grieco M, Fusco A, Vecchio G, Matoskova B, Kraus MH, et al. Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. Science. 1995;267(5196):381–3.
53. Knowles PP, Murray-Rust J, Kjaer S, Scott RP, Hanrahan S, Santoro M, Ibáñez CF, McDonald NQ. Structure and chemical inhibition of the RET tyrosine kinase domain. J Biol Chem. 2006;281(44):33577–87.
54. Plaza-Menacho I, Barnouin K, Goodman K, Martínez-Torres RJ, Borg A, Murray-Rust J, Mouilleron S, Knowles P, McDonald NQ. Oncogenic RET kinase domain mutations perturb the autophosphorylation trajectory by enhancing substrate presentation in trans. Mol Cell. 2014;53(5):738–51.
55. Songyang Z, Carraway 3rd KL, Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Hubbard SR, Smith DP, Eng C, et al. Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. Nature. 1995;373(6514):536–9.
56. Carlomagno F, Vitagliano D, Guida T, Ciardiello F, Tortora G, Vecchio G, Ryan AJ, Fontanini G, Fusco A, Santoro M. ZD6474, an orally available inhibitor of KDR tyrosine kinase activity, efficiently blocks oncogenic RET kinases. Cancer Res. 2002;62(24):7284–90.
57. Wells Jr SA, Robinson BG, Gagel RF, Dralle H, Fagin JA, Santoro M, Baudin E, Elisei R, Jarzab B, Vasselli JR, Read J, Langmuir P, Ryan AJ, Schlumberger MJ. Vandetanib in patients with locally advanced or metastatic medullary thyroid cancer: a randomized, double-blind phase III trial. J Clin Oncol. 2012;30(2):134–41.
58. Elisei R, Schlumberger MJ, Müller SP, Schöffski P, Brose MS, Shah MH, Licitra L, Jarzab B, Medvedev V, Kreissl MC, Niederle B, Cohen EE, Wirth LJ, Ali H, Hessel C, Yaron Y, Ball D, Nelkin B, Sherman SI. Cabozantinib in progressive medullary thyroid cancer. J Clin Oncol. 2013;31(29):3639–46.
59. Nikiforov YE, Nikiforova MN. Molecular genetics and diagnosis of thyroid cancer. Nat Rev Endocrinol. 2011;7(10):569–80.
60. Melillo RM, Castellone MD, Guarino V, De Falco V, Cirafici AM, Salvatore G, Caiazzo F, Basolo F, Giannini R, Kruhoffer M, Orntoft T, Fusco A, Santoro M. The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. J Clin Invest. 2005;115(4):1068–81.
61. Santoro M, Chiappetta G, Cerrato A, Salvatore D, Zhang L, Manzo G, Picone A, Portella G, Santelli G, Vecchio G, Fusco A. Development of thyroid papillary carcinomas secondary to tissue-specific expression of the RET/PTC1 oncogene in transgenic mice. Oncogene. 1996;12(8):1821–6.
62. Nikiforova MN, Stringer JR, Blough R, Medvedovic M, Fagin JA, Nikiforov YE. Proximity of chromosomal loci that participate in radiation-induced rearrangements in human cells. Science. 2000;290(5489):138–41.
63. Gandhi M, Evdokimova VN, Cuenco K, Nikiforova MN, Kelly LM, Stringer JR, Bakkenist CJ, Nikiforov YE. Homologous chromosomes make contact at the sites of double-strand

breaks in genes in somatic G0/G1-phase human cells. Proc Natl Acad Sci USA. 2012; 109(24):9454–9.
64. Williams D. Radiation carcinogenesis: lessons from Chernobyl. Oncogene. 2008;27 Suppl 2:S9–18.
65. Rabes HM, Demidchik EP, Sidorow JD, Lengfelder E, Beimfohr C, Hoelzel D, Klugbauer S. Pattern of radiation-induced RET and NTRK1 rearrangements in 191 post-chernobyl papillary thyroid carcinomas: biological, phenotypic, and clinical implications. Clin Cancer Res. 2000;6(3):1093–103.
66. Ricarte-Filho JC, Li S, Garcia-Rendueles ME, Montero-Conde C, Voza F, Knauf JA, Heguy A, Viale A, Bogdanova T, Thomas GA, Mason CE, Fagin JA. Identification of kinase fusion oncogenes in post-Chernobyl radiation-induced thyroid cancers. J Clin Invest. 2013; 123(11):4935–44.
67. Bongarzone I, Monzini N, Borrello MG, Carcano C, Ferraresi G, Arighi E, Mondellini P, Della Porta G, Pierotti MA. Molecular characterization of a thyroid tumor-specific transforming sequence formed by the fusion of ret tyrosine kinase and the regulatory subunit RI alpha of cyclic AMP-dependent protein kinase A. Mol Cell Biol. 1993;13(1):358–66.
68. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med. 2008;359(13): 1367–80.
69. Ju YS, Lee WC, Shin JY, Lee S, Bleazard T, Won JK, Kim YT, Kim JI, Kang JH, Seo JS. A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. Genome Res. 2012;22(3):436–45.
70. Lipson D, Capelletti M, Yelensky R, Otto G, Parker A, Jarosz M, Curran JA, Balasubramanian S, Bloom T, Brennan KW, Donahue A, Downing SR, Frampton GM, Garcia L, Juhn F, Mitchell KC, White E, White J, Zwirko Z, Peretz T, Nechushtan H, Soussan-Gutman L, Kim J, Sasaki H, Kim HR, Park SI, Ercan D, Sheehan CE, Ross JS, Cronin MT, Jänne PA, Stephens PJ. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. Nat Med. 2012;18(3):382–4.
71. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, Asaka R, Hamanaka W, Ninomiya H, Uehara H, Lim Choi Y, Satoh Y, Okumura S, Nakagawa K, Mano H, Ishikawa Y. RET, ROS1 and ALK fusions in lung cancer. Nat Med. 2012;18(3):378–81.
72. Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nammo T, Sakamoto H, Tsuta K, Furuta K, Shimada Y, Iwakawa R, Ogiwara H, Oike T, Enari M, Schetter AJ, Okayama H, Haugen A, Skaug V, Chiku S, Yamanaka I, Arai Y, Watanabe S, Sekine I, Ogawa S, Harris CC, Tsuda H, Yoshida T, Yokota J, Shibata T. KIF5B-RET fusions in lung adenocarcinoma. Nat Med. 2012;18(3):375–7.
73. Li F, Feng Y, Fang R, Fang Z, Xia J, Han X, Liu XY, Chen H, Liu H, Ji H. Identification of RET gene fusion by exon array analyses in "pan-negative" lung cancer from never smokers. Cell Res. 2012;22(5):928–31.
74. Wang R, Hu H, Pan Y, Li Y, Ye T, Li C, Luo X, Wang L, Li H, Zhang Y, Li F, Lu Y, Lu Q, Xu J, Garfield D, Shen L, Ji H, Pao W, Sun Y, Chen H. RET fusions define a unique molecular and clinicopathologic subtype of non-small-cell lung cancer. J Clin Oncol. 2012;30(35): 4352–9.
75. Dabir S, Babakoohi S, Kluge A, Morrow JJ, Kresak A, Yang M, MacPherson D, Wildey G, Dowlati A. RET mutation and expression in small-cell lung cancer. J Thorac Oncol. 2014;9(9):1316–23.
76. Ballerini P, Struski S, Cresson C, Prade N, Toujani S, Deswarte C, Dobbelstein S, Petit A, Lapillonne H, Gautier EF, Demur C, Lippert E, Pages P, Mansat-De Mas V, Donadieu J, Huguet F, Dastugue N, Broccardo C, Perot C, Delabesse E. RET fusion genes are associated with chronic myelomonocytic leukemia and enhance monocytic differentiation. Leukemia. 2012;26(11):2384–9.
77. Bossi D, Carlomagno F, Pallavicini I, Pruneri G, Trubia M, Raviele PR, Marinelli A, Anaganti S, Cox MC, Viale G, Santoro M, Di Fiore PP, Minucci S. Functional characterization of a novel FGFR1OP-RET rearrangement in hematopoietic malignancies. Mol Oncol. 2014; 8(2):221–31.

78. Wiesner T, He J, Yelensky R, Esteve-Puig R, Botton T, Yeh I, Lipson D, Otto G, Brennan K, Murali R, Garrido M, Miller VA, Ross JS, Berger MF, Sparatta A, Palmedo G, Cerroni L, Busam KJ, Kutzner H, Cronin MT, Stephens PJ, Bastian BC. Kinase fusions are frequent in Spitz tumours and spitzoid melanomas. Nat Commun. 2014;5:3116.
79. Esseghir S, Todd SK, Hunt T, Poulsom R, Plaza-Menacho I, Reis-Filho JS, Isacke CM. A role for glial cell derived neurotrophic factor induced expression by inflammatory cytokines and RET/GFR alpha 1 receptor up-regulation in breast cancer. Cancer Res. 2007;67(24): 11732–41.
80. Boulay A, Breuleux M, Stephan C, Fux C, Brisken C, Fiche M, Wartmann M, Stumm M, Lane HA, Hynes NE. The Ret receptor tyrosine kinase pathway functionally interacts with the ERalpha pathway in breast cancer. Cancer Res. 2008;68(10):3743–51.
81. Gattelli A, Nalvarte I, Boulay A, Roloff TC, Schreiber M, Carragher N, Macleod KK, Schlederer M, Lienhard S, Kenner L, Torres-Arzayus MI, Hynes NE. Ret inhibition decreases growth and metastatic potential of estrogen receptor positive breast cancer cells. EMBO Mol Med. 2013;5(9):1335–50.
82. Stine ZE, McGaughey DM, Bessling SL, Li S, McCallion AS. Steroid hormone modulation of RET through two estrogen responsive enhancers in breast cancer. Hum Mol Genet. 2011;20(19):3746–56.
83. Plaza-Menacho I, Morandi A, Robertson D, Pancholi S, Drury S, Dowsett M, Martin LA, Isacke CM. Targeting the receptor tyrosine kinase RET sensitizes breast cancer cells to tamoxifen treatment and reveals a role for RET in endocrine resistance. Oncogene. 2010; 29(33):4648–57.
84. Morandi A, Martin LA, Gao Q, Pancholi S, Mackay A, Robertson D, Zvelebil M, Dowsett M, Plaza-Menacho I, Isacke CM. GDNF-RET signaling in ER-positive breast cancers is a key determinant of response and resistance to aromatase inhibitors. Cancer Res. 2013;73(12): 3783–95.
85. Wu Q, Ishikawa T, Sirianni R, Tang H, McDonald JG, Yuhanna IS, Thompson B, Girard L, Mineo C, Brekken RA, Umetani M, Euhus DM, Xie Y, Shaul PW. 27-Hydroxycholesterol promotes cell-autonomous, ER-positive breast cancer growth. Cell Rep. 2013;5(3):637–45.
86. Gil Z, Cavel O, Kelly K, Brader P, Rein A, Gao SP, Carlson DL, Shah JP, Fong Y, Wong RJ. Paracrine regulation of pancreatic cancer cell invasion by peripheral nerves. J Natl Cancer Inst. 2010;102(2):107–18.
87. He S, Chen CH, Chernichenko N, He S, Bakst RL, Barajas F, Deborde S, Allen PJ, Vakiani E, Yu Z, Wong RJ. GFRα1 released by nerves enhances cancer cell perineural invasion through GDNF-RET signaling. Proc Natl Acad Sci USA. 2014;111(19):E2008–17.
88. Luo Y, Tsuchiya KD, Il Park D, Fausel R, Kanngurn S, Welcsh P, Dzieciatkowski S, Wang J, Grady WM. RET is a potential tumor suppressor gene in colorectal cancer. Oncogene. 2013;32(16):2037–47.
89. Draht MX, Smits KM, Tournier B, Jooste V, Chapusot C, Carvalho B, Cleven AH, Derks S, Wouters KA, Belt EJ, Stockmann HB, Bril H, Weijenberg MP, van den Brandt PA, de Bruïne AP, Herman JG, Meijer GA, Piard F, Melotte V, van Engeland M. Promoter CpG island methylation of RET predicts poor prognosis in stage II colorectal cancer patients. Mol Oncol. 2014;8(3):679–88.
90. Wood LD, Parsons DW, Jones S, Lin J, Sjöblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, Silliman N, Szabo S, Dezso Z, Ustyanksky V, Nikolskaya T, Nikolsky Y, Karchin R, Wilson PA, Kaminker JS, Zhang Z, Croshaw R, Willis J, Dawson D, Shipitsin M, Willson JK, Sukumar S, Polyak K, Park BH, Pethiyagoda CL, Pant PV, Ballinger DG, Sparks AB, Hartigan J, Smith DR, Suh E, Papadopoulos N, Buckhaults P, Markowitz SD, Parmigiani G, Kinzler KW, Velculescu VE, Vogelstein B. The genomic landscapes of human breast and colorectal cancers. Science. 2007;318(5853):1108–13.
91. Mehlen P, Tauszig-Delamasure S. Dependence receptors and colorectal cancer. Gut. 2014; 63(11):1821–9.
92. Butler Tjaden NE, Trainor PA. The developmental etiology and pathogenesis of Hirschsprung disease. Transl Res. 2013;162(1):1–15.

93. Goldstein AM, Hofstra RM, Burns AJ. Building a brain in the gut: development of the enteric nervous system. Clin Genet. 2013;83(4):307–16.
94. UniProt Consortium. The universal protein resource (UniProt). Nucleic Acids Res. 2008;36(Database issue):D190–5.
95. Stenson PD, Ball EV, Mort M, Phillips AD, Shiel JA, Thomas NS, Abeysinghe S, Krawczak M, Cooper DN. Human Gene Mutation Database (HGMD): 2003 update. Hum Mutat. 2003;21(6):577–81.
96. Carlomagno F, De Vita G, Berlingieri MT, de Franciscis V, Melillo RM, Colantuoni V, Kraus MH, Di Fiore PP, Fusco A, Santoro M. Molecular heterogeneity of RET loss of function in Hirschsprung's disease. EMBO J. 1996;15(11):2717–25.
97. Pasini B, Borrello MG, Greco A, Bongarzone I, Luo Y, Mondellini P, Alberti L, Miranda C, Arighi E, Bocciardi R, et al. Loss of function effect of RET mutations causing Hirschsprung disease. Nat Genet. 1995;10(1):35–40.
98. Pelet A, Geneste O, Edery P, Pasini A, Chappuis S, Atti T, Munnich A, Lenoir G, Lyonnet S, Billaud M. Various mechanisms cause RET-mediated signaling defects in Hirschsprung's disease. J Clin Invest. 1998;101(6):1415–23.
99. Geneste O, Bidaud C, De Vita G, Hofstra RM, Tartare-Deckert S, Buys CH, Lenoir GM, Santoro M, Billaud M. Two distinct mutations of the RET receptor causing Hirschsprung's disease impair the binding of signalling effectors to a multifunctional docking site. Hum Mol Genet. 1999;8(11):1989–99.
100. Ito S, Iwashita T, Asai N, Murakami H, Iwata Y, Sobue G, Takahashi M. Biological properties of Ret with cysteine mutations correlate with multiple endocrine neoplasia type 2A, familial medullary thyroid carcinoma, and Hirschsprung's disease phenotype. Cancer Res. 1997; 57(14):2870–2.
101. Chappuis-Flament S, Pasini A, De Vita G, Ségouffin-Cariou C, Fusco A, Attié T, Lenoir GM, Santoro M, Billaud M. Dual effect on the RET receptor of MEN 2 mutations affecting specific extracytoplasmic cysteines. Oncogene. 1998;17(22):2851–61.
102. Fitze G, Appelt H, König IR, Görgens H, Stein U, Walther W, Gossen M, Schreiber M, Ziegler A, Roesner D, Schackert HK. Functional haplotypes of the RET proto-oncogene promoter are associated with Hirschsprung disease (HSCR). Hum Mol Genet. 2003; 12(24):3207–14.
103. Garcia-Barcelo M, Ganster RW, Lui VC, Leon TY, So MT, Lau AM, Fu M, Sham MH, Knight J, Zannini MS, Sham PC, Tam PK. TTF-1 and RET promoter SNPs: regulation of RET transcription in Hirschsprung's disease. Hum Mol Genet. 2005;14(2):191–204.
104. Griseri P, Bachetti T, Puppo F, Lantieri F, Ravazzolo R, Devoto M, Ceccherini I. A common haplotype at the 5′ end of the RET proto-oncogene, overrepresented in Hirschsprung patients, is associated with reduced gene expression. Hum Mutat. 2005;25(2):189–95.
105. Emison ES, McCallion AS, Kashuk CS, Bush RT, Grice E, Lin S, Portnoy ME, Cutler DJ, Green ED, Chakravarti A. A common sex-dependent mutation in a RET enhancer underlies Hirschsprung disease risk. Nature. 2005;434(7035):857–63.
106. Skinner MA, Kalyanaraman S, Safford SD, Heuckeroth RO, Tourtellotte W, Goyeau D, Goodfellow P, Milbrandt JD, Freemerman A. A human yeast artificial chromosome containing the multiple endocrine neoplasia type 2B Ret mutation does not induce medullary thyroid carcinoma but does support the growth of kidneys and partially rescues enteric nervous system development in Ret-deficient mice. Am J Pathol. 2005;166(1):265–74.
107. Jeanpierre C, Macé G, Parisot M, Morinière V, Pawtowsky A, Benabou M, Martinovic J, Amiel J, Attié-Bitach T, Delezoide AL, Loget P, Blanchet P, Gaillard D, Gonzales M, Carpentier W, Nitschke P, Tores F, Heidet L, Antignac C. Salomon R; Société Française de Foetopathologie. RET and GDNF mutations are rare in fetuses with renal agenesis or other severe kidney development defects. J Med Genet. 2011;48(7):497–504.
108. Chatterjee R, Ramos E, Hoffman M, VanWinkle J, Martin DR, Davis TK, Hoshi M, Hmiel SP, Beck A, Hruska K, Coplen D, Liapis H, Mitra R, Druley T, Austin P, Jain S. Traditional and targeted exome sequencing reveals common, rare and novel functional deleterious

variants in RET-signaling complex in a cohort of living US patients with urinary tract malformations. Hum Genet. 2012;131(11):1725–38.
109. Ishizaka Y, Itoh F, Tahira T, Ikeda I, Sugimura T, Tucker J, Fertitta A, Carrano AV, Nagao M. Human ret proto-oncogene mapped to chromosome 10q11.2. Oncogene. 1989;4(12): 1519–21.
110. Pasini B, Hofstra RM, Yin L, Bocciardi R, Santamaria G, Grootscholten PM, Ceccherini I, Patrone G, Priolo M, Buys CH, et al. The physical map of the human RET proto-oncogene. Oncogene. 1995;11(9):1737–43.
111. Itoh F, Ishizaka Y, Tahira T, Yamamoto M, Miya A, Imai K, Yachi A, Takai S, Sugimura T, Nagao M. Identification and analysis of the ret proto-oncogene promoter region in neuroblastoma cell lines and medullary thyroid carcinomas from MEN2A patients. Oncogene. 1992;7(6):1201–6.
112. Andrew SD, Delhanty PJ, Mulligan LM, Robinson BG. Sp1 and Sp3 transactivate the RET proto-oncogene promoter. Gene. 2000;256(1–2):283–91.
113. Puppo F, Musso M, Pirulli D, Griseri P, Bachetti T, Crovella S, Patrone G, Ceccherini I, Ravazzolo R. Comparative genomic sequence analysis coupled to chromatin immunoprecipitation: a screening procedure applied to search for regulatory elements at the RET locus. Physiol Genomics. 2005;23(3):269–74.
114. Patrone G, Puliti A, Bocciardi R, Ravazzolo R, Romeo G. Sequence and characterisation of the RET proto-oncogene 5′ flanking region: analysis of retinoic acid responsiveness at the transcriptional level. FEBS Lett. 1997;419(1):76–82.
115. Sukumaran M, Waxman SG, Wood JN, Pachnis V. Flanking regulatory sequences of the locus encoding the murine GDNF receptor, c-ret, directs lac Z (beta-galactosidase) expression in developing somatosensory system. Dev Dyn. 2001;222(3):389–402.
116. Zordan P, Ravazzolo R, Bocciardi R. A very short segment of the murine Ret promoter contains elements sensitive to in vitro neural cell differentiation. Int J Mol Med. 2005;16(2): 325–31.
117. Zordan P, Tavella S, Brizzolara A, Biticchi R, Ceccherini I, Garofalo S, Ravazzolo R, Bocciardi R. The immediate upstream sequence of the mouse Ret gene controls tissue-specific expression in transgenic mice. Int J Mol Med. 2006;18(6):601–8.
118. Lorenzo MJ, Eng C, Mulligan LM, Stonehouse TJ, Healey CS, Ponder BA, Smith DP. Multiple mRNA isoforms of the human RET proto-oncogene generated by alternate splicing. Oncogene. 1995;10(7):1377–83.
119. Myers SM, Eng C, Ponder BA, Mulligan LM. Characterization of RET proto-oncogene 3′ splicing variants and polyadenylation sites: a novel C-terminus for RET. Oncogene. 1995; 11(10):2039–45.
120. Ivanchuk SM, Eng C, Cavenee WK, Mulligan LM. The expression of RET and its multiple splice forms in developing human kidney. Oncogene. 1997;14(15):1811–8.
121. Ivanchuk SM, Myers SM, Mulligan LM. Expression of RET 3′ splicing variants during human kidney development. Oncogene. 1998;16(8):991–6.
122. Le Hir H, Charlet-Berguerand N, Gimenez-Roqueplo A, Mannelli M, Plouin P, de Franciscis V, Thermes C. Relative expression of the RET9 and RET51 isoforms in human pheochromocytomas. Oncology. 2000;58(4):311–8.
123. Le Hir H, Charlet-Berguerand N, de Franciscis V, Thermes C. 5′-End RET splicing: absence of variants in normal tissues and intron retention in pheochromocytomas. Oncology. 2002; 63(1):84–91.
124. Fluge O, Haugen DR, Akslen LA, Marstad A, Santoro M, Fusco A, Varhaug JE, Lillehaug JR. Expression and alternative splicing of c-ret RNA in papillary thyroid carcinomas. Oncogene. 2001;20(7):885–92.
125. Carter MT, Yome JL, Marcil MN, Martin CA, Vanhorne JB, Mulligan LM. Conservation of RET proto-oncogene splicing variants and implications for RET isoform function. Cytogenet Cell Genet. 2001;95(3–4):169–76.

126. Lee KY, Samy ET, Sham MH, Tam PK, Lui VC. 3′ Splicing variants of ret receptor tyrosine kinase are differentially expressed in mouse embryos and in adult mice. Biochim Biophys Acta. 2003;1627(1):26–38.
127. Arighi E, Alberti L, Torriti F, Ghizzoni S, Rizzetti MG, Pelicci G, Pasini B, Bongarzone I, Piutti C, Pierotti MA, Borrello MG. Identification of Shc docking site on Ret tyrosine kinase. Oncogene. 1997;14(7):773–82.
128. Lorenzo MJ, Gish GD, Houghton C, Stonehouse TJ, Pawson T, Ponder BA, Smith DP. RET alternate splicing influences the interaction of activated RET with the SH2 and PTB domains of Shc, and the SH2 domain of Grb2. Oncogene. 1997;14(7):763–71.
129. de Graaff E, Srinivas S, Kilkenny C, D′Agati V, Mankoo BS, Costantini F, Pachnis V. Differential activities of the RET tyrosine kinase receptor isoforms during mammalian embryogenesis. Genes Dev. 2001;15(18):2433–44.
130. Jain S, Encinas M, Johnson Jr EM, Milbrandt J. Critical and distinct roles for key RET tyrosine docking sites in renal development. Genes Dev. 2006;20(3):321–33.
131. Lang D, Chen F, Milewski R, Li J, Lu MM, Epstein JA. Pax3 is required for enteric ganglia formation and functions with Sox10 to modulate expression of c-ret. J Clin Invest. 2000;106(8):963–71.
132. Southard-Smith EM, Kos L, Pavan WJ. Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. Nat Genet. 1998;18(1):60–4.
133. Pingault V, Bondurand N, Kuhlbrodt K, Goerich DE, Préhu MO, Puliti A, Herbarth B, Hermans-Borgmeyer I, Legius E, Matthijs G, Amiel J, Lyonnet S, Ceccherini I, Romeo G, Smith JC, Read AP, Wegner M, Goossens M. SOX10 mutations in patients with Waardenburg-Hirschsprung disease. Nat Genet. 1998;18(2):171–3.
134. Herbarth B, Pingault V, Bondurand N, Kuhlbrodt K, Hermans-Borgmeyer I, Puliti A, Lemort N, Goossens M, Wegner M. Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. Proc Natl Acad Sci USA. 1998;95(9): 5161–5.
135. Chi N, Epstein JA. Getting your Pax straight: Pax proteins in development and disease. Trends Genet. 2002;18(1):41–7.
136. Clarke JC, Patel SR, Raymond Jr RM, Andrew S, Robinson BG, Dressler GR, Brophy PD. Regulation of c-Ret in the developing kidney is responsive to Pax2 gene dosage. Hum Mol Genet. 2006;15(23):3420–8.
137. Zhu J, Garcia-Barcelo MM, Tam PK, Lui VC. HOXB5 cooperates with NKX2-1 in the transcription of human RET. PLoS One. 2011;6(6):e20815.
138. Zetterström RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T. Dopamine neuron agenesis in Nurr1-deficient mice. Science. 1997;276(5310):248–50.
139. Castillo SO, Baffi JS, Palkovits M, Goldstein DS, Kopin IJ, Witta J, Magnuson MA, Nikodem VM. Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. Mol Cell Neurosci. 1998;11(1–2):36–46.
140. Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, Burbach JP, Conneely OM. Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc Natl Acad Sci USA. 1998;95(7):4013–8.
141. Galleguillos D, Fuentealba JA, Gómez LM, Saver M, Gómez A, Nash K, Burger C, Gysling K, Andrés ME. Nurr1 regulates RET expression in dopamine neurons of adult rat midbrain. J Neurochem. 2010;114(4):1158–67.
142. Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF. The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature. 1999;399(6734):366–70.
143. Amiel J, Laudier B, Attié-Bitach T, Trang H, de Pontual L, Gener B, Trochet D, Etchevers H, Ray P, Simonneau M, Vekemans M, Munnich A, Gaultier C, Lyonnet S. Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. Nat Genet. 2003;33(4):459–61.

144. Moreau E, Vilar J, Lelièvre-Pégorier M, Merlet-Bénichou C, Gilbert T. Regulation of c-ret expression by retinoic acid in rat metanephros: implication in nephron mass control. Am J Physiol. 1998;275(6Pt 2):F938–45.
145. Oppenheimer O, Cheung NK, Gerald WL. The RET oncogene is a critical component of transcriptional programs associated with retinoic acid-induced differentiation in neuroblastoma. Mol Cancer Ther. 2007;6(4):1300–9.
146. Batourina E, Gim S, Bello N, Shy M, Clagett-Dame M, Srinivas S, Costantini F, Mendelsohn C. Vitamin A controls epithelial/mesenchymal interactions through Ret expression. Nat Genet. 2001;27(1):74–8.
147. Munnes M, Patrone G, Schmitz B, Romeo G, Doerfler W. A 5′-CG-3′-rich region in the promoter of the transcriptionally frequently silenced RET protooncogene lacks methylated cytidine residues. Oncogene. 1998;17(20):2573–83.
148. Angrisano T, Sacchetti S, Natale F, Cerrato A, Pero R, Keller S, Peluso S, Perillo B, Avvedimento VE, Fusco A, Bruni CB, Lembo F, Santoro M, Chiariotti L. Chromatin and DNA methylation dynamics during retinoic acid-induced RET gene transcriptional activation in neuroblastoma cells. Nucleic Acids Res. 2011;39(6):1993–2006.
149. Kjaer S, Kurokawa K, Perrinjaquet M, Abrescia C, Ibáñez CF. Self-association of the transmembrane domain of RET underlies oncogenic activation by MEN2A mutations. Oncogene. 2006;25(53):7086–95.
150. Takahashi M, Buma Y, Taniguchi M. Identification of the ret proto-oncogene products in neuroblastoma and leukemia cells. Oncogene. 1991;6(2):297–301.
151. Takahashi M, Asai N, Iwashita T, Isomura T, Miyazaki K, Matsuyama M. Characterization of the ret proto-oncogene products expressed in mouse L cells. Oncogene. 1993;8(11): 2925–9.
152. Asai N, Iwashita T, Matsuyama M, Takahashi M. Mechanism of activation of the ret proto-oncogene by multiple endocrine neoplasia 2A mutations. Mol Cell Biol. 1995;15(3): 1613–9.
153. Mehlen P, Bredesen DE. Dependence receptors: from basic research to drug development. Sci Signal. 2011;4(157):2.
154. Anders J, Kjar S, Ibáñez CF. Molecular modeling of the extracellular domain of the RET receptor tyrosine kinase reveals multiple cadherin-like domains and a calcium-binding site. J Biol Chem. 2001;276(38):35808–17.
155. Liu X, Vega QC, Decker RA, Pandey A, Worby CA, Dixon JE. Oncogenic RET receptors display different autophosphorylation sites and substrate binding specificities. J Biol Chem. 1996;271(10):5309–12.
156. Kawamoto Y, Takeda K, Okuno Y, Yamakawa Y, Ito Y, Taguchi R, Kato M, Suzuki H, Takahashi M, Nakashima I. Identification of RET autophosphorylation sites by mass spectrometry. J Biol Chem. 2004;279(14):14213–24.
157. Salvatore D, Barone MV, Salvatore G, Melillo RM, Chiappetta G, Mineo A, Fenzi G, Vecchio G, Fusco A, Santoro M. Tyrosines 1015 and 1062 are in vivo autophosphorylation sites in ret and ret-derived oncoproteins. J Clin Endocrinol Metab. 2000;85(10):3898–907.
158. Salvatore D, Melillo RM, Monaco C, Visconti R, Fenzi G, Vecchio G, Fusco A, Santoro M. Increased in vivo phosphorylation of ret tyrosine 1062 is a potential pathogenetic mechanism of multiple endocrine neoplasia type 2B. Cancer Res. 2001;61(4):1426–31.
159. Pandey A, Liu X, Dixon JE, Di Fiore PP, Dixit VM. Direct association between the Ret receptor tyrosine kinase and the Src homology 2-containing adapter protein Grb7. J Biol Chem. 1996;271(18):10607–10.
160. Encinas M, Crowder RJ, Milbrandt J, Johnson Jr EM. Tyrosine 981, a novel ret autophosphorylation site, binds c-Src to mediate neuronal survival. J Biol Chem. 2004;279(18): 18262–9.
161. Donatello S, Fiorino A, Degl'Innocenti D, Alberti L, Miranda C, Gorla L, Bongarzone I, Rizzetti MG, Pierotti MA, Borrello MG. SH2B1beta adaptor is a key enhancer of RET tyrosine kinase signaling. Oncogene. 2007;26(45):6546–59.

162. Borrello MG, Alberti L, Arighi E, Bongarzone I, Battistini C, Bardelli A, Pasini B, Piutti C, Rizzetti MG, Mondellini P, Radice MT, Pierotti MA. The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase Cgamma. Mol Cell Biol. 1996;16(5):2151–63.
163. Iwashita T, Asai N, Murakami H, Matsuyama M, Takahashi M. Identification of tyrosine residues that are essential for transforming activity of the ret proto-oncogene with MEN2A or MEN2B mutation. Oncogene. 1996;12(3):481–7.
164. Asai N, Murakami H, Iwashita T, Takahashi M. A mutation at tyrosine 1062 in MEN2A-Ret and MEN2B-Ret impairs their transforming activity and association with shc adaptor proteins. J Biol Chem. 1996;271(30):17644–9.
165. Coulpier M, Anders J, Ibáñez CF. Coordinated activation of autophosphorylation sites in the RET receptor tyrosine kinase: importance of tyrosine 1062 for GDNF mediated neuronal differentiation and survival. J Biol Chem. 2002;277(3):1991–9.
166. Buckwalter TL, Venkateswaran A, Lavender M, La Perle KM, Cho JY, Robinson ML, Jhiang SM. The roles of phosphotyrosines-294, −404, and −451 in RET/PTC1-induced thyroid tumor formation. Oncogene. 2002;21(53):8166–72.
167. Ichihara M, Murakumo Y, Takahashi M. RET and neuroendocrine tumors. Cancer Lett. 2004;204(2):197–211.
168. Schuringa JJ, Wojtachnio K, Hagens W, Vellenga E, Buys CH, Hofstra R, Kruijer W. MEN2A-RET-induced cellular transformation by activation of STAT3. Oncogene. 2001;20(38): 5350–8.
169. Plaza Menacho I, Koster R, van der Sloot AM, Quax WJ, Osinga J, van der Sluis T, Hollema H, Burzynski GM, Gimm O, Buys CH, Eggen BJ, Hofstra RM. RET-familial medullary thyroid carcinoma mutants Y791F and S891A activate a Src/JAK/STAT3 pathway, independent of glial cell line-derived neurotrophic factor. Cancer Res. 2005;65(5):1729–37.
170. Perrinjaquet M, Vilar M, Ibáñez CF. Protein-tyrosine phosphatase SHP2 contributes to GDNF neurotrophic activity through direct binding to phospho-Tyr687 in the RET receptor tyrosine kinase. J Biol Chem. 2010;285(41):31867–75.
171. Fukuda T, Kiuchi K, Takahashi M. Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase. J Biol Chem. 2002;277(21):19114–21.
172. Schuetz G, Rosário M, Grimm J, Boeckers TM, Gundelfinger ED, Birchmeier W. The neuronal scaffold protein Shank3 mediates signaling and biological function of the receptor tyrosine kinase Ret in epithelial cells. J Cell Biol. 2004;167(5):945–52.
173. Kales SC, Nau MM, Merchant AS, Lipkowitz S. Enigma prevents Cbl-c-mediated ubiquitination and degradation of RETMEN2A. PLoS One. 2014;9(1):e87116.
174. Borrello MG, Mercalli E, Perego C, Degl'Innocenti D, Ghizzoni S, Arighi E, Eroini B, Rizzetti MG, Pierotti MA. Differential interaction of Enigma protein with the two RET isoforms. Biochem Biophys Res Commun. 2002;296(3):515–22.
175. Wong A, Bogni S, Kotka P, de Graaff E, D'Agati V, Costantini F, Pachnis V. Phosphotyrosine 1062 is critical for the in vivo activity of the Ret9 receptor tyrosine kinase isoform. Mol Cell Biol. 2005;25(21):9661–73.
176. Jijiwa M, Fukuda T, Kawai K, Nakamura A, Kurokawa K, Murakumo Y, Ichihara M, Takahashi M. A targeting mutation of tyrosine 1062 in Ret causes a marked decrease of enteric neurons and renal hypoplasia. Mol Cell Biol. 2004;24(18):8026–36.
177. Jijiwa M, Kawai K, Fukihara J, Nakamura A, Hasegawa M, Suzuki C, Sato T, Enomoto A, Asai N, Murakumo Y, Takahashi M. GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. Genes Cells. 2008;13(4):365–74.
178. Ohgami N, Ida-Eto M, Shimotake T, Sakashita N, Sone M, Nakashima T, Tabuchi K, Hoshino T, Shimada A, Tsuzuki T, Yamamoto M, Sobue G, Jijiwa M, Asai N, Hara A, Takahashi M, Kato M. c-Ret-mediated hearing loss in mice with Hirschsprung disease. Proc Natl Acad Sci USA. 2010;107(29):13051–6.
179. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science. 1993;260(5111):1130–2.

180. Trupp M, Arenas E, Fainzilber M, Nilsson AS, Sieber BA, Grigoriou M, Kilkenny C, Salazar-Grueso E, Pachnis V, Arumäe U. Functional receptor for GDNF encoded by the c-ret proto-oncogene. Nature. 1996;381(6585):785–9.
181. Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW, Fox GM. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell. 1996;8(7):1113–24.
182. Treanor JJ, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips HS, Goddard A, Moore MW, Buj-Bello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE, Rosenthal A. Characterization of a multicomponent receptor for GDNF. Nature. 1996;382(6586):80–3.
183. Baloh RH, Tansey MG, Golden JP, Creedon DJ, Heuckeroth RO, Keck CL, Zimonjic DB, Popescu NC, Johnson Jr EM, Milbrandt J. TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. Neuron. 1997;18(5):793–802.
184. Jing S, Yu Y, Fang M, Hu Z, Holst PL, Boone T, Delaney J, Schultz H, Zhou R, Fox GM. GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family. J Biol Chem. 1997;272(52):33111–7.
185. Klein RD, Sherman D, Ho WH, Stone D, Bennett GL, Moffat B, Vandlen R, Simmons L, Gu Q, Hongo JA, Devaux B, Poulsen K, Armanini M, Nozaki C, Asai N, Goddard A, Phillips H, Henderson CE, Takahashi M, Rosenthal A. A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature. 1997;387(6634):717–21.
186. Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson Jr EM, Milbrandt J. Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron. 1998;21(6):1291–302.
187. Enokido Y, de Sauvage F, Hongo JA, Ninkina N, Rosenthal A, Buchman VL, Davies AM. GFR alpha-4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr Biol. 1998;8(18):1019–22.
188. Lindahl M, Poteryaev D, Yu L, Arumae U, Timmusk T, Bongarzone I, Aiello A, Pierotti MA, Airaksinen MS, Saarma M. Human glial cell line-derived neurotrophic factor receptor alpha 4 is the receptor for persephin and is predominantly expressed in normal and malignant thyroid medullary cells. J Biol Chem. 2001;276(12):9344–51.
189. Goodman KM, Kjær S, Beuron F, Knowles PP, Nawrotek A, Burns EM, Purkiss AG, George R, Santoro M, Morris EP, McDonald NQ. RET recognition of GDNF-GFRα1 ligand by a composite binding site promotes membrane-proximal self-association. Cell Rep. 2014;8(6):1894–904.
190. Poteryaev D, Titievsky A, Sun YF, Thomas-Crusells J, Lindahl M, Billaud M, Arumäe U, Saarma M. GDNF triggers a novel ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor alpha1. FEBS Lett. 1999;463(1–2):63–6.
191. Trupp M, Scott R, Whittemore SR, Ibáñez CF. Ret-dependent and -independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells. J Biol Chem. 1999;274(30):20885–94.
192. Paratcha G, Ledda F, Ibáñez CF. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell. 2003;113(7):867–79.
193. Enomoto H, Heuckeroth RO, Golden JP, Johnson EM, Milbrandt J. Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. Development. 2000;127(22):4877–89.
194. Rossi J, Tomac A, Saarma M, Airaksinen MS. Distinct roles for GFRalpha1 and GFRalpha2 signalling in different cranial parasympathetic ganglia in vivo. Eur J Neurosci. 2000;12(11):3944–52.
195. Nishino J, Mochida K, Ohfuji Y, Shimazaki T, Meno C, Ohishi S, Matsuda Y, Fujii H, Saijoh Y, Hamada H. GFR alpha3, a component of the artemin receptor, is required for migration and survival of the superior cervical ganglion. Neuron. 1999;23(4):725–36.

196. Andres R, Forgie A, Wyatt S, Chen Q, de Sauvage FJ, Davies AM. Multiple effects of artemin on sympathetic neurone generation, survival and growth. Development. 2001;128(19): 3685–95.
197. Lindfors PH, Lindahl M, Rossi J, Saarma M, Airaksinen MS. Ablation of persephin receptor glial cell line-derived neurotrophic factor family receptor alpha4 impairs thyroid calcitonin production in young mice. Endocrinology. 2006;147(5):2237–44.
198. Barlow A, de Graaff E, Pachnis V. Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. Neuron. 2003;40(5):905–16.
199. Kruger GM, Mosher JT, Tsai YH, Yeager KJ, Iwashita T, Gariepy CE, Morrison SJ. Temporally distinct requirements for endothelin receptor B in the generation and migration of gut neural crest stem cells. Neuron. 2003;40(5):917–29.
200. Tsui-Pierchala BA, Milbrandt J, Johnson Jr EM. NGF utilizes c-Ret via a novel GFL-independent, inter-RTK signaling mechanism to maintain the trophic status of mature sympathetic neurons. Neuron. 2002;33(2):261–73.
201. Greco A, Miranda C, Pierotti MA. Rearrangements of NTRK1 gene in papillary thyroid carcinoma. Mol Cell Endocrinol. 2010;321(1):44–9.
202. Greco A, Roccato E, Pierotti MA. TRK oncogenes in papillary thyroid carcinoma. Cancer Treat Res. 2004;122:207–19.
203. Hennige AM, Lammers R, Höppner W, Arlt D, Strack V, Teichmann R, Machicao F, Ullrich A, Häring HU, Kellerer M. Inhibition of Ret oncogene activity by the protein tyrosine phosphatase SHP1. Endocrinology. 2001;142(10):4441–7.
204. Qiao S, Iwashita T, Furukawa T, Yamamoto M, Sobue G, Takahashi M. Differential effects of leukocyte common antigen-related protein on biochemical and biological activities of RET-MEN2A and RET-MEN2B mutant proteins. J Biol Chem. 2001;276(12):9460–7.
205. Richardson DS, Lai AZ, Mulligan LM. RET ligand-induced internalization and its consequences for downstream signaling. Oncogene. 2006;25(22):3206–11.
206. Richardson DS, Mulligan LM. Direct visualization of vesicle maturation and plasma membrane protein trafficking. J Fluoresc. 2010;20(1):401–5.
207. Scott RP, Eketjäll S, Aineskog H, Ibáñez CF. Distinct turnover of alternatively spliced isoforms of the RET kinase receptor mediated by differential recruitment of the Cbl ubiquitin ligase. J Biol Chem. 2005;280(14):13442–9.
208. Richardson DS, Gujral TS, Peng S, Asa SL, Mulligan LM. Transcript level modulates the inherent oncogenicity of RET/PTC oncoproteins. Cancer Res. 2009;69(11):4861–9.
209. Pierchala BA, Milbrandt J, Johnson Jr EM. Glial cell line-derived neurotrophic factor-dependent recruitment of Ret into lipid rafts enhances signaling by partitioning Ret from proteasome-dependent degradation. J Neurosci. 2006;26(10):2777–87.
210. Tsui CC, Pierchala BA. CD2AP and Cbl-3/Cbl-c constitute a critical checkpoint in the regulation of ret signal transduction. J Neurosci. 2008;28(15):8789–800.

Chapter 13
The ROR Receptor Family

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Abbreviations

A–P	Anterior–posterior
aPKC	Atypical protein kinase C
ARNT	Arylhydrocarbon receptor nuclear translocator
BDA2	Brachydactyly type A2
BDB	Brachydactyly type B
BDC	Brachydactyly type C
BRI	Bone morphogenetic protein receptor type I
CAM-1	CAN abnormal migration 1
CaSR	Calcium-sensing receptor
CE	Convergent extension
Chip	Chromatin-immunoprecipitation
CKIε	Casein kinase Iε
CLL	Chronic lymphocytic leukemia
CPZ	Carboxypeptidase Z
CRD	Cysteine-rich domain
Cthrc1	Collagen triple-helix repeat-containing protein 1
Ddr	Discoidin-like domain receptor
DKK	Dickkopf
DMZ	Dorsal marginal zone
DRM	Detergent-resistant microdomains
DRS	Dominant form of Robinow syndrome
Dvl	Dishevelled

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ECM	Extracellular matrix
EF	Electrical field
EMT	Epithelial–mesenchymal transition
ER	Endoplasmic reticulum
ERDA	ER-associated degradation
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FLNa	Filamin A
Fzd	Frizzled
GAS	γ-interferon activation sequence
GRK2	G protein-coupled receptor kinase 2
GSK3	Glycogen synthase kinase 3
HH-stage	Hamburger and Hamilton stage
HIF	Hypoxia-inducible factor
TGF-β	Transforming growth factor-β
Ig	Immunoglobulin
IHC	Inner hair cell
JNK	c-Jun N-terminal kinase
KD	Kinase-dead
LD	LIM domain
LEF	Lymphoid enhancer factor
MAP3K	MAP kinase kinase kinase
MMP	Matrix metalloproteinase
MTOC	Microtubule-organizing center
MuSK	Muscle-specific receptor tyrosine kinase
NMJ	Neuromuscular junction
OHC	Outer hair cell
PAPC	Paraxial protocadherin
PC	Prostate carcinoma
PCP	Planar cell polarity
PGC	Primordial germ cell
PRD	Proline-rich domain
PTA	Persistent truncus arteriosus
RCC	Renal cell carcinoma
RRS	Recessive form of Robinow syndrome
RTK	Receptor tyrosine kinase
S/TRD	Serine/threonine-rich domain
SCF	Stem cell factor
SFK	Src-family kinase
sFRP	Secreted Fzd-related protein
Smo	Smoothened
STAT	Signal transducer and activator of transcription
TAK1	TGF-β-activated kinase-1
TCF	T-cell factor
TCL1	T-cell leukemia 1
TEP	Transepithelial potential difference

TK	Tyrosine kinase
Trk	Tropomyosin-receptor-kinase
UTR	Untranslated region, Ser/Thr, serine/threonine
Vangl2	Vang-like 2
VHL	von Hippel-Lindau
VSD	Ventricular septal defect
Wnts	Wnt-family proteins
WT	Wild-type
WT1	Wilms tumor protein 1

13.1 Introduction to the ROR Receptor Tyrosine Kinase Family

The Ror family of RTKs are type I transmembrane protein tyrosine kinases, originally identified as RTK-like orphan receptors in a PCR-based screen for novel RTKs [1]. The Ror-family RTKs are characterized by their extracellular Frizzled (Fzd)-like cysteine-rich domain (CRD) and membrane-proximal kringle domain (Fig. 13.1). The Ror-family RTKs are evolutionarily conserved in invertebrate and vertebrate, including *Nematostella vectensis* (sea anemone), *Hydra magnipapillata*,

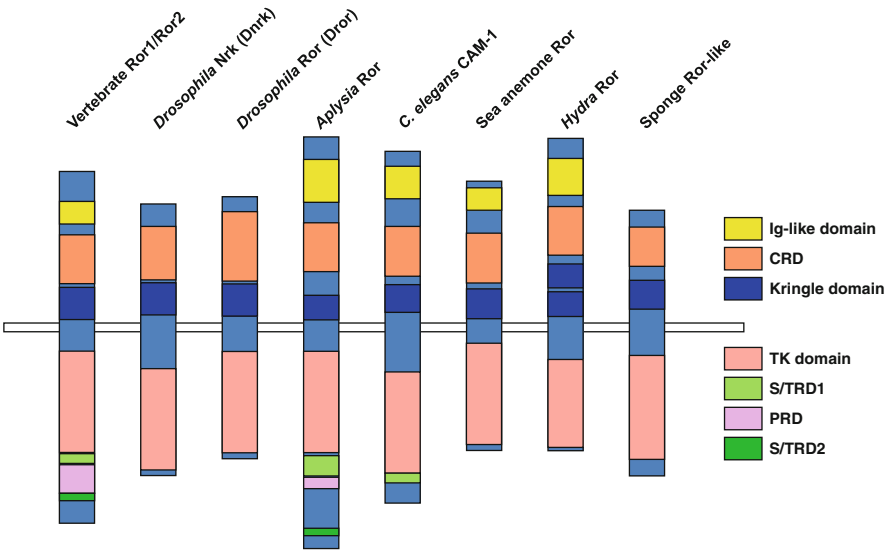

Fig. 13.1 Schematic representation of the structure of Ror-family RTKs in vertebrate and invertebrate [modified from Dev. Dyn. 239:1–15, 2010]. Immunoglobulin-like (Ig-like) domains, Frizzled-like cysteine-rich (CRD) domains, kringle domains, tyrosine kinase (TK) domains, proline-rich domains (PRD) and serine/threonine-rich domains (S/TRD1 and 2) are indicated

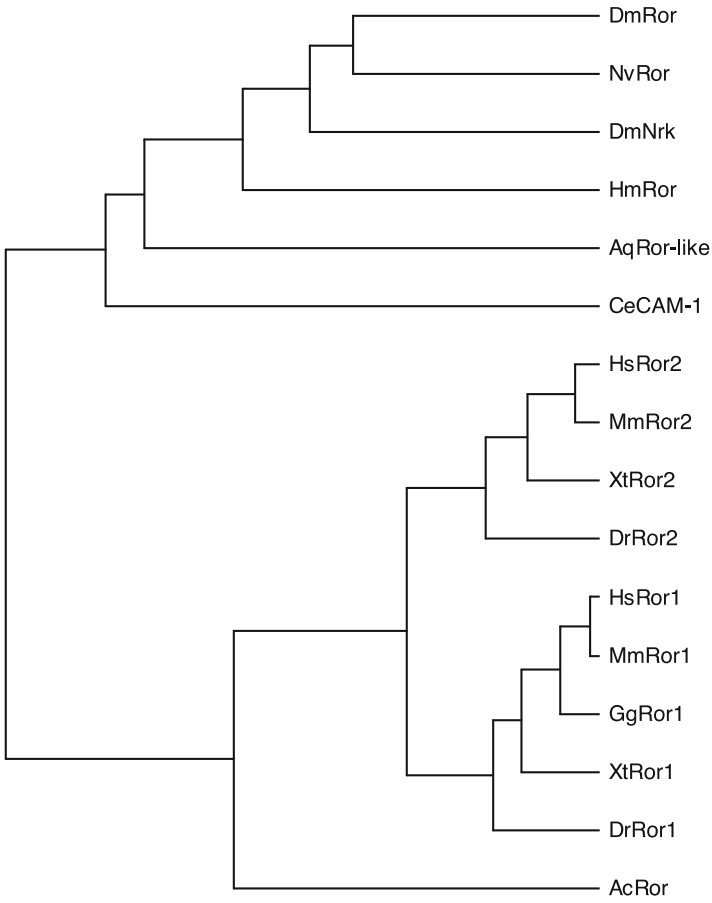

Fig. 13.2 A phylogenetic tree for the Ror-family RTKs. The tree was generated using the protein alignment program of the GeneWorks software (IntelliGenetics). The length of the horizontal lines indicates the estimated genetic distance between sequences. The aligned sequences are from *Amphimedon queenslandica* (Aq), *Nematostella vectensis* (Nv), *Hydra magnipapillata* (Hm), *Caenorhabditis elegans* (Ce), *Aplysia californica* (Ac), *Drosophila melanogaster* (Dm), *Danio rerio* (Dr), *Xenopus tropicalis* (Xt), *Gallus gallus* (Gg), *Mus musculus* (Mm), and *Homo sapiens* (Hs). It should be noted that in *Xenopus laevis* (Xl) only *Ror2* ortholog, *Xror2*, has been reported in the literature and database thus far

Caenorhabditis elegans, *Aplysia californica*, *Drosophila melanogaster*, *Danio rerio*, *Xenopus laevis*, *Gallus gallus*, *Mus musculus*, and *Homo sapiens* (Fig. 13.2). In addition, the sponge *Amphimedon queenslandica* also has a *Ror*-like gene. In vertebrate, the Ror-family RTKs consist of two structurally related members, Ror1 and Ror2.

The CRDs of the Ror-family RTKs exhibit similarities to the CRDs found in the Fzd family of seven transmembrane receptors for Wnt-family proteins (Wnts). Wnts are secreted cysteine-rich glycoproteins with lipid modifications, consisting

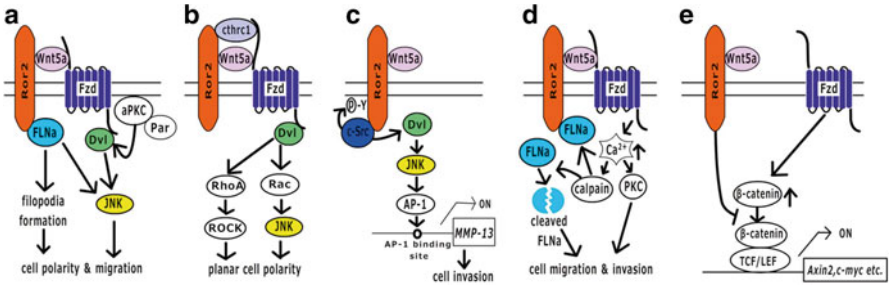

Fig. 13.3 The roles of Ror2 in noncanonical Wnt signaling. (**a**) Wnt5a-Ror2 signaling induces filopodia formation and JNK activation leading to the regulation of cell polarity and migration, through a process involving the association between Ror2 and FLNa. The Par–aPKC pathway cooperates with Wnt5a-Ror2 signaling to activate JNK, thereby promoting cell polarization. (**b**) Cthrc1 can associate with multiple Wnt and Fzd proteins, as well as with Ror2, and acts to enhance the formation of the Wnt-Fzd-Ror2 complex, thereby selectively activating the PCP pathway through activation of RhoA and Rac. (**c**) The binding of Wnt5a to Ror2 results in recruitment and activation of the protein-tyrosine kinase c-Src that in turn phosphorylates Ror2. In human osteosarcoma cells, constitutive expression of Wnt5a and Ror2 leads to sustained activation of noncanonical Wnt5a-Ror2 signaling in a cell-autonomous manner, and this confers invasive properties on the cells by inducing expression of the matrix metalloprotease *MMP-13* through activation of c-Src, Dvl, JNK, and AP-1. (**d**) Wnt5a can also trigger intracellular Ca^{2+} mobilization, leading to the activation of PKC and the cleavage of FLNa by calpain in a Ror2-dependent manner—key processes required for migration and invasion of melanoma cells. However it remains unclear whether Ror2-bound FLNa can be cleaved by Wnt5a stimulation. (**e**) In canonical Wnt signaling, accumulated β-catenin translocates to the nucleus, that in turn associates with TCF/LEF-family transcription factors and stimulates the expression of their target genes, such as *Axin2*, *c-Myc*, and *cyclin D1*. Wnt5a–Ror2 signaling inhibits canonical Wnt signaling at the level of TCF/LEF-mediated transcription

of 19 highly conserved members in mouse and human [2]. A series of studies demonstrate that Ror proteins can interact with several Wnts both physically and functionally, and the CRDs play a central role for these interactions [3–9]. In mammals, Ror2 acts as a receptor or co-receptor for Wnt5a and the CRD of Ror2 is required for binding to Wnt5a and mediating Wnt5a signaling to cell interior [4, 8]. In addition, Ror2 binds to Fzds through their CRDs, highlighting the importance of this domain in the function of the Ror-family RTKs [4, 10] (Fig. 13.3).

Recent evidence suggests that Ror1 also acts as a receptor for Wnt5a [11–15]. However, the signaling properties of Ror1 are largely unknown compared to those of Ror2. Ror2 mediates Wnt5a signaling by activating the β-catenin-independent pathways (noncanonical Wnt signaling pathways), known as planar cell polarity (PCP) pathway and Wnt–Ca^{2+} pathway [16–18]. The PCP pathway is known to regulate convergent extension (CE), a process driven by polarized cell migration, which leads to tissue narrowing along one axis and concomitant elongation along a perpendicular axis during embryogenesis [19]. In fact, both *Wnt5a-* and *Ror2-knockout* mice show a shortened anterior–posterior (A–P) body axis (CE defects) in addition to the PCP defects in ciliary bundles of cochlear hair cells [20, 21]. Furthermore, Ror2 mediates Wnt5a signaling to regulate polarized migration of

cultured cells [22, 23]. Importantly, Wnt5a–Ror2 signaling has both permissive and instructive roles in polarized cell migration depending on the cell types [24–26]. Wnt5a–Ror2 signaling also plays a crucial role in inhibiting the β-catenin-dependent pathway (canonical Wnt signaling pathway) [8, 27], which might play roles in embryogenesis and cancer invasion [28, 29].

Mutations within the *Ror2* gene in human cause skeletal disorders, autosomal dominant brachydactyly type B (BDB) and autosomal recessive form of Robinow syndrome (RRS) [30–33]. On the other hand, sustained or increased expression of Wnt5a and/or Ror2, which results in constitutive activation of Wnt5a–Ror2 signaling, induces expression of matrix metalloproteinases (MMPs), and thereby confers invasive properties on several types of cancer cells, including osteosarcoma, prostate carcinoma, renal cell carcinoma, and melanoma cells [29, 34–36]. Furthermore, expression of both Wnt5a and Ror2 is upregulated during epithelial–mesenchymal transition (EMT) of carcinoma cells. Consequently activated Wnt5a–Ror2 signaling confers highly motile and invasive properties on cancer cells by inducing expression of MMPs. On the other hand, *Ror1* is highly expressed in most of human B-cell chronic lymphocytic leukemia (CLL), but not in mature normal B cells, and is implicated in survival of the cells [11, 37, 38].

13.2 The Role of the ROR Receptor Tyrosine Kinase Family in Embryonic Development

13.2.1 Expression Patterns

Gene expression patterns of the Ror-family RTKs during development have been investigated in *C. elegans*, *Aplysia*, *D. melanogaster*, *X. laevis*, chicken, and mouse.

13.2.1.1 *C. elegans*

C. elegans ortholog of the Ror-family RTKs, *cam-1* (*CAN abnormal migration 1*), is expressed in the nervous system, intestinal cells, hypodermal cells, muscles in the head, pharynx, body wall, and vulval precursor cells [9, 39–41].

13.2.1.2 *Aplysia*

Aplysia ortholog of the Ror-family RTKs, *Apror*, is expressed in most developing neurons and some adult neuronal populations, including the neuroendocrine bag-cell neurons [42].

13.2.1.3 *D. melanogaster*

The expression patterns of *Dror* and *Dnrk* have been determined by in situ hybridization and Northern blot analysis [43, 44]. Although there is no apparent expression of *Dror* and *Dnrk* in the early embryos, both *Dror* and *Dnrk* are expressed by the germ-band extension stage. Unlike *C. elegans* and mouse genes encoding the Ror-family RTKs, the expression of *Dror* and *Dnrk* is restricted to the developing nervous systems. In addition, the highest expression of *Dnrk* is observed at pupal stage, when the nervous system is reconstructed.

13.2.1.4 *X. laevis*

Transcripts of *Xror2*, encoding the *Xenopus* ortholog of the mammalian Ror2, are detected in the dorsal mesoderm and ectoderm of a gastrula stage embryo, and then found in the notochord, neuroectoderm, and neural crest in late gastrula to neurula embryos [3]. The expression of *Xror2* is restricted to the pharyngeal arches in tail bud stage embryos.

13.2.1.5 Chicken

Chicken *Ror1* (*cRor1*) and *Ror2* (*cRor2*) are expressed in the developing limbs. Expression of *cRor1* is restricted to the proximal limb region until Hamburger and Hamilton stage (HH-stage) 25 and is expanded toward the distal region at later stages [45]. In early limb buds (HH-stage 23), *cRor2* expression is detected throughout the limb bud with a stronger expression in the anterior and posterior areas. At later stages, *cRor2* expression in central mesenchyme of the limb becomes weakened, while its expression in the anterior and posterior margins of the limb stays prominent. *cRor2* is also detected in several organs, including the nervous system, cartilage, muscles, mesonephros, heart, digestive system, lung, and liver [46]. These expression patterns of chicken *Rors* are essentially similar to those of mice.

13.2.1.6 Mouse

The expression patterns of mouse *Ror1* and *Ror2* at embryonic stages have been analyzed and compared by in situ hybridization [47, 48].

Gastrulation Stage *Ror2* is expressed in the entire primitive streak, while the expression of *Ror1* is localized in the anterior part of the embryo.

E8.5–E10.5 The expression of *Ror1* is partly overlapped with that of *Ror2*, but tends to localize more restricted regions at earlier stages of organogenesis. At E8.5, both *Ror1* and *Ror2* are expressed in the cephalic mesenchyme, predominantly in the cephalic neural crest cells. *Ror2* is also expressed in the dorsal part of the trunk

neural tube and the entire primitive streak regions. At later stages (E9.5–E10.5), both *Ror1* and *Ror2* exhibit similar expression patterns in the craniofacial region, including the medial and lateral processes and pharyngeal arches, which are originated from the cephalic neural crest cells. Specific expression of *Ror2* is found in the presomitic mesoderm.

Nervous System Mouse *Ror1* and *Ror2* are also expressed in the developing nervous systems. At E9.5–E10.5, *Ror1* is expressed in the dorsal part of the diencephalons and mid-hind brain boundary, while expression of *Ror2* is found in the forebrain and midbrain. At E12.5–E14.5, high levels of *Ror1* expression in the developing brain are detected in the terminal lamina of the hypothalamus, in the choroidal plexus of the forebrain, in the mesencephalic–metencephalic isthmus, and in the rostral and dorsal region of the myelencephalon. Low levels of *Ror1* expression are also detected in the ventricular zone of the telencephalon at E14.5. In contrast, *Ror2* is strongly expressed in the telencephalon at E12.5, and the expression is largely confined to the ventricular zone at E14.5. Slight and moderate levels of *Ror2* expression are observed in the myelencephalon and in the choroidal plexus of the forebrain, respectively. *Ror2* expression is also observed in the dorsal root ganglia, the trigeminal ganglia, the mesenchyal tissues within the mesencephalic flexure, the mammillary area, the vomeronasal organs, and the meninges. The distribution of *Ror1* and *Ror2* expression within the nervous system (rhinencephalon, cerebrum, mesencephalon, cerebellum, medulla oblongata, spinal cord) at a later stage of embryogenesis (E18) and after birth (P0, P8, P23) has been analyzed by Northern blot analysis [49]. At E18, P0, and P8, *Ror1* and *Ror2* exhibit similar distributions within the neural tissues, although the distribution of *Ror2* expression is rather widely observed. By P23, the expression of *Ror2* is hardly detectable in the neural tissues, while the sustained expression of *Ror1* is observed at this stage. At the cellular level, *Ror1* and *Ror2* are expressed in neural progenitor cells, neurons, and astrocytes, in which *Ror1* is more abundant than *Ror2* [12, 15, 50–52].

Eye At E12.5 and E14.5, *Ror1* is abundantly expressed in the mesenchymal tissue surrounding the eye, which gives rise to the scleral cartilage. *Ror2* is also expressed in mesenchyme adjacent to the eye at these stages, but at lower levels than *Ror1*. Interestingly, the expression of *Ror1*, but not *Ror2*, is also observed in the developing lens epithelium [48, 49].

Ear Expression of both *Ror1* and *Ror2* is observed in the otic capsule, external auditory meatus, and within all components of the inner ear at E12.5 and E14.5. Widespread expression of *Ror2* in E18.5 cochlea is observed, but restricted to the sensory hair cells of the organ of Corti [53].

Limb Strong expression of *Ror1* and *Ror2* is observed from E9.5 onward. At E10.5, the expression of *Ror1* is restricted to the proximal regions of the limb buds, while that of *Ror2* appears to extend throughout the limbs. Both of them are expressed in the limb mesenchyme, but not in the ectoderm. *Ror1* expression becomes restricted to the anterior and posterior regions (E12.5), and to the interdigital region (E13.5). *Ror2* expression is observed in the perichondrium of the digits and the marginal regions of the limbs (E12.5–E13.5).

Heart Both *Ror1* and *Ror2* are expressed in the myocardium, interventricular septum, aortic valve, and atrium at E13.5, but not in the epicardium.

Lung The expression of both *Ror1* and *Ror2* genes is detected in primitive alveoli of the developing lungs. *Ror1* expression is prominent in mesenchyme, whereas *Ror2* expression is primarily restricted to the outer walls of the bronchi.

Gut Expression of *Ror1* is detected in the stomach, intestines, and pancreatic primordium. The expression of Ror2 is detected in both the epithelium and mesenchyme of the developing gut in a region-specific manner [54].

Tooth *Ror2* expression is detectable in the tooth germs of the molars [55]. Detailed expression patterns in the developing tooth have been documented [56]. At the E13.5 bud stage, *Ror2* is expressed in the dental epithelium and the condensed dental mesenchyme, with stronger expression in the tip of the dental epithelium where the enamel knot can be formed. At the E14.5 cap stage, *Ror2* expression is seen in both the epithelium and mesenchymal compartments, with stronger expression in the enamel knot. At the E17.5 bell stage, *Ror2* transcripts are still detectable in the dental epithelium and dental papilla. At P0, *Ror2* expression becomes downregulated in the developing tooth, but is still detectable in the inner enamel epithelium and the differentiating odontoblasts.

13.2.2 Developmental Functions

13.2.2.1 *C. elegans*

Unlike vertebrates and *D. melanogaster*, the *C. elegans* genome contains only a single Ror gene, *cam-1*. Studies with *cam-1* mutants have shown that CAM-1 plays diverse functions in the developmental processes, including cell migration, asymmetric division, axon outgrowth, synaptic transmission, neurite pruning, and dauer larva formation.

Cell Migration Mutations in *cam-1* disrupt final proper positioning of certain migrating cells, resulting in both incomplete and excessive migrations during embryonic development [39, 57]. Mutations in *cam-1* also disrupt post-embryonic migrations. For example, the post-embryonic migrations of the QR descendants are disrupted in *cam-1* mutants so that the cells sometimes fail to migrate to their proper positions [57].

Asymmetric Cell Division During the post-embryonic larval stages, the six V cells (V1–V6) divide asymmetrically in a polarized manner along the A–P axis, each producing an anterior daughter that joins a growing epithelial syncytium and a posterior blast cell. Mutations in *cam-1* also cause reversals in the polarity of the V1 daughter cell fates [39]. Like V cells, the six P neuroblasts divide asymmetrically in first-larval-stage males to generate two different daughter neurons, the anterior CA neurons and the posterior CP neurons. In *cam-1* mutants, the positions of the most anterior CA neurons and the posterior CP neurons are reversed [39].

Axon Guidance Normally, the posterior CP neurons derived from P neuroblasts extend axons posteriorly. In *cam-1* mutants, the most CP neurons, which are anteriorly misplaced, extend its axon anteriorly to the head [39]. Interestingly, anteriorly directed CP axons and putative CA/CP reversals often occur independently, indicating that *cam-1* is implicated in axon guidance as well as asymmetric cell division. *Cam-1* has also shown to play a role in regulating axon guidance in RME head motor neurons [58].

Dauer Larva Formation Under less favorable conditions, wild-type (WT) animal forms a specialized larva, so-called dauer larva, which is non-feeding, developmentally arrested, and resistant to harsh conditions. The *cam-1* (also called as *kin-8*) mutants show constitutive dauer larva formation in spite of favorable conditions, although WT animals form no dauer under the same condition [40].

Wnt Signaling CAM-1 has been implicated in Wnt signaling. Mutations in *egl-20*, one of five Wnt genes in *C. elegans*, suppress the excessive migration phenotype of *cam-1* mutants, and overexpression of *egl-20*, like *cam-1* mutations, disrupts final proper positioning of certain migrating cells [5]. This finding proposes a model that CAM-1 sequesters EGL-20 possibly through direct binding of the CRD of CAM-1 with EGL-20. The sequestration model is further supported by a study showing that CAM-1 functions in a non-cell-autonomous manner in the development of vulva [9]. EGL-20-CAM-1 signaling also contributes to establish the proper arrangement of vulva precursor cells mediated via the intracellular domain of CAM-1 [59]. In addition, CAM-1 has been shown to act as a receptor of CWN-2, the *C. elegans* homologue of Wnt5, in the nerve ring development and neurite outgrowth [58, 60]. These findings suggest that the function of CAM-1 is not only regulated by the Wnt-binding extracellular domain, but also by the intracellular domain, which contributes to a cell-autonomous action.

Synaptic Transmission *Cam-1* mutants have been reported to have defects in synaptic transmission at the neuromuscular junction (NMJ) [41]. The defects at the NMJ appear to result from mislocalizations of an acetylcholine receptor subunit at the postsynaptic site and of synaptic vesicles at the presynaptic site. *C. elegans* mutants lacking expression of *Rig-3*, a cell surface Ig-superfamily protein, have an exaggerated paralytic response to a cholinesterase inhibitor, aldicarb, through aldicarb-induced increase in muscle acetylcholine receptor abundance, and a subsequent potentiation of postsynaptic responses at NMJs [61]. This effect seems to reflect changes in Wnt signaling, in particular the inhibition of CAM-1 function.

Neurite Pruning Studies with *Cam-1* mutants have also unraveled a novel function of the Ror-family RTKs in the developmental neurite pruning, a fundamental process to establish elaborate neural networks. In the developing nervous system, many of the neurites formed initially are eliminated later on. During subsequent periods of the first larval stage of *C. elegans*, the medially projected neurites are eliminated in a large proportion of individuals. Mutations in *wnt* genes or *cam-1* enhance neurite elimination, whereas overexpression of *cam-1* inhibits neurite elimination in an Wnt-dependent manner [62]. These findings indicate that Wnt-CAM-1 signaling plays an inhibitory role in neurite pruning.

13.2.2.2 *Aplysia* and *D. melanogaster*

The functions of the Ror-family RTKs in these organisms are still unknown, but their spatiotemporal expression patterns (as described above) suggest their functions in the development of the nervous system.

13.2.2.3 *X. laevis*

Convergent Extension Movements and Neural Plate Closure *Xror2* has been implicated in CE morphogenetic movements and neural plate closure during the early embryogenesis [3]. *Xror2* was initially isolated by the screening for genes activated by ectopic expression of *Xlim-1*, a gene encoding the LIM homeobox protein expressed in the Spemann organizer. The organizer is required to coordinate cell fate specification and morphogenetic movements at early stages of development. As a result of the cooperative action of the induction and morphogenetic movements, the organizer can properly execute the body plan. Ectopic expression of *Xror2* in the dorsal marginal zone (DMZ) at four-cell-stage embryos causes a shortened body axis with dorsal bending and abnormalities in head structures by inhibiting CE movements that is characterized by medio-lateral intercalation of the DMZ. The failure of proper CE movements during gastrulation is not due to changes in cell fate, because overexpression of *Xror2* does not affect cell differentiation of neural tissue and the notochord. Surprisingly, ectopic expression of Xror2-TM, a kinase-domain deleted mutant, or Xror2-3I, a kinase activity-dead mutant, can also inhibit CE movements, suggesting Xror2 appears to regulate CE in a kinase activity-independent manner. WT Xror2 and Xror2-3I, but not Xror2-TM, further inhibit neural plate closure during neurulation, suggesting that *Xror2* has distinct roles in CE movements and neural plate closure. Xror2-FZΔ1, a mutant with a small deletion in the extracellular Fzd-like CRD, a predicted binding domain of Xror2 with Wnt, exhibits much less inhibitory effects on CE movements and neural plate closure during gastrulation and neurulation compared with WT Xror2, raising the possibility that Xror2 might interact with a Wnt signaling pathway. *Xenopus* Wnt5a (Xwnt5a) has been shown to regulate CE movements during *Xenopus* development [63, 64]. In fact, Xwnt5a–Xror2 signaling has been implicated in the transcriptional activation of *paraxial protocadherin* (*XPAPC*) whose product plays an important role in the regulation of CE movements during *Xenopus* gastrulation [65]. Xwnt5a-Xror2-mediated transcriptional activation of *XPAPC* seems to require the intrinsic kinase activity of Xror2, indicating that both kinase activity-dependent and -independent functions of Xror2 are involved in the regulation of CE movements during *Xenopus* gastrulation.

Neural Crest Specification *Xror2* has also been implicated in neural crest specification [66]. The Neural crest comprises stem-cell-like cells that are originated from the neural plate border in vertebrate embryos. The neural crest cells migrate to diverse locations in the body and differentiate into various cell types, including face

cartilaginous cells, melanocytes, and peripheral nerve cells. *Xror2* is expressed in early neuroectoderm at the neural plate border, and later in migrating neural crest populations. Ectopic expression of Xror2-TM or suppressed expression of *Xror2* with an antisense morpholino oligonucleotide strongly inhibits expression of *FoxD3* and *Sox8*, neural crest-specific marker genes at premigratory stages, indicating that Xror2 regulates neural crest specification, although the involvement of Xror2 in neural crest cell migration at later stages has not been yet elucidated.

13.2.2.4 Chicken

The role of the chicken homologue of Ror2, cRor2, in skeletogenesis has been examined in the developing chick limbs by using retroviral vector-mediated overexpression of the truncated forms of cRor2, corresponding to mutations in human *Ror2 gene* that cause autosomal recessive form of Robinow syndrome (RS) and autosomal dominant BDB [46] (for details of RS and BDB, see Sect. 13.3). Overexpression of these truncated forms of cRor2 does not show any apparent effects on skeletal patterning, but the cartilage elements in the wings and legs are shorter and thicker compared with the uninfected contralateral control wings and limbs. Once initial cartilaginous condensations are formed, a fine balance between chondrocyte proliferation and differentiation determines the growth of the skeletal elements. Overexpression of the truncated forms of cRor2 appears to disrupt severely the growth plate architecture as well as chondrocyte differentiation, without affecting its proliferation.

13.2.2.5 Mouse

The roles of Ror1 and Ror2 during the development of various organs and tissues have been studied extensively using the mutant mice. It has been shown in mouse that Ror2 binds to Wnt5a via its CRD [4], and that *Ror2* and *Wnt5a* are expressed during the development of various organs and tissues, including the facial primordia, limb mesenchyme, neural crest-derived tissues, and genital tubercle, in a spatiotemporally similar manner [21, 47, 48]. In addition, *Ror2* and *Wnt5a* mutant mice exhibit overall similarities in their phenotypes [4, 21, 67, 68] (see Table 13.1). In fact, it has been well documented that Ror2 acts as a receptor or co-receptor for Wnt5a (see below).

Neonatal Lethality Like *Wnt5a* mutant mice, *Ror1* and *Ror2* mutant mice usually die neonatally (or perinatally) presumably due to respiratory dysfunction and cyanosis [67–69].

Skeletal Phenotypes *Ror2* mutant mice exhibit dwarfism and several skeletal phenotypes, i.e., short limbs, tail, abnormal vertebrae, fusion of ribs, and abnormal facial structures, somewhat similar to those of *Wnt5a* mutant mice, with severer defects in their distal portions [67, 68]. *Ror2* mutant mice also show a unique

Table 13.1 Phenotypes of *Ror1*, *Ror2*, *Ror1/Ror2 and Wnt5a* mutant mice

	Ror1 KO*	*Ror2* KO	*Ror1*/Ror2* KO	*Wnt5a* KO	References
(1) neonatal lethality	+	+	+	+	[4, 21, 67–69]
(2) forced respiration and cyanosis	+	+	+	+	[4, 67–69]
(3) respiratory dysfunction	+	+	+	+	[4, 67–69]
(4) skeletal phenotypes					
(a) facial anomalies	–	+	++	++	[4, 21, 69]
(b) hypoplasia of the maxilla and mandible	–	+	++	++	[4, 21, 69]
(c) short limbs and tail	–	+	++	++	[4, 21, 69]
(d) dysplasia of the proximal long bones	–	+	++	++	[4, 21, 69]
(e) dysplasia of the distal long bones	–	+	+	+	[4, 21, 69]
(f) sternal defect**	+	–	+	N.D.	[69, 75]
(g) dysplasia of symphysis of the pubic bone	–	–	+	N.D.	[69]
(h) osteoclastgenesis defect	N.D.	+	N.D.	+	[71]
(i) other defects**	+	N.D.	N.D.	N.D.	[75]
(5) cardiac phenotypes					
(a) ventricular septal defect (VSD)	–	+	+	+	[4, 68]
(b) persistent truncus arteriosus (PTA)	–	+	N.D.	+	[69, 170]
(c) transposition of the great arteries	–	–	+	+	[4, 69]
(6) lung phenotypes					
truncation of trachea and abnormalities in distal lung architecture	–	+	++	++	[4, 69, 72]
(7) gut phenotype					
gut elongation defects	N.D.	+	N.D.	+	[54, 171]
(8) urogenital phenotypes					
duplicated ureters and kidneys	+	+	N.D.	+	[73–75]
outgrowth defects in the genitals	–	+	++	+++	[4, 21, 55, 76]
(9) somite phenotypes					
reduction of somites in size along the A-P axis and irregular shape	N.D.	+	N.D.	N.D.	[4, 21, 55]
(10) palate phenotype					
cleft palate	–	+	N.D.	+	[26, 55]
(11) tooth phenotypes					
retardation of tooth growth and defective development of odontoblasts and ameloblass	N.D.	+	N.D.	+	[56]
(12) germ cell phenotype					
primordial germ cells (PGC) defects	N.D.	+	N.D.	N.D.	[25]
(13) PCP/CE defect in cochlea	N.D.	+	N.D.	+	[53, 163]
(14) uterine phenotypes after pregnancy					
crypt formation defects for embryo implantation and infertility	–	–	+	+	[79]

Modified from Dev Dyn. 239:1–15, 2010. See Text about current details on * and ** (page 16)

anomaly characterized by mesomelic dysplasia (significant or complete loss of the radius, ulna, tibia, and fibula). In contrast with *Ror2* mutant mice, *Ror1* mutant mice are similar in size to WT mice and do not show any apparent skeletal and morphological abnormalities [69]. However, gross appearances of *Ror1* and *Ror2*-double mutant mice (*Ror1*/*Ror2* mutant mice) exhibit enhanced *Ror2* mutant phenotypes. The extents of the limb and tail shortening and malformation of the facial structures, observed in *Ror2* mutant mice are more profound in *Ror1*/*Ror2* mutant mice [69]. *Ror1*/*Ror2* mutant mice show a drastic enhancement of the skeletal abnormalities observed in *Ror2* mutant mice. Interestingly, dysplasia of the proximal long bones, in addition to dysplasia of the distal long bones, is also observed in *Ror1*/*Ror2* mutant mice, like *Wnt5a* mutant mice. Of interest, *Ror1*/*Ror2* mutant mice exhibit a sternal defect (sternal agenesis) and dysplasia of the symphysis of the pubic bone, skeletal abnormalities, that are not seen in *Ror2* mutant mice [69], indicating that *Ror1* and *Ror2* interact genetically during the development of the skeletal system and that Ror2 can compensate for the lack of Ror1 function in *Ror1* mutant mice. In the cartilage, *Ror2* is expressed in the cartilaginous condensations and throughout the growth plate. Additional expression is seen in the perichondrium and periosteum [55, 67]. Loss of *Ror2* leads to a decrease of chondrocyte differentiation and delay of ossification, eventually leading to mesomelic limb shortening [55]. Two types of cells that are of great importance in bone formation and remodeling are osteoblasts and osteoclasts, which deposit and resorb bone tissues, respectively. Both Wnt5a and Ror2 are expressed in osteoblasts, and osteoblast-lineage cells from *Wnt5a*, but not *Ror2*, mutant mice exhibit impaired osteoblast differentiation due to reduced expression of *Lrp5* and *Lrp6*, required for canonical Wnt signaling, suggesting that Ror2-independent Wnt5a signaling enhances canonical Wnt signaling by inducing expression of *Lrp5* and *Lrp6* during osteoblastogenesis [70]. Osteoblast-lineage cells are required for differentiation of monocyte/macrophage-lineage osteoclast precursors into multinucleated osteoclasts. Mice deficient in either *Wnt5a* or *Ror2*, and those with either osteoclast precursor-specific *Ror2* mutation or osteoblast-lineage cell-specific *Wnt5a* mutation, show impaired osteoclastogenesis, suggesting that Wnt5a produced by osteoblast-lineage cells activates Ror2 signaling in osteoclast precursors to induce osteoclastogenesis [71].

Cardiac Phenotypes *Ror1* mutant mice do not show any apparent abnormalities in their hearts, while *Ror2* mutant mice have ventricular septal defects (VSD) and persistent truncus arteriosus (PTA) without any other abnormalities in the heart, i.e., malformation of valves, aortic arch, and great vessels [68]. Intriguingly, in addition to VSD and PTA, *Ror1*/*Ror2* mutant mice show complete transposition of the great arteries, a phenotype observed in *Wnt5a* mutant mice but not in *Ror2* mutant ones, indicating that *Ror1* and *Ror2* interact genetically in regulating the development of the cardiac system.

Lung Phenotypes *Ror1* mutant mice do not show any morphological abnormalities in their lungs, while *Ror2* mutant mice show abnormalities in their lungs with foreshortened trachea along the proximal–distal axis and a reduced number of cartilage rings, similar to *Wnt5a* mutant mice [4, 72]. Expansion of the alveoli in *Ror1* and

Ror2 mutant newborns is found to be incomplete, suggesting that *Ror1* and *Ror2* mutants die due to difficulty in breathing [69].

Gut Phenotypes At E11.5, Ror2 mutant mouse embryos exhibit a shorter middle midgut with a larger diameter and more accumulation of epithelial cells in the middle midgut than WT mouse embryos, while the total cell numbers remain unaltered, suggesting that Ror2 plays important roles in midgut elongation presumably through the mechanism of CE movements of epithelial cells [54].

Urogenital Phenotypes Both *Ror2* and *Wnt5a* mutant mice exhibit duplicated ureters and kidneys due to ectopic formation of the ureteric buds [73, 74]. The findings that the phenotypes are severer in *Wnt5a* mutants than in *Ror2* mutants, and that the loss of one allele of *Wnt5a* enhances the phenotypes observed in *Ror2* mutants [73, 74], indicate a genetic interaction between *Wnt5a* and *Ror2* during the kidney development. Since *Ror1* mutant mice also exhibit duplicated ureters and kidneys [75], Ror1 and Ror2 might function redundantly to mediate Wnt5a signaling in the developing kidney. Consistent with the expression of *Ror2* in the genital tubercle at E13.5 [55], *Ror2* mutant mice exhibit outgrowth defects in the genitals, yet their genital hypoplasia is somewhat modest compared with *Wnt5a* mutant mice [4, 55, 76].

Somite Phenotypes Expression of *Ror2* is seen in the presomitic mesoderm, the somitomeres of the presomitic mesoderm, and the epithelium of the differentiating somites, but not in the forming somite. Somites of *Ror2* mutant mice are reduced in size along the anteroposterior axis and show an irregular cone-like shape at E13.5 [55]. Cells constituting the epithelium and the basolateral region of the somites show an irregular size and shape, whereas cells in the center of the somite appear normal.

Palate Phenotypes Like *Wnt5a* mutant mice, it has been shown that *Ror2* mutant mice exhibit a cleft palate [55]. *Ror2* is indeed expressed in a graded manner along the anteroposterior axis in the epithelium and mesenchyme of the palate. It has been demonstrated that Ror2 plays a role in mediating Wnt5a signaling in the regulation of cell proliferation and migration during the palate development [26].

Tooth Phenotypes It has been reported that *Ror2* mutant mice exhibit normal size, shape, and number of molar at E16.5 and E17.5 [55]. At P0, however, retarded tooth growth and defective development of odontoblasts and ameloblasts are found in *Ror2* mutant mice, similar to *Wnt5a* mutant mice [56].

Germ Cell Phenotypes *Ror2Y324C* mutant mice has been identified in a genome-wide recessive ethylnitrosourea mutagenesis screen for primordial germ cell (PGC) defects in E9.5 embryos [25]. This missense mutation of *Ror2* gene results in Y to C amino acid substitution at residue 324 within the kringle domain, a conserved structural motif in the extracellular domain of Ror2. *Ror2Y324C* mutant embryos exhibit defects in tail elongation and somite segmentation and die perinatally, similar to the *Ror2* targeted deletion allele. In *Ror2Y324C* mutants, polarized migration and survival of PGCs are dysregulated, resulting in a diminished number of PGCs in the embryonic gonad. It has been demonstrated by *ex vivo* culture that Ror2 acts

autonomously in PGCs to enhance their polarized response to the chemoattractant Stem Cell Factor (SCF, or secreted KitL). Mutations in *Wnt5a* phenocopy *Ror2Y324C*.

Inner Ear Phenotypes PCP refers to coordinated polarization of cells in the plane of a cell sheet. In the organ of Corti of the cochlea in the inner ears, each hair cell is intrinsically polarized, and its polarity is displayed by the orientation of the stereociliary bundle and the position of the kinocilium. The vertices of all the hair bundles point toward the periphery of cochlear spiral, manifesting a distinct form of PCP parallel to the plane of the epithelial sheet. In addition, hair cells are precisely patterned into one row of inner (IHCs) and three rows of outer hair cells (OHCs). The organ of Corti appears to undergo cellular rearrangements characteristic of CE movements during the terminal differentiation of the hair cells when they acquire PCP [77]. Like *Wnt5a* mutant mice, *Ror2* mutant mice exhibit misoriented stereocilia and cochlear phenotypes (a shortened and widened cochlea) [53], indicating that Ror2 is required for proper PCP and CE movements during the cochlear development.

Phenotypes in the Nervous System Expression patterns of *Ror1* and *Ror2* emphasize possible roles of the Ror-family RTKs in regulating the formation and function of the mammalian central nervous system. Indeed, it has been shown that both Ror1 and Ror2 play roles in regulating neurite extension and synapse formation in cultured hippocampal neurons [12, 52]. Expression of *Ror1* and *Ror2* within the developing cerebral cortex is mainly localized in neural progenitor cells that are distributed within the ventricular zone [15]. Wnt5a–Ror1 and Wnt5a–Ror2 signalings have been shown to play roles in regulating neocortical neurogenesis through the maintenance of proliferative and neurogenic state of neural progenitor cells [15].

Phenotypes Observed in Ror1 Mutant Mice It has been reported that *Ror1* mutant mice have neonatal or perinatal lethality due to respiratory defects, but that these mice lack any abnormalities in skeletogenesis as described above [69]. However, it has recently been found that the *Ror1* mutant mice have subtle skeletal defects at birth; they show fusions of the sternebrae, a cleft in the basisphenoid bone, and abnormal development of the cervical vertebral element C2 [75]. In this report, homozygous mice have been shown to survive and display abnormal synchondrosis in the cranial base, postnatal growth retardation, and age-related skeletal changes. Furthermore, *Ror1*$^{-/-}$ mutants exhibit additional phonotypic defects, such as female infertility probably due to an imperforated hymen, kidney defects, and occasionally enlarged seminal vesicles in *Ror1*$^{-/-}$ males. More recently, Dr. Greenberg's group has found that *Ror1* mutant mice still expressed a truncated Ror1 protein [78] (personal communication). We have also found that *Ror1* mutant mice currently available in our facility expressed the two transcripts, i.e., one transcript lacks the exons 3 and 4, and another one lacks the exon 3 [288 bp, corresponding to the 96 amino acid residues within the Ig-like domain of Ror1 (unpublished data)]. While the former transcript possesses a very early termination codon due to a frameshift in the exon 5, the latter transcript can produce a truncated Ror1 protein with 96 a.a. deletion within its Ig-like domain. These observations suggest that *Ror1* mutant mice might exhibit hypomorphic phenotypes, and that Ig-like domain of Ror1 appears to play a role for the function of Ror1.

Phenotypes in the Uterus After Pregnancy It has been reported that female mice with uterine deletion of both *Ror1* and *Ror2* exhibit severely compromised fertility [79]. In *Ror1/Ror2* mutant female mice mated with WT male mice, 29% of plug-positive female mice produce a small number of pups. In the uterus after pregnancy of *Ror1/Ror2* mutant female mice, villi-like epithelial projections from the main uterine lumen toward the antimesometrial pole at regularly spaced intervals are disrupted, leading to the malformation of crypts for embryo implantation. It has been shown that *Bmp2*, which is critical for crypt formation, is downregulated and its downstream effectors Smad1/5/8 are not activated in the uterus of *Ror1/Ror2* mutant female mice. Moreover, female mice with deletion or overexpression of *Wnt5a* in the uterus exhibit similar phenotypes. These findings suggest that regulated activation of Wnt5a–Ror signaling via BMP–Smad signaling in the uterine is critical for crypt formation.

13.3 The Role of the ROR Receptor Tyrosine Kinase Family in Human Disease

13.3.1 Brachydactyly Type B

Autosomal dominant BDB is the most severe of the brachydactylies characterized by hypoplasia/aplasia of distal phalanges and nails and caused by mutations within the *Ror2* gene [30, 33]. All of the reported mutations in BDB cause truncation of the predicted Ror2 protein before and after the tyrosine kinase (TK) domain within its cytoplasmic portion, indicating dominant negative functions of mutant Ror2 proteins. Some of the mutations in BDB patients not only cause truncation of the protein, but also produce a novel C-terminal polypeptide (reviewed in [80], raising a question of the possible significance of the novel C-terminal peptides in BDB. *Ror2W749X*, resulting from a 2246G to A substitution mapping downstream of the TK domain, is linked to BDB in humans. Interestingly, although mice heterozygous for *Ror2W749FLAG* (the mutation of W749X is introduced into the mouse *Ror2* gene) are normal and do not show brachydactyly, homozygous mice exhibit phenotypes resembling human RRS [81].

13.3.2 Robinow Syndrome

Recessive RS (RRS) is characterized by mesomelic shortening, abnormal morphogenesis of the face (fetal facies), hemivertebrae, and genital hypoplasia [31, 32] reviewed in [80, 82] and caused by homozygous mutations within the *Ror2* gene, indicating loss-of-function effects of mutant Ror2 proteins. Consistent with this observation, it has been appreciated that *Ror2* mutant mice (*Ror2-/-* mice) can be a

model for the developmental pathology of RRS in humans [55]. Distinct missense, nonsense, and frameshift mutations in various domains of Ror2 protein, including Ig-like, CRD, Kringle, and TK domains, have been found in patients with RRS [31, 32, 83, 84]. It has been reported that missense mutations within the *Ror2* gene causing RRS are retained in the endoplasmic reticulum (ER) presumably due to improper folding of mutant Ror2 proteins and subjected to ER-associated degradation (ERDA) [83, 85]. In comparison with BDB, the range of severity in RRS is broad. Clinical and molecular examinations of two adults with RRS identify nephrological abnormalities (hydronephrosis, nephrocalcinosis, and renal failure) and endocrinological abnormalities (elevated gonadotropic hormones), respectively [84]. In addition, patients with RRS caused by a novel intragenic *Ror2* deletion, involving exons 6 and 7, exhibit cleft lip, cleft palate, and cardiac anomalies, and one patient has syringomyelia as well [86]. While phenotypes of RRS are more severe than those of autosomal dominant form of Robinow syndrome (DRS) in general, their phenotypic features overlap with each other, suggesting that the same signaling pathway is involved in both forms RS. In fact, heterogeneous missense mutations in *Wnt5a* gene, although found in only a subset of patients, are associated with DRS [87].

13.3.3 Cancer Progression

13.3.3.1 Ror2 and Osteosarcomas

Osteosarcoma is a malignant bone tumor with poor prognosis due to its propensity for metastasis. *Ror2* is expressed at high levels in both human osteosarcoma specimens and cell lines [29, 88]. Human osteosarcoma SaOS-2 and U2OS cells also express Wnt5a, and thereby manifest constitutively activated Wnt5a–Ror2 signaling, which results in the formation of invadopodia and confers invasiveness on these cells [29]. Invadopodia are actin-rich protrusions, observed in highly invasive cancer cells, that degrade the extracellular matrix (ECM) [89]. It was found that Wnt5a–Ror2 signaling, via activation of c-Src, induces expression of MMP-13, which is required for the formation of invadopodia, and that tyrosine kinase activity of Ror2 is indispensable for this process [29]. Furthermore, Wnt5a–Ror2 signaling activates c-Jun N-terminal kinase (JNK) and thereby induces phosphorylation of and direct binding of the transcription factor AP-1 (c-Jun and ATF2) to the AP-1 binding site within the promoter region of *MMP-13* gene [90]. Wnt5b, another Wnt protein with structurally highly related to Wnt5a, is also overexpressed in human osteosarcoma specimens and can bind to Ror2 [88].

13.3.3.2 Ror2 and Prostate Carcinomas

Prostate carcinomas (PCs) are prevalent cancers in men, which develop in an androgen-dependent manner and often progress in an androgen-independent manner following treatment with androgen receptor antagonist. It has been shown that

Wnt5a is expressed at high levels both in human prostate carcinoma cell lines, DU145 and LNCap cells, and in tumor regions of patients with PCs. This indicates that Wnt5a-mediated signaling plays an important role in a pathology of PCs [34]. Indeed, constitutively active Wnt5a–Ror2 signaling enhances cell migration and confers invasiveness on these cells [34]. It was found that Wnt5a–Ror2 signaling activates JNK via protein kinase D, resulting in the expression of a gene encoding MMP-1, which is required for the Wnt5a-dependent migration and invasion of prostate cancer cells [34].

13.3.3.3 Ror2 and Renal Cell Carcinomas

Renal cell carcinomas (RCCs) are common tumors of the kidney with poor prognosis. It has been shown that Ror2 is heavily phosphorylated in the kidney of patients with RCCs and is expressed highly in human RCC cell lines [36], indicating that Ror2 is implicated in a pathology of RCCs. In fact, suppressed expression of *Ror2* results in reduced expression of *MMP-2*, whose upregulation correlates with advanced stages of RCCs [91].

13.3.3.4 Ror2 and Melanomas

Wnt5a–Ror2 signaling has also been shown to play important roles in invasion and metastasis of melanoma cells [92, 93]. In fact, pulmonary metastases are formed highly in nude mice injected with human melanoma cells in a Ror2-dependent manner [93]. Wnt5a–Ror2 signaling contributes to melanoma cell invasiveness not only by activating PKC but also by activating a protease calpain through an increase in intracellular Ca^{2+} concentration (Fig. 13.3d) [94]. Indeed, Wnt5a induces calpain-mediated filamin A (FLNa) cleavage in melanoma cells, contributing to the high motility and invasiveness of melanoma cells (Fig. 13.3d), although the underlying mechanism responsible for this remains unclear. Interestingly, Ror2 is expressed in highly invasive melanoma cells, while Ror1 is expressed in proliferative, poorly invasive cells, and its expression is decreased in metastatic cells [95]. Furthermore, hypoxic condition initiates a shift of Ror1-positive melanomas to a more invasive, Ror2-positive phenotype in a manner depending on Wnt5a whose expression is also upregulated by hypoxia [95]. Interestingly, a study using a Wnt5a antagonist delivered to melanoma lines *in vitro* has shown that Wnt5a–Ror2 signaling might be a suitable therapeutic target for the treatment of malignant melanomas [96].

13.3.3.5 Ror2 and Colon Carcinomas

The calcium-sensing receptor (CaSR) is a G protein-coupled receptor, which senses extracellular Ca^{2+} levels. It has been reported that activation of CaSR in colonic myofibroblasts cells and colon carcinoma cells results in induced expression of both

Wnt5a and Ror2, which potentiates Wnt5a–Ror2 signaling in the colon carcinoma cells [97]. Activation of Wnt5a–Ror2 signaling in the colon carcinoma cells increases expression of CDX2, an intestinal-specific homeobox domain transcription factor known to play a critical role in the differentiation and maintenance of intestinal epithelial functions. Because Ca^{2+} is a chemoprotective agent for colon cancer, these results suggest that Wnt5a–Ror2 signaling may regulate calcium-mediated chemoprevention of colon cancer by inducing CDX2 expression. With this respect, it is worth noting that reduced levels of CDX2 in $Cdx2^{+/-}$ mice result in increased colon tumor progression [98, 99]. Furthermore, epigenetic repression of *Ror2* has been reported in colon carcinomas [100]. *Ror2* is frequently repressed by aberrant promoter hypermethylation in human colon cancer cell lines and primary tumors. Restoration of *Ror2* expression impairs cancer cell growth both *in vitro* and *in vivo*, suggesting that the epigenetical loss of *Ror2* has a pro-tumorigenic role in colon carcinomas.

13.3.3.6 Ror2 and EMT

EMT is a fundamental process during embryonic development, by which epithelial cells acquire mesenchymal, fibroblast-like phenotypes with reduced cell–cell adhesion, loss of cell polarity, and increased migratory and invasive properties [101, 102]. EMT also contributes to the progression of epithelial tumors (carcinomas) by endowing cancer cells with migratory and invasive properties, leading to invasion and metastasis [101, 102]. Members of the Snail family of transcription factors play critical roles in EMT during both embryonic development and cancer progression [103–105]. It was found that Snail induces expression of both Wnt5a and Ror2, and thereby activates Wnt5a–Ror2 signaling in A431 epidermoid carcinoma cells [106]. Activation of Wnt5a–Ror2 signaling is dispensable for Snail-induced down- and upregulated expression of E-cadherin and vimentin, respectively, characteristics of mesenchymal cells, but is essential for Snail-induced expression of MMP-2, which is critically required for highly migratory and invasive properties of Snail-expressing A431 cells [106].

13.3.3.7 Ror1 and Malignancies

Recent molecular investigations have demonstrated that overexpression of a set of tumor-associated antigens can be correlated with malignancies. Overexpression of *Ror1* has been reported in B-cell CLL [11, 37, 38, 107, 108]. Ror1 and Wnt5a can physically interact and cooperatively activate NF-κB when overexpressed in HEK293 cells [11]. Wnt5a enhances the survival of CLL cells in vitro, an effect that could be neutralized by anti-Ror1 antisera. These findings suggest that Ror1 functions as an oncofetal surface antigen through which Wnt5a activates NF-κB-dependent survival signaling in CLL. Furthermore, Ror1 can interact with T-cell leukemia 1 (TCL1) that is overexpressed in CLL cells. Transgenic mice with

B-cell-specific expression of human *Ror1* develop a B-cell leukemia with features of human CLL. When these mice are crossed with transgenic mice with B-cell-specific expression of human *TCL1*, *Ror1* accelerates development and progression of leukemia [109]. Therefore, Ror1 might be used as a diagnostic marker and a therapeutic target for CLL. It has been shown that the expression of Ror1 is correlated with disease-free survival and overall survival rates in patients with ovarian cancer, indicating that Ror1 might serve as a prognostic marker for ovarian cancer [110]. Ror1 is also expressed in other types of human cancer cell lines, including melanoma, cervical, breast, lung, and gastric cancer cell lines, and is implicated in the proliferation, survival, and/or therapy resistance of these cancer cells [95, 111–116]. Knockdown of *Ror1* can sensitize HeLa cervical cancer cells to chemotherapy [111]. Furthermore, oncogenic receptor tyrosine kinase Met can transphosphorylate Ror1 to mediate Met-driven transformation in HS746T gastric carcinoma and NCI-H1993 lung adenocarcinoma cells [112]. NKX2-1 (also known as TITF1 and TTF-1), a lineage-survival oncogene in lung adenocarcinomas, induces the expression of *Ror1*, which in turn sustains a favorable balance between prosurvival PI3-kinase/Akt and pro-apoptotic p38 signaling [115]. Importantly, inhibition of Ror1 appears to be effective in treatment of gefitinib-resistant lung adenocarcinomas with various resistance mechanisms, including a secondary EGFR mutation, Met amplification, and HGF overexpression [115]. In the case of breast cancers, Ror1 might regulate cell migration and invasion in addition to cell proliferation, through the induction of EMT [116, 117].

13.3.4 Tissue Damage, Repair, and Inflammation

A recent study has shown that Wnt5a is required for intestinal crypt regeneration after experimental colonic damage in mice [118]. *Wnt5a* is induced in stromal cells in the colonic wound bed, and Wnt5a inhibits proliferation of intestinal epithelial stem cells by mediating activation of transforming growth factor-β (TGF-β) signaling, leading to the proper regeneration of crypts after damage, possibly through the formation of clefts [118]. In vitro analyses using intestinal organoid culture system have shown that activation of TGF-β signaling pathway by Wnt5a is mediated through Ror2, although Ror2 does not seem to mediate Wnt5a- and TGF-β-induced growth inhibition [118]. It has also been shown that expression of both Wnt5a and Ror2 is induced in a damaged mouse kidney after unilateral ureteral obstruction treatment [119]. Ror2 expression is induced in tubular epithelial cells that express Snail and vimentin (mesenchymal markers) during renal fibrosis, suggesting that Ror2 might be induced in epithelial cells undergoing EMT. Activation of Wnt5a–Ror2 signaling in epithelial cells undergoing EMT may play an important role in disrupting tubular basement membrane via MMP-2 induction, leading to the generation of myofibroblasts during renal fibrosis [119]. In adult tissues, repair is generally accompanied by a robust inflammatory response following the damage of various tissues. Importantly, transient or reversible inflammation contributes to

tissue repair, while persistent or repeated inflammation is involved in severe pathological conditions, including tissue fibrosis. Therefore, Wnt5a–Ror2 signaling may be involved in inflammatory responses following tissue damages or in irreversible pathological conditions. With this respect, it is noteworthy that expression of *Wnt5a* and *Ror2* can be induced in human mesenchymal stem cells by stimulation with IL-1β, a pro-inflammatory cytokine [120].

13.3.5 Mental Disorder

The *Ror1* promoter has been identified to exert high genetic background effects from a genome-wide association study of bipolar disorder [121]. It has been reported that insomnia-associated genotypic differences are highly concentrated within genes, including *Ror1*, involved in neural function [122]. It has been shown that mutations in *Ror2* cause RRS (as described above), and that some of RRS patients exhibit mental retardation [123, 124].

13.4 Domain Structure of the ROR Receptor Tyrosine Kinase Family

The Ror-family RTKs share a conserved domain structure. In general, the extracellular regions are predicted to contain the immunoglobulin (Ig)-like, CRD, and kringle domains (see Fig. 13.1), all of which are thought to mediate protein–protein interactions. Typically, the intracellular region of the Ror-family RTKs is predicted to contain the TK domain, serine/threonine-rich domain (S/TRD1), proline-rich domain (PRD), and another serine/threonine-rich domain (S/TRD2) (Fig. 13.1).

CRD
The extracellular CRDs of the Ror-family RTKs have 10 conserved cysteine residues within ~130 amino acids and exhibit similarities to the CRDs found in the Fzd family of seven transmembrane Wnt receptors. In addition, the CRDs can also be found in the various soluble and transmembrane proteins which play crucial roles during the developmental processes, including secreted Fzd-related proteins (sFRPs); muscle-specific receptor tyrosine kinase (MuSK); Smoothened (Smo), a co-receptor of Hedgehog signaling; carboxypeptidase Z (CPZ), a secreted metallocarboxypeptidase; and collagen XVIII (C18) [125–128].

Kringle Domain
The membrane proximal kringle domains, consisting of ~80 amino acids, are also highly conserved throughout the Ror-family RTKs. The kringle domain is characterized by a triple loop via triple disulfide bridges and is thought to play a role in binding to peptides, proteins, membranes, or phospholipids. This domain has been

found in a number of proteins such as serine proteases involved in blood-clotting cascade, apolipoprotein(s), hepatocyte growth factor, and transmembrane Kremen proteins [129–133]. Kremen proteins were identified as the receptors for Dickkopf (Dkk), which acts as an inhibitor of Wnt/β-catenin signaling [134]. Although the function of the kringle domain in the Ror-family RTKs and Kremens is still elusive, the kringle domains in Ror proteins are assumed to function as recognition modules for binding to another Wnt regulatory proteins.

Ig-like Domain

In addition to the CRDs and kringle domains, most of the Ror-family members, except *Drosophila* Rors (Dror and Dnrk) and the sponge *Amphimedon* Ror-like protein, possess the Ig-like domains in their N-terminal extracellular regions. The Ig-like domains are found in a large number of proteins and frequently involved in protein–protein and protein–ligand interactions. The Ig-like domains of the Ror-family RTKs may also contribute to binding to their ligands and extracellular or transmembranous signaling molecules.

Tyrosine Kinase Domain

The cytoplasmic regions of the Ror-family RTKs contain conserved TK domains which exhibit highest similarities to those of the tropomyosin-receptor-kinase (Trk) family, MuSK family, and discoidin-like domain receptor (Ddr) family [135–137]. Tyrosine kinase activity of Ror2 can be assessed by autophosphorylation and tyrosine phosphorylation of its substrate, a scaffold protein 14-3-3β [6, 7, 138]. Similar to other RTKs, forced homodimerization of Ror2 or stimulation with Wnt5a have been shown to enhance tyrosine kinase activity of Ror2, indicating that Ror2 can transduce signals by mediating tyrosine phosphorylation of Ror2 by itself and its downstream substrates [138–140].

Serine/Threonine-Rich and Proline-Rich Domains

Both Ror1 and Ror2 also possess the S/TRD1, PRD, and S/TRD2 at their C-terminal to the TK domains [1, 49, 141]. Domains similar to the S/TRDs or PRDs of the Ror-family RTKs have not been found in any other proteins. The S/TRD1s of Ror1 and Ror2 show a higher degree of homology (~67% identity), while their PRDs show a relatively lower degree of homology (~30% identity). Interestingly, their S/TRD2s do not exhibit any apparent homology. These cytoplasmic domains are thought to be involved in the functions of the Ror-family RTKs by interacting with signaling mediators.

13.5 ROR1

13.5.1 ROR1 Gene

13.5.1.1 Promoter Structure

γ-interferon activation sequence (GAS)-like elements, a known signal transducer and activator of transcription (STAT) binding site, have been identified in the ROR1 promoter by sequence analysis [142]. Furthermore, a database search (http://www.cbrc.jp/research/db/TFSEARCH.html) for transcription factor binding sites shows that several consensus sequences recognized by transcription factors are present within the 5'-untranslated region (UTR) (+100 to -3000) of human *Ror1* (Table 13.2).

Table 13.2 Promoter structure of *Ror1*

Name	Target sequence	Location
ATF-2	TGACGTYA	–207/–214, –318/–325, –1959/–1966, –2529/–2536, –2876/–2883
C/EBP	TKTGGWNA	–44/–57, –140/–152, –391/–404, –939/–951, –987/–1000, –1605/–1617, –1783/–1796, –2215/–2227, –2402/–2414, –2450/–2461
CREB	TGACGTMA	–2876/–2883
CUX1	ATYGATSSS	–2790/–2799, –2820/–2829
DBP	GTTACRTMAK	–2528/–2537
E2F	TTTSGCGC	–2313/–2320
E4BP4	RTTAYGTAAY	–2527/–2538
ETS1	AMMGGAWRW	–1864/–1876
EVI-1	ACAAGATAA	–1225/–1235, –2729/–2737
FOXA2	TRTTTRYTY	–511/–522, –1393/–1404, –1509/–1520, –1792/–1807
FOXD3	NAWTGTTTRTTT	+53/+41, –6/–7, –511/–522, –1792/–1807, –1902/–1913
GATA	GATAAGNM	+78/+65, –27/–35, –215/–224, –225/–238, –354/–363, –528/–537, –578/–587, –892/–900, –916/–931, –954/–963, –1001/–1009, –1225/–1239, –1315/–1325, –1379/–1388, –1528/–1537, –1561/–1570, –1631/–1644, –1919/–1928, –2085/–2094, –2248/–2257, –2266/–2275, –2477/–2489, –2534/–2543, –2668/–2677, –2766/–2779, –2891/–2899
IKZF	YGGGAW	–466/–477, –595/–606/–607/–618, –1254/–1262, –2154/–2165, –2166/–2177, –2253/–2262, –2720/–2731
IRF	SAAAAGYGAAASY	–2341/–2353

(continued)

Table 13.2 (continued)

Name	Target sequence	Location
MYB	NSYAACGGN	–312/–320
MYC	ANCACGTGNNW	–2358/–2369
MYCN	CACGTG	–2358/–2369
MYOD	SRACAGGTGKYG	–1029/–1040
MZF1	NGNGGGGA	–143/–250, –1747/–1754, –2812/–2819, –2899/–2906
NFE2	TGCTGASTCAY	–1696/–1706, –2012/–2022
NKX-2_5	TYAAGTG	–833/–840, –1438/–1449, –1597/–1604, –1675/–1682, –1999/–2005, –2472/–2478, –2491/–2498, –2771/–2778, –2948/–2954
OCT	RTAATNA	+45/+31, –653/–665, –771/–786, –1094/–1106, –1480/–1493, –1512/–1527, –1629/–1641, –1798/–1812, –1824/–1838, –2830/–2842
p300	RGGAGTNNNNS	–562/–575, –2119/–2132
PBX-1	ANCAATCAW	+47/+37, –216/–224, –1790/–1804
PRRX2	ANYYAATTANY	–272/–287, –735/–750, –918/–933, –1591/–1606, –2769/–2784
RUNX	TGCGGT	–392/–397, –533/–538, –1038/–1043, –1178/–1183, –1348/–1353,–1609/–1614, –1895/–1900, –2878/–2883
SOX	AACAAT	+50/+39, +37/+28, –663/–672
SP1	GRGGCRGGGW	–2565/–2581
SRY	AAACWAM	+50/+39, –274/–280, –283/–289, –513/–524, –662/–673, –802/–808, –1101/–1108, –1302/–1308/–1398/–1404, –1451/–1462, –1658/–1669, –2213/–2219, –2308/–2314,–2907/–2913
STAT	TTCCCRKAA	–2461/–2469
TATA	NTATAAAAR	–192/–201, –1647/–1655, –2372/–2386
TFCP2	GCNMNAMCMAA	–2315/–2324
USF	CACGTG	–252/–259, –1021/–1041, –2360/–2367, –2982/–2989

13.5.1.2 Transcriptional Regulation

IL-6 stimulation of human multiple myeloma MM1 cells induces phosphorylation of the STAT3, which in turn activates transcription of *Ror1* gene by directly binding to the STAT3-binding sites of *Ror1* promoter [142]. In CLL cells, STAT3 is constitutively phosphorylated, which results in sustained binding of phosphorylated STAT3 to the *Ror1* promoter and transcriptional activation of *Ror1* gene [142].

13.5.2 ROR1 Protein

13.5.2.1 Amino Acid Sequence

Human Ror1 (hRor1) and mouse Ror1 (mRor1) proteins consist of 937 amino acids (914 amino acids after cleavage of their signal peptides). hRor1 and mRor1 share 97% amino acid identity overall. The amino acid sequence of hRor1 is as follows:

MHRPRRRGTRPPLLALLAALLLAARGAAAQETELSVSAELVPTS
SWNISSELNKDSYLTLDEPMNNITTSLGQTAELHCKVSGNPPPTI
RWFKNDAPVVQEPRRLSFRSTIYGSRLRIRNLDTTDTGYFQCVA
TNGKEVVSSTGVLFVKFGPPPTASPGYSDEYEEDGFCQPYRGIA
CARFIGNRTVYMESLHMQGEIENQITAAFTMIGTSSHLSDKCSQ
FAIPSLCHYAFPYCDETSSVPKPRDLCRDECEILENVLCQTEYIF
ARSNPMILMRLKLPNCEDLPQPESPEAANCIRIGIPMADPINKN
HKCYNSTGVDYRGTVSVTKSGRQCQPWNSQYPHTHTFTALRF
PELNGGHSYCRNPGNQKEAPWCFTLDENFKSDLCDIPACDSKD
SKEKNKMEILYILVPSVAIPLAIALLFFFICVCRNNQKSSSAPVQ
RQPKHVRGQNVEMSMLNAYKPKSKAKELPLSAVRFMEELGE
CAFGKIYKGHLYLPGMDHAQLVAIKTLKDYNNPQQWTEFQQ
EASLMAELHHPNIVCLLGAVTQEQPVCMLFEYINQGDLHEFL
IMRSPHSDVGCSSDEDGTVKSSLDHGDFLHIAIQIAAGMEYLS
SHFFVHKDLAARNILIGEQLHVKISDLGLSREIYSADYYRVQS
KSLLPIRWMPPEAIMYGKFSSDSDIWSFGVVLWEIFSFGLQPY
YGFSNQEVIEMVRKRQLLPCSEDCPPRMYSLMTECWNEIPSR
RPRFKDIHVRLRSWEGLSSHTSSTTPSGGNATTQTTSLSASPV
SNLSNPRYPNYMFPSQGITPQGQIAGFIGPPIPQNQRFIPINGY
PIPPGYAAFPAAHYQPTGPPRVIQHCPPPKSRSPSSASGSTSTGH
VTSLPSSGSNQEANIPLLPHMSIPNHPGGMGITVFGNKSQKPY
KIDSKQASLLGDANIHGHTESMISAEL

13.5.2.2 Posttranscriptional Modification

Phosphorylation Wnt5a stimulation of HCC1500 and HCC3153 breast cancer cells induces phosphorylation of Ror1 on serine/threonine (Ser/Thr) residue(s) in a glycogen synthase kinase 3 (GSK3)-dependent manner [13]. Ser/Thr phosphorylation of Ror1 is also detected in unstimulated human CLL cells [143]. In human CLL cells, treatments with anti-Ror1 monoclonal antibodies against the extracellular kringle domain and CRD reduce phosphorylation of Ror1 and subsequently induce their apoptosis [144]. On the other hand, oncogenic receptor tyrosine kinase Met can transphosphorylate Ror1 to mediate Met-driven transformation of HS746T gastric carcinoma and NCI-H1993 lung adenocarcinoma cells [112]. A recent study has identified three tyrosine residues (Y641, Y645, and Y646) within the TK domain and five tyrosine residues (Y786, Y789, Y822, Y828, and Y836) within the PRD as

possible Met-dependent phosphorylation sites within Ror1, although the three tyrosine residues within the TK domain appear to be phosphorylated indirectly by Src that binds to the PRD of Ror1 [145].

Glycosylation Ror1 is modified by N-linked glycosylation in human CLL cells [143]. Tunicamycin treatment, which blocks the synthesis of N-linked glycosylation, suppresses trafficking of Ror1 onto cell surface in human CLL cells and Ror1-induced filopodia formation in HEK293 cells [143], suggesting an important role of the N-linked glycosylation of Ror1 in its trafficking and/or function. In fact, the glycosylated Ror1 is detected more frequently in CLL cells from progressive patients than in those from nonprogressive patients [144].

Ubiquitination Although Ror1 is ubiquitinated when coexpressed with HA-tagged ubiquitin in CHO cells [143], the sites of ubiquitination and its role have not been determined yet.

13.5.3 ROR1 Ligands

It has been reported that Wnt5a can interact physically with Ror1 in vitro [11] and in the mouse brain [12], and that Ror1 is phosphorylated on Ser/Thr residue(s) following Wnt5a stimulation [13], suggesting that Wnt5a is a ligand for Ror1. In fact, Ror1 is required for Wnt5a-induced Dishevelled (Dvl) phosphorylation and Rac activation [14, 15]. Resistin, a cysteine-rich protein primarily secreted from mature adipocytes, also binds to Ror1 through the CRD and kringle domain of Ror1 and inhibits tyrosine phosphorylation of Ror1 in mouse 3T3-L1 preadipocytes [146]. Resistin can modulate adipogenesis and glucose uptake in 3T-L1 cells through its binding to Ror1 [146].

13.5.4 ROR1 Activation and Signaling

13.5.4.1 Dimerization

Ror1 can form complex with both Wnt5a and Ror2 in the mouse brain [12]. It is proposed that Ror1-Ror2 heterodimers might mediate Wnt5a signaling, leading to synapse formation in hippocampal neurons [12].

13.5.4.2 Phosphorylation

GSK3-dependent phosphorylation of Ror1 is implicated in Wnt5a signaling [13]. Transphosphorylation of Ror1 by oncogenic receptor tyrosine kinase Met can mediate Met-driven transformation of HS746T gastric carcinoma and NCI-H1993 lung adenocarcinoma cells [112].

13.5.4.3 Pathway Activation

Recent findings showing that Ror1 is required for Wnt5a-induced Dvl phosphorylation and Rac activation [14, 15] indicate that Ror1 can mediate Dvl–Rac pathway in noncanonical Wnt signaling. It has also been reported that coexpression of Wnt5a and Ror1 activates NF-kB in HEK293 cells, suggesting an involvement of NF-kB pathway in Wnt5a–Ror1 signaling [11].

13.5.4.4 Cross talk with Other Receptor Systems

The Met RTK has been shown to cross talk with other RTKs, including EGFR, HER3, and Ron [147]. As mentioned above, Ror1 is transphosphorylated by Met and thereby mediates Met-driven transformation of HS746T gastric carcinoma and NCI-H1993 lung adenocarcinoma cells, in which Met is overexpressed due to *Met* gene amplification [112]. Although active forms of EGFR (EGFRL858R) and ErbB2 (ErbB2V659E), in addition to Met, can be co-immunoprecipitated with Ror1 when overexpressed in COS-7 cells, among them only Met induces phosphorylation of Ror1 [112].

13.6 ROR2

13.6.1 ROR2 Gene

13.6.1.1 Promoter Structure

A database search (http://www.cbrc.jp/research/db/TFSEARCH.html) for transcription factor binding sites shows that several consensus sequences recognized by transcription factors are present within the 5'-UTR (+100 to -3000) of human *Ror2* (Table 13.3).

13.6.1.2 Transcriptional Regulation

The transcriptional regulation of *Ror2* via hypoxia-inducible factor (HIF)-1α and -2α has been shown in renal cell carcinoma that harbors inactivating mutations in the tumor suppressor von Hippel-Lindau (*VHL*) leading to the subsequent stabilization of HIF-1α and HIF-2α [148]. Furthermore, it has been found that HIF-2α and arylhydrocarbon receptor nuclear translocator (ARNT, also known as HIF-1β) can bind to a restricted region of the immediate *Ror2* promoter region, which lacks any known hypoxia response element binding sequence, in cells lacking expression of *VHL* and overexpressing only HIF-2α. Consistent with this finding, *C. elegans* homologue of *Ror2*, *cam-1*, has been identified as a HIF-dependent hypoxia target gene in *C. elegans* [149].

Table 13.3 Promoter structure of *Ror2*

Name	Target sequence	Location
AP-1	RSTGASTNMNW	–1042/–1052, –1649/–1664, –1690/–1698, –1989/–1999
ARNT	CACGTG	–2261/–2276
ATF2	TGACGTYA	–1653/–1660
C/EBP	TKTGGWNA	–468/–481, –908/—920, –114/–1127, –1213/–1229, –1604/–1616, –2000/–2011
CREB	TGACGTMA	–274/–285, –1653/–1660
CUX1	ATYGATSSS	–877/–886, –1994/–2003
E2F1	TTTSGCGC	–11/–18
EGR	NTGCGTRGGCGK	–299/–310
ETS-1	AMMGGAWRW	+42/+33, –474/–486, –1088/–1097, –1642/–1654, –2751/–2760
EVI-1	ACAAGATAA	–2776/–2790, –2888/–2902
FOXA2	TRTTTRYTY	–494/–504, –687/–698, –767/–778, –1880/–1891, –2403/–2414
FOXD3	NAWTGTTTRTTT	–494/–504, –1008/–1020
GATA	GATAAGNM	+41/+32, –107/–116, –607/–621, –851/–860, –1093/–1106, –1122/–1132, –1317/–1327, –1656/–1676, –1876/–1886, –2101/–2111, –2159/–2173, –2240/–2249, –2280/–2300, –2335/–2348, –2466/–2478, –2634/–2643, –2695/–2714, –2754/–2763
HSF	RGAANNWTC	–712/–721, –1121/–1130, –1465/–1475, –2169/–2178
IKZF	YGGGAW	–955/–963, –1437/–1449, –2242/–2253, –2308/–2316, –2638/–2649, –2981/–2992
IRF	SAAAAGYGAAASY	–459/–471
MYB	NSYAACGGN	–749/–757, –2797/–2805
MYC	ANCACGTGNNW	–2263/–2275
MYCN	CACGTG	–2263/–2275
MYOD	SRACAGGTGKYG	–1451/–1461, –2497/–2508
MZF1	NGNGGGGA	+3/–5, –92/–99, –533/–540, –1189/–1201, –1293/–1300, –2506/–2513
NFKB1	GGGAMTTYCC	–2/–11, –1330/–1339, –1437/–1450, –1487/–1496
NHLH1	GGGNCGCAGCTGCG NCCC	–184/–205
NKX2-5	TYAAGTG	–732/–738, –1408/–1414, –1570/–1576, –2388/–2395, –2728/–2735, –2936/–2942
OCT	RTAATNA	–753/–767, –774/–788, –889/–901, –1213/–1225, –2735/–2749
PPRX2	ANYYAATTANY	–887/–901, –938/–953
REL	SGGRNWTTCC	–2/–11, –1330/–1339, –1438/–1451, –1487/–1496
RORa	WAWNNAGGTCA	–361/–374
RUNX	TGCGGT	–1298/–1303, 1330/–1339, –1655/–1660, –1925/–1930, –2493/–2498, –2734/–2739

(continued)

Table 13.3 (continued)

Name	Target sequence	Location
SP1	GRGGCRGGGW	–83/–92, –423/–432
SRY	AAACWAM	–491/–502/, –635/–642, –651/–657, –725/–731, –787/–793, –906/–912, –1561/–1567, –1725/–1731, –1816/–1822, –1878/–1895, –2010/–2016, –2442/–2448, –2888/–2894
STAT	TTCCCRKAA	–828/–836
TATA	NTATAAAAR	–1373/–1382, –1806/–1815, –2351/–2366
TFCP2	GCNMNAMCMAA	–12/–22, –168/–178, –1100/1110, –1756/–1766
USF	CACGTG	–800/–807, –1587/–1594, –1686/–1694, –2212/–2219, –2262/–2275, –2378/–2390
ZBTB6	GGCTCYATCAYC	–1592/–1604

13.6.2 ROR2 Protein

13.6.2.1 Amino Acid Sequence

Human Ror2 (hRor2) protein consists of 943 amino acids (916 amino acids after cleavage of its signal peptide). Mouse Ror2 (mRor2) is one amino acid longer than hRor2. hRor2 and mRor2 share 92% amino acid identity overall. The amino acid sequence of hRor2 is as follows:

MARGSALPRRPLLCIPAVWAAAALLLSVSRTSGEVEVLDPNDPLGPLDG
QDGPIPTLKGYFLNFLEPVNNITIVQGQTAILHCKVAGNPPPNVRWLKN
DAPVVQEPRRIIIRKTEYGSRLRIQDLDTTDTGYYQCVATNGMKTITAT
GVLFVRLGPTHSPNHNFQDDYHEDGFCQPYRGIACARFIGNRTIYVDS
LQMQGEIENRITAAFTMIGTSTHLSDQCSQFAIPSFCHFVFPLCDARSRT
PKPRELCRDECEVLESDLCRQEYTIARSNPLILMRLQLPKCEALPMPES
PDAANCMRIGIPAERLGRYHQCYNGSGMDYRGTASTTKSGHQCQPW
ALQHPHSHHLSSTDFPELGGGHAYCRNPGGQMEGPWCFTQNKNVRM
ELCDVPSCSPRDSSKMGILYILVPSIAIPLVIACLFFLVCMCRNKQKASAS
TPQRRQLMASPSQDMEMPLINQHKQAKLKEISLSAVRFMEELGEDRF
GKVYKGHLFGPAPGEQTQAVAIKTLKDKAEGPLREEFRHEAMLRARL
QHPNVVCLLGVVTKDQPLSMIFSYCSHGDLHEFLVMRSPHSDVGSTD
DDRTVKSALEPPDFVHLVAQIAAGMEYLSSHHVVHKDLATRNVLVYD
KLNVKISDLGLFREVYAADYYKLLGNSLLPIRWMAPEAIMYGKFSIDS
DIWSYGVVLWEVFSYGLQPYCGYSNQDVVEMIRNRQVLPCPDDCPA
WVYALMIECWNEFPSRRPRFKDIHSRLRAWGNLSNYNSSAQTSGASN
TTQTSSLSTSPVSNVSNARYVGPKQKAPPFPQPQFIPMKGQIRPMVPPP
QLYVPVNGYQPVPAYGAYLPNFYPVQIPMQMAPQQVPPQMVPKPSSH
HSGSGSTSTGYVTTAPSNTSMADRAALLSEGADDTQNAPEDGAQSTV
QEAEEEEEGSVPETELLGDCDTLQVDEAQVQLEA

13.6.2.2 Posttranscriptional Modification

Phosphorylation Ror2 is phosphorylated by several Ser/Thr kinases and tyrosine kinases. Casein kinase Iε (CKIε), a member of the CKI-family protein Ser/Thr kinases, is known to be able to phosphorylate Ror2 on Ser/Thr residues, within its C-terminal S/TRD2 [6]. This phosphorylation results in the autophosphorylation of Ror2 on tyrosine residue(s) within its cytoplasmic PRD. Forced homodimerization of Ror2 or stimulation with Wnt5a have been shown to enhance tyrosine kinase activity of Ror2 and increase tyrosine phosphorylation of Ror2 [138, 140]. When the cytoplasmic domains of Ror2 mutants fused with the dimeric Fc portion of human IgG (Fc-Ror2) were expressed in T/C28a2 human chondrocytes, robust tyrosine phosphorylation of Fc-Ror2 WT was detected in contrast with a markedly reduced tyrosine phosphorylation of Fc-Ror2 kinase-dead (KD) mutant [139]. However, the remnant of receptor tyrosine phosphorylation was observed in cells expressing Fc-Ror2 KD, raising the possibility that there may be other tyrosine kinases or co-receptors involved in the tyrosine phosphorylation of Ror2. Indeed, non-receptor tyrosine kinase Src has been shown to associate with Ror2 at the C-terminal PRD and/or S/TRD1 and phosphorylate Ror2 on tyrosine residues following Wnt5a stimulation [139]. Wnt5a stimulation has been shown to induce phosphorylation of Ror2 on Ser/Thr residues in a manner dependent on GSK3, but not CKIε [150]. Ror2 Ser-864 was identified as a critical residue phosphorylated by GSK3 in response to Wnt5a [13]. Furthermore, this phosphorylation at Serine-864 is required for Wnt5a-induced cell migration in a Ror2 tyrosine kinase activity-independent manner.

***O*-sulfonation** The study aiming to identify posttranslational modification of proteins with *O-sulfonation* on serine and threonine residues has revealed that this modification was observed in the cytoplasmic juxtamembrane region of Ror2 at Ser-469 or Ser-471, although its functional significance has not been identified [151].

13.6.3 ROR2 Ligands

Wnts are classified into several subfamilies depending on the signaling pathways they activate. The Wnt1 subfamily (e.g., Wnt1, Wnt3a, Wnt8) activates the canonical Wnt/β-catenin pathway, whereas the Wnt5a subfamily (e.g., Wnt5a, Wnt11) activates the noncanonical pathways, including the G protein-mediated Wnt–Ca^{2+} pathway and the small GTPase/JNK pathway. Ror2 has been shown to interact with several canonical and noncanonical Wnts, including Wnt3a and Wnt5a, both physically and functionally, and the CRD plays a central role for these interaction [3, 4, 7, 8]. It is well established that Ror2 acts as a receptor or co-receptor for Wnt5a (see Pathway activation Section).

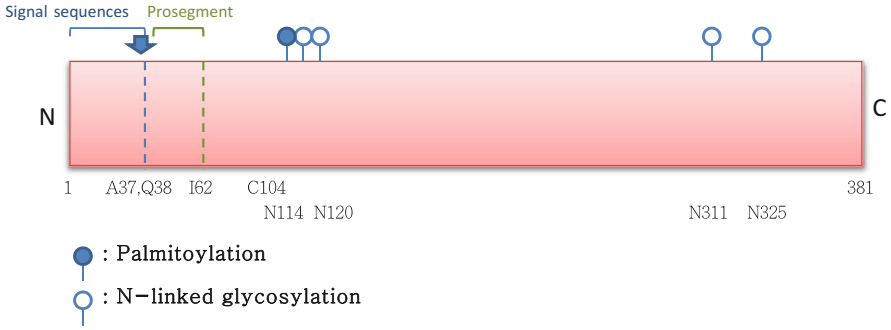

Fig. 13.4 Posttranslational modification of human Wnt5a. The *arrow* indicates the predicted cleavage site of signal peptidase between residues 37 and 38. *A* Ala, *C* Cys, *I* Ile, *N* Asn, *Q* Gln

13.6.3.1 Ligand Structure

A Ror2 ligand, Wnt5a, is a member of the Wnt-family proteins with an apparent molecular mass of 44-50 kDa. A schematic structure and posttranslational modification of human Wnt5a protein is depicted in Fig. 13.4 [Accession # P41221 (human); P22725 (mouse)]. A precursor human Wnt5a contains 381 amino acids, with a 37 amino acids signal peptide, a 25 amino acids prosegment which is not seen in purified mature Wnt5a [8], and a 319 amino acids mature protein which starts with Ile-62 [152]. Like other members of Wnt-family proteins, mature Wnt5a contains 24 cysteine residues that seem to be involved in intramolecular disulfide bonding, four N-linked glycosylation sites (Asn-114, Asn-120, Asn-311, and Asn-325), and a palmitate adduct site (Cys-104). Although palmitoylation of Wnt5a at Cys-104 is dispensable for the secretion of Wnt5a, it is critically required to elicit Wnt5a signaling at the cell surface [153]. On the other hand, N-linked glycosylation of Wnt5a at four Asn residues is required for the secretion of Wnt5a, but is dispensable for its biological functions [153].

13.6.4 ROR2 Activation and Signaling

13.6.4.1 Dimerization

Wnt5a-induced homodimerization of Ror2 has been reported [140]. In this report, a chimeric receptor consisting of the extracellular domain of Ror2 fused to the transmembrane and intracellular domains of TrkB was used. Consistent with the fact that TrkB signaling is activated by receptor homodimerization, Wnt5a stimulation increases the cAMP-response element-mediated transcription, an intracellular signaling event elicited by TrkB, in cells expressing the Ror2-TrkB chimeric receptor, suggesting that Wnt5a binds to the extracellular domain of Ror2 and induces its

homodimerization. The homodimerization of Ror2 following the treatment with Wnt5a appears to enhance Ror2 tyrosine kinase activity [28].

As mentioned above, Ror1 and Ror2 can form heterodimers to mediate Wnt5a-induced signaling in hippocampal neurons [12].

13.6.4.2 Phosphorylation

Ror2 has been shown to associate with and phosphorylate G protein-coupled receptor kinase 2 (GRK2) following activation of Ror2 by CKIε [6]. Of interest, the developmental expression patterns of GRK2 and CKIε are similar to that of Ror2 in mouse embryos [6], suggesting the role of GRK2 and CKIε in Ror2-mediated signaling during embryogenesis.

Ror2 also associates directly with and phosphorylates 14-3-3β which negatively regulates osteogenesis [138]. Furthermore, the finding that Wnt5a stimulation augments phosphorylation of 14-3-3β [140] leads to a notion that Wnt5a–Ror2 signaling promotes osteogenesis by preventing the 14-3-3β-mediated inhibition by means of phosphorylation.

13.6.4.3 Pathway Activation

Roles of Wnt5a–Ror2 Signaling in Polarized Cell Migration The presence of PCP and CE defects in *Ror2*$^{-/-}$ embryos suggests that Ror2 mediates Wnt5a signaling to regulate polarized cell migration. Indeed, Wnt5a induces migration of embryonic fibroblasts from WT but not *Ror2*$^{-/-}$ mice, indicating an essential role for Ror2 in Wnt5a-induced cell migration [22]. Interestingly, Ror2 can mediate the formation of filopodia by associating with the actin-binding protein FLNa through its PRD, an event crucial for Wnt5a-induced cell migration (Fig. 13.3a). In vitro wound-healing assays show that Wnt5a stimulates lamellipodia formation and reorientation of the microtubule-organizing center (MTOC), processes that are essential to polarized cell migration, through the Ror2–FLNa–JNK pathway [23] (Fig. 13.3a). Furthermore, Wnt5a-induced JNK activation and MTOC reorientation require the activity of atypical protein kinase C (aPKC), suggesting a functional link between the Wnt5a–Ror2–FLNa–JNK and Par–aPKC pathways in polarized cell migration (Fig. 13.3a). It is unclear how aPKC regulates JNK activity in Wnt5a–Ror2 signaling. However, it has recently been reported that aPKC associates with Dvl [154, 155], functioning upstream of JNK, and that Dvl is required for Wnt5a-induced cell migration [22, 155], raising the possibility that aPKC might regulate Wnt5a-induced JNK activation through Dvl in wound-healing cells. Importantly, a uniform concentration of Wnt5a can stimulate polarization of cells at the wound edge, where wound-induced loss of cell–cell contacts serves as the instructive polarity cue, whereas Wnt5a has no effect on cell polarity in confluent cells [23], suggesting that the role of Wnt5a in cell polarity is permissive rather than instructive. Consistent with this, Wnt5a is required for polarization of melanoma cells in

response to a chemokine gradient, but exposure of the cells to Wnt5a alone has little effect [24]. By contrast, mesenchymal cells can migrate toward the source of Wnt5a in the developing palate tissues in a Ror2-dependent manner, suggesting that Wnt5a plays an instructive role acting as a chemoattractant for palate mesenchymal cells through a Ror2-mediated noncanonical Wnt signaling pathway [26]. Thus, Wnt5a might have different roles in polarized cell migration (i.e., permissive and instructive roles) depending on the cell/tissue type.

Roles of Wnt5a–Ror2 Signaling in Epithelial Cell Polarization Polarized intestinal epithelial cells have a distinctive apico-basal axis, in which the apical membrane faces the luminal side of the intestine. The apical surface of epithelial cells is covered with microvilli, actin-rich membrane protrusions. Wnt5a stimulation has been shown to upregulate expression of villin, an actin-regulatory protein involved in the formation of microvilli, via Ror2-mediated activation of extracellular signal-regulated kinase 1 and 2 (Erk1/2) in intestinal epithelial cells [156]. An electrical transepithelial potential difference (TEP) across an epithelium provides a directional signal for epithelial cells. When a physiological electrical field (EF), which mimics the natural EF created by TEP, was applied to intestinal epithelial cells, apical membrane proteins, including actin and CD66, were reoriented to the cathodal side of cells in a manner dependent on the expression of LKB1, a key molecule in apical membrane formation [157]. Interestingly, a physiological EF activates LKB1 via Ror2-mediated activation of Erk1/2 [157]. These findings suggest that Wnt5a–Ror2 signaling might regulate apical membrane formation by upregulating expression of villin and activating LKB1 via Erk1/2.

Specificity of Wnt5a Signaling Pathways Wnt5a can activate various signaling pathways, including PCP pathway and Wnt–Ca^{2+} pathway. In the PCP pathway, Wnt5a binding to Fzd protein results in activation of Dvl, which in turn activates Rho-family small GTPases, including RhoA and Rac. These Rho GTPases then activate the Rho-associated protein kinase ROCK and JNK to regulate cell polarity and migration. Wnt5a can bind to several receptors, including Fzds and Ror2, and it has therefore been proposed that these receptors, at least partly, trigger distinct signaling pathways to induce specific outcomes [8]. It has been shown that Wnt5a induces the internalization of Fzd2 through a clathrin-mediated route, which is required for Wnt5a-induced activation of Rac [14]. Knockdown of either *Ror1* or *Ror2* suppresses the Wnt5a-induced internalization of Fzd2 and activation of Rac [14], suggesting that Ror1 and Ror2 have a function in these processes. In addition to the receptor context, specific signaling seems to be achieved by an extracellular cofactor, collagen triple-helix repeat-containing protein 1 (Cthrc1), that binds to Wnts, Fzds, and Ror2, and selectively activates the PCP pathway by stabilizing the Wnt-receptor complex [53] (Fig. 13.3b). Importantly, Cthrc1 can associate with Ror2 lacking the CRD, indicating that Cthrc1 and Wnt5a interact with distinct domains of Ror2. Furthermore, Cthrc1 enhances not only binding of Wnt5a but also that of Wnt3a to Ror2 results in the activation of PCP pathway. Thus, cell/tissue-dependent expression of Wnt receptors and their cofactors, such as Cthrc1, in

addition to intracellular signaling molecules, might determine which signaling pathways are preferentially activated in response to Wnts.

Inhibition of β-catenin-Dependent Canonical Wnt Signaling Wnt5a–Ror2 signaling plays a crucial role in inhibiting canonical Wnt signaling at the level of T-cell factor (TCF)/lymphoid enhancer factor (LEF)-mediated transcription [8, 27] (Fig. 13.3e). Wnt5a inhibits Wnt3a-induced activation of a TCF/LEF-driven reporter gene in a Ror2-dependent manner, without inducing Ca^{2+} mobilization [8]. Structure–function analysis of Ror2 reveals that an intrinsic kinase activity of Ror2 is necessary for its inhibitory function [28]. The role of endogenous Ror2 has also been studied by using *Ror2*$^{-/-}$ mice crossed with *Axin2–LacZ* mice, where one copy of the *Axin2* gene, a direct Wnt target gene in canonical Wnt signaling, was replaced by the *LacZ* gene to allow visualization of canonical Wnt signaling activity *in vivo*. Compared with *Ror2*$^{+/-}$ embryos, *Ror2*$^{-/-}$ embryos exhibits increased LacZ expression in specific regions where cells lost Ror2 expression in the lung, indicating that Ror2 indeed inhibits canonical Wnt signaling at least in the developing lung [28]. During early embryogenesis, the ECM protein Del1 promotes forebrain development in the *Xenopus* embryos. Del1 function in the neural patterning has been shown to be mediated by the inhibition of canonical Wnt signaling through Ror2-mediated signaling pathway [158]. Furthermore, suppression of either Ror2 or Wnt5a expression in human osteosarcoma SaOS-2 cells results in the inhibition of invasiveness with a concomitant enhancement of TCF/LEF-mediated transcription without affecting β-catenin levels [29], indicating that Wnt5a–Ror2 signaling is constitutively activated in SaOS-2 cells, thereby inhibiting the canonical Wnt pathway. Collectively, inhibition of canonical Wnt signaling pathway by Wnt5a–Ror2 signaling might play roles in embryogenesis and tumor invasion. The identity of downstream effector(s) of Ror2 in Wnt5a-Ror2 signaling for the inhibition of the canonical Wnt pathway is currently unknown.

Potentiation of *β*-catenin-Dependent Canonical Wnt Signaling Although Ror2 can antagonize canonical Wnt signaling, other studies indicate that Ror2 potentiates β-catenin-dependent TCF/LEF pathway in multiple cell types. In osteosarcoma U2OS cells, Ror2 potentiates Wnt1-induced TCF/LEF-driven reporter activity in a Ror2 kinase activity-dependent manner [7]. In lung carcinoma H441 cells, Ror2 cooperates with the receptor Fzd2 to activate canonical Wnt signaling in response to Wnt3a [27]. Thus, Ror2 can both inhibit and potentiate canonical Wnt signaling in cellular context-dependent manner; however, the molecular mechanism underlying Ror2-mediated opposing effects on canonical Wnt signaling is currently unknown. TGF-β-activated kinase-1 (TAK1), a MAP kinase kinase kinase (MAP3K), has been shown to interact with the C-terminal PRD and/or S/TRD1 of Ror2 and phosphorylate the C-terminal region of Ror2 in a manner dependent on the kinase activity of TAK1 [159]. This study demonstrates that expression of Ror2 in HEK293T cells potentiates Wnt1-dependent canonical signaling and TAK1 inhibits the Ror2-mediated enhancement of canonical Wnt signaling.

13.6.4.4 Major Genes Regulated

In *Xenopus* embryos Wnt5a–Ror2–JNK signaling pathway upregulates expression of the paraxial protocadherin *XPAPC* gene through c-Jun and ATF2 transcription factors [65]. Induction of *XPAPC* is required for CE movements during gastrulation of *Xenopus* embryos. Treatment with cycloheximide, an inhibitor of protein synthesis, fails to suppress *XPAPC* induction by Wnt5a, suggesting a direct regulation of *XPAPC* gene expression by Wnt5a–Ror2–JNK signaling pathway through c-Jun/ATF2. XShcA, a phospho-tyrosine binding protein, has been shown to be required for upregulation of *XPAPC* by Wnt5a–Ror2 signaling in *Xenopus* embryos [160]. XShcA binds to a conserved Shc-SH2-binding motif in Ror2 via its SH2 domain and XWnt5a induces clustering of XRor2 in the cell membrane and recruitment of XShcA to the XRor2 receptor complex [160]. The Shc-SH2-binding motif of Ror2 is evolutionarily conserved among all the Ror-family members, suggesting that signaling through ShcA might represent an evolutionarily conserved biochemical event.

Another target gene, known to be regulated by Wnt5a–Ror2 signaling, is *MMP-13* gene. Suppressed expression of either Ror2 or Wnt5a results in inhibition of *MMP-13* expression in SaOS2 cells, and conversely, stimulation of serum-starved SaOS2 cells with Wnt5a leads to induction of *MMP-13* [29]. Expression of *MMP-13* by Wnt5a–Ror2 signaling can be abrogated by an inhibitor of the Src-family kinases (SFKs), suggesting the role of the SFK(s) in *MMP-13* expression by Wnt5a–Ror2 signaling (Fig. 13.3c). The promoter region within *MMP-13* gene contains an AP-1 binding site. Chromatin-immunoprecipitation (Chip) assays reveals that c-Jun and ATF2 are crucial transcription factors recruited to the AP-1 binding site in the *MMP-13* gene promoter during Wnt5a–Ror2 signaling in SaOS-2 cells (Fig. 13.3c) [90]. Dvl2 and JNK are required for *MMP-13* induction presumably via phosphorylation of c-Jun and ATF2 in SaOS-2 cells (Fig. 13.3c). Interestingly, Dvl2 and Rac1, but not Dvl3, are required for *MMP-13* expression in SaOS-2 cells, whereas Dvl3, but not Dvl2 and Rac1, is required for its expression in U2OS cells [90], indicating the presence of distinct intracellular signaling machineries leading to expression of the same gene, in this case *MMP-13* gene in different osteosarcoma cell lines.

13.6.4.5 Binding Proteins

As mentioned above, Ror2 interacts with various proteins, including Wnts, CKIε, Src, 14-3-3β, GRK2, FLNa, Fzd, Cthrc1, and TAK1 through its extracellular or intracellular regions (Table 13.4). In addition to these proteins, several other proteins have been shown to interact with the intracellular regions of Ror2 (Table 13.4).

Ror2 associates with the melanoma-associated antigen-family protein Dlxin-1 (also called as NRAGE) through its C-terminal portion of Ror2, containing the PRD and S/TRDs [161]. Dlxin-1 is known to bind to the homeodomain transcription factors Msx2 and Dlx5 and regulate their transcriptional functions. In the presence of Ror2, Dlxin-1 is colocalized with Ror2 at the membranous compartments and Msx2 is retained in the nuclei. Furthermore, transcriptional activity of Msx2 is regulated

Table 13.4 Ror2-binding proteins

Protein	Binding domain of Ror2	Function(s) in Ror2-mediated signaling	References
Wnt5a	CRD	A secreted glycoprotein that acts as a ligand for Ror2.	[8]
Fzd	CRD	Wnt5a-induced receptor complex.	[4, 10]
CKIε	S/TRD	Ror2 phosphorylation on Ser/Thr residues. Enhancement of Ror2 autophosphorylation.	[6]
c-Src	PRD and/or S/TRD1	Ror2 phosphorylation on tyrosine residues following Wnt5a stimulation. MMP13 induction and cell invasion.	[29, 90, 139]
14-3-3β	Intracellular region	A scaffold protein that are phosphorylated by Ror2 following Wnt5a stimulation. Osteogenesis.	[138]
GRK2	N.D.	Tyrosin phosphorylated following the activation of Ror2 by CKIε.	[6]
FLNa	PRD	Filopodia formation. JNK activation following Wnt5a stimulation. Polarized cell migration.	[22, 23]
Cthrc1	Extracellular region	Wnt-Fzd-Ror2 complex formation. PCP pathway activation.	[53]
TAK1	PRD and/or S/TRD1	Inhibition of canonical Wnt signaling.	[159]
XShcA	SH2-binding motif in TK domain	Wnt5a-induced upregulation of *XPAPC*.	[160]
Dlxin-1	PRD and/or S/TRDs	Msx2-dependent transcriptional regulation.	[161]
Dvl	PRD and/or S/TRDs	Inhibition of canonical Wnt signaling.	[162]
Vangl2	N.D.	Wnt5a-induced receptor complex formation. PCP pathway activation.	[164]
Wtip	S/TRD1	Inhibition of canonical Wnt signaling.	[165]
BRIb	CRD	Chondrogenesis.	[166, 168]

by ectopic expression of Ror2, irrespective of its kinase activity. Thus, Ror2 might affect transcriptional functions of Msx2 by regulating intracellular distribution of Dlxin-1 in a tyrosine kinase-independent manner.

Dvl is phosphorylated by both canonical and noncanonical Wnt signalings via CKIε. Phosphorylated Dvl has been shown to interact with the C-terminal PRD and S/TRDs of Ror2, which is required for efficient inhibition of canonical Wnt signaling [162].

PCP, which originally refers to the polarity of epithelial cells within a plane orthogonal to their apico-basal axis, is well characterized genetically in *Drosophila* and is regulated by a group of evolutionarily conserved core PCP components, including a four-pass transmembrane protein, Van Gogh. Its vertebrate homologue, *Vang-like 2* (*Vangl2*), genetically interacts with *Wnt5a* [163]. It has recently been shown that Ror2 and Vangl2 form an Wnt-induced receptor complex that is essential to establish PCP [164]. Wnt5a induces Vangl2 phosphorylation on two clusters

of Ser and Thr residues through Ror2, and Wnt5a-induced Vangl2 phosphorylation is essential for its function regulating PCP [164].

The WT1-interacting protein Wtip has been shown to interact with the S/TRD1 of Ror2 [165]. Wtip, originally identified as an interactor of the Wilms tumor protein 1 (WT1), contains three LIM domains (LDs) and exhibits high degree of homology with the Ajuba/Zyxin family of LD proteins. The interaction of Wtip and Ror2 is mediated by the first and/or second LD of Wtip. Full-length Ror2 recruits Wtip to the cell membrane, whereas a truncated mutant found in BDB patients, which lacks the PRD and S/TRDs, fails to do so. Both transcripts and proteins of Wtip and Ror2 show overlapping expression in the mouse embryo, indicating a functional interaction of these proteins *in vivo*. Ectopic expression of *Wtip* can also antagonize canonical Wnt signaling in mammalian cells and in *Xenopus* embryos.

13.6.4.6 Cross Talk with Other Receptor Systems

A Cross talk between Ror2 and the bone morphogenetic protein receptor type Ib (BRIb) has been reported [166]. Mutations in *GDF5*, a gene encoding a member of BMP/TGF-β ligand family, cause brachydactyly type C (BDC), whereas mutations in *BRIb*, encoding the receptor for GDF5, cause brachydactyly type A2 (BDA2). Because there is considerable phenotypic overlap among BDB, BDC, and BDA2, the molecular mechanisms responsible for BDB, caused by *Ror2* mutation (see Sect. 13.3), might be shared among these forms of brachydactyly. Ror2 has been shown to form a complex with BRIb in a ligand-independent manner, thereby inhibiting subsequent GDF5/BRIb-induced Smad1/5 signaling cascade [166]. In addition, the inactivation of one *Ror2* allele in *Gdf5*$^{-/-}$ mice, as well as inactivation of one *GDF5* allele or two *BRIb* alleles in *Ror2*$^{-/-}$ mice, results in a significant reduction in bone length, a phenotype which is not seen in the single mutants, indicating that *Ror2* genetically interacts with *BRIb* and *GDF5* [166]. GDF5 binds to BRIb with a high affinity, while BMP2 preferentially binds to BRIa [167]. Interestingly, Ror2 is associated specifically with BRIb, and any apparent cross talk between Ror2 and BRIa or low-affinity type II receptor (BRII) cannot be observed [168]. Since Ror2/BRIb complex can be detected in distinct detergent-resistant microdomains (DRMs) of the plasma membrane, from which BMP-induced Smad-dependent signaling emanates [168], it can be envisaged that Ror2 might interfere with GDF5/BRIb signaling complexes within the membrane domains.

13.6.5 Unique Features of the ROR2

It has been shown that the cytoplasmic region of Ror2, but not its intrinsic kinase activity, is indispensable for Wnt5a-induced cell migration [22]. In contrast, the cytoplasmic region of Ror2 is dispensable for Wnt5a-induced phosphorylation and polymerization of Dvl2 [10]. Moreover, Wnt5a–Ror2 signaling induces

invadopodia formation and inhibits canonical Wnt signaling by mechanisms that require the intrinsic kinase activity of Ror2 [28, 29]. Thus, Ror2 seems to mediate Wnt5a signaling through at least three distinct mechanisms, i.e., (1) the cytoplasmic region-dependent, kinase activity-independent mechanism, (2) the cytoplasmic region-independent mechanism, and (3) the kinase activity-dependent mechanism. Importantly, the *C. elegans* CAM-1 also exhibits the kinase activity-dependent and -independent functions [39, 59, 62], as well as the cytoplasmic region-independent functions [9, 41, 169].

Receptor at a glance; *Ror1*

Chromosome location	1p32-p31 (human), 4 49.6 cM (mouse)
Gene Size (bp)	405018 bp (human), 346755 bp (mouse)
Intron/exon numbers	Intron: 8/exon: 9 (human and mouse)
mRNA size (ORF)	3382 bases (ORF: 2814 bases) (human), 3542 bases (ORF: 2814 bases) (mouse)
Amino acid number	937 (human and mouse)
kDa	MW: 104 kDa, SDS-PAGE: 135 kDa
Posttranslational modifications	phosphorylation, glycosylation, ubiquitination
Domains	immunoglobulin-like domain, cysteine-rich domain, kringle domain, tyrosine kinase domain, serine/threonine-rich domain, proline-rich domain
Ligands	Wnt5a (see text for details)
Known dimerizing partners	Ror2
Pathways activated	See text for details
Tissues expressed	See text for details
Human Diseases	malignancy
Knockout Mouse phenotype	See Table 13.1

Receptor at a glance; *Ror2*

Chromosome location	9q22.31 (human), 13 34.2 cM (mouse)
Gene Size (bp)	227567 bp (human), 176793 bp (mouse)
Intron/exon numbers	Intron: 8/exon: 9 (human and mouse)
mRNA size	4099 bases (ORF: 2832 bases) (human), 3987 bases (ORF: 2835 bases) (mouse)
Amino acid number	943 (human), 944 (mouse)
kDa	MW: 105 kDa, SDS-PAGE: 135 kDa
Posttranslational modifications	phosphorylation, *O*-sulfonation
Domains	immunoglobulin-like domain, cysteine-rich domain, kringle domain, tyrosine kinase domain, serine/threonine-rich domain, proline-rich domain

(continued)

Ligands	Wnt5a (see text for details)
Known dimerizing partners	See Table 13.4
Pathways activated	See text for details
Tissues expressed	See text for details
Human Diseases	autosomal dominant Brachydactyly type B (BDB), autosomal recessive Robinow syndrome (RRS)
Knockout Mouse phenotype	See Table 13.1

References

1. Masiakowski P, Carroll RD. A novel family of cell surface receptors with tyrosine kinase-like domain. J Biol Chem. 1992;267(36):26181–90.
2. Wodarz A, Nusse R. Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol. 1998;14:59–88.
3. Hikasa H, Shibata M, Hiratani I, Taira M. The Xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling. Development. 2002;129(22):5227–39.
4. Oishi I, Suzuki H, Onishi N, Takada R, Kani S, Ohkawara B, et al. The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. Genes Cells. 2003;8(7):645–54.
5. Forrester WC, Kim C, Garriga G. The Caenorhabditis elegans Ror RTK CAM-1 inhibits EGL-20/Wnt signaling in cell migration. Genetics. 2004;168(4):1951–62.
6. Kani S, Oishi I, Yamamoto H, Yoda A, Suzuki H, Nomachi A, et al. The receptor tyrosine kinase Ror2 associates with and is activated by casein kinase Iepsilon. J Biol Chem. 2004;279(48):50102–9.
7. Billiard J, Way DS, Seestaller-Wehr LM, Moran RA, Mangine A, Bodine PV. The orphan receptor tyrosine kinase Ror2 modulates canonical Wnt signaling in osteoblastic cells. Mol Endocrinol. 2005;19(1):90–101.
8. Mikels AJ, Nusse R. Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS Biol. 2006;4(4):e115.
9. Green JL, Inoue T, Sternberg PW. The C. elegans ROR receptor tyrosine kinase, CAM-1, non-autonomously inhibits the Wnt pathway. Development. 2007;134(22):4053–62.
10. Nishita M, Itsukushima S, Nomachi A, Endo M, Wang Z, Inaba D, et al. Ror2/Frizzled complex mediates Wnt5a-induced AP-1 activation by regulating dishevelled polymerization. Mol Cell Biol. 2010;30(14):3610–9.
11. Fukuda T, Chen L, Endo T, Tang L, Lu D, Castro JE, et al. Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. Proc Natl Acad Sci USA. 2008;105(8):3047–52.
12. Paganoni S, Bernstein J, Ferreira A. Ror1-Ror2 complexes modulate synapse formation in hippocampal neurons. Neuroscience. 2010;165(4):1261–74.
13. Grumolato L, Liu G, Mong P, Mudbhary R, Biswas R, Arroyave R, et al. Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. Genes Dev. 2010;24(22):2517–30.
14. Sato A, Yamamoto H, Sakane H, Koyama H, Kikuchi A. Wnt5a regulates distinct signalling pathways by binding to Frizzled2. EMBO J. 2010;29(1):41–54.
15. Endo M, Doi R, Nishita M, Minami Y. Ror family receptor tyrosine kinases regulate the maintenance of neural progenitor cells in the developing neocortex. J Cell Sci. 2012;125(Pt 8):2017. -29.

16. Kikuchi A, Yamamoto H. Tumor formation due to abnormalities in the beta-catenin-independent pathway of Wnt signaling. Cancer Sci. 2008;99(2):202–8.
17. Veeman MT, Axelrod JD, Moon RT. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev Cell. 2003;5(3):367–77.
18. Kohn AD, Moon RT. Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium. 2005;38(3–4):439–46.
19. Seifert JR, Mlodzik M. Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility. Nat Rev Genet. 2007;8(2):126–38.
20. Minami Y, Oishi I, Endo M, Nishita M. Ror-family receptor tyrosine kinases in noncanonical Wnt signaling: their implications in developmental morphogenesis and human diseases. Dev Dyn. 2010;239(1):1–15.
21. Yamaguchi TP, Bradley A, McMahon AP, Jones S. A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development. 1999;126(6):1211–23.
22. Nishita M, Yoo SK, Nomachi A, Kani S, Sougawa N, Ohta Y, et al. Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration. J Cell Biol. 2006;175(4):555–62.
23. Nomachi A, Nishita M, Inaba D, Enomoto M, Hamasaki M, Minami Y. Receptor tyrosine kinase Ror2 mediates Wnt5a-induced polarized cell migration by activating c-Jun N-terminal kinase via actin-binding protein filamin A. J Biol Chem. 2008;283(41):27973–81.
24. Witze ES, Litman ES, Argast GM, Moon RT, Ahn NG. Wnt5a control of cell polarity and directional movement by polarized redistribution of adhesion receptors. Science. 2008; 320(5874):365–9.
25. Laird DJ, Altshuler-Keylin S, Kissner MD, Zhou X, Anderson KV. Ror2 enhances polarity and directional migration of primordial germ cells. PLoS Genet. 2011;7(12):e1002428.
26. He F, Xiong W, Yu X, Espinoza-Lewis R, Liu C, Gu S, et al. Wnt5a regulates directional cell migration and cell proliferation via Ror2-mediated noncanonical pathway in mammalian palate development. Development. 2008;135(23):3871–9.
27. Li C, Chen H, Hu L, Xing Y, Sasaki T, Villosis MF, et al. Ror2 modulates the canonical Wnt signaling in lung epithelial cells through cooperation with Fzd2. BMC Mol Biol. 2008;9:11.
28. Mikels A, Minami Y, Nusse R. Ror2 receptor requires tyrosine kinase activity to mediate Wnt5A signaling. J Biol Chem. 2009;284(44):30167–76.
29. Enomoto M, Hayakawa S, Itsukushima S, Ren DY, Matsuo M, Tamada K, et al. Autonomous regulation of osteosarcoma cell invasiveness by Wnt5a/Ror2 signaling. Oncogene. 2009; 28(36):3197–208.
30. Oldridge M, Fortuna AM, Maringa M, Propping P, Mansour S, Pollitt C, et al. Dominant mutations in ROR2, encoding an orphan receptor tyrosine kinase, cause brachydactyly type B. Nat Genet. 2000;24(3):275–8.
31. Afzal AR, Rajab A, Fenske CD, Oldridge M, Elanko N, Ternes-Pereira E, et al. Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2. Nat Genet. 2000;25(4):419–22.
32. van Bokhoven H, Celli J, Kayserili H, van Beusekom E, Balci S, Brussel W, et al. Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome. Nat Genet. 2000;25(4):423–6.
33. Schwabe GC, Tinschert S, Buschow C, Meinecke P, Wolff G, Gillessen-Kaesbach G, et al. Distinct mutations in the receptor tyrosine kinase gene ROR2 cause brachydactyly type B. Am J Hum Genet. 2000;67(4):822–31.
34. Yamamoto H, Oue N, Sato A, Hasegawa Y, Yamamoto H, Matsubara A, et al. Wnt5a signaling is involved in the aggressiveness of prostate cancer and expression of metalloproteinase. Oncogene. 2010;29(14):2036–46.
35. Dissanayake SK, Wade M, Johnson CE, O'Connell MP, Leotlela PD, French AD, et al. The Wnt5A/protein kinase C pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors and initiation of an epithelial to mesenchymal transition. J Biol Chem. 2007;282(23):17259–71.

36. Wright TM, Brannon AR, Gordan JD, Mikels AJ, Mitchell C, Chen S, et al. Ror2, a developmentally regulated kinase, promotes tumor growth potential in renal cell carcinoma. Oncogene. 2009;28(27):2513–23.
37. Baskar S, Kwong KY, Hofer T, Levy JM, Kennedy MG, Lee E, et al. Unique cell surface expression of receptor tyrosine kinase ROR1 in human B-cell chronic lymphocytic leukemia. Clin Cancer Res. 2008;14(2):396–404.
38. Daneshmanesh AH, Mikaelsson E, Jeddi-Tehrani M, Bayat AA, Ghods R, Ostadkarampour M, et al. Ror1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy. Int J Cancer. 2008;123(5):1190–5.
39. Forrester WC, Dell M, Perens E, Garriga GA. C elegans Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division. Nature. 1999;400(6747):881–5.
40. Koga M, Take-uchi M, Tameishi T, Ohshima Y. Control of DAF-7 TGF-(alpha) expression and neuronal process development by a receptor tyrosine kinase KIN-8 in Caenorhabditis elegans. Development. 1999;126(23):5387–98.
41. Francis MM, Evans SP, Jensen M, Madsen DM, Mancuso J, Norman KR, et al. The Ror receptor tyrosine kinase CAM-1 is required for ACR-16-mediated synaptic transmission at the C. elegans neuromuscular junction. Neuron. 2005;46(4):581–94.
42. McKay SE, Hislop J, Scott D, Bulloch AG, Kaczmarek LK, Carew TJ, et al. Aplysia ror forms clusters on the surface of identified neuroendocrine cells. Mol Cell Neurosci. 2001; 17(5):821–41.
43. Wilson C, Goberdhan DC, Steller H. Dror, a potential neurotrophic receptor gene, encodes a Drosophila homolog of the vertebrate Ror family of Trk-related receptor tyrosine kinases. Proc Natl Acad Sci USA. 1993;90(15):7109–13.
44. Oishi I, Sugiyama S, Liu ZJ, Yamamura H, Nishida Y, Minami Y. A novel Drosophila receptor tyrosine kinase expressed specifically in the nervous system. Unique structural features and implication in developmental signaling. J Biol Chem. 1997;272(18):11916–23.
45. Rodriguez-Niedenfuhr M, Prols F, Christ B. Expression and regulation of ROR-1 during early avian limb development. Anat Embryol. 2004;207(6):495–502.
46. Stricker S, Verhey van Wijk N, Witte F, Brieske N, Seidel K, Mundlos S. Cloning and expression pattern of chicken Ror2 and functional characterization of truncating mutations in Brachydactyly type B and Robinow syndrome. Dev Dyn. 2006;235(12):3456–65.
47. Al-Shawi R, Ashton SV, Underwood C, Simons JP. Expression of the Ror1 and Ror2 receptor tyrosine kinase genes during mouse development. Dev Genes Evol. 2001;211(4):161–71.
48. Matsuda T, Nomi M, Ikeya M, Kani S, Oishi I, Terashima T, et al. Expression of the receptor tyrosine kinase genes, Ror1 and Ror2, during mouse development. Mech Dev. 2001; 105(1–2):153–6.
49. Oishi I, Takeuchi S, Hashimoto R, Nagabukuro A, Ueda T, Liu ZJ, et al. Spatio-temporally regulated expression of receptor tyrosine kinases, mRor1, mRor2, during mouse development: implications in development and function of the nervous system. Genes Cells. 1999; 4(1):41–56.
50. Paganoni S, Anderson KL, Ferreira A. Differential subcellular localization of Ror tyrosine kinase receptors in cultured astrocytes. Glia. 2004;46(4):456–66.
51. Paganoni S, Ferreira A. Expression and subcellular localization of Ror tyrosine kinase receptors are developmentally regulated in cultured hippocampal neurons. J Neurosci Res. 2003;73(4):429–40.
52. Paganoni S, Ferreira A. Neurite extension in central neurons: a novel role for the receptor tyrosine kinases Ror1 and Ror2. J Cell Sci. 2005;118(Pt 2):433–46.
53. Yamamoto S, Nishimura O, Misaki K, Nishita M, Minami Y, Yonemura S, et al. Cthrc1 selectively activates the planar cell polarity pathway of Wnt signaling by stabilizing the Wnt-receptor complex. Dev Cell. 2008;15(1):23–36.
54. Yamada M, Udagawa J, Matsumoto A, Hashimoto R, Hatta T, Nishita M, et al. Ror2 is required for midgut elongation during mouse development. Dev Dyn. 2010;239(3):941–53.

55. Schwabe GC, Trepczik B, Suring K, Brieske N, Tucker AS, Sharpe PT, et al. Ror2 knockout mouse as a model for the developmental pathology of autosomal recessive Robinow syndrome. Dev Dyn. 2004;229(2):400–10.
56. Lin M, Li L, Liu C, Liu H, He F, Yan F, et al. Wnt5a regulates growth, patterning, and odontoblast differentiation of developing mouse tooth. Dev Dyn. 2011;240(2):432–40.
57. Forrester WC, Garriga G. Genes necessary for C elegans cell and growth cone migrations. Development. 1997;124(9):1831–43.
58. Song S, Zhang B, Sun H, Li X, Xiang Y, Liu Z, et al. A Wnt-Frz/Ror-Dsh pathway regulates neurite outgrowth in Caenorhabditis elegans. PLoS Genet. 2010;6(8):e1001056.
59. Green JL, Inoue T, Sternberg PW. Opposing Wnt pathways orient cell polarity during organogenesis. Cell. 2008;134(4):646–56.
60. Kennerdell JR, Fetter RD, Bargmann CI. Wnt-Ror signaling to SIA and SIB neurons directs anterior axon guidance and nerve ring placement in C elegans. Development. 2009;136(22): 3801–10.
61. Babu K, Hu Z, Chien SC, Garriga G, Kaplan JM. The immunoglobulin super family protein RIG-3 prevents synaptic potentiation and regulates Wnt signaling. Neuron. 2011;71(1): 103–16.
62. Hayashi Y, Hirotsu T, Iwata R, Kage-Nakadai E, Kunitomo H, Ishihara T, et al. A trophic role for Wnt-Ror kinase signaling during developmental pruning in Caenorhabditis elegans. Nat Neurosci. 2009;12(8):981–7.
63. Moon RT, Campbell RM, Christian JL, McGrew LL, Shih J, Fraser S. Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of Xenopus laevis. Development. 1993;119(1):97–111.
64. Yamanaka H, Moriguchi T, Masuyama N, Kusakabe M, Hanafusa H, Takada R, et al. JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. EMBO Rep. 2002;3(1):69–75.
65. Schambony A, Wedlich D. Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway. Dev Cell. 2007;12(5):779–92.
66. Ossipova O, Sokol SY. Neural crest specification by noncanonical Wnt signaling and PAR-1. Development. 2011;138(24):5441–50.
67. DeChiara TM, Kimble RB, Poueymirou WT, Rojas J, Masiakowski P, Valenzuela DM, et al. Ror2, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development. Nat Genet. 2000;24(3):271–4.
68. Takeuchi S, Takeda K, Oishi I, Nomi M, Ikeya M, Itoh K, et al. Mouse Ror2 receptor tyrosine kinase is required for the heart development and limb formation. Genes Cells. 2000;5(1): 71–8.
69. Nomi M, Oishi I, Kani S, Suzuki H, Matsuda T, Yoda A, et al. Loss of mRor1 enhances the heart and skeletal abnormalities in mRor2-deficient mice: redundant and pleiotropic functions of mRor1 and mRor2 receptor tyrosine kinases. Mol Cell Biol. 2001;21(24):8329–35.
70. Okamoto M, Udagawa N, Uehara S, Maeda K, Yamashita T, Nakamichi Y, et al. Noncanonical Wnt5a enhances Wnt/beta-catenin signaling during osteoblastogenesis. Sci Rep. 2014;4:4493.
71. Maeda K, Kobayashi Y, Udagawa N, Uehara S, Ishihara A, Mizoguchi T, et al. Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. Nat Med. 2012;18(3):405–12.
72. Li C, Xiao J, Hormi K, Borok Z, Minoo P. Wnt5a participates in distal lung morphogenesis. Dev Biol. 2002;248(1):68–81.
73. Nishita M, Qiao S, Miyamoto M, Okinaka Y, Yamada M, Hashimoto R, et al. Role of Wnt5a-Ror2 signaling in morphogenesis of the metanephric mesenchyme during ureteric budding. Mol Cell Biol. 2014;34(16):3096–105.
74. Yun K, Ajima R, Sharma N, Costantini F, Mackem S, Lewandoski M, et al. Non-canonical Wnt5a/Ror2 signaling regulates kidney morphogenesis by controlling intermediate mesoderm extension. Hum Mol Genet. 2014;23:6807–14.

75. Lyashenko N, Weissenbock M, Sharir A, Erben RG, Minami Y, Hartmann C. Mice lacking the orphan receptor ror1 have distinct skeletal abnormalities and are growth retarded. Dev Dyn. 2010;239(8):2266–77.
76. Suzuki K, Bachiller D, Chen YP, Kamikawa M, Ogi H, Haraguchi R, et al. Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling [corrected]. Development. 2003;130(25):6209–20.
77. Chen P, Johnson JE, Zoghbi HY, Segil N. The role of Math1 in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell fate determination. Development. 2002;129(10):2495–505.
78. Ho HY, Susman MW, Bikoff JB, Ryu YK, Jonas AM, Hu L, et al. Wnt5a-Ror-Dishevelled signaling constitutes a core developmental pathway that controls tissue morphogenesis. Proc Natl Acad Sci USA. 2012;109(11):4044–51.
79. Cha J, Bartos A, Park C, Sun X, Li Y, Cha SW, et al. Appropriate crypt formation in the uterus for embryo homing and implantation requires Wnt5a-ROR signaling. Cell Rep. 2014;8(2): 382–92.
80. Afzal AR, Jeffery S. One gene, two phenotypes: ROR2 mutations in autosomal recessive Robinow syndrome and autosomal dominant brachydactyly type B. Hum Mutat. 2003; 22(1):1–11.
81. Raz R, Stricker S, Gazzerro E, Clor JL, Witte F, Nistala H, et al. The mutation ROR2W749X, linked to human BDB, is a recessive mutation in the mouse, causing brachydactyly, mediating patterning of joints and modeling recessive Robinow syndrome. Development. 2008;135(9):1713–23.
82. Patton MA, Afzal AR. Robinow syndrome. J Med Genet. 2002;39(5):305–10.
83. Ali BR, Jeffery S, Patel N, Tinworth LE, Meguid N, Patton MA, et al. Novel Robinow syndrome causing mutations in the proximal region of the frizzled-like domain of ROR2 are retained in the endoplasmic reticulum. Hum Genet. 2007;122(3–4):389–95.
84. Tufan F, Cefle K, Turkmen S, Turkmen A, Zorba U, Dursun M, et al. Clinical and molecular characterization of two adults with autosomal recessive Robinow syndrome. Am J Med Genet A. 2005;136(2):185–9.
85. Chen Y, Bellamy WP, Seabra MC, Field MC, Ali BR. ER-associated protein degradation is a common mechanism underpinning numerous monogenic diseases including Robinow syndrome. Hum Mol Genet. 2005;14(17):2559–69.
86. Brunetti-Pierri N, Del Gaudio D, Peters H, Justino H, Ott CE, Mundlos S, et al. Robinow syndrome: phenotypic variability in a family with a novel intragenic ROR2 mutation. Am J Med Genet A. 2008;146A(21):2804–9.
87. Person AD, Beiraghi S, Sieben CM, Hermanson S, Neumann AN, Robu ME, et al. WNT5A mutations in patients with autosomal dominant Robinow syndrome. Dev Dyn. 2010;239(1): 327–37.
88. Morioka K, Tanikawa C, Ochi K, Daigo Y, Katagiri T, Kawano H, et al. Orphan receptor tyrosine kinase ROR2 as a potential therapeutic target for osteosarcoma. Cancer Sci. 2009; 100(7):1227–33.
89. Weaver AM. Invadopodia: specialized cell structures for cancer invasion. Clin Exp Metastasis. 2006;23(2):97–105.
90. Yamagata K, Li X, Ikegaki S, Oneyama C, Okada M, Nishita M, et al. Dissection of Wnt5a-Ror2 signaling leading to matrix metalloproteinase (MMP-13) expression. J Biol Chem. 2012;287(2):1588–99.
91. Slaton JW, Inoue K, Perrotte P, El-Naggar AK, Swanson DA, Fidler IJ, et al. Expression levels of genes that regulate metastasis and angiogenesis correlate with advanced pathological stage of renal cell carcinoma. Am J Pathol. 2001;158(2):735–43.
92. Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, et al. Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell. 2002;1(3): 279–88.

93. O'Connell MP, Fiori JL, Xu M, Carter AD, Frank BP, Camilli TC, et al. The orphan tyrosine kinase receptor, ROR2, mediates Wnt5A signaling in metastatic melanoma. Oncogene. 2010;29(1):34–44.
94. O'Connell MP, Fiori JL, Baugher KM, Indig FE, French AD, Camilli TC, et al. Wnt5A activates the calpain-mediated cleavage of filamin A. J Invest Dermatol. 2009;129(7):1782–9.
95. O'Connell MP, Marchbank K, Webster MR, Valiga AA, Kaur A, Vultur A, et al. Hypoxia induces phenotypic plasticity and therapy resistance in melanoma via the tyrosine kinase receptors ROR1 and ROR2. Cancer Discov. 2013;3:1378–93.
96. Jenei V, Sherwood V, Howlin J, Linnskog R, Safholm A, Axelsson L, et al. A t-butyloxycarbonyl-modified Wnt5a-derived hexapeptide functions as a potent antagonist of Wnt5a-dependent melanoma cell invasion. Proc Natl Acad Sci USA. 2009;106(46): 19473–8.
97. Pacheco II, Macleod RJ. CaSR stimulates secretion of Wnt5a from colonic myofibroblasts to stimulate CDX2 and sucrase-isomaltase using Ror2 on intestinal epithelia. Am J Physiol Gastrointest Liver Physiol. 2008;295(4):G748–59.
98. Aoki K, Tamai Y, Horiike S, Oshima M, Taketo MM. Colonic polyposis caused by mTOR-mediated chromosomal instability in Apc+/Delta716 Cdx2+/- compound mutant mice. Nat Genet. 2003;35(4):323–30.
99. Bonhomme C, Duluc I, Martin E, Chawengsaksophak K, Chenard MP, Kedinger M, et al. The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. Gut. 2003;52(10):1465–71.
100. Lara E, Calvanese V, Huidobro C, Fernandez AF, Moncada-Pazos A, Obaya AJ, et al. Epigenetic repression of ROR2 has a Wnt-mediated, pro-tumourigenic role in colon cancer. Mol Cancer. 2010;9:170.
101. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139(5):871–90.
102. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol. 2006;172(7):973–81.
103. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol. 2000;2(2):84–9.
104. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol. 2000;2(2):76–83.
105. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development. 2005;132(14):3151–61.
106. Ren D, Minami Y, Nishita M. Critical role of Wnt5a-Ror2 signaling in motility and invasiveness of carcinoma cells following Snail-mediated epithelial-mesenchymal transition. Genes Cells. 2011;16(3):304–15.
107. Shabani M, Asgarian-Omran H, Jeddi-Tehrani M, Vossough P, Faranoush M, Sharifian RA, et al. Overexpression of orphan receptor tyrosine kinase Ror1 as a putative tumor-associated antigen in Iranian patients with acute lymphoblastic leukemia. Tumour Biol. 2007;28(6): 318–26.
108. Shabani M, Asgarian-Omran H, Vossough P, Sharifian RA, Faranoush M, Ghragozlou S, et al. Expression profile of orphan receptor tyrosine kinase (ROR1) and Wilms' tumor gene 1 (WT1) in different subsets of B-cell acute lymphoblastic leukemia. Leuk Lymphoma. 2008;49(7):1360–7.
109. Widhopf 2nd GF, Cui B, Ghia EM, Chen L, Messer K, Shen Z, et al. ROR1 can interact with TCL1 and enhance leukemogenesis in Emu-TCL1 transgenic mice. Proc Natl Acad Sci USA. 2014;111(2):793–8.
110. Zhang H, Qiu J, Ye C, Yang D, Gao L, Su Y, et al. ROR1 expression correlated with poor clinical outcome in human ovarian cancer. Sci Rep. 2014;4:5811.

111. MacKeigan JP, Murphy LO, Blenis J. Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. Nat Cell Biol. 2005;7(6):591–600.
112. Gentile A, Lazzari L, Benvenuti S, Trusolino L, Comoglio PM. Ror1 is a pseudokinase that is crucial for Met-driven tumorigenesis. Cancer Res. 2011;71(8):3132–41.
113. Klemm F, Bleckmann A, Siam L, Chuang HN, Rietkotter E, Behme D, et al. Beta-catenin-independent WNT signaling in basal-like breast cancer and brain metastasis. Carcinogenesis. 2011;32(3):434–42.
114. Hojjat-Farsangi M, Ghaemimanesh F, Daneshmanesh AH, Bayat AA, Mahmoudian J, Jeddi-Tehrani M, et al. Inhibition of the receptor tyrosine kinase ROR1 by anti-ROR1 monoclonal antibodies and siRNA induced apoptosis of melanoma cells. PLoS One. 2013;8(4):e61167.
115. Yamaguchi T, Yanagisawa K, Sugiyama R, Hosono Y, Shimada Y, Arima C, et al. NKX2-1/TITF1/TTF-1-Induced ROR1 is required to sustain EGFR survival signaling in lung adenocarcinoma. Cancer Cell. 2012;21(3):348–61.
116. Zhang S, Chen L, Cui B, Chuang HY, Yu J, Wang-Rodriguez J, et al. ROR1 is expressed in human breast cancer and associated with enhanced tumor-cell growth. PLoS One. 2012;7(3):e31127.
117. Cui B, Zhang S, Chen L, Yu J, Widhopf 2nd GF, Fecteau JF, et al. Targeting ROR1 inhibits epithelial-mesenchymal transition and metastasis. Cancer Res. 2013;73(12):3649–60.
118. Miyoshi H, Ajima R, Luo CT, Yamaguchi TP, Stappenbeck TS. Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury. Science. 2012;338(6103):108–13.
119. Li X, Yamagata K, Nishita M, Endo M, Arfian N, Rikitake Y, et al. Activation of Wnt5a-Ror2 signaling associated with epithelial-to-mesenchymal transition of tubular epithelial cells during renal fibrosis. Genes Cells. 2013;18(7):608–19.
120. Sonomoto K, Yamaoka K, Oshita K, Fukuyo S, Zhang X, Nakano K, et al. Interleukin-1beta induces differentiation of human mesenchymal stem cells into osteoblasts via the Wnt-5a/receptor tyrosine kinase-like orphan receptor 2 pathway. Arthritis Rheum. 2012;64(10):3355–63.
121. Smith EN, Bloss CS, Badner JA, Barrett T, Belmonte PL, Berrettini W, et al. Genome-wide association study of bipolar disorder in European American and African American individuals. Mol Psychiatry. 2009;14(8):755–63.
122. Ban HJ, Kim SC, Seo J, Kang HB, Choi JK. Genetic and metabolic characterization of insomnia. PLoS One. 2011;6(4):e18455.
123. Butler MG, Wadlington WB. Robinow syndrome: report of two patients and review of literature. Clin Genet. 1987;31(2):77–85.
124. Nowakowska B, Kutkowska-Kazmierczak A, Stankiewicz P, Bocian E, Obersztyn E, Ou Z, et al. A girl with deletion 9q22.1–q22.32 including the PTCH and ROR2 genes identified by genome-wide array-CGH. Am J Med Genet A. 2007;143A(16):1885–9.
125. Masiakowski P, Yancopoulos GD. The Wnt receptor CRD domain is also found in MuSK and related orphan receptor tyrosine kinases. Curr Biol. 1998;8(12):R407.
126. Rehn M, Pihlajaniemi T, Hofmann K, Bucher P. The frizzled motif: in how many different protein families does it occur? Trends Biochem Sci. 1998;23(11):415–7.
127. Saldanha J, Singh J, Mahadevan D. Identification of a Frizzled-like cysteine rich domain in the extracellular region of developmental receptor tyrosine kinases. Protein Sci. 1998;7(8):1632–5.
128. Xu YK, Nusse R. The frizzled CRD domain is conserved in diverse proteins including several receptor tyrosine kinases. Curr Biol. 1998;8(12):R405–6.
129. Patthy L. Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules. Cell. 1985;41(3):657–63.
130. McLean JW, Tomlinson JE, Kuang WJ, Eaton DL, Chen EY, Fless GM, et al. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. Nature. 1987;330(6144):132–7.
131. Furie B, Furie BC. The molecular basis of blood coagulation. Cell. 1988;53(4):505–18.

132. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature. 1989;342(6248):440–3.
133. Nakamura T, Aoki S, Kitajima K, Takahashi T, Matsumoto K. Molecular cloning and characterization of Kremen, a novel kringle-containing transmembrane protein. Biochim Biophys Acta. 2001;1518(1–2):63–72.
134. Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, et al. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. Nature. 2002;417(6889): 664–7.
135. Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. Oncogene. 2000;19(49):5548–57.
136. Sossin WS. Tracing the evolution and function of the Trk superfamily of receptor tyrosine kinases. Brain Behav Evol. 2006;68(3):145–56.
137. Green JL, Kuntz SG, Sternberg PW. Ror receptor tyrosine kinases: orphans no more. Trends Cell Biol. 2008;18(11):536–44.
138. Liu Y, Ross JF, Bodine PV, Billiard J. Homodimerization of Ror2 tyrosine kinase receptor induces 14-3-3(beta) phosphorylation and promotes osteoblast differentiation and bone formation. Mol Endocrinol. 2007;21(12):3050–61.
139. Akbarzadeh S, Wheldon LM, Sweet SM, Talma S, Mardakheh FK, Heath JK. The deleted in brachydactyly B domain of ROR2 is required for receptor activation by recruitment of Src. PLoS One. 2008;3(3):e1873.
140. Liu Y, Rubin B, Bodine PV, Billiard J. Wnt5a induces homodimerization and activation of Ror2 receptor tyrosine kinase. J Cell Biochem. 2008;105(2):497–502.
141. Yoda A, Oishi I, Minami Y. Expression and function of the Ror-family receptor tyrosine kinases during development: lessons from genetic analyses of nematodes, mice, and humans. J Recept Signal Transduct Res. 2003;23(1):1–15.
142. Li P, Harris D, Liu Z, Liu J, Keating M, Estrov Z. Stat3 activates the receptor tyrosine kinase like orphan receptor-1 gene in chronic lymphocytic leukemia cells. PLoS One. 2010;5(7): e11859.
143. Kaucka M, Krejci P, Plevova K, Pavlova S, Prochazkova J, Janovska P, et al. Post-translational modifications regulate signalling by Ror1. Acta Physiol (Oxf). 2011;203(3):351–62.
144. Hojjat-Farsangi M, Khan AS, Daneshmanesh AH, Moshfegh A, Sandin A, Mansouri L, et al. The tyrosine kinase receptor ROR1 is constitutively phosphorylated in chronic lymphocytic leukemia (CLL) cells. PLoS One. 2013;8(10):e78339.
145. Gentile A, Lazzari L, Benvenuti S, Trusolino L, Comoglio PM. The ROR1 pseudokinase diversifies signaling outputs in MET-addicted cancer cells. Int J Cancer. 2014;135(10): 2305–16.
146. Sanchez-Solana B, Laborda J, Baladron V. Mouse resistin modulates adipogenesis and glucose uptake in 3T3-L1 preadipocytes through the ROR1 receptor. Mol Endocrinol. 2012; 26(1):110–27.
147. Lai AZ, Abella JV, Park M. Crosstalk in Met receptor oncogenesis. Trends Cell Biol. 2009;19(10):542–51.
148. Wright TM, Rathmell WK. Identification of Ror2 as a hypoxia-inducible factor target in von Hippel-Lindau-associated renal cell carcinoma. J Biol Chem. 2010;285(17):12916–24.
149. Shen C, Nettleton D, Jiang M, Kim SK, Powell-Coffman JA. Roles of the HIF-1 hypoxia-inducible factor during hypoxia response in Caenorhabditis elegans. J Biol Chem. 2005;280(21):20580–8.
150. Yamamoto H, Yoo SK, Nishita M, Kikuchi A, Minami Y. Wnt5a modulates glycogen synthase kinase 3 to induce phosphorylation of receptor tyrosine kinase Ror2. Genes Cells. 2007;12(11):1215–23.
151. Medzihradszky KF, Darula Z, Perlson E, Fainzilber M, Chalkley RJ, Ball H, et al. O-sulfonation of serine and threonine: mass spectrometric detection and characterization of a new posttranslational modification in diverse proteins throughout the eukaryotes. Mol Cell Proteomics. 2004;3(5):429–40.

152. Clark CC, Cohen I, Eichstetter I, Cannizzaro LA, McPherson JD, Wasmuth JJ, et al. Molecular cloning of the human proto-oncogene Wnt-5A and mapping of the gene (WNT5A) to chromosome 3p14-p21. Genomics. 1993;18(2):249–60.
153. Kurayoshi M, Yamamoto H, Izumi S, Kikuchi A. Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. Biochem J. 2007;402(3):515–23.
154. Zhang X, Zhu J, Yang GY, Wang QJ, Qian L, Chen YM, et al. Dishevelled promotes axon differentiation by regulating atypical protein kinase C. Nat Cell Biol. 2007;9(7):743–54.
155. Schlessinger K, McManus EJ, Hall A. Cdc42 and noncanonical Wnt signal transduction pathways cooperate to promote cell polarity. J Cell Biol. 2007;178(3):355–61.
156. Cheung R, Kelly J, Macleod RJ. Regulation of villin by wnt5a/ror2 signaling in human intestinal cells. Front Physiol. 2011;2:58.
157. Cao L, McCaig CD, Scott RH, Zhao S, Milne G, Clevers H, et al. Polarizing intestinal epithelial cells electrically through Ror2. J Cell Sci. 2014;127(Pt 15):3233–9.
158. Takai A, Inomata H, Arakawa A, Yakura R, Matsuo-Takasaki M, Sasai Y. Anterior neural development requires Del1, a matrix-associated protein that attenuates canonical Wnt signaling via the Ror2 pathway. Development. 2010;137(19):3293–302.
159. Winkel A, Stricker S, Tylzanowski P, Seiffart V, Mundlos S, Gross G, et al. Wnt-ligand-dependent interaction of TAK1 (TGF-beta-activated kinase-1) with the receptor tyrosine kinase Ror2 modulates canonical Wnt-signalling. Cell Signal. 2008;20(11):2134–44.
160. Feike AC, Rachor K, Gentzel M, Schambony A. Wnt5a/Ror2-induced upregulation of xPAPC requires xShcA. Biochem Biophys Res Commun. 2010;400(4):500–6.
161. Matsuda T, Suzuki H, Oishi I, Kani S, Kuroda Y, Komori T, et al. The receptor tyrosine kinase Ror2 associates with the melanoma-associated antigen (MAGE) family protein Dlxin-1 and regulates its intracellular distribution. J Biol Chem. 2003;278(31):29057–64.
162. Witte F, Bernatik O, Kirchner K, Masek J, Mahl A, Krejci P, et al. Negative regulation of Wnt signaling mediated by CK1-phosphorylated dishevelled via Ror2. FASEB J. 2010;24(7):2417–26.
163. Qian D, Jones C, Rzadzinska A, Mark S, Zhang X, Steel KP, et al. Wnt5a functions in planar cell polarity regulation in mice. Dev Biol. 2007;306:121–33.
164. Gao B, Song H, Bishop K, Elliot G, Garrett L, English MA, et al. Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. Dev Cell. 2011;20(2):163–76.
165. van Wijk NV, Witte F, Feike AC, Schambony A, Birchmeier W, Mundlos S, et al. The LIM domain protein Wtip interacts with the receptor tyrosine kinase Ror2 and inhibits canonical Wnt signalling. Biochem Biophys Res Commun. 2009;390(2):211–6.
166. Sammar M, Stricker S, Schwabe GC, Sieber C, Hartung A, Hanke M, et al. Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2. Genes Cells. 2004;9(12):1227–38.
167. Nishitoh H, Ichijo H, Kimura M, Matsumoto T, Makishima F, Yamaguchi A, et al. Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. J Biol Chem. 1996;271(35):21345–52.
168. Sammar M, Sieber C, Knaus P. Biochemical and functional characterization of the Ror2/BRIb receptor complex. Biochem Biophys Res Commun. 2009;38:1–6.
169. Kim C, Forrester WC. Functional analysis of the domains of the C elegans Ror receptor tyrosine kinase CAM-1. Dev Biol. 2003;264(2):376–90.
170. Schleiffarth JR, Person AD, Martinsen BJ, Sukovich DJ, Neumann A, Baker CV, et al. Wnt5a is required for cardiac outflow tract septation in mice. Pediatr Res. 2007;61(4):386–91.
171. Cervantes S, Yamaguchi TP, Hebrok M. Wnt5a is essential for intestinal elongation in mice. Dev Biol. 2009;326(2):285–94.

Chapter 14
The ROS1 Receptor Family

Alain Charest

14.1 Introduction to the Receptor Tyrosine Kinase ROS1

ROS1 was originally discovered more than 30 years ago as the *onc* gene portion of an avian sarcoma RNA tumor virus named UR2 (*U*niversity of *R*ochester tumor virus 2), which had been isolated 20 years earlier by R. E. Luginbuhl in 1963 at the University of Connecticut Poultry Diagnostic Lab [1–3]. In the early 1980s, Balduzzi and colleagues observed that this highly invasive fibrosarcoma-derived virus was efficient at transforming chicken embryo fibroblasts (CEFs) [2, 4–7] and chicken embryo neuroretinal cells [8]. These observations were later followed by deciphering the amino acid sequence coded from the *onc* portion of UR2 (a 68 kDa polypeptide named p68v-ros) which demonstrated a significant homology to analogous regions from other RNA tumor viruses (*src, yes, fps, fms, erb B, fgr, abl*) and that it is similarly coded for a protein with tyrosine kinase activity [4, 6, 9, 10]. At the same time, Wigler's group identified an additional oncogenic form of ROS1 during human genomic DNA transfer experiments in NIH-3T3 cells designed to uncover novel transforming genes [11]. Recovery of tumor-inducing genomic DNA uncovered the mcf3 oncogene, later shown to be a truncated c-ROS1 product similar in structure to the avian UR2 viral p68v-ros [12]. Sequence comparison of these oncogenic forms of ROS1 to its then recently available full-length sequence led to the suggestion that loss of the extracellular domain may result in the activation of this proto-oncogene [12–18]. In fact, like many RNA tumor viruses, the oncogenic

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potential of p68v-ros resides in the fusion of the viral genome-coded structural p19gag protein to the intracellular kinase domain region of avian c-Ros1 and subsequent activation of tyrosine kinase activity [19–22]. To this date, the identity of the sequence upstream to c-ROS1 in the mcf3 oncogene locus remains unknown. The full-length receptor is a large, orphan ~280 kDa protein made up of over 2,000 amino acid residues with architectural features that are unique among the entire RTK family.

14.1.1 ROS1 Is an Evolutionarily Conserved Receptor

The advent of whole genome sequencing in multiple species has led to the identification of ROS1 genes in many species. ROS1 is found within the genomes of *H. sapiens* (human), *M. musculus* (mouse), *R. norvegicus* (rat), *C. griseus* (Chinese hamster), *G. gallus* (chicken), *P. troglodytes* (chimpanzee), *N. leucogenys* (White-cheeked Gibbon), *C. jacchus* (common marmoset), *M. mulatta* (rhesus macaque), *C. lupus familiaris* (dog), *A. melanoleuca* (giant panda), *O. anatinus* (duckbill platypus) and *E. caballus* (horse). ROS1 is also present in worm (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*) and fish (*Danio rerio*) species. In all species, the molecular architecture of the c-ROS1 gene product consists of an extracellular or ectodomain, a hydrophobic stretch corresponding to a single pass transmembrane spanning region, an intracellular portion containing the tyrosine kinase domain and a carboxyl-terminal tail [18, 23, 24] (see Fig. 14.1). The percent identity of the intracellular kinase domain remains highly conserved throughout evolution when the kinase domain amino acid sequence of worm, fly, zebrafish and human ROS1 are compared (Fig. 14.2). ROS1's structural organization is similar to those of other type I growth and differentiation factor receptors (e.g., EGFR, INSR, FGFR). What impart uniqueness to ROS1 is its ectodomain composition, the lack of a known ligand in vertebrate species and the remote similarity of its catalytic domain sequence when compared to other tyrosine kinase domains. Phylogenic relationship analysis of the kinase domains of all 58 known human RTKs demonstrates that ROS1 is a distinct receptor which is distantly related to the ALK/LTK and INSR families [25]. Clues to ROS1 ligand identity and ensuing functions in mammalian systems may be obtained from its non-vertebrate orthologues, the *C. elegans* ROL-3 and the *D. melanogaster* SEVENLESS receptors.

In *C. elegans*, the ROS1 homolog, encoded by the ORF C16D9.2 or ROL-3, was originally identified in a screening effort to isolate and analyze chemically induced mutants that appear to have alterations in cuticle structure [26, 27]. The nematode cuticle or exoskeleton is an impervious barrier between the animal and its environment that is essential for the maintenance of body morphology. It is a highly structured extracellular matrix (ECM) that is composed predominantly of cross-linked collagens, insoluble proteins termed cuticlins, associated glycoproteins, and lipids (for a review, see [28]). During the synthesis of the cuticle, these ECM components are secreted from the apical epithelial membranes of the hypodermis and then

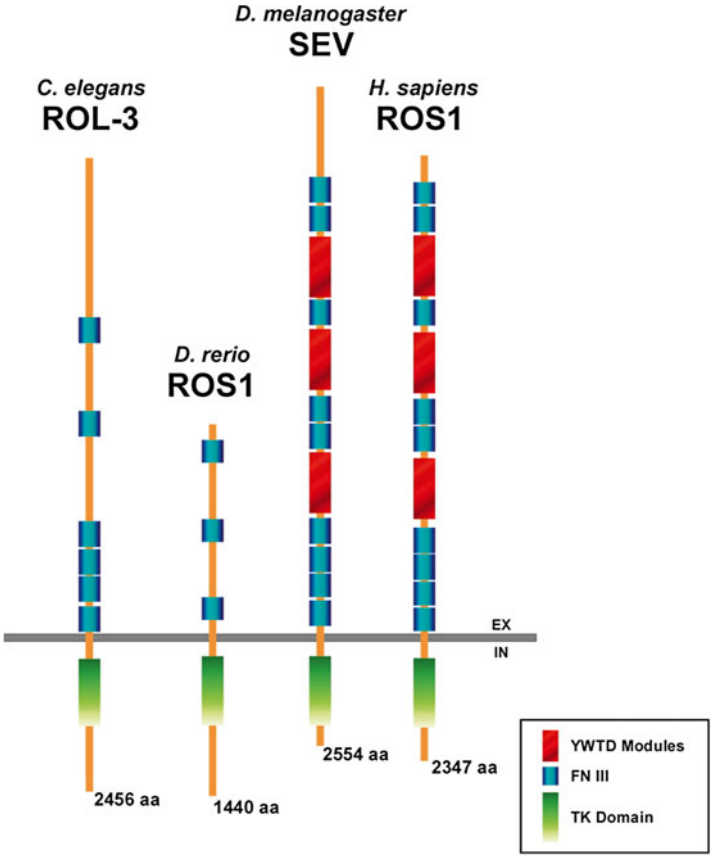

Fig. 14.1 ROS1 is an evolutionarily conserved receptor tyrosine kinase. Schematic representation of ROS1 and its homologues ROL-3, zebrafish ROS1, and SEV. The figure is drawn to scale in relation to the position of the FN-III-like repeats, YWTD modules, the transmembrane region, and the kinase domain. The length of each receptor (number of amino acids) is also indicated

Fig. 14.2 Alignment of the kinase domain sequences of human and zebrafish ROS1, ROL-3, and SEV demonstrating that they are highly similar to each other. Shown is a matrix of percent identity between each pair of kinase domain as calculated by the Clustal W method

Identity %

Divergence	ROL-3	SEV	zROS1	hROS1
ROL-3		24.7	30.6	21.8
SEV	173.7		57.4	42.3
zROS1	136.1	63.4		45.8
hROS1	135.0	59.1	50.9	

polymerize only to remain in intimate contact with the outer surface of these membranes.

The rol-3(e754) mutation is a member of a general class of mutations affecting gross morphology through disruption of the nematode cuticle. It was found that worms homozygous for rol-3(e754) display abnormally left-hand twisted cuticles, body musculature, gut and ventral nerve cords, as well as an aberrant left-handed rotation during locomotion [29]. Barbazuk et al. further generated recessive alleles of rol-3 alleles including a temperature-sensitive rol-3(s1040ts) allele lethal at high temperature but viable albeit weak at low temperature [30]. The identity of each of these allelic mutants of Rol-3 at the molecular level still remains undetermined.

Experiments aimed at solving ROL-3's function in cuticle structure genesis and maintenance led to the delineation of ROL-3 expression patterns. Using a transgene consisting of the Rol-3 promoter driving the expression of eGFP, Hunt-Newbury found that during embryo and larval stages, the Rol-3 promoter is active in the body wall muscle and the epidermal layer of the hypodermis [31]. In the adult, the promoter is active in the body wall muscle, the vulval muscle of the reproductive system, and in unidentified head neuron of the nervous system. More recently, it was shown that ROL-3 is expressed in a very dynamic fashion and exclusively in the major outer epithelial tissues of the worm. It was also found that ROL-3 is required for the fidelity of elongation and morphogenesis of seam cells [32] and for the maintenance of seam cell identity. Animals carrying mutations in rol-3 synthesize a disorganized cuticle and are defective in molting as the development of the seam syncytium requires ROL-3 function [32]. These experiments demonstrate that ROL-3 expression and presumably signaling events are necessary for proper developmentally regulated cues that give rise to the morphology of the animal.

Genetic screens for suppressor phenotypes of rol-3(s1040ts) mutation generated two loci; suppressor of roller lethal srl-I and srl-2. The molecular identity of these two loci remains to be determined. Interestingly, srl-I localizes to linkage group (LG) II, the same LG where the ptp-2 gene, the *C. elegans* orthologue of SHP-2, a known direct downstream effector of ROS1, is found. Deciphering the structure–function relationship of this ROS1 analog from these rol-3 mutants would certainly further our understanding of ROS1 regulation and function in *C. elegans* development and could shed light on mammalian ROS1 functions otherwise unnoticed.

Similar to ROL-3, ROS1 and SEVENLESS exhibit an unusually high degree of gross structural and sequence similarities (Fig. 14.1) [18, 23, 24]. During the development of the compound eye of drosophila, differentiation of the various cell types in each ommatidium is controlled by at least two RTKs, the drosophila EGF receptor (DER) and the SEVENLESS receptor (SEV) [33–35]. DER is known to control the differentiation of most cells in the developing eye and to provide additional proliferation and cell survival cues. [36–41]. SEV, however, is only required for specification of the R7 photoreceptor cell even though it is dynamically expressed in subpopulations of other ommatidial cells [42]. A loss of function mutation in the sev gene results in the failure of the R7 precursor cells to fully differentiate into photoreceptor cells. Large-scale genetic screens conducted in this system unraveled many steps in the SEV signaling cascade. Like many RTKs, activated SEV

transduces signals through the small GTPase RAS1 through the activation of the RAS guanine exchange factor protein son of sevenless (SOS). Supporting and surrounding this RTK-RAS1 pathway are many additional signaling molecules such as the tyrosine phosphatase CORKSCREW, downstream of receptor kinases (DRK) and daughter of sevenless (DOS), and invertebrate homologues of the signaling proteins SHP-2, GRB2, and GAB-1/IRS1, respectively. From these studies emerged a complex system of protein tyrosine kinases and phosphatases acting upon various enzymes and scaffolding proteins designed to transduce an SEV signal into changes in gene expression which leads to neuronal differentiation (for a review, see [43]).

The exclusive specificity of SEV signaling to the R7 cells was molecularly uncovered through genetic studies which have revealed that the ligand for SEV is a seven-transmembrane G-protein coupled receptor (GPCR) which is located on the surface of the adjacent R8 cells. This protein, termed bride of sevenless (BOSS), represents an unusual ligand for an RTK. Positional proximity of the already differentiated R8 to the noncommitted R7 cells result in the activation of SEV in the R7 precursor population by binding to the BOSS protein located on the surface of R8 cells. The ensuing activation of SEV result in the differentiation of the R7 cells to their final state of photoreceptor [44, 45]. In the absence of BOSS, the R7 precursor cells fail to differentiate into photoreceptors but rather develop into a non-neuronal cone cell [42, 46]. A major question remains to be addressed. For example, why is it that R7 cells need two RTKs to accomplish differentiation when other photoreceptor cells can perform with only DER signaling? In this system, it appears that SEV activity act in conjunction with another RTK to drive a very specific cell type into a differentiation path. Despite major inroads into the deciphering of SEV signaling, much remains to be clarified in this system. No doubt that uncovering additional SEV-driven biological processes will help elucidate ROS1's function in vertebrate systems.

To this day, there has been no mutation reported within the ROS1 homologue of zebrafish (*Danio rerio*) nor has the expression levels and tissue specificity for ROS1 determined in this species. Despite the unusual levels of architectural and sequence similarities, parallels between ROL-3, SEV, and ROS1 functions remained to be established experimentally. Vertebrate homologues of drosophila and worm genes can have conceptually similar functions when compared to their invertebrate counterparts. To help understand the function of ROS1 in vertebrate species, several groups have conducted detailed ROS1 expression pattern studies.

14.2 ROS1 Expression in Vertebrates

Expression of ROS1 was examined in detail in chicken, mouse, and rat tissues at various stages of development through adulthood. Northern blot analysis, RNAse protection assays, and in situ hybridization demonstrate that *c-Ros1* is expressed in a spatial, temporal, and cell-type-specific manner. Expression of *c-Ros1* is found in kidneys, small intestines, lungs, heart, and male reproductive organs and is highly restricted to epithelial cells.

Embryonic chicken, mouse, and rat kidneys contain moderate levels of *c-Ros1* mRNA. Levels of *c-Ros1* expression rise during the transition period from development to early postnatal and remain high (chicken and rat) or tapered down (mouse) to low levels in adult kidneys [47–49]. In situ RNA hybridization of chicken kidney at various stages of development demonstrated *c-Ros1* expression to be initially localized to the tips of the collecting ducts [47]. As development progresses, *c-Ros1* expression is detected in the entire epithelial layer of the collecting duct system and progresses to involve the epithelial cells of the larger tubules and even the proximal part of the ureters [47]. This is in contrast to in situ hybridization data obtained from mice where in early embryos, *c-Ros1* expression was confined to the early ureteric bud epithelium as it proliferates and connects with the developing metanephrogenic mesenchyme.

RNAse protection assay of *c-Ros1* expression in intestinal tissue from mice shows that expression of *c-Ros1* is restricted to epithelial cells and corresponds appropriately to the process of induction induced by epithelial–mesenchymal interaction in the developing intestine. Expression of ROS1 is first detected during embryogenesis, and significant levels are exhibited during the neonatal period with relative resolution by postnatal week three [49]. This pattern of *c-Ros1* expression correlates with the gross differentiation of the epithelium into the various brush border cells. In situ hybridization data show that expression of *c-Ros1* is first detected in epithelial cells of the villi at the stage in gut development when the stratified epithelium begins the process of terminal differentiation into columnar epithelium [48].

In the lungs, all three species studied reveal different kinetics of *c-Ros1* expression. In mice, the levels of ROS1 mRNA are relatively low in embryogenesis, rise slightly after birth then decline and remain low into adulthood [48]. In rats, levels of ROS1 mRNA in the lung are detected during embryogenesis, decrease postnatally, and remain constant in the adult [23]. In chickens, levels were shown to increase after birth and remain high in adult tissues [47]. Discrepancies in the pattern of *c-Ros1* expression in the lung between the two rodent species are most likely due to the differences in levels of sensitivity in detection modalities (RNAse protection assays versus northern blot analysis) inherent to the methods utilized. Nevertheless, ROS1 is expressed in adult lungs in these species albeit at relatively low levels. In addition, the levels of ROS1 mRNA in human lungs are also higher than in other human tissues [50].

Characterization of the transcripts found in rat lungs revealed multiple messages. The predominant message codes for a protein analogous to the previously elucidated and characterized ROS1 mRNAs. Interestingly, one lung ROS1 transcript clone contained a 171 base pair insertion just downstream of the transmembrane domain, which is likely to be the result of an alternative splicing event. Significantly, this insertion sequence contains a stop codon, which, if translated, would result in a receptor membrane protein without the cytoplasmic tyrosine kinase portion [23]. Further elucidation of this observation is needed especially since this may represent a potential artifact that arose during the cDNA library construction. Confirmation of a ROS1 splice variant, which creates a membrane-bound

receptor uncoupled to kinase activity, would represent an additional mode of functioning for this still elusive receptor.

In mice, testicular expression of ROS1 is detected only in adults and the transcript has been shown to be approximately 4.5 kb [48, 49]. Of note, the length of the testicular transcript is not sufficient to correspond with the full ROS1 protein, and its length differs from the transcripts found in other tissue (8.3 kb). In situ hybridization of adult testes showed *c-Ros1* expression to be present only in mature stages of germ cell development, namely, spermatids and spermatozoa [49]. This suggests that the expression of *c-Ros1* may correspond with the onset of male sexual maturity. There are numerous examples of truncated mRNA transcripts in adult testes. The purpose of testicular mRNA variants remains to be established, but it has been suggested that alternate transcripts may result in changes in localization, domain composition, or creation of a functionally different protein compared to its somatic cell counterpart [51]. However, the nature of this shortened *c-Ros1* transcript and evidence of ROS1 protein expression of any form in spermatozoa remain to be determined.

14.3 The Role of ROS1 During Development

Little is known of the function of ROS1 during mammalian development. Most of our knowledge comes from kidney explant experiments and in characterizing constitutive knockout alleles of mouse *c-Ros1*. As the kidney develops, high levels of ROS1 message are found predominantly at the terminal ends of the budding ureteric epithelium, suggesting a role for ROS1 in reciprocal epithelial–mesenchymal interactions [48, 49, 52]. Reciprocal epithelial–mesenchymal induction interaction describes a well-established process in kidney development where mesenchymal cells cause the ureters to branch and its epithelial cells to differentiate. At the same time, the differentiating epithelial cells induce the differentiation of mesenchymal cells into epithelial cells of the renal tubules and glomeruli [53]. The expression pattern of ROS1 in the developing rodent kidney coincides with differentiation events driven by epithelial–mesenchymal interactions that are essential to major morphological and differentiation events that occur during kidney development. In addition, it has been observed that downregulation of *c-Ros1* expression in an embryonic kidney culture system disrupted the morphology of ureteric bud branches and interfered with development of tubular and glomerular elements [52, 54]. These observations lead the authors to postulate that ROS1 functions in mesenchymal–epithelial transition (MET) during kidney development. However, these results are in contrast with the *c-Ros1* null knockout mice phenotype (see below) where kidney development is unimpaired [55]. This perhaps reflects the presence of compensatory mechanisms that are induced during the development of the kidneys to counteract a constitutive absence of ROS1 expression in these mice.

To gain a better understanding of ROS1 function, mice with a full deletion of the *c-Ros1* gene [55] and mice with a knockin allele of a kinase inactive *c-Ros1* gene

[56] have been generated and characterized. The latter was generated by mutating the ATP-binding lysine residue within the catalytic domain to methionine (K1973M also referred to as KM) on the endogenous *c-Ros1* gene by homologous recombination in embryonic stem cells. In both mice, the mutations resulted in a gender- and tissue-specific defect. Female ROS1$^{-/-}$ mice or homozygous for the kinase dead allele (ROS1$^{KM/KM}$) do not display any detectable abnormalities nor do heterozygous mice of either sex. Homozygous ROS1$^{-/-}$ or ROS1$^{KM/KM}$ male mice, however, are infertile but otherwise healthy. Further studies demonstrated that ROS1 is not needed for sperm production or sperm function as evidenced by investigations using chimeric mice consisting of wild-type and homozygous mutant ES cells [55]. In these ROS1$^{-/-}$/ROS1$^{+/+}$ chimeric mice, precursor germ cells are able to produce functional sperm when given a wild-type environment. Moreover, spermatocytes isolated from homozygous males are able to fertilize in vitro demonstrating that the infertility present in nullizygous c-Ros males is not sperm cell autonomous.

In situ hybridization data show c-Ros mRNA present in the initial segment (IS) of the epididymis in adult mice and demonstrate that c-Ros expression is restricted to epithelial cells of the IS [55]. In mammals, spermatozoa produced in the testis are immature and gain motility and fertilizing capacity during their transit through the epididymis, which provides an appropriate environment and supplies several of the molecules required for spermatozoa maturation [57–59]. The epididymis is a single, highly convoluted tubule that is composed of a pseudostratified epithelial layer of several cell types (principal, basal, clear and narrow cells) attached to a basement membrane and surrounded by contractile cells. The structure of the epididymis in a variety of species is functionally and structurally divided into four distinctive regions: initial segment (IS), caput, corpus, and cauda. The epididymal epithelial cells vary in number and size along the length of the epididymal duct and are equipped to perform specific functions. Each region expresses, synthesizes, and secretes a specific set of proteins resulting in unique luminal microenvironments that are essential for the sperm maturation process [60, 61].

At birth, the epididymis is still immature but continues to develop after birth with the differentiation of the epithelial cells into principal, basal, and narrow/clear cells [61–66]. At the onset of puberty and spermatogenesis, the epididymal epithelium develops segment-specific gene expression [61, 67] and gains regionalized functions aimed towards proper sperm concentration, maturation, and storage. Genetic and experimental surgical studies demonstrated that the most proximal segments (IS and caput) are essential for sperm maturation since disrupted development or function often leads to male infertility [64, 68–75]. Several lines of evidence revealed that the principal cells of the IS in postnatal, pre-pubescent mice require both testicular luminal fluid factors (or lumicrine factors) and androgen for terminal differentiation into fully functional, tall, columnar epithelial cells.

Puberty in mice is defined by full spermatogenic activity that occurs between 34 and 38 days postpartum. Prior to puberty, at day 17 to 21 days postpartum, the cuboidal IS epithelium differentiates into a tall columnar epithelium and gains enhanced secretory and absorptive features that are necessary for sperm maturation (for reviews see [60, 76]). We recently determined using qRT-PCR and Western

blotting that this process coincides with the expression of active ROS1 and that ROS1 kinase activity is required for the differentiation of these cells [56]. It has been postulated that the emergence of androgen production at puberty triggers the cuboidal epithelia of the IS of the epididymis to differentiate into tall columnar epithelia and to gain enhanced secretory features. Since this process coincides with the expression of c-Ros, it is not surprising therefore that c-Ros expression has been shown to be responsive to androgens in vitro [77]; however, c-Ros expression was retained in the IS of androgen receptor (AR) knocked out specifically in the epididymis [68], suggesting that c-ROS expression is not under the control of androgens in vivo.

Further studies in the $ROS1^{-/-}$ and $ROS1^{KM/KM}$ mice demonstrated that the source of infertility lies in the improper maturation of the spermatocytes as they migrate through the convoluted network of tubules of the epididymis. Conversion of immature to mature spermatozoa requires a well-balanced osmolarity and composition of the luminal epididymal fluid, which in turn is regulated by the highly secretory epithelial layer of the epididymis (for a review see [78]). In $ROS1^{-/-}$ null homozygous and $ROS1^{KM/KM}$ male mice, failure of the IS epithelia to function normally results in an imbalance in the osmolarity and composition of the luminal fluid with consequences of improper spermatocyte maturation [56, 79–83].

Quantitative analysis of signaling pathways downstream of ROS1 in IS demonstrate a dominant MEK/ERK signaling program [56], and pharmacological inhibition of MEK or ROS1 kinase activity during the sensitive 17–21 days postpartum result in a failure of the IS epithelium to differentiate, phenocopying the genetic ablation of ROS1 kinase activity [56].

14.4 ROS1 Expression in *Homo sapiens*

There has been very limited data reported on ROS1 expression in humans. The finding of *c-Ros1* expression in the proximal murine epididymis along with subsequent studies showing *c-Ros1* knockout mice and kinase dead mice to be healthy but infertile has inspired investigations into the expression of ROS1 in human epididymis. The rationale for these experiments is the concept of developing a male contraceptive based on inhibition of ROS1 function. Expression of ROS1 in the human epididymis as analyzed by RT-PCR and in situ hybridization demonstrated that ROS1 mRNA is present throughout the human epididymis and expression levels varied among epididymal segments and is absent from the proximal caput [84]. This is in stark contrast to what is observed in mice where *c-Ros1* expression is restricted exclusively to the caput epididymis. Interestingly, immunohistochemical staining indicates that ROS1 is localized to the cytoplasm of basal cells and in the supranuclear cytoplasmic compartment of principal cells within the epididymal corpus [84]. This is a surprising observation given that full-length ROS1 protein is a membrane-bound receptor, questioning the validity of these results. In order to consider ROS1 inhibitors as contraceptive agents, evidence demonstrating that inhibition of ROS1

function (through genetic or chemical methods) in adult animals (i.e., after the differentiation program has taken place) results in diminished sperm maturation and functionality is an absolute necessity. We recently have demonstrated that pharmacological inhibition of ROS1 in adult mice does not lead to decrease fertility, thus eliminating ROS1 as a target for reversible male contraceptive development [56].

To gain insight into ROS1 expression in human, we have previously performed a northern blot analysis on RNA isolated from various adult human organs and determined that c-ROS expression is detectable mostly in the lungs [50]. Size variants are also detected in RNA isolated from placenta and skeletal muscle tissues. Although very informative vis-à-vis transcript sizes, northern blots notoriously suffer from low sensitivity of detection for low abundant messages. This drawback is overcome with the advent of microarray gene expression studies where the expression patterns of any given gene are readily ascertained from various sources. This very informative, quantitative type of in silico analysis complement expression information obtained from northern blot analyses where ROS1 expression is consistently found at the highest levels in adult lung tissues [50, 85]. The recent development of a commercially available high-affinity rabbit monoclonal antibody capable of detecting ROS1 protein in immunohistochemistry-based analyses of human tissues will greatly facilitate the elucidation of ROS1 function in tissues [86].

Overall, expression of ROS1 is found to be both temporally and spatially regulated. Its patterned localization of expression during embryogenesis and in various adult organs suggests that ROS1 may play a role in the mature function of these organ systems beyond development. The parallels between the role of ROS1 in drosophila photoreceptor R7 cell differentiation and the expression of ROS1 during terminal differentiation of various tissues strongly suggest that ROS1 initiates signaling events that are a key component of a differentiation program in epithelial tissues.

14.5 The Role of ROS1 in Diseases

14.5.1 Cancers of the Central Nervous System

Early studies on the expression of human c-ROS1 in cancers pointed to events leading to the discovery of aberrant expression in tumors of the CNS, specifically gliomas. A survey of 45 human cell lines from various normal and cancer tissues by RNase protection assays revealed ROS1 transcript expression in 56 % of the glioblastoma cell lines analyzed ranging from 10 to 60 transcripts per cell [87]. In these experiments, ROS1 expression was not detected in normal brain tissue or in cell lines from 16 other types of cancer. Southern blot analysis showed that the ROS1 gene is present in normal copy number making gene amplification an unlikely event to account for the observed aberrant expression. This suggests that ectopic expression of ROS1, without gene amplification, may contribute to tumorigenesis. Two independent analyses of surgical specimens for ROS1 expression by RNase

protection and cDNA hybridization techniques have yielded similar results. High levels of ROS1 expression in 33 % and 40 % of glioblastoma surgical tumors have been reported [88, 89]. However, these results were not corroborated by the recent TCGA efforts in glioblastoma, where ROS1 overexpression is reported in a lower percentage of tumors [90]. This discrepancy may be due to the differences in the sensitivities of the detection methods, gene expression profiling by microarrays vs. RNase protection. The lack of c-ROS1 detection in lower-grade astrocytomas suggests that expression of c-ROS1 may be an event important for tumor progression [88]. However, there has been no definitive study performed demonstrating a prognostic value to c-ROS1 expression in gliomas. In other studies, high levels of c-ROS transcripts were detected in meningioma tumor specimens [91, 92], indicating that ectopic c-ROS expression in different cell types can give rise to histopathologically distinct tumors. Combined, these studies clearly demonstrate that c-ROS1 is aberrantly expressed in 33 % to 56 % of glioblastoma tumors and up to 55 % of meningeal tumors. These observations prompted an in-depth analysis of the c-ROS1 promoter in human glioblastoma tumors where it was shown that a CpG island within the human c-ROS1 gene promoter was highly demethylated in tumors that expressed c-ROS1 [93]. These observations most certainly warrant further investigations into the potential role(s) that full-length ROS1 plays in gliomagenesis.

14.5.2 Lung Cancer

Aberrant expression of c-Ros1 was also detected in cancerous lung tissue in mice. Microarray analysis of carcinogen-induced murine lung carcinomas and adenocarcinomas showed a threefold increase in c-Ros1 expression compared to normal lung tissue. c-Ros1 expression was elevated in both early and late stage lung tumors suggesting that ROS1 is important for lung tumor development and/or maintenance rather than progression [94]. Elevated c-Ros1 expression is also observed in non-small cell lung cancer (NSCLC) tumors in an oncogenic K-Ras initiated mouse model of lung cancer [95, 96]. This suggests that in these tumors, ROS1 may work in unison with activated K-Ras to establish signaling pathways with transforming activities. Increasing evidence shows that deregulated expression of ROS1 may be important to the pathogenesis of human lung cancer (see below).

Several microarray analyses of tumor specimens revealed significantly elevated c-ROS1 expression levels in 20–30 % of patients with NSCLC [97–99]. Using hierarchical clustering, one study demonstrated that elevated c-ROS1 expression was part of a molecular signature of lung adenocarcinoma subclasses, which is associated with well-differentiated tumors with a more favorable outcome [97]. However, a more recent immunohistochemistry-based study revealed that, overall, 22 % of primary and recurrent NSCLC overexpresses ROS1 protein and that ROS1 expression was an independent prognostic factor for overall survival in adenocarcinomas of stage I NSCLC [100].

Finally, to identify common genetic variants that contribute to lung cancer susceptibility, a large multistage genome-wide association study of lung cancer in Asian women who never smoked was conducted. 5,510 never-smoking female lung cancer cases and 4,544 controls drawn from 14 studies from different Southeast Asian centers were scanned by SNP genotyping. In doing so, the authors identified three new susceptibility loci including one within the c-ROS1 locus [101]. However, it remains to be seen if the association involves ROS1 function directly.

14.5.3 Chronic Myelomonocytic Leukemia and Breast, Stomach, Kidney, and Colon Cancers

Chronic myelomonocytic leukemia (CMML) is a clonal disorder that shares features with myelodysplastic syndromes and chronic myeloproliferative neoplasms. In a recent CMML study, ROS1 was shown to be overexpressed by qRT-PCR and constitutively phosphorylated in approximately 70 % of CMML patients tested [102]. It was further demonstrated that ROS1 is highly expressed in a CD34+ cell compartment from CMML patients and not expressed in their normal counterparts, supporting the idea that ROS1 overexpression arises early and that it does not occur during monocyte differentiation. Overexpression and activation of ROS1 in CMML patients was shown to result in the activation of the Erk/Akt pathways through a Grb2/SOS complex [102].

In a gene expression profile survey of 20 fibroadenomas of the breast, a common benign tumor, c-ROS1 was found overexpressed at levels more than twofold higher than normal tissues [103]. Fibroadenoma is the most common types of benign breast tumor in young women and patients with such tumors have a twofold increase in the relative risk to develop malignant breast cancer. It is thought to arise from a disrupted developmental program. It is conceivable that overexpression of c-ROS1 in these tumors may be attributable to a role in tumorigenesis as a function of improper developmental processes. A recent follow-up study demonstrated that in invasive ductal carcinoma (IDC) of the breast, ROS1 expression was significantly higher in lower histologic grade tumors suggesting that high ROS1 expression may be associated with favorable prognostic factors of IDC [104]. This research is certainly worth pursuing especially in the context of ROS1 function in the various differentiation programs mentioned above.

It has recently been shown in a carcinogen-induced rat stomach cancer model that c-Ros1 expression, along with a handful of other genes, is induced upon carcinogen treatment, and this expression persists long after chemical exposure [105]. This led the authors to suggest that such persistent changes, of which ROS1 figures prominently, are drivers of tumorigenesis. Similarly, Yovchev et al. recently discovered that de novo c-Ros1 expression is associated with induction of hepatic progenitor cell activation in a rat model of liver injury and that c-Ros1 expression is high in a rat hepatoma cell line [106, 107]. These observations further the need to address the

function of ROS1 signaling in the context of cell differentiation and transformation. More recently, a global sequencing survey of all tyrosine kinases in 254 established cell lines uncovered three new ROS1 mutations in two colon adenocarcinoma and one kidney carcinoma cell lines [108]. Interestingly, the two colon carcinoma mutations in c-ROS1 result in exon skipping which leads the authors to suggest that translation of these c-ROS1 species are prematurely terminated and are predicted to give rise to secreted (non-membrane bound) form of the receptor. This raises an interesting concept worth pursuing especially with regard to ligand identification. Unfortunately, the authors did not address the causative nature of these observations. Given the fact that both cell lines are known to contain chromosomal rearrangements at 6q22 (http://www.ncbi.nlm.nih.gov) where c-ROS1 is located [109–111] (http://www.ensembl.org/Homo_sapiens/contigview?l=6:117716223-117853711), it is quite possible that these observed ROS1 aberrations are the result of translocations and/or deletions which begs the question, what is the status of the carboxyl-terminal kinase domain of ROS1 in these cell lines?

Collectively, these observations highlight the importance of elucidating ROS1 function in cancer genesis and maintenance especially in the context of co-expression with other activated oncogenes. The use of animal models, which offer genetically tractable platforms, will certainly advance our understanding of ROS1 function in tumor initiation and progression. It is interesting to draw parallels with the drosophila SEV role in terminal differentiation during development and what is observed in human lung adenocarcinomas. At which point and under what circumstances, if any, does c-ROS1 expression trigger differentiation cues during the transformation process? These questions need to be addressed experimentally, and the results will certainly further our understanding of cancer especially in the context of developing therapeutically active ROS1-specific kinase inhibitors.

14.5.4 Heart Disease

Recent studies have suggested a link between specific c-ROS1 alleles and heart disease. A gene-centric association study of more than 11,000 single nucleotide polymorphisms (SNPs) spanning approximately 7,000 genes uncovered a c-ROS1 allelic variant that had statistically significant association with myocardial infarctions (MI) [112]. These results were directly confirmed in a prospective population-based study where an increased risk for MI was associated with the same variant c-ROS1 genotype identified by Zee et al. [113]. Interestingly, a recent retrospective study [114] failed to validate these studies. This seeming discrepancy emphasizes the need for further investigations to verify the applicability of c-ROS1 polymorphisms in cardiovascular medicine. A separate study showed that the same c-ROS1 SNP is significantly associated with the incidence of restenosis after coronary stenting [115]. A similar association study has linked a c-ROS1 polymorphism to increased blood pressure and a factor in the development of hypertension in Japanese individuals [116]. The nature of the c-ROS1 polymorphism further indicates a role

for ROS1 in cardiac function, assuming this SNP is functionally associated to the c-ROS1 gene rather than other distant genes. This SNP is a coding polymorphism changing amino acid residue 2213 of ROS1 from asparagine to aspartic acid. This residue is located immediately next to the kinase domain of the receptor, which allows one to speculate that these two allelic variants of ROS1 may be endowed with altered intrinsic kinase activities. However, the location and levels of c-ROS1 expression in cardiac tissues still remains to be determined and should be the focus of intense research. Once these basics are established, experiments aimed at obtaining mechanistic insights into the manner in which these alleles affect ROS1 function may lead to a better understanding of the role of ROS1 in heart disease.

14.6 ROS1

14.6.1 c-ROS1 Gene

14.6.1.1 Gene Structure

The human c-ROS1 gene is composed of 44 exons [117] that span over 137 kbp on chromosome 6 (6q22). The current NCBI annotation for human c-Ros1 lists the gene at 43 exons (http://www.ncbi.nlm.nih.gov/gene/6098). This is incorrect for there is a short unannotated exon present after exon three, thus shifting the exon numbering by one. Note that this exon four is correctly annotated in the mouse c-Ros1 gene. Different isoforms of c-ROS1 mRNA have been described in different tissues with little information on the resulting coded receptors. It remains to be demonstrated whether these variants are functionally relevant.

14.6.1.2 Promoter Structure

The initiation of transcription of the c-ROS1 gene in the glioblastoma multiforme cell line SW1088 was determined to take place 700 nucleotides upstream of the ATG translation initiation codon [93]. Taking this residue as the +1 position, a "TATA"-like sequence and a "CAAT" motif are present at positions –22 and –55, respectively. Thus, the c-ROS1 promoter contains canonical elements for basic transcriptional activity [93]. Jun and colleagues determined the transcriptional strength of the upstream sequences by reporter gene assays (firefly luciferase) and reported that in SW1088 cells, a construct composed of 2 kb of upstream sequences generated reporter activity fourfold greater than that of control constructs. From these results, the authors concluded that the sequences contained within this –2 kb construct and comprising the transcription initiation site have promoter activity in SW1088.

14.6.1.3 Transcriptional Regulation

Studies on the transcriptional regulation of c-ROS1 in normal tissues remain inexistent. In malignant glioma, it was found that a CpG island (a 253-bp fragment from nucleotides −132 to −384 bp relative to the transcription initiation site) resides within the promoter sequence of the c-ROS1 gene [93]. This led the authors to demonstrate that c-ROS1 expression in gliomas results from changes in promoter methylation. The methylation status of the c-ROS1 promoter CpG island is associated with expression and, more importantly, c-ROS1 expression can be acquired from a demethylation event [93].

14.6.2 ROS1 Protein

14.6.2.1 Amino Acid Sequence

The human ROS1 receptor is a large protein composed of 2,347 amino acids. ROS1 receptors in other species are similar except for zebrafish ROS1, which appears to be smaller.

14.6.2.2 Processing

Several transmembrane proteins, including RTKs, can be proteolytically processed to release all or a portion of their extracellular domain. There is no evidence that this is the case for ROS1. This suggests then that ROS1 is translated as a full-length protein that does not undergo proteolytic modification.

14.6.2.3 Domain Structure

Perhaps the most striking feature of ROS1 is its extracellular domain structure. In humans and other vertebrates, it is composed of a tandem of nine repeat motifs with high sequence homology to the type III repeat of fibronectin (FN-III) (Fig. 14.1). Fibronectin is an extracellular matrix and plasma protein that plays a critical role in cell adhesion. It contains three kinds of repeats: type I–III [118]. Amino acid sequences analogous to those of the third fibronectin repeat (FN-III-like repeat) are present in a variety of cell surface and extracellular matrix (ECM) proteins from invertebrates to vertebrates. In addition to FN-III domains, the extracellular domains of both ROS1 and SEV contain three YWTD β-propeller modules (Fig. 14.1).

The YWTD domain is a very abundant motif found in functionally diverse cell surface proteins and extracellular matrix proteins [119]. It has recently been shown that most YWTD repeats exist in groups of six contiguous repeats that fold into a

compact structure known as a six-bladed β-propeller domain [120]. YWTD modules are found in a diverse group of cell receptors including LDLR, LRP1/2, Yolkless, EGF precursor protein, and LR8/11. The YWTD modules for some of these have been shown to be involved in ligand binding, receptor internalization, and recycling [120]. The function of YWTD modules in ROS1 or SEV is yet to be determined.

The presence of multiple fibronectin type III-like repeats distributed throughout the ectodomain of ROS1 is highly reminiscent of many cell adhesion molecules (CAMs). However, unlike most CAMs, ROS1 also contains an intracellular domain possessing kinase activity. This combination makes ROS1 unusual in its potential to directly translate adhesion engagement to phosphotyrosine-mediated intracellular signaling pathways.

Among other members of the RTK family harboring FN-III-like repeats are members of the insulin receptor family (INSR, IGF-1R, and IRR), Axl family (MER and TYRO3), TIE family (TEK), and the ephrin receptor (EPH) family. In these receptors, data pertaining to functional attributes of their FN-III-like repeats is scarce. For the INSR and IGF-1R, it appears that the C-terminal portion of the first FN-III-like repeat is involved in mediating a structural requirement for insulin binding [121, 122]. For the EPHA3 receptor, binding of the EphrinA5 ligand is mediated through the membrane proximal FN-III-like repeat [123]. To this date, no role for FN-III-like repeats from any of the other RTKs has been reported. Given the importance of these receptors in many biological systems and in various diseases, elucidating FN-III-like repeat structure–function relationships represents a legitimate area to focus research on especially in the context of ROS1 function.

14.6.2.4 Posttranslational Modifications

Similar to many CAMs, the extracellular domain of ROS1 was found to contain 30 potential sites of N-linked glycosylation and shown to be glycosylated [18, 24, 52, 124]. These structural features suggest that perhaps ROS1 has the unique ability to directly couple extracellular adhesion-mediated events to tyrosine phosphorylation-based intracellular signaling pathways.

14.6.2.5 Phosphorylation Sites and Known Functions

Like other RTKs, ROS1 becomes phosphorylated on tyrosine residues upon receptor activation. These phosphorylation sites have been mapped to two main residues on the human ROS1 receptor, Y2274 and Y2334. These phosphorylation sites serve as docking sites for Src homology-2 (SH2) and phosphotyrosine-binding (PTB) domain-containing proteins. There are several known SH2 and PTB domain-containing proteins that have been shown to recognize and bind to these sites, as described below.

14.6.3 ROS1 Activation and Signaling

14.6.3.1 Dimerization

Biochemical, cell biology, and X-ray crystallographic studies have made it clear by now that RTKs are activated by homo- or heterodimerization mechanisms. There is little reason to suspect that ROS1 behave otherwise. Evidence based on chimeric Ins-ROS1 [125], EGFR-ROS1 [126], and TRK-ROS1 [124, 127] receptors suggest that ROS1 kinase domain can be activated by ligand-mediated homodimerization. Until a ligand is identified, the structural mechanisms by which ROS1 kinase is activated will remain speculative. Recently, a 2.2 A crystal structure of ROS1 bound to the small-molecule inhibitor crizotinib (wwPDB: entry ID 3zbf) has been solved, and ROS1 kinase domain structure was shown to be similar to the closely related ALK kinase [128].

14.6.3.2 Oncogenic ROS1 Fusion Kinases

Cancer is an opportunistic disease. Throughout its progression, individual tumor cells select for genetic events, which gives them net growth and survival advantages. Chromosomal rearrangements in the form of translocations, deletions, and amplifications are very commonly observed in tumors. This is because, all too often, the result of these events is the creation of gene fusion with potent oncogenic activities. Over 35 distinct members from ten RTK families have been observed in such chromosomal rearrangements in various kinds of malignancies (discussed in other chapters). For the most part, these genomic events lead to the juxtaposition of novel sequences to those coding for the kinase domain of a given RTK. Production of fusion kinases in this manner results in chimeric kinases that are ligand independent and are constitutively activated counterparts to their ligand-regulated full-length, wild-type receptors. Creation of these fusion kinases leads to unchecked signaling events and cellular transformation ensues. For most of all known RTK fusion proteins, the non-RTK fusion partner contains protein dimerization domains (e.g., coiled-coils, leucine zippers), which force the fusion kinase monomers into homodimers leading to a constitutively activated kinase state. Interestingly, approximately half of ROS1 fusion kinases (see below) retain ROS1's transmembrane region suggesting that ROS1 fusion kinases may signal from the plasma membrane.

14.6.3.3 V-ROS

The oncogenic DNA of the avian sarcoma virus UR2 is a rearranged product of the chicken c-Ros1 gene. Comparison of the UR2 v-ros and *Gallus gallus* c-Ros1 genes revealed structural differences in p68v-ros that are important mediators of oncogenic transformation (Table 14.1): (1) v-ros is joined in frame to the gag sequence

Table 14.1 ROS1 fusion kinases. List of all known ROS1 fusion events with their corresponding 5′ protein fusion partners

5′ fusion	Chrm	Type	Clinical	Freq.	References
FIG	6q22.1	Deletion	Glioblastoma	–	[18, 117, 134]
			NSCLC	2 %	[137, 142]
			Cholangiocarcinoma	9 %	[136]
			Ovarian cancer		[143]
CD74	5q32	Interchromosomal	NSCLC		[142, 144–149]
SLC34A2	4p15.2	Interchromosomal	NSCLC		[142, 144–149]
			Gastric cancer		[150]
			Colorectal cancer		[151]
TPM3	1q21.2	Interchromosomal	NSCLC		[148, 149]
			Spitzoid neoplasm		[152]
SDC4	20q12	Interchromosomal	NSCLC		[148, 149]
EZR	6q25.3	Paracentric inv.	NSCLC		[148, 149]
LRIG3	12q14.1	Interchromosomal	NSCLC		[148]
KDELR2	7p22.1	Interchromosomal	NSCLC		[144]
MSN	Xq11.1	Interchromosomal	NSCLC		[149]
LIMA1	12q13.12	Interchromosomal	NSCLC		[149]
CCDC6	10q21	Interchromosomal	NSCLC		[147]
CEP85L	6q22.31	Deletion	Angiosarcoma	3 %	[153]
			Glioblastoma		[154]
YWHAE (14-3-3 ε)	17p13.3	Interchromosomal	Infl. myofibroblastic	3.5 %	[155]
TFG	3q12.2	Interchromosomal	Infl. myofibroblastic	3.5 %	[155]
HLA-A	6p21.3	Intrachromosomal	Spitzoid neoplasm		[152]
MYO5A	15q21	Interchromosomal	Spitzoid neoplasm		[152]
PPFIBP1	12p12.1	Interchromosomal	Spitzoid neoplasm		[152]
ERC1	12p13.3	Interchromosomal	Spitzoid neoplasm		[152]
PWWP2A	5q33.3	Interchromosomal	Spitzoid neoplasm		[152]
CLIP1	12q24.3	Interchromosomal	Spitzoid neoplasm		[152]
ZCCHC8	12q24.3	Interchromosomal	Spitzoid neoplasm		[152]
KIAA1598	10q25.3	Interchromosomal	Spitzoid neoplasm		[152]

of UR2 seven amino acids upstream of the TM domain of c-Ros1, (2) there is a three-amino-acid insertion within the TM domain of v-ros, and (3) c-Ros1 sequences coding for the 3′ carboxyl-terminal tail are truncated in v-ros. Structure–activity relationship experiments demonstrated that the TM domain of p68v-ros is functional and that targeting of p68v-ros to the plasma membrane is necessary for the transformation of CEFs by UR2 [21, 129]. Moreover, it was shown that the p19gag sequence does not mediate oligomerization of the p68v-ros protein at the plasma membrane, leading the authors to suggest that membrane targeting rather than

homodimerization was sufficient for transformation [20, 130]. Further work provided insight into the significance of membrane targeting for cellular transformation. First, the additional three amino acid residues within the TM domain of p68v-ros proved to be necessary for the transformation activity of v-ros [131], and second, chimeric EGFR-ROS1 receptors differing only by their TM domains demonstrated that receptor recycling modalities, signaling pathways activation, and ensuing cell transformation activities were significantly dictated by sequences within the TM domain [126]. Finally, swapping of the 3′ carboxyl-terminal tail sequence of v-ROS to that of c-ROS1, which essentially add an autophosphorylation site, does not modify the transforming potential of p68v-ros [132]. A great deal of information pertaining to ROS1-mediated mechanisms of cellular transformation transpired from the seminal biochemical work on UR2's p68v-ros. This information paved the way to a better understanding of ROS1 signaling networks induced during transformation.

14.6.3.4 FIG-ROS1

We have characterized an aberrant c-ROS1 transcript in glioblastoma cell lines [117]. We have shown that this transcript is the result of a small intrachromosomal deletion that fuses a then uncharacterized gene that we termed FIG (fused in glioblastoma) to c-ROS1 sequence (Table 14.1) [133]. FIG is a membrane-bound protein that localizes to the Golgi apparatus [133]. The FIG-ROS1 transcript, which is encoded by seven FIG exons and nine ROS1 exons, also localizes to the Golgi apparatus. We investigated the biological activities of the FIG-ROS1 fusion protein and found that FIG-ROS1 is a potent oncogene with a unique mechanism of tyrosine kinase activation. We demonstrated that it is this localization to endomembranes that is responsible for the oncogenic potential of FIG-ROS1 [134]. Finally, expression of FIG-ROS1 in the CNS of an adult genetically engineered mouse model readily induces glioblastoma formation [135]. Recently, FIG-ROS1 fusion transcripts were reported in ~11 % of human cholangiocarcinoma [136], in 1–2 % of NSCLC [137–142], and in ~2 % of ovarian cancer [143].

14.6.3.5 Other ROS1 Fusion Kinases

A large-scale survey of tyrosine kinase activity in lung cancer uncovered two novel ROS1 fusion proteins associated with NSCLC [145]. Analogous to p68v-ros in UR2 ASV and FIG-ROS1 in glioblastoma, cholangiocarcinoma, ovarian cancer, and NSCLC cells, ROS1 is activated by genetic translocation rearrangements that result in fusion proteins between the membrane-associated protein solute carrier SLC34A2 and the type II transmembrane protein CD74 to ROS1 kinase domain (Table 14.1). Takeuchi and colleagues have uncovered additional ROS1 fusion kinases in lung cancer patients [148]. The genes TPM3, SDC4, LRIG3, and EZR were observed fused to ROS1 (Table 14.1). In addition, further in-depth

characterization of NSCLC genomes has revealed ROS1 fused to KDELR2 [144] and CCDC6 [147]. Recently, using an anchored multiplex PCR method, Shaw and colleagues have uncovered ROS1 fusion partners MSN and LIMA1 [149]. CEP85L was observed as a fusion to ROS1 in an angiosarcoma by massive genomic and transcriptional profile screen of multiple human cancer types [153] and by in-depth analysis of cancer RNA-seq data [154]. Next-generation sequencing of inflammatory myofibroblastic tumors uncovered fusion transcripts of YWHAE-ROS1 and TFG-ROS1 [156], and Wiesner and colleagues have uncovered eight new ROS1 fusion partners (HLA-A, MYO5A, PPFIBP1, ERC, PWWP2A, CLIP1, ZCCHC8, and KIAA1598) in spitzoid neoplasms, a group of melanocytic tumors with distinctive histopathological features and various malignancies [152].

Interestingly, all of these fusion events occur at exons 33, 35, or 36 of ROS1. This observation perhaps underlines the presence of a hotspot for genomic rearrangements within that region of ROS1 or given the high oncogenicity of the resulting fusion kinases, are selected during tumorigenesis.

SLC34A2 is a member of the sodium phosphate cotransporter solute carrier group of membrane transport proteins (for a review see [157]). Members of the SLC34 family are topologically arranged as eight TM domain receptors with intracellular NH_2^- and $COOH^-$ termini [158]. SLC34A2 is expressed in the small intestine, lung, testis, liver, and secreting mammary gland where it plays a role in the homeostasis of inorganic phosphate [159–161]. In the lung, SLC34A2 is localized at the apical pole of alveolar type II epithelial cells [162]. In HCC78 cells, a translocation between chromosomes 4p15 and 6q22 causes an in-frame fusion of exon 4 of SLC34A2 to exons 33 and 35 of ROS1, the latter being the result of an alternative splicing event skipping exons 33 and 34 (Table 14.1). The SLC34A2-ROS1 protein has been shown to localize to membrane fractions and displays constitutive kinase activity. siRNA-mediated downregulation of SLC34A2-ROS1 induced apoptosis in transformed cells demonstrating that ROS1 signaling is essential for survival of these NSCLC cells [145]. Since its original discovery in NSCLC, SLC34A2-ROS1 fusion transcripts have also been observed in gastric adenocarcinoma samples [150] and in a colorectal cancer sample [151].

CD74 is an integral membrane protein shown to function as the receptor for the macrophage migration inhibitory factor (MIF) [163]. CD74 is also known as an MHC class II-associated invariant chain that plays a critical role during peptide presentation to CD4-positive lymphocytes [164]. CD74 is ubiquitously expressed, with increased levels of CD74 at the surface of multiple malignant cells [165–172]. A t(5;6)(q32;q22) translocation in a NSCLC tumor from a patient gives rise to an in-frame fusion of exon 6 of CD74 to exon 35 of ROS1 (Table 14.1).

TPM3 is a member of the tropomyosin family of actin-binding proteins. It is involved in the contractile system of striated and smooth muscles and is part of the cytoskeleton of non-muscle cells. Architecturally, tropomyosins are dimers of coiled-coil proteins that polymerize end-to-end along the major groove in most actin filaments [173]. Chromosomal translocations involving this locus result to the creation of oncogenes associated with cancer. TPM3 has been found fused to ALK, PDGFRβ, and NTRK1 in various cancers [174–176]. An essential structural feature

of the TPM3 protein is the coiled-coil domain, which contributes to the dimerization and activation of tyrosine kinase fusion proteins. A t(1;6)(q21.2;q22) translocation in a NSCLC tumor from a patient gives rise to an in-frame fusion of exon 8 of TPM3 to exon 36 of ROS1 (Table 14.1). More recently, a similar translocation in a spitzoid melanoma tumor was observed, which fuses exon 3 of TPM3 to exon 37 of ROS1 [152] (Table 14.1).

SDC4 or syndecan 4 is a member of the syndecan proteoglycan family. It is an integral plasma membrane (type I) heparan sulfate proteoglycan that functions as a receptor in intracellular signaling. This receptor is found to homodimerize [177, 178] and has not been implicated in chromosomal translocation until now. A t(6;20)(q22;q12) from three NSCLC patients give rise to three in-frame fusion of SDC4 to ROS1 (Table 14.1).

The **LRIG3** gene codes for an integral membrane protein with an extracellular domain consisting of a leucine-rich repeat (LRR) domain and three immunoglobulin-like domains, followed by a transmembrane region and a cytoplasmic tail [179]. A t(6;12)(q22;q14.1) from a patient's tumor resulted in an in-frame fusion of LRIG3's exon 16 to ROS1's exon 36 (Table 14.1).

The **EZR** gene codes for the EZRIN protein, a cytoplasmic peripheral membrane protein that functions as a protein-tyrosine kinase substrate in microvilli. EZRIN is a member of the ERM (ezrin, radixin, and moesin) protein family and serves as an intermediate between the plasma membrane and the actin cytoskeleton. EZRIN plays a key role in cell adhesion, migration, and organization [180]. An inversion on chromosome 6 inv(6)(q22q25.3) found in a NSCLC patient's tumor resulted in an in-frame fusion of EZR exon 10 to ROS1's exon 35 (Table 14.1).

KDELR2 is seven-transmembrane-spanning receptor that functions to retrotransport chaperones from the Golgi complex to the endoplasmic reticulum. The tetrapeptide signal lys–asp–glu–leu (KDEL) motif is found within the C-terminal of the chaperones and acts as an ER retention/retrieval signal to keep the chaperones in the ER. The precise KDELR2-ROS1 fusion breakpoints have not been published [144].

CCDC6 is a substrate of the ataxia telangiectasia mutated (ATM) kinase able to sustain DNA damage checkpoint in response to genotoxic stress [181–183]. CCDC6 has been shown to be commonly rearranged in malignancies upon fusion with different partners. CCDC6 was originally identified by a rearrangement with RET in thyroid and lung tumors [184, 185] and with genes other than RET in solid tumors and hematological cancers [148, 186–188]. CCDC6 contains coiled coil domains that are retained in the CCDC6-ROS1 fusion kinase. A t(10;6)(q22;q22) in a NSCLC patient tumor creates an in-frame fusion of exon 6 of CCDC6 to exon 35 of ROS1 [147].

Little is known about the function of **CEP85L** (centrosomal protein 85 kDa-like a.k.a. C6orf204). CEP85L was originally identified as a breast cancer antigen [189] and recently discovered as one of the 59 partners fused to PDGFRβ receptor in hematological malignancies [190]. Structural analysis of CEP85L reveals the presence of coiled coil domains within its sequence, which are retained in the CEP85L–ROS1 fusion. An intrachromosomal rearrangement between CEP85L

(6q23) and ROS1 (6q22) leads to the in-frame fusion of CEP85L exon 12 to ROS1 exon 36 in an angiosarcoma tumor [153] and CEP85L exon 8 to ROS1 exon 37 in a glioblastoma [154].

YWHAE (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon) also known as 14-3-3ε is a member of the highly conserved 14-3-3 family of proteins. 14-3-3s are small acidic molecules that interact physically with over 300 proteins and are involved in cell cycle and apoptosis pathways [191]. **TFG** (TRK-fused gene) is an endoplasmic reticulum-associated protein that functions in controlling the export of cargoes from the ER to the Golgi apparatus. TFG was shown to form hexamers and interacts directly with SEC-16 [192]. Both YWHAE and TFG were found fused to ROS1 in two inflammatory myofibroblastic tumors, which is a rare mesenchymal neoplasm [155]. Exon 4 of YWHAE and exon 4 of TFG were found to be fused to exon 36 of ROS1.

MOESIN (MSN) is a member of the ERM family that is essential for linking the actin cytoskeleton to the cell membrane. They are key organizers of submembrane domains (e.g., microvilli, lamellipodia, and filopodia), and they can also function in signaling pathways that are fundamental in cell adhesion, migration, and morphogenesis. ERM proteins bind PtInsP2 and are recruited to the cell membrane through their N-terminal FERM domain [193]. Fusion of MSN to ROS1 retains the FERM domain suggesting that an MSN-ROS1 fusion kinase would be plasma membrane bound.

LIMA1 (LIM domain and actin binding 1) or EPLIN (epithelial protein lost in neoplasm) is a gene product that was originally identified as a downregulated gene in prostate and breast cancer cell lines. LIMA1 localizes to the cortical cytoskeleton or to actin stress fibers and focal adhesions depending on the cell type. The only recognizable domain of LIMA1 is a centrally located, 54-residue LIM domain, a protein–protein interaction module that may contribute to LIMA1 to homodimerize or associate with other proteins [194].

PWWP2A is a PWWP domain containing, nuclear protein with no known function. The PWWP domain is a small domain composed of 100–150 amino acids that is found in numerous proteins involved in cellular division, growth, and differentiation. Most PWWP domain containing proteins are localized within the nucleus that often function as transcription factors capable of regulating a variety of developmental processes. In a spitzoid tumor from a patient, an interchromosomal translocation t(5;6)(q33.3;q22) fuses exon 1 of PWWP2A to exon 37 of ROS1 (Table 14.1).

PPFIBP1 is a member of the liprin (LAR protein-tyrosine phosphatase-interacting) family of proteins. Liprins interact with members of LAR family of transmembrane protein tyrosine phosphatases. Liprins are multivalent proteins that form multiprotein complex structures that act as scaffolds for the recruitment and anchoring of LAR tyrosine phosphatases. PPFIBP1 codes for liprin beta 1 expressed in the lymphatic vasculature [195]. PPFIBP1 contains five coiled-coil domains [196] coded for by amino acid sequences in exons 5 through 12, and three coiled-coil domains are retained in t(12;6)(p12.1;q22) that fuses exon 9 of PPFIBP1 to exon 36 of ROS1 [152] (Table 14.1).

ERC1 is a member of the RIM-binding family of proteins. RIMs are proteins that regulate neurotransmitter release within the active zone, which is a specialized presynaptic plasma membrane region where synaptic vesicles dock and fuse. ERC1 has been found fused to the receptor tyrosine kinase gene RET by a translocation t(10;12)(q11;p13) in thyroid papillary carcinoma [197] and to PDGFRβ t(5;12)(q33;p13) in acute myeloid leukemia [198]. A t(12;6)(p13;q22) rearrangement fuses exon 11 of ERC1 to exon 37 of ROS1 in a spitzoid tumor (Table 14.1).

MYO5A is one of three myosin V heavy-chain genes that is part of the myosin gene superfamily. The myosin V subclass are actin-based ATP binding motor proteins involved in cytoplasmic vesicle transport and anchorage as well as spindle-pole alignment and mRNA translocation. Myosin5A is a large homodimer with an N-terminal motor domain that binds actin and ATP, followed by a neck domain containing six IQ motifs and a long coiled-coil domain in the tail that mediates dimerization. A t(15;6)(q21;q22) translocation fuses exon 23 of MYO5A to exon 36 of ROS1 (Table 14.1).

CLIP1 is a cytoplasmic linker protein that links endocytic vesicles to microtubules [199]. CLIP1 consists of an N-terminal microtubule-binding region, a central α-helical coiled-coil domain, and a C-terminal metal-binding motif. Fusion of exon 20 of CLIP1 to exon 37 of ROS1 in a spitzoid melanoma forms a fusion kinase that retains a portion of the coiled-coil domain of CLIP1.

The **HLA-A** protein belongs to the class I major histocompatibility complex protein. It exists as a heterodimer bound to a light chain (beta-2 microglobulin). The HLA-A protein is anchored in the plasma membrane. It is composed of eight exons and an intrachromosomal rearrangement fuses exon 7 of HLA-A to exon 35 of ROS1 (Table 14.1).

Very little is known about the product of the gene **KIAA1598**. KIAA1598 has recently been shown to be fused to FGFR2 in a cholangiocarcinoma [200]; however, its function in that event remains uncharacterized. In a spitzoid melanoma, a t(10;6)(q25.3;q22) fuses exon 11 of KIAA1598 to exon 37 of ROS1 [152].

The **ZCCHC8** gene codes for an uncharacterized zinc finger transcription factor. It has been shown to be phosphorylated by GSK-3 and to be interacting with proteins that are involved with RNA processing and degradation [201]; however, its true function remains to be determined. A translocation between t(12;6)(q24.31;q22) creates a fusion of exon 2 of ZCCHC8 to exon 37 of ROS1 (Table 14.1).

It is of interest to decipher the subcellular location and topology of these various ROS1 fusion kinases. Detailed biochemical experimentations are necessary and worth pursuing in order to determine the membrane insertion topology for these ROS1 fusion proteins and the exact cytoplasmic localization for the non-membrane associated fusions. Many RTKs activated by genomic rearrangements have several different fusion partners. Therefore, it is not surprising to observe multiple ROS1 fusion events, and it is conceivable that additional oncogenic ROS1 fusion kinases exist in other human cancers. It is becoming increasingly evident that activation of ROS1 kinase by genetic rearrangement is an important event in human cancers. It will be interesting to compare the signaling effector proteins that are activated by those ROS1 rearrangement products in NSCLC, cholangiocarcinoma,

and glioblastoma. Recent work from our laboratory on CD74-ROS1 and FIG-ROS1 demonstrates that different ROS1 fusion kinases have unique transforming activities [202]. It remains to be seen whether additional and/or different signaling events specifically emanate from these fusion kinases when compared to full-length, ligand-regulated ROS1. If so, determining these differences in pathway utilization would have profound value in terms of cancer therapeutics development.

14.6.3.6 Pathway Activation

ROS1 activates several signaling pathways that are known to be important for cell growth and survival. Activation of these pathways is mediated by the kinase activity of ROS1 on specific substrates and by the formation of phosphotyrosine recruitment sites within ROS1's carboxyl-terminal tail. Figure 14.3 schematically depicts the

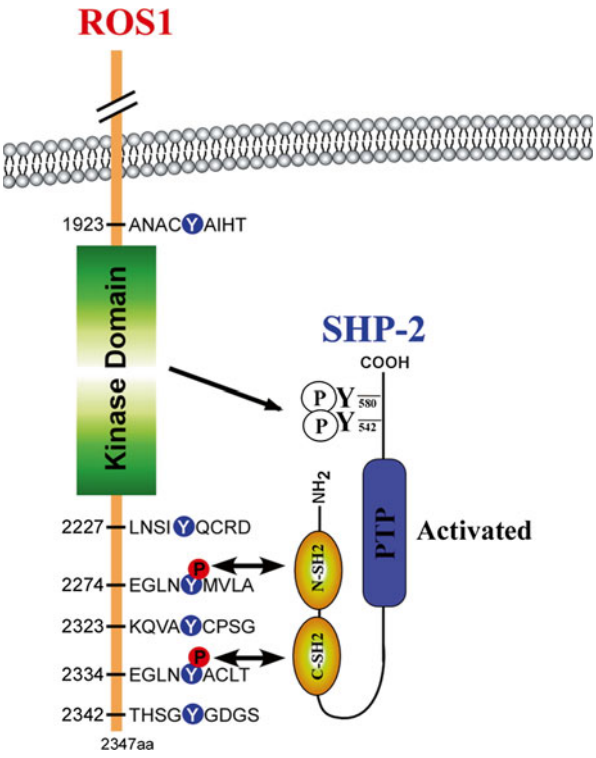

Fig. 14.3 ROS1 signals through SHP-2. Schematic representation of the positions of intracellular tyrosine residues of human ROS1. Excluded from the schematics are those tyrosine residues within the kinase domain. Tyrosine residues 2274 and 2334 have been demonstrated to be phosphorylated and correspond to target sequences for the SH2 domains of the SHP-2 tyrosine phosphatase. Activated ROS1 kinase can also phosphorylate SHP-2's two C-terminal tyrosine residues (Y542 and Y580), which are also target sequences for its own SH2 domains. Activation of ROS1 strongly activates the SHP-2 phosphatase activity. Figure not drawn to scale

tyrosine residues of the juxtamembrane domain and the carboxyl-terminal tail of human ROS1. Tyrosine residues 2274 and 2334 have been shown to be the only two major sites of phosphorylation and substitution of both residues to phenylalanine abrogates the transforming activity of oncogenic ROS1 [134].

The study of ROS1-induced signaling pathways and transformation continues to be hampered by the absence of a known ligand for c-ROS and the inability to overexpress full-length, wild-type c-ROS in different types of mammalian cells. A simple strategy employed over the years to overcome these obstacles has been the development and utilization of ROS1 chimeric receptors where the extracellular ectodomain of ligand-activated RTKs such as the insulin receptor [125], EGF receptor [126], or NGF receptor [124, 127] are fused to the intracellular kinase domain of ROS1, thus rendering ROS1 activity ligand-regulated. These ligand-regulated ROS1 chimeric species were shown to activate several signaling pathways. It has been demonstrated that ROS1 activity can lead to activation of the SH2 domain tyrosine phosphatases SHP-1 and SHP-2, the mitogen-activated protein kinase ERK1/2, insulin receptor substrate 1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), STAT3, and VAV3 signaling pathways (Fig. 14.4) [126, 135, 203, 204].

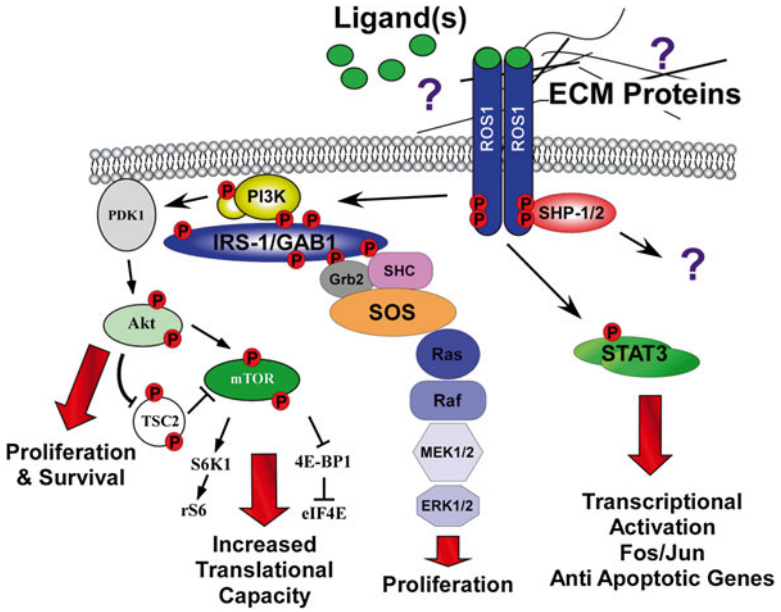

Fig. 14.4 Schematic representation of signaling pathways activated by ROS1. The map is an amalgamation of observations obtained from chimeric EGFR-ROS1, TRK-ROS1 membrane-bound receptors expressed in fibroblasts, as well as V-ROS- and FIG-ROS1-derived signaling events. Putative ligands are depicted as soluble molecules, and hypothetical activation of ROS1 through contact with ECM proteins is also indicated. ROS1 autophosphorylation sites are docking sites for the tyrosine phosphatases SHP-1 and SHP-2. ROS1 activates PI3K through the scaffolding proteins IRS-1 and/or GAB1. Phosphorylated IRS1/Gab1 are targets for Grb-2/SHC/SOS complexes, which in turn activate a RAS/RAF/MAPK pathway. Activated PI3K in turn activates an AKT/mTOR signaling axis. STAT3 is phosphorylated directly by ROS1 kinase activity

14.6.3.7 SH2 Domain-Containing Tyrosine Phosphatases SHP-1 and SHP-2

Insights into ROS1 signaling pathways involved in terminal differentiation of the IS epididymis came from the observation that, analogous to ROS1 null animals, male mice carrying the "viable motheaten" (mev) mutation are also sterile due to a similar defect in differentiation of the epididymis which result in impaired sperm maturation [205]. This observation suggested that ROS1 and SHP-1 function in signaling pathways responsible for terminal differentiation of the caput epithelia. mev is a naturally occurring splice site mutation in the gene coding for SHP-1. The mev allele is hypomorphic and codes for two SHP-1 protein variants both with aberrant phosphatase domains, one with a small deletion and the other with a small insertion [206]. Together, these mutant proteins have been shown to retain approximately 20 % of wild-type SHP-1 phosphatase activity [207].

SHP-1 activity was coupled to ROS1 signaling when it was demonstrated that SHP-1 (Ptpn6) is co-expressed with c-Ros in the caput epididymis of adult mice and elevated phosphorylation of ROS1 was observed in the epididymis of mev mice. Furthermore, in vitro biochemical experiments demonstrated that SHP-1 directly binds autophosphorylated ROS1 via an interaction between the amino-terminal SH2 domain of SHP-1 and the 2274 autophosphorylated phosphotyrosine residue in the carboxyl-terminal end of ROS1 [205]. It is known that engagement of the SH2 domain of SHP-1 activates its catalytic phosphatase activity, and thus, physical interaction between ROS1 and SHP-1 is thought to activate the phosphatase activity of SHP-1. Furthermore, substrate sequence specificity of SHP-1 matches the same site (Y2274) of ROS1 that interacts with the SH2 domain of SHP-1. These studies suggest that under normal circumstances SHP-1 activation is under the control of ROS1 signaling in vivo and that activated SHP-1 triggers or leads to a differentiation program. This is further strengthened by the fact that in mev/mev mice, ROS1 is hyperphosphorylated, and yet the differentiation program remains abrogated suggesting that hyperphosphorylated ROS1 does not signal through pathways other than SHP-1 in this tissue [205]. Parallel observations from the mev mice hematopoietic system has led to the dogma that SHP-1 acts as a negative regulator of RTKs, cytokine and chemokine receptors, and integrin signaling [208]. Given the information at hand, it appears that in epididymal epithelial tissues, SHP-1 acts as a positive effector of the RTK ROS1 since inactivation of either ROS1 or SHP-1 independently result in the same phenotype, that is, failure of the epididymal epithelia to differentiate. It has recently been shown in epithelial cells that SHP-1 can act as a positive regulator of RTK signaling [209]. It seems that the results of SHP-1 engagement upon growth factor receptor activation are context dependent. During development of the epididymis, the evidences suggest that SHP-1 activation is a pro-differentiation event. Perhaps, in different context, engaging SHP-1 may lead to an alternative outcome. Regardless, the studies conducted in both c-Ros knockout mice and viable motheaten mice have demonstrated the importance of regulating ROS1 signaling for the proper differentiation and development of the caput epididymis.

In addition to the aforementioned activation of SHP-1 in the differentiation program of the epididymal epithelia, ROS1 has been shown to interact with, phosphorylate, and activate the SH2 domain containing tyrosine phosphatase SHP-2 [135]. Germ line missense mutations of PTPN11, the gene coding for SHP-2, are responsible for the majority of Noonan syndrome cases, an autosomal dominant disorder characterized by a short stature, typical facial dysmorphology, congenital heart defects, and associated with an increased incidence of leukemia [208, 210, 211]. In addition, several somatic Ptpn11 mutations have been reported in a high percentage of hematological malignancies [212–215] and in solid tumors of the colon, lungs, skin, and peripheral nervous system [216]. All of these PtpN11 mutations predict an activated form of SHP-2, which strongly suggest that constitutive activation of SHP-2 is an oncogenic event. Consistent with this concept, transduction of mouse bone marrow cells with a constitutively activated SHP-2 protein cause a fatal JMML-like disorder in vivo [217]. Constitutively active SHP-2 is considered an oncogene since it is known that SHP-2 positively regulates the ability of several RTKs to activate the Ras/Raf/MAPK signaling cascade [218–223]. In most cases, the catalytic activity of SHP-2 is required to propagate receptor-mediated signaling to MAPK [224, 225]. Further biochemical and genetic evidences of SHP-2 acting as a positive regulator of growth factor receptors come from studies on *D. melanogaster* SHP-2 homologue corkscrew (*csw*).

The oncogenic activity of FIG-ROS1 in fibroblasts is mediated by an interaction with and the phosphorylation of SHP-2, suggesting that activation of SHP-2 is a crucial event in FIG-ROS1 transformation [135]. Interestingly, phosphopeptides corresponding to the tyrosyl phosphorylation sites of SHP-2 were recovered in tumor samples and cell lines that contained the rearranged SLC34A2-ROS1 and CD74-ROS1 products [145] suggesting that constitutively active ROS1 may preferentially signal through the SHP-2 tyrosine phosphatase. Given the retention of C-terminal tail sequences and phosphorylation sites in ROS1 fusion kinases, it is likely that full-length ROS1 receptors are also preferentially signaling through SHP-2. Activation of SHP-2 phosphatase activity is achieved in two ways: (1) through the engagement of its two SH2 domains binding to phosphotarget sites and (2) by phosphorylation of its C-terminal tyrosine 525 and 580 residues, which are high-affinity binding sites for its own SH2 domains. The interaction between ROS1 and SHP-2 results in both events suggesting a strong SHP-2 activation by ROS1 RTK (Fig. 14.3). The remaining question is, what are then the signaling events downstream of SHP-2 activation? Perhaps the recently identified link between SPROUTY proteins and SHP-2 activity will shed light on SHP-2 signaling pathways [226].

14.6.3.8 MAP Kinase

Furthering the homology between SEV and ROS1, analysis of ligand stimulated EGFR-ROS1 and TRKA-ROS1 chimeric receptors demonstrated a temporal activation of ERK1/2 in NIH-3T3 cells [126, 205]. Similarly, oncogenic V-ROS also

activates ERK1/2 in chicken embryonic fibroblasts (CEFs) [204]. Surprisingly, chemical inhibition of the MAPK pathway by the MEK inhibitor PD98059 had no effect on the transforming potency of chimeric EGFR-ROS1 on CEFs or NIH-3T3 cells [227]. In addition, in FIG-ROS1-induced transformed fibroblasts, elevated levels of phosphorylated ERK1/2 are modest (Charest, A. unpublished data). Taken together, these observations suggest that although activated under certain circumstances, the MAPK pathway may play little, if any, role in the transformation of these cell types by activated ROS1.

Fibroblasts expressing either v-ROS or FIG-ROS1 are capable of growing in an anchorage-independent fashion, a feature intrinsic to many cancer cells. It is known that powerful reorganization of cytoskeletal structures is necessary for this kind of morphological transformation. Not surprisingly, v-ROS expressing cells demonstrated phosphorylated versions of a series of cytoskeletal proteins involved in the formation of focal adhesions and cell–cell interactions [204] suggesting that constitutively activated ROS1 can signal to elicit structural rearrangements of the cytoskeleton as well as modulate biochemical events necessary for cell–cell interactions. It remains to be determined if wild-type ROS1 signaling is also capable of modulating cell–ECM and cell–cell interactions especially in the context of epithelial differentiation as seen in the epididymis, small intestine, and ureteric buds.

14.6.3.9 The PI3K and Akt Pathways

The PI3K signaling pathway promotes growth factor-mediated cell survival in many cell types. PI3K also has key regulatory functions in cellular proliferation, differentiation, apoptosis, glucose metabolism, and vesicle trafficking. PI3K, a lipid kinase, is activated by RTKs through recruitment at the plasma membrane, a process fulfilled by the engagement of the SH2 domain of its p85α regulatory subunit to phosphorylated tyrosine residues on the receptors themselves or on scaffold proteins such as IRS-1 or GAB-1 [228]. This binding then elicits activation of PI3K catalytic subunit, which, in turn phosphorylates inositol lipids to produce various phosphatidylinositol phosphates (reviewed in [229]). The latter serve as second messengers to recruit and activate a multitude of proteins that contain pleckstrin homology domains or FYVE fingers. Among these, several kinases (PDK1, PDK2, and Akt/PKB), nucleotide exchange factors (TIAM1, VAV, and SOS), GTPase-activating proteins, and phospholipases have been shown to respond to PI3K activity. The AKT kinase is a major downstream target of PI3K activation and has functions both in cell growth and survival (for reviews see [230, 231]). The list of AKT substrates is ever growing and comprises substrates that have been shown to be involved in cellular growth, survival, and metabolism (for reviews see [232–235]).

Activation of the PI3K/AKT pathway has been demonstrated to be an important event for ROS-induced transformation. Chemical and genetic inhibition of the PI3K pathway significantly attenuated the ability of activated ROS1 to induce colony formation and anchorage-independent growth of fibroblasts [227, 236]. In these cells,

activation of PI3K by ROS1 is thought to be mediated by the scaffold protein IRS-1 since PI3K is immunoprecipitated using anti IRS-1 antibodies in V-ROS expressing CEFs [204, 236]. Activation of AKT is frequently associated with malignant astrocytomas in humans, and hyperactivation of its downstream effector, mTOR, is found in human and mouse brain cancer [237–240]. We have shown that the oncogenic fusion protein FIG-ROS1 can activate a PI3K/AKT/mTOR signaling axis in FIG-ROS1-induced brain tumors [135]. In addition, the mTOR inhibitor, rapamycin, induced dose-dependent growth inhibition in FIG-ROS1 brain tumor cell cultures [135], which suggest that mTOR inhibitors may be effective in treating malignancies harboring activated ROS1. Although detailed mechanistic features of ROS1-mediated PI3K activation remain to be established in different cell types, the data reported in the literature strongly support the view that ROS1 signals through a PI3K/AKT axis.

14.6.3.10 STAT3 Activation

Signal transducers and activators of transcription (STATs) are transcription factors involved in cytokine and growth factor receptor-induced gene expression. STATs are activated by tyrosine phosphorylation, which result in their dimerization and translocation to the nucleus where they regulate the transcription of target genes by binding to specific DNA-response elements. STATs have been found to be activated in a wide variety of tumors and in oncogene-transformed cell lines (reviewed in [241, 242]). It is becoming increasingly clear that STAT3, in particular, is constitutively activated in oncogenic tyrosine kinase transformed cells and in certain instances shown to be essential for their transformation activity [243, 244]. Interestingly, constitutive activation of STAT3 has been reported in malignant brain cancer tumors with overexpression of the EGF receptor [245]. Many STAT3 transcriptional target genes are antiapoptotic, for example, survivin, MCL-1, BCL-x, and BCL-2 have been shown to be transcriptionally responsive to STAT3 activation. Not surprisingly, STAT3 activation has been associated with resistance to chemo- and radiation therapy, a salient feature of malignant brain cancer. Stimulation of STAT3 signaling is an additional event for oncogenic transformation by activated ROS1 in fibroblasts [246]. It has been shown that activation of STAT3 is required for ROS1-mediated transformation in vitro. Expression of dominant negative STAT3 partially suppressed ROS1-induced anchorage-independent growth of NIH-3T3 cells and was shown to inhibit both initiation and maintenance of the transformed state [227, 246]. The transcriptional program initiated by a ROS1-STAT3 signal axis in these cells remains to be established and is certainly worth pursuing in other tissues especially in the context of changes in gene expression profiles that are associated with ROS1-mediated anchorage-independent growth.

14.6.4 Crosstalk with Other Receptor Systems

There are numerous examples of cross-family RTK heterodimerization and signaling cross-talk. This phenomenon has not been reported for ROS1 but is a distinct possibility. It is possible that ROS1 is a ligand-less receptor much like ErbB2 (Her2), which heterodimerizes with other members of the ErbB family of receptors. It is therefore not inconceivable that ROS1 is a co-receptor for another RTK or even for a non-RTK cell surface receptor. This concept is worth pursuing and will certainly lead to the discovery of a role for ROS1 in an already established signaling system.

14.6.5 ROS1 Internalization, Processing and Attenuation

There has not been any exhaustive report of the dynamics of ROS1 internalization and signal attenuation so far in the literature. Xiong and colleagues have demonstrated differential rates of internalization and slight variations in temporal signaling between EGFR-ROS1 chimeric proteins containing either the ROS1 transmembrane domain or that of EGFR [126]. Despite the paucity of hard evidence, one can still infer similar modes of ligand-activated internalization and endosomal processing of ROS1, as with other RTKs.

14.6.6 Unique Features of ROS

14.6.6.1 Ligand(s)

Perhaps the most obstructing element of ROS1 research has been the inability to identify ROS1's natural ligand(s). The high degree of parallelism between ROS1 and SEV suggests that a mammalian homologue of BOSS would represent a valid candidate. Unlike other receptors whose ligands are conserved throughout evolution, there is a lack of BOSS conservation in more distantly related genomes. This may be the result of an evolutionary replacement of SEV ligands. Sequence alignment of BOSS to mammalian genomes revealed the presence of a single orphan seven-transmembrane G-protein coupled receptor gene with weak overall homology to BOSS [247]. GPRC5B is related to the metabotropic glutamate receptor family [248, 249]. GPRC5B is expressed in the peripheral and central nervous system, kidney, pancreas, testis [248–250], and placenta and yolk sac during gestation in mice [251]. The expression of this receptor in kidney and testis is interesting in the context of ROS1 expression and function in these tissues. Experiments aimed at validating the potential for GPRC5B to activate ROS1 should be performed.

Another source for the search of a putative ligand is the testicular lumicrine space. Pre-pubescent efferent duct ligation in mice prevents testicular exocrine secretion and leads to a failure of the IS epididymis epithelia to differentiate much like the c-Ros knockout phenotype. This suggests that factors within this fluid act upon ROS1 as ligands [252]. Given ROS1 function in the differentiation of the IS epididymis, one can therefore conceive that a ligand(s), produced within the testes, is present within the testicular fluid flowing through the epididymis and referred to as lumicrine factors [78, 252]. These observations suggest that the testicular exocrine fluid may harbor molecules capable of activating ROS1 and thus represent a valid source of material to search for ligand species. Finally, it is quite possible that ROS1 has more than one ligand. Experiments aimed at elucidating the identity of ROS1 ligand(s) are deeply needed if we are to fully understand the role and biological significance of ROS1 activation in normalcy and disease.

14.7 Conclusion

Since its discovery over 30 years ago, research on ROS1 has been dampened by the lack of a known ligand and the inability to express the full-length, wild-type receptor both in vivo and in different cell types in vitro. Nevertheless, a great deal of information gravitates ROS1. Genetic screens in invertebrate models led to the deciphering of many signaling components of ROS1. Biochemical approaches in mammals furthered our knowledge on how ROS1 triggers specific pathways, which ultimately leads to cellular transformation and cancer. There are, however, many more questions to be answered. What function does ROS1 play in intestinal and urological tissue development? Is there a role for ROS1 in cardiac function, and if so, what are the biological effect(s) of various c-ROS alleles in this system? Is the aberrant expression of full-length, wild-type ROS1 observed in human cancers a causative or by-stander phenomenon? No doubt the field is at a standstill, awaiting the identification of a ligand and the development of the necessary tools to address these questions. For example, the creation of a Cre/Lox-based conditional knockout or overexpressing transgenic mouse strains would certainly benefit and undoubtedly advance ROS1 research. The recent publications on chromosomal rearrangement events creating oncogenic ROS1 variants in brain, lung, and gall bladder cancers point to the undeniable fact that ROS1 activating mutations are now part of the panoply of cancer-causing mutations with potential therapeutic involvement. The coming years should witness the production of small chemical kinase inhibitor of ROS1 along with preclinical validations in appropriate and relevant animal models.

Receptor at a glance: ROS1

Chromosome location	6q22
Gene size (bp)	137,489 bp
Intron/exon numbers	44 exons
mRNA size (5′, ORF, 3′)	7,375 nt
Amino acid number	2,347 amino acids
kDa	~260 kD
Posttranslational modifications	30 potential N-linked (GlcNAc), Phosphorylation
Domains	9 FN-III, 3 YWTD modules, extracellular, transmembrane domain, kinase domain
Ligands	Unknown
Known dimerizing partners	N/A
Pathways activated	RAS/MEK/ERK, STAT3, PI3K-AKT-mTOR, VAV3, SHP-1/2
Tissues expressed	Mostly in lungs
Human diseases	Cancer, heart
Knockout mouse phenotype	Male sterility, lack of epididymal function

References

1. Balduzzi PC, Notter MF, Morgan HR, Shibuya M. Some biological properties of two new avian sarcoma viruses. J Virol. 1981;40(1):268–75.
2. Shibuya M, Hanafusa H, Balduzzi PC. Cellular sequences related to three new onc genes of avian sarcoma virus (fps, yes, and ros) and their expression in normal and transformed cells. J Virol. 1982;42(1):143–52.
3. Wang LH, Hanafusa H, Notter MF, Balduzzi PC. Genetic structure and transforming sequence of avian sarcoma virus UR2. J Virol. 1982;41(3):833–41.
4. Feldman RA, Wang LH, Hanafusa H, Balduzzi PC. Avian sarcoma virus UR2 encodes a transforming protein which is associated with a unique protein kinase activity. J Virol. 1982;42(1):228–36.
5. Macara IG, Marinetti GV, Balduzzi PC. Transforming protein of avian sarcoma virus UR2 is associated with phosphatidylinositol kinase activity: possible role in tumorigenesis. Proc Natl Acad Sci USA. 1984;81(9):2728–32.
6. Neckameyer WS, Wang LH. Molecular cloning and characterization of avian sarcoma virus UR2 and comparison of its transforming sequence with those of other avian sarcoma viruses. J Virol. 1984;50(3):914–21.
7. Notter MF, Balduzzi PC. Cytoskeletal changes induced by two avian sarcoma viruses: UR2 and Rous sarcoma virus. Virology. 1984;136(1):56–68.
8. Notter MF, Navon SE, Fung BK, Balduzzi PC. Infection of neuroretinal cells in vitro by avian sarcoma viruses UR1 and UR2: transformation, cell growth stimulation, and changes in transducin levels. Virology. 1987;160(2):489–93.
9. Maytin EV, Balduzzi PC, Notter MF, Young DA. Changes in the synthesis and phosphorylation of cellular proteins in chick fibroblasts transformed by two avian sarcoma viruses. J Biol Chem. 1984;259(19):12135–43.
10. Neckameyer WS, Wang LH. Nucleotide sequence of avian sarcoma virus UR2 and comparison of its transforming gene with other members of the tyrosine protein kinase oncogene family. J Virol. 1985;53(3):879–84.
11. Fasano O, Birnbaum D, Edlund L, Fogh J, Wigler M. New human transforming genes detected by a tumorigenicity assay. Mol Cell Biol. 1984;4(9):1695–705.

12. Birchmeier C, Birnbaum D, Waitches G, Fasano O, Wigler M. Characterization of an activated human ros gene. Mol Cell Biol. 1986;6(9):3109–16.
13. Shibuya M, Matsushime H, Yamazaki H, Wang LH, Fukui Y, Ueyama Y, et al. Analysis of structure and activation of some receptor-type tyrosine kinase oncogenes. Princess Takamatsu Symp. 1986;17:195–202.
14. Matsushime H, Wang LH, Shibuya M. Human c-ros-1 gene homologous to the v-ros sequence of UR2 sarcoma virus encodes for a transmembrane receptor like molecule. Mol Cell Biol. 1986;6(8):3000–4.
15. Neckameyer WS, Shibuya M, Hsu MT, Wang LH. Proto-oncogene c-ros codes for a molecule with structural features common to those of growth factor receptors and displays tissue specific and developmentally regulated expression. Mol Cell Biol. 1986;6(5):1478–86.
16. Podell SB, Sefton BM. Chicken proto-oncogene c-ros cDNA clones: identification of a c-ros RNA transcript and deduction of the amino acid sequence of the carboxyl terminus of the c-ros product. Oncogene. 1987;2(1):9–14.
17. Wang LH, Hanafusa H. Avian sarcoma viruses. Virus Res. 1988;9(2–3):159–203.
18. Birchmeier C, O'Neill K, Riggs M, Wigler M. Characterization of ROS1 cDNA from a human glioblastoma cell line. Proc Natl Acad Sci USA. 1990;87(12):4799–803.
19. Das KS, Christensen JR, Balduzzi PC. Transfection and recombination with molecularly cloned derivatives of avian sarcoma virus UR2. Virology. 1986;154(2):415–9.
20. Jong SM, Wang LH. Role of gag sequence in the biochemical properties and transforming activity of the avian sarcoma virus UR2-encoded gag-ros fusion protein. J Virol. 1990;64(12): 5997–6009.
21. Jong SM, Wang LH. Two point mutations in the transmembrane domain of P68gag-ros inactive its transforming activity and cause a delay in membrane association. J Virol. 1991; 65(1):180–9.
22. Jong SM, Zong CS, Dorai T, Wang LH. Transforming properties and substrate specificities of the protein tyrosine kinase oncogenes ros and src and their recombinants. J Virol. 1992; 66(8):4909–18.
23. Matsushime H, Shibuya M. Tissue-specific expression of rat c-ros-1 gene and partial structural similarity of its predicted products with sev protein of Drosophila melanogaster. J Virol. 1990;64(5):2117–25.
24. Chen JM, Heller D, Poon B, Kang L, Wang LH. The proto-oncogene c-ros codes for a transmembrane tyrosine protein kinase sharing sequence and structural homology with sevenless protein of Drosophila melanogaster. Oncogene. 1991;6(2):257–64.
25. Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. Oncogene. 2000;19(49):5548–57.
26. Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Berghe PV, Kamath RS, et al. Genome-wide RNAi of C. elegans using the hypersensitive rrf-3 strain reveals novel gene functions. PLoS Biol. 2003;1(1):E12.
27. Brenner S. The genetics of Caenorhabditis elegans. Genetics. 1974;77(1):71–94.
28. Page AP, Johnstone IL. The cuticle. WormBook. 2007;1–15.
29. Cox GN, Laufer JS, Kusch M, Edgar RS. Genetic and phenotypic characterization of roller mutants of Caenorhabditis elegans. Genetics. 1980;95(2):317–39.
30. Barbazuk WB, Johnsen RC, Baillie DL. The generation and genetic analysis of suppressors of lethal mutations in the Caenorhabditis elegans rol-3(V) gene. Genetics. 1994;136(1): 129–43.
31. Hunt-Newbury R, Viveiros R, Johnsen R, Mah A, Anastas D, Fang L, et al. High-throughput in vivo analysis of gene expression in Caenorhabditis elegans. PLoS Biol. 2007;5(9):e237.
32. Jones MR, Rose AM, Baillie DL. The ortholog of the human proto-oncogene ROS1 is required for epithelial development in C. elegans. Genesis. 2013;51(8):545–61.
33. Hafen E, Basler K, Edstroem JE, Rubin GM. Sevenless, a cell-specific homeotic gene of Drosophila, encodes a putative transmembrane receptor with a tyrosine kinase domain. Science. 1987;236(4797):55–63.

34. Price JV, Clifford RJ, Schupbach T. The maternal ventralizing locus torpedo is allelic to faint little ball, an embryonic lethal, and encodes the Drosophila EGF receptor homolog. Cell. 1989;56(6):1085–92.
35. Schejter ED, Shilo BZ. The Drosophila EGF receptor homolog (DER) gene is allelic to faint little ball, a locus essential for embryonic development. Cell. 1989;56(6):1093–104.
36. Bergmann A, Agapite J, McCall K, Steller H. The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling. Cell. 1998;95(3):331–41.
37. Freeman M. Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. Cell. 1996;87(4):651–60.
38. Kumar JP, Tio M, Hsiung F, Akopyan S, Gabay L, Seger R, et al. Dissecting the roles of the Drosophila EGF receptor in eye development and MAP kinase activation. Development. 1998;125(19):3875–85.
39. Kurada P, White K. Ras promotes cell survival in Drosophila by downregulating hid expression. Cell. 1998;95(3):319–29.
40. Spencer SA, Powell PA, Miller DT, Cagan RL. Regulation of EGF receptor signaling establishes pattern across the developing Drosophila retina. Development. 1998;125(23): 4777–90.
41. Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult Drosophila tissues. Development. 1993;117(4):1223–37.
42. Tomlinson A, Ready DF. Sevenless: a cell-specific homeotic mutation of the Drosophila eye. Science. 1986;231(4736):400–2.
43. Raabe T. The sevenless signaling pathway: variations of a common theme. Biochim Biophys Acta. 2000;1496(2–3):151–63.
44. Hart AC, Kramer H, Van Vactor Jr DL, Paidhungat M, Zipursky SL. Induction of cell fate in the Drosophila retina: the bride of sevenless protein is predicted to contain a large extracellular domain and seven transmembrane segments. Genes Dev. 1990;4(11):1835–47.
45. Kramer H, Cagan RL, Zipursky SL. Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine-kinase receptor. Nature. 1991;352(6332):207–12.
46. Reinke R, Zipursky SL. Cell-cell interaction in the Drosophila retina: the bride of sevenless gene is required in photoreceptor cell R8 for R7 cell development. Cell. 1988;55(2):321–30.
47. Chen J, Zong CS, Wang LH. Tissue and epithelial cell-specific expression of chicken protooncogene c-ros in several organs suggests that it may play roles in their development and mature functions. Oncogene. 1994;9(3):773–80.
48. Sonnenberg E, Godecke A, Walter B, Bladt F, Birchmeier C. Transient and locally restricted expression of the ros1 protooncogene during mouse development. EMBO J. 1991;10(12): 3693–702.
49. Tessarollo L, Nagarajan L, Parada LF. c-ros: the vertebrate homolog of the sevenless tyrosine kinase receptor is tightly regulated during organogenesis in mouse embryonic development. Development. 1992;115(1):11–20.
50. Acquaviva J, Wong R, Charest A. The multifaceted roles of the receptor tyrosine kinase ROS in development and cancer. Biochim Biophys Acta. 2009;1795(1):37–52.
51. Eddy EM. Male germ cell gene expression. Recent Prog Horm Res. 2002;57:103–28.
52. Kanwar YS, Liu ZZ, Kumar A, Wada J, Carone FA. Cloning of mouse c-ros renal cDNA, its role in development and relationship to extracellular matrix glycoproteins. Kidney Int. 1995;48(5):1646–59.
53. Saxen L. Organogenesis of the kidney. Cambridge: Cambridge University Press; 1987.
54. Liu ZZ, Wada J, Kumar A, Carone FA, Takahashi M, Kanwar YS. Comparative role of phosphotyrosine kinase domains of c-ros and c-ret protooncogenes in metanephric development with respect to growth factors and matrix morphogens. Dev Biol. 1996;178(1):133–48.
55. Sonnenberg-Riethmacher E, Walter B, Riethmacher D, Godecke S, Birchmeier C. The c-ros tyrosine kinase receptor controls regionalization and differentiation of epithelial cells in the epididymis. Genes Dev. 1996;10(10):1184–93.
56. Jun HJ, Roy J, Smith TB, Wood LB, Lane K, Woolfenden S, et al. ROS1 signaling regulates epithelial differentiation in the epididymis. Endocrinology. 2014;155(9):3661–73.

57. Bedford JM. Maturation, transport and fate of spermatozoa in the epididymis. In: Hamilton DW, Greep RO, editors. Handbook of physiology. Washington DC: American Physiological Society; 1975. p. 303–17.
58. Hinton BT. What does the epididymis do and how does it do it? In: Hinton BT, Robaire B, Prryor JL, Trasler JM, editors. Handbook of andrology. Lawrence, KS: American Society of Andrology; 1995. p. 1–5.
59. Orgebin-Crist M-C, Danzo BJ, Davies J. Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. In: Hamilton DW, Greep RO, editors. Handbook of physiology. Washington DC: American Physiological Society; 1975. p. 319–38.
60. Cornwall GA. New insights into epididymal biology and function. Hum Reprod Update. 2009;15(2):213–27.
61. Robaire B, Syntin P, Jervis K. The coming of age of the epididymis. In: Jegou B, editor. Testis, epididymis and technologies in the year 2000. New York: Springer; 2000. p. 229–62.
62. Da Silva N, Silberstein C, Beaulieu V, Pietrement C, Van Hoek AN, Brown D, et al. Postnatal expression of aquaporins in epithelial cells of the rat epididymis. Biol Reprod. 2006;74(2): 427–38.
63. Hermo L, Barin K, Robaire B. Structural differentiation of the epithelial cells of the testicular excurrent duct system of rats during postnatal development. Anat Rec. 1992;233(2):205–28.
64. Rodriguez CM, Kirby JL, Hinton BT. The development of the epididymis. In: Robaire B, Hinton BT, editors. The epididymis: from molecules to clinical practice. New York: Kluwer Academic/Plenum; 2002. p. 251–67.
65. Shum WW, Hill E, Brown D, Breton S. Plasticity of basal cells during postnatal development in the rat epididymis. Reproduction. 2013;146(5):455–69.
66. Sun EL, Flickinger CJ. Development of cell types and of regional differences in the postnatal rat epididymis. Am J Anat. 1979;154(1):27–55.
67. Kirchhoff C. Gene expression in the epididymis. Int Rev Cytol. 1999;188:133–202.
68. Krutskikh A, De Gendt K, Sharp V, Verhoeven G, Poutanen M, Huhtaniemi I. Targeted inactivation of the androgen receptor gene in murine proximal epididymis causes epithelial hypotrophy and obstructive azoospermia. Endocrinology. 2011;152(2):689–96.
69. Bjorkgren I, Saastamoinen L, Krutskikh A, Huhtaniemi I, Poutanen M, Sipila P. Dicer1 ablation in the mouse epididymis causes dedifferentiation of the epithelium and imbalance in sex steroid signaling. PLoS One. 2012;7(6):e38457.
70. Hoshii T, Takeo T, Nakagata N, Takeya M, Araki K, Yamamura K. LGR4 regulates the postnatal development and integrity of male reproductive tracts in mice. Biol Reprod. 2007; 76(2):303–13.
71. McPhaul MJ. Androgen receptor mutations and androgen insensitivity. Mol Cell Endocrinol. 2002;198(1–2):61–7.
72. Mendive F, Laurent P, Van Schoore G, Skarnes W, Pochet R, Vassart G. Defective postnatal development of the male reproductive tract in LGR4 knockout mice. Dev Biol. 2006; 290(2):421–34.
73. Murashima A, Miyagawa S, Ogino Y, Nishida-Fukuda H, Araki K, Matsumoto T, et al. Essential roles of androgen signaling in Wolffian duct stabilization and epididymal cell differentiation. Endocrinology. 2011;152(4):1640–51.
74. O'Hara L, Welsh M, Saunders PT, Smith LB. Androgen receptor expression in the caput epididymal epithelium is essential for development of the initial segment and epididymal spermatozoa transit. Endocrinology. 2011;152(2):718–29.
75. Sipila P, Cooper TG, Yeung CH, Mustonen M, Penttinen J, Drevet J, et al. Epididymal dysfunction initiated by the expression of simian virus 40 T-antigen leads to angulated sperm flagella and infertility in transgenic mice. Mol Endocrinol. 2002;16(11):2603–17.
76. Shum WW, Ruan YC, Da Silva N, Breton S. Establishment of cell-cell cross talk in the epididymis: control of luminal acidification. J Androl. 2011;32(6):576–86.

77. Britan A, Lareyre JJ, Lefrancois-Martinez AM, Manin M, Schwaab V, Greiffeuille V, et al. Spontaneously immortalized epithelial cells from mouse caput epididymidis. Mol Cell Endocrinol. 2004;224(1–2):41–53.
78. Cooper TG. Sperm maturation in the epididymis: a new look at an old problem. Asian J Androl. 2007;9(4):533–9.
79. Cooper TG, Wagenfeld A, Cornwall GA, Hsia N, Chu ST, Orgebin-Crist MC, et al. Gene and protein expression in the epididymis of infertile c-ros receptor tyrosine kinase-deficient mice. Biol Reprod. 2003;69(5):1750–62.
80. Cooper TG, Yeung CH, Wagenfeld A, Nieschlag E, Poutanen M, Huhtaniemi I, et al. Mouse models of infertility due to swollen spermatozoa. Mol Cell Endocrinol. 2004;216(1–2): 55–63.
81. Yeung CH, Anapolski M, Setiawan I, Lang F, Cooper TG. Effects of putative epididymal osmolytes on sperm volume regulation of fertile and infertile c-ros transgenic mice. J Androl. 2004;25(2):216–23.
82. Yeung CH, Breton S, Setiawan I, Xu Y, Lang F, Cooper TG. Increased luminal pH in the epididymis of infertile c-ros knockout mice and the expression of sodium-hydrogen exchangers and vacuolar proton pump H+-ATPase. Mol Reprod Dev. 2004;68(2):159–68.
83. Yeung CH, Cooper TG. Developmental changes in signalling transduction factors in maturing sperm during epididymal transit. Cell Mol Biol (Noisy-le-grand). 2003;49(3):341–9.
84. Legare C, Sullivan R. Expression and localization of c-ros oncogene along the human excurrent duct. Mol Hum Reprod. 2004;10(9):697–703.
85. Shyamsundar R, Kim YH, Higgins JP, Montgomery K, Jorden M, Sethuraman A, et al. A DNA microarray survey of gene expression in normal human tissues. Genome Biol. 2005;6(3):R22.
86. Sholl LM, Sun H, Butaney M, Zhang C, Lee C, Janne PA, et al. ROS1 immunohistochemistry for detection of ROS1-rearranged lung adenocarcinomas. Am J Surg Pathol. 2013; 37(9):1441–9.
87. Birchmeier C, Sharma S, Wigler M. Expression and rearrangement of the ROS1 gene in human glioblastoma cells. Proc Natl Acad Sci USA. 1987;84(24):9270–4.
88. Mapstone T, McMichael M, Goldthwait D. Expression of platelet-derived growth factors, transforming growth factors, and the ros gene in a variety of primary human brain tumors. Neurosurgery. 1991;28(2):216–22.
89. Watkins D, Dion F, Poisson M, Delattre JY, Rouleau GA. Analysis of oncogene expression in primary human gliomas: evidence for increased expression of the ros oncogene. Cancer Genet Cytogenet. 1994;72(2):130–6.
90. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. Cell. 2013;155(2):462–77.
91. Zhao JF, Sharma S. Expression of the ROS1 oncogene for tyrosine receptor kinase in adult human meningiomas. Cancer Genet Cytogenet. 1995;83(2):148–54.
92. Girish V, Sachdeva N, Minz RW, Radotra B, Mathuria SN, Arora SK. Bcl2 and ROS1 expression in human meningiomas: an analysis with respect to histological subtype. Indian J Pathol Microbiol. 2005;48(3):325–30.
93. Jun HJ, Woolfenden S, Coven S, Lane K, Bronson R, Housman D, et al. Epigenetic regulation of c-ROS receptor tyrosine kinase expression in malignant gliomas. Cancer Res. 2009; 69(6):2180–4.
94. Bonner AE, Lemon WJ, Devereux TR, Lubet RA, You M. Molecular profiling of mouse lung tumors: association with tumor progression, lung development, and human lung adenocarcinomas. Oncogene. 2004;23(5):1166–76.
95. Sweet-Cordero A, Mukherjee S, Subramanian A, You H, Roix JJ, Ladd-Acosta C, et al. An oncogenic KRAS2 expression signature identified by cross-species gene-expression analysis. Nat Genet. 2005;37(1):48–55.
96. Sweet-Cordero A, Tseng GC, You H, Douglass M, Huey B, Albertson D, et al. Comparison of gene expression and DNA copy number changes in a murine model of lung cancer. Genes Chromosomes Cancer. 2006;45(4):338–48.

97. Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, et al. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc Natl Acad Sci USA. 2001;98(24):13790–5.
98. Bild AH, Yao G, Chang JT, Wang Q, Potti A, Chasse D, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature. 2006;439(7074):353–7.
99. Garber ME, Troyanskaya OG, Schluens K, Petersen S, Thaesler Z, Pacyna-Gengelbach M, et al. Diversity of gene expression in adenocarcinoma of the lung. Proc Natl Acad Sci USA. 2001;98(24):13784–9.
100. Lee HJ, Seol HS, Kim JY, Chun SM, Suh YA, Park YS, et al. ROS1 receptor tyrosine kinase, a druggable target, is frequently overexpressed in non-small cell lung carcinomas via genetic and epigenetic mechanisms. Ann Surg Oncol. 2012;20(1):200–8.
101. Lan Q, Hsiung CA, Matsuo K, Hong YC, Seow A, Wang Z, et al. Genome-wide association analysis identifies new lung cancer susceptibility loci in never-smoking women in Asia. Nat Genet. 2012;44(12):1330–5.
102. Cilloni D, Carturan S, Bracco E, Campia V, Rosso V, Torti D, et al. Aberrant activation of ROS1 represents a new molecular defect in chronic myelomonocytic leukemia. Leuk Res. 2013;37(5):520–30.
103. Eom M, Han A, Yi SY, Shin JJ, Cui Y, Park KH. RHEB expression in fibroadenomas of the breast. Pathol Int. 2008;58(4):226–32.
104. Eom M, Lkhagvadorj S, Oh SS, Han A, Park KH. ROS1 expression in invasive ductal carcinoma of the breast related to proliferation activity. Yonsei Med J. 2013;54(3):650–7.
105. Yamashita S, Nomoto T, Abe M, Tatematsu M, Sugimura T, Ushijima T. Persistence of gene expression changes in stomach mucosae induced by short-term N-methyl-N′-nitro-N-nitrosoguanidine treatment and their presence in stomach cancers. Mutat Res. 2004; 549(1–2):185–93.
106. Yovchev MI, Grozdanov PN, Joseph B, Gupta S, Dabeva MD. Novel hepatic progenitor cell surface markers in the adult rat liver. Hepatology. 2007;45(1):139–49.
107. Yovchev MI, Grozdanov PN, Zhou H, Racherla H, Guha C, Dabeva MD. Identification of adult hepatic progenitor cells capable of repopulating injured rat liver. Hepatology. 2008;47(2):636–47.
108. Ruhe JE, Streit S, Hart S, Wong CH, Specht K, Knyazev P, et al. Genetic alterations in the tyrosine kinase transcriptome of human cancer cell lines. Cancer Res. 2007;67(23):11368–76.
109. Nagarajan L, Louie E, Tsujimoto Y, Balduzzi PC, Huebner K, Croce CM. The human c-ros gene (ROS) is located at chromosome region 6q16–6q22. Proc Natl Acad Sci USA. 1986;83(17):6568–72.
110. Rabin M, Birnbaum D, Young D, Birchmeier C, Wigler M, Ruddle FH. Human ros1 and mas1 oncogenes located in regions of chromosome 6 associated with tumor-specific rearrangements. Oncogene Res. 1987;1(2):169–78.
111. Satoh H, Yoshida MC, Matsushime H, Shibuya M, Sasaki M. Regional localization of the human c-ros-1 on 6q22 and flt on 13q12. Jpn J Cancer Res. 1987;78(8):772–5.
112. Shiffman D, Ellis SG, Rowland CM, Malloy MJ, Luke MM, Iakoubova OA, et al. Identification of four gene variants associated with myocardial infarction. Am J Hum Genet. 2005;77(4): 596–605.
113. Zee RY, Michaud SE, Hegener HH, Diehl KA, Ridker PM. A prospective replication study of five gene variants previously associated with risk of myocardial infarction. J Thromb Haemost. 2006;4(9):2093–5.
114. Horne BD, Carlquist JF, Muhlestein JB, Nicholas ZP, Anderson JL. Associations with myocardial infarction of six polymorphisms selected from a three-stage genome-wide association study. Am Heart J. 2007;154(5):969–75.
115. Oguri M, Kato K, Hibino T, Yokoi K, Segawa T, Matsuo H, et al. Genetic risk for restenosis after coronary stenting. Atherosclerosis. 2007;194(2):e172–8.
116. Yamada Y, Kato K, Yoshida T, Yokoi K, Matsuo H, Watanabe S, et al. Association of polymorphisms of ABCA1 and ROS1 with hypertension in Japanese individuals. Int J Mol Med. 2008;21(1):83–9.

117. Charest A, Lane K, McMahon K, Park J, Preisinger E, Conroy H, et al. Fusion of FIG to the receptor tyrosine kinase ROS in a glioblastoma with an interstitial del(6)(q21q21). Genes Chromosomes Cancer. 2003;37(1):58–71.
118. Ruoslahti E. Fibronectin and its receptors. Annu Rev Biochem. 1988;57:375–413.
119. Bork P, Downing AK, Kieffer B, Campbell ID. Structure and distribution of modules in extracellular proteins. Q Rev Biophys. 1996;29(2):119–67.
120. Springer TA. An extracellular beta-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components. J Mol Biol. 1998;283(4):837–62.
121. McKern NM, Lawrence MC, Streltsov VA, Lou MZ, Adams TE, Lovrecz GO, et al. Structure of the insulin receptor ectodomain reveals a folded-over conformation. Nature. 2006;443(7108):218–21.
122. Ward C, Lawrence M, Streltsov V, Garrett T, McKern N, Lou MZ, et al. Structural insights into ligand-induced activation of the insulin receptor. Acta Physiol (Oxf). 2008;192(1):3–9.
123. Carvalho RF, Beutler M, Marler KJ, Knoll B, Becker-Barroso E, Heintzmann R, et al. Silencing of EphA3 through a cis interaction with ephrinA5. Nat Neurosci. 2006;9(3): 322–30.
124. Riethmacher D, Langholz O, Godecke S, Sachs M, Birchmeier C. Biochemical and functional characterization of the murine ros protooncogene. Oncogene. 1994;9(12):3617–26.
125. Ellis L, Morgan DO, Jong SM, Wang LH, Roth RA, Rutter WJ. Heterologous transmembrane signaling by a human insulin receptor-v-ros hybrid in Chinese hamster ovary cells. Proc Natl Acad Sci USA. 1987;84(15):5101–5.
126. Xiong Q, Chan JL, Zong CS, Wang LH. Two chimeric receptors of epidermal growth factor receptor and c-Ros that differ in their transmembrane domains have opposite effects on cell growth. Mol Cell Biol. 1996;16(4):1509–18.
127. Sachs M, Weidner KM, Brinkmann V, Walther I, Obermeier A, Ullrich A, et al. Motogenic and morphogenic activity of epithelial receptor tyrosine kinases. J Cell Biol. 1996;133(5): 1095–107.
128. Awad MM, Engelman JA, Shaw AT. Acquired resistance to crizotinib from a mutation in CD74-ROS1. N Engl J Med. 2013;369(12):1173.
129. Garber EA, Hanafusa T, Hanafusa H. Membrane association of the transforming protein of avian sarcoma virus UR2 and mutants temperature sensitive for cellular transformation and protein kinase activity. J Virol. 1985;56(3):790–7.
130. Chan JL, Lai M, Wang LH. Effect of dimerization on signal transduction and biological function of oncogenic Ros, insulin, and insulin-like growth factor I receptors. J Biol Chem. 1997;272(1):146–53.
131. Zong CS, Wang LH. Modulatory effect of the transmembrane domain of the protein-tyrosine kinase encoded by oncogene ros: biological function and substrate interaction. Proc Natl Acad Sci USA. 1994;91(23):10982–6.
132. Zong CS, Poon B, Chen J, Wang LH. Molecular and biochemical bases for activation of the transforming potential of the proto-oncogene c-ros. J Virol. 1993;67(11):6453–62.
133. Charest A, Lane K, McMahon K, Housman DE. Association of a novel PDZ domain-containing peripheral Golgi protein with the Q-SNARE (Q-soluble N fusion protein (NSF) attachment protein receptor) protein syntaxin 6. J Biol Chem. 2001;276(31):29456–65.
134. Charest A, Kheifets V, Park J, Lane K, McMahon K, Nutt CL, et al. Oncogenic targeting of an activated tyrosine kinase to the Golgi apparatus in a glioblastoma. Proc Natl Acad Sci USA. 2003;100(3):916–21.
135. Charest A, Wilker EW, McLaughlin ME, Lane K, Gowda R, Coven S, et al. ROS fusion tyrosine kinase activates a SH2 domain-containing phosphatase-2/phosphatidylinositol 3-kinase/mammalian target of rapamycin signaling axis to form glioblastoma in mice. Cancer Res. 2006;66(15):7473–81.
136. Gu TL, Deng X, Huang F, Tucker M, Crosby K, Rimkunas V, et al. Survey of tyrosine kinase signaling reveals ROS kinase fusions in human cholangiocarcinoma. PLoS One. 2011; 6(1):e15640.

137. Suehara Y, Arcila M, Wang L, Hasanovic A, Ang D, Ito T, et al. Identification of KIF5B-RET and GOPC-ROS1 fusions in lung adenocarcinomas through a comprehensive mRNA-based screen for tyrosine kinase fusions. Clin Cancer Res. 2012;18(24):6599–608.
138. Cooper WA, Lam DC, O'Toole SA, Minna JD. Molecular biology of lung cancer. J Thorac Dis. 2013;5 Suppl 5:S479–S90.
139. Shames DS, Wistuba II. The evolving genomic classification of lung cancer. J Pathol. 2014;232(2):121–33.
140. Sequist LV. ROS1-targeted therapy in non-small cell lung cancer. Clin Adv Hematol Oncol. 2012;10(12):827–8.
141. Stumpfova M, Janne PA. Zeroing in on ROS1 rearrangements in non-small cell lung cancer. Clin Cancer Res. 2012;18(16):4222–4.
142. Rimkunas VM, Crosby KE, Li D, Hu Y, Kelly ME, Gu TL, et al. Analysis of receptor tyrosine kinase ROS1-positive tumors in non-small cell lung cancer: identification of a FIG-ROS1 fusion. Clin Cancer Res. 2012;18(16):4449–57.
143. Birch AH, Arcand SL, Oros KK, Rahimi K, Watters AK, Provencher D, et al. Chromosome 3 anomalies investigated by genome wide SNP analysis of benign, low malignant potential and low grade ovarian serous tumours. PLoS One. 2011;6(12):e28250.
144. Govindan R, Ding L, Griffith M, Subramanian J, Dees ND, Kanchi KL, et al. Genomic landscape of non-small cell lung cancer in smokers and never-smokers. Cell. 2012;150(6): 1121–34.
145. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell. 2007;131(6):1190–203.
146. Rimkunas V, Crosby K, Silver M, Hincman K, Kelly M, Li D, et al. Frequencies of ALK and ROS in NSCLC FFPE tumor samples utilizing a highly specific and sensitive immunohistochemistry-based assay and FISH analysis. J Clin Oncol. 2010;28(15):10536.
147. Seo JS, Ju YS, Lee WC, Shin JY, Lee JK, Bleazard T, et al. The transcriptional landscape and mutational profile of lung adenocarcinoma. Genome Res. 2012;22(11):2109–19.
148. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, et al. RET, ROS1 and ALK fusions in lung cancer. Nat Med. 2012;18(3):378–81.
149. Shaw AT, Ou SH, Bang YJ, Camidge DR, Solomon BJ, Salgia R, et al. Crizotinib in ROS1-rearranged non-small-cell lung cancer. N Engl J Med. 2014;27.
150. Lee J, Lee SE, Kang SY, Do IG, Lee S, Ha SY, et al. Identification of ROS1 rearrangement in gastric adenocarcinoma. Cancer. 2013;119(9):1627–35.
151. Aisner DL, Nguyen TT, Paskulin DD, Le AT, Haney J, Schulte N, et al. ROS1 and ALK fusions in colorectal cancer, with evidence of intratumoral heterogeneity for molecular drivers. Mol Cancer Res. 2013;12(1):111–8.
152. Wiesner T, He J, Yelensky R, Esteve-Puig R, Botton T, Yeh I, et al. Kinase fusions are frequent in Spitz tumours and spitzoid melanomas. Nat Commun. 2014;5:3116.
153. Giacomini CP, Sun S, Varma S, Shain AH, Giacomini MM, Balagtas J, et al. Breakpoint analysis of transcriptional and genomic profiles uncovers novel gene fusions spanning multiple human cancer types. PLoS Genet. 2013;9(4):e1003464.
154. Stransky N, Cerami E, Schalm S, Kim JL, Lengauer C. The landscape of kinase fusions in cancer. Nat Commun. 2014;5:4846.
155. Lovly CM, Gupta A, Lipson D, Otto G, Brennan T, Chung CT, et al. Inflammatory myofibroblastic tumors harbor multiple potentially actionable kinase fusions. Cancer Discov. 2014; 4(8):889–95.
156. Lovly CM, Lipson D, Otto G, Brennan T, Sankar S, Stephens PJ, et al. Potentially actionable kinase fusions in inflammatory myofibroblastic tumors. ASCO Meeting Abstracts. 2013; 31(15):10513.
157. Murer H, Forster I, Biber J. The sodium phosphate cotransporter family SLC34. Pflugers Arch. 2004;447(5):763–7.
158. Lambert G, Traebert M, Hernando N, Biber J, Murer H. Studies on the topology of the renal type II NaPi-cotransporter. Pflugers Arch. 1999;437(6):972–8.

159. Hilfiker H, Hattenhauer O, Traebert M, Forster I, Murer H, Biber J. Characterization of a murine type II sodium-phosphate cotransporter expressed in mammalian small intestine. Proc Natl Acad Sci USA. 1998;95(24):14564–9.
160. Feild JA, Zhang L, Brun KA, Brooks DP, Edwards RM. Cloning and functional characterization of a sodium-dependent phosphate transporter expressed in human lung and small intestine. Biochem Biophys Res Commun. 1999;258(3):578–82.
161. Xu H, Collins JF, Bai L, Kiela PR, Ghishan FK. Regulation of the human sodium-phosphate cotransporter NaP(i)-IIb gene promoter by epidermal growth factor. Am J Physiol Cell Physiol. 2001;280(3):C628–36.
162. Traebert M, Hattenhauer O, Murer H, Kaissling B, Biber J. Expression of type II Na-P(i) cotransporter in alveolar type II cells. Am J Physiol. 1999;277(5 Pt 1):L868–73.
163. Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, et al. MIF signal transduction initiated by binding to CD74. J Exp Med. 2003;197(11):1467–76.
164. Badve S, Deshpande C, Hua Z, Logdberg L. Expression of invariant chain (CD 74) and major histocompatibility complex (MHC) class II antigens in the human fetus. J Histochem Cytochem. 2002;50(4):473–82.
165. Lazova R, Moynes R, May D, Scott G. LN-2 (CD74). A marker to distinguish atypical fibroxanthoma from malignant fibrous histiocytoma. Cancer. 1997;79(11):2115–24.
166. Ong GL, Goldenberg DM, Hansen HJ, Mattes MJ. Cell surface expression and metabolism of major histocompatibility complex class II invariant chain (CD74) by diverse cell lines. Immunology. 1999;98(2):296–302.
167. Datta MW, Shahsafaei A, Nadler LM, Freeman GJ, Dorfman DM. Expression of MHC class II-associated invariant chain (Ii;CD74) in thymic epithelial neoplasms. Appl Immunohistochem Mol Morphol. 2000;8(3):210–5.
168. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Iwashige H, Aridome K, et al. Invariant chain expression in gastric cancer. Cancer Lett. 2001;168(1):87–91.
169. Young AN, Amin MB, Moreno CS, Lim SD, Cohen C, Petros JA, et al. Expression profiling of renal epithelial neoplasms: a method for tumor classification and discovery of diagnostic molecular markers. Am J Pathol. 2001;158(5):1639–51.
170. Burton JD, Ely S, Reddy PK, Stein R, Gold DV, Cardillo TM, et al. CD74 is expressed by multiple myeloma and is a promising target for therapy. Clin Cancer Res. 2004;10(19):6606–11.
171. Hustinx SR, Cao D, Maitra A, Sato N, Martin ST, Sudhir D, et al. Differentially expressed genes in pancreatic ductal adenocarcinomas identified through serial analysis of gene expression. Cancer Biol Ther. 2004;3(12):1254–61.
172. Rangel LB, Agarwal R, Sherman-Baust CA, Mello-Coelho V, Pizer ES, Ji H, et al. Anomalous expression of the HLA-DR alpha and beta chains in ovarian and other cancers. Cancer Biol Ther. 2004;3(10):1021–7.
173. Lees JG, Bach CT, O'Neill GM. Interior decoration: tropomyosin in actin dynamics and cell migration. Cell Adh Migr. 2011;5(2):181–6.
174. Beimfohr C, Klugbauer S, Demidchik EP, Lengfelder E, Rabes HM. NTRK1 re-arrangement in papillary thyroid carcinomas of children after the Chernobyl reactor accident. Int J Cancer. 1999;80(6):842–7.
175. Lamant L, Dastugue N, Pulford K, Delsol G, Mariame B. A new fusion gene TPM3-ALK in anaplastic large cell lymphoma created by a (1;2)(q25;p23) translocation. Blood. 1999;93(9):3088–95.
176. Rosati R, La Starza R, Luciano L, Gorello P, Matteucci C, Pierini V, et al. TPM3/PDGFRB fusion transcript and its reciprocal in chronic eosinophilic leukemia. Leukemia. 2006;20(9):1623–4.
177. Multhaupt HA, Yoneda A, Whiteford JR, Oh ES, Lee W, Couchman JR. Syndecan signaling: when, where and why? J Physiol Pharmacol. 2009;60 Suppl 4:31–8.
178. Simons M, Horowitz A. Syndecan-4-mediated signalling. Cell Signal. 2001;13(12):855–62.

179. Guo D, Holmlund C, Henriksson R, Hedman H. The LRIG gene family has three vertebrate paralogs widely expressed in human and mouse tissues and a homolog in Ascidiacea. Genomics. 2004;84(1):157–65.
180. Neisch AL, Fehon RG. Ezrin, Radixin and Moesin: key regulators of membrane-cortex interactions and signaling. Curr Opin Cell Biol. 2011;23(4):377–82.
181. Bartkova J, Rajpert-De Meyts E, Skakkebaek NE, Lukas J, Bartek J. DNA damage response in human testes and testicular germ cell tumours: biology and implications for therapy. Int J Androl. 2007;30(4):282–91. Discussion 91.
182. Celetti A, Cerrato A, Merolla F, Vitagliano D, Vecchio G, Grieco M. H4(D10S170), a gene frequently rearranged with RET in papillary thyroid carcinomas: functional characterization. Oncogene. 2004;23(1):109–21.
183. Merolla F, Pentimalli F, Pacelli R, Vecchio G, Fusco A, Grieco M, et al. Involvement of H4(D10S170) protein in ATM-dependent response to DNA damage. Oncogene. 2007;26(42): 6167–75.
184. Merolla F, Luise C, Muller MT, Pacelli R, Fusco A, Celetti A. Loss of CCDC6, the first identified RET partner gene, affects pH2AX S139 levels and accelerates mitotic entry upon DNA damage. PLoS One. 2012;7(5):e36177.
185. Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, et al. PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell. 1990;60(4):557–63.
186. Schwaller J, Anastasiadou E, Cain D, Kutok J, Wojiski S, Williams IR, et al. H4(D10S170), a gene frequently rearranged in papillary thyroid carcinoma, is fused to the platelet-derived growth factor receptor beta gene in atypical chronic myeloid leukemia with t(5;10)(q33;q22). Blood. 2001;97(12):3910–8.
187. Kulkarni S, Heath C, Parker S, Chase A, Iqbal S, Pocock CF, et al. Fusion of H4/D10S170 to the platelet-derived growth factor receptor beta in BCR-ABL-negative myeloproliferative disorders with a t(5;10)(q33;q21). Cancer Res. 2000;60(13):3592–8.
188. Puxeddu E, Knauf JA, Sartor MA, Mitsutake N, Smith EP, Medvedovic M, et al. RET/PTC-induced gene expression in thyroid PCCL3 cells reveals early activation of genes involved in regulation of the immune response. Endocr Relat Cancer. 2005;12(2):319–34.
189. Scanlan MJ, Gout I, Gordon CM, Williamson B, Stockert E, Gure AO, et al. Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression. Cancer Immun. 2001;1:4.
190. Chmielecki J, Peifer M, Viale A, Hutchinson K, Giltnane J, Socci ND, et al. Systematic screen for tyrosine kinase rearrangements identifies a novel C6orf204-PDGFRB fusion in a patient with recurrent T-ALL and an associated myeloproliferative neoplasm. Genes Chromosomes Cancer. 2012;51(1):54–65.
191. Berg D, Holzmann C, Riess O. 14-3-3 proteins in the nervous system. Nat Rev Neurosci. 2003;4(9):752–62.
192. Witte K, Schuh AL, Hegermann J, Sarkeshik A, Mayers JR, Schwarze K, et al. TFG-1 function in protein secretion and oncogenesis. Nat Cell Biol. 2011;13(5):550–8.
193. Clucas J, Valderrama F. ERM proteins in cancer progression. J Cell Sci. 2014;127(Pt 2): 267–75.
194. Maul RS, Song Y, Amann KJ, Gerbin SC, Pollard TD, Chang DD. EPLIN regulates actin dynamics by cross-linking and stabilizing filaments. J Cell Biol. 2003;160(3):399–407.
195. Norrmen C, Vandevelde W, Ny A, Saharinen P, Gentile M, Haraldsen G, et al. Liprin (beta)1 is highly expressed in lymphatic vasculature and is important for lymphatic vessel integrity. Blood. 2010;115(4):906–9.
196. Takeuchi K, Soda M, Togashi Y, Sugawara E, Hatano S, Asaka R, et al. Pulmonary inflammatory myofibroblastic tumor expressing a novel fusion, PPFIBP1-ALK: reappraisal of anti-ALK immunohistochemistry as a tool for novel ALK fusion identification. Clin Cancer Res. 2011;17(10):3341–8.

197. Nakata T, Kitamura Y, Shimizu K, Tanaka S, Fujimori M, Yokoyama S, et al. Fusion of a novel gene, ELKS, to RET due to translocation t(10;12)(q11;p13) in a papillary thyroid carcinoma. Genes Chromosomes Cancer. 1999;25(2):97–103.
198. Gorello P, La Starza R, Brandimarte L, Trisolini SM, Pierini V, Crescenzi B, et al. A PDGFRB-positive acute myeloid malignancy with a new t(5;12)(q33;p13.3) involving the ERC1 gene. Leukemia. 2008;22(1):216–8.
199. Galjart N. CLIPs and CLASPs and cellular dynamics. Nat Rev Mol Cell Biol. 2005;6(6): 487–98.
200. Ross JS, Wang K, Gay L, Al-Rohil R, Rand JV, Jones DM, et al. New routes to targeted therapy of intrahepatic cholangiocarcinomas revealed by next-generation sequencing. Oncologist. 2014;19(3):235–42.
201. Gustafson MP, Welcker M, Hwang HC, Clurman BE. Zcchc8 is a glycogen synthase kinase-3 substrate that interacts with RNA-binding proteins. Biochem Biophys Res Commun. 2005;338(3):1359–67.
202. Jun HJ, Johnson H, Bronson RT, de Feraudy S, White F, Charest A. The oncogenic lung cancer fusion kinase CD74-ROS activates a novel invasiveness pathway through E-Syt1 phosphorylation. Cancer Res. 2012;72(15):3764–74.
203. Zeng L, Sachdev P, Yan L, Chan JL, Trenkle T, McClelland M, et al. Vav3 mediates receptor protein tyrosine kinase signaling, regulates GTPase activity, modulates cell morphology, and induces cell transformation. Mol Cell Biol. 2000;20(24):9212–24.
204. Zong CS, Chan JL, Yang SK, Wang LH. Mutations of Ros differentially effecting signal transduction pathways leading to cell growth versus transformation. J Biol Chem. 1997; 272(3):1500–6.
205. Keilhack H, Muller M, Bohmer SA, Frank C, Weidner KM, Birchmeier W, et al. Negative regulation of Ros receptor tyrosine kinase signaling. An epithelial function of the SH2 domain protein tyrosine phosphatase SHP-1. J Cell Biol. 2001;152(2):325–34.
206. Bignon JS, Siminovitch KA. Identification of PTP1C mutation as the genetic defect in motheaten and viable motheaten mice: a step toward defining the roles of protein tyrosine phosphatases in the regulation of hemopoietic cell differentiation and function. Clin Immunol Immunopathol. 1994;73(2):168–79.
207. Kozlowski M, Mlinaric-Rascan I, Feng GS, Shen R, Pawson T, Siminovitch KA. Expression and catalytic activity of the tyrosine phosphatase PTP1C is severely impaired in motheaten and viable motheaten mice. J Exp Med. 1993;178(6):2157–63.
208. Neel BG, Gu H, Pao L. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem Sci. 2003;28(6):284–93.
209. Wang N, Li Z, Ding R, Frank GD, Senbonmatsu T, Landon EJ, et al. Antagonism or synergism. Role of tyrosine phosphatases SHP-1 and SHP-2 in growth factor signaling. J Biol Chem. 2006;281(31):21878–83.
210. Choong K, Freedman MH, Chitayat D, Kelly EN, Taylor G, Zipursky A. Juvenile myelomonocytic leukemia and Noonan syndrome. J Pediatr Hematol Oncol. 1999;21(6):523–7.
211. Tartaglia M, Gelb BD. Noonan syndrome and related disorders: genetics and pathogenesis. Annu Rev Genomics Hum Genet. 2005;6:45–68.
212. Loh ML, Vattikuti S, Schubbert S, Reynolds MG, Carlson E, Lieuw KH, et al. Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis. Blood. 2004;103(6): 2325–31.
213. Tartaglia M, Martinelli S, Cazzaniga G, Cordeddu V, Iavarone I, Spinelli M, et al. Genetic evidence for lineage-related and differentiation stage-related contribution of somatic PTPN11 mutations to leukemogenesis in childhood acute leukemia. Blood. 2004;104(2):307–13.
214. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet. 2003;34(2):148–50.
215. Tartaglia M, Niemeyer CM, Shannon KM, Loh ML. SHP-2 and myeloid malignancies. Curr Opin Hematol. 2004;11(1):44–50.

216. Bentires-Alj M, Paez JG, David FS, Keilhack H, Halmos B, Naoki K, et al. Activating mutations of the noonan syndrome-associated SHP2/PTPN11 gene in human solid tumors and adult acute myelogenous leukemia. Cancer Res. 2004;64(24):8816–20.
217. Mohi MG, Williams IR, Dearolf CR, Chan G, Kutok JL, Cohen S, et al. Prognostic, therapeutic, and mechanistic implications of a mouse model of leukemia evoked by Shp2 (PTPN11) mutations. Cancer Cell. 2005;7(2):179–91.
218. Bennett AM, Hausdorff SF, O'Reilly AM, Freeman RM, Neel BG. Multiple requirements for SHPTP2 in epidermal growth factor-mediated cell cycle progression. Mol Cell Biol. 1996;16(3):1189–202.
219. Noguchi T, Matozaki T, Horita K, Fujioka Y, Kasuga M. Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. Mol Cell Biol. 1994;14(10):6674–82.
220. Saxton TM, Ciruna BG, Holmyard D, Kulkarni S, Harpal K, Rossant J, et al. The SH2 tyrosine phosphatase shp2 is required for mammalian limb development. Nat Genet. 2000; 24(4):420–3.
221. Shi ZQ, Yu DH, Park M, Marshall M, Feng GS. Molecular mechanism for the Shp-2 tyrosine phosphatase function in promoting growth factor stimulation of Erk activity. Mol Cell Biol. 2000;20(5):1526–36.
222. Yamauchi K, Milarski KL, Saltiel AR, Pessin JE. Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling. Proc Natl Acad Sci USA. 1995;92(3):664–8.
223. Zhan Y, O'Rourke DM. SHP-2-dependent mitogen-activated protein kinase activation regulates EGFRvIII but not wild-type epidermal growth factor receptor phosphorylation and glioblastoma cell survival. Cancer Res. 2004;64(22):8292–8.
224. Barford D, Neel BG. Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2. Structure. 1998;6(3):249–54.
225. Neel BG, Tonks NK. Protein tyrosine phosphatases in signal transduction. Curr Opin Cell Biol. 1997;9(2):193–204.
226. Jarvis LA, Toering SJ, Simon MA, Krasnow MA, Smith-Bolton RK. Sprouty proteins are in vivo targets of Corkscrew/SHP-2 tyrosine phosphatases. Development. 2006;133(6): 1133–42.
227. Nguyen KT, Zong CS, Uttamsingh S, Sachdev P, Bhanot M, Le MT, et al. The role of phosphatidylinositol 3-kinase, rho family GTPases, and STAT3 in Ros-induced cell transformation. J Biol Chem. 2002;277(13):11107–15.
228. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet. 2006;7(8):606–19.
229. Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002;296(5573):1655–7.
230. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev. 2004;30(2):193–204.
231. Osaki M, Oshimura M, Ito H. PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis. 2004;9(6):667–76.
232. Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene. 2005;24(50):7455–64.
233. Plas DR, Thompson CB. Akt-dependent transformation: there is more to growth than just surviving. Oncogene. 2005;24(50):7435–42.
234. Ruggero D, Sonenberg N. The Akt of translational control. Oncogene. 2005;24(50): 7426–34.
235. Testa JR, Tsichlis PN. AKT signaling in normal and malignant cells. Oncogene. 2005; 24(50):7391–3.
236. Uttamsingh S, Zong CS, Wang LH. Matrix-independent activation of phosphatidylinositol 3-kinase, Stat3, and cyclin A-associated Cdk2 Is essential for anchorage-independent growth of v-Ros-transformed chicken embryo fibroblasts. J Biol Chem. 2003;278(21):18798–810.

237. Dasgupta B, Yi Y, Chen DY, Weber JD, Gutmann DH. Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in neurofibromatosis 1-associated human and mouse brain tumors. Cancer Res. 2005;65(7):2755–60.
238. Haas-Kogan D, Shalev N, Wong M, Mills G, Yount G, Stokoe D. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. Curr Biol. 1998;8(21):1195–8.
239. Holland EC, Celestino J, Dai C, Schaefer L, Sawaya RE, Fuller GN. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. Nat Genet. 2000;25(1):55–7.
240. Hu X, Pandolfi PP, Li Y, Koutcher JA, Rosenblum M, Holland EC. mTOR promotes survival and astrocytic characteristics induced by Pten/AKT signaling in glioblastoma. Neoplasia. 2005;7(4):356–68.
241. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res. 2002;8(4):945–54.
242. Klampfer L. Signal transducers and activators of transcription (STATs): novel targets of chemopreventive and chemotherapeutic drugs. Curr Cancer Drug Targets. 2006;6(2):107–21.
243. Frank DA. StAT signaling in cancer: insights into pathogenesis and treatment strategies. Cancer Treat Res. 2003;115:267–91.
244. Frank DA. STAT3 as a central mediator of neoplastic cellular transformation. Cancer Lett. 2007;251(2):199–210.
245. Mizoguchi M, Betensky RA, Batchelor TT, Bernay DC, Louis DN, Nutt CL. Activation of STAT3, MAPK, and AKT in malignant astrocytic gliomas: correlation with EGFR status, tumor grade, and survival. J Neuropathol Exp Neurol. 2006;65(12):1181–8.
246. Zong CS, Zeng L, Jiang Y, Sadowski HB, Wang LH. Stat3 plays an important role in oncogenic Ros- and insulin-like growth factor I receptor-induced anchorage-independent growth. J Biol Chem. 1998;273(43):28065–72.
247. Bao R, Friedrich M. Fast co-evolution of sevenless and bride of sevenless in endopterygote insects. Dev Genes Evol. 2008;218(3–4):215–20.
248. Robbins MJ, Michalovich D, Hill J, Calver AR, Medhurst AD, Gloger I, et al. Molecular cloning and characterization of two novel retinoic acid-inducible orphan G-protein-coupled receptors (GPRC5B and GPRC5C). Genomics. 2000;67(1):8–18.
249. Brauner-Osborne H, Krogsgaard-Larsen P. Sequence and expression pattern of a novel human orphan G-protein-coupled receptor, GPRC5B, a family C receptor with a short amino-terminal domain. Genomics. 2000;65(2):121–8.
250. Robbins MJ, Charles KJ, Harrison DC, Pangalos MN. Localisation of the GPRC5B receptor in the rat brain and spinal cord. Brain Res Mol Brain Res. 2002;106(1–2):136–44.
251. Imanishi S, Sugimoto M, Morita M, Kume S, Manabe N. Changes in expression and localization of GPRC5B and RARalpha in the placenta and yolk sac during middle to late gestation in mice. J Reprod Dev. 2007;53(5):1131–6.
252. Avram C, Yeung CH, Nieschlag E, Cooper TG. Regulation of the initial segment of the murine epididymis by dihydrotestosterone and testicular exocrine secretions studied by expression of specific proteins and gene expression. Cell Tissue Res. 2004;317(1):13–22.

Chapter 15
The RYK Receptor Family

Michael M. Halford, Maria L. Macheda, and Steven A. Stacker

Abbreviations

AC	Anterior commissure
AF-6	MLLT4 myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*) translocated to 4 (*Homo sapiens* (human)), ALL-1 fused gene from chromosome 6
AL	*Drosophila* antennal lobe
ALM	Anterior lateral microtubule
AP	Anterior–posterior axis
APP	Amyloid precursor protein
ATP	Adenosine 5′-triphosphate
ATP5O	ATP synthase, H^+ transporting, mitochondrial F1 complex, O subunit
βarr	β-Arrestin
βgeo	β-Galactosidase-neomycin phosphotransferase II fusion protein

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CAM-1	*CA*N cell *m*igration defective-1
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II α
CASK	Calcium/calmodulin-dependent serine protein kinase (MAGUK family)
CCK4	Colon carcinoma kinase 4
Cdc37	Cell division cycle 37
cDNA	Complementary DNA
CE	Convergent extension
CELSR2	Cadherin EGF LAG seven-pass G-type receptor 2
CFZ-2	*Caenorhabditis* frizzled homolog 2
CNS	Central nervous system
CK1α	Casein kinase 1α
COSMIC	Catalogue of Somatic Mutations in Cancer
CST	Corticospinal tract
CWN-1	*C. elegans* WNT family-1
CWN-2	*C.* e*legans* WNT family-2
Da	Dalton
DA	Dopaminergic
DL	Dorsolateral
DLC	Dorsolateral cluster
DNA-PKcs	Catalytic subunit of the DNA-dependent protein kinase
DRG	Dorsal root ganglion
dnt	*doughnut on 2*
drl	*derailed*
Drl-2	*derailed*-2
DRS	Dominant Robinow syndrome
DSG2	Desmoglein 2
DsRed2	*Discosoma* sp. red fluorescent protein variant 2
DV	Dorsal–ventral axis
Dvl	Dishevelled
E	Rodent embryonic day
ECM	Extracellular matrix
EGL-20	Egg-laying defective-20
ERBB3	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3
ERBB4	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4
Eph	Erythropoietin-producing hepatoma
FISH	Fluorescence in situ hybridization
Fzd	Frizzled
GABA	γ-Aminobutyric acid
GFP	Green fluorescent protein
GPR125	G protein-coupled receptor 125
GSK3	Glycogen synthase kinase 3
hpf	Hours postfertilization
Hsp90	Heat shock protein 90 kDa
Htt	Huntingtin
IP3R	Inositol 1,4,5-trisphosphate receptor
JAK2	Janus kinase 2

JH2	Janus homology 2
JNK	c-JUN N-terminal kinase/mitogen-activated protein kinase 8
kb	Kilobase pairs
L	*C. elegans* larval stage
LGE	Lateral ganglionic eminence
LGR	Leucine-rich repeat-containing G protein-coupled receptor
LIN-17	Abnormal cell lineage-17
LIN-18	Abnormal cell lineage-18
lio	*linotte*
LRP	Low-density lipoprotein receptor-related protein
MAP2	Microtubule-associated protein 2
MB	*Drosophila* mushroom body
MGE	Medial ganglionic eminence
MIB1	Mindbomb E3 ubiquitin protein ligase 1
MIG-1	Abnormal cell migration-1
ML	Medial–lateral axis
MOM-2	*mo*re *m*esoderm-2
MOM-5	*mo*re *m*esoderm-5
mRNA	Messenger RNA
MuSK	Muscle, skeletal, receptor tyrosine kinase
NCBI	National Center for Biotechnology Information
NFATc	Nuclear factor of activated T cells
NG2	Neuron–glial antigen 2
NMJ	Neuromuscular junction
NPC	Neural progenitor cell
Nrt	Neurotactin
OR	Odorant receptor
ORN	Olfactory receptor neuron
OT	Optic tectum
P	Postnatal
PC	Posterior commissure
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PDZ	*P*ostsynaptic density protein 95/*d*iscs large-1/*z*onula occludens-1
PK	Protein kinase
PLR-1	Cell *pol*a*r*ity defective-1
PN	Projection neuron
Psen	Presenilin
PTK	Protein tyrosine kinase
PTK7	Protein tyrosine kinase 7
RRS	Recessive Robinow syndrome
Rab5	RAB5A member of the RAS oncogene family
Rβ	Ryk β-amyloid-like fragment
RGC	Retinal ganglion cell
RING	Really interesting new gene

RNF43	Ring finger protein 43
ROCK	Rho-associated coiled-coil containing protein kinase 1 or 2
ROR	Receptor tyrosine kinase-like orphan receptor
RT	Reverse transcription
R spine	Regulatory spine
RTK	Receptor-type protein tyrosine kinase
RWD1	Fully human anti-RYK (WIF domain-specific) monoclonal antibody
Ryk	Related to tyrosine kinase
Ryk-CTF45	Ryk carboxyl-terminal fragment of 45 kDa
Ryk-CTF55	Ryk carboxyl-terminal fragment of 55 kDa
Ryk-FL	Full-length Ryk lacking N-terminal signal peptide
Ryk-ICF	Ryk intracellular fragment
Ryk-NTF	Ryk amino-terminal fragment
S	Svedberg unit
SC	Superior colliculus
Scr	*sex combs reduced*
Sema	Semaphorin
sFRP2	Secreted Fzd-related protein 2
SFK	Src family kinase
SH2	Src homology domain 2
SH3	Src homology domain 3
siRNA	Short interfering RNA
SOD1	Superoxide dismutase 1, soluble
Src	Src proto-oncogene, non-receptor tyrosine kinase
STYK1	Serine/threonine/tyrosine kinase 1
TBC	Tetrabasic cleavage
TCF/LEF	T cell factor/lymphocyte enhancer factor
TGF-β	Transforming growth factor-β
TM	Transmembrane
TN	Temporal–nasal axis
TRPC	Transient receptor potential channel
TUJ1	βIII-tubulin
UBC	Ubiquitin C
Vangl2	Van Gogh-like planar cell polarity protein 2
VM	Ventral midbrain or ventral–medial axis
VNC	*Drosophila* ventral nerve cord
VPC	Vulval precursor cell
VZ	Ventricular zone
WASF1	WAS protein family, member 1
WG	Wingless
WIF	Wnt inhibitor factor
WNK	With-no-lysine protein kinase
Wnt	Wingless-related integration site
XRyk	*Xenopus* Ryk
ZNRF3	Zinc and ring finger 3

15.1 Introduction

Members of the RYK receptor-type tyrosine kinase (RTK) subfamily are metazoan cell surface proteins that exhibit an extracellular amino-terminus, are embedded in the plasma membrane by a single-pass hydrophobic transmembrane helix, and end with an intracellular carboxyl-terminus. A unique extracellular region, single-pass transmembrane topology, and conserved arrangement of intracellular peptide motifs definitive of the protein tyrosine kinase (PTK) group are the signature of the RYK subfamily and collectively distinguish it from the 19 other human RTK subfamilies (Fig. 15.1; Table "Receptor at a Glance"). Through gradually emerging biochemical mechanisms that involve proteolytic processing of—and/or binding of Wnts by—its extracellular region, RYK regulates a wide range of developmental processes, most notably commissural axon pathfinding, topographic mapping, and craniofacial and musculoskeletal morphogenesis.

Studies of human RYK and its orthologs/paralogs in model organisms—brown rat (*Rattus norvegicus*), hamster (*Mesocricetus auratus*), house mouse (*Mus musculus*), African clawed frog (*Xenopus laevis*), zebrafish (*Danio rerio*), fruit fly (*Drosophila melanogaster*), and nematode worms (*Caenorhabditis elegans* and *Pristionchus pacificus*)—form the basis for our present understanding of this idiosyncratic RTK subfamily. In this review, we summarize current knowledge concerning RYK expression, domain organization, binding partners, function, dysfunction, and potential for therapeutic targeting gleaned from studies of these diverse species.

15.2 The RYK Subfamily of RTKs

Complementary DNAs (cDNAs) for the human and mouse *RYK* transcripts were described by multiple teams in the early 1990s. At the time, cloning of these cDNAs relied on the design of degenerate oligonucleotides complementary to cDNA encoding conserved PTK subdomains [1] that could, via the then newly invented polymerase chain reaction (PCR), amplify and facilitate identification of novel sequences from different source tissues. Although different names were initially used to describe these (JTK5, JTK5a, nyk-r, MRK, and vik) [2–6], the acronym *RYK* (for *r*elated to t*y*rosine *k*inase) endured for the mouse and human genes [7, 8] and, ultimately, the subfamily that these genes came to define across all metazoa.

There was no pressure for a more logical name given that RYK subfamily members bore official orphan receptor status for a further 11 years [5, 7, 9]. Additionally, despite the prospective identification of a Wnt inhibitory factor (WIF)-like Wnt-binding protein domain in RYK subfamily members in 2000 [10], Wnt-binding activity within the RTK group was ultimately found shared with the ROR, PTK7/CCK4, and MuSK subfamilies. A current functional comparison (November 2014) of these Wnt-binding RTKs is intriguing in that there is evidence in support of a pseudokinase classification for RYK, ROR1 and ROR2 (but not the *C. elegans* ROR ortholog CAM-1[11]), and PTK7/CCK4 [11, 12].

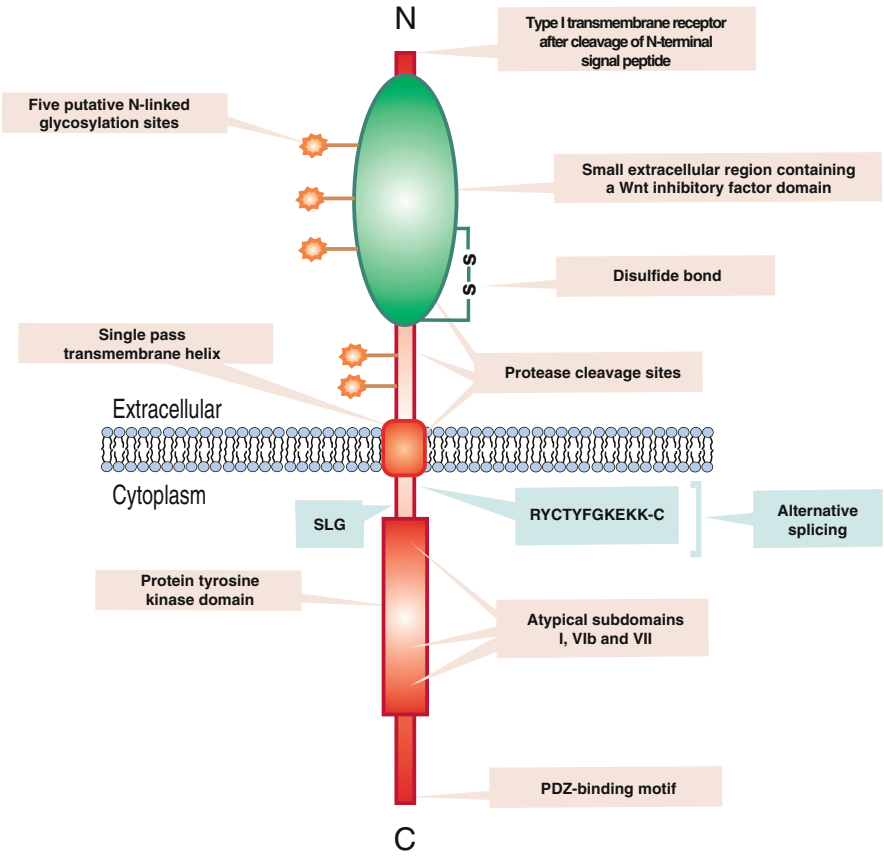

Fig. 15.1 Key features of RYK subfamily members. RYKs are transmembrane proteins with an amino (N)-terminal extracellular region containing a single WIF domain that mediates high-affinity binding of Wnt ligands. Five potential *N*-linked glycosylation sites, a disulfide bond, and three proteolytic cleavage sites are indicated. The intracellular PTK domain contains all sequence motifs characteristic of this group of enzymes; atypical substitutions specific to RYKs are consistent with the apparent lack of intrinsic kinase activity. The extreme carboxyl (C)-terminus represents a PDZ domain-binding motif. The sequences of a tripeptide insertion ("SLG") and alternative intracellular region ("RYCTYFGKEKK") encoded by alternative splicing or 3′-end processing of human mRNAs, respectively, are shown next to the intracellular juxtamembrane region. Note that not all depicted features have been demonstrated in all RYK subfamily members

15.3 *RYK* Genes

15.3.1 Chromosomal Localization, Paralogs, and Pseudogenes

One active *RYK* gene is identifiable in every completed metazoan genome sequence, with the notable exception of *Drosophila* species in which one ortholog and two paralogs are present (Table "Receptor at a Glance"; http://www.ensembl.org,

viewed November 2014; 68 orthologs). Fluorescence in situ hybridization (FISH) originally revealed two separate locations for *RYK* in the human genome. One signal localized to chromosome 3q22 and another to the short arm of chromosome 17 [8, 13]. Two *Ryk* loci were also identified in the mouse genome, on chromosomes 9 and 12, by linkage analysis using recombinant inbred mouse strains [14]. Subsequent analysis of the human kinome [15] established that 3q22 harbored the functional *RYK* gene (NCBI Gene ID 6259) and that the FISH signal on 17p13.3 was a non-transcribed, fully processed *RYK* pseudogene (NCBI Gene ID 6260). Analysis of the mouse kinome [16] established that chromosome 9 harbored the functional *Ryk* gene at cytogenetic band D1 (NCBI Gene ID 20187) and that a fully processed *Ryk* pseudogene (*Ryk-ps1*; NCBI Gene ID 111178) was located on chromosome 12 at cytogenetic band B1. *Drosophila* genomes contain a *RYK* ortholog, *derailed* (*drl* [17]; formerly known as *linotte* (*lio*) [18, 19]), and the paralogs *Derailed-2* (*Drl-2* [20]) and *doughnut on 2* (*dnt* [21, 22]).

15.3.2 Exon/Intron Structure

The genomic organization of *Drosophila drl* was the first of the *RYK* subfamily to be described and comprises four exons distributed over 18 kilobase pairs (kb) [17, 19]. The mouse *Ryk* mRNA is encoded by 15 exons distributed over a region of approximately 73 kb [23]. A notable feature is that the variant adenosine 5′-triphosphate (ATP)-binding motif of the mouse Ryk PTK domain (glycine-rich loop or subdomain I) is encoded by exons 8 and 9. This vital motif is encoded by a single exon in most RTKs.

15.3.3 Promoter Structures

RYK subfamily promoters are largely uncharacterized. The human *RYK* transcription start site has not been mapped experimentally but is predicted to lie within a CpG island that contains the translation initiation methionine codon, a feature shared with the mouse *Ryk* gene [23]. The mouse *Ryk* gene promoter overlaps an unmethylated CpG island that encompasses exon 1 and contains a TATA box, putative transcription factor-binding sites, and a single transcription start site [23]. The human *RYK* and mouse *Ryk* pseudogenes are not transcribed [15, 16].

Drosophila drl enhancers that drive reporter gene expression in four distinct subtypes of embryonic neurons, all of which project axons in the anterior commissure (AC) of the developing central nervous system (CNS), were identified by Bonkowsky and Thomas [24]. Distinct enhancers responsible for driving *drl* expression in muscles and epidermal attachment cells were also defined [24]. Expression of *drl* mRNA was regulated by the homeotic *Sex combs reduced* (*Scr*) gene and its downstream target gene, the transcription factor *fork head (fkh)*, in salivary placodes [25]. The *nerfin-1* gene, required for proper development of commissural and connective axon fascicles, was essential for the proper expression of *drl* and *Wnt5a* [26].

15.3.4 RYK *mRNA Structure and Alternative Splicing*

Two mature polyadenylated transcript variants, each approximately 3.4 kb, were found to be generated by alternative splicing of the human *RYK* primary RNA transcribed from the active locus at 3q22 [8, 27]. Additional hybridization signals, possibly representing significantly longer *RYK* transcripts, were seen on a Northern blot of human placental RNA [27]. Use of alternative splice acceptor sites in the same (type I) reading frame at the junction of intron 8 and exon 9 of both human and mouse *RYK* primary RNAs results in the splicing in (NCBI Reference Sequences NM_001005861.2 and NM_013649.3, respectively) or splicing out (NCBI Reference Sequences: NM_002958.3 and NM_001042607.1, respectively) of RNA encoding the tripeptide (bolded) PITS^{297}**SLG**YPTL (human) or PITS^{281}**SSG**YPTL (mouse) in the cytoplasmic juxtamembrane region (Fig. 15.1) [27]. Use of an alternative, in-frame, and noncanonical yet conserved TG 3′-splice acceptor site generated the mRNA encoding a tripeptide insertion in *Ryk* transcripts from at least 10 vertebrate species [28]. The functional significance of this alternative splicing event—if any—is unknown.

An alternatively spliced mouse *Ryk* mRNA (NCBI Reference Sequence NM_001284258.1) encoding a predicted mouse isoform 3 precursor resulted from skipping of exon 2. Predicted in-frame initiation of translation at exon 3M117 may result in an N-terminally truncated Ryk isoform. Two alternative polyadenylation signals, both of which perfectly match the consensus sequence AATAAA, are used in 3′-end processing of the mouse *Ryk* primary transcript and reside in exon 15 [2, 23].

Vorlová et al. identified a splice variant of human *RYK* resulting from the retention of intron 6 and subsequent use of an alternative polyadenylation signal [29]. This was a regulated feature of splicing and mRNA 3′-end processing common to human RTKs that led to the expression of soluble decoy or membrane-anchored PTK domain-negative receptor chains. In the case of the truncated human *RYK* transcript, exon 6 sequences encoding the transmembrane helix were present and an alternative polyadenylation signal in intron 6 was utilized. The resulting transcript was predicted to encode a 313-residue transmembrane chain (isoform 3 precursor; Fig. 15.1) with the unique carboxyl-terminus RYCTYFGKEKK-COOH [29], which may represent a forward signaling-defective RYK isoform.

15.3.5 mRNA Expression Patterns

15.3.5.1 Mammals

Northern blot analyses of human *RYK* mRNA demonstrated expression in melanocytes, pancreas, kidney, skeletal muscle, liver, lung, brain, heart, and placenta [13, 27, 30]. In situ hybridization analysis of human *RYK* localized mRNA to the colonic epithelium and stroma; mammary epithelium; alveolar epithelium and the smooth muscle and epithelium of larger airways in the developing lung; cortical layers of the embryonic brain; and the renal cortex [30].

Northern blot analysis of *Ryk* transcripts from adult rat tissues showed broad expression, with the notable absence of mRNA from liver and spleen [31]. *Ryk* mRNA expression in the rat brain during development showed abundant mRNA at embryonic days (E) 13–18, which decreased by E20 [31]. Postnatal cerebrum at week 1 and cerebellum at week 2 were enriched for *Ryk* mRNA relative to later stages [31]. In situ hybridization analysis of embryonic *Ryk* mRNA showed expression throughout the rat central nervous system, with the highest levels in the E11 telencephalon, mesencephalon, branchial arches, tail, dorsal root ganglia and spinal cord, and at E13 in limb buds and retina [31]. In situ hybridization and quantitative reverse transcription (RT)-PCR analyses demonstrated mouse *Ryk* mRNA expression in tyrosine hydroxylase-positive dopaminergic neurons [32].

Embryonic mouse *Ryk* promoter activity, visualized using the surrogate enzymatic activity of a knock-in β-galactosidase-neomycin phosphotransferase II (βgeo) fusion from $Ryk^{\beta geo}$, showed strong and broad activation in most tissues from mid-embryogenesis [33]. βgeo expression in the mouse telencephalon, the embryonic structure from which the mature cerebrum develops, was first detectable at E10.5, peaked at E12.5, and was sustained until E14.5, after which time it decreased and was expressed at the limit of detection by E18.5 [34]. At E12.5 and E14.5, βgeo expression was observed in the dorsal and ventral regions of the ventricular zone (VZ) and the medial and lateral ganglionic eminences (MGE and LGE, respectively). βgeo in the E14.5 cortical plate indicated *Ryk* promoter activity in both mitotic and postmitotic neurons. Colocalization of βgeo with the neural progenitor cell (NPC) marker Nestin in the VZ of the LGE was observed, and also in Nestin-negative cells of the subventricular and mantle zones [34]. In E14.5 $Ryk^{\beta geo/\beta geo}$ limbs, reporter activity was ubiquitous and enriched in chondrocytes and osetoblasts of the humerus [35]. Northern blot and RNase protection analyses of mouse *Ryk* revealed a similar distribution to the human, with mRNA detected in placenta, ovary, testes, thymus (embryonic and adult), lymph node, bone marrow (upregulated by 5-fluorouracil treatment and enriched in the Lin^+ population), liver (embryonic and adult), E9–E11 yolk sac, lung, kidney, spleen, brain (embryonic and adult), placenta (E12-term), salivary gland, heart, skeletal muscle, eye, purified erythroid progenitors, stomach, duodenum, uterus, and tongue [2–6].

In situ hybridization signals for mouse *Ryk* were observed in a differentiation stage-specific manner in various mouse epithelial tissues: crypts and the lower half of villi in the small intestine; the basal layer and replicating papillae of the dorsal tongue; the basal layer of the ventral tongue; the lower, dividing bulb region of the hair follicle; and the embryonic and postnatal dermis [36]. *Ryk* mRNA was seen in the inner cell mass, consistent with *Ryk* expression in embryonic stem cells, but was much more abundant in decidualizing maternal uterine stroma [6, 33, 36]. *Ryk* mRNA was enriched in the microvascular endothelial cell fraction freshly isolated from adult mouse lung [37]. In postnatal rat brain, *Ryk* mRNA was detected in neurons from a variety of locations: the hippocampus; cerebral cortex; Purkinje and granular cells of the cerebellum; the facial, olfactory, pontine, medial vestibular, and spinal vestibular nuclei; and the periaqueductal gray matter [38]. At mouse E14.5 and rat E18 and E20, *Ryk* mRNA and protein were abundant in the VZ and cortical plate of the developing forebrain [31, 39].

15.3.5.2 Nonmammalian Vertebrates

RT-PCR and in situ hybridization demonstrated *Ryk* expression throughout zebrafish development [40]. During somite stages, *Ryk* was expressed strongly in the developing somites and central nervous system. Beyond 30 h postfertilization, *Ryk* was highly expressed in the brain, heart, eyes, and posterior tail. At four days postfertilization, strong *Ryk* expression was observed in the lining of the ventricular zones and in the notochord [40]. In *X. laevis*, *XRyk* mRNA was expressed both maternally and zygotically throughout early development [41, 42]. Dorsal marginal zone cells expressed *XRyk* mRNA during the gastrula stages. At later stages, *XRyk* mRNA was localized to structures in the head including the eyes, otic vesicle, and branchial arches [41] and pronephros [42].

15.3.5.3 Invertebrates

Messenger RNA encoding DRL was detected at ~6 h postfertilization (hpf) in *Drosophila* salivary placodes [25]. At 12 hpf, *Drosophila drl* promoter activity visualized using an enhancer trap or reporter transgene revealed activation in a heterogenous population of ~50 pathfinding embryonic neurons per body segment. Neurons positive for *drl* represented four neuronal populations in which *drl* expression is dependent upon enhancer elements *drlR*, *drlT*, *drlU*, and *drlZ*, all of which project axons across the ventral midline in the AC of each segment [17, 24, 43]. In situ hybridization revealed co-expression of *drl* and *Src64B* mRNAs in the ventral nerve cord (VNC) at the anterior portion of each body segment [44]. Activation of a *drl* enhancer trap was also observed in the actively migrating distal tip cells of embryonic *Drosophila* salivary glands at 12 hpf [25].

The *Drosophila dnt* transcript was detected by in situ hybridization during embryogenesis in a series of dynamic domains in the epidermis. Expression of *dnt* mRNA peaks at 4–6 hpf, 2 h prior to maximal *drl* expression. Notably, *dnt* only partially rescues the *drl* muscle attachment phenotype [21], suggesting diversification of function. A *P*-element insertion in the *dnt* locus affected several body size-related traits, including wing size, thorax length, and head and face width [45]. Expression of both *dnt* and *drl* was detectable throughout subsequent stages of the *Drosophila* life cycle [46]. Many of the embryonic regions of *dnt* expression bordered sites of epithelial invagination, including the ventral and cephalic furrows, foregut, hindgut, optic lobe, and characteristic rings surrounding tracheal primordia that resemble doughnuts [21, 22]. *Drl-2* was expressed in subsets of neurons in the central nervous system, and in antennal lobe (AL) neurons during development [20, 47]. The developmental phenotypes of *drl*, *Drl-2*, and *dnt* mutants have been recently summarized [48, 49].

In the nematode *C. elegans*, transcription of the *RYK* ortholog *lin-18* was revealed by a *lin-18*::*gfp* transgene. LIN-18::GFP expression was observed in neurons, body wall musculature, the vulva [50], and pharyngeal muscle cells [51]. At the L3–L4 stages, the multipotent vulval precursor cells (VPCs) P5.p, P6.p, P7.p and all their

descendants expressed LIN-18::GFP or a nuclear-localized LIN-18::DsRed2 reporter fusion [50]. Although a product of γ-secretase cleavage of LIN-18::GFP was not detectable by Western blotting [51], its labile nature may require the use of a proteasome inhibitor.

15.4 RYK Proteins

15.4.1 Primary Sequence

Translation of the two human *RYK* mRNAs generated by alternative splicing of exon 9 generates two corresponding isoforms of 610 (isoform 1 precursor; NCBI Reference Sequence NP_001005861.1) and 607 (isoform 2 precursor; NCBI Reference Sequence NP_002949.2) residues in length, including the amino-terminal signal peptide. As noted before, retention of intron 6 is coupled with the use of an alternative polyadenylation site that encodes a predicted transmembrane human RYK (isoform 3 precursor) with the predicted cytoplasmic sequence MKRIELDD**RYCTYFGKEKK**-COOH (unique carboxyl-terminus bolded) and no PTK domain [29].

Two mouse Ryk isoforms are encoded by exon 9 splice variants analogous to those in human, resulting in polypeptides of 594 (isoform 1 precursor; NCBI Reference Sequence NP_038677.3) and 591 (isoform 2 precursor; NCBI Reference Sequence NP_001036072.1) residues, respectively. Skipping of exon 2 results in a transcript predicted to encode an N-terminally truncated 475-residue polypeptide (isoform 3 precursor) resulting from initiation of translation at an in-frame ATG codon.

15.4.2 Domain Organization

RYK subfamily members share the same basic domain organization (Figs. 15.1 and 15.2). Like all other RTKs, RYK proteins display a type I transmembrane topology with an extracellular amino-terminus, single-pass hydrophobic transmembrane helix, and intracellular carboxyl-terminus. RYK proteins have a signal peptide at the extreme amino-terminus, predicted to consist of residues 1–46 for human RYK and 1–34 for mouse Ryk (http://www.cbs.dtu.dk/services/SignalP, viewed November 2014; Fig. 15.2). A Wnt inhibitory factor (WIF) domain, so named by virtue of its strong sequence homology with the Wnt-binding domain of the secreted WIF-1 protein [10, 52], encompasses the majority of the RYK extracellular region.

The intracellular region of full-length RYK subfamily members includes a juxtamembrane segment that is longer than that of most RTKs and that contains two serine/threonine-rich clusters (Fig. 15.2) [7]. This is followed by a single PTK domain that exhibits several subtle yet characteristic subfamily-specific sequence

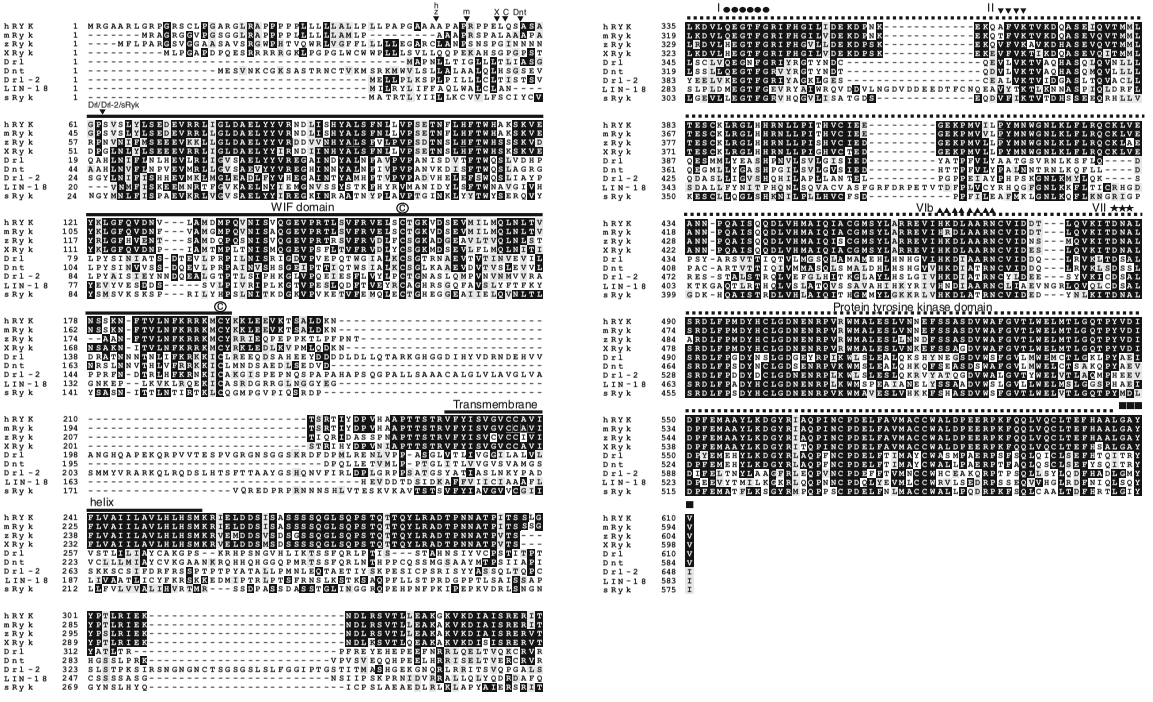

Fig. 15.2 Alignment of human (h), mouse (m), zebrafish (z), and *X. laevis* (X) Ryk; *Drosophila* Derailed (DRL), DRL-2, and Doughnut (DNT); *C. elegans* LIN-18; and *Strongylocentrotus purpuratus* (s; purple sea urchin) Ryk. Amino acid sequences (single letter code) were aligned and gaps (-) were introduced to achieve maximum alignment. Sequences have been shaded to indicate identity between sequences (*dark*) or a conservative amino acid substitution (*light*). Key functional modules are indicated by *solid lines* (WIF domain and transmembrane helix) or a *dotted line* (PTK domain) above the alignment. The signal peptide cleavage site for each protein (located toward the amino-terminus) is shown by a filled inverted triangle. The cysteine residues involved in disulfide bond formation are indicated (©). Atypical PTK domain motifs peculiar to RYK subfamily members are in subdomains I, II, VIb, and VII. The PDZ-binding motif is indicated by filled squares at the extreme carboxyl-terminus. Sequences used in the alignment are NCBI reference sequences: human, NP_001005861.1; mouse, NP_038677.3; zebrafish, NP_001077295.1; *X. laevis*, NP_001089445.1; DRL, NP_477139.1; DRL-2, NP_610835.2; DNT, NP_477341.2; LIN-18, NP_508684.3; *S. purpuratus*, XP_788381.3

variations in catalytically important but dispersed subdomains that are the signature of the conserved PTK fold (Fig. 15.2) [1, 53]. The glycine-rich loop (also referred to as the phosphate-binding loop or subdomain I; sequence consensus GXGXXG), located between protein kinase (PK) strands β1 and β2, normally functions to anchor the phosphate groups of bound ATP through contacts with the peptide backbone. All RYK subfamily members deviate from the glycine-rich loop consensus by virtue of a substitution of the first glycyl with a residue projecting a bulky side chain that is expected to significantly distort the ATP-binding pocket (Fig. 15.2; RYK subfamily consensus: **X**XGXXG, where **X** is **Q** in mammals, DRL and DNT; **K** in DRL-2; **M** in LIN-18; **H** in zebrafish and *X. laevis*; and **L** in *S. purpuratus*). The glycine-rich loops of the human RTKs ROR1, ROR2, CCK4/PTK7, and STYK1 (GH**C**AFG, GH**D**RFG, GH**S**EFG, and **C**SGSCG, respectively) also show substitutions (bolded) in one of the consensus subdomain I glycine residues. Mouse Ryk, DRL, and the human RTKs ROR1, ROR2, and CCK4/PTK7 lack in vitro ATP-binding activity and/or phosphotransferase activity [11, 12, 54, 55], but these functions of STYK1 have not yet been examined.

The catalytic loop (also referred to as subdomain VIb; consensus: HRDLXXXN) lies between PK strands β6 and β7. The asparaginyl (N)-oriented aspartyl (D) of the catalytic loop interacts with the attacking hydroxyl of the phosphoacceptor tyrosine residue. These catalytic residues are embedded within the sequence H(**K/N/R**) DXXXXN in RYK subfamily members (Fig. 15.2; where the second position is R in mouse, N in LIN-18, and K in all others). Substitution of the consensus arginine residue (R) is also present in the catalytic loops of the human pseudokinases ROR1, ROR2, CCK4/PTK7, and STYK1 (H**K**DLAARN, H**K**DLATRN, H**K**DLAARN, and H**G**DVAARN, respectively). However, given that the arginine residue in H**R**DLXXXN is conserved only among eukaryotic protein kinases and the presence of the consensus R residue at position two in mouse Ryk, these substitutions by themselves are not predicted to be inactivating.

The aspartyl (D) of subdomain VII (also known as the Mg^{2+}-positioning loop; consensus DFG), which lies between PK strands β8 and β9 at the beginning of the activation loop, is a residue vital for catalysis that forms polar contacts with all three ATP phosphates, either directly or via coordinating Mg^{2+} ions. In the active ("DFG-in") state of all protein kinases, the consensus phenylalanine residue of the DFG motif packs into a hydrophobic pocket between one residue from the N-lobe and one residue from the C-lobe to form a hydrophobic regulatory (R) spine that orients the aspartyl for catalysis [56]. Assembly of the R spine is dynamically regulated, often by phosphorylation of one or more activation loop residues, but also by other mechanisms. RYK subfamily members display signature substitutions at the second and third positions of subdomain VII (Fig. 15.2) that are of unknown functional significance. For example, human and mouse RYK both exhibit the sequence DNA (Asp-Asn-Ala) in subdomain VII [7, 8].

The extreme carboxyl-terminus of both human and mouse RYK exhibits the sequence GAYV-COOH and acts as a PDZ-binding motif [33, 57]. Similar motifs are conserved in other RYK subfamily members (Fig. 15.2), and deletion of the

putative PDZ-binding peptide from DRL abrogated its interaction with SRC64B and, in a transgenic *Drosophila* gain-of-function axon commissure-switching assay, reduced the frequency of switching by ~35 % [54].

15.4.3 Posttranslational Modifications

15.4.3.1 Sequential Proteolytic Cleavage

The full-length mouse Ryk protein (lacking the signal peptide; Ryk-FL) undergoes sequential proteolytic cleavage in the extracellular and transmembrane regions (Fig. 15.3). A conserved tetrabasic sequence just within the carboxyl-terminal end of the RYK WIF domain represents a predicted substrate for members of the proprotein convertase family that appears to be cleaved during or after transit through the secretory pathway [58–60]. Substitutions in this putative tetrabasic cleavage (TBC) motif interfere with generation of a ~55 kDa carboxy-terminal transmembrane Ryk fragment (Ryk-CTF55) [59] which is likely disulfide bonded to the corresponding amino-terminal fragment [40]. The functional importance of the intra-WIF domain TBC motif—which is not recognizable in WIF1 [61]—is exemplified by the complete loss of activity of the $DRL^{\Delta TBC}$ receptor in a transgenic *Drosophila* gain-of-function axon commissure-switching assay [54]. Ryk-FL is a substrate for a metalloprotease activity that cleaves the extracellular region at a membrane-proximal site to liberate a water-soluble ~30 kDa amino-terminal fragment, termed Ryk-NTF, from the cell surface [39, 59, 62, 63] (Fig. 15.3).

The ~45 kDa carboxy-terminal product of metalloprotease-mediated cleavage of Ryk-FL, termed Ryk-CTF45, remains embedded in the plasma membrane [39, 62]. Ryk-CTF45 is a substrate for intramembranous cleavage by the γ-secretase complex [39]. Type I integral membrane proteins with less than 40 amino acid residues in their extracellular region are potential substrates for γ-secretase [64, 65], but undefined cryptic structural features of genuine substrates appear to restrict cleavage to a select subset [66]. The γ-secretase cleavage sites in Ryk-CTF have not been mapped, but based upon the location of sites in bona fide substrates such as APP, Notch, and E-cadherin [67], they are expected to lie in the hydrophobic transmembrane helix or at the interface of the transmembrane helix and intracellular region. This prediction is consistent with the release of a water-soluble ~42 kDa intracellular fragment, Ryk-ICF, from Ryk-CTF45 [39, 62]. The predicted ~3 kDa peptide amino-terminal to the site(s) of γ-secretase-mediated cleavage (named Rβ by analogy to the Aβ peptides generated by the sequential action of β- and γ-secretases on the amyloid precursor protein APP [67]) is predicted to be released into the extracellular space (Fig. 15.3).

Chemical inhibitors of γ-secretase completely block further proteolytic processing of Ryk-CTF45 [39]. *Psen1*$^{-/-}$ fibroblasts are impaired in processing Ryk-CTF45 [39], but *Psen1*$^{-/-}$;*Psen2*$^{-/-}$ fibroblasts that completely lack γ-secretase activity generate further reduced but nevertheless detectable amounts of Ryk-ICF (MMH, MLM, and SAS, unpublished). These findings indicate that other proteases sensitive to γ-secretase inhibitors can process Ryk-CTF45 into Ryk-ICF and Rβ.

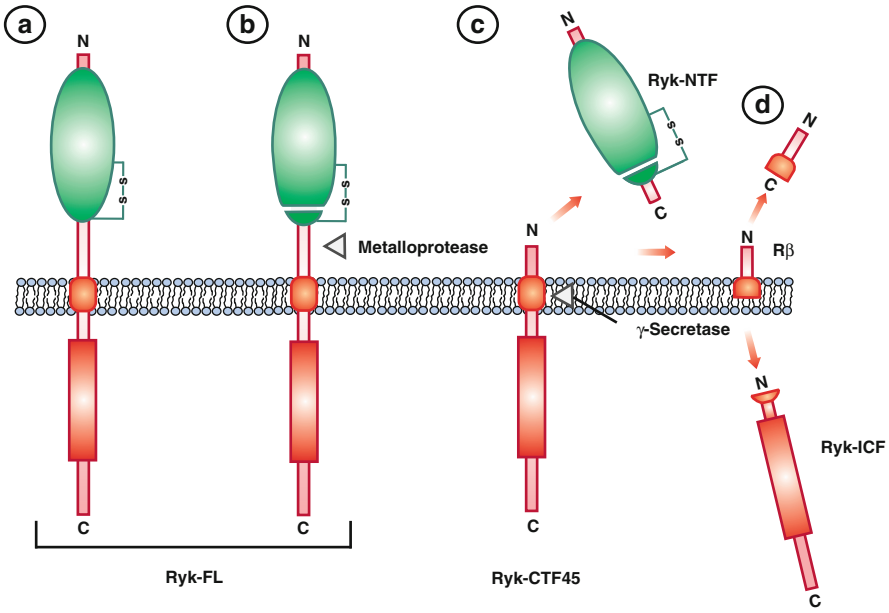

Fig. 15.3 Schematic representation of posttranslational proteolytic cleavage events in RYK subfamily members. (**a**) Cleavage of Ryk-FL between the WIF domain cystine residues, probably by a proprotein convertase, can generate a disulfide-linked heterodimeric receptor. It is unclear whether this is necessary for subsequent cleavage events. (**b**) Metalloprotease-mediated cleavage within the extracellular juxtamembrane region of Ryk-FL generates Ryk-NTF and membrane-bound Ryk-CTF45. (**c**) Ryk-NTF is shed into the extracellular space and studies in *Drosophila* implicate it as a Wnt antagonist via ligand sequestration from receptors. (**d**) The γ-secretase complex cleaves Ryk-CTF45 to release Ryk-ICF into the cytoplasm; the small Rβ peptide is likely shed into the extracellular space

15.4.3.2 Glycosylation

The human RYK extracellular region, following predicted signal peptide cleavage between residues 46 and 47, displays potential *O*-linked glycosylation sites at S57, S59, S204, T210, S211, and T213 (all outside the WIF domain; predicted by NetOGlyc 4.0 Server http://www.cbs.dtu.dk/services/NetOGlyc/; viewed November 2014) and potential *N*-linked glycosylation sites at N139, N174, N178 (all within the WIF domain), N182, and N209 (predicted by NetNGlyc 1.0 Server; http://www.cbs.dtu.dk/services/NetNGlyc/; viewed November 2014; Fig. 15.1; Table "Receptor at a Glance"). Treatment of immunoprecipitated and denatured mouse Ryk with peptide *N*-glycosidase F reduces its apparent molecular weight in SDS-PAGE, indicating that the extracellular region does indeed bear *N*-linked glycans (MMH and SAS, unpublished).

15.4.3.3 Disulfide Bonding

The zebrafish Ryk extracellular region was shown to be disulfide bonded between cystine residues 155 and 188 (corresponding to residues C159 and C194 of human RYK) within the WIF domain, and this disulfide was essential for co-immunoprecipitation of Wnt5b [40]. It is likely that this disulfide bond can form in all RYK orthologs given that these cysteine residues are absolutely conserved in all RYK subfamily members (Fig. 15.2) and all WIF-1 orthologs [61]. The disulfide bond in zebrafish Ryk was shown to maintain covalent interchain association after cleavage of the extracellular region [40] (Fig. 15.3). The cleavage event likely involves the TBC site that lies between C155 and C188 of zebrafish Ryk.

15.4.3.4 Phosphorylation

RYK subfamily members possess many potential phosphorylation sites in their intracellular regions. Although autophosphorylation activity on seryl, threonyl, or tyrosyl has not been reported, mouse Ryk and DRL were phosphorylated on tyrosine residues when co-expressed with EphB2 or EphB3 [33] or SRC64B [44]. In a mouse osteosarcoma xenograft model, strong phosphorylation of human RYK on tyrosine residues was downregulated by imatinib mesylate treatment [68]. The locations of these phosphotyrosine residues have not been mapped and their role in signaling—if any—remains unknown.

15.4.3.5 Ubiquitylation

The labile mouse Ryk-ICF is polyubiquitylated and subsequently degraded by the 26 S proteasome. This catabolic pathway is antagonized by association of Ryk-ICF with Cdc37, via PTK subdomains III–XI (amino acids 367–579) of mouse Ryk, which apparently functions to promote Ryk-ICF translocation to the nucleus [62]. Berndt et al. [69] demonstrated that the ubiquitin E3 ligase Mindbomb 1 (MIB1) induced polyubiquitylation of the human and mouse Ryk intracellular regions and their subsequent degradation by the 26 S proteasome and lysosomes. Ryk turnover was dependent on its extracellular region and an intact MIB1 RING domain, but independent of Ryk cleavage by γ-secretase [69]. In a systematic and quantitative assessment of the ubiquitin-modified proteome, lysyl 356 in the human RYK peptide NH_2-GILIDE**K**DPNKEK-COOH from the PTK domain was identified as a site of polyubiquitylation in HCT116 cells treated with the proteasome inhibitor bortezomib [70].

Moffat et al. [71] identified the *C. elegans* PLR-1 protein, the ancestor of the RNF43 and ZNRF3 transmembrane E3 ubiquitin ligases, as a negative regulator of Wnt signaling in the AVG neuron. The activities of Wnts CWN-1 and CWN-2 via their receptors CAM-1/Ror and LIN-18/Ryk were antagonized by PLR-1 in a manner dependent upon the function of Frizzleds (MIG-1, LIN-17, CFZ-2, and/or MOM-5).

PLR-1 was demonstrated to reduce cell surface levels of Frizzleds, CAM-1/Ror, and LIN-18/Ryk through an endosomal mechanism likely to involve E3 ubiquitin ligase activity towards Frizzleds. In mammals, LGR family receptors function to neutralize RNF43 and ZNFR3 in an R-Spondin-dependent manner by nucleating formation of a ternary complex that is cleared from the cell surface, thereby derepressing Frizzled activity [72].

15.4.4 RYK Subfamily Members are Group 2 Pseudokinases

Sequence alignments reveal that approximately 10 % of human and mouse kinome members display variations in peptide subdomains normally conserved in the protein kinase domain [15, 16]. Extensive structural and biochemical analyses of active PTKs have defined roles for these canonical residues in Mg^{2+}-ATP binding or in catalysis of γ-phosphate group transfer from ATP to the acceptor tyrosine residue in the protein substrate [73]. PTKs with variant subdomains are classified as pseudokinases, until proven otherwise, by virtue of their predicted catalytic inactivity. Inevitably, some of these predictions have proven to be incorrect (e.g., ERBB3 is a low-activity kinase [74]; CASK is a Mg^{2+}-independent kinase [75]; the JH2 domain of JAK2 is a dual-specificity, low-activity protein kinase [76]), and different groups have made contradictory predictions regarding the capacity of variant PTK domains to catalyze phosphoryl transfer. On the basis of current evidence, we endorse classification of members of the RYK subfamily as Group 2 pseudokinases according to the nomenclature system proposed by Zeqiraj and van Aalten [77]. The subclass of Group 2 pseudokinases exemplified by RYK subfamily members is representative of those with an absence of evidence for intrinsic PTK activity but without structural confirmation of this finding.

While some have predictively classified RYK subfamily members as active kinases [15, 16, 78], no credible biochemical evidence of intrinsic kinase activity by a wild-type RYK protein has ever been reported to our knowledge. Overexpression of full-length RYK transformed NIH/3T3 cells and conferred tumorigenicity to xenografts in immunocompromised mice [79, 80]. However, other groups—including our own—have not replicated RYK-mediated transformation of cultured immortalized cells or tumorigenicity in vivo.

The soundest evidence in support of a pseudokinase classification for RYK subfamily members is the study of a K371A substitution within subdomain II of the *Drosophila* RYK ortholog DRL. Substitution of this residue destroyed the phosphoryl transferase activity of most PTKs [81] (although the *w*ith-*n*o-*k*inase (WNK) subfamily is a notable exception [82]), but did not impair the in vivo biological activity of DRL^{K371A} in a dominant gain-of-function commissure-switching assay or in phenotypic rescue of muscle attachment or antennal lobe development in *drl* mutants [83, 84].

Axon guidance defects in the MB of *Drosophila drl/lio* mutants were rescued by expression of DRL^{K371A} or $DRL^{\Delta intra}$ (a truncated receptor lacking the intracellular

region) [85]. When expressed in a wild-type background, however, these proteins caused gain-of-function phenotypes in the MB more potently than overexpression of wild-type Drl protein [85]. While the authors interpreted these findings as evidence for an autoregulatory role for DRL PTK activity (for which no evidence was provided), other functions of the intracellular domain may be affected by K371A and/or increased gene dosage in these transgenic flies. In contrast, Yoshikawa et al. found no effect of overexpression of a similar $DRL^{\Delta intra}$ protein on axon pathfinding in the *Drosophila* VNC [9], suggesting that any autoregulatory role of the DRL intracellular region inferred from gain-of-function phenotypes is specific to the MB.

Detailed biochemical and structural analyses of a representative RYK subfamily PTK domain are needed to conclusively establish whether members of this subfamily are genuine Group 2 pseudokinases or if they possess the structural plasticity to exhibit PTK activity under a highly specific set of conditions [77, 78, 86]. Alternatively, RYK subfamily-specific changes to the PTK domain may indeed reflect the loss of ATP-binding and phosphotransferase activity, but with retention of the capacity to function as an allosteric activator of, or scaffold for, interacting proteins [55]. The nuclear-localized intracellular fragment of the ErbB4 RTK has, for example, been demonstrated to complex with and chaperone the nuclear translocation of transcriptional regulators [87]. Another possibility is that RYK subfamily members have evolved to catalyze an orthogonal biochemical transformation, such as the phosphorylation of nonprotein (e.g., lipid) substrates.

15.4.5 Protein Expression Patterns

15.4.5.1 Mammals

Immunohistochemical analysis of human RYK protein has revealed expression in islets of Langerhans in the pancreas; hepatocytes in the liver; tubular cells in the kidney; enterocytes on the villus tips in the small intestine; epithelium and smooth muscle in the vas deferens; epithelium of the Fallopian tubes; vascular smooth muscle, surface epithelium, smooth muscle, and stroma of the ovary; lobular acini and ductal epithelium of the breast; vascular smooth muscle of the lung; myocardium and blood vessels in the heart; red pulp of the spleen; sinus histiocytes in lymph nodes; squamous epithelium and lymphoid follicles in the tonsil; epithelial cells of the thyroid gland; blood vessels and ependyma of the brain; secretory but not proliferative endometrium; skeletal muscle; and a notable lack of signal in the prostate gland [79].

Immunohistochemical analyses of mouse Ryk revealed a broad distribution of expression [23, 36]. The collecting tubule epithelium, but not glomeruli, immediately beneath the renal cortex displayed strong staining that decreased towards the pelvic region. E16 hair follicles showed strong expression of Ryk. Hepatocytes in liver lobules and E14 myocardium were uniformly stained. Expression in the spleen was restricted

to the red pulp. The small intestine showed staining associated with villi on enterocytes and stroma, but not crypts. The mucosa of the large intestine had staining on connective tissue surrounding intestinal glands. The adrenal cortex, but not medulla, showed strong Ryk expression.

Ryk was expressed on descending corticospinal tract (CST) axons in the neonatal mouse spinal cord [88]. Co-expression of Ryk with mouse TUJ1 (βIII-tubulin), a marker of immature neurons, and rat MAP2 (microtubule-associated protein 2), a marker of mature neurons, was observed in the cortical plate [31, 39, 89]. In 35 % of TUJ1$^+$ mouse neurons, Ryk was localized to the nucleus [39]. Co-expression with the neural stem cell marker Nestin was demonstrated in the VZ [38, 39, 89]. In mouse Nestin$^+$ neural stem cells, Ryk was localized to the nucleus in only 10 % of cells [39]. Rat Ryk protein expression was observed in neural pregenitor cells (NPCs) and neurons in the forebrain (VZ and cortical plate) and embryonic spinal cord [89]. All neurons in primary cultures of rat cerebrum expressed Ryk, as well as many oligodendrocytes, O-2A progenitor cells, and type 2 astrocytes but not type 1 astrocytes or microglia.

Immunofluorescent analysis of pyramidal neurons from sensorimotor cortex isolated from 0- to 3-day-old golden Syrian hamsters and cultured for 2–3 days before staining showed Ryk expression over entire neurons [90]. All layers of the mouse cortex, and callosal axons and fascicles as they cross the midline and enter the contralateral hemisphere, were observed to express Ryk protein at E18 [91].

Analysis of Ryk on cells from adult mouse bone marrow by flow cytometry revealed expression on 23.6 % of hematopoietic stem cells, with subsequent downregulation during the transition to lineage-restricted multipotent progenitors [92]. A notable exception was the megakaryocyte progenitor population, of which 29.6 % maintained Ryk expression. These findings are consistent with transcriptome-profiling studies [93, 94], indicating concordance of *Ryk* mRNA and protein levels and their general decline as hematopoietic stem cells differentiate into lineage-committed progenitor cells.

15.4.5.2 Invertebrates

Third instar *Drosophila* larvae expressed DRL in the muscle at glutamatergic neuromuscular junctions [95]. Mesodermal precursors to *Drosophila* ventral body wall muscles 21–23 in embryonic abdominal hemisegments A2–A7 began to express DRL at 10 hpf. As they extended towards their epidermal attachment sites, they upregulated *drl* mRNA and protein by hour 13. During attachment site selection, DRL was enriched near the tips of muscles 21–23. Following the completion of attachment at 15 hpf, *drl* protein and mRNA were downregulated. Stripes of DRL expression 3–4 cells wide in the epidermis of abdominal hemisegments A2–A7 were detectable at hour 6; these subsequently became restricted to cells near the segmental grooves. Posterior expansion of the DRL expression domain from the lateral aspects of the grooves at hour 9.5 generated broad patches of DRL$^+$

epidermal cells that overlaid the developing progenitors of muscles 21–23. These patches condensed by hour 11, each segregating into two clusters located near the dorsal and ventral attachment sites of muscles 21–23. Further refinement of DRL expression to discrete 15-cell clusters occurred by 12.5 hpf; these clusters abutted and partially overlapped the epidermal attachment cell clusters for muscles 21–23. Expression in the epidermis was undetectable by 15 hpf [96].

Expression of DRL in the developing embryonic brain was observed on commissural tracts close to axonal growth cones from the MB primordia [97]. Interhemispheric DRL^+ glial cells were found in the third instar larval brain, which wrapped around MB lobes [97–99]. At the same stage, DRL expression was also observed in thoracic and abdominal ganglia, the optic lobes and on afferent photoreceptor axons [97].

In the developing pupal olfactory system of *Drosophila*, DRL was expressed on the dendrites of projection neurons (PNs) located within the neuropil of the AL and by glial cells found in the commissure connecting the bilateral ALs [20, 84, 100]. DRL-2 protein was observed on largely distinct cell populations: initially on olfactory receptor neurons (ORNs) navigating towards the AL in a lateral tract and in a distinct region adjacent to the site of ORN exit from the dorsal AL [20]. Increasing numbers of ORNs in both lateral and medial axon tracts expressed DRL-2 at later developmental stages. DRL-2 was also present in several AL glomeruli [20].

15.5 Insights into the Biological Roles of RYK

15.5.1 RYK Extracellular Region

WIF-1 is a secreted protein that binds members of the *W*ingless/*i**nt*-1 (Wnt) family via an amino-terminal domain [52]. Patthy was the first to report significant homology between this region of WIF-1 and a large portion of the extracellular region of RYK subfamily members, dubbing the conserved sequence the WIF domain [10] (Figs. 15.1 and 15.2). This observation implied that the RYK WIF domain would bind Wnt proteins; a prediction resoundingly verified by the landmark identification of WNT5 as a ligand of DRL in *Drosophila* by John B. Thomas and colleagues in 2003 [9].

15.5.1.1 Wnt-Activated Signaling Cascades

Encoded by five genes in *C. elegans*, seven in *Drosophila*, and 19 in mammals, the Wnts are a large family of evolutionarily ancient secreted glycoproteins acylated at one or two residues [101, 102]. Via regulatory interactions with their agonists/antagonists [72, 102, 103] and engagement of cell surface receptors [104], Wnts modulate a wide variety of downstream signaling pathways in a highly context-dependent fashion [105] to achieve pervasive regulatory control over an astonishing variety of biological processes during embryonic development and adult

physiology [106], as well as—when deregulated—in pathologies such as cancer, neurologic and inflammatory conditions, and disorders of endocrine function and bone homeostasis [107–109].

Wnts activate two biochemically distinct classes of cell signaling pathways: those dependent versus those independent of β-catenin as a signaling intermediate. Formation of a cell surface complex between Wnt and transmembrane Frizzled (Fzd) and LRP5/6 chains induces conformational changes in the receptors that are transduced into the cell interior. Subsequent phosphorylation of the LRP cytoplasmic tail leads to inhibition of the activity of a large destruction complex which otherwise functions to create a GSK3- and CK1α-dependent phosphodegron on nascent β-catenin and promote its degradation by the 26 S proteasome [110]. Consequently, β-catenin is transiently stabilized and can translocate to the nucleus where it governs context-dependent transcriptional responses via association with members of the TCF/LEF transcription factor family (see the Wnt homepage for Wnt target genes: http://www.stanford.edu/group/nusselab/cgi-bin/wnt; viewed November 2014).

β-Catenin-independent, also referred to as noncanonical or alternative, Wnt signaling pathways are more heterogeneous with respect to molecular mechanisms and may simultaneously inhibit β-catenin-dependent signaling [111]. In general, these pathways regulate tissue morphogenesis through control of cell migration and polarization, rather than proliferation or fate specification, but there are exceptions. A major determinant of the specific signaling pathway activated and the consequent cellular response to a Wnt is likely conferred by cellular context with respect to factors such as the expressed repertoire of receptors and signal transducers, rather than by a unique property of individual or subclasses of Wnt proteins [102].

15.5.1.2 Insights from Vertebrate Model Organisms

Mouse Ryk co-immunoprecipitated Wnt1 or Wnt3a when both Ryk and Wnt were overexpressed in HEK293T cells, and their presence in a common complex was dependent upon the Ryk extracellular region [57]. The mouse Ryk extracellular region also co-immunoprecipitated Wnt5a in a WIF domain-dependent manner from lysates of transfected HEK293T cells [91]. Biochemical analyses revealed that mouse Ryk bound directly to Wnt1 and Wnt3a with dissociation constants of 7.9 nM and 4.5 nM, respectively [88, 112], demonstrating that Ryk is a high-affinity Wnt receptor. The Ryk extracellular region co-immunoprecipitated the cysteine-rich Wnt-binding domain of mouse Fzd8 in a Wnt1-dependent manner from lysates of transfected HEK293T cells, demonstrating the likely formation of a heteromeric receptor complex [57].

Most mice transgenic for a *Ryk* siRNA died after birth [57] as observed with $Ryk^{-/-}$ neonates [33]. Analysis of *Ryk* siRNA embryos at E10.5 revealed that the glossopharyngeal and vagus nerves were connected prematurely and that axons of craniofacial motor neurons were less fasciculated. Axons in ophthalmic nerves were also less fasciculated and strayed posteriorly in E10.5 *Ryk* siRNA embryos. Dorsal root ganglion (DRG) explants revealed that Ryk function was required for Wnt3a-induced neurite outgrowth.

In the visual system, images are represented by the spatial activation pattern of a two-dimensional sheet of retinal ganglion cells (RGCs). During development, RGCs project axons to the superior colliculus (SC) or its nonmammalian homolog, the optic tectum (OT). Each axis of the retina (dorsal–ventral, DV; temporal–nasal, TN) is mapped to a corresponding axis of these midbrain visual centers (medial–lateral, ML; anterior–posterior, AP; respectively) independently using different receptor/guidance molecule systems expressed in gradients. The outcome is the representation of each point in the retina with a point within the target, thereby forming a retinocollicular or retinotopic map [113]. Positional information is transduced by EphB receptors, expressed on pathfinding RGC axons in a V>D gradient, in response to an attractive M>L gradient of ephrinB in the SC/OT.

However, these gradients are insufficient to account for establishment of the ML map, and in 2006 Yimin Zou and colleagues provided compelling evidence for transduction of a repulsive signal by RGC axons via a V>D gradient of Ryk expression in response to a M>L Wnt3 gradient in the chick OT and mouse SC [112]. In support of their model, Wnt3 overexpression in the chick OT caused RGC axons to avoid the expression zone, while expression in chick RGCs of a dominant-negative Ryk lacking its intracellular region caused their termination zones in the OT to be shifted medially, as predicted, and in a direction opposite to that of RGC axons in which loss of EphB function had been engineered. Thus an attractive ephrinB gradient and a repulsive Wnt3 gradient in the same direction function in a complementary fashion to pattern the ML axis of the SC/OT.

The mammalian spinal cord functions like an axon superhighway, with outbound descending projections providing motor or regulatory innervation and ascending inbound axons delivering sensory input to higher brain centers. Wnts provide important developmental pathfinding signals to navigating axons that direct them to project in an anterior or posterior direction, along the longitudinal axis of the spinal cord. An A>P gradient of mouse Wnt4 in the floor plate was demonstrated to provide an attractive signal for ascending commissural axons transduced by the Fzd3 receptor after midline crossing [114]. In contrast, Wnt1 and Wnt5a were expressed in the neonatal dorsal spinal cord to form an A>P concentration gradient. These latter parallel Wnt gradients repelled descending corticospinal tract (CST) axons, which entered from the brain, in a posterior direction [88]. Insensitive to Wnt due to a lack of Ryk expression during the initial phase of their trajectory, the CST axons crossed the midline, entered the dorsal funiculus of the spinal cord, and then began to express Ryk. Significantly, intrathecal injection of a neutralizing anti-Ryk polyclonal antibody blocked the posterior growth of CST axons [88].

In the mouse corpus callosum, a large forebrain commissure that connects the left and right cerebral hemispheres to facilitate communication between them, *Ryk*$^{-/-}$ axons crossed the midline but then showed aberrant trajectories and failed to fasciculate upon reaching the contralateral side [91]. Wnt5a was expressed in areas around the corpus callosum, and Wnt5a repelled Ryk-expressing but not *Ryk*$^{-/-}$ axons growing from cortical explants in vitro [91]. It may be that Ryk activity is subject to fine temporal regulation in callosal neurons crossing the midline such that while on the ipsilateral side, the chemorepulsive signal it transduces is silenced.

Using cortical slices or dissociated cortical neurons from hamsters in vitro, Wnt5a induced Ryk- and Fzd-dependent repulsive turning and simultaneously increased Ryk-mediated axon outgrowth, revealing a potential mechanism for the directed propulsion of cortical axons in vivo [90, 115].

In addition to the spinal cord and corpus callosum, a Wnt5a gradient is an important cue for the growth and guidance of mouse ventral midbrain (VM) dopaminergic (DA) neurons. Dysfunction of DA neurons, which regulate voluntary movement, emotion, and reward, is implicated in serious human neurological and psychiatric disorders, including Parkinson's disease, schizophrenia, attention-deficit/hyperactivity disorder, addiction, and major depressive disorder. Wnt5a promoted mouse VM DA axon elongation, retraction, and repulsion in a temporally regulated manner, which could be antagonized by a neutralizing anti-Fzd3 antibody or Wnt sink comprising the human RYK extracellular region fused to the hinge and Fc regions of human IgG_1 (RYK-Fc) [116]. A neutralizing anti-Ryk monoclonal antibody was used to demonstrate in vitro a requirement for Ryk in DA progenitor proliferation, differentiation, and connectivity [32]. In agreement, *Ryk*$^{-/-}$ mice showed fewer DA neurons as a result of depleted progenitor and precursor populations, thus revealing Ryk as an important mediator of midbrain DA neuron development.

An examination of neurogenesis in *Ryk*$^{-/-}$ mice revealed strong reduction in the expression of neuronal markers but no change in the number, proliferation, or death of NPCs in the embryonic forebrain [39]. Ryk was demonstrated to be necessary for Wnt3-mediated differentiation of NPCs into neurons in vitro and in vivo. The mode of signaling by Ryk involved sequential proteolysis of its extracellular and transmembrane regions, the latter event mediated by γ-secretase, and Wnt3-dependent nuclear localization of Ryk-ICF. Kamitori et al. [31] demonstrated that overexpression of Ryk in embryonic cortical slice cultures suppressed cell migration from the VZ to the pial surface, revealing an additional role for Ryk in cortical development.

During development of the mouse ventral telencephalon, Ryk was demonstrated to regulate NPC choice between the oligodendrocytic and GABAergic neuronal differentiation pathways in ventral progenitor cells [34]. This function of Ryk was cell autonomous and executed via upregulation or inhibition of expression of the pivotal cell-fate determinants Dlx2 and Olig2, respectively Ryk mediated the Wnt3a-induced promotion of differentiation into GABAergic neurons and inhibition of oligodendrocyte differentiation. The Ryk-ICF was necessary and sufficient for this activity, indicating that its ability to regulate NPC fate was perhaps mediated by a role in modulating nuclear gene expression.

Ryk$^{-/-}$ mice were created by targeted insertion of a promoterless βgeo reporter cassette into the *Ryk* gene, creating a null allele. Knockout mice died on the day of birth; 88 % had a complete cleft of the secondary palate due to abnormal positioning of the tongue at E13.5–14.5 [33]. Initial characterization of *Ryk*$^{-/-}$ postnatal day (P) 0 mice showed growth retardation and skeletal defects, including a smaller and more rounded skull, shortened snout and nasal bones, reduced mandible size, flattened midface, reduced length of long bones, and laterally splayed hind limbs [33].

Planar cell polarity (PCP) refers to the polarization of cells and cell sheets in the plane of a tissue and, by extension, to the signaling pathways controlling it [117]. It is orthogonal and complementary to apico-basal cell polarity, but PCP is not restricted to epithelial tissues and also operates in mesenchymal cells where it can regulate migration and cell intercalation. Two molecular systems regulate PCP behavior: the core and Fat–Dachsous pathways; a central feature of both is the asymmetric distribution of their components [118]. *Ryk* interacted genetically with *Vangl2*, a core PCP signaling component, to generate typical PCP phenotypes during embryonic mouse neural tube closure, elongation of the AP body axis, growth of long bones in the limbs, forelimb digit development, rib/sternum and eyelid development, craniofacial morphogenesis, and orientation of stereociliary bundles in hair cells of the cochlea [35, 119]. Concordance of all phenotypes with those of *Wnt5a*$^{-/-}$ embryos, with the exception of the absence of additional stereociliary hair cell rows and neural tube defects in *Wnt5a*$^{-/-}$ embryos and differences in the severity of skeletal defects, plus the finding that the embryonic *Wnt5a*$^{-/-}$ phenotype was not exacerbated by hetero- or homozygosity for the *Ryk* null allele, indicated that this ligand is the major Wnt regulating PCP via Ryk. Macheda et al. [119] presented evidence for genetic interaction between *Ryk* and *Wnt11* during zebrafish embryonic convergent extension (CE), demonstrated defective orientation of stereociliary bundles in the third outer hair cell row of the cochlea in *Ryk*$^{-/-}$ embryos, and detected genetic interaction of *Ryk* with the hypomorphic *Looptail* allele of *Vangl2*, *Vanlg2*^{Lp}, during neural tube closure and orientation of stereociliary bundles in hair cells of the cochlea.

In *X. laevis*, Ryk was essential for normal CE movements during gastrulation [41]. XRyk co-immunoprecipitated Wnt5a and Wnt11, while *XRyk* knockdown using morpholino oligonucleotides rescued defects resulting from overexpression of Wnt11. In zebrafish, knockdown of *Ryk* produced overlapping phenotypes with those resulting from knockdown of *Wnt5b* and involved defective CE during extension of the AP axis and disrupted retinal development [40]. *Ryk* and *Wnt5b* knockdown morpholinos acted synergistically to perturb these gastrulation-associated CE movements, with Ryk functioning downstream of Wnt5b to transduce signals regulating the polarity of cell projections and directional cell migration [40].

15.5.1.3 Insights from Invertebrate Model Organisms

The *Drosophila* mutants *drl* and *lio* were isolated independently in separate laboratories performing genetic screens, but were subsequently found to be allelic [17, 19]. One of the best-characterized phenotypes of the mutants is the inappropriate projection of interneuron axons across the midline within the VNC. In wild-type flies, axons of *drl*-expressing neurons cross the midline within the AC and then turn anteriorly and fasciculate to form two distinct axon bundles in the longitudinal connectives. In *drl* mutants, axons cross the midline inappropriately via both the AC and the posterior commissure (PC), and the axons subsequently fail to fasciculate [17, 43]. Axons of PC neurons ectopically overexpressing DRL were forced to cross the midline via the AC [43].

In a clever genetic screen for suppressors of the ability of DRL to switch axons to the AC when misexpressed by PC neurons, *Drosophila Wnt5* (formerly known as *Dwnt3*) loss of function was identified [9]. Mutation of *wg* or *Wnt4*, encoding other *Drosophila* Wnts active in the embryonic CNS, did not exhibit any suppression of DRL-mediated axon switching, demonstrating specificity in the interaction of *drl* with *Wnt5* [9]. Like *drl* mutant animals, the Wnt5 phenotype involved misprojection of AC axons [120], and experiments involving ectopic central nervous system (CNS) expression of Wnt5 specifically affected AC axons in a DRL-dependent manner [9]. Physical interaction of the DRL extracellular region with Wnt5, which displayed an expression domain complementary to that of DRL [9, 121], was also demonstrated [9].

Several brain phenotypes have been described in *drl*/*lio* mutants. These include defects in all structures of the adult protocerebrum: the MBs, involved in olfactory learning and memory; the central complex, which connects the left and right hemispheres and may control information transfer between them; and the optic lobes. *drl*/*lio* mutants showed disrupted 3 h memory after olfactory associative learning, without effects on the perception of odors or electroshock [18, 122]. DRL overexpression resulted in the loss of some or all MB lobes, while the *drl* mutant phenotype was rescued by expression of DRL in neurons extrinsic to the MB [85, 97]. *drl* mutants displayed defects in the glial transient interhemispheric fibrous ring, a structure present only during late larval to pupal development and that may serve as a scaffold for brain development [84, 98, 99, 122]. Alterations in activity-dependent dendritic branching of serotonergic neurons in the CNS were phenocopied in *Wnt5* and *drl* mutants [123].

Studies of *Drosophila* olfactory system development have revealed surprising diversity in the modes of signaling employed by RYK subfamily members. Environmental odorants activate combinations of the approximately 1,300 ORNs that innervate the olfactory epithelium of the antennae and the maxillary palps [124]. Each ORN expresses one or two of the repertoire of 50 different odorant receptors (ORs), and ORN axons expressing a given OR together converge onto one of 50 structures termed glomeruli in the AL (equivalent to the vertebrate olfactory bulb) of the brain. This discrete connectivity map represents odor information as a combination of activated glomeruli in the AL [125]. Within glomeruli, ORN axons synapse with local interneurons and dendrites of PNs with high specificity. PNs then relay afferent olfactory information to higher brain centers, including the MB and protocerebrum [124]. The patterning of the mammalian olfactory system is similar to that of *Drosophila*.

Antagonistic roles of WNT5 and its receptor DRL were found necessary for correct formation of the glomerular map in the *Drosophila* AL [84]. A hierarchy of gene activity was established in which glial expression of *drl* was necessary to locally repress *Wnt5* activity on ingrowing ORNs mediated by a Wnt receptor that was unidentified at the time. The DRL WIF domain was essential for this antagonistic activity towards *Wnt5*, but its intracellular region was dispensable, leading the authors to propose a model whereby DRL mediates sequestration and/or endocytosis of WNT5, which was observed to accumulate in *drl* mutants.

Drl-2 mutants, in which expression by subsets of growing ORN axons was lost, had mild AL glomerular defects. However, the glomerular phenotype in *drl*; *Drl-2* double mutants was more severe than that of *drl* mutants and more closely phenocopied the *Wnt5* mutant [20]. The aberrant positioning of glomeruli within the AL induced by overexpression of *Wnt5* in ORNs was ameliorated in a *Drl-2* mutant background, identifying DRL-2 as the receptor for WNT5 on ORNs. Surprisingly, ectopic expression of DRL-2 in the glia of *drl* mutants rescued glomerular phenotypes expected to result from the loss of antagonistic DRL activity towards WNT5 [20]. Thus the closely related DRL and DRL-2 receptors can functionally substitute for each other in glia and perform opposing functions depending upon the cell type in which they are expressed.

DRL was also demonstrated to function in the targeting of the 50 different types of PN dendrites to appropriate glomeruli in the *Drosophila* AL [100]. Positional information provided in a dorsolateral-to-ventromedial (DL>VM) gradient of WNT5 secreted by AL-extrinsic dorsolateral cluster (DLC) neurons, and establishment of a topographically similar gradient of DRL expression on PNs, was necessary for the rotational rearrangement of dendritic arbors at 16–30 h after puparium formation. DRL function was cell autonomous in the PNs and its intracellular region essential for correct dendrite patterning, suggesting the transduction of a forward signal. However, DRL and WNT5 acted antagonistically to position PN dendrites, and a model in which the level of DRL expression is instructive for targeting PN dendrites in a DL>VM gradient of WNT5 and an opposing DL<VM repulsive gradient of SEMA-2A/B was proposed.

Normal neuromuscular junction (NMJ) formation in *Drosophila* larvae at muscles 6 and 7 requires *Wnt5* and *Drl* function [95]. Genetic rescue experiments demonstrated a hierarchical requirement for *Wnt5* in the motor neuron upstream of *drl*, which most likely functioned at the muscle membrane to transduce a forward signal given the requirement for its WIF domain and intracellular region. Inaki et al. [47] studied determinants of motor neuron target specificity on adjacent ventral muscles 12 and 13 and demonstrated a chemorepulsive function for *Wnt4* expressed by muscle 13 in preventing innervation by motor neurons that normally form a NMJ on muscle 12. The activity of *Drl-2*, expression of which was limited to a subset of CNS neurons, was proposed to account for why WNT4 was repulsive to only specific subsets of motor neurons.

RYK subfamily members also control migratory events outside the nervous system during *Drosophila* development. Embryos mutant for *drl*, *dnt*, or *Wnt5*—but not *Drl-2*—all have defects in the guidance and attachment of a subset of lateral transverse muscles (numbers 21–23) to tendon cells in the epidermis, such that attachment sites are more ventral than in wild-type embryos [96, 126]. Overexpression of *dnt* in *drl* mutants only partially rescued the muscle attachment phenotype [21]. Abnormal curvature and positioning of the salivary glands were observed in *drl* mutant embryos as a result of insensitivity of late-stage migrating gland tips to chemorepulsive WNT5 produced by the CNS [25]. Both *dnt* and *Drl-2* single mutants showed similar salivary gland positioning defects with *drl*, though at lower penetrance, while *drl*; *Drl-2* double heterozygotes had a similar penetrance of salivary gland positioning defects as embryos homozygous for *drl* or *Drl-2*.

The *drl* mutant showed a strong genetic interaction with *Neurotactin* (*Nrt*), which encodes a cell adhesion protein involved in neural development [127]. In *drl*; *Nrt* embryos, severe misguidance and stalling phenotypes in both DRL$^+$ and DRL$^-$ axons were observed in many segments, revealing a nonautonomous requirement for DRL in the latter. A genetic interaction between *lio/drl* and the *no bridge* (*nob*) mutant, so named on the basis of a structural defect in the protocerebral bridge of the central complex affecting locomotion, was described by Hitier et al. [98]. However, the genetic lesion responsible for the *nob* phenotype has not been identified.

The *C. elegans lin-18* mutant was originally identified in a screen for developmental vulval phenotypes [128], but the gene disabled by the mutation was only later identified as a *Ryk* ortholog [50]. An epidermis-derived tube, the hermaphrodite *C. elegans* vulva forms during larval development to provide a conduit for the passage of sperm and eggs by connecting the uterus to the external environment [129]. In response to Notch, Ras, and Wnt signaling pathways, VPCs (P5.p, P6.p, and P7.p) adopt cell fates representing stereotypical lineage patterns (2°, 1°, and 2°, respectively) that together generate 22 progeny. Subsequent differentiation and morphogenetic changes generate the adult vulva, a stack of concentric cellular toroids surrounding a central lumen.

The 1° and 2° fates are distinguished by both the types of terminally differentiated cell types produced and their pattern of cell divisions (with respect to the number and axis of division). The 2° VPCs P5.p and P7.p produce identical lineages except that their patterns are arranged in opposite AP orientations. Genetic studies have revealed that two *C. elegans* Wnt receptors, LIN-17/Fzd and LIN-18/Ryk, function in parallel but largely undefined pathways to specify the division orientation of the 2° lineage of P7.p in response to the Wnts LIN-44 and MOM-2, respectively [50, 130–132]. In *lin-17* or *lin-18* mutants, the P7.p lineage often exhibits a reverse, P5.p-like, orientation such that vulval mirror-image symmetry is lost. This reversal in polarity of the P7.p lineage results in a second invagination posterior to the main vulva, a phenotype originally termed bivulva [128, 130] but now known as *p*osterior-*r*eversed *v*ulval *l*ineage (P-Rvl) to distinguish it from reversal of P5.p lineage polarity (*a*nterior-*r*eversed *v*ulval *l*ineage, A-Rvl) or simultaneous reversal in P5.p and P7.p polarity (the AP-Rvl phenotype) [132]. The *lin-18/Ryk* mutant also displayed reduced migration of anterior lateral microtubule (ALM) neurons [133]. In *lin-18*; *cfz*-2 animals, the ALM neuron undermigration phenotype was suppressed, suggesting activity of LIN-18/Ryk as an antagonist of CFZ-2/Fzd function during ALM neuron migration.

Investigations of diversity in the molecular and genetic control of vulva induction have employed *C. elegans* and the satellite model nematode *P. pacificus* [134]. Three features of vulva induction were found to distinguish *P. pacificus* from *C. elegans*. First, Wnt signaling rather than EGF/RAS and Notch signaling functioned in *P. pacificus* vulva induction [135]. Second, the somatic gonad and the posterior body region were redundantly involved in vulva induction by supplying distinct Wnts [135]. Third, appropriate inductive activity of a posterior source of *Ppa*-EGL-20/Wnt involved refinement of its gradient by an evolutionarily novel ligand sequestration function of

Ppa-LIN-17/Fzd and the acquisition of novel protein-binding motifs in *Ppa*-LIN-18/Ryk [136]. Analysis of double and triple mutants established that *Ppa-egl-20*/*Wnt*, *Ppa-mom-2*/*Wnt*, and *Ppa-lin-18*/*Ryk* functioned redundantly in vulva induction such that triple mutants were vulvaless [135]. Although genetic analysis indicated that *Cel*-MOM-2/Wnt and *Cel*-LIN-18/Ryk form a ligand/receptor pair [50], synergism was detected between *Ppa-mom-2*/*Wnt* and *Ppa-lin-18*/*Wnt*, suggesting that they functioned in parallel pathways during *P. pacificus* vulva induction. A cell-autonomous function of *Ppa*-LIN-18/Ryk in transducing the *Ppa*-EGL-20/Wnt signal during vulva differentiation was established [136]. However, *Ppa*-LIN-18 had only a minor role in polarization of the P7.p lineage [134, 135].

15.5.2 RYK Transmembrane Helix

Evidence for WNT5-stabilized cell surface hetero- and homo-oligomerization among DRL, DRL-2, and DNT was reported by Fradkin and colleagues [54]. These authors demonstrated that the DRL transmembrane (TM) helix translationally fused to a DNA-binding domain could mediate oligomerization in the *Escherichia coli* periplasmic membrane as monitored by activation of a reporter gene. Using the same experimental system to quantify the propensity for self-interaction of all 58 human RTK TM helices, RYK was ranked tenth strongest [137]. However, transgenic expression of DRL^{T245V}, bearing a substitution in the TM helix that abrogated oligomerization in *E. coli*, was phenotypically indistinguishable from wild-type DRL in a *Drosophila* gain-of-function commissure-switching assay. This finding, together with the unexplored roles of other regions of DRL in promoting self-association, leaves the mode of Wnt-stimulated oligomerization unresolved [54].

15.5.3 RYK Intracellular Region

15.5.3.1 Catalytically Competent PTKs

RYK orthologs have been reported to interact genetically and physically with catalytically competent PTKs from the Eph receptor and Src subfamilies [25, 33, 44, 54, 138] (Figs. 15.3b and 15.4). Mammalian Ryk immunoprecipitated with EphB2 and EphB3 from lysates of cotransfected cells or mouse E12.5–13.5 head and adult brain lysates [31, 33, 69]. Under these conditions, Ryk was phosphorylated on tyrosine residues but not when co-expressed with a kinase-inactive EphB3 [33]. Furthermore, cleft palate in EphB2/EphB3-deficient embryos [139] was phenocopied in *Ryk*-deficient mice, suggesting a developmental requirement for these interactions in vivo [33].

Mutations in the *Drosophila Src64B* or *Src42A* genes underlie developmental defects in the MB, salivary glands and AC that closely resemble those of *drl* or *Wnt5* mutants. That Src family kinases (SFKs) operate downstream of DRL is

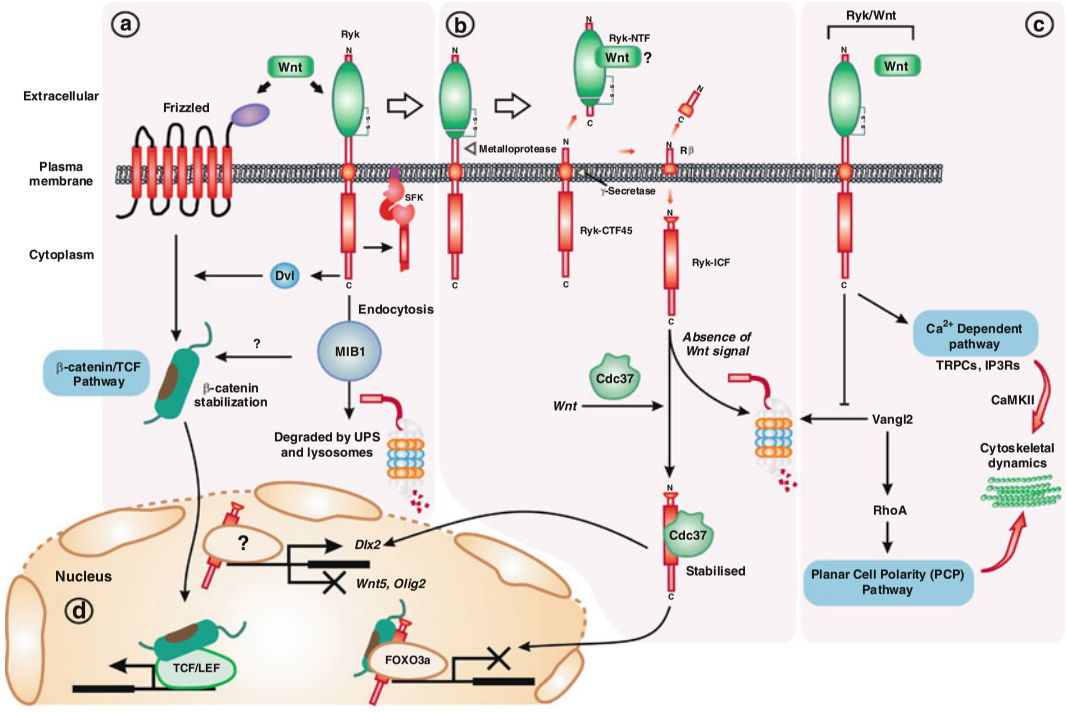

Fig. 15.4 Schematic representation of signal transduction events associated with Ryk subfamily members. (**a**) Ryk binds Wnt ligands to modulate signaling via Fzd receptors, either in a common receptor complex or through the activation of convergent pathways. The involvement of Dvl binding, and MIB1-mediated endocytosis and degradation of RYK, in activating the β-catenin/TCF pathway is shown. Wnt/Ryk-dependent, but Fzd-independent, activation of SFKs operates in *Drosophila* to trigger uncharacterized pathways. (**b**) Sequential proteolysis of Ryk sheds Ryk-NTF (a potential Wnt sink) and liberates Ryk-ICF into the cytoplasm. Uncharacterized Wnt-dependent signals rescue Ryk-ICF from degradation by promoting Cdc37-mediated stabilization and translocation to the cell nucleus. (**c**) Wnt/Ryk complexes can regulate PCP behaviors by stabilizing Vangl2 protein and activating RhoA and/or promoting Ca^{2+}-dependent downstream signaling via the activation of TRPCs and IP3Rs. (**d**) Nuclear consequences of Wnt/Ryk signaling involve induction of transcription at β-catenin/TCF-responsive promoters (*left*); modulation of transcription at the *Drosophila Wnt5* or mouse Dlx2 and Olig2 promoters by Ryk-ICF (*center*); and/or downregulation of transcription from FOXO3a-responsive promoters by Ryk-ICF in a β-catenin-scaffolded complex (*right*)

supported by experiments that demonstrated genetic interaction of *Src64B* with *Wnt5a* and *drl* in MB development [138], salivary gland migration [25] and commissural axon projection within the VNC [44]. In vitro assays demonstrated that SRC64B and DRL or mouse Ryk were co-immunoprecipitated; formation of a complex containing DRL and SRC64B resulted in SRC64B kinase activity-dependent DRL phosphorylation on tyrosine residues and increased SRC64B activation [44]. Recruitment of SRC64B to DRL was promoted by oligomerization of the receptor and required the SH2 and native PTK domains of SRC64B plus the C-terminus and native PTK-like domain of DRL [54]. However, DRL lacking either the WIF domain or the intracellular region did not activate SRC64B [44].

15.5.3.2 The Cdc37 Co-Chaperone Protein

A yeast two-hybrid screen using mouse Ryk-ICF as bait identified binding to Cdc37 [62]. A large fraction of the eukaryotic kinome requires association with the Cdc37–Hsp90 complex for maturation and/or stabilization [140]. Specific recruitment of protein kinases to the Hsp90 chaperone is mediated by a scaffold function of Cdc37. Simultaneous binding of protein kinase and Hsp90 by Cdc37 permits coupling of the chaperone's ATPase cycle to conformational changes in the kinase [141]. The C-lobe of the mouse Ryk PTK domain (residues 367–579) mediates a direct Wnt-dependent interaction with the carboxyl-terminus of Cdc37, which rescues the otherwise highly labile Ryk-ICF from ubiquitylation and degradation by the 26 S proteasome, permitting its translocation to the cell nucleus [62]. A requirement of Hsp90 chaperone function for Ryk-ICF stabilization was demonstrated by the accumulation of Ryk-ICF–polyubiquitin conjugates in the presence of a 20 S proteasome inhibitor or a highly specific inhibitor of the ATPase cycle of Hsp90 [62].

15.5.3.3 Mindbomb 1

Among other proteins, Berndt et al. [69] identified the ubiquitin E3 ligase Mindbomb 1 (MIB1) as an interacting partner of human RYK. Although they did not define the region of the RYK intracellular region that mediates interaction with MIB1, a region of MIB1 including the amino-terminal zinc finger was essential for coprecipitation of the two proteins from HEK293T cell lysates. MIB1 promoted polyubiquitylation of the RYK intracellular region and its subsequent degradation in lysosomes or by the 26 S proteasome. MIB1 mediated the internalization of RYK on Rab5-positive endosomes, and a complex containing the two proteins (MIB1 and RYK) was necessary for events proximal to β-catenin stabilization and signaling in response to Wnt3a ligand. RNAi targeting *Cel-mib-1* generated a bivulva (P-Rvl) phenotype resembling that of the *lin-18*/*Ryk* mutant, and the penetrance of this phenotype was increased in a *lin-18*/*Ryk* background by RNAi targeting *Cel-mib-1* [69].

15.5.3.4 PDZ Domain-Containing Proteins

The PDZ domain derives its name from the three proteins in which the module was first recognized: *P*SD95, *D*lg, and *Z*O1. PDZ domains (80–100 amino acids in size) mediate interprotein interactions, typically by binding short peptide motifs at the extreme carboxyl-termini of target proteins and less commonly by recognizing an internal sequence motif in the target protein [142].

The extreme carboxyl-terminus of RYK orthologs is highly conserved (Fig. 15.2); in mouse Ryk, it was demonstrated to be a ligand for the single PDZ domain of AF-6 (also known as Afadin in mammals or Canoe in *Drosophila*) [33, 143]. The mouse Ryk carboxyl-terminus was also shown to be a ligand for the PDZ domain of the cytoplasmic protein Dishevelled (Dvl)-1, Dishevelled-2, and/or Dishevelled-3, demonstrated by co-immunoprecipitation from HEK293T cells and mouse brain [57].

15.5.3.5 Others

Berndt et al. [69] employed affinity purification of human RYK-interacting proteins and their subsequent identification by mass spectrometry. A total of 66 proteins that passed stringent criteria were identified; most were functionally classified as participating in the cell cycle/apoptosis (e.g., DNA-PKcs), cell adhesion (e.g., β-catenin), desmosomes (e.g., DSG2), cell–cell signaling (e.g., GPR125), ubiquitylation/trafficking (e.g., UBC), PCP (e.g., CELSR2), and actin remodeling (e.g., WASF1). *Xenopus* β-arrestins (βarr) 1 and 2 were found to co-immunoprecipitate with Ryk from cotransfected HEK293T cells and modulate Wnt-dependent endocytic trafficking during convergent extension in *Xenopus* embryos [41, 144].

The *drl* mutant allele *linotte* (*lio*) interacted genetically with *castor*, a zinc finger transcription factor involved in post-embryonic brain development [145]. In a screen for genes that regulated the nuclear–cytoplasmic ratio of GFP-tagged LIN-18-ICF expressed from a transgene, *cdc-37*, *mom-2*/*Wnt*, *egl-20*/*Wnt*, *cwn-2*/*Wnt*, *cam-1*/*Ror*, *apr-1*/*APC*, *mig-5*/*Dvl*, *dsh-2*/*Dvl*, and *par-5*/*14-3-3* were identified [51].

15.6 Mechanisms of RYK Activation and Signaling

15.6.1 The RYK Protein Tyrosine Kinase Domain

The pseudokinase classification of RYK subfamily members primarily reflects the failure to observe transphosphorylation upon clustering of the RYK intracellular region in the context of Wnt binding, chimeric receptor expression and clustering, and overexpression or immunoprecipitation–in vitro protein kinase assays. In addition, substrates for transphosphorylation by RYK—synthetic or cellular—have not been identified, and the human RYK, *Drosophila* DRL, and mouse Ryk Mg^{2+}-ATP-binding clefts are reportedly defective [12, 54, 55]. The mouse Ryk PTK domain was unable

to bind divalent cation, nucleotide, cation–nucleotide complex, or ATP-competitive inhibitor in a sensitive and robust thermal-shift assay. The mouse Ryk TFVK^{232} (subdomain II; consensus VAIK) motif was altered to **VA**VK^{232} to test the hypothesis that the RYK subfamily-specific bulky phenylalanine residue at position 230 might occlude the adenine-binding pocket and prevent nucleotide binding. While the dual substitution did not result in detectable nucleotide binding, the variant was able to engage the promiscuous ATP-competitive inhibitor N2-[4-(aminomethyl)phenyl]-5-fluoro-N4-phenylpyrimidine-2,4-diamine (DAP) while wild-type Ryk did not [55].

As previously discussed, the RYK PTK domain awaits definitive evidence to substantiate or disprove the prediction of its catalytic inactivity and pseudokinase designation. It is notable that the sequence deviations from canonical PTK subdomains I (GXGXXG) and VII (DFG) are characteristic of all RYK orthologs (Fig. 15.2) and do not preclude catalytic activity per se based upon the increasingly well-documented capacity for structural plasticity of the PTK domain. However, in light of the inability of RYK subfamily members from evolutionarily divergent species to bind ATP, it is likely that they have been correctly assigned pseudokinase status and that the PTK-like domain fulfills a catalysis-independent role as a protein interaction scaffold.

15.6.2 RYK Signaling Mechanisms and Outputs

15.6.2.1 Differential Requirement for the RYK Intracellular Region

Genetic studies in *Drosophila* have established that DRL can function cell-nonautonomously at a distance from its site of synthesis in a manner independent of its intracellular region. Sequestration and functional neutralization of WNT5 by the glial-derived DRL WIF domain was likely to be the basis for their antagonistic activities in patterning the glomerular map in the *Drosophila* AL [84]. DRL-2 on ORNs was subsequently identified as the relevant receptor prevented from interaction with WNT5 in a temporospatially regulated fashion by the DRL WIF domain to ensure normal ORN pathfinding [20]. The finding that ectopic DRL-2 could substitute for the cell nonautonomous function of DRL in the glia of *drl* mutants indicated a cell type-specific mechanism for regulation of receptor function that did not distinguish between the two paralogs. A cell nonautonomous function of DRL in axon guidance was revealed in *drl*; *Nrt* mutant embryos [127] and during larval MB development, where *drl* and *Wnt5* again functioned antagonistically [97]. An essential role for commissure-derived DRL in guiding WNT5-expressing MB neurons was identified in which the intracellular region of DRL was dispensable [85, 97]. Overexpression of DRL phenocopied the *Wnt5* mutant MB defects, while WNT5 overexpression produced a *drl*-like mutant MB phenotype [97]. Context-dependent proteolytic cleavage and release of the DRL WIF domain is an attractive mechanism by which it may attenuate the activity of WNT5 and thereby execute a cell nonautonomous function in specific developmental contexts (Fig. 15.4b).

A role for *Cel-lin-18*/*Ryk* in polarizing the 2° P7.p vulva lineage was defined in which its intracellular region was dispensable [50, 51]. The *lin-18*/*Ryk* and *lin-17*/*Fz* genes function in independent and parallel pathways to cooperatively determine the AP orientation of the P7.p cell lineage [50, 51]. While the LIN-18/Ryk intracellular region was dispensable for normal vulval development, deletion of just two highly conserved WIF domain residues (ΔEL^{39}) resulted in LIN-18/Ryk loss of function and reversal of the orientation of the P7.p lineage such that it resembled the P5.p lineage [50, 130]. Anchorage of the LIN-18/Ryk extracellular region to the membrane of the P7.p lineage by a transmembrane helix was also essential [50]. Genetic interaction of *mom-2*/*Wnt* with *lin-18*/*Ryk* [50], together with failure to detect an ICF corresponding to the product of γ-secretase activity on LIN-18 and rescue of the P-Rvl phenotype by expression of a γ-secretase-resistant LIN-18/Ryk [51], indicates that LIN-18/Ryk transduces a Wnt signal in a cell-autonomous mode in the P7.p lineage, without the need for the intracellular region, by an unknown mechanism. In contrast, the intracellular region of *Ppa*-LIN-18/Ryk was necessary for vulva induction in response to EGL-20/Wnt, reflecting the novel wiring of Wnt signaling in *P. pacificus* [136].

In *C. elegans*, overexpression of LIN-18-ICF or the intracellular juxtamembrane region, but not the PTK-like domain, enhanced the frequency of defective P7.p cell polarity (P-Rvl phenotype) in a *lin-18* background, suggesting dominant-negative effects of the LIN-18 intracellular region towards other proteins regulating vulval cell polarity [51]. Like mammalian Ryk-ICF, LIN-18-ICF expressed from a transgene could translocate to the nucleus in a PTK-like domain-dependent manner, but such a phenomenon may be functionally relevant only in non-vulval cell lineages.

15.6.2.2 RYK Receptor-Proximal Phosphorylation Events

The downstream proteins mediating signaling via Eph receptor-dependent phosphorylation of the Ryk intracellular region on tyrosine residues have not been elucidated. However, cross talk between unrelated axon guidance ligand/receptor systems in contexts such as retinotopic/retinocollicular mapping might facilitate the refinement of an initial map and/or the alignment of topographic maps across sensory modalities in higher vertebrates.

In *Drosophila*, compelling genetic and biochemical evidence supports the involvement of SFKs in signaling downstream of DRL (Fig. 15.4a). Using *Drosophila* S2 cells that expressed endogenous *fz* and *fz2* transcripts, cotransfection of plasmids encoding DRL and WNT5 was unable to stimulate TCF-luciferase reporter activity or inhibit reporter activity stimulated by Wg, indicating that DRL is unlikely to signal via the β-catenin pathway in this assay [44]. Similar TCF-specific transcriptional reporter assays have often been used to quantify β-catenin signaling, but they significantly underestimate the contribution of active nuclear β-catenin to the modulation of activity of other transcription factors [108].

During salivary gland positioning, *drl*;*fz* mutants showed an additive phenotype compared to single mutants, suggesting that DRL and FZ were signaling via parallel pathways in this system [25]. Other than the cytoplasmic region of DRL, the relevant substrates of SFKs downstream of DRL/WNT5 have not been identified, nor have any binding partners of phospho-DRL been described. However, the observation that the normally low *Wnt5* mRNA and protein levels in AC neurons both rise significantly upon genetic loss of *drl* suggests that active WNT5/DRL signaling normally functions to repress expression of *Wnt5*a at the transcriptional level [120] (Fig. 15.4d).

15.6.2.3 β-Catenin-Dependent RYK Signaling

Despite the failure to observe coupling of WNT5/DRL signaling and β-catenin/TCF stabilization in *Drosophila*, evidence of engagement of the β-catenin/TCF pathway by mammalian RYK and *C. elegans*/*P. pacificus* LIN-18 has been reported. A synergistic increase in TCF-luciferase reporter activity was observed in HEK293T cells cotransfected with Ryk and Wnt3a expression vectors versus cells transfected with Ryk or Wnt3a alone, while knockdown of endogenous *RYK* using siRNA abrogated the increase in TCF-luciferase activity induced by Wnt1 [57]. Baltimore and colleagues concluded from co-immunoprecipitation studies that the interaction of Ryk with Dvl-2 and Dvl-3 (the only Dvl proteins expressed in HEK293T cells) was the mechanism by which Ryk transduced assembly of a Ryk/Wnt/Fzd ternary complex at the cell surface into activation of the TCF pathway in HEK293T cells [57] (Fig. 15.4a).

Using HEK293T cells, and verified in U2OS and HeLa cells, Berndt et al. demonstrated that MIB1 and RYK were required for events upstream of β-catenin stabilization, such as LRP6 phosphorylation, in response to Wnt3a and that siRNA targeting endogenous *RYK* inhibited Wnt1- or Wnt3a-induced TCF transcriptional reporter activity [69]. Formation of a RYK/MIB1 complex with intact ubiquitin E3 ligase activity was necessary for the activation of β-catenin-dependent Wnt signaling, but further mechanisms proved elusive. Evidence for a genetic interaction between *Cel-mib-1* and *lin-18*/*Ryk* in orienting the divisions of the P7.p lineage implied that their cooperative regulation of the β-catenin/TCF pathway might be conserved in vivo across diverse species (Fig. 15.4a).

Three β-catenin paralogs that function in two distinct pathways are found in *C. elegans*: SYS-1, WRM-1, and BAR-1. The Wnt/β-catenin asymmetry pathway employs SYS-1 and WRM-1 to control both the polarity of mother cells and distinct fates of daughter cells [146]. In VPCs, as in most other tissues, following an asymmetric cell division, a complex of WRM-1 and LIT-1/Nemo-like kinase is enriched in the posterior cell nucleus where it recruits and phosphorylates POP-1/TCF to promote its export from the nucleus. SYS-1, a transcriptional coactivator of POP-1/TCF, is similarly but independently enriched in the posterior cell nucleus. The outcome is thus different ratios of SYS-1 to POP-1/TCF and, consequently, differential activation of Wnt target genes in the daughters of an asymmetric division.

Deshpande et al. discovered that most VPC divisions produced sister cells with nuclear POP-1/TCF enriched in nuclei of the distal granddaughters of P5.p and P7.p (i.e., the asymmetry had opposite orientations in the two mirror-image halves of the vulva) [147]. LIN-17/Fz and LIN-18/Ryk were the receptors that controlled nuclear POP-1/TCF and SYS-1 asymmetry to invert the P7.p lineage from a default orientation exhibited in the anterior half of the vulva [132, 147]. BAR-1, a classic β-catenin that can also function as a transcriptional coactivator with POP-1/TCF, played a minor role in VPC orientation. LIN-17/Fz and LIN-18/Ryk also regulated the asymmetric localization of BAR-1 through differential protein stabilization, with higher nuclear levels in the proximal daughters of P5.p and P7.p [132].

Green et al. [132] proposed a model for orientation of the P5.p and P7.p lineages that involved three distinct cell polarity states. In the absence of Wnt signals, a default state was revealed in which the orientation of VPCs was randomized. Posterior orientation of the P5.p and P7.p lineages in the absence of *lin-17/fz* and *lin-18/Ryk*, ground polarity, was dependent upon the instructive activity of a tail-derived P>A EGL-20/Wnt gradient. A state of refined polarity was generated by the subsequent activation of LIN-17/Fz and LIN-18/Ryk by centrally derived MOM-2/Wnt (and perhaps LIN-44/Wnt) and the resulting orientation of the P5.p and P7.p lineages toward the center to ensure the mirror-image symmetry required for a functional vulva. Although the Wnt/β-catenin asymmetry and BAR-1 pathways were implicated downstream of LIN-17/Fz and LIN-18/Ryk, weakly penetrant loss-of-function phenotypes or discordance between transcriptional activity and morphological phenotypes, respectively, led to speculation that refined polarity may be dependent on signaling to the cytoskeleton, independent of the transcriptional machinery, via the spindle reorientation branch of the Wnt/β-catenin asymmetry pathway.

An evolutionary reconfiguration of Wnt signaling in *P. pacificus* for the regulation of vulva induction was described in which *Ppa*-LIN-17/Fz and *Ppa*-LIN-18/Ryk performed antagonistic roles that contrasted with their cooperative functions during *C. elegans* VPC fate patterning [136]. *Ppa*-LIN-17/Fz antagonized *Ppa*-EGL-20/Wnt activity by ligand sequestration, while *Ppa*-LIN-18/Ryk transduced the *Ppa*-EGL-20/Wnt signal. The *Ppa-axl-1/Axin* gene was epistatic to *Ppa-lin-18/Ryk*, and the Wnt pathway for vulva induction in *P. pacificus* was BAR-1/β-catenin dependent. Transduction of an inductive *Ppa*-EGL-20/Wnt signal by *Ppa*-LIN-18/Ryk required the intracellular region in which two putative SH3 domain-binding motifs (PIGP^{268} and PPFPDLPS^{559}), both absent in *Cel*-LIN-18/Ryk, were identified that functioned to suppress receptor signaling in the absence of ligand.

A caveat to these descriptions of β-catenin-dependent signaling by Wnt/Ryk is the failure of other investigators to observe β-catenin/TCF pathway activation in seemingly similar experimental systems. For example, overexpression of Ryk did not synergize with Wnt3- or Wnt3a-induced TCF-luciferase activity in HEK293T cells [39, 119].

15.6.2.4 β-Catenin-Independent Wnt Signaling by RYK

Recent findings suggest that Ryk and Fzd may coordinately regulate downstream cellular responses in a context-dependent manner via the activation of β-catenin-independent Wnt signaling pathways. Ryk cooperated with Fzd7 to mediate Wnt11-triggered *X. laevis* gastrulation-associated CE movements through the regulation of PCP signaling components [41]. Ryk and Fzd7 were required for Wnt11-stimulated endocytosis, a hallmark of PCP signaling. Ryk formed an endocytic complex containing Fzd7, Dvl, and Rab5, recruited βarr-2 and RhoA to the plasma membrane, promoted hyperphosphorylation of Dvl, and induced the activation of RhoA and JNK. No effect of Wnt11/Ryk/Fz7 signaling on the abundance of β-catenin in animal caps was observed. In ex vivo assays of hamster cortical axon outgrowth, an ungraded source of Wnt5a triggered the Ryk-dependent activation of inositol 1,4,5-trisphosphate receptors (IP3Rs) and transient receptor potential channels (TRPCs), both of which were necessary for the release and influx, respectively, of Ca^{2+} and the promotion of axon outgrowth [90]. In contrast, a gradient of Wnt5a promoted axon outgrowth and repulsion, a complex growth cone response that was activated only after cortical axons had crossed the midline via the corpus callosum [90, 115]. Simultaneous, or perhaps cooperative, signaling by Ryk and unidentified Fzd receptor(s) was necessary for axon repulsion and required Ca^{2+} influx via TRPCs but not release from intracellular stores via IP3Rs [90]. Although the mechanisms of signaling remain unclear, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-mediated phosphorylation of Tau was found to underlie the regulation of microtubule dynamics in the growth cone in response to Wnt5a [148]. Integration of Ca^{2+} and other signals by this pathway may lie downstream of Wnt5a-stimulated Ryk and Fzd receptors to effect simultaneous axon outgrowth and repulsion (Fig. 15.4c).

Wnt5b/Ryk signaling regulated zebrafish gastrulation-associated CE movements through the negative control of cell intermingling [40]. Ryk-expressing cells were polarized by Wnt5b to extend lamellipodia-like protrusions in the direction of migration away from the ligand source. While sequential proteolysis of Ryk was not the basis for transduction of the chemorepulsive Wnt5b signal, internalization of Ryk-FL cleaved between the two disulfide-bonded Cys residues in the WIF domain was critical for signal transduction. Ryk was necessary but not sufficient for Wnt5b-induced Ca^{2+} influx and/or release. Recruitment of Dvl to Wnt5b-activated Fzd, in contrast to Wnt5b-activated Ryk, and the free intermingling of Wnt5b-expressing cells with those expressing Fzd indicated that pathways activated by the two Wnt receptors were distinct. Wnt5b was proposed to be an instructive signal for directed migration when engaged by Ryk and a permissive cue via Fz and core PCP components during zebrafish gastrulation.

Formation of a complex containing Ryk and Van Gogh-like 2 (Vangl2), a core PCP signaling component, was promoted by Wnt5a treatment of cotransfected HEK293T cells [35]. Unlike Ror2, another RTK with a pseudokinase domain that functions as a Wnt receptor, Ryk did not induce Vangl2 phosphorylation. Instead, in vitro and in vivo evidence indicated that lysosomal degradation of Vangl2 protein was inhibited by Wnt5a/Ryk. Macheda et al. [119] also detected coprecipitation of

Ryk and Vangl2, but additionally found that the Ryk PDZ-binding C-terminus was important for their presence in a common complex and that Ryk could activate RhoA [119] (Fig. 15.4c).

15.6.2.5 Proteolytic Cleavage and Nuclear Translocation

Constitutive metalloprotease-mediated shedding of mouse Ryk-NTF was observed to operate in cell culture [59]. This did not require cell stimulation with exogenous Wnt to engage the Ryk WIF domain [39, 59, 62]. A regulated component of Ryk-NTF shedding was revealed upon treatment of cells expressing Ryk with inhibitors of protein tyrosine phosphatases or calmodulin, both of which induced a rapid and large increase in shedding of Ryk-NTF into conditioned medium [59]. While Wnt ligands have not been shown to directly regulate Ryk cleavage events, they dramatically enhance the stability and nuclear translocation of Ryk-ICF [39] (Fig. 15.4b).

Ryk-NTF contains the WIF domain and could therefore function as a soluble antagonist of Wnt activity. Indeed, genetic studies in *Drosophila* indicate that WIF domain-dependent functional antagonism between *drl* and *Wnt5* is essential for normal AL and MB development [20, 84, 97]. These are contexts in which a shed DRL-NTF may function cell nonautonomously to neutralize extracellular WNT5 by sequestration, thus preventing its activation of cell surface receptors (Fig. 15.4b).

Gamma-secretase and a γ-secretase-like activity process Ryk-CTF45 and release Ryk-ICF into the cytoplasm (Fig. 15.3d) ([39]; MMH, MLM, and SAS, unpublished data). Unexpectedly, Wnt stimulation was indirectly required for propagation of the signal initiated by Ryk ectodomain cleavage—at least in a mammalian system—by providing additional but unidentified cues that resulted in the stabilization and nuclear translocation of Ryk-ICF. The finding that an RTK with a Wnt-binding extracellular region relies upon Wnts for signal propagation in specific contexts only after irreversible separation of its extracellular and intracellular domains is counterintuitive and represents a variation on the paradigm established by Notch signaling (Fig. 15.4b).

15.7 Recombinant Protein Antagonists of RYK

To interrogate in vivo the postnatal role of Ryk in CST axon pathfinding, a neutralizing rabbit polyclonal antibody directed to the Ryk extracellular region was developed [88]. Injection of this anti-Ryk antibody into the cervical spinal cord of neonatal P1 and P3 mice, with collection of tissue at P5, showed a decreased area of CST axons posterior to the injection site, but increased area anterior to the injection site [88]. These results suggested that Ryk was required for posterior growth of postnatal CST axons. A similar antibody was used to demonstrate a requirement for mouse Ryk in Wnt3a-induced GABAergic neuronal differentiation and simultaneous inhibition of oligodendrocyte differentiation from cultured NPCs [34].

An increasing number of studies have examined the expression of Ryk after nerve injury in adult rodents. *Ryk* mRNA and protein expression were induced from previously undetectable levels after spinal cord injury in rats and mice, in CST axons in the lesion area by 7 days post-injury [149, 150]. In the rat spinal cord, Ryk expression was detected in neurons, astrocytes, and blood vessels, but not in oligodendrocytes, microglia, neuron–glial antigen 2-positive ($NG2^+$) glial precursor cells, or axonal projections. Following moderate contusive spinal cord damage, Ryk was significantly upregulated after 7 and 14 days and demonstrated an altered expression pattern with reactive astrocytes and microglia/macrophages, $NG2^+$ glial precursors, fibronectin-positive cells, oligodendrocytes, and axons demonstrating injury-induced immunoreactivity [151]. *Ryk* mRNA and protein were induced ipsilaterally in rat dorsal root ganglion neurons following unilateral sciatic nerve injury [152].

The injection or osmotic minipump-mediated release of neutralizing anti-Ryk antibody in rats after spinal cord injury prevented CST axon retraction from the lesion several weeks after injury and caused sprouting of axons at and caudal to the lesion [149, 150]. The ectopic expression of secreted Wnt antagonists (WIF1 or sFRP2) by stromal cells grafted at the site of a lesion to central branch dorsal column axons following a peripheral conditioning injury also enhanced the central regenerative response [153]. These results demonstrate that injury-induced Ryk expression strongly inhibits axon growth and recovery of function after a spinal cord lesion.

A fully human inhibitory monoclonal antibody—RWD1—to the RYK WIF domain [59] may represent an effective therapeutic in the setting of spinal cord and/or peripheral nerve injury. RWD1 inhibited the stimulatory activity of Wnt5a on progenitor proliferation, dopamine (DA) neuron differentiation, and DA axonal connectivity in rat E13.5 ventral midbrain explants [32] and attenuated neurite outgrowth from Wnt5a-stimulated mouse E15.5 cortical neurons [59].

Expression of a dominant-negative zebrafish Ryk-EGFP fusion, in which the intracellular region was replaced with EGFP, in zebrafish habenular commissural axons caused them to reenter the midline after reaching the contralateral habenula [154], reminiscent of the post-crossing defect seen in mouse $Ryk^{-/-}$ embryos [91]. A similar dominant-negative mouse Ryk, in which the intracellular region was deleted, was employed to disrupt lateral-directed interstitial axon branching from RGC neurons during chick retinotectal mapping [112] or inhibit Wnt3-induced nuclear localization of Ryk-ICF in cultured mouse NPCs [39].

15.8 Involvement of RYK in Disease

15.8.1 Cancer

The Catalogue of Somatic Mutations in Cancer (COSMIC; http://www.sanger.ac.uk/genetics/CGP/cosmic; viewed November 2014) documents 35 different non-synonymous alterations in human *RYK* exons (Table 15.1). However, the functional implications of each mutation—if any—are unknown. In a patient with atypical

Table 15.1 Non-synonymous human *RYK* mutations reported in the Catalogue of Somatic Mutations in Cancer; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/ (viewed November 2014) and polymorphisms (dbSNP release 138)

Mutation ID	Polymorphism ID[a]	Carcinoma origin	Nucleotide change(s)[b]	Protein change(s)[a]	Location	Somatic status	Zygosity	Reference
COSM1616953		Liver	c.241_253del13	p.L81fs*15	WIF domain	Unknown	Heterozygous	
	rs374574792 (SNP)		c.284C>G	p.S95C				
COSM149476		Stomach	c.290 G>A	p.S97N		Unknown	Homozygous	[176]
	rs371293491 (SNP)		c.370C>G	p.Q124E				
COSM1038630		Endometrium	c.388G>A	p.A130T		Unknown	Heterozygous	
		Large intestine						
	rs374496055 (SNP)		c.436C>T	p.R146C		Confirmed	Unknown	[177]
COSM1038629[c]		Lung	c.436C>T	p.R146C		Previously reported	Heterozygous	
		Endometrium						
	rs372371526 (SNP)		c.437G>A	p.R146H				
	rs149986169 (SNP)		c.493G>A	p.V165I				
COSM1419221		Large intestine	c.539delA	p.N180fs*5		Unknown	Heterozygous	
	rs140598974 (SNP)		c.571A>G	p.M191V				
COSM1038628		Endometrium	c.629G>T	p.R210I	Extracellular juxtamembrane	Unknown	Heterozygous	
COSM3846278		Breast	c.671C>T	p.T224M		Confirmed		
	rs55740278 (SNP)		c.673C>T	p.R225C				
	rs374337304 (SNP)		c.674G>A	p.R225H				
COSM3944890		Lung	c.674G>C	p.R225P		Confirmed	Unknown	
COSM251114		Liver	c.710 T>G	p.V237G	Transmembrane helix	Unknown	Unknown	[178]
COSM20738		Ovary	c.721G>A	p.V241I		Confirmed	Heterozygous	

(continued)

Table 15.1 (continued)

Mutation ID	Polymorphism ID[a]	Carcinoma origin	Nucleotide change(s)[c]	Protein change(s)[a]	Location	Somatic status	Zygosity	Reference
COSM1419220		Large intestine	c.722 T>C	p.V241A	Transmembrane helix	Unknown	Heterozygous	
	rs191818784 (SNP)		c.755G>A	p.S252N				
	rs201031716 (SNP)		c.797G>A	p.S266N	Intracellular juxtamembrane			
			p.797G>C	p.S266T				
COSM95661		Breast	c.817C>T	p.Q273*		Confirmed	Unknown	[179]
	rs139935782 (SNP)		c.833C>T	p.T278M				
COSM48907		Lung	c.844A>G	p.R282G		Confirmed	Heterozygous	
	rs180735733 SNP		c.871A>G	p.T291A				
COSM3587717		Malignant melanoma	c.892G>A	p.G298S		Confirmed	Unknown	
	rs375652759 SNP		c.901A>G	p.T301A				
	rs372101827 SNP		c.944 T>C	p.L315S				
	rs375133082 SNP		c.990G>C	p.R330S				
	rs370703140 SNP		c.1009C>G	p.L337V	PTK domain			
COSM72514		Ovary	c.1018C>T	p.R340C		Confirmed	Heterozygous	
COSM76315		Ovary	c.1022 T>C	p.I341T		Confirmed	Heterozygous	[180]
COSM1419219		Large intestine	c.1027G>T	p.G343W		Unknown	Heterozygous	
COSM1419218		Large intestine	c.1031G>A	p.R344H		Unknown	Heterozygous	
COSM1419217		Large intestine	c.1034 T>C	p.I345T		Unknown	Heterozygous	
COSM1038626		Endometrium	c.1044G>A	p.G348G		Unknown	Heterozygous	
COSM1419216		Large intestine	c.1058A>G	p.E353G		Unknown	Heterozygous	
	rs187357709 SNP		c.1071 T>A	p.N357K				
COSM204552		Large intestine	c.1077A>C	p.E359D		Confirmed	Unknown	
	rs374029224 SNP		c.1097C>T	p.T366I				

COSM1038625		Endometrium	c.1103A>G	p.K368R		Unknown	Heterozygous	
COSM3587716		Malignant melanoma	c.1123C>T	p.Q375*		Confirmed	Unknown	
	rs199538071 SNP		c.1132A>G	p.M378V				
COSM1419215		Large intestine	c.1192A>G	p.T398A		Unknown	Heterozygous	
COSM125974		Upper aerodigestive tract	c.1201 T>C	p.C401R		Unknown	Unknown	[181]
	rs186853257 SNP		c.1208A>G	p.E403G				
COSM1038623		Endometrium	c.1220A>C	p.K407T		Unknown	Heterozygous	
	rs34226753 INSERTION		c.1249_1250insG	p.N419Efs*3				
	rs369256717 SNP		c.1298A>G	p.D433S				
	rs141092884 SNP		c.1346A>G	p.Q449R				
COSM263480		Large intestine	c.1358G>T	p.G453V		Confirmed	Unknown	[176]
	rs375788400 SNP		c.1367A>G	p.Y456C				
COSM204551		Large intestine	c.1372G>A	p.A458T		Confirmed	Unknown	
	rs116254638 SNP		c.1394A>G	p.K465R				
COSM3373023		Thyroid	c.1459C>G	p.L487V		Confirmed	Unknown	
COSM1419214[c]		Large intestine	c.1522C>T	p.R508C		Unknown	Heterozygous	
	rs144378986 SNP		c.1522C>T	p.R508C				
	rs187302288 SNP		c.1534C>G	p.L512V				
	rs115107686 SNP		c.1546G>A	p.V516I				
	rs375916618 SNP		c.1595C>T	p.T532M				
COSM164135		Breast	c.1615C>G	p.L539V		Confirmed	Unknown	[182]
	rs368912529 SNP		c.1633G>A	p.V545M				

(continued)

Table 15.1 (continued)

Mutation ID	Polymorphism ID[a]	Carcinoma origin	Nucleotide change(s)[b]	Protein change(s)[a]	Location	Somatic status	Zygosity	Reference
	rs375889971 SNP		c.1650C>G	p.F550L				
COSM263479		Large intestine	c.1682G>A	p.R561Q		Confirmed	Unknown	[176]
		Endometrium				Previously reported	Heterozygous	
COSM294791		Large intestine	c.1692G>T	p.Q564H		Confirmed	Unknown	
	rs369924907 SNP		c.1700A>G	p.N567S				
COSM1632844		Liver	c.1733G>T	p.C578F		Unknown	Heterozygous	
	rs374382478 SNP		c.1745 T>C	p.L582S				
	rs56149724 SNP		c.1787G>A	p.C596Y				
	rs368597652 SNP		c.1804G>A	p.A602T	C-terminal tail			
	rs146665782 SNP		c.1816G>C	p.A606P	C-terminal tail/ PDZ-binding motif			
	rs200256181 SNP		c.1822G>A	p.V608I				

[a]From dbSNP (release 138). Sequence variants in the G+C-rich exon 1 are not listed due to their unreliability

[b]Nomenclature follows recommendations for the description of sequence variants [183]. The *RYK* reference transcript is ENST00000296084

[c]Unlikely to represent a somatic mutation given that the same substitution is observed at high frequency in the germline

fs Frameshift;

[*]Stop codon

chronic myeloid leukemia, a 46,XY,del(3)(q22),dup(12)(q13q22),der(21)t(3;21)(q22;q22) karyotype was established [155]. The 3q22 breakpoint was in exon 1 of *RYK*, which was fused with the *ATP5O* gene from 21q22 to generate der(21)t(3;21). DNA sequencing of RT-PCR products indicated that two *RYK–ATP5O* fusion transcripts were expressed. However, due to alternative splicing and a frame shift, both transcripts encoded a protein predicted to contain the initial 77 residues of RYK followed by an ectopic 44 residues from genomic chromosome 21 sequence. The role of the mutation in the etiology of the leukemia—if any—is unlikely but unknown [155].

Altered *RYK* expression was observed in several cancer types, including acute myeloid leukemia, gastrointestinal, breast, testicular, and brain cancers, neuroblastoma, and Wilms and primitive neuroectodermal tumors [2–6, 156, 157]. Normal ovaries and benign ovarian tumors expressed very little *RYK* mRNA; borderline ovarian tumors showed moderate upregulation of *RYK* mRNA in the stromal compartment; and malignant ovarian tumors comprising a variety of histological types and grades showed a variable but distinctive upregulation of *RYK* mRNA, spatially restricted to the epithelial compartment [30]. Strong upregulation of RYK protein expression was observed in borderline and malignant forms of primary human ovarian tumors by immunohistochemistry. The stroma, epithelium, and blood vessels of clear cell and serous histological subtypes showed a higher frequency of increased RYK expression than did endometrioid or mucinous tumors [79]. Significantly, reduced overall survival was associated with overexpression of RYK in blood vessels and malignant epithelium of patients with ovarian cancer [158]. High expression of RYK in tumor-associated blood vessels was also linked to significantly shorter progression-free survival [158]. RYK expression was also observed in the cancer and stromal compartments of breast tumors [59].

Anastas et al. [159] described an autocrine role for WNT5A expression in promoting the growth of naïve human melanoma and found that elevated WNT5A expression upon treatment with a $BRAF^{V600E/K}$ inhibitor was part of a characteristic transcriptional signature clinically correlated with therapy resistance. FZD7 and RYK, mRNA for the latter found to be significantly upregulated in human melanoma samples versus normal skin, were functionally identified as the relevant WNT5A receptors in vitro. RNAi-mediated knockdown of either strongly reduced the activation of AKT and caused decreased viability of both naïve and BRAF inhibitor-resistant melanoma cell lines.

Two insertional mutagenesis experiments using an oncogenic transposon have identified mouse *Ryk* as a common insertion site. In primary but not metastatic medulloblastomas on a *Ptch*$^{+/-}$*/Math1-SB11*/T2Onc genetic background, insertions into *Ryk* were monoallelic [160]. T2Onc insertion into *Ryk* in primary medulloblastomas demonstrated a statistically significant positive association with insertions in *Ipo8*, *Daam1*, *Ehmt1*, *Slc2a4*, *E2f3*, *Ccdc86*, *Polr2d*, *Epha1*, *Pdia4*, *Ptp4a2*, *Nsbp1*, *Baiap2*, *Zmynd11*, *Arrdc3*, *Chd4*, *Elavl1*, *Ski*, *Clstn1*, *Lyst*, *Vps41*, and *Exoc2*. In a mouse model of nonmelanoma skin cancer, mobilization of the T2Onc2 transposon in the skin was also found to result in insertions in *Ryk* more often than by chance [161].

15.8.2 Craniofacial and Skeletal Defects

A patient with non-syndromic cleft lip and/or palate (precise defect not specified) was heterozygous for a *RYK* missense mutation, G1373A, which results in a Y458C substitution in subdomain VIa of the PTK domain. Evidence for a loss-of-function effect of the Y452(458)C substitution was purportedly shown by a reduced capacity for transformation in a NIH/3T3 colony formation assay [80]. Two larger population studies using European subjects showed no association between non-syndromic cleft lip and/or palate with *RYK* polymorphisms [162, 163].

Robinow syndrome is a condition for which the genetic basis is heterogeneous. An autosomal recessive form (RRS) results from homozygous or compound heterozygous mutations in the *ROR2* gene [164, 165], while the autosomal dominant variant (DRS) is caused by heterozygous mutation in the *WNT5A* gene [166]. Clinical malformations common to the recessive and dominant forms are characteristic facial features (hypertelorism, large nasal bridge, short upturned nose, midface hypoplasia), orodental abnormalities, hypoplastic genitalia, and mesomelic limb shortening [167]. The principal discriminating features of RRS are the more severe phenotypes and the additional presence of cleft lip and palate, and multiple rib and vertebral anomalies. Umbilical hernia and supernumerary teeth are seen exclusively in 32 % and 10 % of patients, respectively, with DRS.

Mazzeu [168], motivated by observations of mouse embryos with various loss-of-function *Ryk*; *Vangl2* genotypes and their similarities to those of age-matched *Wnt5a*$^{-/-}$ embryos [35], screened 24 patients with a diagnosis of DRS, for which the genetic basis did not involve *ROR2* or *WNT5A*, for mutations in exons 2–16 of *RYK*. However, no mutations were found and Mazzeu concluded that mutations in *RYK* do not cause DRS [168]. In response, Andre and Yang [169] emphasized that a heterozygous *WNT5A* mutation was established as the genetic etiology in only a small fraction of patients with a clinical diagnosis of DRS [166], indicative of unexplained genetic heterogeneity, and that exon 1 and regulatory regions of the *RYK* gene were not examined for mutation. Furthermore, given that *Ror2*$^{-/-}$ mouse embryos exhibit more severe limb phenotypes and resemble *Wnt5a*$^{-/-}$ embryos more than *Ryk*$^{-/-}$ embryos, human *RYK* mutations are perhaps more likely to be associated with RRS than DRS. Screening Robinow syndrome patients homozygous for wild-type *ROR2* and *WNT5A* alleles for mutations in *RYK* will be required to test this hypothesis.

Activity of the anti-osteoporotic agent strontium ranelate (SrRan) was found to depend upon nuclear translocation of the nuclear factor of activated T cells (NFATc) in mouse osteoblasts, resulting in their increased proliferation and differentiation [170]. *Wnt3a* and *Wnt5a* were upregulated in SrRan-treated osteoblasts and participated in an autocrine signaling loop involving Ryk that was necessary for the biological effects of this therapeutic agent. In contrast, the human immunodeficiency virus (HIV) protease inhibitor ritonavir is known to accelerate the HIV-promoted loss of bone mineral density. Enhanced osteoclast differentiation from mouse and human monocytic precursors, and their subsequent resorption of bone, was found to be the cellular basis for this observation [171]. Ritonavir induced Wnt5a and Wnt5b expression in osteoclast precursors, and Ryk expression was necessary for their subsequent differentiation into mature osteoclasts.

15.8.3 Other Conditions

WNT5A signaling in human airway smooth muscle cells was activated by TGF-β and proved necessary for induction of extracellular matrix (ECM) production [172]. RYK and FZD8 transduced β-catenin-independent WNT5A signals to induce ECM expression as part of the smooth muscle response to TGF-β. Patients with asthma demonstrated twofold higher WNT5A expression in airway smooth muscle, which may be a significant contributor to the fibrotic pathology characteristic of this disease [172]. Activation of rat hepatic stellate cells, a central feature of liver fibrosis, was reported to involve upregulation of mRNAs for *Wnts*, *Fzd* receptors and *Ryk* [173].

Mouse Ryk was expressed with atypical protein kinase C in normal adult motoneurons and oligodendrocytes, but not in astrocytes. In a transgenic mouse $SOD1^{G93A}$ model of amyotrophic lateral sclerosis, Ryk expression was increased in motor neurons and the ventral white matter of the lumbar spinal cord. Colocalization of Ryk with $SOD1^{G93A}$ protein was restricted to choline acetyltransferase-positive motor neurons in the gray matter and axons in the ventral white matter of the lumbar spinal cord [174].

In transgenic animal models of Huntington's disease expressing polyglutamine-expanded Huntingtin (Htt), expression of Ryk was increased during the presymptomatic but pathogenic phase during which neuronal homeostasis is first perturbed [63]. Dysfunction induced by the expression of polyglutamine-expanded Htt was prevented in touch receptor and PLM neurons of the *C. elegans lin-18*/*Ryk* mutant and in mouse striatal cells transfected in vitro with siRNA targeting *Ryk*. In a polyglutamine-expanded Htt context, a protein complex scaffolded by BAR-1/β-catenin facilitated the repression of DAF-16/Foxo3a transcriptional and neuroprotective activities by recruitment of increased quantities of the nuclear LIN-18/Ryk-ICF.

15.9 Concluding Remarks

A combination of unique properties—poor immunogenicity, a Wnt-binding extracellular WIF domain, context-dependent Notch-like proteolytic processing and signaling, and pseudokinase status (Table "Receptor at a Glance")—has conspired to make progress toward a comprehensive understanding of the molecular, cellular, developmental, and pathological roles of RYK subfamily members challenging. However, a powerful mix of genetically tractable model organisms, new technologies, and devoted investigators has recently generated unexpected and exciting insights into fundamental biological phenomena regulated, at least in part, by the activity of RYK subfamily proteins (Fig. 15.5). Although our current understanding of how cellular context influences the coupling of RYK to alternative effector pathways (Fig. 15.4) is limiting, an emergent feature of function is the transduction of positional information provided by Wnt gradients.

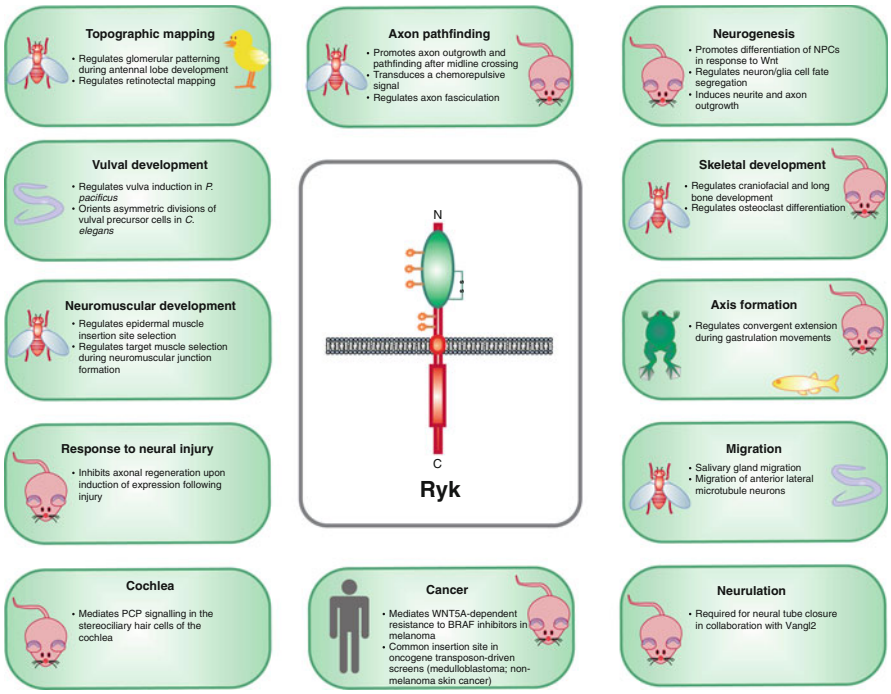

Fig. 15.5 Ryk is functionally important during embryonic development and in adult physiology. Processes dependent on Ryk activity are shown with a depiction of the organism(s) in which relevant studies were performed

Uncovering the diverse pathways and mechanisms by which a Wnt gradient evokes—for example—a repulsive response in navigating growth cones, the orientation of asymmetric cell divisions during organogenesis, or the choice of cell fate in embryonic NPCs will depend in large part on our capacity to develop an integrated model of context-dependent Wnt receptor activity. From a biomedical perspective, this is a formidable task given that the currently recognized human WNT (co)receptors number 17 (ten FZD receptors, LRP5, LRP6, ROR1 and ROR2, RYK, PTK7, and MuSK) [104] and the known complexity in number and activity of extracellular Wnt agonists/antagonists [103]. However, *C. elegans* vulva development provides an inspiring example of how long-term focused interrogation of a well-characterized model system can yield a highly sophisticated molecular understanding of Wnt/receptor-regulated cell behavior [50, 128, 130, 132].

Other important contributions to the understanding of RYK function will emerge from diverse but equally important areas of study. Structural and biochemical studies that address the basis for, or misclassification of, RYK subfamily members as pseudokinases (e.g., [55]) are eagerly anticipated. Conditional mammalian models of Ryk function that circumvent the perinatal lethality of *Ryk* loss of function will provide long-awaited information regarding the roles of Ryk in juvenile, adolescent,

and adult physiology and pathological states when interrogated in appropriate contexts. Potential anti-WNT/RYK therapeutics have been developed, and the results of testing these in appropriate animal models [59] and ongoing clinical trials [175] are of great interest.

The range of identified and potential pathological contexts for evaluating the usefulness of anti-RYK therapeutics in human conditions is particularly diverse. Cancer is an attractive condition for the development of anti-RYK reagents given the established efficacy of small molecule RTK inhibitors and anti-growth factor approaches, but the pseudokinase status of RYK clearly signifies a different but no less relevant class of target. Although speculative, other diverse human conditions—depression, addiction, schizophrenia, spinal cord and peripheral nerve injuries, ADHD, Parkinson's and Huntington's diseases, and disorders of bone metabolism—together represent even greater potential for benefit from targeting Wnt/RYK signaling. While basic research into the fundamental biological roles of RYK subfamily members will undoubtedly continue to surprise, brave investment in targeting Wnt/RYK signaling has great potential to alleviate human suffering.

***RYK* receptor at a glance**

	Human[a]	Mouse[b]
Gene location	3q22	9D1
Gene size (bp)	93,609	≈73 kb
Intron/exon numbers	14/15	14/15
mRNA size (5′, ORF, 3′)	2,942 bp	3,267 bp
Amino acid residues	610 (isoform 1 precursor), 607 (isoform 2 precursor), 273 (isoform 3 precursor)	594 (isoform 1 precursor), 591 (isoform 2 precursor), 475 (isoform 3 precursor)
Mature protein (predicted; kDa)	68 (isoform 1 precursor)	66 (isoform 1 precursor)
Posttranslational modifications	*N*-linked glycosylation, 5 (N139, N174, N178, N182, N209, N291) Phosphorylation Polyubiquitylation Proteolytic cleavages	*N*-linked glycosylation, 5 (N123, N158, N162, N166, N193) Phosphorylation Polyubiquitylation Proteolytic cleavages
Domains	WIF (66–194) Transmembrane helix (228–255) Protein tyrosine kinase (333–606)	WIF (50–178) Transmembrane helix (212–239) Protein tyrosine kinase (317–590)
Extracellular ligands	Wnts	Wnts
Known dimerizing partners	Self	EphB2 EphB3 Fzd8

(continued)

	Human[a]	Mouse[b]
Pathways activated	Wnt/β-catenin Wnt/calcium dependent Wnt/planar cell polarity AKT	Wnt/β-catenin Wnt/calcium dependent Wnt/planar cell polarity
Tissues expressed (mRNA)	Adipose, adrenal gland, blood cells, brain, breast, cerebellum, colon Colorectal adenocarcinoma Endothelial cells, fallopian tube, heart, kidney, liver, lung, lymph node, ovary Ovarian carcinoma, pancreas Placenta, prefrontal cortex Prostate, retina, salivary gland, skeletal muscle Skin, small intestine, smooth muscle, spleen, spinal cord Testis, thymus, thyroid Tongue, tonsil, thyroid, uterus	Adipose, adrenal gland, bladder, bone, bone marrow Brain, cerebellum, colon Embryo, whole Embryo, heart, brain, spinal cord, small intestine, eye, hair follicle Hematopoietic stem cells Hippocampus, kidney, liver Lung, lymph node, mammary gland, ovary, pancreas Placenta, prostate, retina Salivary gland, skeletal muscle Skin, small intestine, spleen Stomach, striatum, testis, tongue, thymus, uterus
Human diseases	Cleft lip/palate, cancer (melanoma)	Cancer (medulloblastoma, non-melanoma skin)
Knockout mouse phenotype	–	Neonatal death on day of birth Cleft of the secondary palate Growth retardation Craniofacial defects Reduced long bone length Defective callosal axon guidance Reduced neuron differentiation and increased oligodendrocyte differentiation in forebrain Open neural tube (craniorachischisis; interaction with *Vangl2*) Open ventral body wall (interaction with *Vangl2*) Defective forelimb digit formation (interaction with *Vangl2*) Sternum and rib defects (interaction with *Vangl2*) Defective polarity of hair cells in cochlea (interaction with *Vangl2*) Additional row of hair cells in middle cochlea (interaction with *Vangl2*) Eyelid closure defects (interaction with *Vangl2*)

[a]Human RYK protein numbering is based on the isoform 1 precursor (NCBI reference sequence NP_001005861.1)

[b]Mouse Ryk protein numbering is based on the isoform 1 precursor (NCBI reference sequence NP_038677.3)

References

1. Hanks SK, Quinn AM, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science. 1988;241(4861):42–52.
2. Kelman Z, Simon-Chazottes D, Guenet JL, Yarden Y. The murine *vik* gene (chromosome 9) encodes a putative receptor with unique protein kinase motifs. Oncogene. 1993;8(1):37–44.
3. Maminta ML, Williams KL, Nakagawara A, Enger KT, Guo C, Brodeur GM, et al. Identification of a novel tyrosine kinase receptor-like molecule in neuroblastomas. Biochem Biophys Res Commun. 1992;189(2):1077–83.
4. Partanen J, Makela TP, Alitalo R, Lehvaslaiho H, Alitalo K. Putative tyrosine kinases expressed in K-562 human leukemia cells. Proc Natl Acad Sci USA. 1990;87(22):8913–7.
5. Paul SR, Merberg D, Finnerty H, Morris GE, Morris JC, Jones SS, et al. Molecular cloning of the cDNA encoding a receptor tyrosine kinase-related molecule with a catalytic region homologous to c-met. Int J Cell Cloning. 1992;10(5):309–14.
6. Yee K, Bishop TR, Mather C, Zon LI. Isolation of a novel receptor tyrosine kinase cDNA expressed by developing erythroid progenitors. Blood. 1993;82(4):1335–43.
7. Hovens CM, Stacker SA, Andres AC, Harpur AG, Ziemiecki A, Wilks AF. RYK, a receptor tyrosine kinase-related molecule with unusual kinase domain motifs. Proc Natl Acad Sci USA. 1992;89(24):11818–22.
8. Stacker SA, Hovens CM, Vitali A, Pritchard MA, Baker E, Sutherland GR, et al. Molecular cloning and chromosomal localisation of the human homologue of a receptor related to tyrosine kinases (RYK). Oncogene. 1993;8(5):1347–56.
9. Yoshikawa S, McKinnon RD, Kokel M, Thomas JB. Wnt-mediated axon guidance via the *Drosophila* Derailed receptor. Nature. 2003;422(6932):583–8.
10. Patthy L. The WIF module. Trends Biochem Sci. 2000;25(1):12–3.
11. Bainbridge TW, DeAlmeida VI, Izrael-Tomasevic A, Chalouni C, Pan B, Goldsmith J, et al. Evolutionary divergence in the catalytic activity of the CAM-1, ROR1 and ROR2 kinase domains. PLoS One. 2014;9(7):e102695.
12. Mendrola JM, Shi F, Park JH, Lemmon MA. Receptor tyrosine kinases with intracellular pseudokinase domains. Biochem Soc Trans. 2013;41(4):1029–36.
13. Lee ST, Strunk KM, Spritz RA. A survey of protein tyrosine kinase mRNAs expressed in normal human melanocytes. Oncogene. 1993;8(12):3403–10.
14. Gough NM, Rakar S, Hovens CM, Wilks A. Localization of two mouse genes encoding the protein tyrosine kinase receptor-related protein RYK. Mamm Genome. 1995;6(4):255–6.
15. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298(5600):1912–34.
16. Caenepeel S, Charydczak G, Sudarsanam S, Hunter T, Manning G. The mouse kinome: discovery and comparative genomics of all mouse protein kinases. Proc Natl Acad Sci USA. 2004;101(32):11707–12.
17. Callahan CA, Muralidhar MG, Lundgren SE, Scully AL, Thomas JB. Control of neuronal pathway selection by a *Drosophila* receptor protein-tyrosine kinase family member. Nature. 1995;376(6536):171–4.
18. Dura JM, Preat T, Tully T. Identification of *linotte*, a new gene affecting learning and memory in *Drosophila melanogaster*. J Neurogenet. 1993;9(1):1–14.
19. Dura JM, Taillebourg E, Preat T. The *Drosophila* learning and memory gene *linotte* encodes a putative receptor tyrosine kinase homologous to the human *RYK* gene product. FEBS Lett. 1995;370(3):250–4.
20. Sakurai M, Aoki T, Yoshikawa S, Santschi LA, Saito H, Endo K, et al. Differentially expressed Drl and Drl-2 play opposing roles in Wnt5 signaling during *Drosophila* olfactory system development. J Neurosci. 2009;29(15):4972–80.
21. Oates AC, Bonkovsky JL, Irvine DV, Kelly LE, Thomas JB, Wilks AF. Embryonic expression and activity of doughnut, a second RYK homolog in *Drosophila*. Mech Dev. 1998;78(1–2):165–9.

22. Savant-Bhonsale S, Friese M, McCoon P, Montell DJ. A *Drosophila derailed* homolog, *doughnut*, expressed in invaginating cells during embryogenesis. Gene. 1999;231(1–2): 155–61.
23. Halford MM, Oates AC, Hibbs ML, Wilks AF, Stacker SA. Genomic structure and expression of the mouse growth factor receptor related to tyrosine kinases (Ryk). J Biol Chem. 1999;274(11):7379–90.
24. Bonkowsky JL, Thomas JB. Cell-type specific modular regulation of *derailed* in the *Drosophila* nervous system. Mech Dev. 1999;82(1–2):181–4.
25. Harris KE, Beckendorf SK. Different Wnt signals act through the Frizzled and RYK receptors during *Drosophila* salivary gland migration. Development. 2007;134(11):2017–25.
26. Kuzin A, Brody T, Moore AW, Odenwald WF. Nerfin-1 is required for early axon guidance decisions in the developing *Drosophila* CNS. Dev Biol. 2005;277(2):347–65.
27. Tamagnone L, Partanen J, Armstrong E, Lasota J, Ohgami K, Tazunoki T, et al. The human ryk cDNA sequence predicts a protein containing two putative transmembrane segments and a tyrosine kinase catalytic domain. Oncogene. 1993;8(7):2009–14.
28. Szafranski K, Schindler S, Taudien S, Hiller M, Huse K, Jahn N, et al. Violating the splicing rules: TG dinucleotides function as alternative 3′ splice sites in U2-dependent introns. Genome Biol. 2007;8(8):R154.
29. Vorlová S, Rocco G, Lefave CV, Jodelka FM, Hess K, Hastings ML, et al. Induction of antagonistic soluble decoy receptor tyrosine kinases by intronic polyA activation. Mol Cell. 2011;43(6):927–39.
30. Wang XC, Katso R, Butler R, Hanby AM, Poulsom R, Jones T, et al. H-RYK, an unusual receptor kinase: isolation and analysis of expression in ovarian cancer. Mol Med. 1996; 2(2):189–203.
31. Kamitori K, Tanaka M, Okuno-Hirasawa T, Kohsaka S. Receptor related to tyrosine kinase RYK regulates cell migration during cortical development. Biochem Biophys Res Commun. 2005;330(2):446–53.
32. Blakely BD, Bye CR, Fernando CV, Prasad AA, Pasterkamp RJ, Macheda ML, et al. Ryk, a receptor regulating Wnt5a-mediated neurogenesis and axon morphogenesis of ventral midbrain dopaminergic neurons. Stem Cells Dev. 2013;22(15):2132–44.
33. Halford MM, Armes J, Buchert M, Meskenaite V, Grail D, Hibbs ML, et al. Ryk-deficient mice exhibit craniofacial defects associated with perturbed Eph receptor crosstalk. Nat Genet. 2000;25(4):414–8.
34. Zhong J, Kim HT, Lyu J, Yoshikawa K, Nakafuku M, Lu W. The Wnt receptor Ryk controls specification of GABAergic neurons versus oligodendrocytes during telencephalon development. Development. 2011;138(3):409–19.
35. Andre P, Wang Q, Wang N, Gao B, Schilit A, Halford MM, et al. The Wnt coreceptor Ryk regulates Wnt/planar cell polarity by modulating the degradation of the core planar cell polarity component Vangl2. J Biol Chem. 2012;287(53):44518–25.
36. Serfas MS, Tyner AL. Ryk is expressed in a differentiation-specific manner in epithelial tissues and is strongly induced in decidualizing uterine stroma. Oncogene. 1998;17(26): 3435–44.
37. Favre CJ, Mancuso M, Maas K, McLean JW, Baluk P, McDonald DM. Expression of genes involved in vascular development and angiogenesis in endothelial cells of adult lung. Am J Physiol Heart Circ Physiol. 2003;285(5):H1917–38.
38. Kamitori K, Machide M, Osumi N, Kohsaka S. Expression of receptor tyrosine kinase RYK in developing rat central nervous system. Brain Res Dev Brain Res. 1999;114(1):149–60.
39. Lyu J, Yamamoto V, Lu W. Cleavage of the Wnt receptor Ryk regulates neuronal differentiation during cortical neurogenesis. Dev Cell. 2008;15(5):773–80.
40. Lin S, Baye LM, Westfall TA, Slusarski DC. Wnt5b-Ryk pathway provides directional signals to regulate gastrulation movement. J Cell Biol. 2010;190(2):263–78.
41. Kim GH, Her JH, Han JK. Ryk cooperates with Frizzled 7 to promote Wnt11-mediated endocytosis and is essential for *Xenopus laevis* convergent extension movements. J Cell Biol. 2008;182(6):1073–82.

42. Zhang B, Tran U, Wessely O. Expression of Wnt signaling components during *Xenopus* pronephros development. PLoS One. 2011;6(10):e26533.
43. Bonkowsky JL, Yoshikawa S, O'Keefe DD, Scully AL, Thomas JB. Axon routing across the midline controlled by the *Drosophila* Derailed receptor. Nature. 1999;402(6761):540–4.
44. Wouda RR, Bansraj MR, de Jong AW, Noordermeer JN, Fradkin LG. Src family kinases are required for WNT5 signaling through the Derailed/RYK receptor in the *Drosophila* embryonic central nervous system. Development. 2008;135(13):2277–87.
45. Carreira VP, Mensch J, Fanara JJ. Body size in *Drosophila*: genetic architecture, allometries and sexual dimorphism. Heredity. 2009;102(3):246–56.
46. The modEncode Consortium, Roy S, Ernst J, Kharchenko PV, Kheradpour P, Negre N, et al. Identification of functional elements and regulatory circuits by Drosophila modENCODE. Science. 2010;330(6012):1787–97.
47. Inaki M, Yoshikawa S, Thomas JB, Aburatani H, Nose A. Wnt4 is a local repulsive cue that determines synaptic target specificity. Curr Biol. 2007;17(18):1574–9.
48. Sopko R, Perrimon N. Receptor tyrosine kinases in *Drosophila* development. Cold Spring Harb Perspect Biol. 2013;5(6).
49. Fradkin LG, Dura JM, Noordermeer JN. Ryks: new partners for Wnts in the developing and regenerating nervous system. Trends Neurosci. 2009;33(2):84–92.
50. Inoue T, Oz HS, Wiland D, Gharib S, Deshpande R, Hill RJ, et al. *C. elegans* LIN-18 is a Ryk ortholog and functions in parallel to LIN-17/Frizzled in Wnt signaling. Cell. 2004;118(6):795–806.
51. Poh WC, Shen Y, Inoue T. Function of the Ryk intracellular domain in C. elegans vulval development. Dev Dyn. 2014;243(9):1074–85.
52. Hsieh JC, Kodjabachian L, Rebbert ML, Rattner A, Smallwood PM, Samos CH, et al. A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature. 1999;398(6726):431–6.
53. Taylor SS, Zhang P, Steichen JM, Keshwani MM, Kornev AP. PKA: lessons learned after twenty years. Biochim Biophys Acta. 2013;1834(7):1271–8.
54. Petrova IM, Lahaye LL, Martianez T, de Jong AW, Malessy MJ, Verhaagen J, et al. Homodimerization of the Wnt receptor DERAILED recruits the Src family kinase SRC64B. Mol Cell Biol. 2013;33(20):4116–27.
55. Murphy JM, Zhang Q, Young SN, Reese ML, Bailey FP, Eyers PA, et al. A robust methodology to subclassify pseudokinases based on their nucleotide-binding properties. Biochem J. 2014;457(2):323–34.
56. Kornev AP, Haste NM, Taylor SS, Eyck LF. Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. Proc Natl Acad Sci USA. 2006;103(47):17783–8.
57. Lu W, Yamamoto V, Ortega B, Baltimore D. Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. Cell. 2004;119(1):97–108.
58. Seidah NG, Prat A. The biology and therapeutic targeting of the proprotein convertases. Nat Rev Drug Discov. 2012;11(5):367–83.
59. Halford MM, Macheda ML, Parish CL, Takano EA, Fox S, Layton D, et al. A fully human inhibitory monoclonal antibody to the Wnt receptor RYK. PLoS One. 2013;8(9):e75447.
60. Halford MM, Stacker SA. Revelations of the RYK receptor. Bioessays. 2001;23(1):34–45.
61. Malinauskas T, Aricescu AR, Lu W, Siebold C, Jones EY. Modular mechanism of Wnt signaling inhibition by Wnt inhibitory factor 1. Nat Struct Mol Biol. 2011;18(8):886–93.
62. Lyu J, Wesselschmidt RL, Lu W. Cdc37 regulates Ryk signaling by stabilizing the cleaved Ryk intracellular domain. J Biol Chem. 2009;284(19):12940–8.
63. Tourette C, Farina F, Vazquez-Manrique RP, Orfila AM, Voisin J, Hernandez S, et al. The Wnt receptor Ryk reduces neuronal and cell survival capacity by repressing FOXO activity during the early phases of mutant Huntingtin pathogenicity. PLoS Biol. 2014;12(6):e1001895.
64. Struhl G, Adachi A. Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. Mol Cell. 2000;6(3):625–36.

65. Shah S, Lee SF, Tabuchi K, Hao YH, Yu C, LaPlant Q, et al. Nicastrin functions as a γ-secretase-substrate receptor. Cell. 2005;122(3):435–47.
66. Hemming ML, Elias JE, Gygi SP, Selkoe DJ. Proteomic profiling of γ-secretase substrates and mapping of substrate requirements. PLoS Biol. 2008;6(10):e257.
67. De Strooper B, Annaert W. Novel research horizons for presenilins and γ-secretases in cell biology and disease. Annu Rev Cell Dev Biol. 2010;26:235–60.
68. Gobin B, Moriceau G, Ory B, Charrier C, Brion R, Blanchard F, et al. Imatinib mesylate exerts anti-proliferative effects on osteosarcoma cells and inhibits the tumour growth in immunocompetent murine models. PLoS One. 2014;9(3):e90795.
69. Berndt JD, Aoyagi A, Yang P, Anastas JN, Tang L, Moon RT. Mindbomb 1, an E3 ubiquitin ligase, forms a complex with RYK to activate Wnt/β-catenin signaling. J Cell Biol. 2011;194(5):737–50.
70. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, et al. Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell. 2011;44(2):325–40.
71. Moffat LL, Robinson RE, Bakoulis A, Clark SG. The conserved transmembrane RING finger protein PLR-1 downregulates Wnt signaling by reducing Frizzled, Ror and Ryk cell-surface levels in *C. elegans*. Development. 2014;141(3):617–28.
72. de Lau W, Peng WC, Gros P, Clevers H. The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. Genes Dev. 2014;28(4):305–16.
73. Huse M, Kuriyan J. The conformational plasticity of protein kinases. Cell. 2002;109(3): 275–82.
74. Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. Proc Natl Acad Sci USA. 2010;107(17):7692–7.
75. Mukherjee K, Sharma M, Urlaub H, Bourenkov GP, Jahn R, Südhof TC, et al. CASK functions as a Mg^{2+}-independent neurexin kinase. Cell. 2008;133(2):328–39.
76. Ungureanu D, Wu J, Pekkala T, Niranjan Y, Young C, Jensen ON, et al. The pseudokinase domain of JAK2 is a dual-specificity protein kinase that negatively regulates cytokine signaling. Nat Struct Mol Biol. 2011;18(9):971–6.
77. Zeqiraj E, van Aalten DM. Pseudokinases—— remnants of evolution or key allosteric regulators? Curr Opin Struct Biol. 2010;20(6):772–81.
78. Boudeau J, Miranda-Saavedra D, Barton GJ, Alessi DR. Emerging roles of pseudokinases. Trends Cell Biol. 2006;16(9):443–52.
79. Katso RM, Manek S, Biddolph S, Whittaker R, Charnock MF, Wells M, et al. Overexpression of H-Ryk in mouse fibroblasts confers transforming ability *in vitro* and *in vivo*: correlation with up-regulation in epithelial ovarian cancer. Cancer Res. 1999;59(10):2265–70.
80. Watanabe A, Akita S, Tin NT, Natsume N, Nakano Y, Niikawa N, et al. A mutation in *RYK* is a genetic factor for nonsyndromic cleft lip and palate. Cleft Palate Craniofac J. 2006; 43(3):310–6.
81. Carrera AC, Alexandrov K, Roberts TM. The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. Proc Natl Acad Sci USA. 1993;90(2):442–6.
82. McCormick JA, Ellison DH. The WNKs: atypical protein kinases with pleiotropic actions. Physiol Rev. 2011;91(1):177–219.
83. Yoshikawa S, Bonkowsky JL, Kokel M, Shyn S, Thomas JB. The Derailed guidance receptor does not require kinase activity *in vivo*. J Neurosci. 2001;21(1):RC119.
84. Yao Y, Wu Y, Yin C, Ozawa R, Aigaki T, Wouda RR, et al. Antagonistic roles of Wnt5 and the Drl receptor in patterning the *Drosophila* antennal lobe. Nat Neurosci. 2007;10(11): 1423–32.
85. Taillebourg E, Moreau-Fauvarque C, Delaval K, Dura JM. *In vivo* evidence for a regulatory role of the kinase activity of the *linotte/derailed* receptor tyrosine kinase, a *Drosophila* Ryk ortholog. Dev Genes Evol. 2005;215(3):158–63.
86. Kornev AP, Taylor SS. Pseudokinases: functional insights gleaned from structure. Structure. 2009;17(1):5–7.

87. Carpenter G, Liao HJ. Receptor tyrosine kinases in the nucleus. Cold Spring Harb Perspect Biol. 2013;5(10):a008979.
88. Liu Y, Shi J, Lu CC, Wang ZB, Lyuksyutova AI, Song XJ, et al. Ryk-mediated Wnt repulsion regulates posterior-directed growth of corticospinal tract. Nat Neurosci. 2005;8(9):1151–9.
89. Kamitori K, Machide M, Tomita K, Nakafuku M, Kohsaka S. Cell-type-specific expression of protein tyrosine kinase-related receptor RYK in the central nervous system of the rat. Brain Res Mol Brain Res. 2002;104(2):255–66.
90. Li L, Hutchins BI, Kalil K. Wnt5a induces simultaneous cortical axon outgrowth and repulsive axon guidance through distinct signaling mechanisms. J Neurosci. 2009;29(18): 5873–83.
91. Keeble TR, Halford MM, Seaman C, Kee N, Macheda M, Anderson RB, et al. The Wnt receptor Ryk is required for Wnt5a-mediated axon guidance on the contralateral side of the corpus callosum. J Neurosci. 2006;26(21):5840–8.
92. Povinelli BJ, Nemeth MJ. Wnt5a regulates hematopoietic stem cell proliferation and repopulation through the Ryk receptor. Stem cells. 2014;32(1):105–15.
93. de Graaf CA, Kauppi M, Baldwin T, Hyland CD, Metcalf D, Willson TA, et al. Regulation of hematopoietic stem cells by their mature progeny. Proc Natl Acad Sci USA. 2010;107(50): 21689–94.
94. Forsberg EC, Passegue E, Prohaska SS, Wagers AJ, Koeva M, Stuart JM, et al. Molecular signatures of quiescent, mobilized and leukemia-initiating hematopoietic stem cells. PLoS One. 2010;5(1):e8785.
95. Liebl FL, Wu Y, Featherstone DE, Noordermeer JN, Fradkin L, Hing H. Derailed regulates development of the *Drosophila* neuromuscular junction. Dev Neurobiol. 2008;68(2): 152–65.
96. Callahan CA, Bonkovsky JL, Scully AL, Thomas JB. *derailed* is required for muscle attachment site selection in Drosophila. Development. 1996;122(9):2761–7.
97. Grillenzoni N, Flandre A, Lasbleiz C, Dura JM. Respective roles of the DRL receptor and its ligand WNT5 in *Drosophila* mushroom body development. Development. 2007;134(17): 3089–97.
98. Hitier R, Simon AF, Savarit F, Preat T. *no-bridge* and *linotte* act jointly at the interhemispheric junction to build up the adult central brain of Drosophila melanogaster. Mech Dev. 2000;99(1–2):93–100.
99. Simon AF, Boquet I, Synguelakis M, Preat T. The *Drosophila* putative kinase Linotte (Derailed) prevents central brain axons from converging on a newly described interhemispheric ring. Mech Dev. 1998;76(1–2):45–55.
100. Wu Y, Helt JC, Wexler E, Petrova IM, Noordermeer JN, Fradkin LG, et al. Wnt5 and Drl/Ryk gradients pattern the *Drosophila* olfactory dendritic map. J Neurosci. 2014;34(45): 14961–72.
101. Bazan JF, Janda CY, Garcia KC. Structural architecture and functional evolution of Wnts. Dev Cell. 2012;23(2):227–32.
102. Willert K, Nusse R. Wnt proteins. Cold Spring Harb Perspect Biol. 2012;4(9):a007864.
103. Cruciat CM, Niehrs C. Secreted and transmembrane wnt inhibitors and activators. Cold Spring Harb Perspect Biol. 2013;5(3):a015081.
104. Niehrs C. The complex world of WNT receptor signalling. Nat Rev Mol Cell Biol. 2012;13(12):767–79.
105. Angers S, Moon RT. Proximal events in Wnt signal transduction. Nat Rev Mol Cell Biol. 2009;10(7):468–77.
106. Nusse R, Varmus H. Three decades of Wnts: a personal perspective on how a scientific field developed. EMBO J. 2012;31(12):2670–84.
107. Clevers H, Nusse R. Wnt/β-catenin signaling and disease. Cell. 2012;149(6):1192–205.
108. Kahn M. Can we safely target the WNT pathway? Nat Rev Drug Discov. 2014; 13(7):513–32.
109. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer. 2013;13(1):11–26.

110. Hernandez AR, Klein AM, Kirschner MW. Kinetic responses of β-catenin specify the sites of Wnt control. Science. 2012;338(6112):1337–40.
111. van Amerongen R. Alternative Wnt pathways and receptors. Cold Spring Harb Perspect Biol. 2012;4(10).
112. Schmitt AM, Shi J, Wolf AM, Lu CC, King LA, Zou Y. Wnt-Ryk signalling mediates medial-lateral retinotectal topographic mapping. Nature. 2006;439(7072):31–7.
113. Feldheim DA, O'Leary DD. Visual map development: bidirectional signaling, bifunctional guidance molecules, and competition. Cold Spring Harb Perspect Biol. 2010;2(11):a001768.
114. Lyuksyutova AI, Lu CC, Milanesio N, King LA, Guo N, Wang Y, et al. Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. Science. 2003;302(5652): 1984–8.
115. Hutchins BI, Li L, Kalil K. Wnt/calcium signaling mediates axon growth and guidance in the developing corpus callosum. Dev Neurobiol. 2011;71(4):269–83.
116. Blakely BD, Bye CR, Fernando CV, Horne MK, Macheda ML, Stacker SA, et al. Wnt5a regulates midbrain dopaminergic axon growth and guidance. PLoS One. 2011;6(3):e18373.
117. Simons M, Mlodzik M. Planar cell polarity signaling: from fly development to human disease. Annu Rev Genet. 2008;42:517–40.
118. Devenport D. The cell biology of planar cell polarity. J Cell Biol. 2014;207(2):171–9.
119. Macheda ML, Sun WW, Kugathasan K, Hogan BM, Bower NI, Halford MM, et al. The Wnt receptor Ryk plays a role in mammalian planar cell polarity signaling. J Biol Chem. 2012; 287(35):29312–23.
120. Fradkin LG, van Schie M, Wouda RR, de Jong A, Kamphorst JT, Radjkoemar-Bansraj M, et al. The *Drosophila* Wnt5 protein mediates selective axon fasciculation in the embryonic central nervous system. Dev Biol. 2004;272(2):362–75.
121. Fradkin LG, Noordermeer JN, Nusse R. The *Drosophila* Wnt protein DWnt-3 is a secreted glycoprotein localized on the axon tracts of the embryonic CNS. Dev Biol. 1995; 168(1):202–13.
122. Bolwig GM, Del Vecchio M, Hannon G, Tully T. Molecular cloning of *linotte* in *Drosophila*: a novel gene that functions in adults during associative learning. Neuron. 1995;15(4): 829–42.
123. Singh AP, VijayRaghavan K, Rodrigues V. Dendritic refinement of an identified neuron in the *Drosophila* CNS is regulated by neuronal activity and Wnt signaling. Development. 2010;137(8):1351–60.
124. Vosshall LB, Stocker RF. Molecular architecture of smell and taste in *Drosophila*. Annu Rev Neurosci. 2007;30:505–33.
125. Luo L, Flanagan JG. Development of continuous and discrete neural maps. Neuron. 2007;56(2):284–300.
126. Lahaye LL, Wouda RR, de Jong AW, Fradkin LG, Noordermeer JN. WNT5 interacts with the Ryk receptors Doughnut and Derailed to mediate muscle attachment site selection in *Drosophila melanogaster*. PLoS One. 2012;7(3):e32297.
127. Speicher S, Garcia-Alonso L, Carmena A, Martin-Bermudo MD, de la Escalera S, Jimenez F. Neurotactin functions in concert with other identified CAMs in growth cone guidance in *Drosophila*. Neuron. 1998;20(2):221–33.
128. Ferguson EL, Horvitz HR. Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. Genetics. 1985;110(1):17–72.
129. Gupta BP, Hanna-Rose W, Sternberg PW. Morphogenesis of the vulva and the vulval-uterine connection. WormBook. 2012:1–20.
130. Ferguson EL, Sternberg PW, Horvitz HR. A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. Nature. 1987;326(6110):259–67.
131. Gleason JE, Szyleyko EA, Eisenmann DM. Multiple redundant Wnt signaling components function in two processes during C. elegans vulval development. Dev Biol. 2006; 298(2):442–57.
132. Green JL, Inoue T, Sternberg PW. Opposing Wnt pathways orient cell polarity during organogenesis. Cell. 2008;134(4):646–56.

133. Zinovyeva AY, Yamamoto Y, Sawa H, Forrester WC. Complex network of Wnt signaling regulates neuronal migrations during *Caenorhabditis elegans* development. Genetics. 2008; 179(3):1357–71.
134. Zheng M, Messerschmidt D, Jungblut B, Sommer RJ. Conservation and diversification of Wnt signaling function during the evolution of nematode vulva development. Nat Genet. 2005;37(3):300–4.
135. Tian H, Schlager B, Xiao H, Sommer RJ. Wnt signaling induces vulva development in the nematode *Pristionchus pacificus*. Curr Biol. 2008;18(2):142–6.
136. Wang X, Sommer RJ. Antagonism of LIN-17/Frizzled and LIN-18/Ryk in nematode vulva induction reveals evolutionary alterations in core developmental pathways. PLoS Biol. 2011;9(7):e1001110.
137. Finger C, Escher C, Schneider D. The single transmembrane domains of human receptor tyrosine kinases encode self-interactions. Sci Signal. 2009;2(89):ra56.
138. Nicolaï M, Lasbleiz C, Dura JM. Gain-of-function screen identifies a role of the Src64 oncogene in *Drosophila* mushroom body development. J Neurobiol. 2003;57(3):291–302.
139. Orioli D, Henkemeyer M, Lemke G, Klein R, Pawson T. Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. EMBO J. 1996;15(22): 6035–49.
140. Mandal AK, Lee P, Chen JA, Nillegoda N, Heller A, DiStasio S, et al. Cdc37 has distinct roles in protein kinase quality control that protect nascent chains from degradation and promote posttranslational maturation. J Cell Biol. 2007;176(3):319–28.
141. Vaughan CK, Gohlke U, Sobott F, Good VM, Ali MM, Prodromou C, et al. Structure of an Hsp90-Cdc37-Cdk4 complex. Mol Cell. 2006;23(5):697–707.
142. Lee HJ, Zheng JJ. PDZ domains and their binding partners: structure, specificity, and modification. Cell Commun Signal. 2010;8:8.
143. Buchert M, Poon C, King JA, Baechi T, D'Abaco G, Hollande F, et al. AF6/s-afadin is a dual residency protein and localizes to a novel subnuclear compartment. J Cell Physiol. 2007; 210(1):212–23.
144. Kim GH, Park EC, Lee H, Na HJ, Choi SC, Han JK. β-Arrestin 1 mediates non-canonical Wnt pathway to regulate convergent extension movements. Biochem Biophys Res Commun. 2013;435(2):182–7.
145. Hitier R, Chaminade M, Preat T. The *Drosophila castor* gene is involved in postembryonic brain development. Mech Dev. 2001;103(1–2):3–11.
146. Sawa H. Control of cell polarity and asymmetric division in *C. elegans*. Curr Top Dev Biol. 2012;101:55–76.
147. Deshpande R, Inoue T, Priess JR, Hill RJ. *lin-17*/*Frizzled* and *lin-18* regulate POP-1/TCF-1 localization and cell type specification during *C. elegans* vulval development. Dev Biol. 2005;278(1):118–29.
148. Li L, Fothergill T, Hutchins BI, Dent EW, Kalil K. Wnt5a evokes cortical axon outgrowth and repulsive guidance by tau mediated reorganization of dynamic microtubules. Dev Neurobiol. 2014;74(8):797–817.
149. Miyashita T, Koda M, Kitajo K, Yamazaki M, Takahashi K, Kikuchi A, et al. Wnt-Ryk signaling mediates axon growth inhibition and limits functional recovery after spinal cord injury. J Neurotrauma. 2009;26(7):955–64.
150. Liu Y, Wang X, Lu CC, Kerman R, Steward O, Xu XM, et al. Repulsive Wnt signaling inhibits axon regeneration after CNS injury. J Neurosci. 2008;28(33):8376–82.
151. Gonzalez P, Fernandez-Martos CM, Arenas E, Rodriguez FJ. The Ryk receptor is expressed in glial and fibronectin-expressing cells after spinal cord injury. J Neurotrauma. 2013; 30(10):806–17.
152. Li X, Li YH, Yu S, Liu Y. Upregulation of Ryk expression in rat dorsal root ganglia after peripheral nerve injury. Brain Res Bull. 2008;77(4):178–84.
153. Hollis 2nd ER, Zou Y. Reinduced Wnt signaling limits regenerative potential of sensory axons in the spinal cord following conditioning lesion. Proc Natl Acad Sci USA. 2012; 109(36):14663–8.

154. Hendricks M, Mathuru AS, Wang H, Silander O, Kee MZ, Jesuthasan S. Disruption of Esrom and Ryk identifies the roof plate boundary as an intermediate target for commissure formation. Mol Cell Neurosci. 2008;37(2):271–83.
155. Micci F, Panagopoulos I, Haugom L, Andersen HK, Tjonnfjord GE, Beiske K, et al. t(3;21)(q22;q22) leading to truncation of the *RYK* gene in atypical chronic myeloid leukemia. Cancer Lett. 2009;277(2):205–11.
156. Muller-Tidow C, Schwable J, Steffen B, Tidow N, Brandt B, Becker K, et al. High-throughput analysis of genome-wide receptor tyrosine kinase expression in human cancers identifies potential novel drug targets. Clin Cancer Res. 2004;10(4):1241–9.
157. Hirano H, Yonezawa H, Yunoue S, Habu M, Uchida H, Yoshioka T, et al. Immunoreactivity of Wnt5a, Fzd2, Fzd6, and Ryk in glioblastoma: evaluative methodology for DAB chromogenic immunostaining. Brain Tumor Pathol. 2013;31(2):85–93.
158. Katso RM, Manek S, Ganjavi H, Biddolph S, Charnock MF, Bradburn M, et al. Overexpression of H-Ryk in epithelial ovarian cancer: prognostic significance of receptor expression. Clin Cancer Res. 2000;6(8):3271–81.
159. Anastas JN, Kulikauskas RM, Tamir T, Rizos H, Long GV, von Euw EM, et al. WNT5A enhances resistance of melanoma cells to targeted BRAF inhibitors. J Clin Invest. 2014;124(7):2877–90.
160. Wu X, Northcott PA, Dubuc A, Dupuy AJ, Shih DJ, Witt H, et al. Clonal selection drives genetic divergence of metastatic medulloblastoma. Nature. 2012;482(7386):529–33.
161. Quintana RM, Dupuy AJ, Bravo A, Casanova ML, Alameda JP, Page A, et al. A transposon-based analysis of gene mutations related to skin cancer development. J Invest Dermatol. 2013;133(1):239–48.
162. Jugessur A, Shi M, Gjessing HK, Lie RT, Wilcox AJ, Weinberg CR, et al. Genetic determinants of facial clefting: analysis of 357 candidate genes using two national cleft studies from Scandinavia. PLoS One. 2009;4(4):e5385.
163. Carter TC, Molloy AM, Pangilinan F, Troendle JF, Kirke PN, Conley MR, et al. Testing reported associations of genetic risk factors for oral clefts in a large Irish study population. Birth Defects Res A Clin Mol Teratol. 2010;88(2):84–93.
164. Afzal AR, Rajab A, Fenske CD, Oldridge M, Elanko N, Ternes-Pereira E, et al. Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of *ROR2*. Nat Genet. 2000;25(4):419–22.
165. van Bokhoven H, Celli J, Kayserili H, van Beusekom E, Balci S, Brussel W, et al. Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome. Nat Genet. 2000;25(4):423–6.
166. Person AD, Beiraghi S, Sieben CM, Hermanson S, Neumann AN, Robu ME, et al. *WNT5A* mutations in patients with autosomal dominant Robinow syndrome. Dev Dyn. 2010;239(1):327–37.
167. Mazzeu JF, Pardono E, Vianna-Morgante AM, Richieri-Costa A, Ae Kim C, Brunoni D, et al. Clinical characterization of autosomal dominant and recessive variants of Robinow syndrome. Am J Med Genet A. 2007;143(4):320–5.
168. Mazzeu JF. *RYK* is not mutated in autosomal dominant Robinow syndrome. J Biol Chem. 2013;288(4):2905.
169. Andre P, Yang Y. Reply to Mazzeu: Human mutations in *RYK* might cause Robinow syndrome. J Biol Chem. 2013;288(4):2906.
170. Fromigue O, Hay E, Barbara A, Marie PJ. Essential role of nuclear factor of activated T cells (NFAT)-mediated Wnt signaling in osteoblast differentiation induced by strontium ranelate. J Biol Chem. 2010;285(33):25251–8.
171. Santiago F, Oguma J, Brown AM, Laurence J. Noncanonical Wnt signaling promotes osteoclast differentiation and is facilitated by the human immunodeficiency virus protease inhibitor ritonavir. Biochem Biophys Res Commun. 2012;417(1):223–30.

172. Kumawat K, Menzen MH, Bos IS, Baarsma HA, Borger P, Roth M, et al. Noncanonical WNT-5A signaling regulates TGF-β-induced extracellular matrix production by airway smooth muscle cells. FASEB J. 2013;27(4):1631–43.
173. Cheng JH, She H, Han YP, Wang J, Xiong S, Asahina K, et al. Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. Am J Physiol Gastrointest Liver Physiol. 2008;294(1):G39–49.
174. Tury A, Tolentino K, Zou Y. Altered expression of atypical PKC and Ryk in the spinal cord of a mouse model of amyotrophic lateral sclerosis. Dev Neurobiol. 2014;74(8):839–50.
175. Lum L, Clevers H. The unusual case of Porcupine. Science. 2012;337(6097):922–3.
176. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487(7407):330–7.
177. Seo JS, Ju YS, Lee WC, Shin JY, Lee JK, Bleazard T, et al. The transcriptional landscape and mutational profile of lung adenocarcinoma. Genome Res. 2012;22(11):2109–19.
178. Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet. 2012;44(6):694–8.
179. Kan Z, Jaiswal BS, Stinson J, Janakiraman V, Bhatt D, Stern HM, et al. Diverse somatic mutation patterns and pathway alterations in human cancers. Nature. 2010;466(7308):869–73.
180. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. Nature. 2011;474(7353):609–15.
181. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157–60.
182. Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC, et al. The landscape of cancer genes and mutational processes in breast cancer. Nature. 2012;486(7403):400–4.
183. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat. 2000;15(1):7–12.

Chapter 16
The TIE Receptor Family

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16.1 Introduction to the TIE Receptor Tyrosine Kinase Family

TIE1 and TIE2 receptor tyrosine kinases (RTK) were isolated in the beginning of the 1990s [1–4]. They constitute a distinct RTK subfamily with a unique extracellular structure consisting of epidermal growth factor, immunoglobulin, and fibronectin type III domains (Fig. 16.1, Table 16.1). Angiopoietin growth

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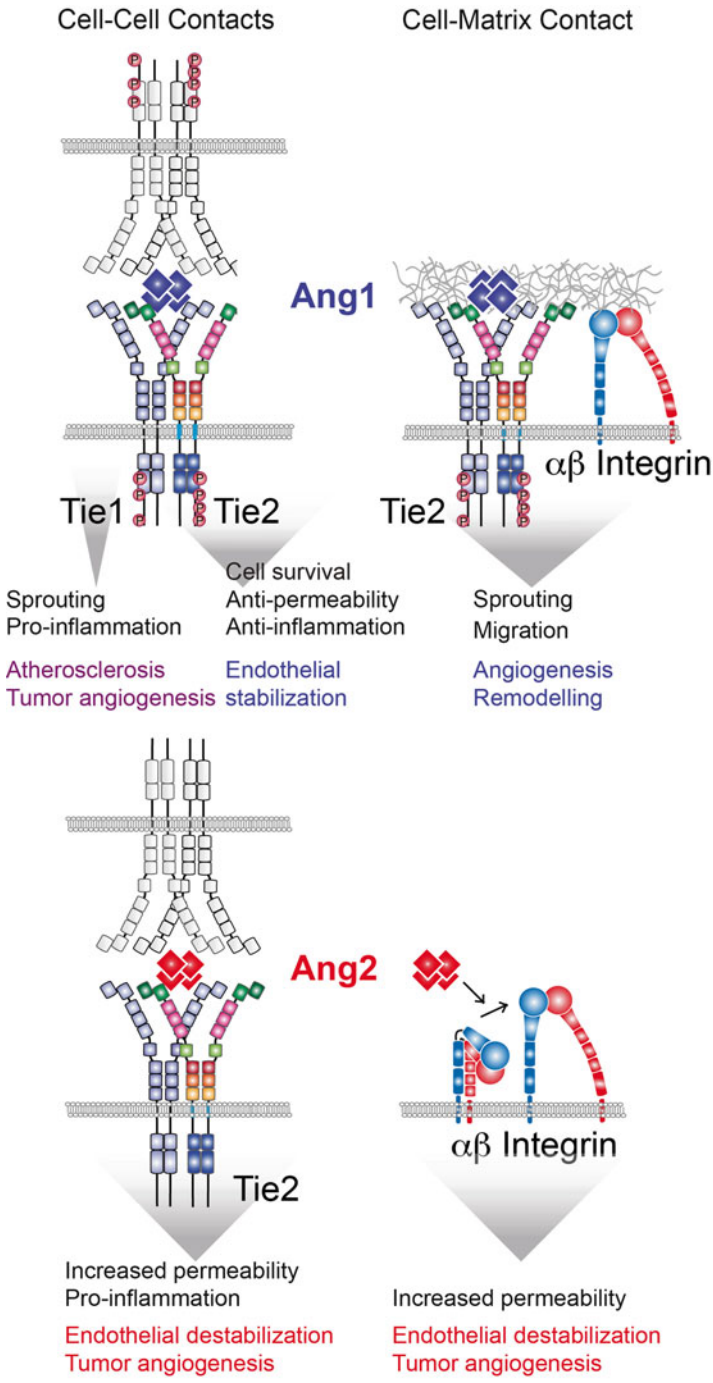

Fig. 16.1 Schematic presentation of the angiopoietin-induced TIE1 and TIE2 receptor signaling complexes. Angiopoietin growth factors bind to the TIE2 receptor tyrosine kinase with similar affinity, inducing TIE receptor clustering. The activating ANG1 ligand induces phosphorylation of TIE2, and to some extent also TIE1 in cell–matrix (*top, right*) and cell–cell

factors (ANGPT1, ANGPT2, ANGPT4, also called ANG1, ANG2, ANG4 in humans) are ligands for TIE2 [5–7], while TIE1 is an orphan receptor. Besides the vascular endothelial growth factors (VEGFs) and their receptors, the angiopoietins and TIE receptors define the second endothelial specific RTK signaling pathway. The ANG-TIE system is important for cardiac, blood vascular, and lymphatic vessel development and for the homeostasis of the mature vasculature. The ANG-TIE system also regulates tumor angiogenesis, lymphangiogenesis, and metastasis as well as pathological endothelial inflammation and vascular leak in numerous diseases (reviewed in [8, 9]).

16.2 The Role of the TIE Receptor Tyrosine Kinase Family in Vascular Development

After the initial assembly of the primary vascular plexus, which requires VEGF-dependent signals, the ANG-TIE system is necessary for cardiac development and for the remodeling of both the blood and lymphatic vasculatures after mid-gestation [8]. Gene-targeted mouse models have revealed a unique function for the ANG1-TIE2 pathway during cardiac development and for TIE1 and TIE2 during later stages of angiogenic capillary growth. Furthermore, TIE1, ANG2 and ANG1 regulate postnatal growth of the retinal vasculature [10–13]. The TIE receptors and their angiopoietin ligands are also required for lymphatic development via effects on vessel remodeling, maturation and valve morphogenesis [14–17].

16.2.1 Ang1 and Tie2 in Cardiac and Vascular Development

Tie2 gene-targeted mouse embryos show severely impaired cardiac development, reduced numbers of endothelial cells, and hemorrhages, resulting in embryonic

Fig. 16.1 (continued) (*top, left*) contacts. In cell–cell contacts, the activated TIE2 receptor signals to stabilize the endothelium after angiogenic processes, and improve the endothelial barrier function, while in cell–matrix contacts, TIE2 stimulates endothelial migration. Pericellular matrix - α5β1-integrin interaction has been reported to promote ANG1-TIE2 signaling for cell survival and cell motility. Vascular sprouting may also involve ANG1 interactions with integrins on non-endothelial cells, such as astrocytes. ANG2 is a weak TIE2 agonist/antagonist, which can counteract ANG1 signaling in cell junctions (*bottom, left*), especially when its expression is increased, for example, in sepsis. High ANG2 levels also activate β1-integrin, which induces further destabilization of endothelial cell junctions, promoting vascular permeability (*bottom, right*). In endothelial tip cells, which express ANG2 but low levels of TIE2, ANG2 signals via integrins to promote vascular sprouting. In the tumor vasculature, and in stressed endothelial cells which are exposed to low levels of ANG1 signaling, ANG2 may provide cell survival signals. Deletion of the ligand-less TIE1 receptor inhibits atherosclerosis and tumor angiogenesis, and results in reduced TIE2 phosphorylation in *Tie1* gene-targeted mice. The *colors* of the TIE receptor domains refer to the corresponding exon colors in Fig. 16.3

Table 16.1 Summary of the TIE1 and TIE2 receptors

	TIE1 (*T*yrosine kinase receptor with *I*mmunoglobulin and *E*GF homology domain 1), also Tie	TIE2, also TEK (*T*unica interna *E*ndothelial cell *K*inase)
Chromosome location, human	1p34–p33	9p21
Gene size (kb), human	22,1	121
Exons (productive, longest transcript), human	23	23
mRNA size (5′ UTR, ORF, 3′ UTR), human	3882 (79, 3417, 386)	4760 (442, 3375, 943)
Amino acid residues, human	1,138	1,124
Molecular weights (kDa), human	125 (without glycosylation), ~135 kDa apparent size on PAGE	126 (without glycosylation), ~150 apparent size on PAGE
Posttranslational modifications	Glycosylation	Glycosylation
Extracellular domains	3 Ig-like (I set), 3 EGF-like, 3 fibronectin type III	3 Ig-like (I set), 3 EGF-like, 3 fibronectin type III
Ligands	Unknown	ANGPT1, ANGPT2, ANGPT4 (mouse ANGPT4 was previously termed ANG3)
Known interaction partners	TIE2	TIE1, TIE2, VE-PTP
Signal transduction pathways activated		PI3-K/Akt (cell–cell contacts), ERK, DokR (cell–matrix contacts), GRB2, ABIN-2, Rac1/ IQGAP1, STAT
Tissues expressed	Endothelial cells, hematopoietic cells (stem cells, some myeloid cells, such as megakaryoblasts)	Endothelial cells, hematopoietic stem cells, megakaryoblasts, TEMs (Tie2-expressing macrophages)
Human diseases	Somatic mutations in cancer and angiosarcoma, implicated in a variety of other diseases	Vascular malformations (germ-line and somatic mutations), implicated in cancer and diabetic retinopathy
Knockout mouse phenotype	Impaired endothelial integrity, hemorrhages, lymphatic vessel defects, edema, death starting at E13.5, reduced atherosclerosis and reduced tumor growth and angiogenesis.	Impaired cardiac development, reduced numbers of endothelial cells, hemorrhages, death by E10.5, lymphatic vessel defects

lethality by E10.5 [18]. Gene-targeted embryos deficient of the Tie2 ligand Ang1 die by E12.5 [5]. They have a very similar phenotype, including impaired cardiac development and less complex vascular structures. In addition, the endothelial cells in the *Ang1*$^{-/-}$ embryos are rounded and poorly associated with basement membranes [5]. Myocardial Ang1 expression appears to stimulate coronary vein formation, by promoting the proliferation, migration, and differentiation of immature endothelial cells derived from the sinus venosus [19]. Interestingly, cardiac-specific *Ang1* deletion during embryogenesis largely phenocopied the ubiquitous loss of Ang1. The deletion of *Ang1* after E13.5 was compatible with life; however, postnatal retinal angiogenesis was defective [11, 12]. Ang1 does not appear to be required for normal homeostasis in the adult vasculature, but it is required to limit pathological angiogenic responses and fibrosis after injury or during microvascular stress [11, 12].

16.2.2 Tie1 Regulates Vascular and Lymphatic Development

Endothelial integrity is impaired in the *Tie1* gene-targeted mouse embryos, resulting in hemorrhages and lethality at E13.5 [20]. Tie1 is required for endothelial cell survival and for capillary growth during late phases of embryonic angiogenesis, especially in the developing kidney and the brain [21]. The deletion of both *Tie1* and *Tie2* causes more severe defects in vascular integrity than single gene deletions, and mosaic analysis has demonstrated that both Tie1 and Tie2 are required in endothelial cells during late phases of embryonic development and in adult tissues [22]. Postnatal conditional deletion of *Tie1* decreased angiogenic sprouting in the developing retinal vasculature, but had little effect on mature vessels in adult tissues [13].

Tie1 is also critical for lymphatic vascular development. Conditionally targeted *Tie1* embryos showed severe edema and abnormal formation of jugular lymph sacs at E13.5–14.5 [14, 15]. A genetic *Tie1* mouse model, with conditionally targeted *Tie1* endodomain, demonstrated abnormal lymphatic collecting vessels and defective lymphatic valve formation between E15.5 and E18.5, and during postnatal life [16].

16.2.3 Ang2/Ang1-Tie2 Pathway Is Critical for Lymphatic Vascular Remodeling

Ang2-deficient mice die postnatally or survive until adulthood, depending on the genetic background [10]. Newborn *Ang2*-deleted mice suffer from generalized lymphatic dysfunction, including subcutaneous edema and chylous ascites [10]. Their collecting lymphatic vessels fail to undergo proper postnatal maturation and have abnormal smooth muscle cell coating, whereas the lymphatic capillaries are hypoplastic and disorganized [10, 23].

Ang2 is indispensable for lymphatic remodeling; genetic deletion or administration of blocking ANG2 antibodies at different stages during embryonic development prevented the maturation of zipper-like junctions to button-like junctions of

the lymphatic capillary endothelium and inhibited the phosphorylation of VE-cadherin at Tyr685, whereas in the collecting lymphatic vessels the adherens junctions were disrupted, resulting in compromised lymph drainage [24]. Embryonic Ang2-blockade suppressed also the formation and maturation of the lymphatic valves [24].

When expressed in the *Ang2* genetic locus, Ang1 was able to complement the lymphatic defects of *Ang2* gene-targeted mice, indicating that Ang2 and Ang1 function similarly in the lymphatic vasculature, likely as Tie2 agonists [10].

Conditional deletion of *Ang1* and *Ang2* in double knockout mice demonstrated lack of Schlemm's canal and lymphatic capillaries in the corneal limbus, leading to a dramatic increase in intraocular pressure and glaucoma [25]. The lymphatic defects in the *Ang1* and *Ang2* double knockout mice were more severe than in the *Ang2* single knockout mice, suggesting that both Ang1 and Ang2 contribute to the formation of the lymphatic vasculature in the eye [25]. Furthermore, deletion of both *Ang1* and *Ang2* at E12.5, but not *Ang2* alone, resulted in subcutaneous edema in the embryos. The lymphatic phenotypes of *Ang1* and *Ang2* double knockout mice resembled those observed upon conditional *Tie2* deletion, suggesting that compensatory mechanisms regulate lymphatic development via Tie2 [25].

Ectopic overexpression of Ang2 in developing mouse embryos resulted in blood vascular defects similar to those in *Ang1- and Tie2*-deficient embryos, which initially suggested that ANG2 acts as a TIE2 antagonist in the vascular endothelium [6]. However, the blood vascular defects of $Ang2^{-/-}$ mice were limited to the development of the postnatal ocular vasculature, where Ang2 was required for the regression of the hyaloid vessels and for sprouting of the retinal vasculature [10]. These results suggest that ANG2 may present its context-dependent antagonistic function during development of the eye vasculature, where it functions to destabilize the existing hyaloid blood vessels.

16.3 The Role of the TIE Receptor Tyrosine Kinase Family in Physiology and Disease

The ANG-TIE system regulates vascular homeostasis and it is also implicated in numerous vascular diseases. Therefore, the ANG-TIE system has become a target of investigational vascular therapies [26]. ANG1 mediates vascular protection via TIE2 activation, and recombinant or viral vector delivery of ANG1 protein has been considered as a potential means for alleviating vascular complications of diabetes and sepsis, which may result in acute lung and kidney injury [27]. The potential of ANG1 to induce organized neovascularization with non-leaky vessels has stimulated research on ANG1 as a therapy for ischemic diseases [28–30]. Reduced TIE receptor expression has been associated with susceptibility to vascular complications caused by hemorrhagic Ebola virus infection, whereas activating TIE2 mutations contribute to the development of cutaneomucosal venous malformations [31, 32]. In contrast, inhibition of ANG2 has shown beneficial

effects in reducing vascular leakage, alleviating e.g. sepsis-induced lung injury and harmful inflammation associated with cardiac transplant rejection [33, 34]. Inhibition of ANG2 [35] and TIE1 may provide protection from atherosclerosis, and inhibition of both ANG2 and TIE1 has been demonstrated to decrease tumor angiogenesis and growth [13, 36–39].

16.3.1 TIE2 Mutations in Vascular Malformations

Vascular anomalies are developmental vascular defects and consist of a variety of hereditary and sporadic disorders. Germline *TIE2* mutations (most common is the point mutation R849W) cause the inherited cutaneomucosal venous malformation (VMCM) syndrome, which is characterized by multifocal small bluish cutaneous and mucosal lesions, composed of enlarged, tortuous venous channels [40]. The patients are also affected by somatic 'second hit' mutations causing loss of function of the second *TIE2* allele [31].

Somatic *TIE2* mutations cause a more common, sporadic form of venous malformation (VM), characterized by extensive unifocal lesions and enlarged venous channels with patchy smooth-muscle cell layers and a thin, continuous TIE2-positive endothelial cell layer [31, 41]. When ectopically expressed in cultured endothelial cells, the VM-associated mutant TIE2 proteins, including the R849W mutant, were hyperphosphorylated. However, the level of hyperphosphorylation did not correlate with the clinical phenotype of the patients [31]. Some of the TIE2 mutations, for example, L914F, affected the subcellular localization of TIE2, resulting in impaired receptor localization on the cell surface, increased Akt and STAT1 phosphorylation, and decreased platelet-derived growth factor B expression, which may contribute to the weak pericyte–endothelial cell association observed in the VM lesions [31, 42].

16.3.2 ANG-TIE System in Cancer

Circulating ANG2 levels have been identified as an independent factor predicting poor prognosis in many human cancers, including metastatic melanoma, colorectal cancer (CRC), and chronic lymphocytic leukemia [26]. Several approaches to target the ANG-TIE system are in clinical development for the treatment of human cancer, some of these have advanced into phase III trials [43]. In contrast to VEGF, which is highly expressed by many tumor cells, tumor cell lines express very little ANG2 [8]. Instead, ANG2 mRNA expression was detected in cells of the tumor stroma in patients with metastatic CRC [44], in line with preclinical data from mouse tumor models [45]. Interestingly, low pre-therapeutic circulating ANG2 levels were associated with a better response to bevacizumab in CRC patients, suggesting that circulating ANG2 may provide a biomarker for anti-angiogenic therapies in CRC, where bevacizumab is used in combination with chemotherapy [44].

In preclinical models, blocking of the ANG-TIE interaction has been demonstrated to inhibit tumor angiogenesis and lymphangiogenesis. These studies used different ANG2 and ANG1/ANG2 blocking agents in various mouse tumor or xenograft models, either as monotherapy or in combination with VEGF signaling inhibitors, which showed synergistic effects (reviewed in [9]). Mechanistically, the function of ANG2 in tumor angiogenesis is not well known. ANG2 expression has been detected as one of the first markers of the activated endothelium of co-opted tumor blood vessels. ANG2 is thought to cause endothelial destabilization, leading to vessel regression, hypoxia, and increased expression of both ANG2 and VEGF, which together induce robust angiogenesis in the tumor (angiogenic switch) [45]. However, ANG2 has been also proposed to act as a TIE2 agonist in tumors, promoting endothelial cell survival in the tumor vasculature, where the ANG2/ANG1 ratio is elevated [46].

In addition, ANG2 appears to contribute to the vascular phenotype of tumors by inducing pericyte detachment and endothelial cell sprouting [47, 48], while blocking ANG2 results in the normalization of tumor vessels with increased levels of adhesion molecules at endothelial cell-cell junctions, increased pericyte coverage, reduced endothelial cell sprouting, and vascular remodeling, producing smaller, more uniform vessels [36]. Some of the vascular normalization effects of ANG2 blocking therapies may be mediated by ANG1 [36]. A chimeric cartilage oligomeric matrix protein-angiopoietin-1 (COMP-ANG1) induced vessel normalization and improved vessel perfusion, potentiating chemotherapy in a syngeneic mouse tumor model [49, 50].

Anti-angiogenic therapies have been suggested to enhance invasive tumor growth [51, 52], while ANG2 blocking inhibits tumor lymphangiogenesis and decreases lymph node and distant metastasis [37, 39]. Anti-ANG2 antibodies inhibited tumor cell extravasation and lung metastasis by improving endothelial cell–cell junctions of tumor-associated pulmonary capillaries [39]. Mechanistically, the ANG2 blocking antibody not only inhibited ANG2 binding to TIE2, but also induced the internalization of the ANG2-TIE2 complexes [39]. Blocking ANG2 was also reported to modulate the functions of TIE2-expressing macrophages (TEMs), which form a subpopulation of tumor-associated proangiogenic myeloid cells [37]. Furthermore, ANG2 neutralization effectively reduced the growth of pancreatic RIP1-Tag2 tumors, which developed evasive resistance to VEGFR-2 inhibitors partly via increased ANG2/TIE2 expression [53].

16.3.3 Tie1 in Cancer and Cardiovascular Disease

TIE1 is expressed in the endothelium of tumor vessels [54]. Recent results demonstrated reduced tumor growth and postnatal angiogenesis in mice with endothelial *Tie1* deletion [13]. Endothelial cell apoptosis was increased in tumors grown in *Tie1*-deficient mice, whereas the normal vasculature was not affected [13]. In addition, *Tie1* deletion reduced tumor growth and angiogenesis to a similar extent as VEGF signaling inhibitors. Interestingly, additive tumor growth inhibition was obtained

with the soluble Tie2 ectodomain capable of neutralizing ANG ligands, but not with VEGF inhibitors when used in the *Tie1*-deficient mouse background [13].

Furthermore, *Tie1* deletion appears to protect mice from atherosclerosis. *Tie1* is induced at sites of turbulent vascular flow in arteries, and the deletion of *Tie1* provided a benefit for *Apoe*$^{-/-}$ mice on regular diet, by decreasing the number of atherosclerosis plaques [38].

16.3.4 ANG-TIE System in Vascular Leak and Therapeutic Angiogenesis

Circulating ANG2 levels are significantly elevated in diseases characterized by endothelial destabilization and vascular leak, such as sepsis, acute lung and kidney injury and acute respiratory distress syndrome (ARDS) [55]. Ang2 appears to contribute to endothelial barrier disruption in sepsis-associated lung injury, but excess systemic Ang2 has been also reported to provoke pulmonary vascular leak and congestion in otherwise healthy adult mice [56].

Mice heterozygous for *Ang2* were protected from sepsis-induced kidney and lung injury, demonstrating less tissue inflammation and vascular leakage compared to wild-type mice, whereas *Tie2* heterozygous mice were more susceptible to endotoxin-induced lung injury [27, 57]. In addition, ANG2 blocking antibodies reduced hemodynamic alterations and mortality rate in mice with lipopolysaccharide (LPS)-induced sepsis [33]. Furthermore, siRNA silencing of *Ang2* in the pulmonary endothelium improved the survival of mice following cecal ligation and puncture, both as a pretreatment and as a rescue intervention [58]. On the other hand, ectopically provided ANG1 alleviated sepsis-induced lung injury [27]. These results suggest that ANG2 promotes, whereas ANG1-TIE2 signaling protects from the drastic consequences of sepsis. The vascular protective signals emanating from ANG1-TIE2 receptor complexes are well studied, whereas ANG2 signaling mechanisms in vascular pathologies are less well understood. However, ANG2 can increase vascular leak induced by many inflammatory cytokines [59]. Decreased Tie2 levels have been reported in the vasculature during septic shock; these may further aggravate the disease [27]. Reduced Tie2 expression was also associated with susceptibility to fatal vascular complications induced by hemorrhagic Ebola virus infection in mice [32]. A recent study demonstrated that when TIE2 levels are reduced or when ANG2 levels are increased, ANG2 can signal via β1-integrin to induce endothelial retraction and destabilization of endothelial cell junctions, a mechanism that may be in place in conditions with increased vascular leak [60] (Fig. 16.2).

ANG2 is also a pro-inflammatory molecule: ANG2 sensitizes endothelial cells to TNF-α signaling to induce expression of endothelial cell adhesion molecules [61]. In a mouse model of chronic airway inflammation, ANG2 blocking agents decreased the remodeling of mucosal capillaries into venules, the amount of leukocyte recruitment, and disease severity [62]. Ang2 is also highly upregulated in the retinas of diabetic rats. Reduced *Ang2* gene dosage inhibited diabetes-induced pericyte loss and the formation of acellular capillary segments, suggesting a critical function for

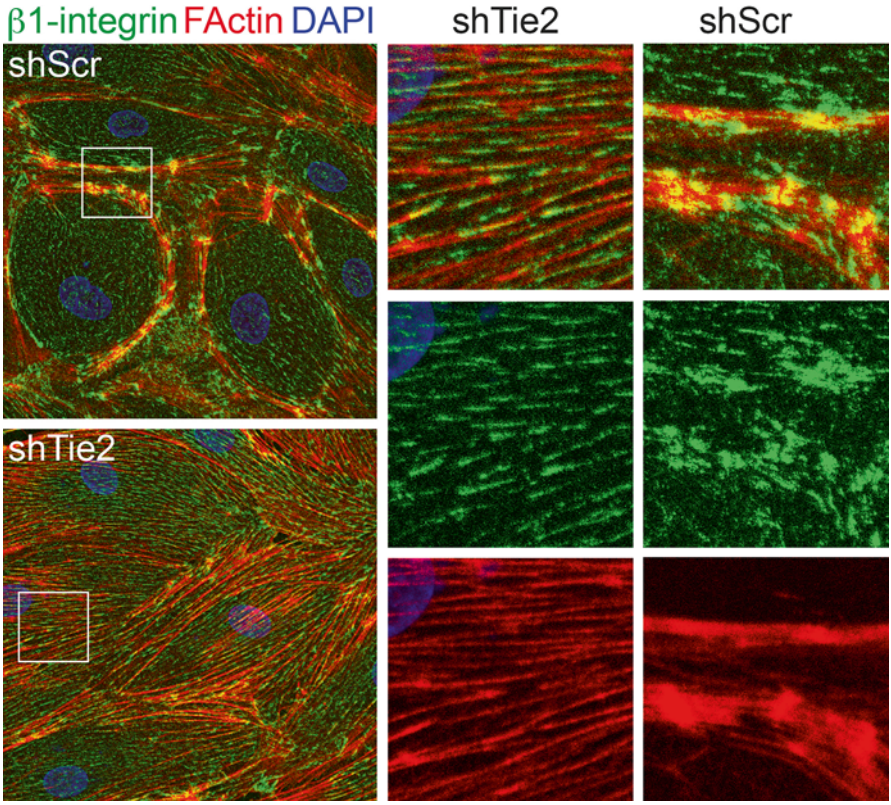

Fig. 16.2 ANG2-β1-integrin signaling promotes endothelial destabilization. In shScramble (shScr) transfected control cells, β1-integrin promotes cell-cell adhesion and cortical actin cytoskeleton in the cell periphery via focal adhesions. In TIE2 silenced (shTIE2) endothelial cells, and in the endothelium of ANG2 transgenic mice [60] β1-integrin is localized in elongated matrix adhesions that connect to actin stress fibres and reduce the integrity of vascular endothelial-cadherin-containing cell–cell junctions. ANG2 was found to signal via β1-integrin when ANG2 levels were elevated or when TIE2 levels were decreased, leading to endothelial β1-integrin activation and cellular tension, which should contribute to reduced barrier function in diseased conditions, such as sepsis [60]. Image provided by Laura Hakanpää

ANG2 in diabetic retinopathy [63]. ANG2-induced pericyte apoptosis seems to occur under high glucose via an integrin-dependent pathway [64].

In contrast to ANG2, ANG1 is anti-inflammatory, stimulates endothelial cell survival, and inhibits vascular permeability via multiple mechanisms including direct effects on the endothelial cell glycocalyx [65]. The potential of ANG1 to promote vascular stability and barrier function as well as to stimulate organized vascular remodeling resulting in non-leaky vessels makes ANG1 a potential therapeutic agent in numerous diseases [28, 30]. For example, ANG1 can improve the integrity of lymphatic vessels in inflamed skin after UVB irradiation by modulating expression of tight junction molecules [66]. The recombinant-soluble ANG1

protein, COMP-ANG1, promoted angiogenesis and suppressed inflammation in sciatic nerves of diabetic (*ob/ob*) mice, suggesting that COMP-ANG1 could improve the morphologic and molecular changes associated with diabetic neuropathy [67]. Adenoviral delivery of COMP-ANG1 also promoted diabetic wound healing by enhancing angiogenesis, lymphangiogenesis, and blood flow [68]. The genetic deletion of *Ang1* in adult mice demonstrated that Ang1 limits pathological tissue fibrosis during wound healing and protects from microvascular disease in streptozotocin-induced diabetes [11]. In preclinical mouse models, endothelial dysfunction during hypertension was reduced by expression of the COMP-ANG1 protein. The treatment reduced the hypertension-associated cardiovascular and renal damage and prevented further elevation of blood pressure [69].

Ischemia followed by reperfusion occurs as a consequence of organ transplantation, treatment of hypotension, and major surgeries and may induce microvascular endothelial cell injury, leading to deregulation of vascular tone, tissue perfusion, permeability, and inflammation. In a renal ischemia–reperfusion model, COMP-ANG1 reduced vascular defects, vascular permeability and interstitial fibrosis, and preserved tissue perfusion, thereby improving renal function [70]. Similar beneficial effects were observed in a renal artery clamping model when rats were treated with simvastatin, a HMG-CoA reductase inhibitor, which inhibits hypoxia-induced release of ANG2 from endothelial Weibel-Palade bodies [71]. During cardiac transplantation, ischemia–reperfusion injury can trigger innate and adaptive immune responses that may lead to rejection, myocardial injury, and death, a series of events associated with increased ANG2 levels [34]. Interestingly, a single ex vivo intracoronary injection of ANG2 blocking antibodies reduced vascular permeability, myocardial injury, and associated inflammation by preventing leukocyte infiltration and expression of endothelial cell adhesion molecules, eventually leading to prolonged allograft survival [34].

Use of ANG1 for therapeutic neovascularization of ischemic tissues has gained interest because initial attempts to sustain neovascularization with VEGF family growth factors resulted in serious side effects. A recombinant protein made by fusion of the receptor-binding domains of VEGF and ANG1 growth factors, the VA1 chimera, was shown to be a potent angiogenic factor that triggers a novel mode of VEGFR-2 activation, promoting less vessel leakiness, less tissue inflammation, and better perfusion in ischemic muscle than VEGF [29]. In contrast, transgenic expression of ANG2 was shown to inhibit collateral artery growth and smooth muscle cell recruitment after arterial occlusion, thereby impairing perfusion and increasing necrosis in the ischemic limb [72].

16.4 TIE1 and TIE2 Genes

The *TIE1* and *TIE2* genes are conserved in numerous species, and their orthologues are present in chordates, but not e.g. in *C. elegans* or *D. melanogaster*. TIE1 and TIE2 are expressed mainly in the endothelial cells, with some expression in certain

hematopoietic cell lineages, including TIE2-positive hematopoietic stem cells and a subpopulation of proangiogenic TIE2-positive macrophages [73–77]. *Tie1* expression is induced during vascular remodeling, in tumors, and by disturbed flow in vascular bifurcations and branching points of arteries, whereas TIE1 expression is reduced by shear stress [54, 78, 79]. *TIE1, ANGPT2*, and *TIE2* mRNAs are strongly expressed also in cells of Kaposi's sarcoma tumor cells, and in cutaneous angiosarcomas [80].

16.4.1 TIE1

The human *TIE1* gene, located in chromosome 1p34-p33, is expressed in an endothelial cell-specific manner [73]. High expression of *Tie1* is found in adult lung, heart, and placenta. Moderate levels are present in the kidney, while skeletal muscle, brain, liver, and pancreas have less prominent expression. *Tie1* expression starts at about E8.5 during mouse embryonic development [78]. *Tie1* mRNA is detected in differentiating angioblasts of the head mesenchyme, in the splanchnopleura and dorsal aorta, as well as in migrating endothelial cells of the developing heart, in the heart endocardium, and in endothelial cells forming the lung vasculature [78].

A *TATA- or CAAT-box* is absent from the *TIE1* promoter. Critical promoter/enhancer elements that determine endothelial expression are located within several hundred nucleotides upstream of the major transcription initiation site. These elements include several binding sites for members of the Ets transcription factor family (NERF-2, ELF-1, and ETS2) and an octamer transcription factor binding site [81]. This part of the promoter is conserved between mice and humans [73], and it contains most of the promoter specificity determining sequences [82]. Hence, it has been frequently utilized to drive endothelial cell-specific expression of transgenes [82, 83].

TIE1 is also expressed in cultured endothelial cells, some hematopoietic progenitor cells, and some myeloid leukemia cell lines having erythroid and megakaryoblastoid characteristics [76] and in adult acute myelogenous leukemia [84].

16.4.2 TIE2

The *TIE2* gene (*TEK*) is very similar to the *TIE1* gene (Fig. 16.3). It features also 23 exons, although they span a much larger genomic region than *TIE1* exons (121 kb on chromosome 9p21 in comparison with 22 kb for the *TIE1* gene). Endothelial specific expression is controlled by transcription factor binding sites in the first intron of the *Tie2* gene [83, 85, 86]. In addition, TIE2 is expressed in several hematopoietic cells, most notably in hematopoietic stem cells (HSCs), where ANG1-induced signals regulate HSC niche regeneration and vascular leakiness [74, 77]. TIE2 is also expressed in a subpopulation of type M2 monocytes (TEMs),

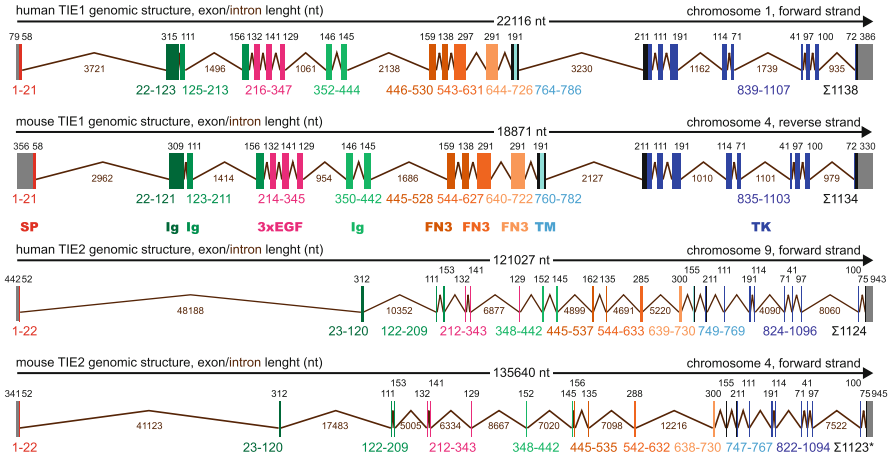

Fig. 16.3 The exon/intron structures of the human and mouse *TIE1* and *TIE2* genes. Exons are shown as *solid boxes* and introns as *brown lines*. Exons are colored according to the domain structure: *red*, signal peptide/SP; *green*, immunoglobulin-like domain/Ig; *magenta*, epidermal growth factor-like domain/EGF; *orange*, fibronectin type 3 domain/FN3; *cyan*, transmembrane domain/TM; *blue*, tyrosine kinase domain/TK; *black*, not assigned to any domain. The *black* numbers above the exons indicate the exon length. The numbers for the first and last exons are split into the coding (*black*) and untranslated sequences (*gray*). The *brown numbers* under the larger introns indicate the intron length. The *colored numbers* under the exons refer to the corresponding amino acid residues. The total number of amino acid residues in the receptors is shown in *black*. *1,123 amino acid residues as deduced from the mouse genome project and one cDNA, and 1,122 amino acid residues according to the UniProtKB/Swiss-Prot database as deduced from three cDNAs. The assignment of the domain structure is according to the SMART protein domain research tool, except for the Ig-like and EGF-like domains, which are according to the TIE2 crystal structure determined by Barton et al. [103]

with tumor and angiogenesis-promoting properties [75] and in the muscle satellite cells, located among skeletal myofibers and associated with the microvasculature [87]. Ang1 derived from the quiescent satellite cells or fibroblasts in the muscle microenvironment promotes satellite cell quiescence for long-term self-renewal of adult muscle stem cells [87].

16.4.3 Alternative Splicing and Conservation

The exon–intron structure is highly conserved between *TIE1* and *TIE2* and also between the mouse and human *TIE* genes. Many alternative splice variants of *TIE1* and *TIE2* have been cloned or are found among EST sequences (see Alternative Splicing Database or Ensemble). Most but not all of the putative proteins resulting from such splice variants are likely not produced due to the lack of a functional signal peptide and thus their significance is unknown [88].

16.4.4 Gene Variants, Polymorphisms, and Somatic Mutations

No variants of the human *TIE1* gene have been reported. However, somatic mutations affecting the extracellular and tyrosine kinase domains have been identified in cancer, including angiosarcoma [89, 90]. In contrast, mouse TIE1 features several sequence variants, which may be polymorphisms [4, 91, 92].

Multiple missense point mutations have been described in the human *TIE2* gene. Most of them are located in sequences encoding the tyrosine kinase domain. They result in increased ligand-independent autophosphorylation and kinase activation and are linked to VMs [31, 39, 93, 94]. The mouse sequences for TIE2 similarly feature plenty of single amino acid variants, some of which correspond to human disease variants (for example, mouse R913G and human R915H). Notable is also a single valine insertion after position 786 of mouse TIE2, which is present in some cDNA sequences [29], but absent from others [2, 3, 34, 66]. This results from alternative splicing at the intron 14–15 splice donor site. Larger variations involving stretches of 7 to 17 amino acid residues likely represent sequencing artifacts since they represent frameshifted versions of the wild-type sequence with junctions at sites of repeated calls of individual bases [70]. Given the critical location of many of these sequence conflicts, the origin, verification, and documentation of TIE sequences used for experimental research are paramount.

16.4.5 Regulation of Transcription

Increased TIE2 expression has been reported in hypoxia in both endothelial cells and TEMs [95, 96]. The response is at least partially mediated by transcriptional activation, presumably involving the HIF-2 transcription factor [97]. However, there seems to be significant heterogeneity in the hypoxic response depending on the origin of the endothelial cells. Also, inflammatory cytokines such as TNFα and IL-1β upregulate TIE2 [95].

TIE1 expression is increased during wound healing, in proliferating ovarian capillaries during hormone-induced superovulation, and in tumor blood vessels [54, 78]. *Tie1* is downregulated by shear stress [98], but induced by disturbed flow in vascular bifurcations and branching points of arteries [79]. *TIE1*, along with *ANGPT2* and *TEK* mRNAs, is strongly expressed in cells of Kaposi's sarcoma tumor cells, and in cutaneous angiosarcomas [80].

16.4.6 TIE Genes in Different Species

The *TIE* family has been considered not only metazoan specific, but also chordate specific as no *TIE* orthologues have been found in *C. elegans* or *D. melanogaster* [86, 99]. Receptor tyrosine kinase genes resembling the *TIE* gene (MBRTK1/

RTKB5 and others) have been identified in the unicellular choanoflagellate *Monosiga brevicollis*. However, the weak similarity with the chordate Tie family might be due to convergent evolution [100, 101].

tie1 and *tie2* orthologues are found in zebrafish, but they appear to have different functions than their mouse counterparts. In contrast to the phenotype of the *Tie2*$^{-/-}$ mouse, *tie2*$^{-/-}$ mutant fish are viable and have no vascular or heart phenotype. However, knockdown of zebrafish *tie1* in the *tie2* mutant background results in phenotypes similar to those of the *Tie2*$^{-/-}$ mouse [102]. This suggests for functional redundancy between the fish tie2 and tie1 proteins. Furthermore, study of *tie1* knockdown in fish suggests that *tie1* but not *tie2* is required for early stages of heart development. However, both *tie1* and *tie2* are indispensable in the later developmental stages for the maintenance of endocardial–myocardial interaction, a phenotype similar to that seen in *Tie2*$^{-/-}$ mice, where poor association between endothelial cells and the myocardium is evident.

16.5 TIE1 and TIE2 Proteins

The TIE RTKs are characterized by a unique extracellular domain (ECD) for ligand binding, a single-pass transmembrane domain, and a cytoplasmic protein tyrosine kinase (TK) domain. The TIE ECDs consist of immunoglobulin, epidermal growth factor-like, and fibronectin type III repeats. Crystal structures of the angiopoietin–TIE2 complexes demonstrate that ANG1 and ANG2 bind to the same sites in TIE2 in a largely similar manner [103]. The mechanism of TIE2 activation remains elusive, although the oligomerization of angiopoietin ligands suggests that TIE2 activation requires its oligomerization [104–106]. Similar to other RTKs, the TIE2 oligomerization is likely to involve interactions between the ECDs and TIE2/TIE1 heterodimerization is mediated by interactions between their ECDs. Activation of the intracellular TIE tyrosine kinase domains is associated with phosphorylation of multiple tyrosine residues in the kinase domains and the C-terminal tail, which couples the TIE receptors to downstream signaling events [107].

16.5.1 Domain Structure and Posttranslational Modifications

The ECD of the TIE receptors is unique and consists of two immunoglobulin (Ig)-like domains, followed by three N-terminal epidermal growth factor (EGF)-like motifs, a third immunoglobulin (Ig)-like domain, and three fibronectin type III (FNIII) domains [1, 3]. The presence of the second Ig-like domain was revealed by the determination of the crystal structure of the TIE2 ligand-binding domain (LBD, residues 23–445) in complex with the ANG2 fibrinogen-like domain [103]. The crystal structure (Fig. 16.4) revealed a compact, arrowhead-shaped molecule containing the Ig-like domains (Ig1–Ig3) and the EGF-like domains (EGF1–EGF3).

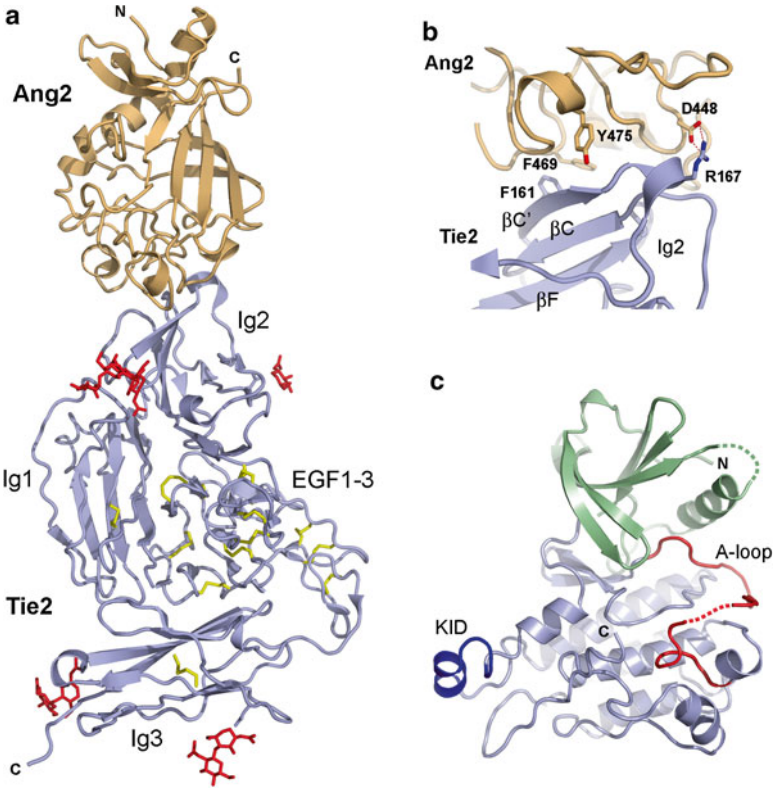

Fig. 16.4 Crystal structures of the ANG2/TIE2 complex and the TIE2 kinase domain. (**a**) ANG2 fibrinogen-like domain in complex with TIE2 ligand-binding domain. TIE2 ligand-binding domain and ANG2 are colored in *light blue* and in *light orange*, respectively. TIE2 asparagine-linked carbohydrate groups are colored in *red* and shown as sticks. (**b**) Close-up view of the ANG2/TIE2 interface in (**a**). TIE2 Ig2 domain interacts with ANG2 P-domain. Hydrophobic residues, including Phe469 and Tyr475 from ANG2 and Phe161 from TIE2, dominate the interface. TIE2 Arg167 and ANG2 Asp448 mediate a salt-bridge. (**c**) TIE2 kinase domain. The N-terminal domain is colored *green*, C-terminal tail in *light blue* and the kinase insert domain (KID) in *blue*. The activation loop (A-loop) is highlighted in *red*

The surface loops of Ig2 are at the tip of the TIE2 arrowhead and alone comprise the ANG2 binding site [103]. Recently, a TIE2 structure including the first FNIII domain (FNIII-1) was solved in complex with the ANG1 fibrinogen-like domain [108]. The inflexible associations between Ig3 and FNIII-1 and the orientation of FNIII-1 to the opposite direction of the ligand-binding site suggest a rigid and elongated molecular architecture for the TIE2 ECD.

The TIE1 and TIE2 ECDs have an amino acid identity of 31 % and homology modeling of TIE1 revealed similar conserved overall folds and hydrophobic surfaces [109]. However, comparison of the surface electrostatic potentials revealed that TIE2 ECD has a slight negative overall charge, whereas TIE1 ECD has a positive overall charge, suggesting that patches of oppositely charged molecular surfaces in TIE1 and TIE2 may be involved in TIE1–TIE2 heterodimerization [109].

The intracellular domains of the TIE receptors are similar to those of VEGFR and PDGFR family members. Overall amino acid sequence identity between TIE1 and TIE2 intracellular domains is 76 %, which is much higher than for their extracellular domains. The kinase domain of human TIE2, residues 808–1124, folds into two domains, with catalysis occurring in a cleft between the two [110]. The structure contains an N-terminal domain (residues 808–904) responsible for ligating ATP and a C-terminal domain (residues 905–1,124) having the catalytic core. A short kinase insert domain (KID) comprised of two α-helical segments pack against the C-terminal tail. The overall fold of the TIE2 kinase domain is similar to that observed in other serine/threonine and tyrosine kinase structures. However, the activation loop (A-loop), the nucleotide-binding loop, and the C-terminal tail in the TIE2 kinase domain structure adopt self-inhibitory conformations [110]. The activation loop in TIE2, residues 982–1,008, contains a single tyrosine at position 992 and adopts an "activated-like" conformation. The nucleotide-binding loop contains residues responsible for binding of the ATP phosphate groups. In the TIE2 kinase domain, this loop, residues 831–836, occupies the ATP binding site. The C-terminal tail adopts an extended conformation to the active site and may inhibit substrate binding. Conformational changes needed for kinase domain activation may represent additional steps in the regulation of TIE2 activity.

TIE1 and TIE2 receptors contain multiple potential N-glycosylation sites. The structure of the TIE2 ligand binding domain revealed N-glycosylation in four of these sites, in Asn140 and Asn158 in Ig2 as well as in Asn399 and Asn438 in Ig3 [103]. Of these, only Asn158 (Asn161) is conserved in TIE1. The human TIE2 sequence contains additional, potential N-glycosylation sites in Asn464, Asn560, Asn596, Asn649, and Asn691. On the other hand, the human TIE1 sequence contains additional, potential N-glycosylation sites in Asn83, Asn503, Asn596, and Asn709. The crystal structure of the TIE2 ligand-binding domain revealed also fourteen disulfide bonds apparently stabilizing the structures of the individual TIE2 domains: one in both Ig1 and Ig3, and four in each of the EGF repeats.

16.5.2 Phosphorylation Sites and Primary Signaling Molecules

A characteristic feature of RTKs, such as VEGFRs, is their dimerization induced by the binding of a dimeric ligand, followed by autophosphorylation of the intracellular kinase domains. TIE receptor function is also regulated by ligand binding to the extracellular domain of the receptor. However, at least a trimeric ligand is required for TIE2 activation in endothelial cells, suggesting that the active TIE2 receptor complex is composed of more than two receptor subunits [104].

Following binding of the activating ANG1 ligand, TIE2 is autophosphorylated and intracellular signaling pathways are activated [111]. The C-terminal tail of TIE2 contains three tyrosine residues. Using a yeast two-hybrid system, it was demonstrated that five molecules, GRB2, GRB7, GRB14, the non-receptor-type protein

tyrosine phosphatase 11 (PTPN11), and the p85 subunit of phosphatidylinositol 3-kinase (PI3K), interact with TIE2 in a phosphotyrosine-dependent manner [112]. Mapping of the binding sites of these molecules on TIE2 revealed a multisubstrate-docking site around Tyr1100 (mouse numbering) and a mutation of this site abolished GRB2 and GRB7 binding to TIE2. The p85 subunit of the PI3K has been shown also to associate with TIE2 Tyr1100 (cited Tyr1101 in [110]). This association results in PI3K activation [113]. Targeted mutation of the Tyr1100 in Tie2 showed impaired cardiac development as well as defective development of hematopoietic and endothelial cells in the mutant mice, but unlike the *Tie2* knockout mice, the perivascular cells were normally associated with the blood vessels [114]. Site-directed mutagenesis of Tyr1100 reduced the association of GRB2, as expected, but PTPN11 association remained intact [115]. Conversely, the Y1111F (cited Y1112F in [110]) mutation did not affect GRB2 association but decreased association of PTPN11, indicating that PTPN11 is a phospho-Tyr1111-specific signaling molecule. On the other hand, Tyr1106 of mouse Tie2 (1,108 in human) was identified as an ANG1-dependent autophosphorylation site that is required for the binding and phosphorylation of the docking protein Dok-R [116]. Notably, phosphorylation of this Tie2 residue was reduced in *Tie1*-deficient mice [13]. No tyrosine residue equivalent to Tyr1106 is present in TIE1.

16.6 Ligands of the Tie Receptor Family

Angiopoietin growth factors (ANG1/ANGPT1, ANGPT2, and ANGPT4) are ligands for the TIE2 receptor, whereas TIE1 is an orphan receptor [117]. Yet, ANG1 activates TIE1, likely via an interaction with TIE2 [118, 119]. Angiopoietins have a unique structure, with a C-terminal TIE2 binding fibrinogen-like domain and N-terminal coiled-coil and superclustering domains that mediate angiopoietin oligomerization into dimers, trimers, and higher-order oligomers [105]. ANG1 is a strong activating ligand, whereas ANG2 is a weak ligand, despite homologous receptor-binding mechanisms [6]. Angiopoietin oligomerization is required for efficient TIE2 activation and clustering. The degree of angiopoietin oligomerization varies, and the possible regulation of various angiopoietin forms in vivo remains to be determined.

16.6.1 Angiopoietin Structure and Receptor Binding

The angiopoietin growth factors (ANGPT1, ANGPT2, and ANGPT4) consist of an N-terminal region lacking homology to any other structures, a coiled-coil domain (ANG1; residues 79–263) similar to structures found in proteins that induce multimerization, and a C-terminal fibrinogen-like region (ANG1, residues 284–498), which contains the TIE2-binding portion [6, 117, 120]. Indeed, crystal structure of the ANG2 fibrinogen-like region revealed a fibrinogen fold with a unique C-terminal

P domain [121]. Furthermore, conservation analysis and structure-based mutagenesis identified a groove on the P domain surface that mediates TIE2 recognition and binding. The mechanism of ligand binding to TIE2 was confirmed by the determination of the crystal structures of the ANG1/TIE2 ECD and ANG2/TIE2 ECD complexes [103, 108]. Both ANG1 and ANG2 interact exclusively with the Ig2 domain of TIE2 via their C-terminal P domains.

Comparison of the free and receptor-bound ANG1 and ANG2 structures indicates that both ANG1 and ANG2 undergo only minor structural changes upon TIE2 binding and that the ANG1/TIE2 interface is very similar to the ANG2/TIE2 interface [108]. The TIE2 ligand-binding interface of about 1,300 $Å^2$ is dominated by van der Waals interactions between non-polar side chains. In addition, several hydrogen bonds and salt bridges are also involved in stabilizing the ANG1/TIE2 and ANG2/TIE2 complexes. Structure-based mutagenesis of TIE2 abolished both ANG1 and ANG2 binding to TIE2 [103].

The angiopoietin fibrinogen-like domains are responsible for receptor recognition and binding, and it has been suggested that the coiled-coil motif mediates homo- or heterodimerization of angiopoietin monomers [104, 122, 123]. Mass spectroscopic analysis of fragments generated proteolytically from native ANG1 demonstrated that Cys265 in the coiled-coil domain is involved in covalent homodimerization by an intermolecular disulfide bridge [104]. ANG1 and ANG2 were observed as trimers and dimers, and especially ANG1 is further clustered to form higher-order oligomers, such as tetramers and hexamers, and even higher multimeric forms, in solution [104, 105, 123]. It is thought that the N-terminal region serves as a superclustering region assembling the angiopoietin coiled-coil dimers into higher-order oligomers. The angiopoietin fibrinogen-like domain has been found to bind TIE2 in a 1:1 stoichiometry [104, 121]. Because of the dimeric, trimeric, or multimeric structures of angiopoietins, the expected stoichiometry of the angiopoietin-receptor complex in vivo would be thus 2:2 or higher.

The angiopoietin fibrinogen-like regions contain also two other surface patches of particularly conserved residues, suggesting that these might be also involved in biologically important interactions [121]. However, mutations within these conserved surface regions in the fibrinogen-like A and B domains did not affect TIE2 binding [122].

16.6.2 Tie Receptor Specificity

All three angiopoietins bind to TIE2 [6, 117, 120]. Interestingly, ANG1 and ANG4 are TIE2 agonists, while ANG2 induces only weak TIE2 phosphorylation and can inhibit ANG1-induced TIE2 activation. The oligomeric structure of angiopoietins suggests that ANG1 and ANG4 activate TIE2 by bridging receptor molecules. However, simply bridging receptor molecules appears to be insufficient for TIE2 activation, because ANG2 activates TIE2 only poorly [106]. Also, ANG1 and ANG2 bind in a very similar manner to TIE2, indicating that altered ligand presentation is not sufficient to explain the differences in angiopoietin biological activities [108].

ANG1 oligomerization has been associated with enhanced TIE2 activity [49, 104, 105]. Monomeric and dimeric ANG1 proteins lacking the coiled-coil and superclustering domains or the superclustering domain alone were able to bind to but not activate TIE2 [104]. An ANG1 variant, ANG1-F1-Fc-F1, with four fibrinogen domains could phosphorylate TIE2 with a similar efficiency as native ANG1 [104]. To improve protein solubility, additional dimeric, trimeric, and pentameric ANG1 fusion proteins lacking the superclustering and coiled-coiled domains have been designed [49]. The pentameric ANG1 chimeric protein, COMP-Ang1, showed enhanced activity in comparison to native ANG1 [49]. The dimeric GCN4 fusion failed to activate the TIE2/Akt pathway, whereas the trimeric Matrilin fusion protein, Mat-ANG1, induced TIE2/Akt phosphorylation similarly as native ANG1. The Mat-ANG1 activity suggests that TIE2 could be activated already by trimeric ANG1 variants. The angiopoietin oligomerization state has been found to regulate subcellular trafficking of Tie2-ANG2 and TIE2-ANG1 complexes and ANG2-mediated cell-matrix interactions [106, 124]. Importantly, it is still not understood which oligomerization state of ANG1 is optimal for TIE2 activation, which ANG1 forms predominate in vivo, or whether the different forms have different signaling functions.

Despite extensive efforts to identify TIE1 ligands, TIE1 is still considered an orphan receptor. However, it has been shown that both native ANG1 and COMP-Ang1 stimulate TIE1 phosphorylation in primary endothelial cells expressing also TIE2 [119]. TIE1 phosphorylation occurs with same kinetics and doses of the stimulating angiopoietins as TIE2 phosphorylation, but is clearly weaker in intensity [119]. Also, TIE1 has been suggested to differentially modulate the binding of ANG1 and ANG2 to TIE2 [109, 125]. These data suggest the possibility that TIE1 is unable to bind a ligand in an independent manner, but acts as a co-receptor with TIE2.

16.7 TIE-1 and TIE-2 Activation and Signaling

ANG1 stimulates endothelial cell survival and migration, and promotes endothelial barrier function via TIE2 [112, 113, 126]. ANG1 activates the TIE receptors via a unique mechanism, which is not used by other growth factor receptors and which is dependent on the cellular microenvironment [106, 127]. In contacting cells, ANG1 stimulates TIE receptor complexes, which form in *trans* across the cell–cell junctions promoting cell–cell adhesion and cell survival (Fig. 16.1). In isolated cells, matrix-bound ANG1 promotes cell–matrix adhesion via the TIE receptor complexes, which accumulate at the rear of migrating cells [106, 127]. The vascular endothelial protein tyrosine phosphatase, VE-PTP, dephosphorylates TIE2 at cell–cell junctions, and VE-PTP inhibitors have shown promise in alleviating pathological retinal vascularization via vessel stabilization [128, 129]. The junctional TIE2 complexes contain also TIE1 and are induced not only by ANG1 but also by ANG2 [106]. ANG2 can inhibit ANG1-induced TIE2 activation, but its downstream signaling cascades are incompletely understood. Integrins, which regulate endothelial cell–cell and cell–matrix interactions, have been implicated in angiopoietin

signaling, and likely regulate the context-dependent vascular functions of the angiopoietin growth factors during vascular destabilization and sprouting [60, 130–133].

16.7.1 Context-Dependent ANG-TIE Signaling

The binding of angiopoietins to TIE2 results in receptor activation via a unique mechanism, which is not used by other soluble growth factor ligands [106, 127]. When contacting endothelial cells are stimulated with angiopoietin ligands, TIE RTKs are rapidly translocated to cell-cell junctions. ANG1-TIE2 complexes reach in *trans* across the endothelial cell-cell junction and preferentially activate the PI3K–Akt pathway to promote cell survival, endothelial monolayer stability and barrier function [106, 127]. In mobile endothelial cells, matrix-bound ANG1 activates TIE2 in endothelial cell–matrix contacts to induce matrix adhesion and cell migration via activation of the extracellular-regulated kinases (ERK) [127] and the adaptor protein DokR [106].

The ANG1-activated Akt pathway, downstream of PI3K activation, enhances cell survival via phosphorylation and inhibition of the transcription factor forkhead box O1 (FOXO1) function [134]. FOXO1 induces the expression of genes associated with vascular destabilization and remodeling, such as ANG2, and inhibits the expression of, e.g., survivin, an apoptosis inhibitor [135]. ANG1 also induces the interaction of TIE2 with ABIN-2 (A20 binding inhibitor of NF-kappaB activation-2) which may promote cell survival and inflammatory signals [136].

In the stalk cells of sprouting vessels, ANG1 may engage TIE2 at cell-cell junctions to mediate vascular stabilization. ANG2 stimulation results also in TIE2 translocation to endothelial cell junctions, but while ANG1 induces TIE2 activation, ANG2 induces only weak TIE2 tyrosine phosphorylation, and may act as an antagonist to inhibit ANG1-induced TIE2 activation [106]. ANG2 is stored in the endothelial Weibel–Palade bodies from where it can be secreted in response to e.g. inflammatory stimuli [137]. Increased ANG2 levels promote endothelial destabilization, whereas in concert with VEGF, ANG2 induces angiogenesis [45]. However, ANG2 has been also suggested to function as an endothelial cell survival factor in stressed cells, where ANG1-stimulated Akt activation is low. In this setting, ANG2 would be induced, via the transcription factor FOXO1 to function as a TIE2 agonist, augmenting Akt activity to provide negative feedback to FOXO1-regulated transcription and apoptosis [138].

Multiple signaling mechanisms have been reported to mediate the barrier promoting functions of ANG1. ANG1 stabilizes the cortical actin cytoskeleton, via the IQ domain GTPase-activating protein 1 (IQGAP1) and Rac1 [139]. ANG1 also induces the expression of Kruppel-like factor 2, a transcription factor involved in vascular quiescence [140], upregulation of the tight junction protein zonula occludens protein-2 (ZO-2) [141], and activation of sphingosine kinase-1 (SK-1) [142]. ANG1 counteracts VEGF-induced permeability and VE-cadherin internalization by inhibiting VEGF-activated Src via mDia [143]. Another study shows that

ANG1 inhibits VEGF-induced nitric oxide (NO) increase and permeability via activation of the atypical protein kinase C-ζ (PKC- ζ (zeta)), which phosphorylates the inhibitory Thr497 residue in endothelial nitric oxide synthetase (eNOS) [144], while in some studies ANG1 has been shown to induce phosphorylation of the activating Ser1177 in eNOS and NO production [145]. These results highlight the complexity of ANG1-mediated improvement of endothelial barrier function in response to various permeability increasing agents.

16.7.2 Negative Regulation of ANG-TIE Signaling

VE-PTP (also known as HPTPβ, PTPRB, RPTPβ) is an endothelial cell-specific protein tyrosine phosphatase, and a negative regulator of TIE2. VE-PTP is indispensable during mouse vascular development, and it has an important function in promoting endothelial barrier function in part by associating with VE-cadherin [146–149]. Furthermore, the localization of VE-PTP in cell–cell junctions is promoted by ANG1 [106]. Inhibition of VE-PTP using function-blocking antibodies resulted in enlarged vascular structures in the developing embryonic allantoic membrane [128], as well as in experimental tumors [150]. In addition, a small molecule inhibitor of VE-PTP increased Tie2 activity and suppressed neovascularization in a mouse model of neovascular age-related macular degeneration by stabilizing retinal and choroidal blood vessels [129]. As the TIE receptors undergo significantly less ubiquitinylation following ligand binding than the VEGFRs and are less efficiently internalized and degraded [29], transmembrane phosphatase-mediated dephosphorylation of TIE2 may be a significant mechanism of TIE2 regulation.

16.7.3 Mechanisms of ANG2/TIE1 Signaling

ANG2 signaling mechanisms are not well understood. Although ANG2 can inhibit the ANG1-induced TIE2 activation, it may, depending on the context, such as incubation time and ligand concentration, also act as an agonist. ANG2 has been suggested to support endothelial cell migration and cell survival under stressed conditions, in the absence of ANG1 and in the tumor vasculature [46, 138, 151], and to induce actin stress fiber formation and endothelial gap formation via myosin light chain (MLC) phosphorylation [55]. Furthermore, genetic mouse models have revealed that Ang2 functions as a Tie2 agonist in the lymphatic vasculature [10, 25]. In addition, ANG2 has been found to signal via integrins, independently of Tie2, promoting endothelial monolayer destabilization and cell migration (see 16.7.4).

The signaling mechanisms of TIE1 have remained poorly characterized. TIE1 can interact with TIE2 [109, 118, 119], and it is phosphorylated in an

ANG1-dependent manner [119, 152]. TIE1 has been proposed to function as an inhibitor of TIE2 activation [109]; however, the results from a *Tie1* genetic mouse model did not support this possibility [13]. Furthermore, it has been reported that a chimeric c-fms-TIE1 receptor with the TIE1 intracellular domain can activate the PI3K-Akt pathway [153].

TIE1 is regulated by proteolytic cleavages, and these cleavages are enhanced by phorbol 12-myristate 13-acetate (PMA) and VEGF [154]. The first cleavage occurs close to the plasma membrane and requires a metalloprotease, resulting in the release of the TIE1 ectodomain. The second, γ-secretase-dependent cleavage, releases the membrane-bound endodomain [155]. TIE2 has been also reported to undergo ectodomain cleavage, but with different kinetics than TIE1, affecting the relative levels of TIE1 and TIE2 and thereby possibly contributing to the ANG1 ligand responsiveness of endothelial cells [156, 157].

16.7.4 ANG-TIE System and Integrins

Multiple integrin heterodimers, which regulate cell–cell and cell–matrix interactions, are expressed in endothelial cells and are known to cross talk with the ANG-TIE signaling system. Angiopoietins have been reported to signal directly via the integrins or via TIE receptor-integrin complexes. Integrin α5β1 was found to co-immunoprecipitate with TIE2, sensitizing TIE2 to ANG1 signals, when cells adhered on fibronectin [130]. On the other hand, ANG2 was reported to induce interaction of TIE2 with the αvβ3 integrin, stimulating the proteosomal degradation of αvβ3, an event that may be linked to endothelial destabilization [131].

In addition, both ANG1 and ANG2 have been shown to directly interact with different integrin subunits, also in the absence of TIE2. In the tip cells of vascular sprouts, which express ANG2, but less TIE2, ANG2 is expected to stimulate sprouting via integrins [132]. Recently, clues to the binding of ANG2 to α5β1 have been elucidated. ANG2 is able to bind α5β1 integrin in the absence of TIE2, and the molecular mechanism seems to require both integrin α and β subunits. ANG2 was found to interact with the tailpiece of the α5 subunit via a region around Gln362 in the C-terminal ANG2 fibrinogen-like domain [133]. In another study, the ANG2 N-terminal domain, but not that of ANG1, was found to induce α5β1-integrin activation [60]. In high glucose environments, such as those observed in diabetic retinopathy, ANG2 stimulated pericyte apoptosis in a process that required the α3β1-integrin, but not TIE2 [64]. ANG1 regulates postnatal retinal angiogenesis; these signals appear to be mediated via astrocyte expressed αvβ5 integrin [12]. During vascular sprouting, the retinal astrocytes secrete fibronectin, a matrix molecule that guides endothelial tip cell migration. Intravitreal ANG1 injection resulted in increased fibronectin deposition, astrocyte distribution, and FAK activation in astrocytes in an αvβ5 integrin-dependent manner, guiding directional sprouting into the avascular region of the retina [12]. In endothelial monolayers silenced for Tie2, and in aortic endothelium of Ang2

transgenic mice, ANG2 activation of β1-integrin resulted in altered endothelial cell-matrix adhesion and the formation of actin stress fibers that promote endothelial retraction and junction destabilization. This alternative Ang2-β1-integrin signalling pathway is likely activated in diseases with increased vascular leak, where Ang2 levels are elevated and Tie2 levels reduced [60]. The integrin dependent functions of angiopoietins should be considered when blocking reagents targeting Ang2 are developed for the treatment of cancer and other human diseases characterized with pathological vascular leakage.

16.8 Conclusions

The ANG-TIE system is an important regulator of endothelial cell functions. It provides critical signals for embryonic and postnatal cardiovascular and lymphatic development. Due to the vascular stabilization and vessel remodeling signals induced by the ANG1-TIE2 pathway, ways to reinforce this signaling axis are being investigated, including recombinant ANG1 proteins and small molecule inhibitors of the TIE2 inactivating phosphatase, VE-PTP, for alleviating pathological vascularization and endothelial destabilization, as well as stimulating neovascularization of ischemic tissues. In contrast, approaches to neutralize increased ANG2 levels are being developed to counteract its increased activity during inflammation and injury, and in tumors. Furthermore, TIE1 may provide a target for inhibition of atherosclerosis and tumor angiogenesis. However, future work is required to better understand the signaling mechanisms of the TIE1 receptor. In addition, increased understanding of the interplay of the ANG–TIE system with integrins may open up novel ways to better exploit the ANG-TIE system as a therapeutic target.

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References

1. Partanen J, Armstrong E, Makela TP, Korhonen J, Sandberg M, Renkonen R, et al. A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. Mol Cell Biol. 1992;12(4):1698–707.
2. Dumont DJ, Yamaguchi TP, Conlon RA, Rossant J, Breitman ML. tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. Oncogene. 1992;7(8):1471–80.

3. Dumont DJ, Gradwohl GJ, Fong GH, Auerbach R, Breitman ML. The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. Oncogene. 1993;8(5):1293–301.
4. Iwama A, Hamaguchi I, Hashiyama M, Murayama Y, Yasunaga K, Suda T. Molecular cloning and characterization of mouse TIE and TEK receptor tyrosine kinase genes and their expression in hematopoietic stem cells. Biochem Biophys Res Commun. 1993;195(1):301–9.
5. Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell. 1996; 87(7):1171–80.
6. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science. 1997;277(5322):55–60.
7. Lee HJ, Cho CH, Hwang SJ, Choi HH, Kim KT, Ahn SY, et al. Biological characterization of angiopoietin-3 and angiopoietin-4. FASEB J. 2004;18(11):1200–8.
8. Augustin HG, Koh GY, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. Nat Rev. 2009;10(3):165–77.
9. Saharinen P, Eklund L, Pulkki K, Bono P, Alitalo K. VEGF and angiopoietin signaling in tumor angiogenesis and metastasis. Trends Mol Med. 2011;17(7):347–62.
10. Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, et al. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev Cell. 2002;3(3):411–23.
11. Jeansson M, Gawlik A, Anderson G, Li C, Kerjaschki D, Henkelman M, et al. Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. J Clin Invest. 2011;121(6):2278–89.
12. Lee J, Kim KE, Choi DK, Jang JY, Jung JJ, Kiyonari H, et al. Angiopoietin-1 guides directional angiogenesis through integrin alphavbeta5 signaling for recovery of ischemic retinopathy. Sci Transl Med. 2013;5(203):203ra127.
13. D'Amico G, Korhonen EA, Anisimov A, Zarkada G, Holopainen T, Hagerling R, et al. Tie1 deletion inhibits tumor growth and improves angiopoietin antagonist therapy. J Clin Invest. 2014;124(2):824–34.
14. D'Amico G, Korhonen EA, Waltari M, Saharinen P, Laakkonen P, Alitalo K. Loss of endothelial Tie1 receptor impairs lymphatic vessel development-brief report. Arterioscler Thromb Vasc Biol. 2010;30(2):207–9.
15. Qu X, Tompkins K, Batts LE, Puri M, Baldwin S. Abnormal embryonic lymphatic vessel development in Tie1 hypomorphic mice. Development. 2010;137(8):1285–95.
16. Shen B, Shang Z, Wang B, Zhang L, Zhou F, Li T, et al. Genetic dissection of tie pathway in mouse lymphatic maturation and valve development. Arterioscler Thromb Vasc Biol. 2014;34(6):1221–30.
17. Qu X, Zhou B, Baldwin SH. Tie1 is required for lymphatic valve and collecting vessel development. Dev Biol. 2015; 399(1):117–28.
18. Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A, et al. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev. 1994;8(16):1897–909.
19. Arita Y, Nakaoka Y, Matsunaga T, Kidoya H, Yamamizu K, Arima Y, et al. Myocardium-derived angiopoietin-1 is essential for coronary vein formation in the developing heart. Nat Commun. 2014;5:4552.
20. Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. EMBO J. 1995;14(23): 5884–91.
21. Partanen J, Puri MC, Schwartz L, Fischer KD, Bernstein A, Rossant J. Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development. Development. 1996;122(10): 3013–21.

22. Puri MC, Partanen J, Rossant J, Bernstein A. Interaction of the TEK and TIE receptor tyrosine kinases during cardiovascular development. Development. 1999;126(20):4569–80.
23. Dellinger M, Hunter R, Bernas M, Gale N, Yancopoulos G, Erickson R, et al. Defective remodeling and maturation of the lymphatic vasculature in Angiopoietin-2 deficient mice. Dev Biol. 2008;319(2):309–20.
24. Zheng W, Nurmi H, Appak S, Sabine A, Bovay E, Korhonen EA, et al. Angiopoietin 2 regulates the transformation and integrity of lymphatic endothelial cell junctions. Genes Dev. 2014;28(14):1592–603.
25. Thomson BR, Heinen S, Jeansson M, Ghosh AK, Fatima A, Sung HK, et al. A lymphatic defect causes ocular hypertension and glaucoma in mice. J Clin Invest. 2014;124(10): 4320–4.
26. Eklund L, Saharinen P. Angiopoietin signaling in the vasculature. Exp Cell Res. 2013; 319(9):1271–80.
27. McCarter SD, Mei SH, Lai PF, Zhang QW, Parker CH, Suen RS, et al. Cell-based angiopoietin-1 gene therapy for acute lung injury. Am J Respir Crit Care Med. 2007;175(10): 1014–26.
28. Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science. 1999; 286(5449):2511–4.
29. Anisimov A, Tvorogov D, Alitalo A, Leppanen VM, An Y, Han EC, et al. Vascular endothelial growth factor-angiopoietin chimera with improved properties for therapeutic angiogenesis. Circulation. 2013;127(4):424–34.
30. Koh GY. Orchestral actions of angiopoietin-1 in vascular regeneration. Trends Mol Med. 2013;19(1):31–9.
31. Limaye N, Wouters V, Uebelhoer M, Tuominen M, Wirkkala R, Mulliken JB, et al. Somatic mutations in angiopoietin receptor gene TEK cause solitary and multiple sporadic venous malformations. Nat Genet. 2009;41(1):118–24.
32. Rasmussen AL, Okumura A, Ferris MT, Green R, Feldmann F, Kelly SM, et al. Host genetic diversity enables Ebola hemorrhagic fever pathogenesis and resistance. Science. 2014;346:987–91.
33. Ziegler T, Horstkotte J, Schwab C, Pfetsch V, Weinmann K, Dietzel S, et al. Angiopoietin 2 mediates microvascular and hemodynamic alterations in sepsis. J Clin Invest. 2013;123: 3436–45.
34. Syrjala SO, Tuuminen R, Nykanen AI, Raissadati A, Dashkevich A, Keranen MA, et al. Angiopoietin-2 inhibition prevents transplant ischemia-reperfusion injury and chronic rejection in rat cardiac allografts. Am J Transplant. 2014;14(5):1096–108.
35. Theelen TL, Lappalainen JP, Sluimer JC, Gurzeler E, Cleutjens JP, Gijbels MJ, et al. Angiopoietin-2 blocking antibodies reduce early atherosclerotic plaque development in mice. Atherosclerosis. 2015;241(2):297–304.
36. Falcon BL, Hashizume H, Koumoutsakos P, Chou J, Bready JV, Coxon A, et al. Contrasting actions of selective inhibitors of angiopoietin-1 and angiopoietin-2 on the normalization of tumor blood vessels. Am J Pathol. 2009;175(5):2159–70.
37. Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, et al. Targeting the ANG2/TIE2 Axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. Cancer Cell. 2011;19(4):512–26.
38. Woo KV, Qu X, Babaev VR, Linton MF, Guzman RJ, Fazio S, et al. Tie1 attenuation reduces murine atherosclerosis in a dose-dependent and shear stress-specific manner. J Clin Invest. 2011;121:1624–35.
39. Holopainen T, Saharinen P, D'Amico G, Lampinen A, Eklund L, Sormunen R, et al. Effects of angiopoietin-2-blocking antibody on endothelial cell–cell junctions and lung metastasis. J Natl Cancer Inst. 2012;104(6):461–75.
40. Vikkula M, Boon LM, Carraway 3rd KL, Calvert JT, Diamonti AJ, Goumnerov B, et al. Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. Cell. 1996;87(7):1181–90.

41. Soblet J, Limaye N, Uebelhoer M, Boon LM, Vikkula M. Variable Somatic TIE2 Mutations in Half of Sporadic Venous Malformations. Mol Syndromol. 2013;4(4):179–83.
42. Uebelhoer M, Natynki M, Kangas J, Mendola A, Nguyen HL, Soblet J, et al. Venous malformation-causative TIE2 mutations mediate an AKT-dependent decrease in PDGFB. Hum Mol Genet. 2013;22(17):3438–48.
43. Monk BJ, Poveda A, Vergote I, Raspagliesi F, Fujiwara K, Bae DS, et al. Anti-angiopoietin therapy with trebananib for recurrent ovarian cancer (TRINOVA-1): a randomised, multicentre, double-blind, placebo-controlled phase 3 trial. Lancet Oncol. 2014;15:799–808.
44. Goede V, Coutelle O, Neuneier J, Reinacher-Schick A, Schnell R, Koslowsky TC, et al. Identification of serum angiopoietin-2 as a biomarker for clinical outcome of colorectal cancer patients treated with bevacizumab-containing therapy. Br J Cancer. 2010;103(9): 1407–14.
45. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science. 1999;284(5422):1994–8.
46. Daly C, Eichten A, Castanaro C, Pasnikowski E, Adler A, Lalani AS, et al. Angiopoietin-2 functions as a Tie2 agonist in tumor models, where it limits the effects of VEGF inhibition. Cancer Res. 2013;73(1):108–18.
47. Nasarre P, Thomas M, Kruse K, Helfrich I, Wolter V, Deppermann C, et al. Host-derived angiopoietin-2 affects early stages of tumor development and vessel maturation but is dispensable for later stages of tumor growth. Cancer Res. 2009;69(4):1324–33.
48. Reiss Y, Knedla A, Tal AO, Schmidt MH, Jugold M, Kiessling F, et al. Switching of vascular phenotypes within a murine breast cancer model induced by angiopoietin-2. J Pathol. 2009;217(4):571–80.
49. Cho CH, Kammerer RA, Lee HJ, Steinmetz MO, Ryu YS, Lee SH, et al. COMP-Ang1: a designed angiopoietin-1 variant with nonleaky angiogenic activity. Proc Natl Acad Sci USA. 2004;101(15):5547–52.
50. Hwang JA, Lee EH, Kim HW, Park JB, Jeon BH, Cho CH. COMP-Ang1 potentiates the antitumor activity of 5-fluorouracil by improving tissue perfusion in murine Lewis lung carcinoma. Mol Cancer Res. 2009;7(12):1920–7.
51. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell. 2009;15(3):232–9.
52. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell. 2009;15(3):220–31.
53. Rigamonti N, Kadioglu E, Keklikoglou I, Wyser Rmili C, Leow CC, De Palma M. Role of angiopoietin-2 in adaptive tumor resistance to VEGF signaling blockade. Cell Rep. 2014; 8(3):696–706.
54. Kaipainen A, Vlaykova T, Hatva E, Bohling T, Jekunen A, Pyrhonen S, et al. Enhanced expression of the tie receptor tyrosine kinase messenger RNA in the vascular endothelium of metastatic melanomas. Cancer Res. 1994;54(24):6571–7.
55. Parikh SM, Mammoto T, Schultz A, Yuan HT, Christiani D, Karumanchi SA, et al. Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. PLoS Med. 2006;3(3):e46.
56. Parikh SM. Dysregulation of the angiopoietin-Tie-2 axis in sepsis and ARDS. Virulence. 2013;4(6):517–24.
57. David S, Mukherjee A, Ghosh CC, Yano M, Khankin EV, Wenger JB, et al. Angiopoietin-2 may contribute to multiple organ dysfunction and death in sepsis*. Crit Care Med. 2012;40(11):3034–41.
58. Stiehl T, Thamm K, Kaufmann J, Schaeper U, Kirsch T, Haller H, et al. Lung-targeted RNA interference against angiopoietin-2 ameliorates multiple organ dysfunction and death in sepsis. Crit Care Med. 2014;42(10):e654–62.

59. Benest AV, Kruse K, Savant S, Thomas M, Laib AM, Loos EK, et al. Angiopoietin-2 is critical for cytokine-induced vascular leakage. PLoS One. 2013;8(8):e70459.
60. Hakanpaa L, Sipila T, Leppanen VM, Gautam P, Nurmi H, Jacquemet G et al. Endothelial destabilization by angiopoietin-2 via integrin β1 activation. Nat Commun. 2015; 6:5962.
61. Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, et al. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med. 2006;12(2):235–9.
62. Tabruyn SP, Colton K, Morisada T, Fuxe J, Wiegand SJ, Thurston G, et al. Angiopoietin-2-driven vascular remodeling in airway inflammation. Am J Pathol. 2010;177(6):3233–43.
63. Hammes HP, Lin J, Wagner P, Feng Y, Vom Hagen F, Krzizok T, et al. Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. Diabetes. 2004;53(4):1104–10.
64. Park SW, Yun JH, Kim JH, Kim KW, Cho CH, Kim JH. Angiopoietin 2 induces pericyte apoptosis via alpha3beta1 integrin signaling in diabetic retinopathy. Diabetes. 2014;10.
65. Salmon AH, Neal CR, Sage LM, Glass CA, Harper SJ, Bates DO. Angiopoietin-1 alters microvascular permeability coefficients in vivo via modification of endothelial glycocalyx. Cardiovasc Res. 2009;83(1):24–33.
66. Kajiya K, Kidoya H, Sawane M, Matsumoto-Okazaki Y, Yamanishi H, Furuse M, et al. Promotion of lymphatic integrity by angiopoietin-1/Tie2 signaling during inflammation. Am J Pathol. 2012;180(3):1273–82.
67. Kosacka J, Nowicki M, Kloting N, Kern M, Stumvoll M, Bechmann I, et al. COMP-angiopoietin-1 recovers molecular biomarkers of neuropathy and improves vascularisation in sciatic nerve of ob/ob mice. PLoS One. 2012;7(3):e32881.
68. Cho CH, Sung HK, Kim KT, Cheon HG, Oh GT, Hong HJ, et al. COMP-angiopoietin-1 promotes wound healing through enhanced angiogenesis, lymphangiogenesis, and blood flow in a diabetic mouse model. Proc Natl Acad Sci USA. 2006;103(13):4946–51.
69. Lee JS, Song SH, Kim JM, Shin IS, Kim KL, Suh YL, et al. Angiopoietin-1 prevents hypertension and target organ damage through its interaction with endothelial Tie2 receptor. Cardiovasc Res. 2008;78(3):572–80.
70. Jung YJ, Kim DH, Lee AS, Lee S, Kang KP, Lee SY, et al. Peritubular capillary preservation with COMP-angiopoietin-1 decreases ischemia-reperfusion-induced acute kidney injury. Am J Physiol Renal Physiol. 2009;297(4):F952–60.
71. Tuuminen R, Nykänen AI, Saharinen P, Gautam P, Keränen MA, Arnaudova R, et al. Donor simvastatin treatment prevents ischemia-reperfusion and acute kidney injury by preserving microvascular barrier function. Am J Transplant. 2013;13(8):2019–34.
72. Reiss Y, Droste J, Heil M, Tribulova S, Schmidt MH, Schaper W, et al. Angiopoietin-2 impairs revascularization after limb ischemia. Circ Res. 2007;101(1):88–96.
73. Korhonen J, Lahtinen I, Halmekyto M, Alhonen L, Janne J, Dumont D, et al. Endothelial-specific gene expression directed by the tie gene promoter in vivo. Blood. 1995;86(5):1828–35.
74. Zhou BO, Ding L, Morrison SJ. Hematopoietic stem and progenitor cells regulate the regeneration of their niche by secreting Angiopoietin-1. Elife. 2015 Mar 30;4. doi: 10.7554/eLife.05521.
75. De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell. 2005;8(3):211–26.
76. Batard P, Sansilvestri P, Scheinecker C, Knapp W, Debili N, Vainchenker W, et al. The Tie receptor tyrosine kinase is expressed by human hematopoietic progenitor cells and by a subset of megakaryocytic cells. Blood. 1996;87(6):2212–20.
77. Yano M, Iwama A, Nishio H, Suda J, Takada G, Suda T. Expression and function of murine receptor tyrosine kinases, TIE and TEK, in hematopoietic stem cells. Blood. 1997;89(12):4317–26.
78. Korhonen J, Partanen J, Armstrong E, Vaahtokari A, Elenius K, Jalkanen M, et al. Enhanced expression of the tie receptor tyrosine kinase in endothelial cells during neovascularization. Blood. 1992;80(10):2548–55.

79. Porat RM, Grunewald M, Globerman A, Itin A, Barshtein G, Alhonen L, et al. Specific induction of tie1 promoter by disturbed flow in atherosclerosis-prone vascular niches and flow-obstructing pathologies. Circ Res. 2004;94(3):394–401.
80. Brown LF, Dezube BJ, Tognazzi K, Dvorak HF, Yancopoulos GD. Expression of Tie1, Tie2, and angiopoietins 1, 2, and 4 in Kaposi's sarcoma and cutaneous angiosarcoma. Am J Pathol. 2000;156(6):2179–83.
81. Iljin K, Dube A, Kontusaari S, Korhonen J, Lahtinen I, Oettgen P, et al. Role of ets factors in the activity and endothelial cell specificity of the mouse Tie gene promoter. FASEB J. 1999;13(2):377–86.
82. Iljin K, Petrova TV, Veikkola T, Kumar V, Poutanen M, Alitalo K. A fluorescent Tie1 reporter allows monitoring of vascular development and endothelial cell isolation from transgenic mouse embryos. FASEB J. 2002;16(13):1764–74.
83. White SJ, Papadakis ED, Rogers CA, Johnson JL, Biessen EA, Newby AC. In vitro and in vivo analysis of expression cassettes designed for vascular gene transfer. Gene Ther. 2008;15(5):340–6.
84. Kivivuori SM, Siitonen S, Porkka K, Vettenranta K, Alitalo R, Saarinen-Pihkala U. Expression of vascular endothelial growth factor receptor 3 and Tie1 tyrosine kinase receptor on acute leukemia cells. Pediatr Blood Cancer. 2007;48(4):387–92.
85. Schlaeger TM, Qin Y, Fujiwara Y, Magram J, Sato TN. Vascular endothelial cell lineage-specific promoter in transgenic mice. Development. 1995;121(4):1089–98.
86. Minami T, Kuivenhoven JA, Evans V, Kodama T, Rosenberg RD, Aird WC. Ets motifs are necessary for endothelial cell-specific expression of a 723-bp Tie-2 promoter/enhancer in Hprt targeted transgenic mice. Arterioscler Thromb Vasc Biol. 2003;23(11):2041–7.
87. Abou-Khalil R, Le Grand F, Pallafacchina G, Valable S, Authier FJ, Rudnicki MA, et al. Autocrine and paracrine angiopoietin 1/Tie-2 signaling promotes muscle satellite cell self-renewal. Cell Stem Cell. 2009;5(3):298–309.
88. Jin P, Zhang J, Sumariwalla PF, Ni I, Jorgensen B, Crawford D, et al. Novel splice variants derived from the receptor tyrosine kinase superfamily are potential therapeutics for rheumatoid arthritis. Arthritis Res Ther. 2008;10(4):R73.
89. Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, et al. Patterns of somatic mutation in human cancer genomes. Nature. 2007;446(7132):153–8.
90. Antonescu CR, Yoshida A, Guo T, Chang NE, Zhang L, Agaram NP, et al. KDR activating mutations in human angiosarcomas are sensitive to specific kinase inhibitors. Cancer Res. 2009;69(18):7175–9.
91. Sato TN, Qin Y, Kozak CA, Audus KL. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc Natl Acad Sci USA. 1993;90(20):9355–8.
92. Gerhard DS, Wagner L, Feingold EA, Shenmen CM, Grouse LH, Schuler G, et al. The status, quality, and expansion of the NIH full-length cDNA project: the mammalian gene collection (MGC). Genome Res. 2004;14(10B):2121–7.
93. Wang H, Zhang Y, Toratani S, Okamoto T. Transformation of vascular endothelial cells by a point mutation in the Tie2 gene from human intramuscular haemangioma. Oncogene. 2004;23(53):8700–4.
94. Wouters V, Limaye N, Uebelhoer M, Irrthum A, Boon LM, Mulliken JB, et al. Hereditary cutaneomucosal venous malformations are caused by TIE2 mutations with widely variable hyper-phosphorylating effects. Eur J Hum Genet. 2009;18(18):141–20.
95. Willam C, Koehne P, Jurgensen JS, Grafe M, Wagner KD, Bachmann S, et al. Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells. Circ Res. 2000;87(5):370–7.
96. Murdoch C, Tazzyman S, Webster S, Lewis CE. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. J Immunol. 2007;178(11):7405–11.
97. Schlaeger TM, Bartunkova S, Lawitts JA, Teichmann G, Risau W, Deutsch U, et al. Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. Proc Natl Acad Sci USA. 1997;94(7):3058–63.

98. Chen-Konak L, Guetta-Shubin Y, Yahav H, Shay-Salit A, Zilberman M, Binah O, et al. Transcriptional and post-translation regulation of the Tie1 receptor by fluid shear stress changes in vascular endothelial cells. FASEB J. 2003;17(14):2121–3.
99. Manning G, Plowman GD, Hunter T, Sudarsanam S. Evolution of protein kinase signaling from yeast to man. Trends Biochem Sci. 2002;27(10):514–20.
100. King N, Carroll SB. A receptor tyrosine kinase from choanoflagellates: molecular insights into early animal evolution. Proc Natl Acad Sci USA. 2001;98(26):15032–7.
101. Manning G, Young SL, Miller WT, Zhai Y. The protist, Monosiga brevicollis, has a tyrosine kinase signaling network more elaborate and diverse than found in any known metazoan. Proc Natl Acad Sci USA. 2008;105(28):9674–9.
102. Gjini E, Hekking LH, Kuchler A, Saharinen P, Wienholds E, Post JA, et al. Zebrafish Tie-2 shares a redundant role with Tie-1 in heart development and regulates vessel integrity. Dis Model Mech. 2011;4(1):57–66.
103. Barton WA, Tzvetkova-Robev D, Miranda EP, Kolev MV, Rajashankar KR, Himanen JP, et al. Crystal structures of the Tie2 receptor ectodomain and the angiopoietin-2-Tie2 complex. Nat Struct Mol Biol. 2006;13(6):524–32.
104. Davis S, Papadopoulos N, Aldrich TH, Maisonpierre PC, Huang T, Kovac L, et al. Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. Nat Struct Biol. 2003;10(1):38–44.
105. Kim KT, Choi HH, Steinmetz MO, Maco B, Kammerer RA, Ahn SY, et al. Oligomerization and multimerization is critical for angiopoietin-1 to bind and phosphorylate tie2. J Biol Chem. 2005;280:20126–131.
106. Saharinen P, Eklund L, Miettinen J, Wirkkala R, Anisimov A, Winderlich M, et al. Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nat Cell Biol. 2008;10(5):527–37.
107. Peters KG, Kontos CD, Lin PC, Wong AL, Rao P, Huang L et al. Functional significance of Tie2 signaling in the adult vasculature. Recent Prog Horm Res. 2004;59:51–71.
108. Yu X, Seegar TC, Dalton AC, Tzvetkova-Robev D, Goldgur Y, Rajashankar KR, et al. Structural basis for angiopoietin-1-mediated signaling initiation. Proc Natl Acad Sci USA. 2013;110(18):7205–10.
109. Seegar TC, Eller B, Tzvetkova-Robev D, Kolev MV, Henderson SC, Nikolov DB, et al. Tie1–Tie2 interactions mediate functional differences between angiopoietin ligands. Mol Cell. 2010;37(5):643–55.
110. Shewchuk LM, Hassell AM, Ellis B, Holmes WD, Davis R, Horne EL, et al. Structure of the Tie2 RTK domain: self-inhibition by the nucleotide binding loop, activation loop, and C-terminal tail. Structure. 2000;8(11):1105–13.
111. Khan AA, Sandhya VK, Singh P, Parthasarathy D, Kumar A, Advani J, et al. Signaling network map of endothelial TEK tyrosine kinase. J Signal Transduct. 2014;2014:173026.
112. Jones N, Master Z, Jones J, Bouchard D, Gunji Y, Sasaki H, et al. Identification of Tek/Tie2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration. J Biol Chem. 1999;274(43):30896–905.
113. Kontos CD, Stauffer TP, Yang WP, York JD, Huang L, Blanar MA, et al. Tyrosine 1101 of Tie2 is the major site of association of p85 and is required for activation of phosphatidylinositol 3-kinase and Akt. Mol Cell Biol. 1998;18(7):4131–40.
114. Tachibana K, Jones N, Dumont DJ, Puri MC, Bernstein A. Selective role of a distinct tyrosine residue on Tie2 in heart development and early hematopoiesis. Mol Cell Biol. 2005; 25(11):4693–702.
115. Huang L, Turck CW, Rao P, Peters KG. GRB2 and SH-PTP2: potentially important endothelial signaling molecules downstream of the TEK/TIE2 receptor tyrosine kinase. Oncogene. 1995;11(10):2097–103.
116. Jones N, Chen SH, Sturk C, Master Z, Tran J, Kerbel RS, et al. A unique autophosphorylation site on Tie2/Tek mediates Dok-R phosphotyrosine binding domain binding and function. Mol Cell Biol. 2003;23(8):2658–68.

117. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, et al. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell. 1996;87(7):1161–9.
118. Marron MB, Hughes DP, Edge MD, Forder CL, Brindle NP. Evidence for heterotypic interaction between the receptor tyrosine kinases TIE-1 and TIE-2. J Biol Chem. 2000;275(50): 39741–6.
119. Saharinen P, Kerkela K, Ekman N, Marron M, Brindle N, Lee GM, et al. Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. J Cell Biol. 2005;169(2):239–43.
120. Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, et al. Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. Proc Natl Acad Sci USA. 1999; 96(5):1904–9.
121. Barton WA, Tzvetkova D, Nikolov DB. Structure of the angiopoietin-2 receptor binding domain and identification of surfaces involved in Tie2 recognition. Structure. 2005;13(5): 825–32.
122. Procopio WN, Pelavin PI, Lee WM, Yeilding NM. Angiopoietin-1 and −2 coiled coil domains mediate distinct homo-oligomerization patterns, but fibrinogen-like domains mediate ligand activity. J Biol Chem. 1999;274(42):30196–201.
123. Fiedler U, Krissl T, Koidl S, Weiss C, Koblizek T, Deutsch U, et al. Angiopoietin-1 and angiopoietin-2 share the same binding domains in the Tie-2 receptor involving the first Ig-like loop and the epidermal growth factor-like repeats. J Biol Chem. 2003;278(3):1721–7.
124. Pietilä R, Nätynki M, Tammela T, Kangas J, Pulkki KH, Limaye N, et al. Ligand oligomerization state controls Tie2 receptor trafficking and angiopoietin-2-specific responses. J Cell Sci. 2012;125:2212–23.
125. Hansen TM, Singh H, Tahir TA, Brindle NP. Effects of angiopoietins-1 and −2 on the receptor tyrosine kinase Tie2 are differentially regulated at the endothelial cell surface. Cell Signal. 2010;22(3):527–32.
126. Gamble JR, Drew J, Trezise L, Underwood A, Parsons M, Kasminkas L et al. Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res. 2000;87(7):603–7.
127. Fukuhara S, Sako K, Minami T, Noda K, Kim HZ, Kodama T, et al. Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. Nat Cell Biol. 2008;10(5):513–26.
128. Winderlich M, Keller L, Cagna G, Broermann A, Kamenyeva O, Kiefer F, et al. VE-PTP controls blood vessel development by balancing Tie-2 activity. J Cell Biol. 2009;185(4): 657–71.
129. Shen J, Frye M, Lee BL, Reinardy JL, McClung JM, Ding K, et al. Targeting VE-PTP activates TIE2 and stabilizes the ocular vasculature. J Clin Invest. 2014;124(10):4564–76.
130. Cascone I, Napione L, Maniero F, Serini G, Bussolino F. Stable interaction between {alpha}5{beta}1 integrin and tie2 tyrosine kinase receptor regulates endothelial cell response to ang-1. J Cell Biol. 2005;170:993–1004.
131. Thomas M, Felcht M, Kruse K, Kretschmer S, Deppermann C, Biesdorf A, et al. Angiopoietin-2 stimulation of endothelial cells induces alphavbeta3 integrin internalization and degradation. J Biol Chem. 2010;285(31):23842–9.
132. Felcht M, Luck R, Schering A, Seidel P, Srivastava K, Hu J, et al. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. J Clin Invest. 2012; 122(6):1991–2005.
133. Lee HS, Oh SJ, Lee KH, Lee YS, Ko E, Kim KE, et al. Gln362 of Angiopoietin-2 Mediates Migration of Tumor and Endothelial Cells through Association with alpha5beta1 Integrin. J Biol Chem. 2014;289:31330–40.
134. Daly C, Wong V, Burova E, Wei Y, Zabski S, Griffiths J, et al. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). Genes Dev. 2004;18(9):1060–71.

135. Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, et al. Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. J Biol Chem. 2000;275(13):9102–5.
136. Hughes DP, Marron MB, Brindle NP. The antiinflammatory endothelial tyrosine kinase Tie2 interacts with a novel nuclear factor-kappaB inhibitor ABIN-2. Circ Res. 2003;92(6):630–6.
137. Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, et al. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood. 2004;103(11):4150–6.
138. Daly C, Pasnikowski E, Burova E, Wong V, Aldrich TH, Griffiths J, et al. Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. Proc Natl Acad Sci USA. 2006;103(42):15491–6.
139. David S, Ghosh CC, Mukherjee A, Parikh SM. Angiopoietin-1 requires IQ domain GTPase-activating protein 1 to activate Rac1 and promote endothelial barrier defense. Arterioscler Thromb Vasc Biol. 2011;31(11):2643–52.
140. Sako K, Fukuhara S, Minami T, Hamakubo T, Song H, Kodama T, et al. Angiopoietin-1 induces Kruppel-like factor 2 expression through a phosphoinositide 3-kinase/AKT-dependent activation of myocyte enhancer factor 2. J Biol Chem. 2009;284(9):5592–601.
141. Lee SW, Kim WJ, Jun HO, Choi YK, Kim KW. Angiopoietin-1 reduces vascular endothelial growth factor-induced brain endothelial permeability via upregulation of ZO-2. Int J Mol Med. 2009;23(2):279–84.
142. Li X, Stankovic M, Bonder CS, Hahn CN, Parsons M, Pitson SM, et al. Basal and angiopoietin-1-mediated endothelial permeability is regulated by sphingosine kinase-1. Blood. 2008;111(7):3489–97.
143. Gavard J, Patel V, Gutkind JS. Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. Dev Cell. 2008;14(1):25–36.
144. Oubaha M, Gratton JP. Phosphorylation of endothelial nitric oxide synthase by atypical PKC zeta contributes to angiopoietin-1-dependent inhibition of VEGF-induced endothelial permeability in vitro. Blood. 2009;114(15):3343–51.
145. Babaei S, Teichert-Kuliszewska K, Zhang Q, Jones N, Dumont DJ, Stewart DJ. Angiogenic actions of angiopoietin-1 require endothelium-derived nitric oxide. Am J Pathol. 2003; 162(6):1927–36.
146. Nawroth R, Poell G, Ranft A, Kloep S, Samulowitz U, Fachinger G, et al. VE-PTP and VE-cadherin ectodomains interact to facilitate regulation of phosphorylation and cell contacts. EMBO J. 2002;21(18):4885–95.
147. Nottebaum AF, Cagna G, Winderlich M, Gamp AC, Linnepe R, Polaschegg C, et al. VE-PTP maintains the endothelial barrier via plakoglobin and becomes dissociated from VE-cadherin by leukocytes and by VEGF. J Exp Med. 2008;205(12):2929–45.
148. Broermann A, Winderlich M, Block H, Frye M, Rossaint J, Zarbock A, et al. Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. J Exp Med. 2011;208(12):2393–401.
149. Carra S, Foglia E, Cermenati S, Bresciani E, Giampietro C, Lora Lamia C, et al. Ve-ptp modulates vascular integrity by promoting adherens junction maturation. PLoS One. 2012; 7(12):e51245.
150. Li Z, Huang H, Boland P, Dominguez MG, Burfeind P, Lai KM, et al. Embryonic stem cell tumor model reveals role of vascular endothelial receptor tyrosine phosphatase in regulating Tie2 pathway in tumor angiogenesis. Proc Natl Acad Sci USA. 2009;106:22399–404.
151. Yuan HT, Khankin EV, Karumanchi SA, Parikh SM. Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. Mol Cell Biol. 2009;29(8):2011–22.
152. Yuan HT, Venkatesha S, Chan B, Deutsch U, Mammoto T, Sukhatme VP, et al. Activation of the orphan endothelial receptor Tie1 modifies Tie2-mediated intracellular signaling and cell survival. FASEB J. 2007;21:3171–83.
153. Kontos CD, Cha EH, York JD, Peters KG. The endothelial receptor tyrosine kinase Tie1 activates phosphatidylinositol 3-kinase and Akt to inhibit apoptosis. Mol Cell Biol. 2002; 22(6):1704–13.

154. McCarthy MJ, Burrows R, Bell SC, Christie G, Bell PR, Brindle NP. Potential roles of metalloprotease mediated ectodomain cleavage in signaling by the endothelial receptor tyrosine kinase Tie-1. Lab Invest. 1999;79(7):889–95.
155. Marron MB, Singh H, Tahir TA, Kavumkal J, Kim HZ, Koh GY, et al. Regulated proteolytic processing of Tie1 modulates ligand responsiveness of the receptor-tyrosine kinase Tie2. J Biol Chem. 2007;282(42):30509–17.
156. Findley CM, Cudmore MJ, Ahmed A, Kontos CD. VEGF induces Tie2 shedding via a phosphoinositide 3-kinase/Akt dependent pathway to modulate Tie2 signaling. Arterioscler Thromb Vasc Biol. 2007;27(12):2619–26.
157. Singh H, Hansen TM, Patel N, Brindle NP. The molecular balance between receptor tyrosine kinases Tie1 and Tie2 is dynamically controlled by VEGF and TNFalpha and regulates angiopoietin signalling. PLoS One. 2012;7(1):e29319.

Chapter 17
The TrK Receptor Family

Hubert Hondermarck, Yohann Demont, and Ralph A. Bradshaw

17.1 Introduction to the Trk Receptor Tyrosine Kinase Family

17.1.1 Trk Discovery

The Trk family of receptors is named for the oncogene, which was first isolated from a colon carcinoma, that is made up of the first 7 of 8 exons of non-muscle tropomyosin joined to the transmembrane and cytoplasmic domains of a kinase that was unknown at the time; consequently, it was named tropomyosin-related kinase or Trk [1]. There are three principal members of the family with several isoforms of each. In the early 1990s, the neurotrophins, a family of four factors, of which nerve growth factor (NGF) is the prototype, were shown to be the ligands of the Trk receptors; NGF was identified as the ligand of TrkA [2–4], BDNF and neurotrophin 4/5 (NT-4/5) were identified as ligands of TrkB [5–7] and neurotrophin 3 (NT-3) was identified as the ligand of TrkC [4, 8]. The discovery of the Trk family resolved a controversy that had been generated by previous descriptions of NGF receptors of lower molecular weight (~75 kDa), which did not possess obvious intracellular

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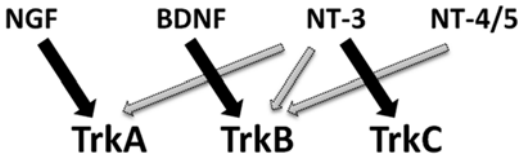

Fig. 17.1 Neurotrophin-binding specificity for the Trk receptors. TrkA is the principal receptor for NGF, TrkB for BDNF, and TrkC for NT-3 (*black arrows*). If there is an insert in the extracellular part of the receptor, NT-3 can also bind to TrkA and TrkB, and NT-4/5 can bind to TrkB (*grey arrows*)

signaling domains, and confirmed several previous reports that NGF did utilize, at least in part, a higher molecular weight entity [9]. It is now established that the neurotrophins (and their unprocessed pro-forms) use two principal receptor types: the Trks and a pan-neurotrophin receptor generally designated $p75^{NTR}$. These can function independently or in concert and can involve additional membrane-bound proteins in some activities.

17.1.2 Interaction with Neurotrophins

NGF basically binds to TrkA, BDNF to TrkB, and NT-3 to TrkC. Under certain conditions (see below), NT-4/5 can bind to TrkB and NT-3 to TrkA and TrkB (Fig. 17.1). By itself, TrkA binds NGF with low affinity (10^{-9} M), but a high affinity site is created (10^{-11} M) when $p75^{NTR}$ is also expressed [10]. During this association, the neurotrophins may bind first to $p75^{NTR}$, which may then present them to the various Trks [11, 12]. Furthermore, in the presence of $p75^{NTR}$, the activation of TrkA and TrkB by NT-3 and the activation of TrkB by NT-4/5 are diminished. Thus, it appears that $p75^{NTR}$ increases the specificity of TrkA and TrkB for their primary ligands [13–17].

17.2 The Role of the Trk Receptor Tyrosine Kinase Family in Embryonic Development and Adult Physiology

Trk receptors have essential functions during the development of both the central and peripheral nervous system. Trk expression in developing neurons, and the concomitant release of neurotrophins by organ targets of innervation, constitutes the basis for the control of neuronal precursor cell survival and differentiation, including the outgrowth of dendrites and axons. These Trk-driven neurotrophic activities [18] result in the progressive establishment of the neuronal circuitry throughout all organs and tissues, but they are not the ultimate function of Trk receptors in the nervous system. Indeed, the assembly and maintenance of synapses also requires

Trk receptors as demonstrated in several populations of neurons [19]. NGF was first identified by its activity on sympathetic and select sensory neurons that were a manifestation of its interaction with TrkA [2–4], but it was eventually shown to be present in and active on select cholinergic neurons [20]. In contrast, TrkB and TrkC are not widely found in the peripheral nervous system, and their activities are generally more related to the central nervous system. In the adult, the Trks are mediators of pain sensation [21]. All of the Trks and $p75^{NTR}$ are expressed on sensory neurons and transduce pain signals to the central nervous system via various signaling pathways including phosphoinositide-3-kinase (PI3K) and the activation of the membrane ion channel TRPV1 [22]. In addition, there are a number of nonnervous system tissues, including the blood cells, lungs, bones, prostate, and pancreas, that also express the Trks [23].

17.3 The Role of the Trk Receptor Tyrosine Kinase Family in Human Disease

17.3.1 Cancer

Historically, NGF was first identified as a factor secreted by two murine sarcomas that stimulated the growth of nerve fibers, and the earliest attempts to purify it were done with these tissues until far richer sources were identified [24]. Since that time, the expression of both neurotrophins and their receptors in a wide variety of neoplasias has been reported. It most cases they appear to be involved in one or another responses of the tumor cells. The most extensive characterization of Trk in neoplastic tissues is with breast tumors [25], which is likely representative of its involvement with other transformed cells.

17.3.1.1 Breast Cancer

In breast cancer, an autocrine loop involving NGF stimulates cancerous epithelial mammary cell growth; it has no effect on normal breast cells, which do, however, express both TrkA and $p75^{NTR}$ [26, 27]. The pro-tumoral effect that NGF exerts on breast cancer cells is twofold: tumor cell proliferation is mediated by TrkA and MAPKs, and their survival depends on $p75^{NTR}$ and NFκB [28–30]. The other neurotrophins (BDNF, NT-3, and NT-4/5) also enable resistance to cancer cell apoptosis via $p75^{NTR}$, but they have no effect on proliferation [31].

Clinicopathologically, the amounts of TrkA and $p75^{NTR}$ mRNA in homogenates of tumoral resections are correlated with a good prognosis [32]. On the other hand, the active form of TrkA that has been phosphorylated on select tyrosine residues is associated with aggressive tumors; it is found more frequently in effusions and local regional recurrences compared to primary tumors [33]. $p75^{NTR}$ is also correlated with tumors presenting less effusion and invasion of the lymph nodes. It is especially

expressed in normal myoepithelial cells and has been suggested as a possible marker to exclude invasive cancers, except in good prognosis basal type of tumors [34, 35]. Finally, compared to the normal epithelium, NGF is overexpressed in almost all mammary tumors [36] and the activation of TrkA is frequently observed [37].

From a therapeutic point of view, it has been shown that targeting HER2 of the EGF receptor family that is overexpressed in 20 % of cancers prevents NGF from promoting the growth of cancer cells [38], suggesting a crosstalk between the two receptors. Similarly, tamoxifen, an antiestrogenic drug commonly used in breast cancer treatment, inhibits the mitogenic effect of NGF [39] and the use of antibodies that neutralize NGF, anti-NGF siRNA, or a pharmacological TrkA inhibitor induces a great reduction in tumor growth in xenograft models [36, 37].

A subgroup of rare invasive ductal carcinomas—secretory cancers—are often (in 90 % of cases) characterized by an ETV6-NTRK3 genetic rearrangement resulting in a fusion protein. This translocation, where the N-terminus of the transcription factor Ets (variant 6) is fused with the kinase domain of TrkC, is the source of an oncogene, which is able to transform normal mammary cells [40].

17.3.1.2 Other Cancers

There have also been a number of observations concerning the involvement of Trks in other cancers. In some cases a role is implied from the expression of, or response to, one or more of the neurotrophins (or their proforms). In many cases, $p75^{NTR}$ expression and activation has also been observed. These studies include melanoma [41, 42], lymphoma [43, 44], and leukemia [45] as well as cancers of the stomach [46, 47], liver [48, 49], pancreas ([285, 50]), prostate [51, 52], thyroid [53–56], lung [57, 58], and ovary [59, 60]. There have also been extensive descriptions of Trk expression and activation in neuroblastoma ([61–69]; [286]), and cultured cell lines from this cancer have provided useful models for studying NGF action.

17.3.2 Trks in Other Pathologies

Early studies have shown severe sensory neuropathies in mice carrying a disrupted *TRK* gene [70]. *TRK* loss-of-function mutations have been described in hereditary sensory and autonomic neuropathies [71] and can result in a wide variety of effects including energy imbalance, loss of appetite control and obesity, and memory impairment [72]. In addition, Trk receptors are noticeably involved in both acute and chronic pain [287]. Both local and systemic administration of neurotrophins have been shown to elicit pain [73], and targeting them, or Trks, with blocking antibodies or small inhibitors has been developed as a pain treatment [21]. The effect is broadly analgesic and these are in clinical trials (phase II and III) for the treatment of acute and chronic pain [74]. Humanized blocking antibodies for NGF have been

developed for commercial purposes, the most advanced being Tanezumab from Pfizer and Eli Lilly in phase III clinical trials, but other companies, such as Johnson & Johnson and Abbott, have developed their own inhibitory molecules [75, 76]. It is noteworthy that anti-neurotrophin drugs could also be of interest for the treatment of cancer pain, a frequently reported symptom in clinical oncology [77].

Aside from pain, Trk receptors have been implicated in various neurodegenerative disorders such as Alzheimer's disease [78] and spongiform encephalopathy where proNGF expression is increased [79]. This suggests that in addition to appearing as biomarkers of these pathologies, pro-neurotrophins could play a biological role. In this respect, proNGF isolated in the brain of patients suffering from Alzheimer's disease induces the death of PC12 cells (the paradigm of choice for studying the effects and signaling of both pro-neurotrophins and their mature forms; see below), whereas NGF enables their survival and differentiation [80]. Beside these effects on cells in culture, it has been shown that proNGF is responsible for neuronal death in various models of nervous system conditions in vivo. Thus, mice or rats with a nervous system lesion show an increase in secreted proNGF, which induces neuronal death [81–84]. Importantly, proNGF can induce neuronal death despite trophic NGF stimulation, suggesting that the signal from pro-neurotrophins may potentially override the signal from the corresponding neurotrophins [85].

17.4 Trk Receptors

17.4.1 Trk Genes

Phylogenetic studies of receptor tyrosine kinases show that the genes (*NTRK*, neurotrophin receptor kinase) for the Trk receptors have probably been formed from just one ancestral gene, which gave rise, via duplication in agnathans, to TrkB and the ancestor of TrkA/TrkC; TrkA and TrkC then split in chondrichthyans [86]. Table 17.1 presents the characteristics of the Trk receptor genes.

Table 17.1 Genetic characteristics of human Trk tyrosine kinases

			mRNA
Gene name	Location	Exons	NCBI no.
NTRK1	1q21–q22	19	NM_002529.3
NTRK2	9q22.1	24	NM_006180.3
NTRK3	15q25	21	NM_002530.2

The genes for TrkA, TrkB, and TrkC; their chromosomal location; and the number of exons they have. NCBI accession numbers for the major transcript of each gene is given

17.4.1.1 Promoter Structure

TrkA promoter sequences are GC rich, lack genuine TATA or CAAT boxes, and are contained within a CpG island, which extends over the entire first coding exon [87]. The TrkA transcription start site is located upstream to the AUG translation initiation codon, with a 150 bp long DNA segment, immediately upstream to the start site. Loss of promoter methylation in frequently found in glioma [88] childhood neuroblastoma [89]. All TRKs have been shown to be hypomethylated in various cancers including hepatocellular carcinomas then contributing to their overexpression [90].

17.4.1.2 Transcriptional Regulation

The TrkA promoter binds to the Sp1 transcription factor [87]. TrkA is transcriptionally induced by IL-13 [91]. The transcriptional downregulation of TrkA by mutant TATA binding protein (TBP) contributes to spinocerebellar ataxia type 17 pathogenesis [92]. The transcription factor Brn3a plays an important role as an enhancer of TrkA transcription during development [93]. DeltaNp73, a truncated form of the p53 family of proteins, directly binds to the TrkA promoter and transcriptionally represses TrkA expression, which in turn attenuates the NGF-mediated MAPK pathway [94].

TrkB expression has been shown to be Ca^{2+} dependent [95]. The cAMP/CREB pathways [96, 97] and hypoxia-inducible factor-1 (HIF-1) [98] are transcriptional activators of TrkB. Furthermore, the histone deacetylase 1 (HDAC1) and HDAC2 are recruited by DeltaNp73 to the TrkA promoter and act as corepressors to suppress TrkA expression [96]. In addition, thyroid hormone (T3) downregulates the expression of the TrkB gene through the active repression of a negative response element located downstream of its transcription initiation site during the development of the brain [99]. TrkC was shown to be transcriptionally repressed by Runx3, a Runt domain transcription factor [100].

17.4.2 Trk Proteins

17.4.2.1 General Organization

Trk receptors share the same overall structure. They are transmembrane, N-glycosylated type I proteins composed of around 800 amino acids (Fig. 17.2). Their extracellular domains (~400 amino acids) contain two cystine-rich regions surrounding a leucine-rich domain, followed in the external juxtamembrane region by two immunoglobulin domains (Ig-C1 and Ig-C2) and an insert segment, which may or may not be present, modifying the receptor's specificity for its ligands. The various regions of the extracellular domain of the Trk receptors control

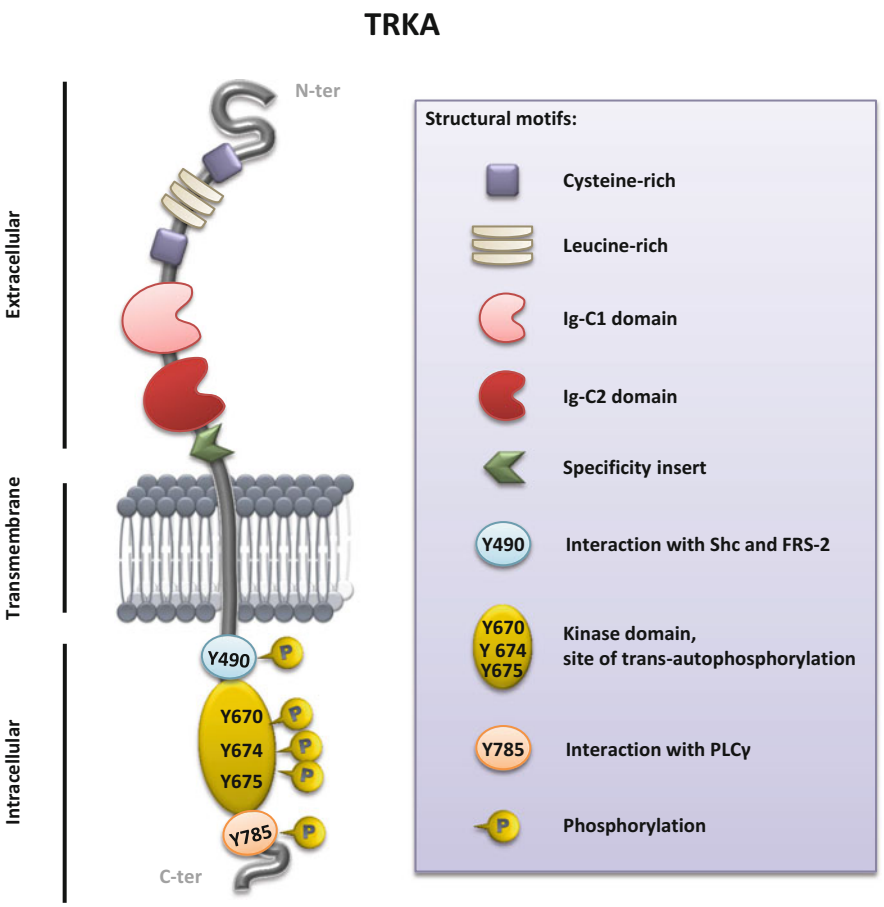

Fig. 17.2 Organization of Trk receptors. Trk receptors are composed of an extracellular domain, formed by a leucine-rich domain surrounded by two cystine-rich regions, followed by two immunoglobin domains (Ig-C1 and Ig-C2) and an insert involved in the specificity for their primary ligands. In the intracellular domain, Trk receptors have a tyrosine kinase domain, which is responsible for their trans-autophosphorylation, a juxtamembrane region, and a C-terminal extension. For TrkA, the tyrosines, following phosphorylation, involved in trans-autophosphorylation are the Y670, Y674, and Y675, whereas recruitment of the adaptor/scaffold proteins Shc and FRS-2 occurs at Y490 and the recruitment of PLCγ is at Y785

ligand-independent dimerization. Deletion of the domains Ig-C1 and/or Ig-C2 reinforce auto-dimerization and the spontaneous activation of Trk receptors, suggesting that these domains could inhibit dimerization in the absence of a ligand. Conversely, the leucine-rich domain seems to promote these auto-associations [101]. Similarly, in addition to enabling membrane targeting, Trk receptor glycosylation seems to prevent spontaneous activation in the absence of a ligand [102].

17.4.2.2 Trk Sequences and Transient PTMs

Sequence alignments of the main forms of human TrkA, TrkB, and TrkC are presented in Fig. 17.3. The three sequences differ in length and gaps have been inserted by the Align program (Uniprot) to compensate for these insertions/deletions.

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SP|P04629|NTRK1_HUMAN MLRGGRRGQLGWHSWAAGPGSLLAWLI-------LA-SAGAAPCPDACCPHGSSGLRCTR 52
SP|Q16620|NTRK2_HUMAN --------MSSWIRWH-GPAMARLWGFCW---LVVGFWRAAFACPTSCK-CSASRIWCSD 47
SP|Q16288|NTRK3_HUMAN --------MD---VSL-CPAKCSFWRIFLLGSVWLDYVGSVLACPANCV-CSKTEINCRR 47

SP|P04629|NTRK1_HUMAN DG--AL----------------DSLHHLPGAENLTELYIENQQHLQHLELRDLRGLGELR 94
SP|Q16620|NTRK2_HUMAN PSPGIVAFPRLEPNSV-------------DPENITEIFIANQKRLEIINEDDVEAYVGLR 94
SP|Q16288|NTRK3_HUMAN PDDGNL-FPLLEGQDSGNSNGNASINITDISRNITSIHIENWRSLHTLNAVDMELYTGLQ 106

SP|P04629|NTRK1_HUMAN NLTIVKSGLRFVAPDAFHFTPRLSRLNLSFNALESLSWKTVQGLSLQELVLSGNPLHCSC 154
SP|Q16620|NTRK2_HUMAN NLTIVDSGLKFVAHKAFLKNSNLQHINFTRNKLTSLSRKHFRHLDLSELILVGNPFTCSC 154
SP|Q16288|NTRK3_HUMAN KLTIKNSGLRSIQPRAFAKNPHLRYINLSSNRLTTLSWQLFQTLSLRELQLEQNFFNCSC 166

SP|P04629|NTRK1_HUMAN ALRWLQRWEEEGLGGVPEQKLQCHG----QGPLAHMPNASCGVPTLKVQVPNASVDVGDD 210
SP|Q16620|NTRK2_HUMAN DIMWIKTLQEAK-SSPDTQDLYCLNESSKNIPLANLQIPNCGLPSANLAAPNLTVEEGKS 213
SP|Q16288|NTRK3_HUMAN DIRWMQLWQEQGEAKLNSQNLYCINADGSQLPLFRMNISQCDLPEISVSHVNLTVREGDN 226

SP|P04629|NTRK1_HUMAN VLLRCQVEGRGLEQAGWILTELEQSATV---MKSGGLPSLGLTLANVTSDLNRKNVTCWA 267
SP|Q16620|NTRK2_HUMAN ITLSCSVAGDPVPNMYWDVGNLVSKHMNET-----SHTQGSLRITNISSDDSGKQISCVA 268
SP|Q16288|NTRK3_HUMAN AVITCNGSGSPLPDVDWIVTGLQSINTHQTNLNWTNVHAINLTLVNVTSEDNGFTLTCIA 286

SP|P04629|NTRK1_HUMAN ENDVGRAEVSVQVNVSFPASVQ-LHTAVEMHHWCIPFSVDGQPAPSLRWLFNGSVLNETS 326
SP|Q16620|NTRK2_HUMAN ENLVGEDQDSVNLTVHFAPTITFLESPTSDHHWCIPFTVKGNPKPALQWFYNGAILNESK 328
SP|Q16288|NTRK3_HUMAN ENVVGMSNASVALTVYYPPRVVSLEEPELRLEHCIEFVVRGNPPPTLHWLHNGQPLRESK 346

SP|P04629|NTRK1_HUMAN FIFTEFLEPAANETVRHGCLRLNQPTHVNNGNYTLLAANPFGQASASIMAAFMDNPFEFN 386
SP|Q16620|NTRK2_HUMAN YICTKIHVT--NHTEYHGCLQLDNPTHMNNGDYTLIAKNEYGKDEKQISAHFMGWPGIDD 386
SP|Q16288|NTRK3_HUMAN IIHVEYYQ---EGEISEGCLLFNKPTHYNNGNYTLIAKNPLGTANQTINGHFLKEPFPES 403

SP|P04629|NTRK1_HUMAN PEDPIP--VSFSPVD-------------TNSTSGDPVEKKDETPFGVSVAVGLAVFACLF 431
SP|Q16620|NTRK2_HUMAN GANPNYPDVIYEDYGTAANDIGDTTNRSNEIPSTDVTDKTGREHLSVYAVVVIASVVG-F 445
SP|Q16288|NTRK3_HUMAN TDNF----ILFDEV--------------SPTPPITVTHKPEEDTFGVSIAVGLAAFACVL 445

SP|P04629|NTRK1_HUMAN LSTLLLVLNKCGRRNKFGINRPAVLA-PEDGLAMSLHFMTLGGSSLSPTEGKGSG----- 485
SP|Q16620|NTRK2_HUMAN CLLVMLFLLKLARHSKFGMKGPASVISNDDDSASPLHHISNGSNTPSSSEGGPDAVIIGM 505
SP|Q16288|NTRK3_HUMAN LVVLFVMINKYGRRSKFGMKGPVAVISGEEDSASPLHHINHGITTPSSLDAGPDTVVIGM 505

SP|P04629|NTRK1_HUMAN LQGHIIENPQYFS--------DACVHHIKRRDIVLKWELGEGAFGKVFLAECHNLLPEQD 537
SP|Q16620|NTRK2_HUMAN TKIPVIENPQYFGITNSQLKPDTFVQHIKRHNIVLKRELGEGAFGKVFLAECYNLCPEQD 565
SP|Q16288|NTRK3_HUMAN TRIPVIENPQYFRQGHNCHKPDTYVQHIKRRDIVLKRELGEGAFGKVFLAECYNLSPTKD 565

SP|P04629|NTRK1_HUMAN KMLVAVKALKEASESARQDFQREAELLTMLQHQHIVRFFGVCTEGRPLLMVFEYMRHGDL 597
SP|Q16620|NTRK2_HUMAN KILVAVKTLKDASDNARKDFHREAELLTNLQHEHIVKFYGVCVEGDPLIMVFEYMKHGDL 625
SP|Q16288|NTRK3_HUMAN KMLVAVKALKDPTLAARKDFQREAELLTNLQHEHIVKFYGVCGDGDPLIMVFEYMKHGDL 625

SP|P04629|NTRK1_HUMAN NRFLRSHGPDAKLLAGGE-DVAPGPLGLGQLLAVASQVAAGMVYLAGLHFVHRDLATRNC 656
SP|Q16620|NTRK2_HUMAN NKFLRAHGPDAVLMAEGNPPT---ELTQSQMLHIAQQIAAGMVYLASQHFVHRDLATRNC 682
SP|Q16288|NTRK3_HUMAN NKFLRAHGPDAMILVDGQPRQAKGELGLSQMLHIASQIASGMVYLASQHFVHRDLATRNC 685

SP|P04629|NTRK1_HUMAN LVGQGLVVKIGDFGMSRDIYSTDYYR--------------VGGRTMLPIRWMPPESILYR 702
SP|Q16620|NTRK2_HUMAN LVGENLLVKIGDFGMSRDVYSTDYYR--------------VGGHTMLPIRWMPPESIMYR 728
SP|Q16288|NTRK3_HUMAN LVGANLLVKIGDFGMSRDVYSTDYYRLFNPSGNDFCIWCEVGGHTMLPIRWMPPESIMYR 745

SP|P04629|NTRK1_HUMAN KFTTESDVWSFGVVLWEIFTYGKQPWYQLSNTEAIDCITQGRELERPRACPPEVYAIMRG 762
SP|Q16620|NTRK2_HUMAN KFTTESDVWSLGVVLWEIFTYGKQPWYQLSNNEVIECITQGRVLQRPRTCPQEVYELMLG 788
SP|Q16288|NTRK3_HUMAN KFTTESDVWSFGVILWEIFTYGKQPWFQLSNTEVIECITQGRVLERPRVCPKEVYDVMLG 805

SP|P04629|NTRK1_HUMAN CWQREPQQRHSIKDVHARLQALAQAPPVYLDVLG 796
SP|Q16620|NTRK2_HUMAN CWQREPHMRKNIKGIHTLLQNLAKASPVYLDILG 822
SP|Q16288|NTRK3_HUMAN CWQREPQQRLNIKEIYKILHALGKATPIYLDILG 839
```

Fig. 17.3 Alignment of the sequences of the human TrkA, TrkB, and TrkC proteins. The alignment was generated using the Align program of Uniprot

The intracellular domains, which are highly conserved in the various Trks, contain a number of tyrosines that are subject to phosphorylation. (Nb. The numbering of the amino acids in the Trk sequences varies by both species and isoform; the numbering in Fig. 17.2, which gives the most commonly used identifications of the five most important tyrosines involved in signaling, differs slightly from the human alignments shown in Fig. 17.3). In human TrkA (and in the other human Trks in slightly different positions) (Fig. 17.3), there are three tyrosines at positions 676, 680, and 681 in the activation loop that are presumably responsible for stabilizing the active form of the receptor. They may also provide docking sites. Y496, in the juxtamembrane domain, forms a binding site for the adaptor/scaffold proteins Shc and FRS-2 in its modified state and the C-terminal extension contains Y791, which, when phosphorylated, recruits and activates PLCγ (see below). There are two other tyrosines at Y701 and Y757 that have also been reported to be signaling sites. When Y701 is replaced by aspartate or phenylalanine, receptor internalization decreases due to a reduction in the association of TrkA with clathrin supporting the view that the Tyr-Arg-Lys-Phe sequence at residues 701–704 is a motif in the lysosomal targeting of TrkA receptors [103]. The Y757 is found in a PI3K binding motif and evidence has been presented that it can participate in the activation of this effector [104–106]. It may also be involved in the activation of Nck [107] (see below). However, evidence for NGF-directed phosphorylation of either of these two sites is lacking.

Both TrkA and B have been shown to be ubiquinated, and these modifications are apparently mainly involved in receptor turnover [108–112]. The presence of $p75^{NTR}$ appears to attenuate this modification for both TrkA and B and thus prolong the lifetime of the Trks [108]. The sites of the ubiquitin modifications have not been reported.

17.4.2.3 Trk Isoforms

Each of the Trk family members can be expressed as isoforms that differ in length and sequence from the parent structure. These have various properties and their physiological roles and importance are not fully understood. Some of these appear to be especially important in cancer tissues.

TrkA

There are two isoforms very close to TrkA called TrkA-I and TrkA-II (Table 17.2). TrkA-I (originally cloned as TrkA) is ubiquitously expressed in nonneuronal tissues, whereas TrkA-II is found preferentially in neuronal tissues and in small amounts in the kidneys and lungs [113]. A new isoform of TrkA, namely, TrkA-III, was identified more recently in neuroblastomas and in the thymus [65, 114]. It is also expressed in pluripotent neural stem and neural crest progenitors.

TrkA-I and TrkA-II differ only by 18 bp in the alternative splicing of exon 11 (Fig. 17.4). TrkA-II, which contains the sequence coded by exon 11, has a 6-amino

Table 17.2 Characteristics of the isoforms of TrkA

Isoforms	mRNA NCBI no.	Exons	Size	Protein Uniprot no.	Size
TrkA-I	NM_001012331.1	16	2647	P04629-2	790
TrkA-II	NM_002529.3	17	2663	P04629-1	796
TrkA-III	[65]	14			698

The three isoforms of TrkA with the NCBI transcript accession number, the number of exons they have and their size in bp, as well as the Uniprot accession number for the proteins that they code and their size in amino acids

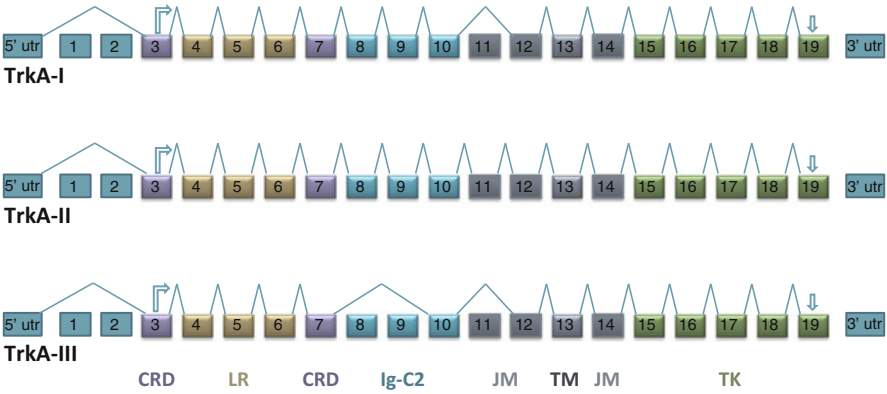

Fig. 17.4 Isoforms of TrkA. The *squares* represent the 19 exons of the gene for TrkA; the *rectangles* represent the 5′ and 3′ utr (untranslated region). TrkA-II is the longest isoform, TrkA-I is spliced at exon 9 and TrkA-III is devoid of exons 8, 9, and 11. The *arrow* pointing *right* indicates the translation initiation point, while the *arrow* pointing *down* marks its end. The various *colors* are a diagrammatic representation of the part of the protein coded by the exon. *CRD* cystine-rich domain, *Ig-C2* immunoglobulin-C2 domain, *JM* juxtamembrane, *LR* leucine-rich domain, *TK* tyrosine kinase domain, *TM* transmembrane domain

acid insert in its extracellular domain. This insert does not affect the affinity of TrkA for NGF or its signal transduction properties [113]. However, it may affect the selectivity of TrkA for NT-3, since NT-3 preferentially activates the form of TrkA with this insert. [16] The isoform TrkA-III, induced in conditions of hypoxia, was only recently discovered. This isoform may contribute to the aggressiveness of neuroblastomas [65] and may be involved in the development of thymocytes [114].

TrkB

There are three major isoforms of TrkB in humans, which are described in Table 17.3 and stem from alternative splicing of the mRNA of the gene NTRK2 (Fig. 17.5). They lead to the formation of full-length TrkB (TrkB-FL), TrkB-T1, and TrkB-T-shc.

Table 17.3 Characteristics of the isoforms of TrkB

	mRNA			Protein	
Isoform	NCBI no.	Exons	Size	Uniprot no.	Size
TrkB-FL	NM_006180.3	21	5,560	Q16620-1	838
TrkB-T1	NM_001007097.1	13	7,111	Q16620-2	477
TrkB-T-shc	NM_001018066.2	14	8,292	Q16620-3	537

The three isoforms of TrkB with their NCBI transcript accession numbers, the number of exons and their size in bp, as well as the Uniprot accession number of the proteins that they code and their size in amino acids

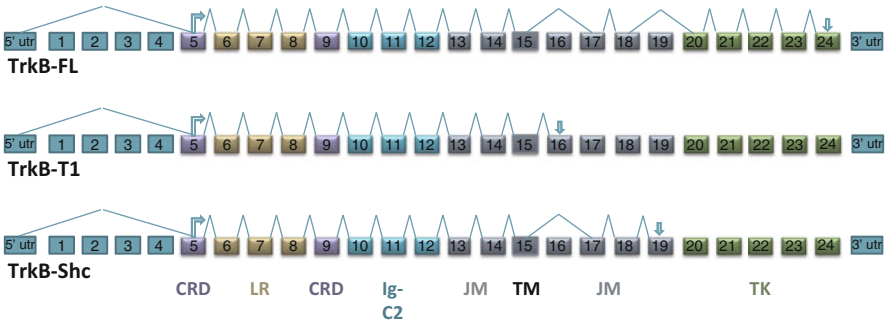

Fig. 17.5 Isoforms of TrkB. The *squares* represent the 24 exons of the gene for TrkB; the *rectangles* represent the 5′ and 3′ utr. TrkB-FL is the longest isoform, while TrkB-T1 is the shortest, being devoid of a large part of its intracellular domain (kinase domain and interaction sites with Shc and FRS-2 and with PLCγ). TrkB-Shc is of intermediate size and still has its binding site for the signaling proteins Shc and FRS-2. The *arrow* pointing *right* indicates the translation initiation point, while the *arrow* pointing *down* marks the end. *CRD* cystine-rich domain, *Ig-C2* immunoglobulin-C2 domain, *JM* juxtamembrane, *LR* leucine-rich domain, *TK* tyrosine kinase domain, *TM* transmembrane domain

The isoform TrkB-T1 has been described in humans and is conserved in evolution since rats and mice share the intracellular region [115]. It is expressed ubiquitously, being present predominantly in the bone marrow [116], brain, heart, skeletal muscles, lungs, kidneys, and pancreas [117]. TrkB-T1 is missing almost all of its intracellular part, but its extracellular domain remains intact [118]. The role played by TrkB-T1 is not clear; it may enable astrocytes to regulate the level of BDNF in the neuronal microenvironment by sequestering and internalizing it [288]; it may be a dominant negative protein, inhibiting the autophosphorylation of TrkB-FL [119]; and/or it may mediate particular cell signaling despite the small size of its intracellular fragment [120].

Like TrKB-T1, the cytoplasmic portion of TrkB-T-shc does not have the kinase domain, but, contrary to TrKB-T1, this isoform still possesses the binding site for the protein Shc. The protein produced is a membrane-bound protein, located exclusively in the brain [117]. Its expression varies in the same way as TrkB-FL and its biological activity is yet to be investigated [121].

TrkC

As with the two other Trk receptors, TrkC has several isoforms, the characteristics of which are summarized in Table 17.4.

The human TrkC transcript is subject to various alternative-splicing events leading to four isoforms (Fig. 17.6). One of these isoforms, TrkC-A, compared to the isoform of normal TrkC (TrkC-C), has a 14-amino acid insert located in the kinase domain, inhibiting the recruitment of the proteins Shc and PLCγ [122, 123]. Another isoform, TrkC-D, also has this 14-amino acid insert, but loses nine residues

Table 17.4 Characteristics of the isoforms of TrkC

	mRNA			Protein	
Isoform	NCBI no.	Exons	Size	Uniprot no.	Size
TrkC-A	NM_001012338.1	19	2860	Q16288-1	839
TrkC-B	NM_001007156.1	15	3997	Q16288-2	612
TrkC-C	NM_002530.2	18	2818	Q16288-3	825
TrkC-D	[115]	18		Q16288-4	830

The three isoforms of TrkC with their NCBI transcript accession numbers, the number of exons and their size in bp, as well as the Uniprot accession number for the proteins that they code and their size in amino acids

Fig. 17.6 The isoforms of TrkC. The *squares* represent the 21 exons of the gene for TrkC; the *rectangles* represent the 5′ and 3′ utr. TrkC-C is the isoform of normal TrkC. TrkC-A and TrkC-D have a 14-amino acid insert coded by exon 20 in their kinase domain, preventing Shc, FRS-2, and PLCγ recruitment. Compared to TrkC-D, TrkC-A has a 9-amino acid insert coded by exon 10, responsible for greater affinity for NT-3. TrkC-B is the shortest form, being devoid of its kinase domain and its interaction site with PLCγ. The *arrow* pointing *right* indicates the translation initiation point, while the *arrow* pointing *down* marks its end. *CRD* cystine-rich domain, *Ig-C2* immunoglobulin-C2 domain, *JM* juxtamembrane, *LR* leucine-rich domain, *TK* tyrosine kinase domain, *TM* transmembrane domain

from its juxtamembrane extracellular domain at the exact location of the insert described for TrkA [115]. Finally, a non-catalytic form of TrkC, TrkC-B, has been described; it is devoid of the tyrosine kinase domain but has an alternative 83-amino acid intracellular domain instead [115]. Mice under-expressing this truncated receptor show a loss of sensory neurons and display cardiac defects similar to those observed in NT-3 knockout mice, suggesting that this receptor has a dominant negative-type inhibitory activity [124]. Moreover, this inhibitory effect is found in neuroblastoma cells where the normal TrkC receptor is expressed in differentiated cancerous cells and the truncated isoform is correlated with more aggressive cancers [69].

17.4.2.4 Trks at a Glance

See Tables "Receptor at a glance*: TrkA—NTRK1*", "Receptor at a glance*: TrkB—NTRK2*", "Receptor at a glance*: TrkC—NTRK3"*.

17.4.3 Trk Ligands (Neurotrophins)

NGF, the first growth factor to be described and the eponym of this class of regulators, was identified in the early 1950s [24] and subsequently characterized, primarily from material isolated from the mouse submandibular gland [125], including sequence analysis (Angeletti and Bradshaw 1971) and three-dimensional structure [126] The other members of the neurotrophin family were discovered subsequently; BDNF was purified in 1982 from pig brain tissue [127], and NT-3 [128–131] and 4/5 [132–134] were subsequently identified by cloning techniques. Although it was known that pro-forms of each neurotrophin existed from their gene structures, it was not until the early 2000s that the pro-neurotrophins were "rediscovered" as entities with functions other than simply being precursors of the mature forms. Lee and his colleagues demonstrated that pro-neurotrophins induced apoptosis, while neurotrophins interacted with $p75^{NTR}$ and Trks to promote survival and neuronal differentiation [135]. In 2004, sortilin was identified as a third receptor that participated in neurotrophin/pro-neurotrophin activities [136] (see below).

The neurotrophin gene family probably stems from the duplication of just one ancestral gene ([289, [86]) that has formed the four modern genes that in humans are now distributed on chromosomes 1, 11, 12, and 19 for the NGF, BDNF, NT-3, and NT-4/5 gene, respectively. All four neurotrophin gene products are translated from just one coding exon, into similar sized precursors with high sequence identity (Table 17.5).

17.4.3.1 Gene Products

The gene products of the neurotrophin family are synthesized as pre-pro-neurotrophins that undergo similar proteolytic processing (Fig. 17.7). As exported proteins, they are separated from their signal peptide (pre-peptide) in the endoplasmic reticulum by

Table 17.5 Characteristics of pro-neurotrophins and their genes

Gene name	Location	Exon(s)	mRNA NCBI no.	Exon(s)	Protein Uniprot no.	Size	Homology
NGF	1p13, 1	3	NM_002506.2	3	P01138	241	84
BDNF	11p13	11	NM_170735.5	1	P23560	247	83
NTF3	12p13	2	NM_002527.4	1	P20783	257	91
NTF4/5	19q13, 3	2	NM_006179.4	2	P34130	210	82

NGF, BDNF, NT3, and NT4/5 genes, their chromosomal location, and the number of exons they have. NCBI accession number for the major transcript with the number of exons. Uniprot accession number of the pre-pro-neurotrophin that it codes followed by its size in amino acids and its family peptide sequence homology percentage

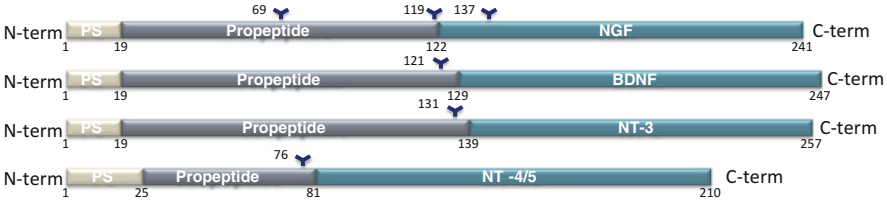

Fig. 17.7 Schematic representation of the pre-pro-neurotrophin family. After transcription and translation, pro-neurotrophin genes produce pre-pro-neurotrophins possessing a signal peptide (PS, *gray*), a pro-peptide (*purple*), and the mature protein (*blue*). The signal peptide (18–24 amino acids) is cleaved after protein sequestration in the ER/Golgi. The neurotrophin is obtained after the pro-peptide is cleaved by specific enzymes (furin, convertase, plasmin, and metalloproteases). The **Y** symbols represent potential N-glycosylation sites on pro-neurotrophins produced in large quantities during its exogenous expression [137]. They have also been observed in the media of certain cancer cells

signal peptidase. The pro-neurotrophins can then be cleaved at a dibasic amino acid site by intracellular proteases such as furin or proconvertases ([290, 291]) or, after secretion, by extracellular proteases such as plasmin and some metalloproteases (MMP-3 and MMP-7) resulting in the corresponding mature neurotrophins [135, 138].

17.4.3.2 Pro-neurotrophin Structure

The neurotrophins all share the same main chain folding as NGF. Each neurotrophin monomer is composed of three variable loops (V1–V3), four ß-sheets (β1–β4), and six conserved half-cystines that are paired in the same fashion. These disulfide bridges constitute a "cystine knot," which, along with the ß-sheets, makes them rigid and with an elongated shape [126]. The neurotrophins are members of a super family of cystine knot proteins that includes the transforming growth factor-β (TGFβ) family, the platelet-derived growth factor (PDGF) family and the human chorionic gonadotropin [139, 140]. Although these are all ligands for membrane-bound receptors, except for the PDGF family, they have evolved to utilize different receptor classes than the receptor

tyrosine kinases. In solution, neurotrophins exist as homodimers bound together non-covalently, where two protomers are mirror images of each other [126]. When isolated, the released pro-peptide is monomeric in solution; it therefore does not appear to contribute to dimerization; rather it is the contacts between the β-sheets of the mature neurotrophins that form and stabilize the dimer interface [141, 142]. Pro-neurotrophin heterodimerization was shown to be possible in vitro [143, 144], but these synthetic isoforms are less stable and readily revert to their respective homodimers [145]. Moreover, the biological role of pro-neurotrophin heterodimers in vivo and, in fact, their very existence remain uncertain.

Although it does not appear to contribute to dimerization directly, the pro-peptide does play a crucial role in the acquisition of pro-neurotrophin conformation. The pro-peptide of proNGF facilitates the establishment of disulfide bridges [146–148] but lacks a stable conformation alone in solution; however, when associated with NGF, it adopts a more stable conformation [141]. Thus, the pro-peptide and mature NGF stabilize each other. This interaction within proNGF involves the residues W19 to A40 of the pro-peptide and the tryptophan at position 21 of NGF (or position 142 of proNGF); the pro-peptide masks this tryptophan, which is involved in the interaction of NGF with TrkA, and may indicate why proNGF is a poor ligand for TrkA [142, 149].

Although modeling does not provide an explanation for why pro-neurotrophins bind with more affinity to $p75^{NTR}$ than neurotrophins do, it may be that the pro-peptide causes a structural change to the mature portion of the molecule, increasing its affinity for this receptor. Moreover, this hypothesis is in line with the observations made by Nykjaer and his colleagues, who showed that the pro-peptide of NGF does not bind to $p75^{NTR}$ itself. [136] In contrast, sortilin binds pro-neurotrophins by their pro-peptide [136, 150]. It thus appears that pro-neurotrophins and neurotrophins have unique conformations, which allow them to interact differently with Trk, $p75^{NTR}$, and sortilin.

The secretion of pro-neurotrophins as physiologically relevant entities has long been the subject of debate [151]. Nonetheless, it appears that proNGF is the NGF gene product form primarily found in numerous organs [152] Furthermore, proNGF is secreted by numerous cells such as cortical neurons [153], sympathetic neurons [154], microglial cells, [155] and astrocytes [156]; its in vivo secretion has also been demonstrated [82]. Similarly, proBDNF is secreted by B lymphocytes [157] and cortical neurons [158] and in vivo in the brain [138]. It is not well understood under what conditions the precursor is extensively if not completely processed to the mature form and under what conditions the pro-form becomes the dominant species.

17.4.3.3 Receptor Interactions with Neurotrophins and Their Precursors

Chimeric constructions of the Trk receptors have shown that the immunoglobulin-like juxtamembrane domain (Ig-C2) is vital to ligand binding ([292, 293]), although the Ig-C1 domain is also important [101]. Structural analyses of these domains from the various Trks and of their ligands [126, 137, 159, 160], as well as the structure of

NGF associated with TrkA binding domains [12, 161] have identified the amino acids involved in complex formation. A "conserved patch" is found in the C-terminus of the Trk Ig-C2 domains and in the central zone of the neurotrophins. This "conserved patch" contains 8 of 15 identical residues in the three Trk receptors and 14 of 23 identical residues in the four neurotrophins (among these 14 residues, W21 is vital to the specific binding between TrkA and proNGF). The second region, or "specificity patch," is located in the N-terminus of the neurotrophins; it is characteristic to each of the neurotrophins and constitutes the main determining element of binding specificity. NT-4/5 and BDNF share 5 of the 7 residues of the specificity patch, explaining their affinity for the same receptor (TrkB).

In addition to the "specificity patch," it has been shown that a small insert in the juxtamembrane region of the extracellular domain of the Trks is also responsible for the specificity of the various receptors for their ligands. Thus, the isoform TrkA-II, which has only nine residues more than TrkA-1, can bind to NT-3 in addition to NGF. [16, 162] Similarly, an isoform of TrkB without such an insert only binds to BDNF, whereas if it has this insert, it can also bind to NT-4/5 and NT-3 [163, 164].

Binding of the pro-neurotrophins to the Trk receptors is somewhat controversial. It has been shown that proNGF induces the death of sympathetic neurons in the superior cervical ganglion (SCG), does not activate TrkA, and does not induce neurite outgrowth in PC12 cells, the opposite effect of NGF in every case [135]. In contrast, Fahnestock et al. [165] demonstrated that proNGF is co-immunoprecipitated with TrkA and that it does induce neuritogenesis of SCG (sympathetic) neurons, although less effectively than mature NGF. Furthermore, Nykjaer et al. showed that TrkA binds to proNGF but with ten times less affinity than NGF (K_d of 20 nM for proNGF, compared to 2 nM for NGF) [136]. Nonetheless, the same authors observed that proNGF leads to apoptosis, whereas NGF increases survival of neuronal SCG cells. In breast cancer cells, proNGF has been shown to activate TrkA phosphorylation via interaction with sortilin [166].

Some clarification of these conflicting results was provided by the work of Al-Shawi et al. [167], who showed that SCG neurons from young mice respond to proNGF by increased survival and neuritogenesis but that SCG neurons from elderly mice die by apoptosis when they are similarly treated. In contrast, NGF promotes their survival. Furthermore, it was observed that sortilin expression is increased in the elderly population compared to the neurons from young individuals, although the amount of $p75^{NTR}$ does not vary. Similarly Masoudi and his colleagues [168] showed that proNGF can induce phosphorylation of TrkA and underlying MAPKs, leading to survival and differentiation of PC12 cells, although to a lesser extent than NGF. However, they also noted that in a PC12 subpopulation, namely, unprimed PC12 cells (which die by apoptosis when the serum is removed from their culture medium), proNGF induces apoptosis whereas NGF rescues them. It was reported that the $p75^{NTR}$/TrkA ratio is increased for unprimed PC12 cells compared to the other PC12 cells. Finally, advances in the structural characterization of the pro-neurotrophins indicated that the pro-peptide of proNGF reduces its association with TrkA compared to NGF. In particular, the pro-peptide masks the tryptophan in position 142 on proNGF (or in position 21 on mature NGF), which is part of the

"conserved patch" (see above) enabling the interaction between the neurotrophins and the Trk receptors [142, 149, 161].

In summary, proNGF is a weak ligand for TrkA, and the level of expression of the various receptors (TrkA, $p75^{NTR}$ and sortilin; see below) directs the cells to the same pathway as NGF (survival and neuritogenesis) when TrkA is abundant, but less effectively, or even to an opposing pathway (apoptosis) when $p75^{NTR}$ and/or sortilin dominate. These observations could therefore explain why pro-neurotrophins are reported to be neurotrophic or neurotoxic, depending on the study.

17.4.4 Trk Activation and Signaling

17.4.4.1 Dimerization and Activation

It is well established that RTKs as a class are activated as the result of the phosphorylation of a varying number of tyrosine residues occurring in the receptor endodomain. Furthermore, it is generally accepted that these modifications result from autocatalytic events carried out in trans by the kinases of the receptor protomers. The initial hypothesis, put forward by Ullrich and Schlessinger [169], called for the dimerization of receptor monomers, induced by the germane ligand binding, which brought the kinase domains into appropriate juxtaposition to carry out these reactions. The phosphorylations involved tyrosines in the activation loop as well as other locations on the kinase and in the flanking sequences. These latter sites then became docking sites for various effector, scaffold, or adaptor proteins [170] that in turn were modified in the course of transmitting and amplifying the induced signals. Subsequent observations have suggested that not all of the modifications are necessarily autocatalytic, i.e., some may be caused by soluble kinases like Src, and the activation loop tyrosines may act in some cases as docking sites. A greater challenge to this model was suggested initially by Gadella and Jovin [171], who hypothesized that dimerization was constitutive, i.e., preformed, and that ligand activation was induced by a rotational (or other) conformational change that effectively released the kinase domains of the receptor from an inactive or inhibited state. This is usually explained by a movement of the activation loop from its closed conformation to an open one, followed by the phosphorylation of the activation loop tyrosines, an event that effectively freezes them in this form. Support for this concept came from a number of biophysical measurements and from observations from other receptor systems, such as cytokine and G-protein receptors, that supported the view that the existence of pre-existing oligomers was a widespread phenomenon [172]. Evidence has mounted in support of both models and it has not been definitively resolved. In the case of the Trks, evidence to support the constitutive model has been reported [173–175], and it seems clear that at least a portion of the Trk receptors do exist in the absence of ligand in a dimerized state. However, it has also been argued that the very tight association of the neurotrophin (or pro-neurotrophin) dimer would certainly facilitate receptor dimerization [176].

17.4.4.2 Downstream Pathways

The principal docking sites on the endodomain of TrkA are Y490 and Y785 (Fig. 17.2). Mutation of these two sites to phenylalanines eliminates essentially all of the neurite proliferation activity as measured with PDGFR/TrkA chimeras stably transfected into PC12 cells [177], a rat pheochromocytoma cell line [178]. Most signaling studies with TrkA have been conducted with this cell line. Downstream phosphoproteomic analyses of TrkA in PC12 cells 20 min after stimulation documented over 4,000 sites of phosphorylation on serine and threonine residues [179]. When derivatives containing Y490F and Y490/785 F were analyzed at the same time point, several classes of responses were observed, the most interesting of which were phosphorylations that were not affected by eliminating both Y490 and Y785 [107]. The most prominent entity identified in this group of proteins was Nck. It has been reported that overexpression of Nck in PC12 cells caused continued proliferation even in the presence of NGF and inhibited neurite outgrowth [180] suggesting its involvement in signaling. Both Nck and a related molecule Nck2 has been reported to be activated by TrkB [181]. The site(s) of these activations has not been identified; both the activation loop tyrosines and Y751 have been implicated.

Among the signaling pathways activated by the Trk receptors in response to neurotrophins, the MAPK, PI3K, and PLCγ-PKC pathways have been the most extensively described (Fig. 17.8) [182]. The signaling of the various Trk receptors has mostly been extrapolated from that of TrkA [183].

Mitogen-Activated Protein Kinases

The activation of mitogen-activated protein kinases (MAPKs) by TrkA can be either of a transitory or prolonged nature, and the physiological consequences are significantly different. In PC12 cells, transitory activation of RTKs will not produce sustained neurite outgrowth; the EGF receptor that is endogenously expressed in these cells will rapidly activate the MAPKs but does not lead to neurite growth because the activation is not sustained unless it, or its expression, has been modified [184].

Transitory activation of MAPKs depends on the activation of the GTPase Ras [185]. The first proof that Ras was involved in this pathway appeared in 1986. Hagag and his colleagues showed that microinjections of antibodies directed against Ras into PC12 cells caused delay in neurite growth [186]. Ras switches between an active state, where it is bound to GTP, and an inactive state, where it is bound to GDP; this switch is facilitated by guanine nucleotide exchange factors (GEFs) and guanosine triphosphatases activating proteins (GAPs).

Phosphorylated Y490 is recognized by the phosphotyrosine-binding (PTB) domain of the protein Src homology 2 domain-containing-transforming protein C1 (Shc) [187], which enables recruitment of the protein growth factor receptor-bound protein 2 (Grb2) [188]. As Grb2 is complexed to the GEF son of sevenless (SOS), its recruitment to TrkA results in the activation of Ras via GTP binding [189]. Activation of proteins from the Raf family (Raf-1 and B-Raf) results from the

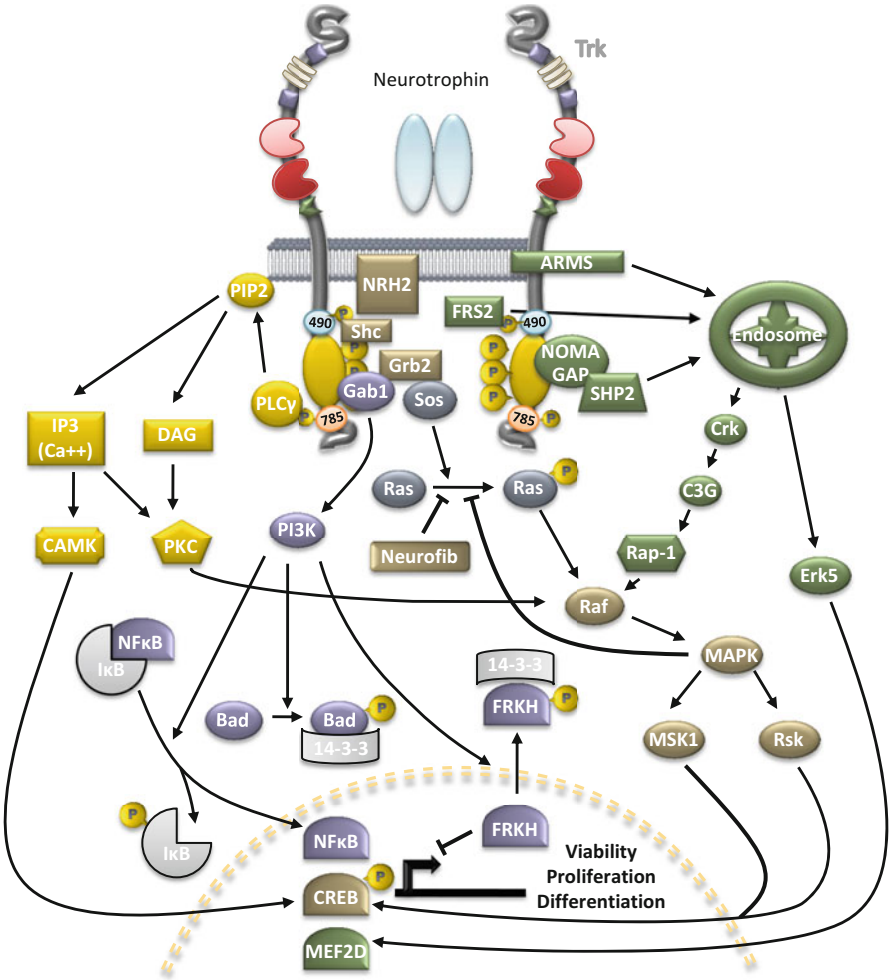

Fig. 17.8 Diagrammatic representation of signaling pathways activated by Trk receptors. Activation of Trk receptors leads to activation of MAPKs, PI3K, and PLCγ-PKC signaling pathways, resulting in neuron survival, proliferation, and differentiation. *ARMS* ankyrin repeat-rich membrane spanning, *Bad* BCL2 antagonist of cell death, *C3G* Crk SH3-domain-binding guanine-nucleotide releasing factor, *CAMK* Ca^{2+}-calmodulin-regulated kinase, *CREB* cAMP response element binding, *DAG* diacyl glycerol, *Erk5* extracellular signal-regulated kinase 5, *FRKH* forkhead, *FRS2* FGF receptor substrate 2, *Gab1* Grb2-associated binding protein 1, *Grb2* growth factor receptor-bound protein 2, *IP3* inositol 1,4,5-trisphosphate, *IκB* inhibitor of κB, *MAPK* mitogen-activated protein kinases, *MEF2D* MADS box transcription enhancer factor 2 polypeptide D, *MSK1* mitogen- and stress-activated protein kinase-1, *Neurofib* neurofibromin, *NF-κB* nuclear factor κappa B, *NOMA-GAP* neurite outgrowth multi-adaptor guanosine triphosphatases activating protein, *NRH2* neurotrophin receptor homolog 2, *PI3K* phosphoinositide 3 kinase, *PIP2* phosphatidylinositol 4,5 bisphosphate/Akt, *PKC* protein kinase C, *PLCγ* phospholipase C γ, *Rsk* ribosomal protein S6 kinase, *Shc* Src homology 2 domain-containing-transforming protein C1, *SHP2* Src homology 2-containing tyrosine phosphatase 2, *Sos* son of sevenless

activation of Ras and leads to the activation of MAPKs [190, 191]. The protein neurofibromin, the GAP of Ras, appears to be involved in inhibiting this pathway [192]. Furthermore, negative feedback from the pathway itself has also been shown, since MAPK activation leads to the phosphorylation of the GEF SOS, which dissociates the Grb2-SOS complex. [193] Activated MAPKs lead to the recruitment of the effector kinases ribosomal protein S6 kinase (Rsk) and mitogen- and stress-activated protein kinase-1 (MSK1), which then phosphorylate the transcription factor cAMP response element binding (CREB) [194, 195]. CREB then stimulates the transcription of genes involved in neuronal growth and differentiation [196]. It has also been shown that neurotrophin receptor homolog 2 (NRH2) is vital to the activation of Shc and to the MAPKs [197].

Prolonged MAPK activation by neurotrophins involves Crk adaptors, the GEF Crk SH3-domain-binding guanine-nucleotide releasing factor (C3G), and the small GTPases Rap-1 and B-Raf. Activation of TrkA by NGF leads to activation of C3G by Crk, which then activates Rap-1. Activated Rap-1 then forms a complex with B-Raf, which results in prolonged MAPK activation [198]. This pathway requires TrkA endocytosis [199, 200]. The inaugural phenomenon leading to the activation of Crk remains unclear [201]. It has been suggested that recruitment of the adaptor Crk to the receptor takes place via the scaffold protein FGF receptor substrate-2 (FRS-2) [193], whereas others believe that prolonged MAPK activation may not involve FRS-2 [199, 202]. However, the protein ankyrin repeat-rich membrane spanning (ARMS) apparently plays a vital role since it binds to Trk receptors by transmembrane interactions [203], it is phosphorylated for hours in response to neurotrophin treatment [204], and, when it is phosphorylated on its tyrosine Y1096, it recruits Crk [109, 110]. Thus, ARMS may enable Crk to be recruited to TrkA. The protein tyrosine phosphatase Src homology 2-containing tyrosine phosphatase 2 (SHP2) also seems to be essential for prolonged MAPK activation [205]. It is recruited to the receptor via the adaptor NOMA-GAP and enables long-term extracellular signal-regulated kinase 5 (ERK5) activation after receptor endocytosis [206]. Activation of ERK5 in turn activates the transcription factor MADS box transcription enhancer factor 2 polypeptide D (MEF2D), leading to expression of the antiapoptotic protein bcl-w, which enables neuron survival [207].

Phosphoinositide-3-Kinase

Neurotrophins play an important role in the survival of numerous types of neurons, and the PI3K pathway is essential to this function. The Shc/Grb2 complex, in addition to activating MAPKs, can also lead to the activation of PI3K. In this pathway, Gab1 is recruited by Grb2, and it has been shown that the association of the protein Gab1 and PI3K is vital since the expression of Gab1 with a mutation in its PI3K binding site reduces the NGF survival effect [208]. However, PI3K can also be activated at the alternative site, Y751, although how this relates to the activation at Y490 is not really known.

The effectors of the survival effect mediated by PI3K have been identified as the protein Bad, and the transcription factors forkhead and NFκB. Direct phosphorylation of Bad by PI3K causes phosphorylation by other kinases, leading to its sequestration by 14-3-3 proteins and inhibiting its proapoptotic action [209]. Similarly, forkhead, phosphorylated by PI3K, leaves the nucleus and is sequestrated in the cytoplasm by 14-3-3 proteins, preventing it from activating the transcription of proapoptotic proteins [210–212]. Conversely, NFkB is translocated in the nucleus following phosphorylation of IκB by PI3K; it can then activate the transcription of various antiapoptotic factors [213].

Phospholipase C γ-Protein Kinase C (PLCγ-PKC)

Even before NGF had been identified as the ligand of TrkA, it had already been shown that this later activates the phosphorylation of PLCγ [214]. PLCγ, bound and activated by complexation with Y785 [104, 105], then hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 mediates the release of internal calcium stores in the cytoplasm, which stimulate the isoforms of PKC that are regulated by Ca^{2+} and activate Ca^{2+}-calmodulin-regulated kinases (CAMK); DAG binds and activates isoforms of the C protein kinases. In PC12 cells stimulated by NGF, PKC activates Raf and then MAPKs, which results in neurite growth [215]. High levels of phosphorylated PLCγ are found in the brain of patients suffering from Alzheimer's disease [216]. More recently, Matrone et al. have shown that following long-lasting NGF depletion, TrkA autophosphorylates by means of an as yet unknown mechanism that leads to the phosphorylation of PLCγ, concomitant to the inactivation of Akt, to the segregation of Aβ peptides (molecular markers of Alzheimer's disease) with the receptor, and to neuronal death [217]. As the absence of NGF signaling is considered to be one of the possible causes of Alzheimer's disease [218], it has been suggested that this abnormal phosphorylation of PLCγ via TrkA reflects a major molecular event in this disease.

17.5 Other Neurotrophin Receptors

17.5.1 p75^{NTR} Receptor

17.5.1.1 Discovery/Properties

Early studies on peripheral neurons in primary culture and on PC12 cells showed that NGF bound to both low-affinity (10^{-9} M) and high-affinity (10^{-11} M) sites [219]. When the pan-neurotrophin receptor, $p75^{NTR}$, was independently cloned [220, 221], transfection experiments identified it as being the low-affinity receptor [222]. When the other members of the neurotrophin family were subsequently identified, it was

shown that they all bound to $p75^{NTR}$ with the same affinity [7, 223, 224]. It was not until 2001 that $p75^{NTR}$ was identified as a receptor for pro-neurotrophins with an affinity five times greater (2×10^{-10} M) than that of the mature neurotrophins [135].

The human gene for $p75^{NTR}$ is comprised of 6 exons located on chromosome 17 in the region q12–q22 and covers around 23 kb. This gene is transcribed into a 3.4 kb mRNA with a short noncoding region (≈100 nucleotides) at the 5′-end and a longer noncoding region at the 3′-end (≈2,000 nucleotides), which contains a single consensus signal sequence for polyadenylation [222]. The NGFR promoter sequence is very similar in rats, mice, and humans [225]. It does not have any TATA or CAAT consensus sequences, but conserved sequences rich in GC near to the transcription initiation site, which form a response element for Sp1 transcription factor [226, 227]. Several E-boxes are also present in the NGFR promoter and may bind to the repressor transcription factor ME1 [228] or the activator transcription factor NeuroD [225]. Retinoic acid enables neuronal differentiation by activating NGFR transcription directly because of response elements in the $p75^{NTR}$ promoter and indirectly due to retinoic acid activation of NeuroD synthesis ([229, 294]).

$p75^{NTR}$ is a type I transmembrane receptor composed of 399 amino acids with three domains: extracellular (222 amino acids), transmembrane (22 amino acids), and intracellular (155 amino acids) (Fig. 17.9). It also has a signal peptide (28 amino acids). The extracellular domain has an N-glycosylation site on N31 and is also O-glycosylated in its juxtamembrane domain, increasing its molecular mass somewhat [231]. However, its designation suggesting a mass of 75 kDa does not represent its true mass but rather its apparent size as observed on SDS-gel electrophoresis. It appears that $p75^{NTR}$ glycosylation plays an important role in its conformation, its membrane targeting [232], and its ligand binding [233]. The $p75^{NTR}$ extracellular region also contains four cystine-rich domains (CRDs) that places it in the superfamily of tumor necrosis factor (TNF) receptors [234–236]. These CRDs (numbered from 1 to 4 from the N-terminus) give the receptor an elongated conformation due to three disulfide bridges in each CRD [11, 237]. The transmembrane domain is formed of a single 22-amino acid transmembrane helix in which the C257 is well; it plays a key role in signal transduction [238]. Finally, the intracellular domain does not contain any obvious effector domain, comparable to the kinase domains of the Trks. It has a palmitoylation site at C279 [239], which seems to play a role in the regulation of $p75^{NTR}$ cleavage [240, 241]. The receptor is also phosphorylated on serine and threonine residues [242, 243], which may contribute to its aggregation and its location in the lipid rafts [242, 244]. The function of these posttranslational modifications has not been fully clarified; they could also play a role in protein–protein interactions, endocytosis, vesicular transportation, and preferential signaling in certain cell compartments [241, 242, 245, 246].

One of the main characteristics of the intracellular domain of $p75^{NTR}$ is, as with the other members of the TNF receptor family, the presence of a death domain (DD) [234] (Fig. 17.9). Based on sequence analyses, this DD is classified as a type II DD protein [230]. The DD is a compact globular structure made up of around 80 amino acids, containing two perpendicular bundles each with three α helices, and enabling the interaction with the adaptor proteins responsible for activating the caspase

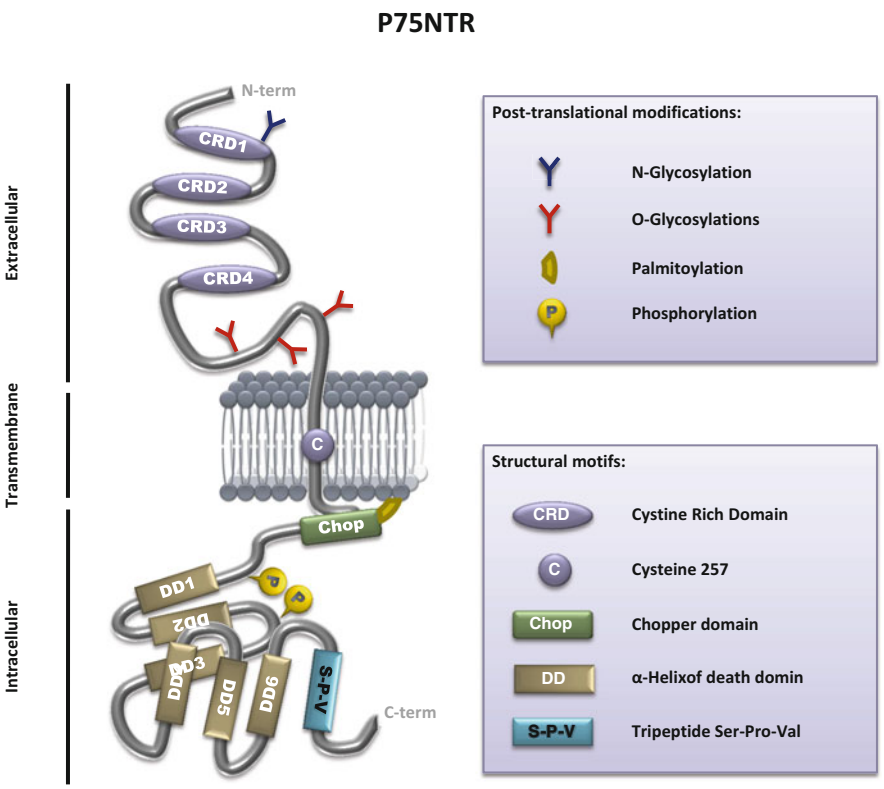

Fig. 17.9 Diagrammatic representation of the p75^{NTR} receptor. The p75^{NTR} receptor is composed of an extracellular domain with four cystine-rich domains (CRD), a single transmembrane domain containing a conserved cysteine, and an intracellular domain including a juxtamembrane Chopper domain, a death domain (DD), and the tripeptide SPV, a binding consensus site for PDZ-domain proteins. It contains several posttranslational modifications such as N- and O-glycosylations, palmitoylation, and phosphorylations [230]

pathway and inducing apoptosis. The p75^{NTR} DD may be phosphorylated on two tyrosine residues, Y337 and Y366, after binding to its ligands. The phosphorylated receptor may then regulate the activity of the GTPase Rho A (Ras homolog gene family, member A) and play a role in neurite growth [247, 248]. p75^{NTR} also possesses a small 29 amino acid domain, located in its intracellular juxtamembrane region called "Chopper" (for a famous Australian criminal) because it was shown to be necessary and sufficient to initiate neuronal death. The Chopper domain is not conserved in the other members of the TNFR family [249]. Lastly, the tripeptide Ser-Pro-Val, which is a consensus binding site for proteins with PDZ (postsynaptic disc-large zona) domains [250], is located at the C-terminus.

Via both alternative splicing and proteolysis, the p75^{NTR} receptor can exhibit different isoforms. Alternative splicing of exon 3 of the p75^{NTR} gene can generate an isoform without CRDs 2, 3, or 4 in its ectodomain, which is unable to bind to any

known ligand [251]. The intracellular and transmembrane domains nevertheless remain intact and functional, although the biological function of this truncated $p75^{NTR}$ remains unknown. The full-length $p75^{NTR}$ receptor can be cleaved by the extracellular metalloproteases ADAM (a disintegrin and metalloprotease) 10 or 17, releasing an extracellular fragment composed of four CRDs. This fragment, called $p75^{NTR}$-ECD (extracellular domain), is soluble and is still able to bind the neurotrophins. The other half of the receptor, including the transmembrane and intracellular domains, termed CTF (for C terminal fragment), undergoes a second cleavage by an intracellular γ-secretase complex, PS (presenilin-dependent γ-secretase). TRAF6 is essential for this step because it causes receptor ubiquitination and PS recruitment; an active intracellular fragment known as $p75^{NTR}$-ICD (intracellular domain) is generated. The complex formed in this way leads to the activation of the transcription factor NFκB via degradation of its inhibitor IκB [252, 253]; this fragment has also been identified in cell nuclei, suggesting a direct transcriptional regulator role [254].

$P75^{NTR}$ binds both neurotrophins and pro-neurotrophins but shows a greater affinity for the latter. An initial model suggested a $p75^{NTR}$–ligand interaction with an asymmetrical 2:1 ratio where a homodimeric ligand bound to a monomeric $p75^{NTR}$ molecule [255]. However, a different structure of the extracellular domain of an N-glycosylated $p75^{NTR}$ clearly showed a 2:2 $p75^{NTR}$–ligand complex ratio [256]. The differences observed between these studies suggest that in addition to CRDs, $p75^{NTR}$ glycosylation plays a key role in its ligand binding. The dimeric structure of $p75^{NTR}$ is supported by the observation that a disulfide bridge forms between two $p75^{NTR}$ monomers at the conserved C257 in the transmembrane domain, without which the receptor cannot be activated by pro-neurotrophins [238].

17.5.1.2 Signaling Pathways Activated by $p75^{NTR}$

As with the other members of the TNF receptor family, $p75^{NTR}$ is devoid of intrinsic catalytic activity. Consequently, $p75^{NTR}$ signaling takes place via the recruitment of intracellular adaptor proteins, leading to the activation of various signaling pathways, which have mainly been established in neuronal models and in PC12 cells. They lead predominantly to survival, cell death via apoptosis, neurite elongation, cycle progression, and migration, depending on the cellular context.

When neurotrophins bind to the $p75^{NTR}$ receptor, they activate the transcription factor NFκB, leading to cell survival [257]. This pathway involves the phosphorylation IκB releasing NFκB and enabling its nuclear translocation and the activation of genes involved in cell survival [253]. Various adaptors are involved in this activation of NFκB by $p75^{NTR}$ including TRAF proteins (TRAF2 promotes apoptosis, TRAF6 enables survival, but TRAF4 is without effect), which are trimeric proteins that bind the TNF receptor superfamily on signals from JNK and NFκB [230, 258]; RIP2, a serine/threonine kinase with a CARD (caspase recruitment domain), enabling it to bind to the $p75^{NTR}$ death domain; FAP1 (Fas-associated protein 1), a protein tyrosine phosphatase (PTP) that is known to interact with the Fas receptor and to inhibit the

proapoptotic signals that it mediates [234, 259]; TRADD (TNF receptor associated via death domain), an adaptor from the TNF receptor family, which leads to caspase activation (although it described as not being involved in $p75^{NTR}$ signaling in neurons) [230, 260, 261]; and NRH2 (neurotrophin receptor homolog 2), a protein, which, as its name suggests, is very similar to the $p75^{NTR}$ receptor. NRH2 has a type two DD, but unlike $p75^{NTR}$, it cannot bind to pro- or mature neurotrophins because its extracellular domain is too short. It has recently been shown that it binds to Trk receptors and that this interaction enables the creation of a high-affinity binding site and leads to signaling that allows neuronal survival [197, 262].

The cell death induced by $p75^{NTR}$ depends both on the Chopper domain [249] and on the its DD [261]. Apoptosis is triggered by the phosphorylation of JNK that activates the transcription factors c-jun and p53, BH3 domain proteins (Bcl-2 homology), and the mitochondrial translocation of Bax. This is followed by cytochrome c release, which in turn stimulates caspases 9, 6, and 3 [263]. Adaptors that induce $p75^{NTR}$-dependent apoptosis included NRIF (neurotrophin-receptor-interacting factor), a primarily nuclear zinc finger protein [264]; NRAGE (neurotrophin-receptor-interacting MAGE homolog), which binds to the juxtamembrane domain [265]; NADE (neurotrophin-associated cell death executor) that induces 14-3-3ε protein-dependent cell death [266, 267]; and NRH2 that enables $p75^{NTR}$ to interact with sortilin, an association essential to transduction of pro-neurotrophin proapoptotic signals [268].

In parallel with the pathways controlling neuronal survival and death, signal transduction by $p75^{NTR}$ can also involve the "ceramide synthesis" pathway. Activation of $p75^{NTR}$ stimulates sphingomyelinases, hydrolyzing sphingomyelins and leading to ceramide release [269] that is correlated with activation of the protein JNK and with nerve cell apoptosis [270].

The $p75^{NTR}$ receptor is also known to modulate cell cycle progression; it recruits various adaptors involved in stopping the cell cycle including Sc1, a zinc finger transcriptional repressor protein [271], and Sall2, a recently described transcription factor that interacts directly with $p75^{NTR}$ and is involved in cell cycle progression and neurite growth.

17.5.2 Sortilin

Sortilin is also a receptor shared by the pro-neurotrophins. It was discovered almost simultaneously at the end of the 1990s by three different laboratories that were pursuing quite different objectives. Hence, it was identified with three quite different names: sortilin, NTSR3 (for neurotensin receptor 3), and GP110 (for *glyco*protein 110). Sortilin was reported as a 100 kDa protein that showed great affinity for the protein RAP (receptor-associated protein)—an LDL receptor chaperone [272]. NTSR3 was discovered by Mazella et al. [273] while purifying neurotensin receptors (NTR1 and 2) in mice and newborn human brains, as a 100 kDa protein that bound to neurotensin on an affinity column. Sequence analysis showed it to be identical to sortilin.

gp110 was observed as one of a group of four glycoproteins found in vesicles containing the glucose transporter type 4 (GLUT4) following insulin treatment [274]. Cloning of the protein confirmed that gp110 was indeed rat sortilin [275].

In situ hybridization has located the sortilin gene on segment p21.3–p13.1 of chromosome 1 [272]. In silico analysis has shown that it contains 20 exons which are spread over 50 kb [276]. Moreover, northern blots performed in humans indicate that its transcript is present quasi-ubiquitously in numerous tissues [272].

Once released from its signal peptide (33 amino acids), sortilin is an N-glycosylated (N162 and N163) [277, 278], type 1 protein of 798 amino acids with a transmembrane domain (23 amino acids), a short cytosolic tail (53 amino acids), and a long N-terminal portion (722 amino acids) (Fig. 17.10) [279]. Sortilin was

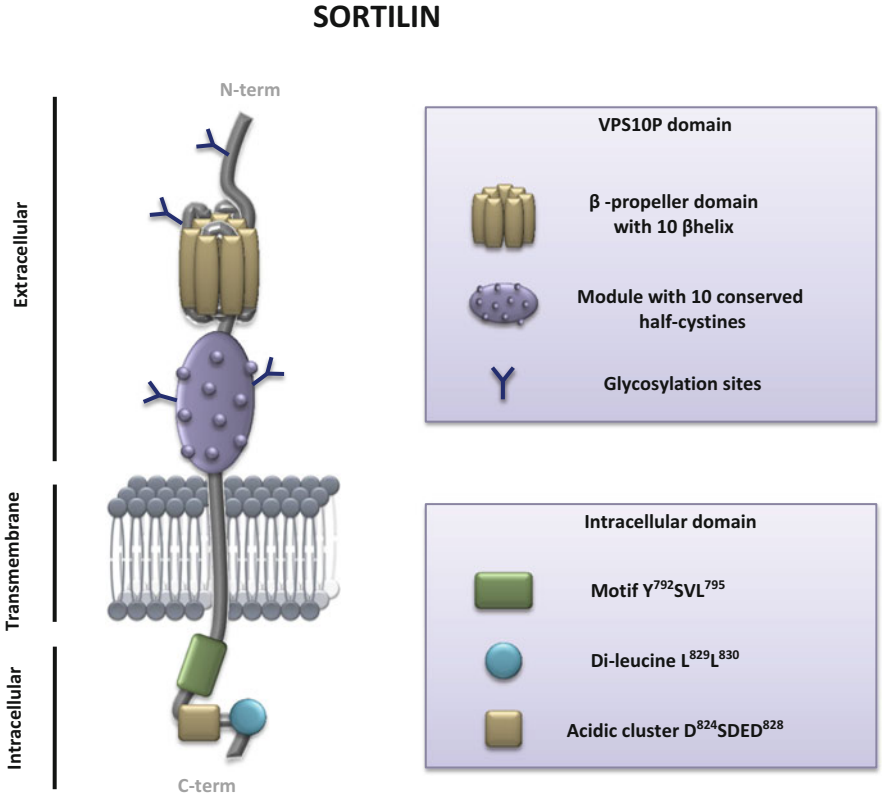

Fig. 17.10 Diagrammatic representation of sortilin. Sortilin is a type 1 glycoprotein with 798 amino acids. Its VPS10P domain (≈700 amino acids) forms almost the whole of its N-terminus. This domain is essential for ligand binding. It is composed of a 10 CC module, where ten conserved half-cystines establish five disulfide bridges, as well as a β propeller domain formed of ten β blades. The C-terminal part is very short with only 53 amino acids. It includes three sequences implicated in its internalization and its intracellular trafficking, namely, the motif YSVL (792–795), the phosphorylatable acidic cluster DSDED (824–828), and the di-leucine L829-L830

initially located in the vesicles of primary neuronal cultures [280]. Similarly, in adipocytes, sortilin is a vesicular protein, which can be translocated to the membrane by insulin [275]. Finally, transfected into CHO cells, it is essentially located in the endoplasmic reticulum and the Golgi apparatus, and only 8 % of sortilin is expressed in the plasma membrane [281].

The extracellular domain, or the VPS10P (vacuolar protein sorting ten protein) domain, was first identified in *Saccharomyces cerevisiae*, where the protein of the same name is a receptor that guides lysosomal enzyme traffic from the Golgi apparatus to the vacuole [282]. In humans it is found in five proteins, namely, sortilin, SORLA (sortilin-related receptor, L [DLR class] A repeats-containing), and SORCS 1-3 (sortilin-related receptor CNS expressed). These proteins are expressed in the central and peripheral nervous systems where they appear to be involved in various pathologies. The VPS10P domain contains a 10 CC (C612–C740) module where ten conserved half-cystines form intramolecular junctions [283] as well as a ten-bladed β-propeller domain. These two regions may be involved in the interaction of sortilin with its ligands [150, 283]. The C-terminal tail has structural characteristics close to the cytosolic tail of the receptor CI-M6P/IGF2R (cation independent–mannose 6-phosphate/insulin growth factor 2 receptor). This receptor is known for its involvement in trans-Golgi intracellular traffic and internalization processes [284]. Like the CI-M6P/IGF2R receptor, it has three characteristic sequences, which are involved in these biological phenomena.

Sortilin interacts with numerous ligands indicating that it is involved in diverse biological phenomena. These include RAP (receptor-associated protein), an intracellular protein residing in the endoplasmic reticulum and the Golgi apparatus that chaperones the class A domains of LDL receptors; neurotensin, a 13-amino acid neuropeptide found that acts via the intermediary of G protein-coupled receptors; lipoprotein lipase, a protein involved in the regulation of plasma lipid levels; and apoA-V (apolipoprotein A-V), a protein that enables the regulation of plasma triglyceride levels and its own pro-peptide [279]. It also binds proNGF and proBDNF via their pro-domains. ProNGF binds to sortilin with high affinity ($K_d = 5$ nM), similar to its pro-peptide ($K_d = 8$ nM), whereas mature NGF has a low affinity for this receptor ($K_d = 87$ nM). Complex formation leads to endocytosis, which depends on the Tyr-Ser-Val-Leu sequence in the cytoplasmic tail, leading to neuronal cell death by apoptosis [136]. Similarly, it has been shown that proBDNF induces neuronal apoptosis via its interaction with sortilin [158]. Besides apoptosis induction, Chen and his colleagues have demonstrated that sortilin plays a role in intracellular proBDNF traffic by directing it to a regulated rather than a constitutive secretory pathway (Chen et al. 2005). Also, using directed mutagenesis of proBDNF followed by co-immunoprecipitation, it has been shown that a region between amino acids 44 and 103 (with V66 playing a key role) is essential for the regulation of proBDNF secretion. A genetic variant of proBDNF (V66M) predisposes individuals to depression and anxiety [295] and mice with this variant show a reduction in the regulated secretion of immunoreactive forms of pro/BDNF [296]. Given that the mutation V66M of proBDNF is needed for its reaction with sortilin, deregulation of proBDNF secretion by sortilin could be the cause of disease [151]. ProNT-4/5 does not bind to

sortilin (Chen et al. 2005); it is not known whether proNT-3 binds to sortilin or not, although given the similarities in its pro-peptide to that of proBDNF, it is likely that it does.

Sortilin recruits various signaling partners, which lead variously to death or migration or which play a role in cell traffic. However, for the most part these are involved with Trk or $p75^{NTR}$ signaling. Sortilin is essential to the apoptosis mediated by pro-neurotrophins [83, 136], but little is known about the molecular actors connecting sortilin to apoptosis. NRH2 appears to be essential to this pathway since it enables intracellular sortilin to be redistributed on the membrane surface [268]. Moreover, NRH2 enables the functional association of $p75^{NTR}$ and sortilin because it binds to $p75^{NTR}$ by its DD and to sortilin by its juxtamembrane domain.

Receptor at a glance*: TrkA—NTRK1*

Chromosome location	1q21q22
Gene size (bp)	66,211
Exon numbers	17
mRNA size (5′, ORF, 3′) (bp)	2,663
Amino acid number	Depending on isoform (See Table 17.2)
Molecular mass (kDa)	~140
Posttranslational modifications	Glycosylation, phosphorylation, ubiquitination
Domains	Extracellular, transmembrane, tyrosine kinase
Ligands	NGF, NTF3
Known dimerizing partners	$P75^{NTR}$, TrkB, TrkC
Pathways activated	MAP kinases, PLCγ, Src, PI3K
Tissues expressed	Broad spectrum of normal/pathological neural and nonneural tissues
Human diseases	Neurodegenerative diseases, cancer
Knockout mouse phenotype	CNS deficit with lack of both nociceptive and superior cervical ganglion neurons

Receptor at a glance*: TrkB—NTRK2*

Chromosome location	9q22.1
Gene Size (bp)	355,040
Exon numbers	24
mRNA size (5′, ORF, 3′)(bp)	2,663
Amino acid number	Depending on isoform (see Table 17.3)
Molecular mass (kDa)	~140
Posttranslational modifications	Glycosylation, phosphorylation, ubiquitination
Domains	Intracellular tyrosine kinase
Ligands	BDNF, NTF4/5
Known dimerizing partners	$P75^{NTR}$, TrkA, TrkC

(continued)

Pathways activated	MAP kinases, PLCγ, Src, PI3K
Tissues expressed	Broad spectrum of normal/pathological neural and nonneural tissues
Human diseases	Neurodegenerative diseases, cancer
Knockout mouse phenotype	CNS deficit with lack of nodose, vestibular, and cochlear neurons; mice display CNS deficits

Receptor at a glance*: TrkC—NTRK3*

Chromosome location	15q25
Gene size (bp)	397,018
Exon numbers	20
mRNA size (5′, ORF, 3′) (bp)	2,818
Amino acid number	Depending on isoform (See Table 17.4)
Molecular mass (kDa)	~140
Post-translational modifications	Glycosylation, phosphorylation
Domains	Intracellular tyrosine kinase
Ligands	NTF3
Known dimerizing partners	$P75^{NTR}$, TrkA, TrkB
Pathways activated	MAP kinases, PLCγ, Src, PI3K
Tissues expressed	CNS, liver, prostate, and breast cancer
Human diseases	Neurodegenerative disease, cancer
Knockout mouse phenotype	CNS deficits with lack of proprioceptive and cochlear neurons; reduction in vestibular neurons

References

1. Martin-Zanca D, Mitra G, Long LK, Barbacid M. Molecular characterization of the human trk oncogene. Cold Spring Harb Symp Quant Biol. 1986;51(Pt 2):983–92.
2. Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science. 1991;252:554–8.
3. Kaplan DR, Martin-Zanca D, Parada LF. Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature. 1991;350:158–60.
4. Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M. The trk proto-oncogene encodes a receptor for nerve growth factor. Cell. 1991;65:189–97.
5. Klein R, Lamballe F, Bryant S, Barbacid M. The trkB tyrosine protein kinase is a receptor for neurotrophin-4. Neuron. 1992;8:947–56.
6. Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, Nikolics K, Parade LF. The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell. 1991;65:895–903.
7. Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, et al. trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell. 1991;65:885–93.
8. Lamballe F, Klein R, Barbacid M. trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell. 1991;66:967–79.
9. Kouchalakos RN, Bradshaw RA. Nerve growth factor receptor from rabbit sympathetic ganglia membranes: relationship between subforms. J Biol Chem. 1986;261:16054–9.

10. Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature. 1991;350:678–83.
11. Barker PA. High affinity not in the vicinity? Neuron. 2007;53:1–4.
12. Wehrman T, He X, Raab B, Dukipatti A, Blau H, Garcia KC. Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. Neuron. 2007;53:25–38.
13. Benedetti M, Levi A, Chao MV. Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci USA. 1993;90:7859–63.
14. Bibel M, Hoppe E, Barde YA. Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J. 1999;18:616–22.
15. Brennan C, Rivas-Plata K, Landis SC. The p75 neurotrophin receptor influences NT-3 responsiveness of sympathetic neurons in vivo. Nat Neurosci. 1999;2:699–705.
16. Clary DO, Reichardt LF. An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. Proc Natl Acad Sci USA. 1994; 91:11133–7.
17. Kuruvilla R, Zweifel LS, Glebova NO, Lonze BE, Valdez G, Ye H, Ginty DD. A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell. 2004;118:243–55.
18. Skaper SD. The neurotrophin family of neurotrophic factors: an overview. Methods Mol Biol. 2012;846:1–12.
19. Sharma N, Deppmann CD, Harrington AW, St. Hillaire C, Chen ZY, Lee FS, Ginty DD. Long-distance control of synapse assembly by target-derived NGF. Neuron. 2010;67:422–34.
20. Holtzman DM, Li Y, Parada LF, Kinsman S, Chen CK, Valletta JS, Zhou J, Long JB, Mobley WC. p140trk mRNA marks NGF-responsive forebrain neurons: evidence that trk gene expression is induced by NGF. Neuron. 1992;9:465–78.
21. Pezet S, McMahon SB. Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci. 2006;29:507–38.
22. Zhuang ZY, Xu H, Clapham DE, Ji RR. Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. J Neurosci. 2004;24:8300–9.
23. Shibayama E, Koizumi H. Cellular localization of the Trk neurotrophin receptor family in human non-neuronal tissues. Am J Pathol. 1996;148:1807–18.
24. Levi-Montalcini R. The nerve growth factor 35 years later. Science. 1987;237:1154–62.
25. Hondermarck H. Neurotrophins and their receptors in breast cancer. Cytokine Growth Factor Rev. 2012;23:357–65.
26. Descamps S, Lebourhis X, Delehedde M, Boilly B, Hondermarck H. Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells. J Biol Chem. 1998;273:16659–62.
27. Dolle L, El Yazidi-Belkoura I, Adriaenssens E, Nurcombe V, Hondermarck H. Nerve growth factor overexpression and autocrine loop in breast cancer cells. Oncogene. 2003;22: 5592–601.
28. Descamps S, Toillon RA, Adriaenssens E, Pawlowski V, Cool SM, Nurcombe V, Le Bourhis X, Boilly B, Peyrat JP, Hondermarck H. Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways. J Biol Chem. 2001;276:17864–70.
29. El Yazidi-Belkoura I, Adriaenssens E, Dolle L, Descamps S, Hondermarck H. Tumor necrosis factor receptor-associated death domain protein is involved in the neurotrophin receptor-mediated antiapoptotic activity of nerve growth factor in breast cancer cells. J Biol Chem. 2003;278:16952–6.
30. Verbeke S, Meignan S, Lagadec C, Germain E, Hondermarck H, Adriaenssens E, Le Bourhis X. Overexpression of p75 (NTR) increases survival of breast cancer cells through p21(waf1). Cell Signal. 2010;22:1864–73.

31. Vanhecke E, Adriaenssens E, Verbeke S, Meignan S, Germain E, Berteaux N, Nurcombe V, Le Bourhis X, Hondermarck H. Brain-derived neurotrophic factor and neurotrophin-4/5 are expressed in breast cancer and can be targeted to inhibit tumor cell survival. Clin Cancer Res. 2011;17:1741–52.
32. Descamps S, Pawlowski V, Revillion F, Hornez L, Hebbar M, Boilly B, Hondermarck H, Peyrat JP. Expression of nerve growth factor receptors and their prognostic value in human breast cancer. Cancer Res. 2001;61:4337–40.
33. Davidson B, Reich R, Lazarovici P, Ann Florenes V, Nielsen S, Nesland JM. Altered expression and activation of the nerve growth factor receptors TrkA and p75 provide the first evidence of tumor progression to effusion in breast carcinoma. Breast Cancer Res Treat. 2004;83:119–28.
34. Popnikolov NK, Cavone SM, Schultz PM, Garcia FU. Diagnostic utility of p75 neurotrophin receptor (p75NTR) as a marker of breast myoepithelial cells. Mod Pathol. 2005;18:1535–41.
35. Reis-Filho JS, Steele D, Di Palma S, Jones RL, Savage K, James M, Milanezi F, Schmitt FC, Ashworth A. Distribution and significance of nerve growth factor receptor (NGFR/p75NTR) in normal, benign and malignant breast tissue. Mod Pathol. 2006;19:307–19.
36. Adriaenssens E, Vanhecke E, Saule P, Mougel A, Page A, Romon R, Nurcombe V, Le Bourhis X, Hondermarck H. Nerve growth factor is a potential therapeutic target in breast cancer. Cancer Res. 2008;68:346–51.
37. Lagadec C, Meignan S, Adriaenssens E, Foveau B, Vanhecke E, Romon R, Toillon RA, Oxombre B, Hondermarck H, Le Bourhis X. TrkA overexpression enhances growth and metastasis of breast cancer cells. Oncogene. 2009;28:1960–70.
38. Tagliabue E, Castiglioni F, Ghirelli C, Modugno M, Asnaghi L, Somenzi G, Melani C, Menard S. Nerve growth factor cooperates with p185(HER2) in activating growth of human breast carcinoma cells. J Biol Chem. 2000;275:5388–94.
39. Chiarenza A, Lazarovici P, Lempereur L, Cantarella G, Bianchi A, Bernardini R. Tamoxifen inhibits nerve growth factor-induced proliferation of the human breast cancerous cell line MCF-7. Cancer Res. 2001;61:3002–8.
40. Tognon C, Knezevich SR, Huntsman D, Roskelley CD, Melnyk N, Mathers JA, Becker L, Carneiro F, MacPherson N, Horsman D, Poremba C, Sorensen PH. Expression of the ETV6-NTRK3 gene fusion as a primary event in human secretory breast carcinoma. Cancer Cell. 2002;2:367–76.
41. Shonukan O, Bagayogo I, McCrea P, Chao M, Hempstead B. Neurotrophin-induced melanoma cell migration is mediated through the actin-bundling protein fascin. Oncogene. 2003;22:3616–23.
42. Truzzi F, Marconi A, Lotti R, Dallaglio K, French LE, Hempstead BL, Pincelli C. Neurotrophins and their receptors stimulate melanoma cell proliferation and migration. J Invest Dermatol. 2008;128:2031–40.
43. Renne C, Willenbrock K, Kuppers R, Hansmann ML, Brauninger A. Autocrine- and paracrine-activated receptor tyrosine kinases in classic Hodgkin lymphoma. Blood. 2005; 105:4051–9.
44. Renne C, Minner S, Kuppers R, Hansmann ML, Brauninger A. Autocrine NGFbeta/TRKA signalling is an important survival factor for Hodgkin lymphoma derived cell lines. Leuk Res. 2008;32:163–7.
45. Koch A, Scherr M, Breyer B, Mancini A, Kardinal C, Battmer K, Eder M, Tamura T. Inhibition of Abl tyrosine kinase enhances nerve growth factor-mediated signaling in Bcr-Abl transformed cells via the alteration of signaling complex and the receptor turnover. Oncogene. 2008;27:4678–89.
46. Du JJ, Dou KF, Peng SY, Qian BZ, Xiao HS, Liu F, Wang WZ, Guan WX, Gao ZQ, Liu YB, Han ZG. Expression of NGF family and their receptors in gastric carcinoma: a cDNA microarray study. World J Gastroenterol. 2003;9:1431–4.
47. Jin H, Pan Y, Zhao L, Zhai H, Li X, Sun L, He L, Chen Y, Hong L, Du Y, Fan D. p75 neurotrophin receptor suppresses the proliferation of human gastric cancer cells. Neoplasia. 2007;9:471–8.

48. Tokusashi Y, Asai K, Tamakawa S, Yamamoto M, Yoshie M, Yaginuma Y, Miyokawa N, Aoki T, Kino S, Kasai S, Ogawa K. Expression of NGF in hepatocellular carcinoma cells with its receptors in non-tumor cell components. Int J Cancer. 2005;114:39–45.
49. Yuanlong H, Haifeng J, Xiaoyin Z, Jialin S, Jie L, Li Y, Huahong X, Jiugang S, Yanglin P, Kaichun W, Jie D, Daiming F. The inhibitory effect of p75 neurotrophin receptor on growth of human hepatocellular carcinoma cells. Cancer Lett. 2008;268:110–9.
50. Mijatovic T, Gailly P, Mathieu V, De Neve N, Yeaton P, Kiss R, Decaestecker C. Neurotensin is a versatile modulator of in vitro human pancreatic ductal adenocarcinoma cell (PDAC) migration. Cell Oncol. 2007;29:315–26.
51. Miknyoczki SJ, Wan W, Chang H, Dobrzanski P, Ruggeri BA, Dionne CA, Buchkovich K. The neurotrophin-trk receptor axes are critical for the growth and progression of human prostatic carcinoma and pancreatic ductal adenocarcinoma xenografts in nude mice. Clin Cancer Res. 2002;8:1924–31.
52. Festuccia C, Gravina GL, Muzi P, Pomante R, Ventura L, Ricevuto E, Vicentini C, Bologna M. In vitro and in vivo effects of bicalutamide on the expression of TrkA and P75 neurotrophin receptors in prostate carcinoma. Prostate. 2007;67:1255–64.
53. Greco A, Mariani C, Miranda C, Lupas A, Pagliardini S, Pomati M, Pierotti MA. The DNA rearrangement that generates the TRK-T3 oncogene involves a novel gene on chromosome 3 whose product has a potential coiled-coil domain. Mol Cell Biol. 1995;15:6118–27.
54. Greco A, Miranda C, Pagliardini S, Fusetti L, Bongarzone I, Pierotti MA. Chromosome 1 rearrangements involving the genes TPR and NTRK1 produce structurally different thyroid-specific TRK oncogenes. Genes Chromosomes Cancer. 1997;19:112–23.
55. Butti MG, Bongarzone I, Ferraresi G, Mondellini P, Borrello MG, Pierotti MA. A sequence analysis of the genomic regions involved in the rearrangements between TPM3 and NTRK1 genes producing TRK oncogenes in papillary thyroid carcinomas. Genomics. 1995; 28:15–24.
56. McGregor LM, McCune BK, Graff JR, McDowell PR, Romans KE, Yancopoulos GD, Ball DW, Baylin SB, Nelkin BD. Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression. Proc Natl Acad Sci USA. 1999;96:4540–5.
57. Ricci A, Greco S, Mariotta S, Felici L, Bronzetti E, Cavazzana A, Cardillo G, Amenta F, Bisetti A, Barbolini G. Neurotrophins and neurotrophin receptors in human lung cancer. Am J Respir Cell Mol Biol. 2001;25:439–46.
58. Perez-Pinera P, Hernandez T, Garcia-Suarez O, de Carlos F, Germana A, Del Valle M, Astudillo A, Vega JA. The Trk tyrosine kinase inhibitor K252a regulates growth of lung adenocarcinomas. Mol Cell Biochem. 2007;295:19–26.
59. Odegaard E, Staff AC, Abeler VM, Kopolovic J, Onsrud M, Lazarovici P, Davidson B. The activated nerve growth factor receptor p-TrkA is selectively expressed in advanced-stage ovarian carcinoma. Hum Pathol. 2007;38:140–6.
60. Davidson B, Reich R, Lazarovici P, Nesland JM, Skrede M, Risberg B, Trope CG, Florenes VA. Expression and activation of the nerve growth factor receptor TrkA in serous ovarian carcinoma. Clin Cancer Res. 2003;9:2248–59.
61. Nakagawara A, Brodeur GM. Role of neurotrophins and their receptors in human neuroblastomas: a primary culture study. Eur J Cancer. 1997;33:2050–3.
62. Brodeur GM, Minturn JE, Ho R, Simpson AM, Iyer R, Varela CR, Light JE, Kolla V, Evans AE. Trk receptor expression and inhibition in neuroblastomas. Clin Cancer Res. 2009;15:3244–50.
63. Kogner P, Barbany G, Dominici C, Castello MA, Raschella G, Persson H. Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. Cancer Res. 1993;53:2044–50.
64. Nakagawara A, Arima M, Azar CG, Scavarda NJ, Brodeur GM. Inverse relationship between trk expression and N-myc amplification in human neuroblastomas. Cancer Res. 1992;52:1364–8.
65. Tacconelli A, Farina AR, Cappabianca L, Desantis G, Tessitore A, Vetuschi A, Sferra R, Rucci N, Argenti B, Screpanti I, Gulino A, Mackay AR. TrkA alternative splicing: a regulated tumor-promoting switch in human neuroblastoma. Cancer Cell. 2004;6:347–60.

66. Tacconelli A, Farina AR, Cappabianca L, Gulino A, Mackay AR. TrkAIII. A novel hypoxia-regulated alternative TrkA splice variant of potential physiological and pathological importance. Cell Cycle. 2005;4:8–9.
67. Eggert A, Grotzer MA, Ikegaki N, Liu XG, Evans AE, Brodeur GM. Expression of the neurotrophin receptor TrkA down-regulates expression and function of angiogenic stimulators in SH-SY5Y neuroblastoma cells. Cancer Res. 2002;62:1802–8.
68. Ho R, Eggert A, Hishiki T, Minturn JE, Ikegaki N, Foster P, Camoratto AM, Evans AE, Brodeur GM. Resistance to chemotherapy mediated by TrkB in neuroblastomas. Cancer Res. 2002;62:6462–6.
69. Laneve P, Di Marcotullio L, Gioia U, Fiori ME, Ferretti E, Gulino A, Bozzoni I, Caffarelli E. The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells. Proc Natl Acad Sci USA. 2007;104:7957–62.
70. Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M. Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature. 1994;368:246–9.
71. Indo Y, Tsuruta M, Hayashida Y, Karim MA, Ohta K, Kawano T, Mitsubuchi H, Tonoki H, Awaya Y, Matsuda I. Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. Nat Genet. 1996;13:485–8.
72. Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, Joyner AL, Barbacid M. Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell. 1993;75:113–22.
73. Lewin GR, Rueff A, Mendell LM. Peripheral and central mechanisms of NGF-induced hyperalgesia. Eur J Neurosci. 1994;6:1903–12.
74. Longo FM, Massa SM. Small-molecule modulation of neurotrophin receptors: a strategy for the treatment of neurological disease. Nat Rev Drug Discov. 2013;12:507–25.
75. McKelvey L, Shorten GD, O'Keeffe GW. Nerve growth factor-mediated regulation of pain signalling and proposed new intervention strategies in clinical pain management. J Neurochem. 2013;124:276–89.
76. Cattaneo A. Tanezumab: A recombinant humanized mAb against nerve growth factor for the treatment of acute and chronic pain. Curr Opin Mol Ther. 2010;12:94–106.
77. Schmidt BL. The neurobiology of cancer pain. Neuroscientist. 2014;20:546–62.
78. Fahnestock M, Michalski B, Xu B, Coughlin MD. The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol Cell Neurosci. 2001;18:210–20.
79. Stoica G, Lungu G, Kim HT, Wong PK. Up-regulation of pro-nerve growth factor, neurotrophin receptor p75, and sortilin is associated with retrovirus-induced spongiform encephalomyelopathy. Brain Res. 2008;1208:204–16.
80. Pedraza CE, Podlesniy P, Vidal N, Arevalo JC, Lee R, Hempstead B, Ferrer I, Iglesias M, Espinet C. Pro-NGF isolated from the human brain affected by Alzheimer's disease induces neuronal apoptosis mediated by p75NTR. Am J Pathol. 2005;166:533–43.
81. Beattie MS, Harrington AW, Lee R, Kim JY, Boyce SL, Longo FM, Bresnahan JC, Hempstead BL, Yoon SO. ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. Neuron. 2002;36:375–86.
82. Harrington AW, Leiner B, Blechschmitt C, Arevalo JC, Lee R, Morl K, Meyer M, Hempstead BL, Yoon SO, Giehl KM. Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury. Proc Natl Acad Sci USA. 2004;101:6226–30.
83. Volosin M, Song W, Almeida RD, Kaplan DR, Hempstead BL, Friedman WJ. Interaction of survival and death signaling in basal forebrain neurons: roles of neurotrophins and proneurotrophins. J Neurosci. 2006;26:7756–66.
84. Volosin M, Trotter C, Cragnolini A, Kenchappa RS, Light M, Hempstead BL, Carter BD, Friedman WJ. Induction of proneurotrophins and activation of p75NTR-mediated apoptosis via neurotrophin receptor-interacting factor in hippocampal neurons after seizures. J Neurosci. 2008;28:9870–9.

85. Sobottka B, Reinhardt D, Brockhaus M, Jacobsen H, Metzger F. ProNGF inhibits NGF-mediated TrkA activation in PC12 cells. J Neurochem. 2008;107:1294–303.
86. Hallbook F. Evolution of the vertebrate neurotrophin and Trk receptor gene families. Curr Opin Neurobiol. 1999;9:616–21.
87. Sacristán MP, de Diego JG, Bonilla M, Martín-Zanca D. Molecular cloning and characterization of the 5′ region of the mouse trkA proto-oncogene. Oncogene. 1999;18:5836–42.
88. Palani M, Arunkumar R, Vanisree AJ. Methylation and expression patterns of tropomyosin-related kinase genes in different grades of glioma. Neuromolecular Med. 2014;16:529–39.
89. Lau DT, Hesson LB, Norris MD, Marshall GM, Haber M, Ashton LJ. Prognostic significance of promoter DNA methylation in patients with childhood neuroblastoma. Clin Cancer Res. 2012;18:5690–700.
90. Jin W, Lee JJ, Kim MS, Son BH, Cho YK, Kim HP. DNA methylation-dependent regulation of TrkA, TrkB, and TrkC genes in human hepatocellular carcinoma. Biochem Biophys Res Commun. 2011;406:89–95.
91. Rochman M, Kartashov AV, Caldwell JM, Collins MH, Stucke EM, Kc K, Sherrill JD, Herren J, Barski A, Rothenberg ME. Neurotrophic tyrosine kinase receptor 1 is a direct transcriptional and epigenetic target of IL-13 involved in allergic inflammation. Mucosal Immunol. 2014. Nov 12 [Epub ahead of print].
92. Shah AG, Friedman MJ, Huang S, Roberts M, Li XJ, Li S. Transcriptional dysregulation of TrkA associates with neurodegeneration in spinocerebellar ataxia type 17. Hum Mol Genet. 2009;18:4141–52.
93. Valderrama X, Misra V. Novel Brn3a cis-acting sequences mediate transcription of human trkA in neurons. J Neurochem. 2008;105:425–35.
94. Zhang J, Chen X. DeltaNp73 modulates nerve growth factor-mediated neuronal differentiation through repression of TrkA. Mol Cell Biol. 2007;27:3868–80.
95. Kingsbury TJ, Murray PD, Bambrick LL, Krueger BK. Ca(2+)-dependent regulation of TrkB expression in neurons. J Biol Chem. 2003;278:40744–8.
96. Kingsbury TJ, Krueger BK. Ca2+, CREB and krüppel: a novel KLF7-binding element conserved in mouse and human TRKB promoters is required for CREB-dependent transcription. Mol Cell Neurosci. 2007;35:447–55.
97. Deogracias R, Espliguero G, Iglesias T, Rodríguez-Peña A. Expression of the neurotrophin receptor trkB is regulated by the cAMP/CREB pathway in neurons. Mol Cell Neurosci. 2004;26:470–80.
98. Martens LK, Kirschner KM, Warnecke C, Scholz H. Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator of the TrkB neurotrophin receptor gene. J Biol Chem. 2007; 282:14379–88.
99. Pombo PM, Barettino D, Espliguero G, Metsis M, Iglesias T, Rodriguez-Pena A. Transcriptional repression of neurotrophin receptor trkB by thyroid hormone in the developing rat brain. J Biol Chem. 2000;275:37510–7.
100. Inoue K, Ito K, Osato M, Lee B, Bae SC, Ito Y. The transcription factor Runx3 represses the neurotrophin receptor TrkB during lineage commitment of dorsal root ganglion neurons. J Biol Chem. 2007;282:24175–84.
101. Arevalo JC, Conde B, Hempstead BL, Chao MV, Martin-Zanca D, Perez P. TrkA immunoglobulin-like ligand binding domains inhibit spontaneous activation of the receptor. Mol Cell Biol. 2000;20:5908–16.
102. Watson FL, Porcionatto MA, Bhattacharyya A, Stiles CD, Segal RA. TrkA glycosylation regulates receptor localization and activity. J Neurobiol. 1999;39:323–36.
103. de Pablo Y, Perez-Garcia MJ, Georgieva MV, Sanchis D, Lindqvist N, Soler RM, Comella JX, Llovera M. Tyr-701 is a new regulatory site for neurotrophin receptor TrkA trafficking and function. J Neurochem. 2008;104:124–39.
104. Obermeier A, Halfter H, Wiesmuller KH, Jung G, Schlessinger J, Ullrich A. Tyrosine 785 is a major determinant of Trk–substrate interaction. EMBO J. 1993;12:933–41.
105. Obermeier A, Lammers R, Wiesmüller KH, Jung G, Schlessinger J, Ullrich A. Identification of Trk binding sites for SHC and phosphatidylinositol 3′-kinase and formation of a multimeric signaling complex. J Biol Chem. 1993;268:22963–6.

106. Zhang X, Huang J, McNaugton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J. 2005;24:4211–23.
107. Biarc J, Chalkley RJ, Burlingame AL, Bradshaw RA. Dissecting the roles of tyrosines 490 and 785 of TrkA in the induction of downstream protein phosphorylation using chimeric receptors. J Biol Chem. 2013;288:16606–18.
108. Makkerh JPS, Ceni C, Auld DS, Vaillancourt F, Dorval G, Barker PA. p75 neurotrophin receptor reduces ligand-induced Trk receptor ubiquitination and delays Trk receptor internalization and degradation. EMBO Rep. 2005;6:936–41.
109. Arevalo JC, Pereira DB, Yano H, Teng KK, Chao MV. Identification of a switch in neurotrophin signaling by selective tyrosine phosphorylation. J Biol Chem. 2006;281:1001–7.
110. Arevalo JC, Waite J, Rajagopal R, Beyna M, Chen Z-Y, Lee FS, Chao MV. Cell survival through Trk neurotrophin receptors is differentially regulated by ubiquitination. Neuron. 2006;50:549–59.
111. Yu T, Calvo L, Anta B, Lopez-Benito S, Lopez-Bellido R, Vicente-García C, Tessarollo L, Rodriguez RE, Arevalo JC. In vivo regulation of NGF-mediated functions by Nedd4-2 ubiquitination of TrkA. J Neurosci. 2014;34:6098–106.
112. Kiris E, Wang T, Yanpallewar S, Dorsey SG, Becker J, Bavari S, Palko ME, Coppola V, Tessarollo L. TrkA in vivo function is negatively regulated by ubiquitination. J Neurosci. 2014;34:4090–8.
113. Barker PA, Lomen-Hoerth C, Gensch EM, Meakin SO, Glass DJ, Shooter EM. Tissue-specific alternative splicing generates two isoforms of the trkA receptor. J Biol Chem. 1993;268:15150–7.
114. Tacconelli A, Farina AR, Cappabianca L, Cea G, Panella S, Chioda A, Gallo R, Cinque B, Sferra R, Vetuschi A, Campese AF, Screpanti I, Gulino A, Mackay AR. TrkAIII expression in the thymus. J Neuroimmunol. 2007;183:151–61.
115. Shelton DL, Sutherland J, Gripp J, Camerato T, Armanini MP, Phillips HS, Carroll K, Spencer SD, Levinson AD. Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. J Neurosci. 1995;15:477–91.
116. Labouyrie E, Dubus P, Groppi A, Mahon FX, Ferrer J, Parrens M, Reiffers J, de Mascarel A, Merlio JP. Expression of neurotrophins and their receptors in human bone marrow. Am J Pathol. 1999;154:405–15.
117. Stoilov P, Castren E, Stamm S. Analysis of the human TrkB gene genomic organization reveals novel TrkB isoforms, unusual gene length, and splicing mechanism. Biochem Biophys Res Commun. 2002;290:1054–65.
118. Biffo S, Offenhauser N, Carter BD, Barde YA. Selective binding and internalisation by truncated receptors restrict the availability of BDNF during development. Development. 1995; 121:2461–70.
119. Haapasalo A, Koponen E, Hoppe E, Wong G, Castren E. Truncated trkB.T1 is dominant negative inhibitor of trkB.TK+-mediated cell survival. Biochem Biophys Res Commun. 2001;280:1352–8.
120. Ohira K, Kumanogoh H, Sahara Y, Homma KJ, Hirai H, Nakamura S, Hayashi M. A truncated tropomyosin-related kinase B receptor, T1, regulates glial cell morphology via Rho GDP dissociation inhibitor 1. J Neurosci. 2005;25:1343–53.
121. Nishida Y, Adati N, Ozawa R, Maeda A, Sakaki Y, Takeda T. Identification and classification of genes regulated by phosphatidylinositol 3-kinase- and TRKB-mediated signalling pathways during neuronal differentiation in two subtypes of the human neuroblastoma cell line SH-SY5Y. BMC Res Notes. 2008;1:95.
122. Guiton M, Gunn-Moore FJ, Glass DJ, Geis DR, Yancopoulos GD, Tavare JM. Naturally occurring tyrosine kinase inserts block high affinity binding of phospholipase C gamma and Shc to TrkC and neurotrophin-3 signaling. J Biol Chem. 1995;270:20384–90.
123. McGregor LM, Baylin SB, Griffin CA, Hawkins AL, Nelkin BD. Molecular cloning of the cDNA for human TrkC (NTRK3), chromosomal assignment, and evidence for a splice variant. Genomics. 1994;22:267–72.
124. Palko ME, Coppola V, Tessarollo L. Evidence for a role of truncated trkC receptor isoforms in mouse development. J Neurosci. 1999;19:775–82.

125. Shooter EM. Early days of the nerve growth factor proteins. Annu Rev Neurosci. 2001;24: 601–29.
126. McDonald NQ, Lapatto R, Murray-Rust J, Gunning J, Wlodawer A, Blundell TL. New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. Nature. 1991;354:411–4.
127. Barde YA, Edgar D, Thoenen H. Purification of a new neurotrophic factor from mammalian brain. Embo J. 1982;1:549–53.
128. Hohn A, Leibrock J, Bailey K, Barde YA. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature. 1990;344:339–41.
129. Jones KR, Reichardt LF. Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc Natl Acad Sci USA. 1990;87:8060–4.
130. Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science. 1990;247: 1446–51.
131. Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, Laramee GR, Nikolics K, Winslow JW. Primary structure and biological activity of a novel human neurotrophic factor. Neuron. 1990;4:767–73.
132. Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A. Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron. 1991;7: 857–66.
133. Hallbook F, Ibanez CF, Persson H. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron. 1991;6:845–58.
134. Ip NY, Ibanez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa 3rd R, Squinto SP, et al. Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci USA. 1992; 89:3060–4.
135. Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. Science. 2001;294:1945–8.
136. Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM. Sortilin is essential for proNGF-induced neuronal cell death. Nature. 2004;427:843–8.
137. Robinson RC, Radziejewski C, Spraggon G, Greenwald J, Kostura MR, Burtnick LD, Stuart DI, Choe S, Jones EY. The structures of the neurotrophin 4 homodimer and the brain-derived neurotrophic factor/neurotrophin 4 heterodimer reveal a common Trk-binding site. Protein Sci. 1999;8:2589–97.
138. Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B. Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science. 2004;306:487–91.
139. Murray-Rust J, McDonald NQ, Blundell TL, Hosang M, Oefner C, Winkler F, Bradshaw RA. Topological similarities in TGF-β2, PDGF-BB, and NGF Define a Superfamily of Polypeptide Growth Factors. Structure. 1993;1:153–9.
140. McDonald NQ, Hendrickson WA. A structural superfamily of growth factors containing a cystine knot motif. Cell. 1993;73:421–4.
141. Kliemannel M, Rattenholl A, Golbik R, Balbach J, Lilie H, Rudolph R, Schwarz E. The mature part of proNGF induces the structure of its pro-peptide. FEBS Lett. 2004;566: 207–12.
142. Paoletti F, Covaceuszach S, Konarev PV, Gonfloni S, Malerba F, Schwarz E, Svergun DI, Cattaneo A, Lamba D. Intrinsic structural disorder of mouse proNGF. Proteins. 2009;75: 990–1009.
143. Arakawa T, Haniu M, Narhi LO, Miller JA, Talvenheimo J, Philo JS, Chute HT, Matheson C, Carnahan J, Louis JC, et al. Formation of heterodimers from three neurotrophins, nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor. J Biol Chem. 1994;269:27833–9.

144. Heymach Jr JV, Shooter EM. The biosynthesis of neurotrophin heterodimers by transfected mammalian cells. J Biol Chem. 1995;270:12297–304.
145. Radziejewski C, Robinson RC. Heterodimers of the neurotrophic factors: formation, isolation, and differential stability. Biochemistry. 1993;32:13350–6.
146. Hauburger A, Kliemannel M, Madsen P, Rudolph R, Schwarz E. Oxidative folding of nerve growth factor can be mediated by the pro-peptide of neurotrophin-3. FEBS Lett. 2007;581:4159–64.
147. Rattenholl A, Lilie H, Grossmann A, Stern A, Schwarz E, Rudolph R. The pro-sequence facilitates folding of human nerve growth factor from Escherichia coli inclusion bodies. Eur J Biochem. 2001;268:3296–303.
148. Rattenholl A, Ruoppolo M, Flagiello A, Monti M, Vinci F, Marino G, Lilie H, Schwarz E, Rudolph R. Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. J Mol Biol. 2001;305:523–33.
149. Kliemannel M, Golbik R, Rudolph R, Schwarz E, Lilie H. The pro-peptide of proNGF: structure formation and intramolecular association with NGF. Protein Sci. 2007;16:411–9.
150. Paiardini A, Caputo V. Insights into the interaction of sortilin with proneurotrophins: a computational approach. Neuropeptides. 2008;42:205–14.
151. Willnow TE, Petersen CM, Nykjaer A. VPS10P-domain receptors - regulators of neuronal viability and function. Nat Rev Neurosci. 2008;9:899–909.
152. Bierl MA, Jones EE, Crutcher KA, Isaacson LG. 'Mature' nerve growth factor is a minor species in most peripheral tissues. Neurosci Lett. 2005;380:133–7.
153. Bruno MA, Cuello AC. Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. Proc Natl Acad Sci USA. 2006;103:6735–40.
154. Hasan W, Pedchenko T, Krizsan-Agbas D, Baum L, Smith PG. Sympathetic neurons synthesize and secrete pro-nerve growth factor protein. J Neurobiol. 2003;57:38–53.
155. Srinivasan B, Roque CH, Hempstead BL, Al-Ubaidi MR, Roque RS. Microglia-derived pro-nerve growth factor promotes photoreceptor cell death via p75 neurotrophin receptor. J Biol Chem. 2004;279:41839–45.
156. Domeniconi M, Hempstead BL, Chao MV. Pro-NGF secreted by astrocytes promotes motor neuron cell death. Mol Cell Neurosci. 2007;34:271–9.
157. Fauchais AL, Lalloue F, Lise MC, Boumediene A, Preud'homme JL, Vidal E, Jauberteau MO. Role of endogenous brain-derived neurotrophic factor and sortilin in B cell survival. J Immunol. 2008;181:3027–38.
158. Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen ZY, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL. ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci. 2005;25:5455–63.
159. Butte MJ, Hwang PK, Mobley WC, Fletterick RJ. Crystal structure of neurotrophin-3 homodimer shows distinct regions are used to bind its receptors. Biochemistry. 1998;37:16846–52.
160. Ultsch MH, Wiesmann C, Simmons LC, Henrich J, Yang M, Reilly D, Bass SH, de Vos AM. Crystal structures of the neurotrophin-binding domain of TrkA, TrkB and TrkC. J Mol Biol. 1999;290:149–59.
161. Wiesmann C, Ultsch MH, Bass SH, de Vos AM. Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. Nature. 1999;401:184–8.
162. Ivanisevic L, Zheng W, Woo SB, Neet KE, Saragovi HU. TrkA receptor "hot spots" for binding of NT-3 as a heterologous ligand. J Biol Chem. 2007;282:16754–63.
163. Boeshore KL, Luckey CN, Zigmond RE, Large TH. TrkB isoforms with distinct neurotrophin specificities are expressed in predominantly nonoverlapping populations of avian dorsal root ganglion neurons. J Neurosci. 1999;19:4739–47.
164. Strohmaier C, Carter BD, Urfer R, Barde YA, Dechant G. A splice variant of the neurotrophin receptor trkB with increased specificity for brain-derived neurotrophic factor. EMBO J. 1996;15:3332–7.

165. Fahnestock M, Yu G, Michalski B, Mathew S, Colquhoun A, Ross GM, Coughlin MD. The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor. J Neurochem. 2004;89:581–92.
166. Demont Y, Corbet C, Page A, Ataman-Onal Y, Choquet-Kastylevsky G, Fliniaux I, Le Bourhis X, Toillon RA, Bradshaw RA, Hondermarck H. Pro-nerve growth factor induces autocrine stimulation of breast cancer cell invasion through tropomyosin-related kinase A (TrkA) and sortilin protein. J Biol Chem. 2012;287:1923–31.
167. Al-Shawi R, Hafner A, Olsen J, Chun S, Raza S, Thrasivoulou C, Lovestone S, Killick R, Simons P, Cowen T. Neurotoxic and neurotrophic roles of proNGF and the receptor sortilin in the adult and ageing nervous system. Eur J Neurosci. 2008;27:2103–14.
168. Masoudi R, Ioannou MS, Coughlin MD, Pagadala P, Neet KE, Clewes O, Allen SJ, Dawbarn D, Fahnestock M. Biological activity of nerve growth factor precursor is dependent upon relative levels of its receptors. J Biol Chem. 2009;284:18424–33.
169. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell. 1990;61:203–12.
170. Pawson T. Protein modules and signalling networks. Nature. 1995;373:573–80.
171. Gadella Jr T, Jovin T. Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. J Cell Biol. 1995;129:1543–958.
172. Tyson D, Bradshaw RA. Transmembrane receptor oligomerization. In: Bradshaw RA, Dennis EA, editors. Handbook of cell signaling, vol. 1. San Diego, CA: Elsevier Academic Press; 2003. p. 361–6.
173. Mischel PS, Umbach JA, Eskandari S, Smith SG, Gundersen CB, Zampighi GA. Nerve growth factor signals via preexisting TrkA receptor oligomers. Biophys J. 2002;83:968–76.
174. Shen J, Maruyama IN. Nerve growth factor receptor TrkA exists as a preformed, yet inactive, dimer in living cells. FEBS Lett. 2011;585:295–9.
175. Shen J, Maruyama IN. Brain-derived neurotrophic factor receptor TrkB exists as a preformed dimer in living cells. J Mol Signal. 2012;7:2.
176. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010;141: 1117–34.
177. Obermeier A, Bradshaw RA, Seedorf K, Choidas A, Schlessinger J, Ullrich A. Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC gamma. EMBO J. 1994;13:1585–90.
178. Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA. 1976;73:2424–8.
179. Biarc J, Chalkley RJ, Burlingame AL, Bradshaw RA. The induction of serine/threonine protein phosphorylations by a PDGFR/TrkA chimera in stably transfected PC12 cells. Mol Cell Proteomics. 2012;11:15–30.
180. Rockow S, Tang J, Xiong W, Li W. Nck inhibits NGF and basic FGF induced PC12 cell differentiation via mitogen-activated protein kinase-independent pathway. Oncogene. 1996; 12:2351–9.
181. Suzuki S, Mizutani M, Suzuki K, Yamada M, Kojima M, Hatanaka H, Koizumi S. Brain-derived neurotrophic factor promotes interaction of the Nck2 adaptor protein with the TrkB tyrosine kinase receptor. Biochem Biophys Res Commun. 2002;294:1087–92.
182. Huang B, Eberstadt M, Olejniczak ET, Meadows RP, Fesik SW. NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. Nature. 1996;384:638–41.
183. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci. 2003;4:299–309.
184. Raffioni S, Bradshaw RA. Staurosporine causes epidermal growth factor to induce differentiation in PC12 cells via receptor up-regulation. J Biol Chem. 1995;270:7568–72.
185. Qiu MS, Green SH. NGF and EGF rapidly activate p21ras in PC12 cells by distinct, convergent pathways involving tyrosine phosphorylation. Neuron. 1991;7:937–46.
186. Hagag N, Halegoua S, Viola M. Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to ras p21. Nature. 1986;319:680–2.

187. Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, Kaplan DR. Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron. 1994;12:691–705.
188. Dikic I, Batzer AG, Blaikie P, Obermeier A, Ullrich A, Schlessinger J, Margolis B. Shc binding to nerve growth factor receptor is mediated by the phosphotyrosine interaction domain. J Biol Chem. 1995;270:15125–9.
189. Nimnual AS, Yatsula BA, Bar-Sagi D. Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. Science. 1998;279:560–3.
190. Thomas SM, DeMarco M, D'Arcangelo G, Halegoua S, Brugge JS. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell. 1992;68:1031–40.
191. Troppmair J, Bruder JT, App H, Cai H, Liptak L, Szeberenyi J, Cooper GM, Rapp UR. Ras controls coupling of growth factor receptors and protein kinase C in the membrane to Raf-1 and B-Raf protein serine kinases in the cytosol. Oncogene. 1992;7:1867–73.
192. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ. First-line Herceptin monotherapy in metastatic breast cancer. Oncology. 2001;61 Suppl 2:37–42.
193. Kao S, Jaiswal RK, Kolch W, Landreth GE. Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells. J Biol Chem. 2001;276:18169–77.
194. Deak M, Clifton AD, Lucocq LM, Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J. 1998;17:4426–41.
195. Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science. 1996;273:959–63.
196. Lonze BE, Ginty DD. Function and regulation of CREB family transcription factors in the nervous system. Neuron. 2002;35:605–23.
197. Wong AW, Willingham M, Xiao J, Kilpatrick TJ, Murray SS. Neurotrophin receptor homolog-2 regulates nerve growth factor signaling. J Neurochem. 2008;106:1964–76.
198. York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJ. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature. 1998;392:622–6.
199. Wu C, Lai CF, Mobley WC. Nerve growth factor activates persistent Rap1 signaling in endosomes. J Neurosci. 2001;21:5406–16.
200. York RD, Molliver DC, Grewal SS, Stenberg PE, McCleskey EW, Stork PJ. Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. Mol Cell Biol. 2000;20:8069–83.
201. Dixon SJ, MacDonald JI, Robinson KN, Kubu CJ, Meakin SO. Trk receptor binding and neurotrophin/fibroblast growth factor (FGF)-dependent activation of the FGF receptor substrate (FRS)-3. Biochim Biophys Acta. 2006;1763:366–80.
202. Postigo A, Calella AM, Fritzsch B, Knipper M, Katz D, Eilers A, Schimmang T, Lewin GR, Klein R, Minichiello L. Distinct requirements for TrkB and TrkC signaling in target innervation by sensory neurons. Genes Dev. 2002;16:633–45.
203. Arevalo JC, Yano H, Teng KK, Chao MV. A unique pathway for sustained neurotrophin signaling through an ankyrin-rich membrane-spanning protein. Embo J. 2004;23:2358–68.
204. Kong H, Boulter J, Weber JL, Lai C, Chao MV. An evolutionarily conserved transmembrane protein that is a novel downstream target of neurotrophin and ephrin receptors. J Neurosci. 2001;21:176–85.
205. Wright JH, Drueckes P, Bartoe J, Zhao Z, Shen SH, Krebs EG. A role for the SHP-2 tyrosine phosphatase in nerve growth-induced PC12 cell differentiation. Mol Biol Cell. 1997;8:1575–85.
206. Rosario M, Franke R, Bednarski C, Birchmeier W. The neurite outgrowth multiadaptor RhoGAP, NOMA-GAP, regulates neurite extension through SHP2 and Cdc42. J Cell Biol. 2007;178:503–16.
207. Pazyra-Murphy MF, Hans A, Courchesne SL, Karch C, Cosker KE, Heerssen HM, Watson FL, Kim T, Greenberg ME, Segal RA. A retrograde neuronal survival response: target-derived neurotrophins regulate MEF2D and bcl-w. J Neurosci. 2009;29:6700–9.

208. Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ. Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. Proc Natl Acad Sci USA. 1997;94:12419–24.
209. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91:231–41.
210. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999;96:857–68.
211. Brunet A, Kanai F, Stehn J, Xu J, Sarbassova D, Frangioni JV, Dalal SN, DeCaprio JA, Greenberg ME, Yaffe MB. 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. J Cell Biol. 2002;156:817–28.
212. Zheng WH, Kar S, Quirion R. FKHRL1 and its homologs are new targets of nerve growth factor Trk receptor signaling. J Neurochem. 2002;80:1049–61.
213. Foehr ED, Lin X, O'Mahony A, Geleziunas R, Bradshaw RA, Greene WC. NF-kappa B signaling promotes both cell survival and neurite process formation in nerve growth factor-stimulated PC12 cells. J Neurosci. 2000;20:7556–63.
214. Kim UH, Fink Jr D, Kim HS, Park DJ, Contreras ML, Guroff G, Rhee SG. Nerve growth factor stimulates phosphorylation of phospholipase C-gamma in PC12 cells. J Biol Chem. 1991;266:1359–62.
215. Corbit KC, Foster DA, Rosner MR. Protein kinase Cdelta mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells. Mol Cell Biol. 1999;19:4209–18.
216. Zhang D, Dhillon H, Prasad MR, Markesbery WR. Regional levels of brain phospholipase Cgamma in Alzheimer's disease. Brain Res. 1998;811:161–5.
217. Matrone C, Marolda R, Ciafre S, Ciotti MT, Mercanti D, Calissano P. Tyrosine kinase nerve growth factor receptor switches from prosurvival to proapoptotic activity via Abeta-mediated phosphorylation. Proc Natl Acad Sci USA. 2009;106:11358–63.
218. Covaceuszach S, Capsoni S, Ugolini G, Spirito F, Vignone D, Cattaneo A. Development of a non invasive NGF-based therapy for Alzheimer's disease. Curr Alzheimer Res. 2009;6: 158–70.
219. Raffioni S, Bradshaw RA, Buxser SE. The receptors for nerve growth factor and other neurotrophins. Ann Rev Biochem. 1993;62:823–50.
220. Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, Buck CR, Sehgal A. Gene transfer and molecular cloning of the human NGF receptor. Science. 1986;232:518–21.
221. Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature. 1987;325:593–7.
222. Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M. Expression and structure of the human NGF receptor. Cell. 1986;47:545–54.
223. Rodriguez-Tebar A, Dechant G, Barde YA. Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron. 1990;4:487–92.
224. Rodriguez-Tebar A, Dechant G, Gotz R, Barde YA. Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J. 1992;11:917–22.
225. Metsis M. Genes for neurotrophic factors and their receptors: structure and regulation. Cell Mol Life Sci. 2001;58:1014–20.
226. Ramos A, Ho WC, Forte S, Dickson K, Boutilier J, Favell K, Barker PA. Hypo-osmolar stress induces p75NTR expression by activating Sp1-dependent transcription. J Neurosci. 2007;27: 1498–506.
227. Sehgal A, Patil N, Chao M. A constitutive promoter directs expression of the nerve growth factor receptor gene. Mol Cell Biol. 1988;8:3160–7.
228. Chiaramello A, Neuman K, Palm K, Metsis M, Neuman T. Helix-loop-helix transcription factors mediate activation and repression of the p75LNGFR gene. Mol Cell Biol. 1995;15:6036–44.

229. Metsis M, Timmusk T, Allikmets R, Saarma M, Persson H. Regulatory elements and transcriptional regulation by testosterone and retinoic acid of the rat nerve growth factor receptor promoter. Gene. 1992;121:247–54.
230. Roux PP, Barker PA. Neurotrophin signaling through the p75 neurotrophin receptor. Prog Neurobiol. 2002;67:203–33.
231. Large TH, Weskamp G, Helder JC, Radeke MJ, Misko TP, Shooter EM, Reichardt LF. Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. Neuron. 1989;2:1123–34.
232. Monlauzeur L, Breuza L, Le Bivic A. Putative O-glycosylation sites and a membrane anchor are necessary for apical delivery of the human neurotrophin receptor in Caco-2 cells. J Biol Chem. 1998;273:30263–70.
233. Gong Y, Cao P, Yu HJ, Jiang T. Crystal structure of the neurotrophin-3 and p75NTR symmetrical complex. Nature. 2008;454:789–93.
234. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol. 2003;3:745–56.
235. Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell. 1994;76:959–62.
236. Yan H, Chao MV. Disruption of cysteine-rich repeats of the p75 nerve growth factor receptor leads to loss of ligand binding. J Biol Chem. 1991;266:12099–104.
237. Barker PA. A p75(NTR) pivoting paradigm propels perspicacity. Neuron. 2009;62:3–5.
238. Vilar M, Charalampopoulos I, Kenchappa RS, Simi A, Karaca E, Reversi A, Choi S, Bothwell M, Mingarro I, Friedman WJ, Schiavo G, Bastiaens PI, Verveer PJ, Carter BD, Ibanez CF. Activation of the p75 neurotrophin receptor through conformational rearrangement of disulphide-linked receptor dimers. Neuron. 2009;62:72–83.
239. Barker PA, Barbee G, Misko TP, Shooter EM. The low affinity neurotrophin receptor, p75LNTR, is palmitoylated by thioester formation through cysteine 279. J Biol Chem. 1994;269:30645–50.
240. Mirnics ZK, Yan C, Portugal C, Kim TW, Saragovi HU, Sisodia SS, Mirnics K, Schor NF. P75 neurotrophin receptor regulates expression of neural cell adhesion molecule 1. Neurobiol Dis. 2005;20:969–85.
241. Underwood CK, Reid K, May LM, Bartlett PF, Coulson EJ. Palmitoylation of the C-terminal fragment of p75(NTR) regulates death signaling and is required for subsequent cleavage by gamma-secretase. Mol Cell Neurosci. 2008;37:346–58.
242. Grob PM, Ross AH, Koprowski H, Bothwell M. Characterization of the human melanoma nerve growth factor receptor. J Biol Chem. 1985;260:8044–9.
243. Taniuchi M, Johnson Jr EM, Roach PJ, Lawrence Jr JC. Phosphorylation of nerve growth factor receptor proteins in sympathetic neurons and PC12 cells. In vitro phosphorylation by the cAMP-independent protein kinase FA/GSK-3. J Biol Chem. 1986; 261:13342–9.
244. Higuchi H, Yamashita T, Yoshikawa H, Tohyama M. PKA phosphorylates the p75 receptor and regulates its localization to lipid rafts. EMBO J. 2003;22:1790–800.
245. Bronfman FC, Fainzilber M. Multi-tasking by the p75 neurotrophin receptor: sortilin things out? EMBO Rep. 2004;5:867–71.
246. Haase G, Pettmann B, Raoul C, Henderson CE. Signaling by death receptors in the nervous system. Curr Opin Neurobiol. 2008;18:284–91.
247. Blochl A, Blochl R. A cell-biological model of p75NTR signaling. J Neurochem. 2007;102: 289–305.
248. Blochl A, Blumenstein L, Ahmadian MR. Inactivation and activation of Ras by the neurotrophin receptor p75. Eur J Neurosci. 2004;20:2321–35.
249. Coulson EJ, Reid K, Baca M, Shipham KA, Hulett SM, Kilpatrick TJ, Bartlett PF. Chopper, a new death domain of the p75 neurotrophin receptor that mediates rapid neuronal cell death. J Biol Chem. 2000;275:30537–45.
250. Sheng M, Sala C. PDZ domains and the organization of supramolecular complexes. Annu Rev Neurosci. 2001;24:1–29.

251. von Schack D, Casademunt E, Schweigreiter R, Meyer M, Bibel M, Dechant G. Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system. Nat Neurosci. 2001;4:977–8.
252. Kanning KC, Hudson M, Amieux PS, Wiley JC, Bothwell M, Schecterson LC. Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. J Neurosci. 2003;23:5425–36.
253. Powell JC, Twomey C, Jain R, McCarthy JV. Association between Presenilin-1 and TRAF6 modulates regulated intramembrane proteolysis of the p75NTR neurotrophin receptor. J Neurochem. 2009;108:216–30.
254. Bronfman FC. Metalloproteases and gamma-secretase: new membrane partners regulating p75 neurotrophin receptor signaling? J Neurochem. 2007;103 Suppl 1:91–100.
255. He XL, Garcia KC. Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. Science. 2004;304:870–5.
256. Aurikko JP, Ruotolo BT, Grossmann JG, Moncrieffe MC, Stephens E, Leppanen VM, Robinson CV, Saarma M, Bradshaw RA, Blundell TL. Characterization of symmetric complexes of nerve growth factor and the ectodomain of the pan-neurotrophin receptor, p75NTR. J Biol Chem. 2005;280:33453–60.
257. Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, Davies AM. p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. Mol Cell Neurosci. 1999;14:28–40.
258. Karin M, Gallagher E. TNFR signaling: ubiquitin-conjugated TRAFfic signals control stop-and-go for MAPK signaling complexes. Immunol Rev. 2009;228:225–40.
259. Sato T, Irie S, Kitada S, Reed JC. FAP-1: a protein tyrosine phosphatase that associates with Fas. Science. 1995;268:411–5.
260. Nichols A, Martinou I, Maundrell K, Martinou JC. The p75 neurotrophin receptor: effects on neuron survival in vitro and interaction with death domain-containing adaptor proteins. Apoptosis. 1998;3:289–94.
261. Wang X, Bauer JH, Li Y, Shao Z, Zetoune FS, Cattaneo E, Vincenz C. Characterization of a p75(NTR) apoptotic signaling pathway using a novel cellular model. J Biol Chem. 2001; 276:33812–20.
262. Murray SS, Perez P, Lee R, Hempstead BL, Chao MV. A novel p75 neurotrophin receptor-related protein, NRH2, regulates nerve growth factor binding to the TrkA receptor. J Neurosci. 2004;24:2742–9.
263. Nykjaer A, Willnow TE, Petersen CM. p75NTR–live or let die. Curr Opin Neurobiol. 2005;15:49–57.
264. Casademunt E, Carter BD, Benzel I, Frade JM, Dechant G, Barde YA. The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. EMBO J. 1999;18:6050–61.
265. Salehi AH, Xanthoudakis S, Barker PA. NRAGE, a p75 neurotrophin receptor-interacting protein, induces caspase activation and cell death through a JNK-dependent mitochondrial pathway. J Biol Chem. 2002;277:48043–50.
266. Kimura MT, Irie S, Shoji-Hoshino S, Mukai J, Nadano D, Oshimura M, Sato TA. 14-3-3 is involved in p75 neurotrophin receptor-mediated signal transduction. J Biol Chem. 2001;276: 17291–300.
267. Mukai J, Hachiya T, Shoji-Hoshino S, Kimura MT, Nadano D, Suvanto P, Hanaoka T, Li Y, Irie S, Greene LA, Sato TA. NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated by the common neurotrophin receptor p75NTR. J Biol Chem. 2000;275:17566–70.
268. Kim T, Hempstead BL. NRH2 is a trafficking switch to regulate sortilin localization and permit proneurotrophin-induced cell death. EMBO J. 2009;28:1612–23.
269. Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA. Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science. 1994;265:1596–9.
270. Casaccia-Bonnefil P, Carter BD, Dobrowsky RT, Chao MV. Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature. 1996;383:716–9.

271. Chittka A, Chao MV. Identification of a zinc finger protein whose subcellular distribution is regulated by serum and nerve growth factor. Proc Natl Acad Sci USA. 1999;96:10705–10.
272. Petersen CM, Nielsen MS, Nykjaer A, Jacobsen L, Tommerup N, Rasmussen HH, Roigaard H, Gliemann J, Madsen P, Moestrup SK. Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. J Biol Chem. 1997;272:3599–605.
273. Mazella J, Chabry J, Kitabgi P, Vincent JP. Solubilization and characterization of active neurotensin receptors from mouse brain. J Biol Chem. 1988;263:144–9.
274. Kandror K, Pilch PF. Identification and isolation of glycoproteins that translocate to the cell surface from GLUT4-enriched vesicles in an insulin-dependent fashion. J Biol Chem. 1994;269:138–42.
275. Lin BZ, Pilch PF, Kandror KV. Sortilin is a major protein component of Glut4-containing vesicles. J Biol Chem. 1997;272:24145–7.
276. Hampe W, Rezgaoui M, Hermans-Borgmeyer I, Schaller HC. The genes for the human VPS10 domain-containing receptors are large and contain many small exons. Hum Genet. 2001;108:529–36.
277. Lewandrowski U, Moebius J, Walter U, Sickmann A. Elucidation of N-glycosylation sites on human platelet proteins: a glycoproteomic approach. Mol Cell Proteomics. 2006;5:226–33.
278. Wollscheid B, Bausch-Fluck D, Henderson C, O'Brien R, Bibel M, Schiess R, Aebersold R, Watts JD. Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. Nat Biotechnol. 2009;27:378–86.
279. Munck Petersen C, Nielsen MS, Jacobsen C, Tauris J, Jacobsen L, Gliemann J, Moestrup SK, Madsen P. Propeptide cleavage conditions sortilin/neurotensin receptor-3 for ligand binding. EMBO J. 1999;18:595–604.
280. Chabry J, Gaudriault G, Vincent JP, Mazella J. Implication of various forms of neurotensin receptors in the mechanism of internalization of neurotensin in cerebral neurons. J Biol Chem. 1993;268:17138–44.
281. Nielsen MS, Gustafsen C, Madsen P, Nyengaard JR, Hermey G, Bakke O, Mari M, Schu P, Pohlmann R, Dennes A, Petersen CM. Sorting by the cytoplasmic domain of the amyloid precursor protein binding receptor SorLA. Mol Cell Biol. 2007;27:6842–51.
282. Marcusson EG, Horazdovsky BF, Cereghino JL, Gharakhanian E, Emr SD. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the VPS10 gene. Cell. 1994; 77:579–86.
283. Westergaard UB, Sorensen ES, Hermey G, Nielsen MS, Nykjaer A, Kirkegaard K, Jacobsen C, Gliemann J, Madsen P, Petersen CM. Functional organization of the sortilin Vps10p domain. J Biol Chem. 2004;279:50221–9.
284. Puertollano R, Aguilar RC, Gorshkova I, Crouch RJ, Bonifacino JS. Sorting of mannose 6-phosphate receptors mediated by the GGAs. Science. 2001;292:1712–6.
285. Zhu Z, Friess H, diMola FF, Zimmermann A, Graber HU, Korc M, Büchler MW Nerve growth factor expression correlates with perineural invasion and pain in human pancreatic cancer. .J Clin Oncol. 1999 Aug;17(8):2419-28.
286. Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-B and BDNF in human neuroblastomas. Mol Cell Biol. 1994 Jan;14(1):759-67.
287. Siniscalco D, Giordano C, Rossi F, Maione S, de Novellis V. Role of neurotrophins in neuropathic pain. Curr Neuropharmacol. 2011 Dec;9(4):523-9.
288. Fryer RH, Kaplan DR, Kromer LF. Truncated trkB receptors on nonneuronal cells inhibit BDNF-induced neurite outgrowth in vitro. Exp Neurol. 1997 Dec;148(2):616-27.
289. Bothwell M. Evolution of the neurotrophin signaling system in invertebrates. Brain Behav Evol. 2006;68(3):124-32.
290. Bresnahan PA, Leduc R, Thomas L, Thorner J, Gibson HL, Brake AJ, Barr PJ, Thomas G. Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-beta-NGF in vivo. J Cell Biol. 1990 Dec;111(6 Pt 2):2851-9.

291. Seidah NG, Benjannet S, Pareek S, Savaria D, Hamelin J, Goulet B, Laliberte J, Lazure C, Chrétien M, Murphy RA. Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. Biochem J. 1996 Mar 15;314 (Pt 3):951-60.
292. Pérez P, Coll PM, Hempstead BL, Martín-Zanca D, Chao MV. NGF binding to the trk tyrosine kinase receptor requires the extracellular immunoglobulin-like domains. Mol Cell Neurosci. 1995 Apr;6(2):97-105.
293. Urfer R, Tsoulfas P, O'Connell L, Shelton DL, Parada LF, Presta LG. An immunoglobulin-like domain determines the specificity of neurotrophin receptors. EMBO J. 1995 Jun 15;14(12):2795-805.
294. Zhang W, Zeng YS, Wang JM, Ding Y, Li Y, Wu W. Neurotrophin-3 improves retinoic acid-induced neural differentiation of skin-derived precursors through a p75NTR-dependent signaling pathway. Neurosci Res. 2009 Jun;64(2):170-6. doi: 10.1016/j.neures.2009.02.010.
295. Martinowich K, Manji H, Lu B. New insights into BDNF function in depression and anxiety. Nat Neurosci. 2007 Sep;10(9):1089-93.
296. Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science. 2006 Oct 6;314(5796):140-3.

Chapter 18
The VEGF Receptor Family

Guanglei Zhuang and Napoleone Ferrara

18.1 Introduction

The formation of an intact vascular system is indispensible for embryonic development. The vasculature continues to remodel dynamically in the adult. In pathological states, abnormal neovascularization may occur and often contributes to human pathogenesis [1, 2]. It is now well established that vessel growth, remodeling, and maturation are highly complex and coordinated processes, engaging numerous molecular machineries to spatially and temporally regulate each step [3, 4]. Over the past decades, the roles of VEGF ligands and receptors have been intensely investigated and largely elucidated.

The mammalian VEGF ligands consist of five glycoproteins referred to as VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor (PlGF). These ligands bind to three related type III receptor tyrosine kinases (RTKs) known as VEGF receptor 1 (VEGFR1), VEGFR2, and VEGFR3 [5]. Different VEGF ligands have distinctive binding specificity and affinity for each of these VEGF receptors. VEGFA, VEGFB, and PlGF bind to VEGFR1; VEGFA binds to VEGFR2; VEGFC and VEGFD bind to VEGFR3 [6]. Proteolytic processing of VEGFC and VEGFD allows for binding to VEGFR2, albeit with lower affinity compared to VEGFR3 [7]. Ligand-bound VEGFRs share similar regulatory mechanisms with other well-characterized RTKs such as epidermal growth factor receptors (EGFRs) and platelet-derived growth factor receptors (PDGFRs). Receptors undergo dimerization and activation of the intracellular tyrosine kinase and provide docking sites for signal transducers [8].

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The downstream signaling events coordinately modulate cell proliferation, survival, and migration in endothelial and hematopoietic cells [9].

Aberrant angiogenesis contributes to many disease states, including inflammation, psoriasis, rheumatoid arthritis, intraocular neovascular disorders, and cancer [10]. Due to the important role of VEGF signaling in pathological angiogenesis, several VEGF-targeted agents have been developed and approved in an increasing number of human diseases [11–13]. Studies on these VEGF inhibitors in both the laboratory and the clinic have further expanded our knowledge of the VEGF pathway, but also raised a number of questions. Better understanding of the VEGF family of ligands and receptors holds great promise to improve current antiangiogenic medicines and to develop novel efficacious regimens.

18.2 The VEGF Ligands

18.2.1 VEGFA

VEGFA is a key regulator of blood vessel growth. The importance of VEGFA in early vasculogenesis and angiogenesis is underscored by embryonic lethality of mice lacking a single *vegfa* allele. The heterozygous mutants exhibit growth retardation and die between day 11 and 12 due to impaired blood island formation and angiogenesis [14, 15]. In addition, the dependence on VEGFA remains in early postnatal development and is eventually lost when the mice mature and reach adulthood [16]. During pathological angiogenesis associated with intraocular neovascular disorders and the vast majority of human tumors, VEGFA is often highly expressed and contributes to abnormal neovascularization [4].

Alternative exon splicing of VEGFA gene gives rise to several different isoforms [17, 18]. The major human isoforms are denoted VEGFA121, VEGFA165, VEGFA189, and VEGFA206, having 121, 165, 189, and 206 amino acids, respectively. Less frequent splice variants have been reported including VEGFA145 and VEGFA183. VEGFA isoforms have different affinities for heparan sulfate proteoglycans (HSPGs) on cell surface and in the extracellular matrix (ECM). The shortest form, VEGFA121, is a freely diffusible protein that does not bind to heparin or HSPGs [19]. In contrast, VEGFA189 and VEGFA206 bind to heparin with high affinity and consequently are almost completely sequestered in the extracellular matrix [19]. Native VEGFA, a diffusible heparin-binding protein, corresponds to the 165-amino acid isoform (VEGFA165). VEGFA165 has intermediary properties, as it is secreted, but a significant fraction remains bound to the cell surface and ECM. The bioavailability of VEGFA isoforms is also regulated by proteolytic processing. For example, plasmin has been reported to cleave VEGFA165 protein at the C-terminus to generate bioactive VEGFA110 [20]. Matrix metalloproteinases (MMPs) may also cleave VEGFA165 at the C-terminus to produce diffusible, non-heparin-binding

fragments [21]. Most cell types express several VEGFA variants simultaneously, with VEGFA121 and VEGFA165 being the predominant isoforms [22].

Genetic studies employing isoform-specific knockouts in mice have indicated that diffusible and HSPG-bound VEGFA isoforms have different roles in the development and patterning of the vascular system. The heparin-binding isoforms are indispensible because mice expressing only VEGFA120 (mouse VEGFA is one amino acid shorter than human VEGFA) die early postnatally and display impaired myocardial angiogenesis and ischemic cardiomyopathy [23]. Importantly, VEGFA120 alone was sufficient to support both vasculogenesis and angiogenesis to sustain embryonic development. However, the loss of heparin-binding VEGFA caused a striking reduction in vascular branching complexity due to abnormal localization of secreted VEGFA [24]. In sharp contrast, VEGFA188 induced the formation of ectopic and abnormally thin vessel branches during brain and retina vascularization. Mice that express solely VEGFA188 were underrepresented at birth, were less fertile, and had smaller litter sizes [25]. In agreement with these findings, mice expressing only VEGFA164 or VEGFA120/188 double heterozygotes are viable with no overt vascular branching abnormalities [24, 26]. Therefore, both heparin-binding and soluble VEGFA proteins are required for the formation of a normal branching vessel network.

18.2.2 VEGFB and PlGF

VEGFB is highly expressed in the myocardium, oxidative skeletal muscle, and brown adipose tissue. Human VEGFB is expressed as two different isoforms, VEGFB167 and VEGFB186 [27]. VEGFB167 is non-glycosylated, binds HSPGs, and is mostly sequestered in the extracellular matrix, while VEGFB186 is O-glycosylated and freely diffusible.

PlGF is predominantly expressed in the placenta, heart, and lungs [28]. In humans, four PlGF isoforms (PlGF1–4) have been described, whereas mice only express the equivalent of PlGF2 [29]. PlGF1 and PlGF3 are diffusible isoforms. PlGF2 and PlGF4 have heparin-binding domains [30]. It is noteworthy that both VEGFB and PlGF may form heterodimers with VEGFA.

Unlike VEGFA, neither VEGFB nor PlGF seems to play a major role in vascular development. Mice lacking VEGFB are viable and fertile, with no obvious morphological changes other than slightly reduced heart sizes [31]. Interestingly, a recent study has shown that VEGFB has an unexpected role in endothelial targeting of lipids to peripheral tissues, via transcriptional regulation of vascular fatty acid transport proteins [32]. Subsequently, the same group reported that targeting VEGFB restores insulin sensitivity and improves glucose tolerance by inhibiting endothelial-to-tissue lipid transport, opening promising avenues for diabetes therapy [33]. PlGF-deficient mice exhibit normal growth, viability, and health, indicating that PlGF is dispensable for vascular development and homeostasis [34].

The role of PlGF in pathological angiogenesis, particularly tumor angiogenesis, is controversial. It has been suggested that PlGF plays a role in tumor angiogenesis because *plgf* null embryonic stem cells developed small hypovascularized teratomas when implanted in plgf knockout mice [34]. In addition, treatment with an anti-PlGF monoclonal antibody (mAb) was reported to inhibit F4/80+ macrophage recruitment, angiogenesis, and tumor growth in multiple models. Importantly, anti-PlGF and anti-VEGFR2 mAb DC101 were reported to have additive effects in VEGFR2 antibody-resistant tumors, suggesting that anti-PlGF may be useful as an adjunct to VEGF pathway inhibitors [35]. However, in contrast to such findings, PlGF blockade using a panel of neutralizing antibodies did not result in significant inhibition of primary tumor growth or angiogenesis in 15 models [36]. Furthermore, mFlt(1-3)-IgG, a truncated soluble VEGFR1 variant that potently blocks VEGFA, VEGFB, and PlGF [37], has similar antitumor efficacy to an anti-VEGFA mAb [38]. Although small subsets of tumors did respond to anti-PlGF mAb, the efficacy correlated with VEGFR1 expression in tumor cells, but not with antiangiogenesis [39]. Collectively, these findings suggest that the role of PlGF in tumorigenesis largely consists of promoting autocrine/paracrine growth of tumor cells expressing a functional VEGFR1 rather than stimulation of angiogenesis. Further meticulous investigations are required to elucidate these discrepancies and to clarify the role of PlGF in tumor angiogenesis. Notably, a humanized anti-PlGF mAb developed by Roche (RO5323441) has been recently evaluated in multiple phase I clinical trials, and early results in recurrent glioblastoma suggest that anti-PlGF treatment does not appear to add on clinical activity observed for single-agent bevacizumab (ASCO annual meeting 2013; abstract 2092).

18.2.3 VEGFC and VEGFD

VEGFC plays a key role during embryonic and postnatal lymphatic angiogenesis. Homozygous deletion of the *vegfc* gene in mice leads to a complete absence of lymph vessels and embryonic lethality due to fluid accumulation in tissues, whereas blood vasculature appears to develop normally. Even heterozygous deletion results in postnatal defects associated with impaired lymphatic development, such as cutaneous lymphatic hypoplasia and lymphedema [40]. On the other hand, *vegfd* null mice displayed normal embryonic and postnatal lymphangiogenesis and efficient lymphatic function [41, 42], suggesting that, unlike VEGFC, VEGFD does not play a major role in lymphatic development or that its function is redundant. Both exogenous VEGFC and VEGFD induce sprouting lymphangiogenesis when overexpressed in transgenic mice or via viral transduction [43–46]. They are also upregulated in various tumor types, which may contribute to lymph node metastasis [47–49]. Therefore, it has been proposed that pro-lymphangiogenic therapies based

on VEGFC or VEGFD may be effective for treatment of lymphedema; conversely inhibition of VEGFC or VEGFD may prove crucial for inhibition of lymphatic metastasis in cancer [50].

18.3 The VEGF Receptors

VEGFR1 (Flt-1 or Fms-like tyrosine kinase-1), VEGFR2 (denoted KDR in the human and Flk-1 in the mouse), and VEGFR3 (Flt-4 or Fms-like tyrosine kinase-4) are separated into three major regions: an extracellular portion consisting of seven immunoglobulin (Ig)-like domains, a single transmembrane domain, and an intracellular region containing the tyrosine kinase domain [51]. The fifth Ig-like domain of VEGFR3 is proteolytically processed and the resulting subunits are held together by a disulfide bond [52]. The ligand-binding region in the extracellular domain is localized within the second and third Ig-like domains of VEGF receptors [51].

18.3.1 VEGFR1

VEGFR1 is expressed in vascular endothelial cells and in a range of non-endothelial cells, including hematopoietic stem cells, dendritic cells, osteoclasts, and monocytes/macrophages [53–56]. Furthermore, VEGFR1 expression has been observed in tumor cells [39]. VEGFR1 activation, using a variety of ligands, rarely results in effective endothelial cell mitogenesis [57, 58]. However, VEGFR1 stimulation has been reported to mediate paracrine release of growth factors such as HGF and CTGF from liver sinusoidal endothelial cells, independent of mitogenesis or angiogenesis [59]. This paracrine function has been shown to mediate liver regeneration following injury [59]. The biological significance of VEGFR1 expression in non-endothelial cells is not clear, although some studies have suggested that the receptor might play a regulatory role in cell proliferation and migration under some circumstances [51].

Gene targeting studies demonstrated that *vegfr1*$^{-/-}$ mice die in utero between days 8.5 and 9.5 [60]. Endothelial cell differentiation is normal in the homozygous embryos, but these cells do not organize into functional vascular channels. According to these studies, excessive density of endothelial progenitors leading to the vascular disorganization was responsible for the lethality [61]. These results indicate that VEGFR1 is a negative regulator of vascular development, at least during embryonic development. Interestingly, a targeted mutation in VEGFR1 resulting in lack of intracellular tyrosine kinase but intact extracellular and transmembrane domains does not result in lethality or any overt defect in the vasculature [62], suggesting that the VEGFR1 kinase activity is dispensable during embryogenesis. Therefore, the ligand-binding domain mediates the negative regulatory role of VEGFR1, most likely by trapping VEGFA

and thereby preventing activation of VEGFR2. In contrast, VEGFR1 kinase activity is required for VEGFA-stimulated migration of hematopoietic cells and PlGF- or VEGFA-induced proliferation of tumor cells [39, 54, 63].

Such a "decoy" function of VEGFR1 is also relevant to human diseases. In hemangioma endothelial cells and hemangioma tissues, expression of VEGFR1 is markedly reduced compared to normal controls. As a consequence of reduced VEGFR1 decoy function, VEGFR2 and its downstream targets are activated in a VEGFA-dependent manner, implying that inhibition of VEGFA-VEGFR2 signaling may be effective in hemangioma treatment [64]. Another example is peripartum cardiomyopathy (PPCM) that affects pregnant women who are near delivery. It has been suggested that PPCM is associated with abnormally high levels of sFLT-1 (a soluble isoform of VEGFR1), which may lead to reduced microvascular density and thereby induce cardiac dysfunction [65].

18.3.2 VEGFR2

There is now agreement that VEGFR2 mediates the majority of downstream effects of VEGFA, including microvascular permeability, endothelial cell proliferation, migration, and survival. The key role of VEGFR2 signaling in development is evidenced by lack of vasculogenesis and very poor hematopoiesis in *vegfr2* null mice, resulting in death in utero between day 8.5 and 9.5 [66]. Blood islands and organized blood vessels were completely absent in *vegfr2*-deficient embryos, indicating that VEGFR2 is required very early in the development of the endothelial lineage. The number of hematopoietic cells was significantly reduced, suggesting that VEGFR2 may be important for hematopoiesis as well [66]. Of note, the hematopoietic lineages were only derived from *vegfr2* wild-type, but not *vegfr2*$^{-/-}$, embryonic stem cells in chimeras, arguing that the hematopoietic defect in *vegfr2* null mice is not secondary to the lack of a suitable endothelial-lined microenvironment [67].

VEGFA signaling through VEGFR2 is thought to be the major pathway that activates and regulates angiogenesis. In sprouting angiogenesis, specialized endothelial tip cells, which produce dynamic filopodia, lead the outgrowth of blood vessel sprouts toward gradients of VEGFA. The stalk cells follow tip cells and proliferate to form lumenized network [68]. VEGFR2 is expressed in both subpopulations of endothelial cells and mediates both tip cell migration and stalk cell proliferation [69]. In addition, by shaping the Notch pathway that inhibits tip cell fate, VEGFR2 also controls the specification of endothelial cells into tip and stalk cells [70, 71]. On the other hand, Notch negatively regulates VEGFR2 expression in endothelial stalk cells [71, 72], thus forming a negative feedback loop. As a result, endothelial cells stimulated by VEGFA compete for the tip cell position via VEGFR2-induced Dll4 expression. The cell that produces more Dll4 can suppress VEGFR2 in its neighbor cells via activation of Notch signaling and will eventually become tip cells. Therefore, tip cells are distinguished from stalk cells by their stronger expression of VEGFR2 [69].

18.3.3 VEGFR3

VEGFR3 is initially widely distributed in all endothelia during early embryogenesis, but later in development, its expression becomes more restricted to lymphatic endothelial cells, with the exception of fenestrated capillaries and tumor vessels [73]. Because VEGFC is a prominent lymphangiogenic factor and shows adjacent expression pattern with VEGFR3 [45, 73, 74], it has been speculated that VEGFR3 mainly functions in the developing and adult lymphatic system. Indeed, human hereditary lymphedema is associated with heterozygous missense mutations of Flt-4, which inactivate the tyrosine kinase and lead to insufficient VEGFR3 signaling [75, 76]. Surprisingly, targeted inactivation of the gene encoding VEGFR3 resulted in defective blood vessel development and cardiovascular failure, suggesting that VEGFR3 has an essential role in the formation of the embryonic cardiovascular system before the emergence of the lymphatic vessels [77]. Although VEGFR3 can heterodimerize with VEGFR2 in endothelial cells, the null phenotype of VEGFR3 was distinct from that of VEGFR2, e.g., no major defects were observed in vasculogenesis or angiogenesis. Additionally, compound depletion of both VEGFC and VEGFD failed to recapitulate the early embryonic lethality observed in *vegfr3* null mice [78]. Therefore, VEGFR3 might be activated by means other than VEGFC or VEGFD, possibly due to as yet unidentified factor(s) or ligand-independent signaling mechanisms. Along this line, several studies using genetic models indicate that VEGFR3 can be phosphorylated in the absence of its ligands and has passive signaling modality, which regulates vascular remodeling in certain contexts [79–81]. Recently, by specifically deleting the ligand-binding domain of VEGFR3, Zhang et al. verified that the ligand-dependent VEGFR3 signaling pathway is only required for lymphatic, but not blood, vascular development [82].

Increasing interest in targeting VEGFR3 in cancer stems from the observation that both tumor lymphatic vessels and endothelial sprouts express high levels of VEGFR3 [50]. It has been shown that blocking VEGFR3 can suppress tumor lymphangiogenesis and thus lymph node metastasis [83–85]. Furthermore, Tammela and colleagues reported that VEGFR3 inhibition impairs angiogenic sprouting in tumors and may have addictive effects with VEGFR2 blockers [86]. Despite these promising results, other evidence suggests that the notion of targeting VEGFR3 as antiangiogenic or antimetastatic therapy needs to be more rigorously evaluated. For example, endothelial deletion of *vegfr3*, but not VEGFR3 antibodies, led to excessive angiogenic sprouting and branching, via decreasing the level of Notch signaling [79]. Therefore, VEGFR3 tyrosine kinase inhibitors or monoclonal antibodies blocking ligand binding may have opposite effects on tumor angiogenesis. In addition, different kinase inhibitors are not equivalent either. For instance, although both cediranib and vandetanib potently inhibit VEGFR3, cediranib significantly reduced lymphatic metastases, while vandetanib had no effect [87]. Clearly, further work is required to understand the effects of VEGFR3-targeted agents on tumor metastasis and angiogenesis.

18.4 VEGF Receptor Signal Transduction

VEGFR1 shows weaker kinase activity and signaling properties as compared with VEGFR2 (or VEGFR3) [58, 88], possibly due, at least in part, to an inhibitory sequence in the juxtamembrane domain that represses its activity [89]. As already noted, VEGFR1 stimulation results in little or absent endothelial cell mitogenesis. The tyrosine kinase domain of VEGFR1 is dispensable for embryonic angiogenesis, but necessary for monocyte/macrophage migration in response to VEGFA or PlGF [62]. VEGFR1 activation in monocytes results in activation of phosphatidylinositol-3 kinase (PI3K)-Akt, extracellular signal-regulated kinase (ERK), and nuclear factor of activated T cells (NFAT) pathways, leading to chemotaxis as well as to the induction of a series of inflammatory cytokines [90, 91]. Additionally, VEGFR1 is detectable in some tumor cell lines and appears to drive proliferation in vitro and in vivo. In such tumor cells, it has been shown that VEGFR1-mediated activation of mitogen-activated protein kinase (MAPK) induces cell proliferation and migration [39, 92, 93].

VEGFR2 undergoes dimerization and ligand-dependent tyrosine phosphorylation in endothelial cells. Several tyrosine residues have been shown to be phosphorylated, including Y801 in the juxtamembrane domain, Y951 in the kinase insert domain, Y1054 and Y1059 within the kinase domain, as well as Y1175 and Y1214 in the carboxy-terminal domain (reviewed in [9, 94]). It has been reported that phosphorylation at Y1054 and Y1059 in the kinase domain activation loop, which may be preceded by autophosphorylation at Y801 [95], is crucial for VEGFR2 kinase activity [96]. On the other hand, phosphorylated Y951, Y1054, and Y1059 serve as docking sites for different signal transduction mediators. For example, phosphorylated Y951 binds the T-cell-specific adapter (TSAd), an adaptor molecule implicated in tumor vascularization and growth [97, 98]. Phosphorylation of Y1214 triggers the recruitment of adaptor protein NCK and activation of the Src family kinase member Fyn [99]. Phosphorylation of Y1175 creates a binding site for several signaling mediators such as PLCγ [100, 101], SHB, and SCK [102, 103]. More importantly, genetic studies employing Y1173F knock-in mice revealed the essential role of Y1173 (human Y1175) in endothelial and hematopoietic cell development during embryogenesis [104]. Y1173F homozygotes die between E8.5 and E9.5 from lack of endothelial and hematopoietic progenitors, similar to the phenotype of *vegfr2*-deficient mice. In contrast, mice with Y1212F substitution (human Y1214) are viable and fertile [104].

A wide spectrum of signaling pathways has been identified downstream of VEGFR2. Among these, VEGFR2 induces the activation of the PI3K-Akt pathway which promotes cell survival and vascular permeability, the Raf-MEK-MAPK pathway which activates cell proliferation, the Src-FAK pathway which increases cell motility, and several other signal transduction molecules [6, 105]. Although in some cases the importance of one pathway has been validated by the vascular phenotypes of genetic models, often it remains a major challenge to elucidate the in vivo significance of these interacting signaling cascades. It is even more complex in the context

of pathological angiogenesis, particularly tumor angiogenesis, where dynamic signaling networks and vigorous reciprocal negative feedback loops coexist [106]. Innovative technologies and more systematic investigations will come together to delineate the important biology of VEGFR2, which holds the promises for efficacious therapeutics.

Similar to VEGFR2, ligand binding to VEGFR3 leads to kinase activation and phosphorylation of several tyrosine residues such as Y1230, Y1231, Y1265, Y1337, and Y1363 [107]. Phosphorylated Y1063 and Y1068, located in the kinase domain activation loop, are fundamental for VEGFR3 kinase activity [108]. VEGFR3 phosphorylation has been shown to lead to PI3K-dependent Akt activation and PKC-dependent activation of the MAPK pathway, which protect lymphatic endothelial cells from serum deprivation-induced apoptosis and induce cell proliferation [109].

18.5 VEGF-Targeted Therapies

The hypothesis that blocking angiogenesis could be used as a therapeutic strategy for treating a variety of disorders has been extensively tested and proven to be valid. Several VEGFA-VEGFR2 blockers have been approved for clinical use in cancer and eye diseases. To date, the US Food and Drug Administration (FDA) has approved 12 agents as anti-VEGF therapy: bevacizumab (Avastin, Genentech/Roche), sunitinib (Sutent, Pfizer), sorafenib (Nexavar, Bayer and Onyx), regorafenib (Stivarga, Onyx and Bayer), vandetanib (Caprelsa, AstraZeneca), axitinib (Inlyta, Pfizer), pazopanib (Votrient, GlaxoSmithKline), cabozantinib (Cometriq, Exelixis), ziv-aflibercept (Zaltrap, Regeneron and Sanofi-Aventis), pegaptanib (Macugen, Eyetech), ranibizumab (Lucentis, Genentech/Roche and Novartis), and aflibercept (Eylea, Regeneron and Bayer). These compounds could be roughly divided into three major categories.

18.5.1 Anti-VEGFA Antibodies

Bevacizumab is a recombinant humanized monoclonal anti-VEGFA antibody. It binds to all biologically active isoforms of VEGFA and neutralizes their activities by blocking the binding of VEGFA to VEGFR1 and VEGFR2. In randomized controlled clinical trials, bevacizumab treatment resulted in improved overall survival (OS) and/or progression-free survival (PFS) compared to standard cytotoxic chemotherapy in patients with metastatic colorectal cancer (mCRC) [110], non-small cell lung cancer (NSCLC) [111], recurrent glioblastoma multiforme (rGBM) [112], and metastatic renal cell carcinoma (mRCC) [113] and subsequently was approved for use in these indications. The initial FDA's accelerated approval of bevacizumab for the treatment of metastatic breast cancer (mBC) was revoked because two additional

clinical trials showed a smaller improvement in PFS compared to the first phase III study [114–116].

Despite its strong preclinical antitumor efficacy, bevacizumab does not provide benefit as a monotherapy in patients with advanced cancer, except in a few types of tumor such as glioblastoma multiforme, renal cell carcinoma, and recurrent ovarian carcinoma. However, in most preclinical studies, treatment was initiated shortly after tumor cell inoculation or with low tumor burden. It is possible that blocking VEGFA alone is not sufficient to induce tumor cell death. Indeed, numerous preclinical and clinical studies suggest that antiangiogenic therapy is cytostatic rather than cytocidal [117]. As expected, bevacizumab has proved to be quite effective when used in combination with cytotoxic therapy and/or radiotherapy. However, the mechanistic basis for combinatorial effects is elusive, and several proposed models need to be rigorously tested in clinic [12].

Ranibizumab is a high-affinity Fab variant of bevacizumab. Intravitreal administration of ranibizumab led to substantial preservation of vision in patients with neovascular AMD (age-related macular degeneration), based on which it was approved by the FDA in June 2006 [118, 119]. As a smaller derivative of the full-length antibodies, ranibizumab was designed to have better retina penetration, higher binding affinity for VEGFA, and lower risk to trigger complement-mediated or cell-dependent cytotoxicity caused by interaction of the antibody with Fc receptors in immune cells [13]. Head-to-head clinical trials comparing ranibizumab to bevacizumab suggest that both agents have comparable effects on visual acuity, whereas the rate of serious systemic adverse events is higher with bevacizumab than with ranibizumab [120, 121]. Nevertheless, many patients have been treated with bevacizumab off-label for neovascular AMD.

18.5.2 Small-Molecule VEGF RTK Inhibitors

Sunitinib, sorafenib, regorafenib, vandetanib, axitinib, pazopanib, and cabozantinib are all oral small-molecule tyrosine kinase inhibitors (TKI) targeting VEGFRs and other signaling pathways [122, 123]. These agents have been approved for treating different types of cancer. At present, sunitinib is approved in patients with gastrointestinal stromal tumor (GIST), mRCC, and pancreatic neuroendocrine tumor (PNET); sorafenib is approved in patients with mRCC and unresectable hepatocellular carcinoma; regorafenib is approved in patients with mCRC and advanced GIST; vandetanib is approved in patients with late-stage medullary thyroid carcinoma; axitinib is approved in patients with mRCC after the failure of one prior systemic therapy; pazopanib is approved in patients with mRCC and advanced soft tissue sarcoma; cabozantinib is FDA-approved in patients with medullary thyroid cancer.

In addition to targeting VEGFRs, different TKIs also inhibit other kinases to variable degrees, including PDGFRs, Fms-like tyrosine kinase-3 (Flt-3), c-Kit,

RAF, and RET [122]. Thus, besides antiangiogenic functions, TKIs might have direct antitumor effects by inhibiting oncogenic kinases or influence other stromal cell compartments such as fibroblasts and pericytes. As a result, multiple mechanisms may account for the efficacy of these therapies, and it is important to emphasize the complex mechanism of action in order to investigate drug resistance or to identify predictive biomarkers.

18.5.3 VEGF-Trap

Aflibercept (or ziv-aflibercept) is a VEGF decoy receptor, termed VEGF-Trap, consisting of a chimeric ligand-binding domain from VEGFR1 and VEGFR2 fused to the Fc portion of the human immunoglobulin (Ig) gamma chain, which makes it a disulfide-linked dimer [124]. Aflibercept binds all isoforms of VEGFA as well as PlGF, and thereby inhibits the activation of the cognate VEGF receptors. Two randomized, double-masked, active-controlled trials in patients with neovascular (wet) AMD demonstrated that injections of aflibercept every 8 weeks (after the initial three monthly doses) improve vision comparably to ranibizumab administered every 4 weeks [125, 126]. Consequently, aflibercept (Eylea) has been approved by the FDA for the treatment of patients with wet AMD. Additionally, aflibercept plus FOLFIRI (5-fluorouracil, leucovorin, and irinotecan) significantly prolonged both progression-free survival and overall survival in patients with metastatic colorectal cancer who have progressed on an oxaliplatin-containing chemotherapy regimen [127]. As a result of this double-blinded phase III trial, aflibercept (Zaltrap) received FDA approval in combination with FOLFIRI for mCRC patients. However, unlike bevacizumab, Zaltrap failed to result in a survival benefit in patients with previously untreated mCRC or NSCLC [128].

18.6 Conclusions and Prospects

Research conducted in the past several decades has established that the VEGF family plays an important role in regulating embryogenesis and angiogenesis during normal development and in a number of pathologic conditions. The VEGF ligands and their receptors are indispensible for the development of the blood vascular system, via vasculogenic and angiogenic mechanisms, as well as for the formation of the lymphatic vascular system. These molecules have also been implicated in pathologic processes, such as tumor progression and ocular neovascular-related diseases, leading to the clinical development of a variety of VEGF inhibitors. As anti-VEGF therapies continue to be formulated and developed, a complete and extensive understanding of the VEGF biology will remain crucial for potential breakthroughs in the paradigm of angiogenesis and antiangiogenic treatments.

VEGF inhibitors have had an especially dramatic impact in nonmalignant conditions such as intraocular neovascular disorders. The availability of such agents has changed the course of wet AMD and potentially other intraocular diseases [129]. Whether the high level of VEGF dependence in these disorders as compared to cancer reflects lack of genomic instability and accumulation of mutations remains to be established.

Notwithstanding the clinical success in the treatment of malignancies, an extremely important and timely question is how antiangiogenic medicines can be improved. VEGF inhibitors so far are the only targeted therapies administered without a predictive biomarker. It is widely believed that in the absence of such biomarkers, the benefits of most targeted anticancer therapies would be considerably reduced. While overall benefits of anti-VEGF therapy have been observed in unselected patient populations, identifying patients most likely responsive to such therapy would be a major advantage. This highlights the urgent need to identify predictive biomarkers, which remain elusive despite extensive investigations. Recent reports have suggested several potential candidates including circulating VEGFA [130], tumor expression of neuropilin-1 [130], and a VEGF-dependent vascular gene signature [131], all of which require clinical validation in appropriate prospective studies. Another key aspect toward optimized usage of VEGF pathway inhibitors is a better understanding of the intrinsic or acquired resistance mechanisms that limit their efficacy [132, 133]. In order to identify predictive markers or overcome drug resistance, it is necessary to precisely understand the mechanisms of different VEGF-targeted agents in each specific tumor type.

Most laboratory investigations and clinical studies have focused on the VEGFA-VEGFR2 signaling axis and its inhibition. While the functions of other VEGF ligands and receptors in malignancies and non-neoplastic disorders remain to be fully characterized, they may also represent therapeutic targets. In addition, VEGF signals regulate and crosstalk with multiple signaling pathways, such as Notch, angiopoietin receptors, and integrins, that operate in endothelial cells in a context-dependent manner. Therapeutic agents modulating these pathways have been generated and are currently under clinical assessments. In the future, we will need to design better combination regimens with these new agents, both as a means of increasing antiangiogenic activity and to overcome tumor resistance to anti-VEGF therapies. Furthermore, one major challenge surrounding antiangiogenic therapies is to identify their potential positive or negative interactions with conventional chemotherapy and other targeted cancer drugs. For example, bevacizumab has demonstrated clinical benefits when added to certain cytotoxic agents. However, combining bevacizumab with EGFR antibodies (cetuximab or panitumumab) showed decreased efficacy in colorectal cancer [134, 135]. With more and more oncologic therapies becoming available for clinical development, it should be possible to identify effective combinatorial strategies with VEGF inhibitors, which is expected to improve outcomes of cancer patients.

Receptor at a glance: VEGFR1

Chromosome location	13q12
Gene size (bp)	194,744
Intron/exon numbers	29/30
mRNA size (5′, ORF, 3′)	285, 4017, 2821
Amino acid number	1,338
kDa	151
Posttranslational modifications	Y794, Y1048, Y1053, Y1169, Y1213,
	Y1242, Y1327, Y1333 phosphorylation
Domains	7 immunoglobulin (Ig)-like domains
	1 transmembrane domain
	1 kinase domain
Ligands	VEGFA, VEGFB, PlGF
Known dimerization partners	VEGFR1, VEGFR2,
	neuropilin-1, neuropilin-2
Pathways activated	PI3K, MAPK, NFAT
Tissues expressed	Vascular endothelial cells
	Placental trophoblast cells
	Peripheral blood monocytes
Human diseases	Peripartum cardiomyopathy
	Cancer
Knockout mouse phenotype	Die in utero at mid-somite stages due to abnormal vascular channels

Receptor at a glance: VEGFR2

Chromosome location	4q11–q12
Gene size (bp)	47,113
Intron/exon numbers	29/30
mRNA size (5′, ORF, 3′)	302, 4071, 1982
Amino acid number	1,356
kDa	152
Posttranslational modifications	Y951, Y996, Y1054, Y1059, Y1175,
	Y1214, Y1238 phosphorylation
Domains	7 immunoglobulin (Ig)-like domains
	1 transmembrane domain
	1 kinase domain
Ligands	VEGFA, VEGFC, VEGFD
Known dimerization partners	VEGFR1, VEGFR2, VEGFR3,
	Neuropilin-1, neuropilin-2
Pathways activated	PI3K, MAPK, PLCG1, NOS3, SRC
Tissues expressed	Vascular endothelial cells
	Hematopoietic stem cells
	Endocardial cells

(continued)

Human diseases	Hemangioma capillary infantile
	Cancer
	Vascular diseases
Knockout mouse phenotype	Die in utero due to early defects in the development of hematopoietic and endothelial cells

Receptor at a glance: VEGFR3

Chromosome location	5q35.3
Gene size (bp)	48,119
Intron/exon numbers	29/30
mRNA size (5′, ORF, 3′)	79, 4092, 1686
Amino acid number	1,363
kDa	153
Posttranslational modifications	Y517, T532, Y1230, Y1231, Y1265,
	Y1333, Y1337, Y1363 phosphorylation
Domains	7 immunoglobulin (Ig)-like domains
	1 transmembrane domain
	1 kinase domain
Ligands	VEGFC, VEGFD
Known dimerization partners	VEGFR2, VEGFR3, neuropilin-2
Pathways activated	PI3K, MAPK, JUN
Tissues expressed	Vascular endothelial cells
	Lymphatic endothelial cells
Human diseases	Lymphedema hereditary type 1A
	Hemangioma capillary infantile
	Cancer
Knockout mouse phenotype	Die in utero due to defective blood vessel development and cardiovascular failure

References

1. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med. 1995;1(1):27–31.
2. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000;407(6801):249–57.
3. Chung AS, Ferrara N. Developmental and pathological angiogenesis. Annu Rev Cell Dev Biol. 2011;27:563–84.
4. Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. Nat Rev Cancer. 2010;10(7):505–14.
5. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9(6):669–76.
6. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling—in control of vascular function. Nat Rev Mol Cell biol. 2006;7(5):359–71.
7. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J. 1997;16(13):3898–911.
8. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010; 141(7):1117–34.

9. Matsumoto T, Claesson-Welsh L. VEGF receptor signal transduction. Science's STKE. 2001;2001(112):21.
10. Carmeliet P. Angiogenesis in life, disease and medicine. Nature. 2005;438(7070):932–6.
11. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov. 2004;3(5):391–400.
12. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer. 2008;8(8):579–91.
13. Ferrara N. Vascular endothelial growth factor and age-related macular degeneration: from basic science to therapy. Nat Med. 2010;16(10):1107–11.
14. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature. 1996;380(6573):439–42.
15. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996;380(6573):435–9.
16. Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, et al. VEGF is required for growth and survival in neonatal mice. Development. 1999;126(6):1149–59.
17. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989;246(4935):1306–9.
18. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol. 1991;5(12):1806–14.
19. Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell. 1993;4(12):1317–26.
20. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem. 1992;267(36):26031–7.
21. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol. 2005;169(4):681–91.
22. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 1999;13(1):9–22.
23. Carmeliet P, Ng YS, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, et al. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med. 1999;5(5):495–502.
24. Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, et al. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev. 2002;16(20):2684–98.
25. Stalmans I, Ng YS, Rohan R, Fruttiger M, Bouche A, Yuce A, et al. Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. J Clin Invest. 2002; 109(3):327–36.
26. Maes C, Stockmans I, Moermans K, Van Looveren R, Smets N, Carmeliet P, et al. Soluble VEGF isoforms are essential for establishing epiphyseal vascularization and regulating chondrocyte development and survival. J Clin Invest. 2004;113(2):188–99.
27. Olofsson B, Pajusola K, von Euler G, Chilov D, Alitalo K, Eriksson U. Genomic organization of the mouse and human genes for vascular endothelial growth factor B (VEGF-B) and characterization of a second splice isoform. J Biol Chem. 1996;271(32):19310–7.
28. Persico MG, Vincenti V, DiPalma T. Structure, expression and receptor-binding properties of placenta growth factor (PlGF). Curr Top Microbiol Immunol. 1999;237:31–40.
29. Dewerchin M, Carmeliet P. PlGF: a multitasking cytokine with disease-restricted activity. Cold Spring Harb Perspect Med. 2012;2(8).
30. Yang W, Ahn H, Hinrichs M, Torry RJ, Torry DS. Evidence of a novel isoform of placenta growth factor (PlGF-4) expressed in human trophoblast and endothelial cells. J Reprod Immunol. 2003;60(1):53–60.

31. Bellomo D, Headrick JP, Silins GU, Paterson CA, Thomas PS, Gartside M, et al. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Circ Res. 2000;86(2):E29–35.
32. Hagberg CE, Falkevall A, Wang X, Larsson E, Huusko J, Nilsson I, et al. Vascular endothelial growth factor B controls endothelial fatty acid uptake. Nature. 2010;464(7290):917–21.
33. Hagberg CE, Mehlem A, Falkevall A, Muhl L, Fam BC, Ortsater H, et al. Targeting VEGF-B as a novel treatment for insulin resistance and type 2 diabetes. Nature. 2012; 490(7420):426–30.
34. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med. 2001;7(5):575–83.
35. Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell. 2007;131(3):463–75.
36. Bais C, Wu X, Yao J, Yang S, Crawford Y, McCutcheon K, et al. PlGF blockade does not inhibit angiogenesis during primary tumor growth. Cell. 2010;141(1):166–77.
37. Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, et al. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med. 1998;4(3):336–40.
38. Shojaei F, Wu X, Malik AK, Zhong C, Baldwin ME, Schanz S, et al. Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. Nat Biotechnol. 2007;25(8):911–20.
39. Yao J, Wu X, Zhuang G, Kasman IM, Vogt T, Phan V, et al. Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PlGF antibodies efficacy. Proc Natl Acad Sci USA. 2011;108(28):11590–5.
40. Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol. 2004;5(1):74–80.
41. Baldwin ME, Halford MM, Roufail S, Williams RA, Hibbs ML, Grail D, et al. Vascular endothelial growth factor D is dispensable for development of the lymphatic system. Mol Cell Biol. 2005;25(6):2441–9.
42. Koch M, Dettori D, Van Nuffelen A, Souffreau J, Marconcini L, Wallays G, et al. VEGF-D deficiency in mice does not affect embryonic or postnatal lymphangiogenesis but reduces lymphatic metastasis. J Pathol. 2009;219(3):356–64.
43. Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, et al. VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. Circ Res. 2003;92(10):1098–106.
44. Tammela T, Saaristo A, Holopainen T, Lyytikka J, Kotronen A, Pitkonen M, et al. Therapeutic differentiation and maturation of lymphatic vessels after lymph node dissection and transplantation. Nat Med. 2007;13(12):1458–66.
45. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science. 1997;276(5317):1423–5.
46. Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, et al. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. EMBO J. 2001;20(6):1223–31.
47. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, et al. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med. 2001;7(2):186–91.
48. Kopfstein L, Veikkola T, Djonov VG, Baeriswyl V, Schomber T, Strittmatter K, et al. Distinct roles of vascular endothelial growth factor-D in lymphangiogenesis and metastasis. Am J Pathol. 2007;170(4):1348–61.
49. Kurahara H, Takao S, Maemura K, Shinchi H, Natsugoe S, Aikou T. Impact of vascular endothelial growth factor-C and -D expression in human pancreatic cancer: its relationship to lymph node metastasis. Clin Cancer Res. 2004;10(24):8413–20.
50. Lohela M, Bry M, Tammela T, Alitalo K. VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. Curr Opin Cell Biol. 2009;21(2):154–65.

51. Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp Cell Res. 2006;312(5):549–60.
52. Pajusola K, Aprelikova O, Pelicci G, Weich H, Claesson-Welsh L, Alitalo K. Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. Oncogene. 1994;9(12):3545–55.
53. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, et al. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood. 2001;97(3):785–91.
54. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood. 1996;87(8):3336–43.
55. Dikov MM, Ohm JE, Ray N, Tchekneva EE, Burlison J, Moghanaki D, et al. Differential roles of vascular endothelial growth factor receptors 1 and 2 in dendritic cell differentiation. J Immunol. 2005;174(1):215–22.
56. Kaipainen A, Korhonen J, Pajusola K, Aprelikova O, Persico MG, Terman BI, et al. The related FLT4, FLT1, and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells. J Exp Med. 1993;178(6):2077–88.
57. Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem. 1994;269(41):25646–54.
58. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem. 1994;269(43):26988–95.
59. LeCouter J, Moritz DR, Li B, Phillips GL, Liang XH, Gerber HP, et al. Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. Science. 2003; 299(5608):890–3.
60. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature. 1995;376(6535):66–70.
61. Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development. 1999;126(13):3015–25.
62. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci USA. 1998;95(16):9349–54.
63. Clauss M, Weich H, Breier G, Knies U, Rockl W, Waltenberger J, et al. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. J Biol Chem. 1996;271(30):17629–34.
64. Jinnin M, Medici D, Park L, Limaye N, Liu Y, Boscolo E, et al. Suppressed NFAT-dependent VEGFR1 expression and constitutive VEGFR2 signaling in infantile hemangioma. Nat Med. 2008;14(11):1236–46.
65. Patten IS, Rana S, Shahul S, Rowe GC, Jang C, Liu L, et al. Cardiac angiogenic imbalance leads to peripartum cardiomyopathy. Nature. 2012;485(7398):333–8.
66. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995;376(6535):62–6.
67. Shalaby F, Ho J, Stanford WL, Fischer KD, Schuh AC, Schwartz L, et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell. 1997;89(6): 981–90.
68. Phng LK, Gerhardt H. Angiogenesis: a team effort coordinated by notch. Dev Cell. 2009;16(2):196–208.
69. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol. 2003; 161(6):1163–77.

70. Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, et al. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci USA. 2007;104(9):3219–24.
71. Suchting S, Freitas C, le Noble F, Benedito R, Breant C, Duarte A, et al. The notch ligand delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci USA. 2007;104(9):3225–30.
72. Williams CK, Li JL, Murga M, Harris AL, Tosato G. Up-regulation of the notch ligand delta-like 4 inhibits VEGF-induced endothelial cell function. Blood. 2006;107(3):931–9.
73. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA. 1995;92(8):3566–70.
74. Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V, et al. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development. 1996;122(12):3829–37.
75. Karkkainen MJ, Ferrell RE, Lawrence EC, Kimak MA, Levinson KL, McTigue MA, et al. Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. Nat Genet. 2000;25(2):153–9.
76. Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K, Vikkula M. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. Am J Hum Genet. 2000;67(2):295–301.
77. Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, et al. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science. 1998; 282(5390):946–9.
78. Haiko P, Makinen T, Keskitalo S, Taipale J, Karkkainen MJ, Baldwin ME, et al. Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. Mol Cell Biol. 2008;28(15):4843–50.
79. Tammela T, Zarkada G, Nurmi H, Jakobsson L, Heinolainen K, Tvorogov D, et al. VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. Nat Cell Biol. 2011;13(10):1202–13.
80. Galvagni F, Pennacchini S, Salameh A, Rocchigiani M, Neri F, Orlandini M, et al. Endothelial cell adhesion to the extracellular matrix induces c-Src-dependent VEGFR-3 phosphorylation without the activation of the receptor intrinsic kinase activity. Circ Res. 2010;106(12):1839–48.
81. Benedito R, Rocha SF, Woeste M, Zamykal M, Radtke F, Casanovas O, et al. Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF-VEGFR2 signalling. Nature. 2012;484(7392):110–4.
82. Zhang L, Zhou F, Han W, Shen B, Luo J, Shibuya M, et al. VEGFR-3 ligand-binding and kinase activity are required for lymphangiogenesis but not for angiogenesis. Cell Res. 2010;20(12):1319–31.
83. He Y, Kozaki K, Karpanen T, Koshikawa K, Yla-Herttuala S, Takahashi T, et al. Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J Natl Cancer Inst. 2002;94(11):819–25.
84. Burton JB, Priceman SJ, Sung JL, Brakenhielm E, An DS, Pytowski B, et al. Suppression of prostate cancer nodal and systemic metastasis by blockade of the lymphangiogenic axis. Cancer Res. 2008;68(19):7828–37.
85. Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, Wu Y, et al. Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. Cancer Res. 2006;66(5):2650–7.
86. Tammela T, Zarkada G, Wallgard E, Murtomaki A, Suchting S, Wirzenius M, et al. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature. 2008;454(7204):656–60.
87. Padera TP, Kuo AH, Hoshida T, Liao S, Lobo J, Kozak KR, et al. Differential response of primary tumor versus lymphatic metastasis to VEGFR-2 and VEGFR-3 kinase inhibitors cediranib and vandetanib. Mol Cancer Ther. 2008;7(8):2272–9.

88. Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. Oncogene. 1995;10(1):135–47.
89. Gille H, Kowalski J, Yu L, Chen H, Pisabarro MT, Davis-Smyth T, et al. A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3′-kinase activation and endothelial cell migration. EMBO J. 2000;19(15):4064–73.
90. Selvaraj SK, Giri RK, Perelman N, Johnson C, Malik P, Kalra VK. Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor. Blood. 2003;102(4):1515–24.
91. Ding Y, Huang Y, Song N, Gao X, Yuan S, Wang X, et al. NFAT1 mediates placental growth factor-induced myelomonocytic cell recruitment via the induction of TNF-alpha. J Immunol. 2010;184(5):2593–601.
92. Fan F, Wey JS, McCarty MF, Belcheva A, Liu W, Bauer TW, et al. Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. Oncogene. 2005;24(16):2647–53.
93. Taylor AP, Leon E, Goldenberg DM. Placental growth factor (PlGF) enhances breast cancer cell motility by mobilising ERK1/2 phosphorylation and cytoskeletal rearrangement. Br J Cancer. 2010;103(1):82–9.
94. Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med. 2012;2(7):a006502.
95. Solowiej J, Bergqvist S, McTigue MA, Marrone T, Quenzer T, Cobbs M, et al. Characterizing the effects of the juxtamembrane domain on vascular endothelial growth factor receptor-2 enzymatic activity, autophosphorylation, and inhibition by axitinib. Biochemistry. 2009;48(29):7019–31.
96. Kendall RL, Rutledge RZ, Mao X, Tebben AJ, Hungate RW, Thomas KA. Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. J Biol Chem. 1999;274(10):6453–60.
97. Matsumoto T, Bohman S, Dixelius J, Berge T, Dimberg A, Magnusson P, et al. VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. EMBO J. 2005;24(13):2342–53.
98. Wu LW, Mayo LD, Dunbar JD, Kessler KM, Ozes ON, Warren RS, et al. VRAP is an adaptor protein that binds KDR, a receptor for vascular endothelial cell growth factor. J Biol Chem. 2000;275(9):6059–62.
99. Lamalice L, Houle F, Huot J. Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK2/p38 activation and endothelial cell migration in response to VEGF. J Biol Chem. 2006;281(45):34009–20.
100. Takahashi T, Yamaguchi S, Chida K, Shibuya M. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. EMBO J. 2001;20(11):2768–78.
101. Cunningham SA, Arrate MP, Brock TA, Waxham MN. Interactions of FLT-1 and KDR with phospholipase C gamma: identification of the phosphotyrosine binding sites. Biochem Biophys Res Commun. 1997;240(3):635–9.
102. Holmqvist K, Cross MJ, Rolny C, Hagerkvist R, Rahimi N, Matsumoto T, et al. The adaptor protein shb binds to tyrosine 1175 in vascular endothelial growth factor (VEGF) receptor-2 and regulates VEGF-dependent cellular migration. J Biol Chem. 2004;279(21):22267–75.
103. Warner AJ, Lopez-Dee J, Knight EL, Feramisco JR, Prigent SA. The Shc-related adaptor protein, Sck, forms a complex with the vascular-endothelial-growth-factor receptor KDR in transfected cells. Biochem J. 2000;347(Pt 2):501–9.
104. Sakurai Y, Ohgimoto K, Kataoka Y, Yoshida N, Shibuya M. Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. Proc Natl Acad Sci USA. 2005;102(4):1076–81.
105. Cross MJ, Dixelius J, Matsumoto T, Claesson-Welsh L. VEGF-receptor signal transduction. Trends Biochem Sci. 2003;28(9):488–94.

106. Zhuang G, Yu K, Jiang Z, Chung A, Yao J, Ha C, et al. Phosphoproteomic analysis implicates the mTORC2-FoxO1 axis in VEGF signaling and feedback activation of receptor tyrosine kinases. Sci Signal. 2013;6(271):ra25.
107. Dixelius J, Makinen T, Wirzenius M, Karkkainen MJ, Wernstedt C, Alitalo K, et al. Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem. 2003;278(42):40973–9.
108. Salameh A, Galvagni F, Bardelli M, Bussolino F, Oliviero S. Direct recruitment of CRK and GRB2 to VEGFR-3 induces proliferation, migration, and survival of endothelial cells through the activation of ERK, AKT, and JNK pathways. Blood. 2005;106(10):3423–31.
109. Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, et al. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. EMBO J. 2001;20(17):4762–73.
110. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med. 2004;350(23):2335–42.
111. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 2006; 355(24):2542–50.
112. Friedman HS, Prados MD, Wen PY, Mikkelsen T, Schiff D, Abrey LE, et al. Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. J Clin Oncol. 2009;27(28):4733–40.
113. Escudier B, Pluzanska A, Koralewski P, Ravaud A, Bracarda S, Szczylik C, et al. Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. Lancet. 2007;370(9605):2103–11.
114. Miles DW, Chan A, Dirix LY, Cortes J, Pivot X, Tomczak P, et al. Phase III study of bevacizumab plus docetaxel compared with placebo plus docetaxel for the first-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. J Clin Oncol. 2010;28(20):3239–47.
115. Robert NJ, Dieras V, Glaspy J, Brufsky AM, Bondarenko I, Lipatov ON, et al. RIBBON-1: randomized, double-blind, placebo-controlled, phase III trial of chemotherapy with or without bevacizumab for first-line treatment of human epidermal growth factor receptor 2-negative, locally recurrent or metastatic breast cancer. J Clin Oncol. 2011;29(10): 1252–60.
116. Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med. 2007; 357(26):2666–76.
117. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer. 2002;2(10):727–39.
118. Rosenfeld PJ, Brown DM, Heier JS, Boyer DS, Kaiser PK, Chung CY, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med. 2006;355(14):1419–31.
119. Brown DM, Kaiser PK, Michels M, Soubrane G, Heier JS, Kim RY, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med. 2006;355(14): 1432–44.
120. Martin DF, Maguire MG, Fine SL, Ying GS, Jaffe GJ, Grunwald JE, et al. Ranibizumab and bevacizumab for treatment of neovascular age-related macular degeneration: two-year results. Ophthalmology. 2012;119(7):1388–98.
121. Martin DF, Maguire MG, Ying GS, Grunwald JE, Fine SL, Jaffe GJ. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. N Engl J Med. 2011; 364(20):1897–908.
122. Ivy SP, Wick JY, Kaufman BM. An overview of small-molecule inhibitors of VEGFR signaling. Nat Rev Clin Oncol. 2009;6(10):569–79 [Review].
123. Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. Nat Rev Cancer. 2009;9(1):28–39.

124. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA. 2002;99(17):11393–8.
125. Heier JS, Brown DM, Chong V, Korobelnik JF, Kaiser PK, Nguyen QD, et al. Intravitreal aflibercept (VEGF trap-eye) in wet age-related macular degeneration. Ophthalmology. 2012;119(12):2537–48.
126. Ohr M, Kaiser PK. Intravitreal aflibercept injection for neovascular (wet) age-related macular degeneration. Expert Opin Pharmacother. 2012;13(4):585–91.
127. Van Cutsem E, Tabernero J, Lakomy R, Prenen H, Prausova J, Macarulla T, et al. Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. J Clin Oncol. 2012;30(28):3499–506.
128. Ramlau R, Gorbunova V, Ciuleanu TE, Novello S, Ozguroglu M, Goksel T, et al. Aflibercept and Docetaxel versus Docetaxel alone after platinum failure in patients with advanced or metastatic non-small-cell lung cancer: a randomized, controlled phase III trial. J Clin Oncol. 2012;30(29):3640–7.
129. Campbell JP, Bressler SB, Bressler NM. Impact of availability of anti-vascular endothelial growth factor therapy on visual impairment and blindness due to neovascular age-related macular degeneration. Arch Ophthalmol. 2012;130(6):794–5.
130. Van Cutsem E, de Haas S, Kang YK, Ohtsu A, Tebbutt NC, Ming Xu J, et al. Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a biomarker evaluation from the AVAGAST randomized phase III trial. J Clin Oncol. 2012;30(17):2119–27.
131. Brauer MJ, Zhuang G, Schmidt M, Yao J, Wu X, Kaminker JS, et al. Identification and analysis of in vivo VEGF downstream markers link VEGF pathway activity with efficacy of anti-VEGF therapies. Clin Cancer Res. 2013;19(13):3681–92.
132. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer. 2008;8(8):592–603.
133. Jain RK, Duda DG, Willett CG, Sahani DV, Zhu AX, Loeffler JS, et al. Biomarkers of response and resistance to antiangiogenic therapy. Nat Rev Clin Oncol. 2009;6(6):327–38.
134. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med. 2009; 360(6):563–72.
135. Hecht JR, Mitchell E, Chidiac T, Scroggin C, Hagenstad C, Spigel D, et al. A randomized phase IIIB trial of chemotherapy, bevacizumab, and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer. J Clin Oncol. 2009;27(5):672–80.

Chapter 19
The NOK Receptor Family

Li Liu

Abbreviations

CDK	Cyclin-dependent kinases
CNPC	Castration-naïve prostate cancer
CRPC	Castration-resistant prostate cancer
DFG	Asp-Phe-Gly
EGFR	Epidermal growth factor receptor
EPOR	Erythropoietin receptor
ERα	Estrogen receptor-alpha
ERK1/2	Extracellular signal-regulated kinases 1/2 (ERK1/2)
GSK-3β	Glycogen synthase kinase-3 beta
H&E staining	Hematoxylin-eosin staining
JAK	Janus kinase
NKT	Natural killer T cells
NOK	Novel oncogene with kinase domain
PC	Prostate cancer
PI3K	Phosphoinositide 3 kinase
RAS/MAPK	Ras/mitogen-activated protein kinase (MAPK) pathway
RPTK	Receptor protein tyrosine kinase
Serine/threonine-specific kinase	Akt
STAT	Signal transducers and activators of transcription
STYK1	Serine/threonine/tyrosine kinase 1

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19.1 Introduction to the Novel Oncogene with Kinase Domain Tyrosine Kinase Family

Structurally, the receptor protein tyrosine kinase (RPTK) molecule is composed of three main elements: a complete extracellular domain, a typical transmembrane domain, and an intact intracellular domain [1]. However, the novel identified oncogene with kinase domain (NOK, other aliases: STYK1, SuRTK106, DKFZp761P10101) presents a unique structure feature that it lacks an intact extracellular domain but possesses a typical transmembrane helix and a complete intracellular domain. This gene was first identified as a novel tyrosine kinase molecule [2] and later found to be a potent oncogene with strong tumorigenic and metastatic properties [3]. Although NOK carries a putative transmembrane domain, it is not destined to cell membrane due to the lack of a signal peptide at its amino terminus. The homologous proteins for NOK can be found in at least 16 species, for example, chimpanzee (96 %), mouse (78 %), rat (79 %), cow (77 %), dog (81 %), chicken (56 %), and zebrafish (46 %). Among the members of RPTK superfamily, only FGFRs and PDGFRs have relatively higher homology (20–30%) with NOK. Therefore, NOK is a unique RPTK gene and may stand up for a distinct receptor tyrosine kinase family.

19.2 NOK Gene: Gene/Promoter/mRNA Structures and Transcriptional Regulation

NOK gene is mapped to the minus strand of human chromosome 12 at 12p13.2 and contains at least 11 exons that span more than 55-kb genomic region (Fig. 19.1). The coding sequence of NOK is composed of 1,269 nucleotides that starts at exon 4 and extends to exon 11 (see Table "Receptor at a glance: NOK"). The potential promoter of *NOK* gene was analyzed with a 100-kb genomic sequence (derived from genomic contig with GenBank accession no. NT_009714.16) immediate upstream of the 5′ end of *NOK* cDNA with computational analysis such as PROSCAN and FirstEF programs. The study uncovered a putative polII promoter at the region of nucleotides 16,555–17,124 with high probability ($P = 0.9932$), and the first exon was identified to be a 104-bp fragment between nucleotide 17,055 and nucleotide 17,158 with high probability ($P = 1.0000$) plus consensus splicing donor and acceptor sites. In addition, FirstEF also identified a 200-bp region localized immediate upstream of the first exon to be a CpG island, which is an important signature for the 5′ region of many mammalian genes [4, 5]. This information positively indicates that the *NOK* gene could have a well-defined promoter/enhancer element.

Although six spliced isoforms have been documented in the Ensembl database (asia.ensembl.org/index.html), it is still elusive if the full length of NOK gene with a complete extracellular domain exists in nature. Attempts have been done to address this issue by using 5′ RACE. However, the experiments failed to extend the 5′ end of NOK gene any further. Therefore, the available data might imply that NOK

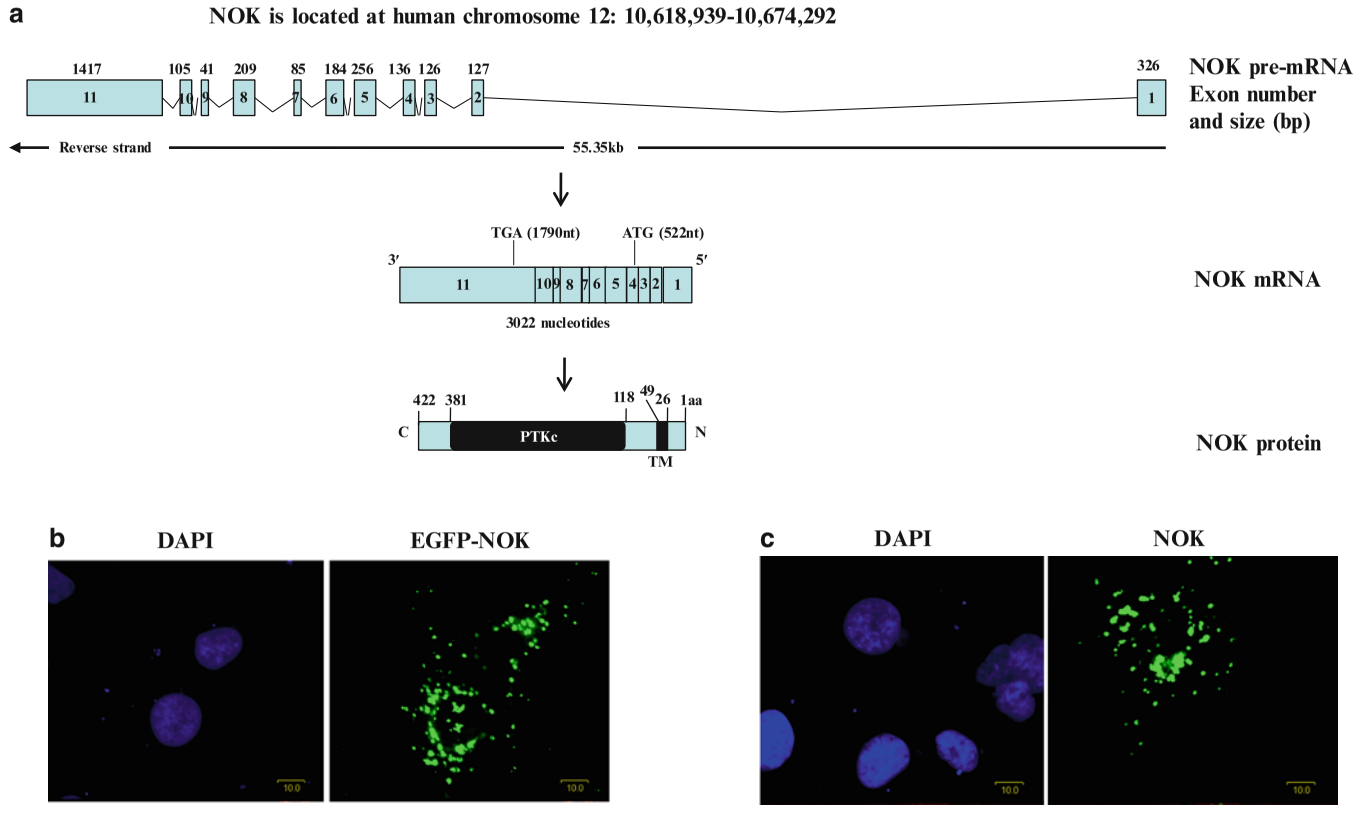

Fig. 19.1 NOK genomic structure and cellular localization. (**a**) Genomic structure of NOK gene on human chromosome 12. The distribution patterns of EGFP-NOK fusion protein (**b**) and NOK protein (**c**) after being transiently transfected into Cos-7 cells

is a nature-occurred aberrant RPTK molecule. Northern blot analysis demonstrated that NOK mRNAs (~3 kbp) could be detected in limited human tissues such as the prostate, colon, brain, and placenta [3]. Using RT-PCR approach, Ye et al. showed that STYK1 (NOK) mRNAs were ubiquitously expressed in the majority of tissues with higher levels of expression in the prostate, brain, and placenta [2]. This inconsistency might be due to the existence of NOK spliced variants that are likely presented in relatively low abundance in the majority of human tissues and is insensitive to Northern blot detection. The expression patterns of NOK gene in different cell lines may provide important clues for elucidating the role of NOK gene in human diseases [3]. Primary study indicates that *NOK* mRNAs could be detected in transformed kidney cell lines (Cos1 and HEK 293T), in various tumor cells such as human hepatoma cells LO2, human cervix carcinoma cells HeLa, human ovary cancer cells Ho8910, and human chronic myelogenous leukemia cells K562, but not in other tumor cells such as macrophage/monocyte lineages (U937, Ana-1, and HL-60) and human epidermoid carcinoma (A431).

19.3 NOK Protein: Translation, Amino Acid Structure, Domain Structure, Posttranslational Modifications

NOK protein is a 422aa long receptor-like molecule. However, subcellular localization of NOK proteins by confocal microscopy reveals its punctuate distribution in cell cytoplasm (Fig. 19.1b–c). Recent study indicates that NOK may be located at early endosome and co-localized with epidermal growth factor receptor (EGFR) upon activation [6]. The amino terminus of this protein possesses an incomplete extracellular domain whose length is 25 amino acids. From aa26 to aa49 is its putative transmembrane domain. The rest 373aa (from aa50 to aa422) of NOK protein is its intracellular domain that harbors a 263aa putative tyrosine kinase domain from aa118 to aa381 (Figs. 19.1a and 19.5). Analysis of protein phosphorylation sites using the NetPhos 2.0 program indicates that there are 4 tyrosine (Y24, Y229, Y327, and Y356), 10 serine (S59, S74, S91, S143, S283, S304, S334, S349, S350, S372), and 5 threonine (T90, T100, T208, T224, and T386) residues that might serve as the potential phosphorylation sites. These potential phosphorylation sites are well conserved between human and mouse NOKs. Sequence analysis reveals that both Y327 and Y356 phosphorylation sites of NOK are likely well conserved among the members of RPTK superfamily and may function as multi-substrate docking sites to control intracellular signaling [7].

Except being phosphorylated, NOK protein may also undergo other types of posttranslational modification such as glycosylation, ubiquitination, and myristoylation. Motif scan analysis indicates that NOK may possess one N-glycosylation site at N418 and three N-myristoylation site at G80 ($_{80}$GGNVAL$_{85}$), G269 ($_{269}$GLGLAY274), and G280 ($_{280}$GASST$_{285}$). UbPred analysis reveals two ubiquitination sites: one at K88 with medium confidence and one at K385 with low confidence. Ubiquitination provides a type of negative regulation to the targeted protein expression.

RPTK adaptor protein c-CBL can function as E3 ligase by recognizing substrates and promoting ubiquitin ligation through its SH2 domain and recruiting an E2 ubiquitin-conjugating enzyme through its RING domain [8]. This information may be useful to study NOK ubiquitination in vivo. However, how NOK gene expression is regulated by ubiquitination is needed to be deeply investigated.

Since NOK contains only VILK and HGD motifs but no DGF motif, it is predicted to be a "pseudokinase" with inactive or reduced kinase activity. However, mutation at lysine 147 in the VILK motif has significantly inhibited its proliferative property, implying that although it has no DGF motif, the "pseudokinase" NOK is still biologically active [9]. Similar situation can be found in other "pseudokinases" such as CASK [10], STRAD [11], Ror1 [12], Erb3 [13], and many others. In the absence of one or more catalytic motif, many of these pseudokinases are still able to phosphorylate their substrates [14–18]. Pseudokinases have attracted more attention recently mainly due to their unusual structural characters and biological functions. Our data indicates that NOK may also represent as an active and functional "pseudokinase" that could induce potent cellular transformation leading to the development of malignant cancer.

19.4 NOK Acts as an Oncogene to Promote Tumorigenesis and Metastasis

From a structural point of view, NOK is an inherently defect RPTK molecule in which it lacks almost complete extracellular domain and might be a "pseudokinase" due to the missing of DFG motif. However, it is surprising that overexpression of NOK causes cellular transformation that converts normal cells into cancer cells.

19.4.1 NOK-Mediated Cellular Transformation: Stable Expression of NOK Results in Anchor-Independent Growth

NOK gene was first transiently expressed in the primary mouse embryonic fibroblast NIH3T3 cells. About 4.2×10^5 NOK-transfected NIH3T3 cells were resuspended into 0.4 % soft agar. After 2 weeks of culture with 400 μg/ml G418, colonies were formed from NOK-transfected NIH3T3 cells but not the control cells NIH3T3-p3 [3]. Stable expression of either *NOK* or the *EPOR/NOK* chimeric construct in BaF3 cells also induced anchorage-independent growth and colony formation in soft agar [3, 7]. The transforming efficiency of BaF3-*NOK* is higher than that of BaF3-*EPOR*/*NOK* as quantitated by the numbers or the sizes of colonies formed after 2 weeks of incubation. All these data indicates that this "pseudokinase" may have potent transformation capacity in vivo.

19.4.2 NOK Induces Tumorigenesis and Metastasis in Animal Model

We subcutaneously injected nude mice with two types of stable cells BaF3-NOK and BaF3-EPOR/NOK. Tumor appeared after 7–10 days post-inoculation (Fig. 19.2a). The animals were then sacrificed 4 weeks later. Tumor samples and various organ sections such as the brain, liver, spleen, kidney, stomach, intestine, lung, skeletal muscle, and colon were collected, fixed, and stained with hematoxylin-eosin (H&E) staining. Staining of a variety of organ sections showed that the invasion of tumor cells was widely spread when nude mice were inoculated with BaF3-*NOK* cells. The metastatic tumor cells were prevalent in mouse liver, spleen, kidney, and skeletal muscle (Fig. 19.2b). In contrast, there were fewer invasive tumor cells and less extensive tissue distributions observed when BaF3-*EPOR*/*NOK* cells were inoculated. The sizes of the liver and spleen in BaF3-*NOK*- and BaF3-EPOR/*NOK*-injected mice were abnormally enlarged as compared with control. Although the spleen is an unusual organ for tumor metastasis, these *NOK*-expressing cells frequently disseminated into the spleen, indicating the aggressive character of these tumor cells in vivo. In addition, the life span of mice that received injections of these cells was significantly shorter than that of control with an average survival time of around 30 days. Thus, these results strongly indicate that the oncogenic properties of NOK are not only associated with cellular transformation and tumorigenesis but also have a striking effect to promote tumor metastasis to a number of distant organs that eventually cause the rapid animal death.

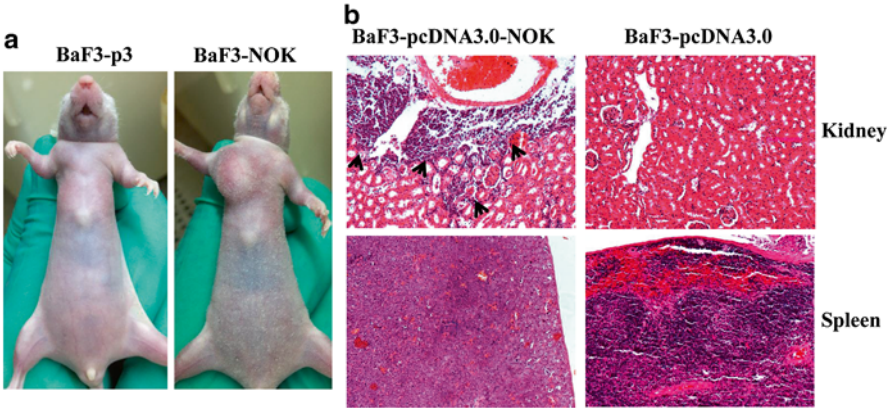

Fig. 19.2 NOK induces tumorigenesis and metastasis in nude mice. (**a**) NOK promotes tumor formation in nude mice. (**b**) H&E staining reveals NOK-mediated metastasis in distant organs such as the kidney and spleen. Tumor invasions are indicated by the *arrows*

19.5 The Mechanisms of NOK Activation and Signaling (Dimerization, Pathways, and Target Genes)

NOK is a receptor-like molecule carrying no extracellular domain. Therefore, the NOK protein may have inherent advantages to directly access and activate multiple intracellular mitogenic signaling cascades that will eventually lead to the transformation of the normal cells into cancer cells.

19.5.1 Oligomerization and Constitutive Activation of NOK

For most of RPTKs, the equilibrium of the monomer-dimer or dimer-higher oligomer transitions can be dynamically regulated in the presence of specific ligands to ensure that a particular steric constraint will be adjusted for the generation of appropriate signaling strength. Ligand-mediated higher-order oligomerization (e.g., tetramerization) of RPTK is most often in its highest activated state [19, 20], while ligand-independent oligomerization may sometimes confer to signaling inhibition [21–23]. However, NOK may represent an exceptional case in which homo-oligomer formation of these orphan receptor-like molecules may result in their constitutive activation when expressed inside the cells [24]. The intermolecular interaction of NOK could be readily detected in cotransfection system. Cross-linking experiments demonstrate that oligomerization of NOK could be induced directly, not relying on a specific ligand, but could be self-aggregated even in the absence of cross-linking reagents. Overexpressing NOK could enhance RAS/MAPK signaling pathway. Overall, these data strongly indicate that NOK is a constitutively active molecule when expressed inside the cells. This activation is presumedly due to the propensity of the self-aggregation in NOK during its homo-oligomer formation.

19.5.2 NOK and RAS/MAPK Signaling Pathway

RPTK can activate RAS/MAPK signaling pathway through at least three mechanisms [25]. The first one is the Raf/MEK/ERK pathway in which the RAS activates RAF kinase family member. RAF kinase phosphorylates and activates MEK that in turn phosphorylates and activates mitogen-activated protein kinase (MAPK). MAPK is also called extracellular signal-regulated kinases (ERK1/2). The activated MAPK then upregulates downstream gene expressions leading to enhanced protein synthesis and cell cycle progression. Second, it is well established that PI3K can be an immediate downstream effector of Ras. More recently, Gupta et al. showed that the activation of PI3K pathway is dependent on the direct interaction between Ras and the p110α PI3K catalytic subunit [26]. The third effector that Ras may activate is RalGDS which in turn activates Ral and subsequent downstream pathway and

eventually leads to antiapoptotic effect [27]. Our studies show that RAS/MAPK pathway is one of the major signaling pathways mediated by NOK, since blocking MEK with chemical inhibitor PD98059 is sufficient to inhibit NOK-mediated cellular transformation and colony formation [3]. Our data also shows that phosphorylated levels of ERK1 and ERK2 are significantly enhanced in BaF3-NOK and BaF3-EPOR/NOK cells versus the control BaF3-p3 cells, indicating that NOK indeed activates RAS/MAPK signaling pathway [3, 7]. Using transient transfection assay, we demonstrate that NOK is also able to activate RAS/MAPK pathway in human HEK 293T cells [24]. In addition, the co-localization of NOK intracellular domain and RAS on cell membrane further highlights the importance of RAS in NOK-mediated cellular signaling [24].

19.5.3 NOK and Akt/PI3K Signaling Pathway

The activation of Class IA PI3K/Akt pathway by RPTK may go through two different mechanisms. After RPTK activation and autophosphorylation, PI3K can be recruited by the activated RPTK to the inner membrane through the SH2 domain of its own or other adaptor protein molecule [28]. The activated PI3K then leads to the production and accumulation of the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 recruits and binds to pleckstrin homology (PH) domain containing proteins such as PDK1 and Akt/PKB on the inner membrane. PDK1 phosphorylates Akt1 at threonine 308 residue (T308) which is required for the activation of Akt1, while phosphorylation at serine 473 (S473) is necessary for the maximal Akt activation [29].

NOK gene might have a pleiotropic effect on the regulation of certain mitogenic signaling pathway such as PI3K/Akt signaling pathway. Our data indicates that PI3K signaling pathway contributes to NOK-mediated cellular transformation since addition of PI3K inhibitor LY294002 markedly inhibits colony formation as assayed using BaF3-NOK stable cells [3]. Interestingly, using cotransfection and coimmunoprecipitation approaches, it has been shown that NOK potentiates PI3K/Akt signaling by direct interaction with Akt in vivo [30]. This interaction does not rely on the presence of either the intact PH domain or a functional Akt kinase. Point mutation at Y356F almost completely abolishes NOK-mediated Akt1 phosphorylation [7], indicating that Y356 might be the potential docking site for Akt. Since NOK is a cytoplasmic protein which is not anchored on cell membrane, the PI3K/Akt activation mediated by NOK might be different from the mode proposed above. In this scenario, NOK and its associated factor may behave like PDK1 and PDK2 that directly phosphorylate Akt. This assumption could be reinforced by the recent finding that NOK could physically interact with and further activate both Akt and glycogen synthase kinase-3 beta (GSK-3β) to account for the NOK-mediated reduction of E-cadherin expression [7, 31]. Alternatively, it might also be true that NOK could interact with the p85 subunit of PI3K to activate the upstream effector of this signaling pathway.

19.5.4 NOK and STAT Signaling Pathway

Different from cytokine receptor-mediated STAT activation, RPTK-mediated STAT activation can be classified into three categories: JAK dependent, JAK independent, and the involvement of both. A portion of RPTKs activates STAT in a JAK-dependent manner as demonstrated by the fact that blocked JAK expression with either dominant negative form or chemically synthesized inhibitor of JAK is sufficient to inhibit STAT activation [32–34]. At least two different pathways account for RPTK-mediated JAK-independent STAT activation. First, the intrinsic kinase domain of RPTK may functionally replace JAK to directly phosphorylate and activate STAT [35–38]. Alternatively, RPTK-mediated STAT activation can be induced by recruiting a non-JAK kinase such as Src [39] or recently MAPK [40] for STAT phosphorylation. In addition, genetic alterations such as gene amplifications or mutations resulting in structural changes in RPTK are important for the generation of both highly potentiated Src and JAK responsible for the constitutively active STATs in certain cancer tissues [41, 42].

Our data shows that NOK is able to activate multiple types of STATs such as STAT1, STAT3, and STAT5 [7, 43, 44] by different mechanisms. The elevated or constitutively active STAT3 mediated by RPTK has been implicated as one of the key events for some human cancer developments [45]. Using coimmunoprecipitation approach, we have shown that NOK is physically associated with both STAT3 and JAK2 but not STAT5. Our results demonstrate that STAT3 may have multiple interfaces (except the coil-coiled domain and C-terminal domain) that are capable of interacting with NOK. The intracellular domain of NOK does readily bind to the full length of STAT3. In addition, dual luciferase assay shows that NOK and JAK2 induce synergistic effect on NOK-mediated STAT3 activation. Although JAK2 can be a common upstream effector for both STAT3 and STAT5, NOK only activates STAT3 but not STAT5 in a JAK2-dependent manner, indicating that other mechanisms may control NOK-mediated STAT5 activation.

However, a remaining question is why NOK alone does not fully activate STAT3 even in the presence of direct protein-protein contact. The answer might be mainly due to the weak kinase property of NOK. In this regard, it is understandable that in order to be fully activated, NOK has to work collaboratively with other kinases such as JAK2 to exert its functional potential.

19.5.5 The Impact of NOK on Cell Cycle Progressions

The influence of NOK on cell cycle progression was evaluated in several mammalian cell lines using transient transfection approach [46]. After transiently transfecting *NOK* into 293T, 293ET, and HeLa cells, the cell cycle distribution in each cell type was determined by flow cytometric analysis. Consistently, overexpression of NOK significantly reduced G1 phase population and at the same time markedly enhanced S population in each cell line tested (Fig. 19.3). The protein expressions of G_1/S check point-related genes such as cyclin D, cyclin E1, CDK2, and CDK4

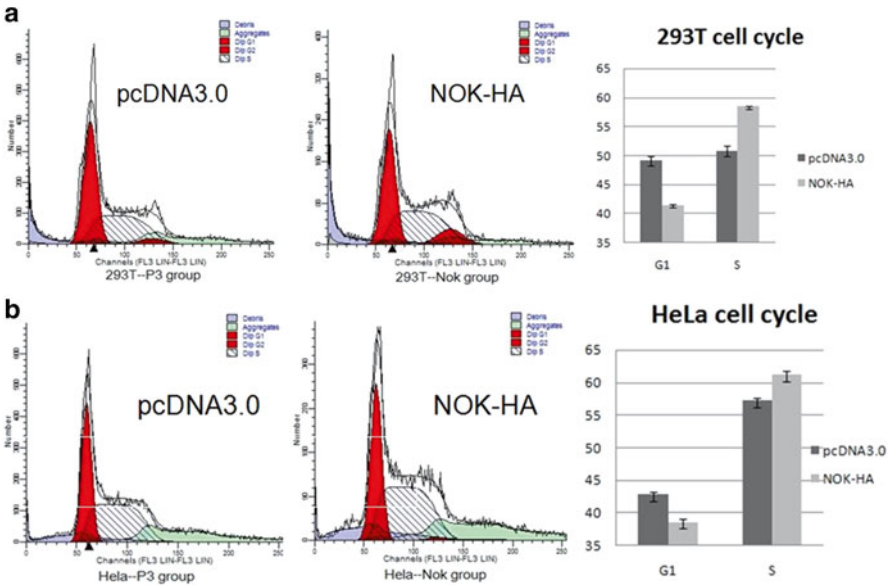

Fig. 19.3 The influence of NOK on the cell cycle distribution was evaluated in mammalian cell lines. Either empty vector (pcDNA3.0) or NOK expression plasmid (pcDNA3.0-NOK-HA) was individually transfected into 293T (A) or HeLa (B) cell lines, respectively. Cell cycle analysis was performed at 48 h posttransfection by flow cytometric analysis

are detected by Western blot analysis. Significant upregulations in the expressions of cyclin D, cyclin E1, p-CDK-2, p-CDK-4, p-Akt, and p-Rb were observed with the increased delivery of *NOK* into 293ET cells. Thus, *NOK* can promote cell cycle progression by rapidly bypassing the G_1/S check point, which may involve the activations of both Akt and CDK2/4 signaling pathways.

19.6 Autoinhibitory Mechanisms of NOK

The expression level of RPTK in human body is tightly regulated by autoinhibitory/activation mechanism so as to finely control its intrinsic kinase activity during normal cellular processes [47–51]. Currently, a few modes may account for RPTK-mediated autoinhibition. First, the sequence element responsible for RPTK autoinhibition is frequently assigned to the juxtamembrane region that is located at a region between the transmembrane helix and the cytoplasmic kinase domain [50, 51]. Second, a CDK/Src-like autoinhibition has been found in the EGFR kinase domain in which the activation loop of EGFR is protected by the N lobe of its kinase domain [48]. Third, tyrosine residue at the carboxyl terminus of RPTK may contribute to autoinhibition. Avian c-erbB is a homologue to the human epidermal growth factor receptor (EGFR).

Mutation at its carboxyl terminal tyrosine residue p5 enhances c-erbB oncogenicity [52], indicating the autoinhibitory role of this tyrosine residue.

Similar to that of avian c-erbB, the terminal tyrosine residue Y417 of NOK also possesses an autoinhibitory role in NOK-mediated cellular signaling [43]. Differently from the carboxyl terminal tyrosines Y770 identified in FGFR3 whose inhibitory effect has no impact on RAS/MAPK and STAT signaling [53], mutation at Y417 residue is able to promote the NOK-mediated activation of RAS/MAPK, STAT1, and STAT3 signaling pathways (Fig. 19.4). The mechanism controlling the autoinhibition by a single C-terminal tyrosine residue such as the Y417 in NOK may be due to the intermolecular interactions between NOK and some key signaling effectors such as the SH2-containing proteins in *trans*. This type of protein-protein interaction may further restrict the access and/or subsequent activation of these signaling effectors. Interestingly, our study indicates that in addition to the Y417, the transmembrane (TM) domain of NOK which is located at the amino terminus of the protein may also contribute to autoinhibitory effect [24]. In this case, NOK aggregates as a tetramer-sized product upon removing its N-terminus (mainly the TM domain) which resulted in the further activation of the intracellular RAS/MAPK signaling pathway (Fig. 19.4), indicating that the TM domain of NOK played an autoinhibitory role in the activation of NOK-mediated signaling transduction.

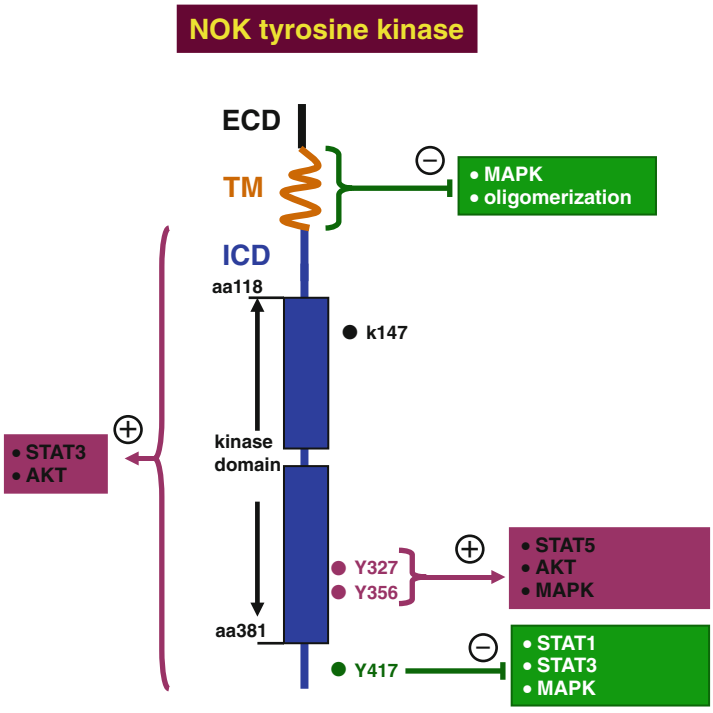

Fig. 19.4 The summary of the major signaling pathways associated with NOK. *Arrow* indicates stimulation (+). A *bar-end arrow* represents inhibition (–)

19.7 The Role of the NOK Receptor Tyrosine Kinase Family in the Regulation of Natural Killer Cells

Natural killer cells have a closer relationship with certain types of innate-like lymphoid cells such as T cells and invariant NKT (iNKT) cells in terms of common expression profiles of cell surface molecules, signaling proteins, and transcriptional factors. Microarray analysis revealed that *NOK/STYK1* is among the few genes being uniquely and preferentially upregulated in the resting NK cells [54]. More recently, *NOK/STYK1* has also been shown to be uniquely associated with the IL-2- and IL-15-mediated NK cell activation [55]. These studies indicate that NOK might be a crucial factor participating in the regulation of NK cell functions.

19.8 The Role of the NOK Receptor Tyrosine Kinase Family in Human Diseases

Recent studies have demonstrated that NOK gene products are frequently overexpressed in certain human cancer tissues such as lung cancer, breast cancer, prostate cancer, colorectal cancer (Fig. 19.5), and leukemia, indicating the potential value of NOK in cancer diagnosis and prognosis [9, 56–60]. NOK mRNAs have been shown to be upregulated in estrogen receptor-alpha (ERα) negative breast cancer cell line (MDA-MB-231) and downregulated in ERα-positive breast cancer cell line (MCF-7) upon estrogen treatment [58]. The study indicates that ER-mediated NOK expression in ERα negative cell lines may be regulated by either ERβ signaling pathways or nonconventional estrogen regulatory pathway. This information might be valuable for the diagnosis and treatment of certain types of breast cancer. Detection of c-erbB2 gene expression has been routinely used for the diagnosis and prognosis of

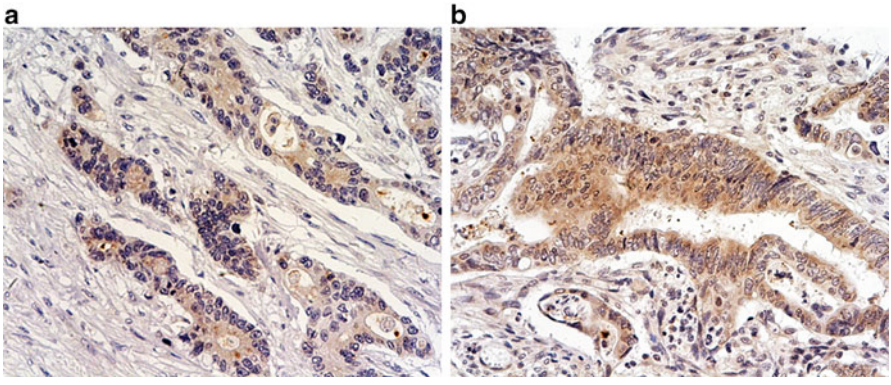

Fig. 19.5 Immunohistochemistry stainings on human colorectal cancer samples were carried out using rabbit anti-NOK antibody on moderately differentiated colon adenocarcinoma (**a**) and low-differentiated rectal adenocarcinoma (**b**). The *yellow brown* staining indicates NOK positive

human breast cancer. However, c-erbB2 mRNA expression was only positive in less than 30 % of breast cancers [58]. This is in contrast with the situation when NOK is used for the detection of human breast cancer in which more than 68 % of cases are identified as positive. In addition, over-expression of NOK gene could be frequently detected in early stages of breast cancers, indicating its potential value for early diagnosis. Recent findings also show that estrogen regulates NOK gene expression through a GPR30 hormone-signaling pathway in ovarian cancer cells [61].

Study by Amachika et al. reveals that *NOK* gene might serve as a diagnosis bio marker for human lung cancer [56]. High level of *NOK* gene expression could also be detected in early stages of lung cancer, indicating that *NOK* gene might be more applicable for the early diagnosis of human lung cancer. Moreover, clinical sample dissections reveal the important roles of NOK in the progressions, metastasis and survival of non-small cell lung cancer, indicating that NOK oncogene might be able to serve as the diagnostic and/or prognostic biomarker for this type of disease [62].

More recently, Kondoh et al. demonstrated that *NOK* mRNA could be detected in the blood samples of ~80 % of acute leukemia patients [60]. Treatments with different combinations of chemotherapies significantly and rapidly decrease *NOK* mRNA expressions, positively indicating that NOK may play a role during acute leukemia development. Acute leukemia patients with high levels of *NOK/STYK1* mRNA before treatment easily develop drug resistance as compared with those patients with low *NOK/STYK1* mRNA levels, indicating that NOK/STYK1 may be a critical factor contributing to drug resistance and therefore may serve as a novel prognostic biomarker for acute leukemia treatment [63].

Prostate cancer (PC) is the leading cause of cancer in men with high mortality worldwide [64]. Advanced and relapsed PCs are difficult to be cured since castration-resistant prostate cancer (CRPC) cells often emerge. NOK gene has been identified as one of the candidate genes that may serve as a molecular target in CRPC cells [65]. Expression analysis indicates that NOK is overexpressed in CRPC versus castration-naïve prostate cancer (CNPC) cells and is essential for maintaining the viability of PC cells [9]. Therefore, NOK could be a novel therapeutic target for drug design to treat CRPC.

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Receptor at a glance: NOK

Chromosome location	Chromosome 12p13.2
Gene size (bp)	1,269
Intron/exon number	10/11
Amino acid number	422
KDa	46.4
Posttranslational modifications	Phosphorylation, N-glycosylation, ubiquitination, and myristoylation

(continued)

Domains	Tyrosine kinase domain, transmembrane domain
Ligands	None
Known dimerizing partners	Homodimer and homotrimer
Pathways activated	RAS/MAPK, PI3K/Akt, STATs
Tissue expressed	Highly expressed in prostate; moderately expressed in the colon, brain, and placenta; can be detected in some cancer cell lines such as hepatoma cells LO2, cervix carcinoma cells HeLa, ovary cancer cells Ho8910 and chronic myelogenous leukemia cells K562; but not be detected in other cancer cell lines such as macrophage/monocyte lineages (U937, Ana-1, and HL-60) and human epidermoid carcinoma A431. Undetectable in most normal lung tissues, widely expressed in lung cancers. Uniquely expressed in the resting and activated NK cells
Human diseases	Breast cancer, lung cancer, ovarian cancer, acute leukemia, prostate cancer, and colorectal cancer
Knockout mouse phenotype	Not known
Cellular localization	Cytoplasm

References

1. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature. 2001;411(6835):355–65.
2. Ye X, Ji C, Huang Q, Cheng C, Tang R, Xu J, et al. Isolation and characterization of a human putative receptor protein kinase cDNA STYK1. Mol Biol Rep. 2003;30(2):91–6.
3. Liu L, Yu XZ, Li TS, Song LX, Chen PL, Suo TL, et al. A novel protein tyrosine kinase NOK that shares homology with platelet- derived growth factor/fibroblast growth factor receptors induces tumorigenesis and metastasis in nude mice. Cancer Res. 2004;64(10):3491–9.
4. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002;16(1):6–21.
5. Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev. 2011;25(10): 1010–22.
6. Ding X, Jiang QB, Li R, Chen S, Zhang S. NOK/STYK1 has a strong tendency towards forming aggregates and colocalises with epidermal growth factor receptor in endosomes. Biochem Biophys Res Commun. 2012;421(3):468–73.
7. Chen Y, Li YH, Chen XP, Gong LM, Zhang SP, Chang ZJ, et al. Point mutation at single tyrosine residue of novel oncogene NOK abrogates tumorigenesis in nude mice. Cancer Res. 2005;65(23):10838–46.
8. Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science. 1999;286(5438):309–12.
9. Chung S, Tamura K, Furihata M, Uemura M, Daigo Y, Nasu Y, et al. Overexpression of the potential kinase serine/threonine/tyrosine kinase 1 (STYK 1) in castration-resistant prostate cancer. Cancer Sci. 2009;100(11):2109–14.
10. Mukherjee K, Sharma M, Urlaub H, Bourenkov GP, Jahn R, Sudhof TC, et al. CASK Functions as a Mg^{2+}-independent neurexin kinase. Cell. 2008;133(2):328–39.
11. Zeqiraj E, Filippi BM, Goldie S, Navratilova I, Boudeau J, Deak M, et al. ATP and MO25alpha regulate the conformational state of the STRADalpha pseudokinase and activation of the LKB1 tumour suppressor. PLoS Biol. 2009;7(6):e1000126.
12. Gentile A, Lazzari L, Benvenuti S, Trusolino L, Comoglio PM. Ror1 is a pseudokinase that is crucial for Met-driven tumorigenesis. Cancer Res. 2011;71(8):3132–41.

13. Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. Proc Natl Acad Sci USA. 2011;107(17):7692–7.
14. Boudeau J, Miranda-Saavedra D, Barton GJ, Alessi DR. Emerging roles of pseudokinases. Trends Cell Biol. 2006;16(9):443–52.
15. Zeqiraj E, van Aalten DM. Pseudokinases-remnants of evolution or key allosteric regulators? Curr Opin Struct Biol. 2010;20(6):772–81.
16. Rajakulendran T, Sicheri F. Allosteric protein kinase regulation by pseudokinases: insights from STRAD. Sci Signal. 2010;3(111):e8.
17. Kannan N, Taylor SS. Rethinking pseudokinases. Cell. 2008;133(2):204–5.
18. Taylor SS, Kornev AP. Yet another "active" pseudokinase, Erb3. Proc Natl Acad Sci USA. 2011;107(18):8047–8.
19. Brennan PJ, Kumagai T, Berezov A, Murali R, Greene MI. HER2/neu: mechanisms of dimerization/oligomerization. Oncogene. 2000;19(53):6093–101.
20. Clayton AH, Walker F, Orchard SG, Henderson C, Fuchs D, Rothacker J, et al. Ligand-induced dimer-tetramer transition during the activation of the cell surface epidermal growth factor receptor-A multidimensional microscopy analysis. J Biol Chem. 2005;280(34):30392–9.
21. Kani K, Warren CM, Kaddis CS, Loo JA, Landgraf R. Oligomers of ERBB3 have two distinct interfaces that differ in their sensitivity to disruption by heregulin. J Biol Chem. 2005;280(9):8238–47.
22. Landgraf R, Eisenberg D. Heregulin reverses the oligomerization of HER3. Biochemistry. 2000;39(29):8503–11.
23. Lackmann M, Oates AC, Dottori M, Smith FM, Do C, Power M, et al. Distinct subdomains of the EphA3 receptor mediate ligand binding and receptor dimerization. J Biol Chem. 1998;273(32):20228–37.
24. Li YH, Wang YY, Zhong S, Rong ZL, Ren YM, Li ZY, et al. Transmembrane helix of novel oncogene with kinase-domain (NOK) influences its oligomerization and limits the activation of RAS/MAPK signaling. Mol Cells. 2009;27(1):39–45.
25. Easty DJ, Gray SG, O'Byrne KJ, O'Donnell D, Bennett DC. Receptor tyrosine kinases and their activation in melanoma. Pigment Cell Melanoma Res. 2011;24(3):446–61.
26. Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, et al. Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. Cell. 2007;129(5):957–68.
27. Rodriguez-Viciana P, McCormick F. RalGDS comes of age. Cancer Cell. 2005;7(3):205–6.
28. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev. 2004;30(2):193–204.
29. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 1996;15(23):6541–51.
30. Li YH, Rong Y, Chang ZJ, Liu L. NOK interacts with Akt and enhances its activation. Prog Biochem Biophys. 2008;35(1):29–34.
31. Li J, Wu F, Sheng F, Li YJ, Jin D, Ding X, et al. NOK/STYK1 interacts with GSK-3beta and mediates Ser9 phosphorylation through activated Akt. FEBS Lett. 2012;586(21):3787–92.
32. Zong CS, Chan J, Levy DE, Horvath C, Sadowski HB, Wang LH. Mechanism of STAT3 activation by insulin-like growth factor I receptor. J Biol Chem. 2000;275(20):15099–105.
33. Lai KO, Chen Y, Po HM, Lok KC, Gong K, Ip NY. Identification of the Jak/Stat proteins as novel downstream targets of EphA4 signaling in muscle: implications in the regulation of acetylcholinesterase expression. J Biol Chem. 2004;279(14):13383–92.
34. Himpe E, Kooijman R. Insulin-like growth factor-I receptor signal transduction and the Janus Kinase/Signal Transducer and Activator of Transcription (JAK-STAT) pathway. Biofactors. 2009;35(1):76–81.
35. Zhang S, Fukuda S, Lee Y, Hangoc G, Cooper S, Spolski R, et al. Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling. J Exp Med. 2000;192(5):719–28.

36. Quelle FW, Thierfelder W, Witthuhn BA, Tang B, Cohen S, Ihle JN. Phosphorylation and activation of the DNA binding activity of purified Stat1 by the Janus protein-tyrosine kinases and the epidermal growth factor receptor. J Biol Chem. 1995;270(35):20775–80.
37. Vignais ML, Sadowski HB, Watling D, Rogers NC, Gilman M. Platelet-derived growth factor induces phosphorylation of multiple JAK family kinases and STAT proteins. Mol Cell Biol. 1996;16(4):1759–69.
38. Chen J, Sadowski HB, Kohanski RA, Wang LH. Stat5 is a physiological substrate of the insulin receptor. Proc Natl Acad Sci USA. 1997;94(6):2295–300.
39. Silva CM. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. Oncogene. 2004;23(48):8017–23.
40. Miranda C, Fumagalli T, Anania MC, Vizioli MG, Pagliardini S, Pierotti MA, et al. Role of STAT3 in in vitro transformation triggered by TRK oncogenes. PLoS One. 2010;5(3):e9446.
41. Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, et al. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene. 2001;20(20):2499–513.
42. Plaza Menacho I, Koster R, van der Sloot AM, Quax WJ, Osinga J, van der Sluis T, et al. RET-familial medullary thyroid carcinoma mutants Y791F and S891A activate a Src/JAK/STAT3 pathway, independent of glial cell line-derived neurotrophic factor. Cancer Res. 2005;65(5):1729–37.
43. Li YH, Zhong S, Rong ZL, Ren YM, Li ZY, Zhang SP, et al. The carboxyl terminal tyrosine 417 residue of NOK has an autoinhibitory effect on NOK-mediated signaling transductions. Biochem Biophys Res Commun. 2007;356(2):444–9.
44. Li YH, Rong Y, Chang ZJ, Liu L. NOK activates STAT3 signaling by a JAK2-dependent mechanism. Prog Biochem Biophys. 2008;35(2):143–50.
45. Johnston PA, Grandis JR. STAT3 signaling: anticancer strategies and challenges. Mol Interv. 2011;11(1):18–26.
46. Liu B, Liu L. Influence and mechanism of NOK oncogene on G1/S distribution in human embryonic kidney 293T cells. Basic Clin Med. 2014;34(4):470–4.
47. Cheetham GM. Novel protein kinases and molecular mechanisms of autoinhibition. Curr Opin Struct Biol. 2004;14(6):700–5.
48. Bose R, Zhang X. The ErbB kinase domain: structural perspectives into kinase activation and inhibition. Exp Cell Res. 2009;315(4):649–58.
49. Schlessinger J. Signal transduction. Autoinhibition control. Science. 2003;300(5620):750–2.
50. Hubbard SR. Juxtamembrane autoinhibition in receptor tyrosine kinases. Nat Rev Mol Cell Biol. 2004;5(6):464–71.
51. Reindl C, Spiekermann K. From kinases to cancer: leakiness, loss of autoinhibition and leukemia. Cell Cycle. 2006;5(6):599–602.
52. Chang CM, Shu HK, Ravi L, Pelley RJ, Shu H, Kung HJ. A minor tyrosine phosphorylation site located within the CAIN domain plays a critical role in regulating tissue-specific transformation by erbB kinase. J Virol. 1995;69(2):1172–80.
53. Hart KC, Robertson SC, Donoghue DJ. Identification of tyrosine residues in constitutively activated fibroblast growth factor receptor 3 involved in mitogenesis, Stat activation, and phosphatidylinositol 3-kinase activation. Mol Biol Cell. 2001;12(4):931–42.
54. Bezman NA, Kim CC, Sun JC, Min-Oo G, Hendricks DW, Kamimura Y, et al. Molecular definition of the identity and activation of natural killer cells. Nat Immunol. 2012;13(10):1000–9.
55. Lotem J, Levanon D, Negreanu V, Leshkowitz D, Friedlander G, Groner Y. Runx3-mediated transcriptional program in cytotoxic lymphocytes. PLoS One. 2013;8(11):e80467.
56. Amachika T, Kobayashi D, Moriai R, Tsuji N, Watanabe N. Diagnostic relevance of overexpressed mRNA of novel oncogene with kinase-domain (NOK) in lung cancers. Lung Cancer. 2007;56(3):337–40.
57. Kimbro KS, Duschene K, Willard M, Moore JA, Freeman S. A novel gene STYK1/NOK is upregulated in estrogen receptor-alpha negative estrogen receptor-beta positive breast cancer cells following estrogen treatment. Mol Biol Rep. 2008;35(1):23–7.

58. Moriai R, Kobayashi D, Amachika T, Tsuji N, Watanabe N. Diagnostic relevance of overexpressed NOK mRNA in breast cancer. Anticancer Res. 2006;26(6C):4969–73.
59. Orang AV, Safaralizadeh R, Hosseinpour Feizi MA, Somi MH. Diagnostic relevance of overexpressed serine threonine tyrosine kinase/novel oncogene with kinase domain (STYK1/NOK) mRNA in colorectal cancer. Asian Pac J Cancer Prev. 2014;15(16):6685–9.
60. Kondoh T, Kobayashi D, Tsuji N, Kuribayashi K, Watanabe N. Overexpression of serine threonine tyrosine kinase 1/novel oncogene with kinase domain mRNA in patients with acute leukemia. Exp Hematol. 2009;37(7):824–30.
61. Jackson KA, Oprea G, Handy J, Kimbro KS. Aberrant STYK1 expression in ovarian cancer tissues and cell lines. J Ovarian Res. 2009;2(1):15.
62. Chen P, Li WM, Lu Q, Wang J, Yan XL, Zhang ZP, et al. Clinicopathologic features and prognostic implications of NOK/STYK1 protein expression in non-small cell lung cancer. BMC Cancer. 2014;14:402.
63. Nirasawa S, Kobayashi D, Kondoh T, Kuribayashi K, Tanaka M, Yanagihara N, et al. Significance of serine threonine tyrosine kinase 1 as a drug resistance factor and therapeutic predictor in acute leukemia. Int J Oncol. 2014;45(5):1867–74.
64. Gronberg H. Prostate cancer epidemiology. Lancet. 2003;361(9360):859–64.
65. Tamura K, Furihata M, Tsunoda T, Ashida S, Takata R, Obara W, et al. Molecular features of hormone-refractory prostate cancer cells by genome-wide gene expression profiles. Cancer Res. 2007;67(11):5117–25.

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