Alfred Wittinghofer *Editor*

Ras Superfamily Small G Proteins: Biology and Mechanisms 1

General Features, Signaling

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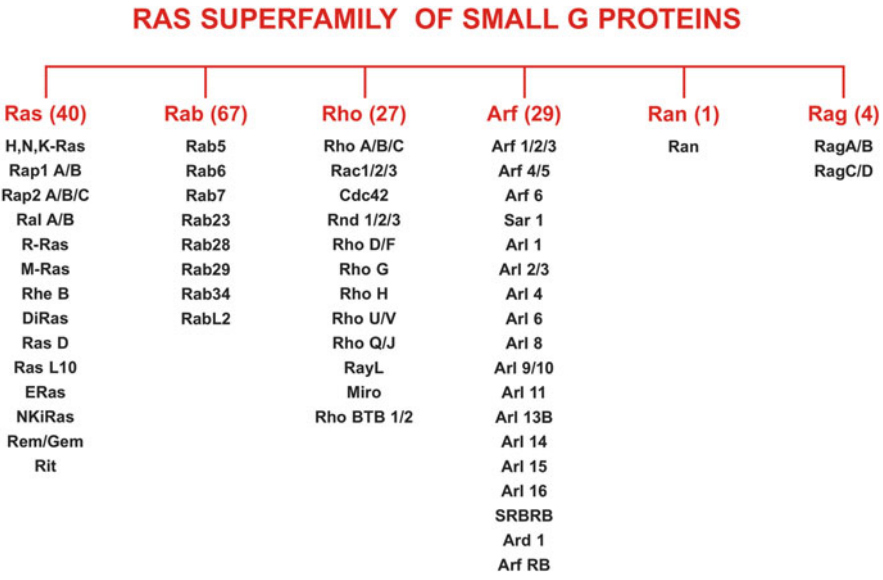
Editorial Introduction

This book deals with the so-called Ras superfamily of guanine nucleotide-binding proteins whose diverse members are between 20 and 25 kDa in size and are classified by a conserved structural domain. These proteins have diverse regulatory functions and act as molecular switches. The superfamily's name derives from the Ras protein which was identified in the 1970s of the last century as a phosphoprotein of 21 kDa encoded by the viral oncogenes found in the Ha-MuSV and Ki-MuSV animal retroviruses (Shih et al. 1979). Shortly thereafter it was found that these oncogenes originated from normal vertebrate genes termed H-ras and K-ras (Ellis et al. 1981). Molecular cloning then led to the isolation and sequencing of the "p21src" genes in 1982 (Dhar et al. 1982; Tsuchida et al. 1982). Starting with human tumors, three different labs were able to isolate transforming genes which turned out to be the homologue of the virally encoded Ras genes (Shih and Weinberg 1982; Pulciani et al. 1982; Goldfarb et al. 1982). Shortly thereafter, normal cellular Ras genes were cloned and sequenced by several laboratories and shown to encode proteins of 21 kDa, now called H-Ras, K-Ras, and N-Ras. Biochemical studies revealed these proteins bind to the guanine nucleotides GDP and GTP, have a slow GTPase activity, and are associated with the plasma membrane. The most exciting aspect of the Ras story was the finding that oncogenic versions of Ras have specific amino acid replacements resulting in a much slower GTP hydrolyzing activity compared to the wild-type version of the protein [for a complete record of the discovery of Ras, see earlier reviews (Malumbres and Barbacid 2003; Cox and Der 2010)]. In 1983 Ras sequences were also identified in the single-celled organism yeast (DeFeo-Jones et al. 1983), and in the following years they were discovered in many other eukaryotes. This demonstrated that Ras proteins are ubiquitous switching devices (see Chap. 1 by Rojas and Valencia in this book).

The first Ras homologue, termed *YPT1*, was discovered already in 1983 in yeast (Gallwitz et al. 1983) and was later shown to share the biochemical properties with Ras (Wagner et al. 1987). The second member of the Rab subfamily was also found

in yeast as the protein encoded by the *SEC4* gene (Salminen and Novick 1987). It was cloned through rescue of the SEC4 mutant which had been isolated earlier in the seminal screen for secretion-defective yeast mutants by Schekman and coworkers (Novick et al. 1980). It became apparent then that Ypt1p and Sec4p are members of the so-called Rab subfamily of proteins, which counts >60 members in higher eukaryotes, all of them acting in intracellular protein trafficking.

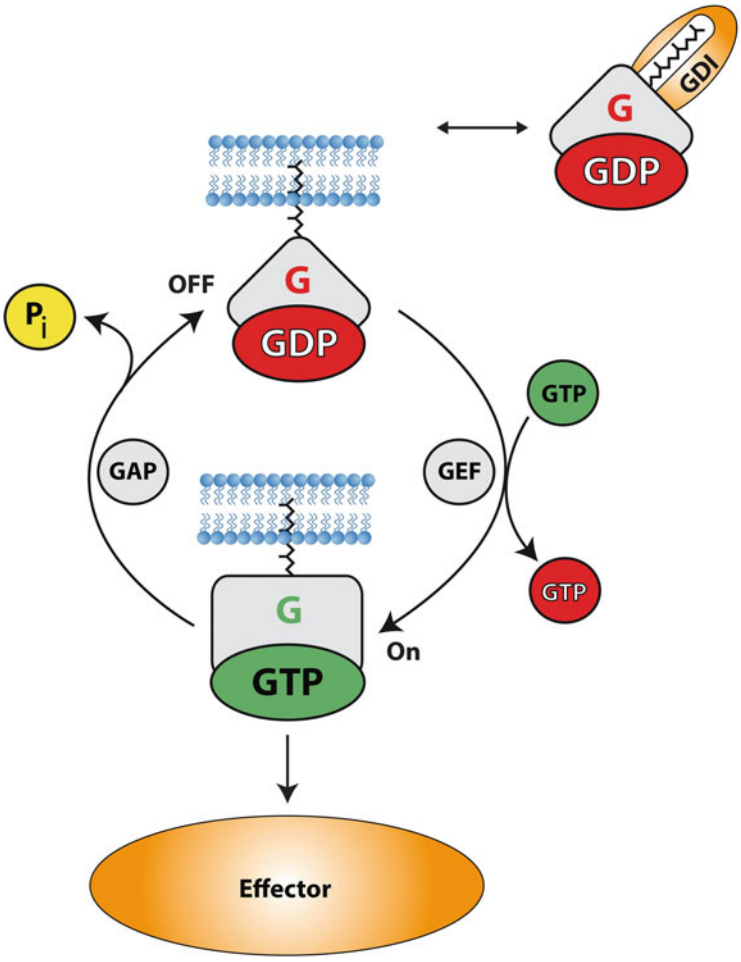
The discovery of yet another novel Ras-related gene family, the Rho family, was first made in Aplysia and later in mice (Madaule and Axel 1985; Madaule et al. 1987). This suggested for the first time that there was a superfamily of Ras-like proteins with several branches (three at the time) (Chardin 1988). Indeed, the sequence homology in certain regions of the proteins thus isolated, now known as the five conserved sequence motifs G1-5, inspired Tavitian and colleagues to design degenerate oligonucleotides and to fish Ral and Rab genes coding for new members of the superfamily (Chardin and Tavitian 1986; Touchot et al. 1987). The complete sequencing of many organisms has allowed us to sample the complete universe of Ras-like GTP-binding proteins. They are classified as the Ras superfamily of small G proteins and can be grouped, by sequence homology, into several subgroups, the most prominent of which are shown in Fig. 1. Incidentally the subgroups also define a more or less similar biological function.



As of today, the protein data base consists of 766 sequences corresponding to 167 human sequences belonging to the Ras superfamily, with the number of subfamily members indicated in Fig. 1, as described in Chap. 1.

The common feature of these proteins is their conserved nucleotide-binding α,β domain, which is called the G domain and consists of approximately 170 residues, with insertions and N- and C-terminal extensions, depending on the subfamily. This is described in detail in Chap. 2. Their common feature is that they act as molecular switches cycling between two conformations, an inactive GDP-bound and an active GTP-bound conformation.

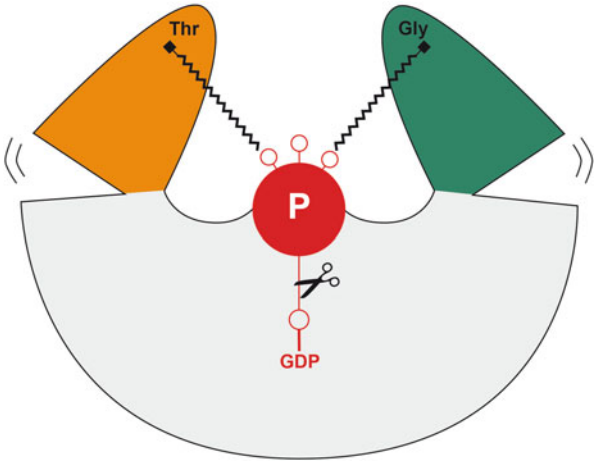
Most of them bind the guanine nucleotides GDP or GTP but not GMP, with high subnanomolar affinity. With a few exceptions they are also very specific for the guanine base and do not bind adenine nucleotides with any reasonable affinity. Where it has been measured, the affinity for ADP/ATP is in the millimolar range (John et al. 1990). As a consequence of the high GDP/GTP affinity, the dissociation rate is very slow and Ras superfamily G proteins thus need a guanine nucleotide exchange factor (GEF) to mediate fast release of GDP and allow loading with GTP. GEFs increase the dissociation of any bound nucleotide, but since GTP is in 10–50 fold excess in living cells, the effect of the GEF interaction is the loading of the protein with GTP. Since Mg^{2+} forms a bi-dentate complex with the β- and γ-phosphates and is required for high affinity of the nucleotide to the protein, addition of EDTA is an easy means of exchanging the bound nucleotide in vitro for biochemical investigations. In the GTP-bound ON state, Ras proteins interact with effectors which are defined as proteins with high affinity to the GTP- and low affinity to the GDP-bound state. To recycle to the inactive state, GTP needs to be hydrolyzed to GDP and Pi. In most cases the reaction is very slow and requires the action of GTPase Activating Proteins (GAPs). Most Ras proteins are incomplete enzymes and require the contribution of residues from GAPs to accelerate the GTPase reaction, up to 10^5–10^6 fold (see Chap. 3 by Cherfils on the action of GEFs and GAPs).



With a few exceptions, like Ran, the regulators of nucleocytoplasmic trafficking, Ras superfamily proteins are posttranslationally modified with lipids either at the N-terminus by acetylation (myristoylation), or C-terminally by prenylation and palmitoylation: While prenylation by farnesyl or geranylgeranyl groups is via a stable thioether linkage, palmitoylation by thioester linkage is reversible and used to dynamically regulate membrane localization (see Chap. 5 on the modification of Ras proteins by Zhou and Cox). Because the lipid moieties are rather hydrophobic, Ras proteins such as Rho and Rab subfamily members use proteins, called RhoGDI and RabGDI, as chaperones for intermembrane transport. A similar GDI-like factor, GDF, has recently been described for Ras subfamily proteins (Chandra et al. 2012).

It had been predicted fairly early that Ras would be structurally similar to elongation factor Tu, the first GTP-binding protein characterized biochemically, and homology models had been presented (McCormick et al. 1985; Jurnak 1985).

Indeed the structure showed Ras to be homologous to the first domain of the three-domain protein EF-Tu (Pai et al. 1989). It also showed that it is homologous to the α subunit of heterotrimeric G proteins, which has an insertion in the G domain (Noel et al. 1993; Coleman et al. 1994). This canonical G domain is a $\alpha - \beta$ protein with six β-strands and five alpha helices. As of February 2014, there are now 558 structures of Ras superfamily proteins in the database, 139 from Ras alone, which confirm the general architecture of the G domain. They also show that there are C- and N-terminal additions and insertions to the general scaffold. It should be stressed however that the G domain scaffold is very dynamic, as evidenced from comparing different X-ray structures or by directly observing NMR and EPR spectra (see Chap. 2 by I. Vetter). The superimposition of structures shows that the proteins are particularly dynamic in the loops, in particular those constituting the switch regions (see below).



The most important aspect of the structure is the nature of the conformational change induced by the presence or absence of the γ-phosphate. The structures of Ras in the different nucleotide states showed that there are two regions in the molecule called switch I and II, which are sensitive to the nature of the nucleotide (Milburn et al. 1990; Schlichting et al. 1990). This conformational change has been compared to a loaded-spring mechanism, whereby switch I and II via their totally conserved and glycine are connected to the γ-phosphate by main chain hydrogen bonds. Threonine GTP hydrolysis and release of γ-phosphate allow the switch regions to relax into a different conformation (Vetter and Wittinghofer 2001).

While the basic feature of this conformational switch is totally conserved, the details vary considerably between the different proteins. It is most dramatic in the case of Arf and Arl proteins, where it involves the rearrangement of two β-strands which detach from the sheet and make a register shift of two residues (Pasqualato et

al. 2002). Another conserved feature of the structural analysis has been the finding that the binding of effectors to the GTP-bound conformation involves either switch I, II or both. Since these are the γ-phosphate sensing regions, the structures confirm and explain the biochemical findings that effectors bind 100 to 1,000 fold tighter to the GTP-bound form of the proteins.

Considering the importance of small G proteins for almost any function of the cell, it is not surprising that pathogens have developed a lot of means to block, modify, or usurp their function. One of these tricks is chemical modification of Ras superfamily proteins, which ranges from glucosylation, ADP-ribosylation, to adenylylation, to name just a few. The other is to use bacterial GEFs and GAPs which in most cases have been developed independently from their eukaryotic counterparts, to change to activation status of the targets. This is described in detail in Chap. 4 by Aktories and Schmid.

Apart from the general introduction in Chaps. 1–5, contributions are presented on the most important subfamilies or individual members of subfamilies by leading authors of the corresponding subjects. The number of chapters devoted to particular subjects may reflect their particular impact in the scientific literature. I thank all the colleagues who enthusiastically agreed to support this project and I sincerely hope that readers will take home what they always wanted to know about Ras superfamily proteins.

Dortmund, Germany Fred Wittinghofer

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Part I
Ras Superfamily, General Topics

Chapter 1
Evolution of the Ras Superfamily of GTPases

Ana M. Rojas and Alfonso Valencia

Abstract The Ras superfamily of small GTPases illustrates a large functional diversification in the context of a preserved structural framework and a prototypic GTP-binding site. The Ras superfamily of small GTP-binding proteins is essential to regulate the cellular organization and signaling in cells. Members of this superfamily contain a structurally and mechanistically preserved GTP-binding core, with considerable functional and sequence divergence. In this chapter we review the evolutionary structure of the superfamily at the organism and sequence level, presenting a representative tree that reflects the history of the Ras superfamily including crucial evolutionary time points and detailed trees for the Rho, Ras, Rab, Arf, and Ran families. Based on this information we discuss some of the complex relationships between the evolution of proteins and the acquisition of distinctive cellular functions.

Keywords Ras phylogeny • Small GTPases • GTP-binding • Multiple sequence alignments

1.1 A Note About the State-of-the-Art in Ras Superfamily Classification

Twenty years ago, Chardin, Sander, Valencia, and Wittinghofer (Valencia et al. 1991) proposed the first classification of the superfamily, which included about 30 sequences in few species. Clearly, this initial classification was largely

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dependent on the availability of sequences—low back then—and on the capabilities of the methods to reconstruct and infer phylogenetic relationships among the protein sequences.

Since then, the numbers of studies involving individual members or particular subfamilies have increased exponentially, particularly due to the fact that most of these proteins have an impact on human disease, and therefore constituted attractive targets for drug design.

It is only recently that the remarkable improvement of sequencing technologies which has produced an incremental data leverage on genomic information, coupled to the development of more powerful and sophisticated phylogenetic methods, has enabled a complete revision of the evolution of the superfamily (Rojas et al. 2012).

The reconstruction of the history of the family can be divided in three steps: (1) collection of the sequences using homology-based procedures, (2) alignment of the large collection of sequences, and (3) inference of the phylogenies. These steps are briefly explained below.

1.2 Sequence Retrieval

The human Ras superfamily contains 167 proteins containing classical families (39 Ras proteins, 30 Arfs, 22 Rhos, 65 Rabs, and 1 Ran family sequence), plus some additional "unclassified" sequences even if for some of them the only evidence of their existence is at the transcriptional level (see the proteins at http://www.cbbio.es/GTPases/and (Rojas et al. 2012).

Although the human proteins provide a general overview it does not account for the specific lineage expansions. To circumvent this limitation we retrieved orthologous proteins that are likely to retain the same functions in other organisms (Altenhoff et al. 2012). We identified a total of 766 sequences from 11 organisms that correspond to orthologues of the 167 human proteins in the superfamily. Orthologues were obtained with the InParanoid software (v.4.0: (Ostlund et al. 2010) from different databases. The selected 11 species correspond to relevant moments in eukaryotic evolution, e.g., *Plantae-Animalia* and *Radiata-Bilateria* by *A. thaliana* and *N. vectensis*, respectively, or the different *Chordata* lineages represented by ascidians (*C. intestinalis*) and lancelets (*B. floridae*) (for an illustration see Fig. 1.1). Not all the genomes were equally annotated and some of them were in draft stage (*e.g., N. vectensis*, *C. intestinalis*, *B. floridae*, *X. tropicalis)* and some of them poorly annotated *(e.g., P. falciparum)*. Since our study of the Ras superfamily uses human sequences as starting point, it is possible that sequences of other species , with no direct representative member in humans, could have been excluded from the analysis.

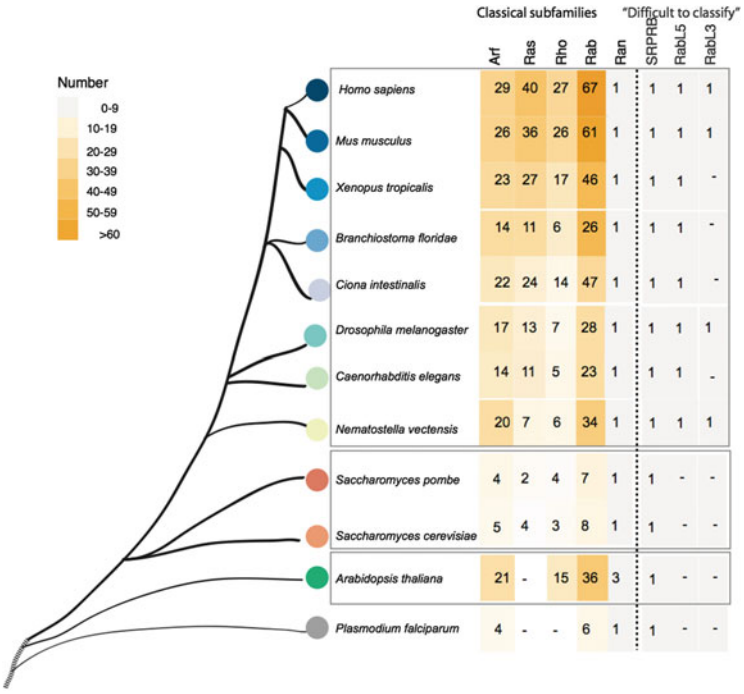

Fig. 1.1 Orthologous sequences for the 167 human sequences along evolution obtained using Inparanoid. The tree at the *left* illustrates the evolutionary timing considered here. *Numbers* indicate the orthologues found in each particular species [for details see (Rojas et al. 2012)]. *Color circles* indicate specific coloring for the family phylogenies and will be used throughout. The sequences for human proteins are available at http://www.cbbio.es/GTPases/

1.3 Multiple Sequence Alignments (MSAs)

The generation of accurate MSAs is a key step in phylogenetic analyses. While standard methods work reasonably well when the sequence similarity is high (over 40 %), very divergent sequences are difficult to align and the MSAs often contain errors (reviewed in Kemena and Notredame, 2009). The GTP–binding domain that is the hallmark of the superfamily constitutes a conserved common core with well-distributed conserved motifs particularly suitable to produce MSAs. Moreover, the existence of structural data provides additional constraints to ensure the quality of the alignments. Figure 1.2 shows the distribution of variable residues within the GTP-binding domain for each family.

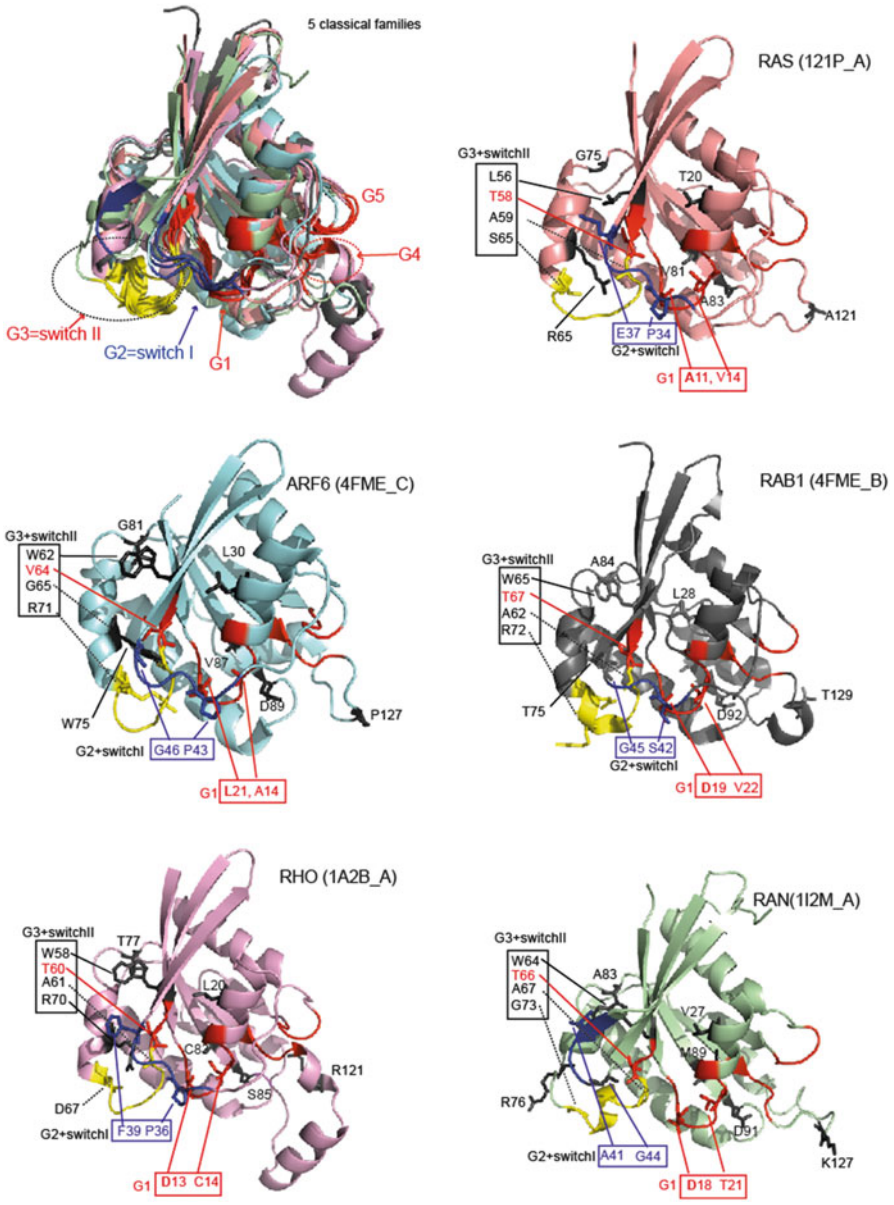

Fig. 1.2 Ras superfamily variable residues. *Top left* shows a multiple structural alignment of one representative structure for each of the five classical families, RAS:121P_A (Krengel 1999), ARF6:4FME_C and RAB1:4FME_C (Dong et al. 2012), RHO:1A2B_A (Ihara et al. 1998), and RAN:1I2M_A (Renault et al. 2001). Conserved regions are indicated. G indicate the conserved G-boxes, with G1, G4, and G5 in *red*, the G2 (switch I) region in *blue*, the G3 (switch II) region in *yellow*. The rest of the figures are the same structures from the multiple structural alignment in the same orientation, of each classical family with variable positions specific for each family according to previous work (Rojas et al. 2012). Numbering of residues corresponds to that found in the particular structure represented

1.4 Phylogenetic Reconstructions

The multiple sequence alignment of G-domains is used as the basis for the phylogenetic analysis. Currently, the best approach for the reconstruction of the phylogeny is the use of statistical Bayesian methods (Holder and Lewis 2003; Lartillot et al. 2007; Ronquist and Huelsenbeck 2003). The downside of the increased accuracy of these new methods is their high computational demands. In the case of the Ras superfamily building trees from a starting alignment of more than 900 divergent sequences is unfeasible, even for large supercomputers (Ernst et al. 2011). Thus, we used an alternative procedure that selects representative sequences from different organisms to build independent trees for each of the five distinct families of the Ras superfamily (Ras, Rho, Rab, Ran and Arf).

1.5 Ras Superfamily: An Overview of the Classical Families

1.5.1 The Rho Family

The Rho family is involved in signaling networks related to actin regulation, cell-cycle progression, and gene expression. In addition to cytoskeletal organization (Heasman and Ridley 2008) and cell polarity (Park and Bi 2007), recent evidence indicates that Rho members may fulfil novel functions in hematopoiesis (Mulloy et al. 2010) as well as in both canonical and noncanonical *wnt* signaling, in particular RAC (Schlessinger et al. 2009). At the sequence level, Rho members are defined by a specific insert located between the G4 and G5 boxes (Freeman et al. 1996). There are 22 human protein isoforms (http://www.cbbio.es/GTPases/) that correspond to 20 different genes, and most of them have orthologues in 10 of the 11 proteomes analyzed (a total of 130 sequences, Fig. 1.3).

Rho is absent from *Eubacteria* and *Archea*, and from the alveolate *Plasmodium falciparum*, whereas these proteins are the only signaling members in plants (Yang 2002). In addition to their role in cytoskeletal reorganization, rho proteins have also been implicated in pathways affecting cell proliferation, apoptosis, adhesion, and vesicular trafficking.

Our analyses distinguish different groups (Fig. 1.3) extending previous observations (Boureux et al. 2007). RHOA is placed at the base of the tree, where it is present in all the genomes except in plants and the parasite *Plasmodium*. This supports the scenario of a spread during the eukaryotic crown radiation [more than 1.5 billion years ago (Hedges and Kumar 2004)]. Our results also support ancestral Rac duplications in fungi/metazoans that likely led to CDC42 that controls cell polarity and to RHO that is active in cytokinesis (Jaffe and Hall 2005). This separation has a probability of 100 % in our analyses (Fig. 1.3). The CDC42

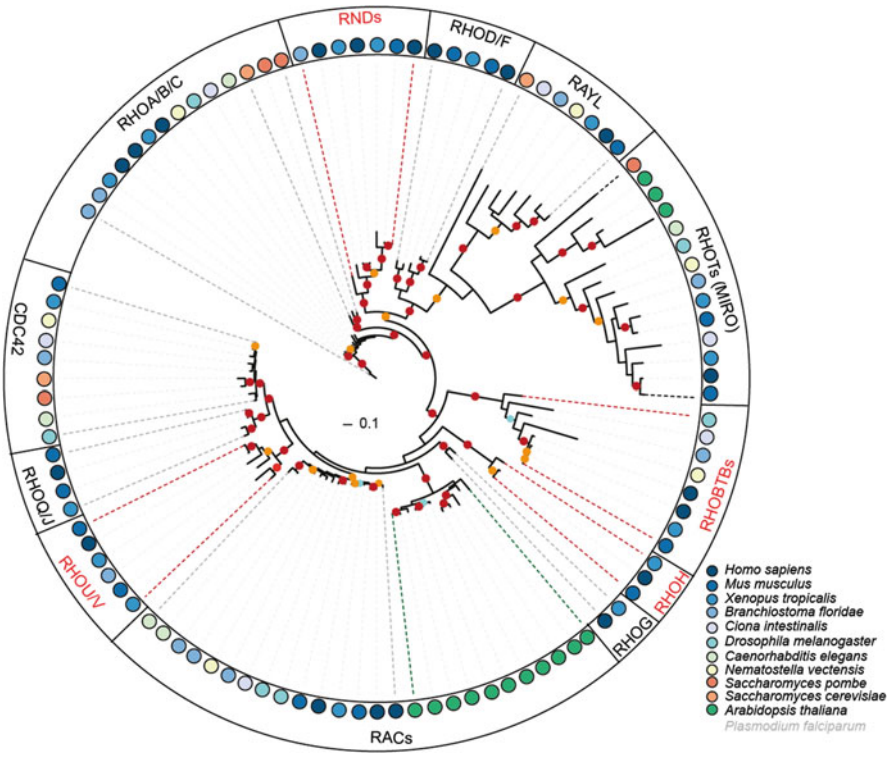

Fig. 1.3 Rho phylogenetic tree. The alignment of the G-binding domain of 105 sequences orthologous to human sequences was used to conduct phylogenies. Analyses were run in four chains and 5,000,000 generations. Trees were sampled after convergence was reached. Color ranges indicate each of the 12 species and are the same as in Fig. 1.1. *Red branches* indicate atypical RHO according to Aspenstrom *et al*. (Aspenstrom et al. 2007). *Red circles* in branches indicate group probabilities of 100 %. *Orange circles* indicate probabilities of 90–100 %. *Cyan circles* indicate group probabilities of: 80 % < Probability ≥90 %. When mouse and human sequences are identical, only mouse sequences are represented in the tree. Rho sequences were not identified in *Plasmodium*

protein, which is involved in promoting actin microspikes and filopodia formation, is conserved throughout all the lineages except plants, with very little divergence. According to the tree, gene duplications of CDC42 gave rise to the vertebrate RHOJ/Q/U/V lineages. The absence of *rho* genes in alveolates indicates that other proteins may play a similar role in cell polarity and cytokinesis.

RAC1 has been shown to promote lamellipodia formation and membrane ruffling. It is present and duplicated in all the organisms studied except fungi, which do not have RAC orthologues. The evolution of the RAC group is supported by a high probability (>90 %, Fig. 1.3), and it appears that RAC has been duplicated several times since speciation as in most genomes all the RAC members group together, especially in plants.

The most studied Rho proteins are RHOA (which promotes actin stress fibber formation and focal adhesion assembly) and constitute the basal group in our tree, with presence in all the organisms analyzed here except plants.

Other Rho members have particular features that make them atypical when compared to RHO, RAC, CDC42, etc. (Aspenstrom et al. 2007). These are RHOBTB, RHOH, RHOU/V, RND (indicated with red lines in Fig. 1.3), since they may not require GEFs or GAPs, may not have been identified for them, or, as in case of RHOBTB, may not even bind GTP (Espinosa et al. 2009). Rather, they are regulated at different levels, such as through their expression and proteasomal degradation (Chardin 2006), and additional types of regulation have been discussed (Aspenstrom et al. 2007; Buchsbaum 2007; Heasman and Ridley 2008).

In the case of RHOBTB the presence of additional BTB domains suggests that they may be regulated by additional protein–protein interactions. These RHOBTB are identified in most of the metazoans investigated here. Its presence in the cnidarian *N. vectensis* indicates that these proteins were present before bilaterians emerged (Fig. 1.1). Unidentified orthologues in other metazoans could be due to the draft state of these genomes, although absences of representatives in *B. floridae* and *C. intestinalis* could indicate that these gene losses may be consistent with the reduced complexity of the adult members of these species and of their smaller genome size, as proposed for the marine chordate *Oikipleura dioica* (Seo et al. 2001).

RHOH are exclusively present in vertebrates and are proposed as hematopoietic-specific GTPases involved in T-cell signaling. They are also viewed as antagonists of the classical Rho GTPases (Ellis et al. 2002). RHOU and RHOV are present from *Chordata* appearance *onwards*, and they are involved in cytoskeletal dynamics and cell adhesion. Interestingly, the RND proteins (proposed to antagonize RHOA) are only present in vertebrates and lanceolets, suggesting that this function appeared later in evolution.

The RHOTs (MIRO) proteins, which were not previously assigned to any particular branch (Stenmark 2009), are frequently grouped with the rho proteins, and for consistency we have done the same. Our results indicate that RHOTs are conserved since Fungi, (Fig. 1.3) and this group supported by a probability of more than 90 %.

1.5.2 The Ras Family

The archetypical Ras family with Ras as the founding member of the superfamily has received much attention due to its importance for signaling and disease. These proteins act trough several pathways. For instance, some bind to RAF and induce gene expression through the MAPK cascade in response to various extracellular signaling molecules, while others signal via PI3K. In general proteins of this family regulates cell proliferation, differentiation, morphology, and apoptosis. The Ras family spanning several organisms contains 178 proteins (Fig. 1.4) and in our

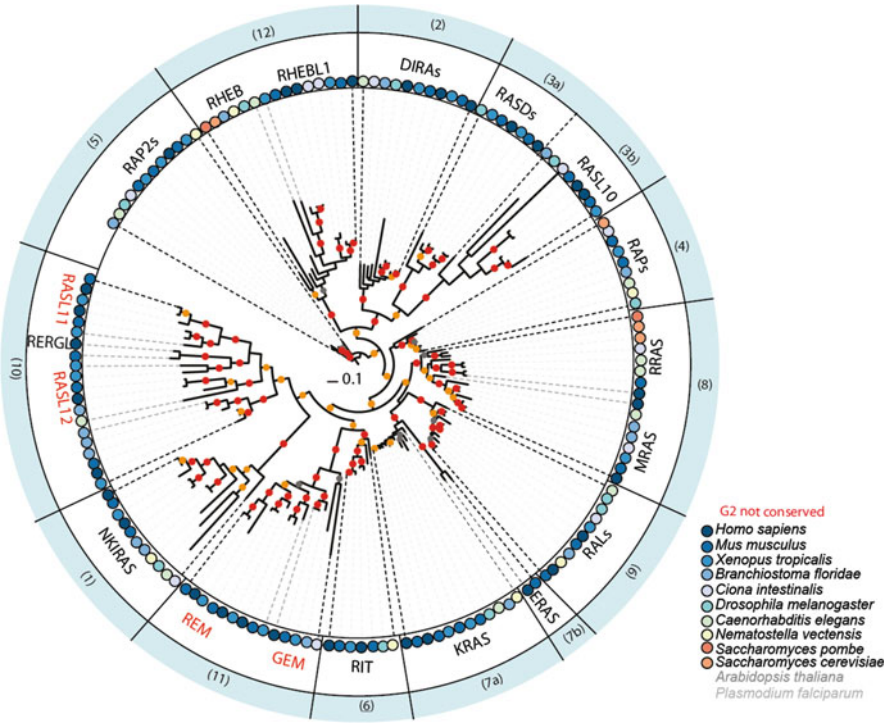

Fig. 1.4 Phylogenetic tree of the Ras family. The alignment of the RAS domain of 155 human orthologous sequences was used to conduct phylogenies. From the 172 RAS sequences, identical sequences were removed for the sake of clarity. Analyses were run in four chains and 1,000,000 generations. Trees were sampled after convergence was reached. Colour ranges indicate each of the species. *Red circles* in branches indicate group probabilities of 100 %. *Orange circles* indicate probabilities of 90–99 %. *Grey circles* indicate group probabilities of: >80–89 %. *Red names* indicate that the G2 motif is not conserved. *Numbers in outer blue circle* indicate groups analyzed previously (Rojas et al. 2012)

classification of human Ras sequences, the family contains 39 members (http://www.cbbio.es/GTPases/).

Our results indicate that this family is entirely absent from *Arabidopsis thaliana*. Thus, alternative members of the RAS superfamily constituting lineage-specific expansions such as the Rop proteins could have replaced the function of some of the Ras proteins in plants.

According to our analyses, we can divide the phylogenetic distribution of Ras domain-containing proteins into 12 stable groups, supported by confident probability values ($p > 0.8$ Fig. 1.4, gray circles).

The inclusion of additional organisms has enabled us to determine the phylogenetic positions of controversial members. For instance, the NKIRAS (KBRAS) protein (Fig. 1.4, group 1), believed to be human-specific (Jiang and Ramachandran 2006), is present in all the eukaryotic lineages except fungi and *Plasmodium*,

showing a large degree of divergence except for the more recent duplications in vertebrates. In addition, some discrepancies were found in the topology of other trees. In the tree generated by Karnoub and Weinberg (2008), the RASL11A (RSLBA) and RASL11B (RSLBB) proteins (Fig. 1.4, group 10) are located at a distance from the NKIRAS proteins (KBRS1 and KBRS2). However, in our study these two groups are found together. Group 7a includes members of the Ras proteins (HRAS, KRAS, etc.), showing little divergence in the different lineages. Thus, it looks like the oncogenic Ras proteins arose by gene duplication in vertebrate genomes, as indicated by their presence in *Xenopus*, and consistent with the hypothesis of major gene duplications. However, in the remaining genomes, only one copy of the ancestral homologue is found (Let60 in *C. elegans* and RAS1 in *D. melanogaster*).

By contrast, fungi do not have any clear orthologue of this particular subgroup although, alternatively, homologues could perform the same function like the case of *S. pombe*.

Group 12 contains the proteins present in all the proteomes analyzed including fungi.

Group 2 contains DIRA proteins, which are absent from fungi but present in the remaining eukaryotic proteomes. These proteins are involved in tumor suppression (Ellis et al. 2002). Group 3 can be divided into two smaller groups. Group 3a contains the RASD1/RASD2 (also called RHES) that are involved in dopamine signaling (Errico et al. 2008) and that are only present in the human, mouse, frog, and fly. Group 3b contains the nucleolar RASL10A/B (RSLAA/B) proteins proposed to be potential tumor suppressors (Elam et al. 2005) and that contain additional insertions in the G2 box. This group is present in the representative chordate species (i.e., fly and vertebrates), while it is absent in the coelomate *C. elegans*.

Groups 4 and 5 contain the RAP1 and RAP2 proteins, respectively, and their presence in the proteomes is variable. These proteins are involved in the signal transduction that regulates cell adhesion via cell surface receptors (Raaijmakers and Bos 2009). They show very little divergence when compared to the other groups in the tree. This suggests potential evolutionary constraints that maintain their function. Group 6 members include the RIT1/RIT2 present in radial animals (*N. vectensis*) and in the fly. On the other hand, RIT1 plays a role in the MAPK14 signaling pathway (Shi and Andres 2005; Shi et al. 2013), and it is found in the *D. melanogaster* and *N. vectensis*, while the RIT2 (a neuron-specific version) is only found in the mouse and human. Group 8 includes RRAS and MRAS. All the lineages except *D. melanogaster* contain homologues of these proteins. Group 9 includes RALA/B. They are present with little divergence in all lineages but fungi.

Group 10 is divided into three subgroups, as supported by high confidence values. The Rergl group is only present in vertebrates and interestingly, the human version does not contain a G1 box, while its mouse and frog homologues do. This suggests a specialization of this protein's activity in humans. The Ris (Raslc) subgroup of group 10 is present in the lanceolet and the worm, and this

protein has been mapped to the TGF-beta pathway (Barrios-Rodiles et al. 2005). Group 11 includes one representative from *Ciona intestinalis* and *B. floridae* and several duplicons that give rise to Rem/Gem/Kik/Rad in all the vertebrates. These so-called RGK proteins have been found to regulate voltage-dependent calcium channels (Correll et al. 2008).

1.5.3 The Rab Family

The Rab family is by far the largest family, and it is present in the 12 proteomes analyzed. Our searches retrieved a total of more than 300 sequences corresponding to Rab proteins. Rab proteins are distributed in several families that are each present in most of the genomes analyzed and they have experienced large expansions through gene duplication (Fig. 1.5a), as seen by the presence of duplicates in all the vertebrate genomes.

Rab GTPases regulate intracellular vesicular transport and the trafficking of proteins between different organelles in the endocytic and secretory pathways (Zerial and McBride 2001). Rab GTPases facilitate budding from the donor compartment, transport to acceptor, vesicle fusion, and cargo release. Rab GTPases serve as determinants of docking sites which integrate both membrane trafficking and intracellular signalling in a temporally and spatially sensitive manner (Bucci and Chiariello 2006). A salient feature of the Rab GTPase family is the distinct intracellular localization of different members which is used to define the nature and quality of a particular membrane domain (reviewed in Stenmark 2009).

Previous phylogenetic analyses (reviewed in Schwartz et al. 2007) divided the Rab family into eight functional groups (Pereira-Leal and Seabra 2001), while later studies classified it into nine groups (Stenmark 2009) and recently published analyses using large-scale comparative genomics comparisons including hundreds of genomes divides the family into six large groups (Klopper et al. 2012). Figure 1.5a–c show the phylogenetic distribution of the Rab repertoire according to our analyses and others indicated by sequential green. In the figures, external circles indicate different classifications. The inner light green indicates previous division in 14 groups (Schwartz et al. 2007). The middle green circle contains the functional family to which each protein belongs to (Stenmark 2009), whereas the outer circle contains the Klopper classification (Klopper et al. 2012) that includes 6 main groups (G1-6). Discrepancies within phylogenies are indicated with asterisks. For instance, group number 10 (Fig. 1.5a, c; inner circle part of the tree) contains RAB18, which is traditionally assigned to an independent family (Stenmark 2009). However, in our analyses RAB18 is grouped within the Stenmark's RAB3 group and also within Group 1 of Klopper (Klopper et al. 2012). A similar scenario was found for Rab family 28 (Fig. 1.5a, b). Magenta groups (Fig. 1.5a) are newly reclassified within Rabs, i.e., Rab20 originally unclassified by Wennerberg and colleagues (2005) appears to be a bonafide Rab protein when information from additional species is included as seen by us and others

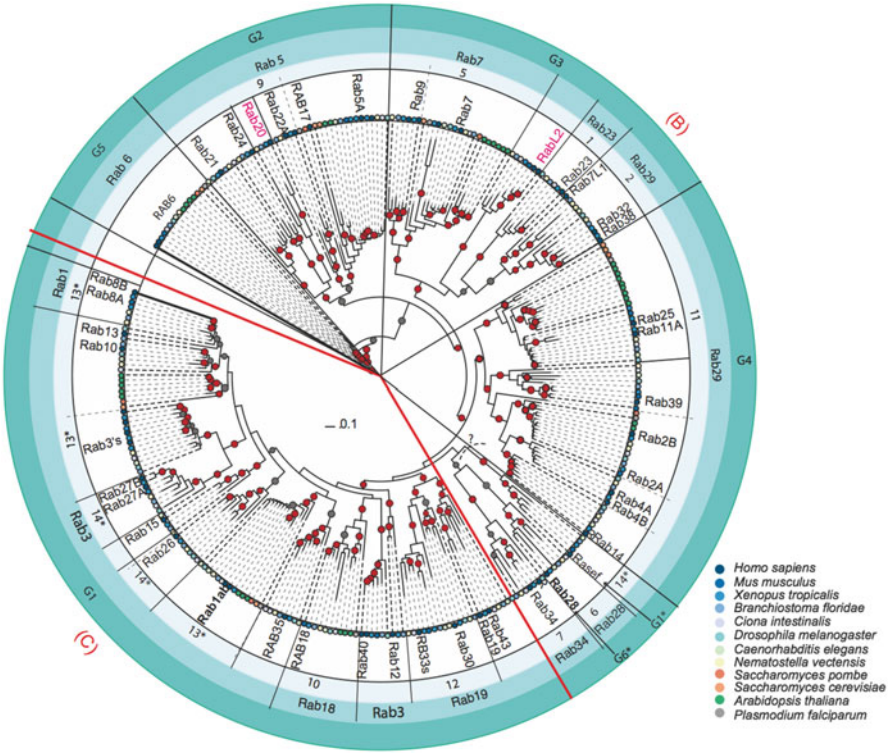

Fig. 1.5 (continued)

(Klopper et al. 2012). The tree was run for five million generations, and 135,190 trees were sampled to obtain the statistical value. When mouse and human sequences are identical, human sequences are removed from the tree to increase clarity.

The most studied Rabs belong to the family RAB7 (Fig. 1.5a, b) that contains the RAB7 and RAB9 proteins. RAB7 localizes to endosomes, lysosomes, and phagosomes. Members of this group also regulate the maturation and biogenesis of phagosomes in both unicellular eukaryotes and macrophages (Saito-Nakano et al. 2007). Taxonomic distributions show that the RAB7 gene arose before the radiation of eukaryotes, and RAB9 must have branched later among the metazoans and their relatives. Interestingly, RAB7B isoforms are only found in representatives of the amphibians, birds, and mammals.

As can be seen in the figure (Fig. 1.5a), the Rab branch has expanded by gene duplication (Mackiewicz and Wyroba 2009), especially in the vertebrate branch. Interestingly, all the different groups have representatives in all the lineages, indicating that the appearance of this family is an old event. Recent analyses of hundreds of genomes indicates that the Rab family history is more complex than

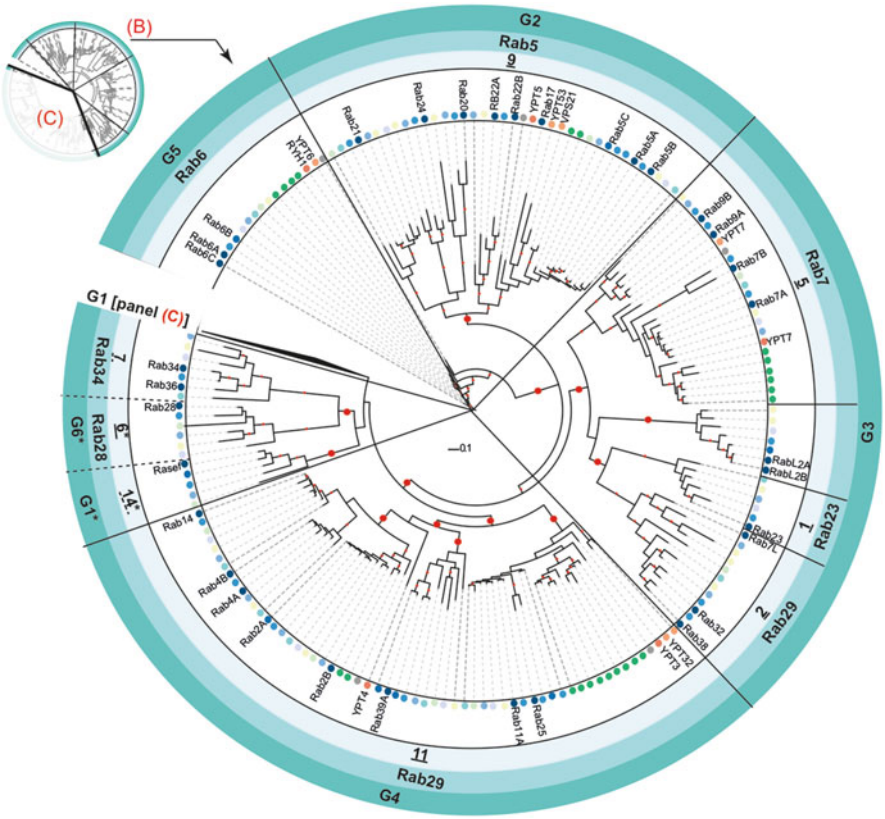

Fig. 1.5 (continued)

anticipated where its evolution may be due to severe gene loses/expansions and strongly correlates with membrane organization (Klopper et al. 2012).

1.5.4 The Arf Family

This family is widely spread throughout evolution, and it is by far the most diverse and divergent family of the RAS superfamily. Due to the wide use of synonyms, a new nomenclature (Kahn et al. 2006) has been agreed upon to name these proteins, the first one characterized being the human ARF1. The Arf family members are ubiquitous regulators of membrane trafficking and phospholipid metabolism in eukaryotic cells (Munro 2005) which translocate to membranes when activated. They also have a wide repertoire of effectors, including coat complexes (COP,

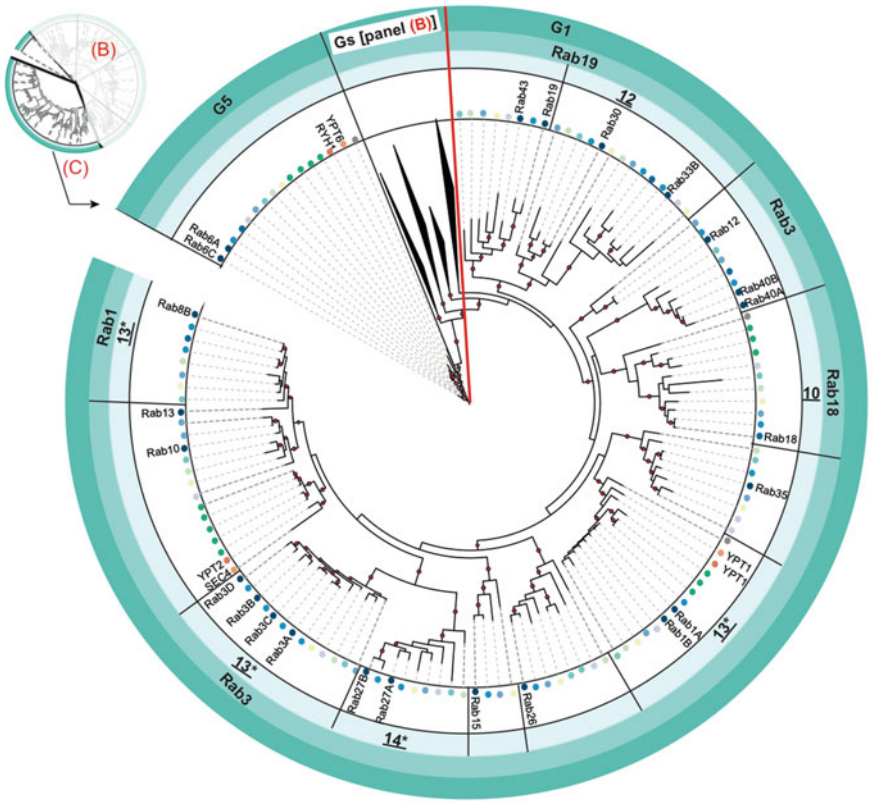

Fig. 1.5 (**a**) The alignment of the G-domain domain of 301 human orthologous sequences was used to conduct phylogenies. From the 390 Rab sequences, identical sequences were removed for the sake of clarity. Analyses were run in four chains and 5,000,000 generations. Trees were sampled after convergence was reached. *Red (B) and (C)* indicate sub-trees depicted in Fig. 5b, c, respectively, to facilitate interpretation of the large tree. Color ranges indicate each of the 12 species. External *green circles* indicate groups according to Schwartz *et al.* (Schwartz et al. 2007) (*lighter inner green*,. Stenmark *et al.* (Stenmark 2009) (*middle green*), and to Klopper et al (Klopper et al. 2012) (*Outer darker green*). *Asterisk* Indicates discrepancies with the aforementioned phylogenies. Magenta names are unclassified sequences. *Red circles* in branches indicate group probabilities higher than 90 %. *Grey circles* indicate group probabilities of: 80 % < Probability ≥90 %. When mouse and human sequences are identical, only the mouse sequences are represented in the tree. (**b**) Rab sub-tree with the G1 group (as depicted in Fig. 1.5a) collapsed to increase the clarity. Colors and elements of the figures are identical as Fig. 1.5a. *Red circles* in branches indicate group probabilities higher than 80 %. When mouse and human sequences are identical, only the mouse sequences are represented in the tree. (**c**) Rab sub-tree with the G2-4 groups, RASEF, RAB28, and RAB34 (as depicted in Fig. 1.5a), collapsed to increase the clarity. Colors and elements of the figure are identical as Fig. 1.5a. *Red circles* in branches indicate group probabilities higher than 80 %. When mouse and human sequences are identical, only the mouse sequences are represented in the tree

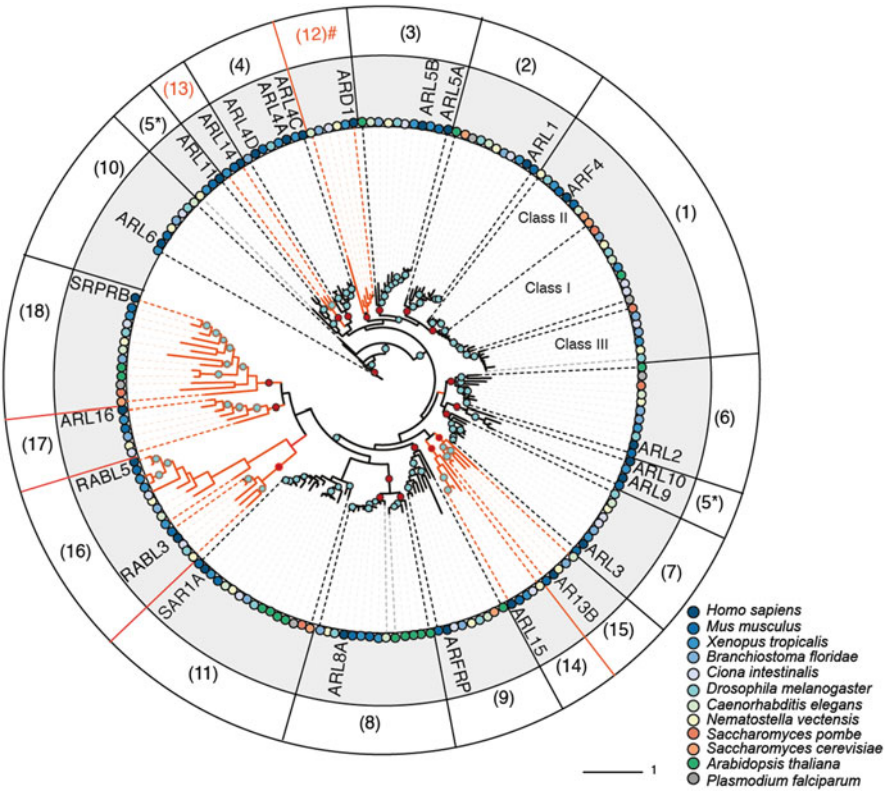

Fig. 1.6 The alignment of the G-domain domain of 183 human orthologous Arf sequences was used to conduct phylogenies. Analyses were run in 4 chains and 5,000,000 generations. Trees were sampled after convergence was reached. Color ranges indicate each of the 12 species. *Numbers in the outer circle in brackets* indicate clustering according to Li *et al*. (Li et al. 2004). *Asterisk* indicates differences with the Li clustering. *Orange clusters* are newly introduced families as a result of a new reclassification. When identical sequences are from human, frog, and mouse, only one is represented. *Red circles* indicate group probabilities higher than 90 %. *Cyan circles* indicate group probabilities of: 80 % < Prob ≥90 %. 180,466 trees were sampled where 99 % credible set contains 174,466 trees. *Hash symbol* are multidomain proteins

AP-1 and AP-3), lipid-modifying enzymes (PLD1, phosphatidylinositol (4,5)-kinase and phosphatidylinositol (4)-kinase), and others.

A previous analyses of the Arf family (Li et al. 2004) proposed a classification in 11 groups: ARFs, ARL1-6, ARL8, ARL10/11, ARFRP, and SAR. A revision to include Ard proteins, that are present in most of the metazoans, creates a new group supported by the sequence data with more than 90 % probability (Fig. 1.6). Ard proteins as other groups in the tree are multidomain proteins containing a Ring domain (SMART), N-terminal Zf_boxes (PFAM PF00643), and a characteristic C-terminal ARF-similarity region. The presence of these domains may point to

functions related to ubiquitination and binding to targets such as DNA, RNA, or proteins.

The analysis of the families in the various species indicates that SRPRBs and RABL5/3 tend to be close and therefore have been included within the Arf proteins (data not shown). The inclusion of additional proteomes and other reassigned sequences, like SRPRB (Fig. 1.6, group18) and RABL5/3 (group 16), support the stable classification of the Arf group (group 1, supported more than 90 % of the times), although in some cases there were discrepancies with previous phylogenies. For instance, ARL11/ARL8B (ARL10) proteins in group 5 (Li et al. 2004) appear in a different grouping in our analyses. siRNA experiments in *C. elegans* (Li et al. 2004) show that targeted knockdown of Arf proteins exhibit embryogenesis defects. By contrast, ARF6 siRNA animals did not show any obvious phenotype, although ARF6 is located at the plasma membranes and involved in endocytosis.

As seen in the tree, the Arf group (group 1) forms a consistent branch that is supported 100 % of times. This main branch contains the three different classes of Arf proteins (Kahn et al. 2006) for which all classes have orthologues in mouse, frog, cnidarian, ascidian, fly, and yeast. Plant representatives are located in the Class I/Class II group albeit with a low confidence value, such that they may be relocated in either class. However, there are no plant representatives for Class III, the most divergent class. The parasitic representative is located deep at the branching of Class I-Class II/Class III.

The Arl (Arf-like) group does not show a consistent phylogeny confirming previous observations (Kahn et al. 2006). Arls contain a glycine in position 2, which is a myristoylation site in Arf proteins. However, despite the conservation of the N-terminal glycine in ARL1,2,3, they are not substrates of N-myristoyltransferases. ARL2 and ARL3 regulate the transport of membrane associate proteins between membrane compartments (Ismail et al. 2011, 2012).

The Sar group is a stable one, with representatives in all 12 proteomes analyzed. The traditional classification of Arf proteins is maintained phylogenetically, with very weak divergence. Interestingly, the multidomain members of Arf that contain Ring and Zn fingers in the N-terminal region (group 12) are only present in metazoans. Human ARD1 presents three different isoforms, indicating either functional enrichment or redundancy. This suggests a specific functional repertoire acquired after divergence. Some vertebrate proteins emerged as duplication events prior to vertebrate speciation events (i.e.: ARL14 and ARL11 are more divergent than would be expected for duplicons). The Sar group is conserved in all the organisms (as supported by higher probabilities than 80 %), suggesting a very ancestral origin that produced ARL6 by duplication after metazoan divergence. The same may have occurred with different groups in the tree.

Finally, we include the previously unclassified SRPRB proteins in this family, given their consistent clustering in various trees (Rojas et al. 2012).

1.5.5 The Ran Family

There is only one copy for the nuclear transport protein RAN in the human genome, although it is the most highly expressed protein in human. In plants there are however several copies of this gene, indicating lineage-specific expansions. This family has been included in the global tree (see next section).

1.6 The Ras Superfamily: A Global View and Possible Novel Families

The systematic use of evolutionary information enabled the classification of a group of difficult sequences (Rojas et al. 2012).

(a) Our data suggested that the RHOT (MIRO) and RAYL proteins are part of a cluster within the Rho family tree (Fig. 1.7). These proteins have probably acquired alternative functions, as indicated by the detailed analysis of their potential functional sites (Rojas et al. 2012).
(b) RAB20 is only present in the human, mouse, frog, lanceolet and cnidarians and clusters within the Rab5 group (Fig. 1.5a, >90 % clade probability). This indicates that this gene may have been lost in coelomates and should be classified within the Rab subfamily.
(c) The RabL5/3 group is found in clearly separated and well-supported clusters (Fig. 1.7), and their key functional positions are also different from those of other groups (Rojas et al. 2012). RABL5 lacks the G4 box, a motif particularly important for the specificity of GDP/GTP binding, which clearly affects its phylogenetic distribution.
(d) SRPRB seems to be the most divergent members of the family and might be considered the oldest family in the tree.

The set of orthologous sequences, along with the estimated point in evolution at which they have diverged, is depicted in Fig. 1.1. The Ras family is entirely absent from plants (*A. thaliana*), in which Rho proteins are the only signaling members of the family (Yang 2002), while no Rho family orthologues are found in alveolates (*P. falciparum*). Although ascidians would be expected to have a similar number of Ras proteins as humans, based on phylogenetic estimates, there is a noticeable decrease in the number of Ras superfamily orthologues for this organism, probably due to the loss of ancestral genes (Hughes and Friedman 2005). Similar observations can be done in coelomates and cnidarians as fewer orthologues are found in worms than in sea anemone (*N. vectensis)*, although more orthologues are detected for the cnidarian than in the coelomate species. This finding is not unexpected, as gene content and genomic structure have been preserved between *N. vectensis* and vertebrates (Putnam et al. 2007), whereas extensive gene loss has occurred in the fruit fly and nematodes (Technau et al. 2005).

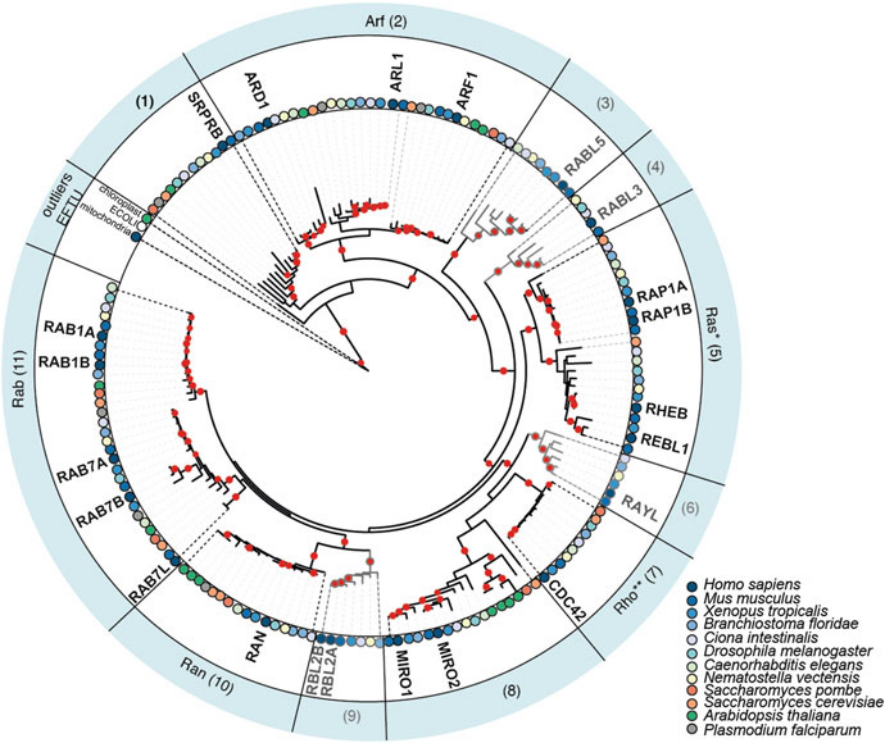

Fig. 1.7 Representative phylogenetic tree of the superfamily including representative sequences. *Names inside the blue circle* groups constitute the classical families, while *numbers in brackets without names* and *gray groups* indicate potential families, related GTPases (EF-TU) are included to root the tree

In some species additional gene duplication events have produced an accumulation of paralogous sequences, which results in variation between species in terms of the numbers of each Ras superfamily member. For example, three copies of the Ran family sequences were detected in *A. thaliana* while only one was found in the other species analyzed (Fig. 1.7). Rho family proteins expanded extraordinarily in plants (Yang 2002), and although plant Racs are homologues of Rac, Rho, and Cdc42, the expansion of Rac in plants after speciation has resulted in the generation of a large number of Rac proteins (RAC1-RAC11) than in other organisms. The gene duplication that generated the Ras family proteins in vertebrate genomes (HRas/ KRas, group 7a in Fig. 1.4) is another example of how variation in the number of Ras superfamily proteins arises. Indeed, while they are present in *Xenopus*, the older genomes only contain one copy (LET60 in *C. elegans* and RAS1 in *D. melanogaster*). Fungal orthologous sequences are only found for RHEB (group 12), RAP (group 4), and RRAS (group 8). Further duplications of these sequences after speciation yielded the Mras and Kras groups. Together, these data are consistent with major gene duplication in vertebrates (Kondrashov

et al. 2002). The vertebrate branch of the Rab protein family has expanded considerably (Fig. 1.5a, see also (Mackiewicz and Wyroba 2009; Klopper et al. 2012) and significantly, we found representatives of each of the different groups of Rab proteins in all lineages, indicating that the appearance of this family was an important evolutionary event. The Arf family of proteins (Kahn et al. 2006) is the most divergent member of the superfamily, and it is associated with recurrent duplication events.

In addition to genome duplications, domain shuffling is an additional source of functional variability whereby the acquisition of additional domains may confer novel capabilities to a protein (Venancio et al. 2009). The balance of losses/gains of domains and the different arrangements of protein domains thus generates alternative functions (Yang and Bourne 2009) and affects the functional repertoire of species (Zmasek and Godzik 2011).

In summary, our comprehensive phylogenetic analyses of selected, well-defined members of each family in representative species, using EFTu as outgroup, points to the Srprb proteins and the Arf family as possible founding members of the superfamily (Fig. 1.7). This implies that the original function of these proteins may have been related to the regulation of membrane trafficking in eukaryotic cells (Munro 2005), a process potentially linked to the emergence of complex intracellular structures. The presence of representative sequences of each family in the selected genomes indicates that divergence occurred prior to the emergence of eukaryotes, and strongly suggests that this superfamily expanded very early to generate the functionally distinct families. It is tempting to propose that this ancestral diversification is related to the increasing complexity of intracellular eukaryotic structures.

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Chapter 2
The Structure of the G Domain of the Ras Superfamily

Ingrid R. Vetter

Abstract Since the first three-dimensional structure of H-Ras has been determined in 1990, the number of solved structures of small GTP-binding proteins has increased tremendously. As of February 2014, 555 structures of Ras-superfamily proteins have been deposited in the protein databank (PDB), either in uncomplexed form or bound to effectors or other regulatory proteins. The 751 chains contain either GTP or a GTP analogue (431 chains) and GDP (320 chains), respectively. This chapter summarizes the most important structural features of single-domain GTP-binding proteins of the Ras superfamily and focuses on the comparison of the solved structures, especially the switch loops, i.e., the regions that change conformation upon nucleotide exchange from GTP to GDP. In particular, the pitfalls of the crystal structure interpretation will be emphasized since flexible protein segments like the switch regions of the G domain are especially prone to crystallization artifacts. Regions that are mobile in solution are commonly "frozen out" into relatively arbitrary conformations that often are dictated by the specifics of the packing against neighboring molecules in the crystals. It requires very careful analysis to decide if the conformations populated in the crystals have any physiological relevance.

Keywords G domain structure • Dynamics of switch regions • Crystallography • Crystal structure interpretation

2.1 Introduction: Structural Elements of the G Domain

The Ras superfamily of small GTP-binding proteins is characterized by the so-called "G domain" that is unique for this superfamily and is one of the most ancient protein domains. Attached to it is a hypervariable C-terminus that can be

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posttranslationally modified to achieve membrane attachment of the G domain via prenyl-, or palmitoyl groups and/or by positive charges. This C-terminus is deleted in most of the crystallized constructs or, if present, disordered in all structures, unless complexed with a stabilizing protein partner. In Arf proteins, the N-terminus can be myristoylated.

The G domain belongs to the fold-family "P-loop containing nucleotide hydrolases" (Saraste et al. 1990, SCOP fold c.37) and is the most common fold in all kingdoms (bacteria, archaea, and eukaryotes) (Wolf et al. 1999). It is also called a "mononucleotide-binding domain" since it is a remote relative of the Rossmann fold. Both fold types might have originated from a common ancestor and are sometimes classified as "Rossmannoids." The CATH database treats the "P-loop containing nucleotide triphosphate hydrolase" superfamily (3.40.50.300) as a subgroup of the Rossmann-fold proteins and accordingly lists it under the "Rossmann fold" topology. The genuine Rossmann fold proteins also have a glycine-rich phosphate-binding loop that is slightly longer than in the "P-loop containing nucleotide hydrolase" fold. In contrast to the genuine Rossmann fold proteins that bind NAD, the "P-loop containing nucleotide triphosphate hydrolases" have to compensate for the charges of the three phosphates, so they feature a magnesium ion and a conserved lysine side chain that contacts the phosphate oxygens. Both are crucial to enable high-affinity nucleotide binding (Kessel and Ben-Tal 2012).

The Ras superfamily is most commonly divided into five major families having related sequences and function as molecular switches in different biological systems: The Ras branch, involved in cell proliferation, gene expression, differentiation, and apoptosis, the Rho family that is involved in the dynamics of the cytoskeleton, the Rab and Arf/Sar families that regulate vesicular transport, and finally the Ran family that determines the direction of nucleocytoplasmic transport and is involved in mitotic spindle organization, with the only member being Ran itself [Table 2.1, (Goitre et al. 2014; Wennerberg 2005; van Dam et al. 2011)]. Since structural data is most abundant for the Ras subfamily, the "extended" Ras family (Di-Ras, Rap, Ral, RheB/RheB-like, Rerg, and the RGK-family (i.e., Rad, Gem/Kir, Rem) will be discussed separately from the core Ras group (formed by H-,K-,N-,M-, and R-Ras). The structurally best-characterized families are the Ras and the Rab families with 139 structures (190 structures including the extended Ras family) and 137 structures, respectively. The Arf and Sar proteins are sometimes assigned to separate families.

There are many more distantly related G-domain structures that contain a wide variety of inserts and deletions, and can also bind adenine nucleotides instead of guanine nucleotides, but they have been extensively reviewed elsewhere (e.g., Vetter and Wittinghofer 1999; Leipe et al. 2002; Wittinghofer and Vetter 2011) and are not covered in this chapter.

This chapter focuses on the single-domain G-proteins of the Ras superfamily, so some multidomain proteins with known structures that have G domains closely related to the Ras superfamily, e.g., the Miro, Roc, Centaurin-γ, and Rag proteins, are also omitted here. The Miro and Rag proteins are discussed in other chapters of this book.

Table 2.1 Structurally characterized families of single-domain small GTPases of the Ras superfamily. PDB accession codes are given where only one or few structures exist

Family	Subfamily	Members (PDB accession codes)
Ras	Ras	H-Ras, K-Ras, N-Ras (3con), M-Ras, R-Ras (2fn4), R-Ras2/TC21 (2ery)
Ras extended	Ral	RalA, RalB
	RheB	RheB, RheBL1 (3oes)
	Rap	Rap1A, Rap1B, Rap2A, Rap2B Ras-3 (4ku4, Ras-like protein from Cryphonectria parasitica) Di-Ras1 (2gf0), Di-Ras2 (2erx)
	RGK	Rad, Gem/Kir, Rem1 (2nzj), Rem2 (3cbq, 3q85, 4aii)
	Rerg	Rerg (2atv), RasL12 (3c5c)
Rho	Rho	RhoA, RhoB, RhoC, EhRho1 (3ref, 3reg, complex with Diaphanous-Protein: 4dvg)
	Rac	Rac1, Rac2, Rac3, Rop4, Rop5, Rop7, Rop9
	Cdc42	Cdc42, RhoUA (2q3h), TC10 (2atx)
	RhoD	RhoD (2j1l), Rnd1 (2cls, complexes with plexin: 2rex, 3q3j), RhoE/Rnd3
Rab	Rab	Rab1,2,3,4,5,6,7,8,9,11,12,14,18,21,22,23,25,26,27,28,30,31,33,35,43
	RasEF	RasEF (2p5s), IFT27 (2yc2/2yc4)
	Ypt	Ypt1,7,8,32,51
	Sec	Sec4
Arf	Arf	Arf1,2,4,5,6,8, Arl2,3,5,6,8,10,13
Sar	Sar	Sar1
Ran	Ran	Ran, RanE (4djt, Nuclear GTP-binding protein from Encephalitozoon cuniculi)

2.2 G Domain Topology and Motifs

The G domain consists of a central β-sheet with six strands that is flanked by five α-helices on both sides (Fig. 2.1). The affinity of the G domain to GMP is usually very low, in contrast to GDP and GTP (John et al. 1990) so that small GTP-binding proteins are most frequently found in complex with GTP or GDP in the cell or after purification in vitro (typical concentrations for GTP and GDP in cells and tissues are 0.3–0.5 mM and 0.03–0.06 mM, respectively (Traut 1994)). Exceptions are found in more distant relatives like hGBP1 that can bind GMP with a comparable affinity to the di- and triphosphate nucleotides by allowing the α-phosphate to shift towards the P-loop so that the position of the α-phosphate of GMP now occupies roughly the position of the β-phosphate (Ghosh et al. 2006). This is achieved by inducing an unusual conformation of the nucleotide.

Comparing the three-dimensional structures with either GDP or GTP bound, large conformational changes are observed in all members of the Ras superfamily. The affected regions are called “switch” regions accordingly since they can switch the interactions of the G-domain with other proteins, e.g., effector proteins, “on” or

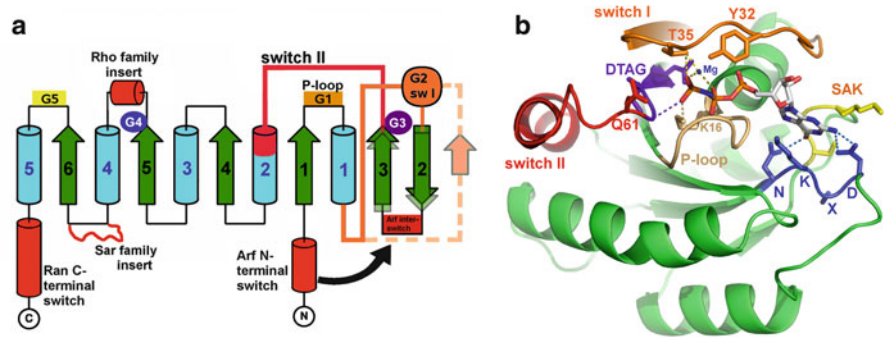

Fig. 2.1 (**a**) Topology diagram for the G-domain of the Ras superfamily. (**b**) Three-dimensional structure of the G-domain with positions of the conserved nucleotide-binding motifs [same color code as in (**a**)]. Side chains of important residues (Lys16, Tyr32, Thr35, and Gln61 of H-Ras) are shown as *sticks*

"off." Usually, the GTP-bound state is the active one that in case of Ras allows activation of downstream kinases and triggers, e.g., cell division.

Since the main characteristic of the G domain is guanine nucleotide binding, it is not surprising that the five conserved fingerprint motifs are located in loops clustered around the nucleotide-binding site and are therefore often called G1-G5 (Fig. 2.1). G1 or the "Walker A motif" is the glycine-rich phosphate-binding loop ("P-loop," GxxxxGKS/T) that gives the fold its name. The P-loop wraps around the phosphates allowing the main chain nitrogen atoms to interact tightly with the negatively charged phosphates. The P-loop lysine directly interacts with the β- and γ-phosphate oxygens and is crucial for nucleotide binding. The hydroxyl group of the serine or threonine contacts the β-phosphate oxygen and the magnesium ion. G2 ("switch I," residues 32–38 in H-Ras) contains a threonine (Thr35 in H-Ras) that is conserved in all members of the Ras superfamily except the RGK family (see also Sect. 2.9). The switch I is one of the regions that changes its conformation upon exchange of GTP and GDP and is also called "effector region" since it is often involved with effector binding when in the GTP state. The conserved threonine is crucial for sensing the presence of the GTP γ-phosphate, and it also contacts the magnesium ion. G3 or the "Walker B motif" is the "DxxG" motif close to the "switch II" region where the D usually sits close to the magnesium ion, but does not necessarily contact it directly. Switch II (residues 59–67 in H-Ras) has no conserved sequence motif besides a glycine (G60 in Ras, conserved in the Ras superfamily except in the RGK family) and also senses the presence of the γ-phosphate. It is often involved in effector interactions also, and plays an important role in nucleotide exchange by GEFs (guanine nucleotide exchange factors) and in stimulation of GTP hydrolysis by GAPs (GTPase activating proteins). G4 is the N/TKxD motif where the aspartate contacts the nitrogen atoms of the base with a bifurcated hydrogen bond, and the asparagine can contact the oxygen of the purine, thus conferring specificity for the guanidinium base. The lysine of this motif stacks along the plane of the base. G5 is the weakly conserved SAK motif: the

backbone amine interacts with the oxygen of the guanine base and the serine side chain helps to stabilize the adjacent loop in a tight turn. The Rho-family insert between β-strand 5 and α-helix 4 (Fig. 2.1a) is missing among the members of this family only in the structure of Rho1 of *Entamoeba histolytica* (see Sect. 2.11). Typically it forms a short helix sticking out from the remainder of the G domain and does not change its position upon nucleotide exchange.

2.3 Structural Changes Upon Nucleotide Exchange

The structural change of the two Ras switch regions can be interpreted as a "loaded spring" mechanism (Vetter and Wittinghofer 2001): The presence of the GTP γ-phosphate causes the switch I and switch II regions to preferentially assume positions close to the nucleotide. The crucial contacts are from the side chain hydroxyl group of the conserved threonine in switch I and the main chain nitrogen of the conserved glycine in switch II to the γ-phosphate oxygens (Fig. 2.2). When the γ-phosphate is cleaved off, the switch regions are thought to become mobile and disordered so that the "open" (GDP) state usually does not have a defined conformation (with the exception of the Arf and Ran families). Instead, the switch regions are dynamic and assumed to fluctuate on a pico- to nanosecond timescale. The "closed" conformation of the switch regions confers a higher affinity to effector molecules when compared to the "open" form since no binding enthalpy has to be expended to fix a highly flexible region. Indeed, if the binding energy of an effector protein is sufficiently high, as, e.g., in case of the A85K mutant of the Ras-binding domain of Raf kinase, it can form a complex even with the GDP form. The switch I region is then forced into the closed conformation by the effector domain (Filchtinski et al. 2010). Generally, in the GDP-bound forms of uncomplexed G domains, the switch regions (and specifically the threonine and the glycine) are distant from the γ-phosphate since the contacts to the threonine and glycine are lost. However, as detailed below, even the GTP state shows intrinsic flexibility.

In the Ras, Rho, and Rab families, the release of the switch regions after GTP hydrolysis commonly leads to a less well-defined position of the switch I region as evidenced by NMR solution studies as well as by the numerous X-ray structures (Figs. 2.2 and 2.3). This situation is different in the Ran and Arf families where the switch I region in the GDP form changes its position and secondary structure in a defined way by forming an additional β-strand that extends the central β-sheet (Fig. 2.1a, orange/dotted position of the switch I loop). In Ran, the position of β-strands 2 and 3 (also called the "interswitch" since they are located between the two switch regions (Pasqualato et al. 2002)), is relatively similar when comparing the GDP and GTP forms. In contrast, in the Arf and Sar proteins β-strands 2 and 3 undergo a register shift of two residues relative to the rest of the β-sheet (Fig. 2.1a). This is probably mediated by strand number 3 moving into the direction of the γ-phosphate, and strand number 2 then adjusts to this movement. The location of this interswitch-β-hairpin in the GDP form opens a hydrophobic groove opposite the nucleotide-binding pocket where the amphipatic N-terminal helix can

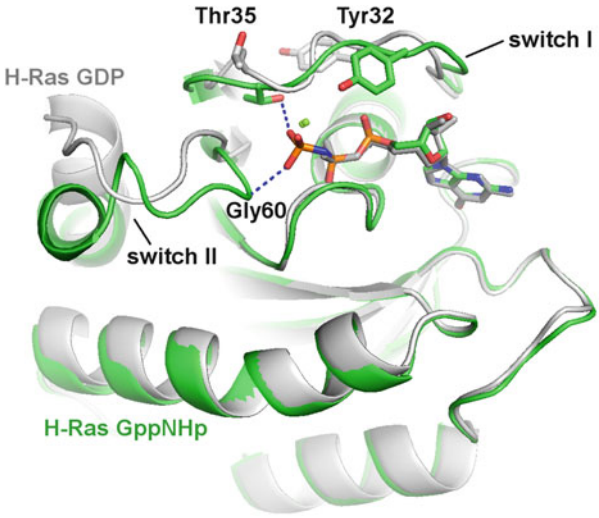

Fig. 2.2 Change of the switch regions in the H-Ras G domain upon nucleotide exchange. H-Ras-GppNHp in *light green* (5p21), H-Ras-GDP in *gray* (4q21). The positions of Thr35, Gly60, and the hydrogen bonds of the switch regions to the γ-phosphate oxygens in the GTP state are indicated. The side chains of Thr35 and Tyr32 are shown as *sticks*, and the magnesium ions as *spheres*

bind (Fig. 2.1a, black arrow). This additional helix is a hallmark of the Arf family. Formation of this pocket in Arf-GDP is only possible because of a much shorter β2-β3-hairpin as compared to Ran and the other members of the Ras superfamily (Pasqualato et al. 2002). In the GTP-bound form, the N-terminal helix (that is myristoylated in most of the Arf proteins at the second glycine) is "pushed away" from the core domain and can then mediate interactions with membranes. This additional switch mechanism is reminiscent of the Ran proteins, where a third switch region has been described that again consists of a helix, but in this case C-terminal to the G-domain (Fig. 2.1a). In the GDP form, the helix is associated with the G domain (Fig. 2.3), whereas in the GTP form the altered position of the switch I region leads to a destabilization of the linker region that precedes the helix, causing the C-terminal helix to detach completely from the core domain. Since the helix is now freely accessible, it can be captured, e.g., by Ran-binding proteins. In addition, the dislocation of the helix uncovers the binding site for karyopherins and allows formation of the high-affinity Ran-karyopherin complexes, thus triggering cargo release in the nucleus. The flexibility of the C-terminal helix apparently interferes with crystallization, so no structures of uncomplexed Ran in the GTP form are available. To date, the Ran-GTP structure has been solved only in complex with other proteins like karyopherins or Ran-binding proteins.

2.4 Dynamics of the Switch Regions

The now numerous crystal structures of the G domain tend to confer the misleading picture that the switch regions are preponderantly in fixed conformations, either in the "closed" conformation that can bind to effectors or in an "open" one that disfavors effector binding.

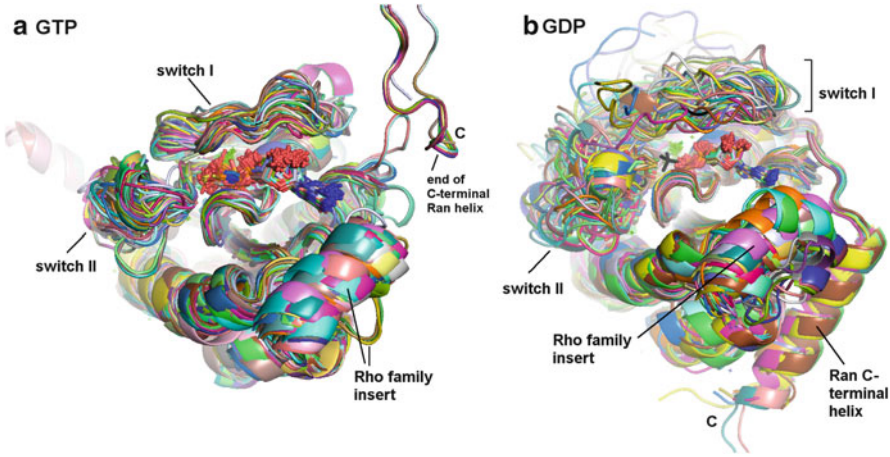

Fig. 2.3 Superimposition of GTP/GppNHp/GppCH2p/GTP-gammaS-bound structures (**a**) and GDP-bound structures (**b**) of the Ras superfamily, highlighting the difference in variability of the positions of the switch I and II regions. H-Ras GppNHp (5p21) is shown in *black* for comparison. The "Rho family insert" is in similar positions in the GTP- and GDP states; it appears to be more variable in the GDP forms since the plant Rop proteins are included here which have a shorter insert helix with a tilted axis relative to the canonical position. The C-terminal helix of Ran is assumed to be flexible in the GTP state in solution

NMR spectroscopy has shown that in solution the switch regions of H-Ras-GDP are disordered and that they show intrinsic mobility on the nanosecond timescale (Kraulis et al. 1994). Likewise, the switch regions of Cdc42-GDP displayed a high level of disorder (Feltham et al. 1997). There were also indications in H-Ras that the helix following the switch II region (α2, Fig. 2.1a) changes its position slightly compared to the X-ray structures of H-Ras-GDP (Kraulis et al. 1994). These observations are nicely corroborated by today's plethora of crystal structures: The "open" conformations of the GDP-bound forms show a much larger variation compared to the GTP forms (Fig. 2.3). Additionally, the switch regions are frequently disordered in the crystals, i.e., they do not show interpretable electron density. In cases where well-defined electron density is observable, they often pack against neighboring molecules in the crystal, so one has to be very careful in interpreting those structures. If a specific conformation of an open switch region is stabilized just because of crystal packing forces, it should be regarded as a crystallization artifact. This is evidenced by the many crystal structures with bound GDP whose switch regions do have well-defined density. For example, in H-Ras-GDP (4q21), there are extensive contacts between the switch regions and neighboring molecules, and, accordingly, both switch regions are reasonably well defined, whereas in a different crystal form of H-Ras-GDP (1ioz), switch II is located next to a relatively wide solvent channel and does not show electron density for residues 61–67. Switch I in 1ioz has crystal contacts only at its ends and correspondingly shows relatively high temperature factors at the tip between

residues 25 and 36. It is evident that the crystallization conditions and the crystal environment have a significant influence when a protein with intrinsically flexible regions is investigated via X-ray structure analysis.

Even in the GTP-bound state, the switch regions are not completely locked down in the "closed" form as evidenced by numerous NMR studies. Phosphorus NMR with H-Ras bound to GppNHp can differentiate between the conformational states as defined by the chemical shifts of the nucleoside phosphates. Especially the γ-phosphate shows a split resonance, indicating the presence of (at least) two different chemical environments of this phosphate, called "state 1" and "state 2," respectively (Geyer et al. 1996). It is likely that the chemical shift change of the γ-phosphate is mainly influenced by a tyrosine in the switch I loop (Tyr32 in H-Ras, Fig. 2.2). The situation with tyrosine distant or close to the γ-phosphate would then correspond to "state 1" and "state 2," respectively. Usually it is assumed that "state 2" to the closed conformation that facilitates effector binding, and that "state 1" consists of a rather undefined ensemble of various "open" conformations (Spoerner et al. 2001). This is also confirmed by a H-Ras-GppCH2p structure (6q21) where the switch I region shows a disordered "open" conformation (see next section).

Only one of the nine crystal forms of H-Ras, [the $P3_221$ space group of the first H-Ras structures, e.g., 5p21 (Pai et al. 1990), does not show the tyrosine 32 in contact with the γ-phosphate, in spite of having a "closed" switch I region. Again, most likely the crystal packing forces are causing the tyrosine to be flipped outwards so that it interacts with the γ-phosphate oxygen of a symmetry-related molecule. In 2007, a new crystal form of H-Ras was published [2rge, in a R32 space group, (Buhrman et al. 2007)] that showed the tyrosine 32 in a position very similar to the effector-bound position [e.g., 1gua, (Nassar et al. 1995)]. In this crystal form, there are no crystal contacts close to the Tyr32, indicating that this might be the most reliable equivalent to the "state 2" observed via NMR spectroscopy. These observations reaffirm the importance of being aware of crystal packing effects.

The equilibrium between the different states of the switch conformations in the GTP form is a delicate balance, fine-tuned to transiently stabilize the active Ras sufficiently to allow activation of downstream effectors without switching it off again via intrinsic GTP hydrolysis, while allowing stabilization of the switch regions into the catalytically competent conformation by the GAP proteins.

2.5 Authentic GTP Versus GTP Analogues

At 298 K, "state 2," corresponding to the "closed" state of truncated H-Ras-GppNHp (residues 1-166), is only slightly preferred with a ratio of approx. 56:44 (Ye et al. 2005), whereas H-Ras with authentic GTP shows a strong preference for the closed state (92:8) (Spoerner et al. 2010). The GTP analogues GppNHp and GppCH2p apparently tend to shift the equilibrium towards the open state, whereas GTP and GTPγS are more efficient in fixing the switch I region (Spoerner

et al. 2010; Long et al. 2013). The equilibrium is strongly temperature dependent; the interconversion rate between the two states increases from 130 s^{-1} at 5 °C to 1,900s^{-1} at 25 °C (Spoerner et al. 2001). This is consistent with the H-Ras-GppCH2p structure 6q21 where one of the four chains in the asymmetric unit (chain D) shows an "open" conformation with a disordered stretch between residues 33 and 36 even in the crystal, whereas chains C and B are in the canonical conformation. Chain A has the threonine 35 in the canonical position, but the preceding part of the switch I region is in a more detached position compared to the other chains. The inherent flexibility of switch I even in the GTP form is usually obscured in other crystal forms since dynamic regions are commonly "frozen out" and artificially stabilized by neighboring molecules in the crystals. NMR studies of H-Ras-GppNHp (Araki et al. 2011) indicate that the switch regions in state 1 move rapidly on a picosecond to nanosecond timescale, and that the mobility is drastically reduced (but still present) in the closed state (state 2).

Of the 65 Ras-superfamily structures with bound GTP in the PDB database, three quarters are in complex with effector molecules or toxins, and three structures involve hydrolysis-impaired mutants [Arf1 Q71L (1o3y), Rab5a A30P (1n6l), Rab7 Q67L (1t91)]. The remaining nine structures are mostly of naturally slow GTPases like the RhoE/Rnd3 constitutively active core domain that has two serines in place of Q61 and A59 of Ras, the extremely slow GTPase Rab6a, and the slow GTPase RheB that has an extension in the switch II region that places the catalytic glutamine away from the γ-phosphate (see also Sect. 2.9 and Fig. 2.6a). The remaining structures are freeze-trapped H-Ras (1qra), the slow GTPase Rap2 (2rap and 3rap), an unpublished structure of the GTPase-deficient Rnd1 (2cls), and an unpublished Arl6 structure (2h57). Detailed analysis of H-Ras-GTP and H-Ras-GppNHp structures at different temperatures has revealed that the nucleotides bind in identical manner to the protein with only slight differences around the bridging NH group (Scheidig et al. 1999), indicating that tiny changes in the structure can still lead to drastic changes in the dynamics of the switch regions.

2.6 Dynamics and Switch States in Other Subfamilies

The dynamic behavior of the switch regions varies drastically between the different members of the Ras superfamily: For example, the Rap proteins (Rap1A and Rap2A) are between 86 and 94 % in the closed form even with GppNHp, whereas RalA-GppNHp is only 40 % closed, and M-Ras-GppNHp is almost completely open (93 % open conformation) (Liao et al. 2008). The corresponding NMR experiments also allowed dissection of the importance of certain residues for the dynamics: Mutation of the conserved switch I Thr35 in Ras [not conserved only in the RGK-family Rad, Gem/Kir, Rem, and in a Ras-like protein (RasL21, 3c5c, unpublished)] to alanine leads to an almost complete shift of the equilibrium towards the open state (>96 % open), and even the mutation T35S shows >78 % open form, suggesting that not only the hydroxy group but also the methyl group of

threonine is important to favor the closed side. The effect of the T35S mutation has been confirmed by solving the X-ray structures of H-Ras T35S, where the switch I regions are either disordered, as expected [1iaq, (Spoerner et al. 2001)], or packing tightly against neighboring molecules, thereby being fixed in a most likely artificial position [3kkn, 3kkm (Shima et al. 2010)]. The effect of the mutation G60A in H-Ras is less drastic: about one-third of H-Ras-G60A-GppNHp is still in the "closed" form (Spoerner et al. 2010).

All crystal structures of members of the RGK family (lacking the T35 and G60) show either completely diordered switch regions or some arbitrary open conformation [Rem-GDP (3cbq, unpublished), Rem-GDP (4aii, (Reymond et al. 2012)]. In some cases the switch regions are only partially visible (Gem-GDP (2cjw, (Splingard et al. 2007), 2ht6, (Opatowsky et al. 2006), 2g3y, unpublished).

A Ras-like protein (RasL12) determined in a structural genomics initiative (3c5c, unpublished) also lacks the conserved threonine and glycine. Interestingly, the switch regions are (even in the GDP form) not too far away from the Ras-GTP position (see Sect. 2.9).

2.7 Influence of Structural Elements on the Intrinsic Hydrolysis Rate

The intrinsic hydrolysis rate of small GTP-binding proteins has to be sufficiently slow so that the biological activity can be accomplished while in the GTP state. GAP proteins accelerate hydrolysis by several orders of magnitude. Interestingly, the intrinsic hydrolysis rates are quite diverse as well, ranging over three orders of magnitude from extremely slow proteins like Ran with $1.8 \cdot 10^{-5}\ s^{-1}$ [25 °C, (Klebe et al. 1995)] or one of the slowest Rabs, Rab6a, with $5 \cdot 10^{-6}$ molecules GTP per second (Bergbrede et al. 2005) to, e.g., Cdc42 with around $1 \cdot 10^{-3}\ s^{-1}$ [20 °C, (Zhang et al. 1997)]. In general, spontaneous hydrolysis of GTP in water is slower than the intrinsic hydrolysis of GTPases. Thus, the presence of the catalytic machinery of the G domain has an accelerating effect. One obvious candidate responsible for this accelerating effect is the catalytic glutamine (Q61 in H-Ras) in the switch II region which, when mutated to, e.g., leucine, reduces the intrinsic hydrolysis of H-Ras by a factor 22–80 (Frech et al. 1994; Smith et al. 2013). But, even if the catalytic glutamine is present, the flexibility of the switch regions implies that only a small fraction of the ensemble of conformational states is in the catalytically competent form at any time. This is strikingly evident from the structure analysis of 151 uncomplexed G-domain structures in the GTP-bound state (222 chains), of which only four (seven chains) show the catalytic glutamine close to a γ-phosphate oxygen (distance cutoff 3.5 Å). Of 120 structures complexed with effector molecules (205 chains), 7 structures (22 chains) show a distance <3.5 Å. In contrast, all transition state structures complexed with GAP and GDP-AlF_3 (19 structures, 47 chains) show a close contact between the glutamine and the

γ-phosphate, with exception of the Gyp1-TBC domain-GAP/Rab33 complex where the catalytic glutamine is supplied by the GAP [2 g77, (Pan et al. 2006)] and the functionally similar complexes of Rab1 with the bacterial GAP proteins VirA and EspG [4fmb, 4fmc, 4fmd, 4fme, (Dong et al. 2012)]. Superimposing the non-transition state structures shows the glutamine side chain pointing in all possible directions. The presence of multiple conformations of the glutamine side chain at room temperature is also supported by the data from non-frozen H-Ras crystals [3tgp, (Fraser et al. 2011)]: here, glutamine 61 is found in two conformations, with one rotamer pointing away from the nucleotide (the major conformation), and one closely resembling the optimal catalytic position found in the transition state complexes, i.e., close to the γ-phosphate oxygens. This highlights a general problem of collecting structural data from cryo-cooled xtals: The cooling leads to a shrinkage of the crystal lattice and thus a compaction of the proteins, resulting in the freeze-out of a particular state of proteins (Halle 2004; Juers and Matthews 2004). Another pitfall are the positions of the (catalytic) water molecules that might change between physiological temperatures and cryo conditions (Scheidig et al. 1999). Since nowadays practically all X-ray structures are solved at low temperatures (around 100 K), one has to be very careful in interpretation of the data, especially from proteins with flexible regions.

Usually, the binding of effector/helper molecules has a stabilizing effect on the switch regions. GTP hydrolysis might be either slightly faster (e.g., in Ran-RanBP1 complexes (Bischoff et al. 1995), remain unchanged [Ras-RafRBD complexes (Spoerner et al. 2010; Herrmann et al. 1995)], or can be completely abolished [e.g., in Ran-karyopherin complexes (Gorlich et al. 1996)]. This has been attributed to the particular position in which the catalytic glutamine is "trapped" by the effector binding (Seewald et al. 2002). The closer the glutamine is to the catalytic position, the faster the hydrolysis. An additional observation concerns the tyrosine of the switch I region (Tyr 32 in Ras): In very slow GTPases like Rab6 and Rab7, this tyrosine covers the phosphates of the triphosphate nucleotide in all known structures, even if not complexed to an effector molecule. The same is observed for the slow GTPases Rap, Rab28, RheB, RheB-like, and the GTPase-deficient RhoE/Rnd1. In complex with effector molecules, the tyrosine is in a similar position in all Ran-GTP complexes and in some Cdc42 (3eg5, 1nf3), Rap (1c1y, 4hdo), Ral (1zc3, 1zc4), and Rho (3a58, 1z2c, 1e96) complexes. Mutating the tyrosine to alanine significantly speeds up hydrolysis in Ran Y39A (Brucker et al. 2010) as well as in RheB Y35A (Mazhab-Jafari et al. 2012). Some of the Rabs with faster intrinsic hydrolysis rate [e.g., Rab3a ($3 \cdot 10^{-4}$ s^{-1}) (Clabecq et al. 2000)] have a phenylalanine instead of the tyrosine, and RheB Y35F is almost as fast as RheB Y35A (Mazhab-Jafari et al. 2012), indicating that the hydroxy group interaction might be more important than the shielding effect. It is thus tempting to assume that the hydroxyl group of the tyrosine unfavorably interferes with the position of the catalytic glutamine. It would seem that the slow GTPases Rab4 and Sec4 would prove this hypothesis wrong since instead of the tyrosine they have a phenylalanine or a serine, respectively. However, Rab4 has a histidine (His39) that occupies exactly the position of the hydroxyl group of the tyrosine, and might thus also

block access for the catalytic glutamate (Huber and Scheidig 2005). Sec4 has a serine (Ser29) in the P-loop that is thought to interfere with the intrinsic hydrolysis rate via a hydrogen bond to the γ-phosphate oxygens (Stroupe and Brunger 2000), which might have an influence either on the positioning or the electronic properties of the γ-phosphate oxygens.

In summary, the flexibility of the switch regions might determine the probability of the catalytic glutamine being in the correct position, and any residue that hinders access of the glutamine to this optimal position is then expected to reduce the intrinsic hydrolysis rate.

2.8 Ras Family

Based on sequence homology, the Ras family in its more narrow definition can be divided into the two subgroups H-Ras/K-Ras/N-Ras and M-Ras/R-Ras, respectively. Among the "canonical" Ras proteins, H-Ras is by far the structurally best-characterized protein, whereas there is only one structure of N-Ras, and until recently there were only two structures of K-Ras. This changed in 2012 and 2013 due to the revived interest in binding small molecules to K-Ras. Still, there is only one wild-type structure of K-Ras in the database. In contrast, the 67 structures of uncomplexed H-Ras in the GTP state have been obtained by growing nine different crystal forms from 31 wild-type and 36 mutant proteins. This large number of structures allows a quite detailed comparison of the switch regions that exemplify various features of crystallization artifacts as explained above. The switch I region tyrosine (Y32 in H-Ras, conserved in the extended Ras subfamily) is in a position close to the γ-phosphate, i.e., in "state 2" in all crystal forms except the trigonal one. The switch regions are in the "closed" position with Thr35 and Gly60 forming the anchor points as expected, with the exceptions of the intrinsically "open" M-Ras, two chains of H-Ras-GppCH2p (6q21) and three structures of H-Ras-GppNHp [4efl (wild type), 4efn (Q61L) and 4efm (G12V)] (Fig. 2.4). The latter were obtained by seeding a H-Ras solution with crystals of the H-Ras-T35S-GppNHp form, causing the H-Ras wild type to crystallize in the orthorhombic I222 space group of the mutant crystals that is otherwise not accessible. Since the crystal form (space group) and thus the crystal packing is now exactly the same, it is not surprising that the switch I region of H-Ras-GppNHp wild type is in exactly the same position as in the H-Ras-T35S-GppNHp crystals. In both mutant and wild type the open state is stabilized by switch I packing tightly to a symmetry-related molecule and pulling away the threonine 35 in the process [2efl compared to 3kkn, (Muraoka et al. 2012)]. Again, this particular manifestation of a "state 1" conformation appears to be an artifact caused by the specifics of the crystal packing. The reverse experiment, i.e., crystallizing H-Ras-T35S-GppNHp in the space group of wild-type H-Ras-GppNHp [rhombohedral crystal form, e.g., 2rge (Buhrman et al. 2007)], illustrates once more the delicate balance of the switch I conformation: in contrast to wild type, only part of the switch I region of the T35S mutant is in the

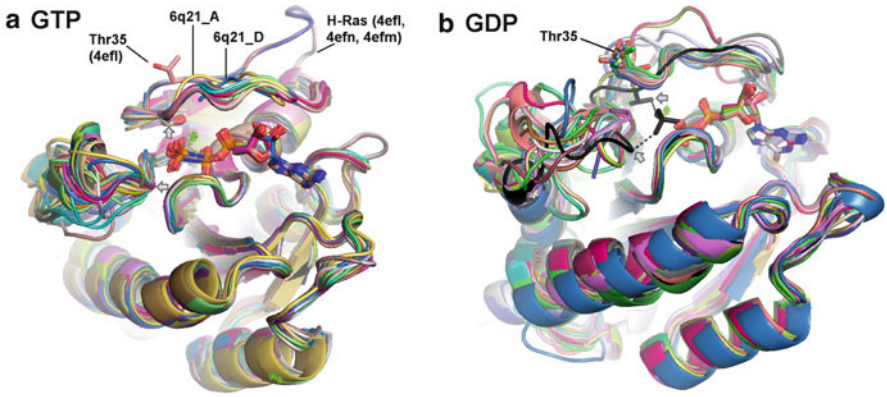

Fig. 2.4 (**a**) Superimposed structures of the GTP state of the H- and K-Ras families (the GTP forms of N-Ras and R-Ras are not available), (67 PDB files, 82 chains). M-Ras-GppNHp is intrinsically in the "open" form of switch I and has been omitted for clarity. Similarly, two "open" H-Ras-Y32F mutants are not shown (3k9l, 3k9n). (**b**) Superimposition of the GDP states, also except M-Ras, and except the K-Ras mutants with bound inhibitors (4luc... and 4m1o.. series) (24 PDB files, 31 chains). H-Ras in the GTP-form (5p21) is shown in *black* for comparison. The hydrogen bonds from the γ-phosphate oxygens to Thr35 and Gly60 are highlighted by *dashed lines*. The *arrows* point to Thr35 of H-Ras (*upper arrow*) and glycine 60 of H-Ras (*lower arrow*). In (**b**), the molecule is rotated slightly upwards and to the right to allow a better view on the threonine (Thr35 in Ras) and the glycine (Gly60 in Ras)

"closed" conformation (i.e., the stretch between residues 32 and 37 slightly deviates in direction of a neighboring molecule), corroborating that the T35S mutant is more predisposed towards the open state than wild type (Shima et al. 2010).

The switch I regions of the GDP state (Fig. 2.4b) appear to be relatively homogeneous which might seem contradictory to the idea that the variability should be higher compared to the well-ordered GTP form. However, there are fewer crystal forms (five) available, and in two of them the switch I regions are disordered and thus not visible. In the other three, switch I is again stabilized to various degrees by neighboring molecules.

M-Ras (also called R-Ras3) has been solved in both nucleotide states, and interestingly, the switch I is open in both nucleotide forms (Fig. 2.5a), with no contacts of the threonine and the glycine to the γ-phosphate at all, corroborating the NMR results which found an almost exclusively "state 1" open conformation for M-Ras-GppNHp (Ye et al. 2005). The sequence of switch I of M-Ras is very similar to H-Ras except for the "DE" (positions 30 and 31 in H-Ras) which is "PD" in M-Ras. Mutational analysis found that replacement of the proline in M-Ras with aspartate leads to a slightly larger percentage of the "closed" state in NMR experiments [13 % in P40D, compared to 7 % in wild-type M-Ras, (Shima et al. 2010)], and additional mutation of the aspartate in M-Ras to glutamate further increased the closed state fraction to about 30 %. Correspondingly, the affinity to the Ras-binding domain of Raf kinase (Raf-RBD) increased from a K_d of 5.6 μM (M-Ras wild type) to 2.5 μM (M-Ras P40D) (Ye et al. 2005). For comparison:

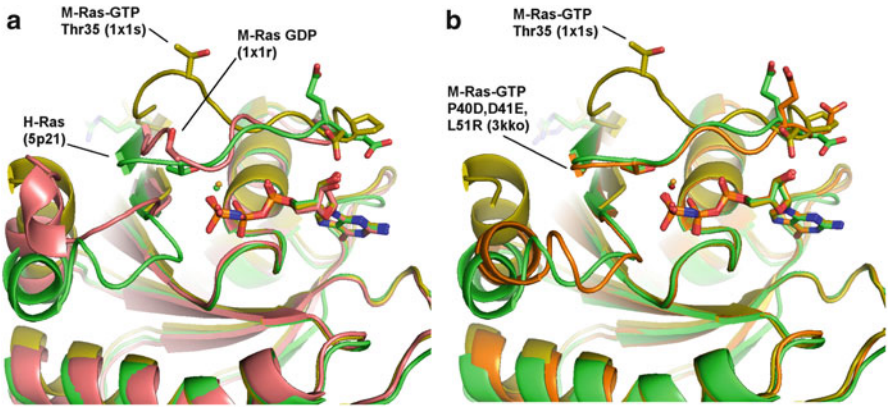

Fig. 2.5 (**a**) M-Ras-GppNHp (*yellow*, 1x1s) and GDP state (*pink*, 1x1r) in comparison with H-Ras-GppNHp (*green*, 5p21). Both GTP- and GDP states of M-Ras show the "open" form of the switch I region. (**b**) M-Ras-GppNHp (*yellow*, 1x1s) in comparison with the M-Ras mutant P40D, D41E,L51R in complex with GppNHp (*orange*, 3kko) and H-Ras-GppNHp (*green*, 5p21). Residues D30, E31 in Ras (*green*), and P/E40, D/E41, and L/R51 in M-Ras (*orange*/*yellow*) are shown as *sticks*. The mutations in M-Ras lead to a "closed" switch I conformation (*orange*, 3kko)

H-Ras - as expected—shows tighter binding to Raf-RBD with a K_d of approximately 0.5 μM (Ye et al. 2005). The M-Ras double mutant P40D, D41E apparently did not crystallize, but the structure of a triple mutant P40D,D41E,L51R (3kko, with about the same closed state fraction as P40D,D41E of approx. 30 %) could be solved (Shima et al. 2010). Indeed, the triple mutant has Thr45 (equivalent to Thr35 in H-Ras) and Gly70 (=Gly60 in H-Ras) in the canonical positions (Fig. 2.5b, orange). Since H-Ras wild type complexed with GppNHp also shows only slightly more than 50 % closed form (see above), the 30 % closed form of M-Ras is not so different, and, accordingly, the mutation of two amino acids (M-Ras P40D,D41E) is sufficient to shift the equilibrium of M-Ras towards the closed form.

2.9 Extended Ras Family

The closest relatives to the core Ras family are Ral, RheB, Rap, Ras-3, Di-Ras, Rerg/RasL12, and, somewhat more distant, the RGK family (Rad, GEM/Kir, Rem).

The Rap (Ras-proximal) structures are very similar to their Ras counterparts, and the first complexes of a small G protein with a Ras-binding domain of an effector have been solved with (mutated) Rap proteins. The Di-Ras proteins are closely related to Rap as indicated by sequence homology and by sharing the same family of activating GAP proteins (Gasper et al. 2010). Like the Rap proteins, they lack the catalytic glutamine; it is replaced by a threonine in Rap and a serine in Di-Ras. The two available structures (2gf0 and 2erx, both unpublished) contain GDP, but, interestingly, 2erx has a phosphate ion bound close to the position where the

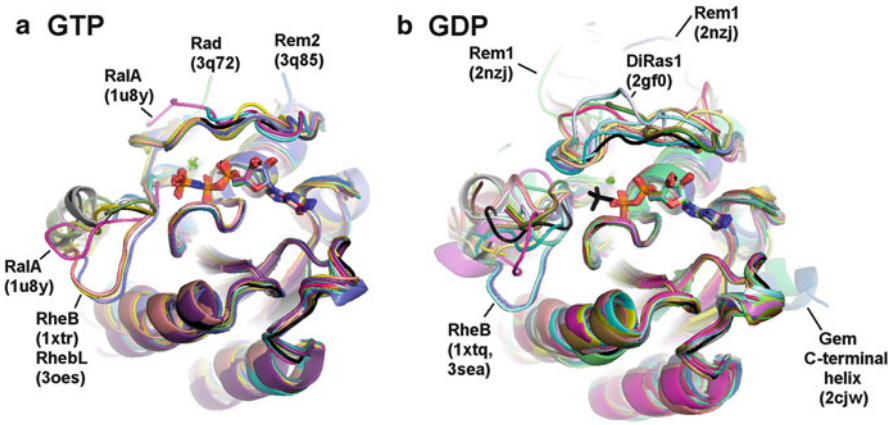

Fig. 2.6 Extended Ras family GTP (**a**) and GDP forms (**b**). All GTP forms are in "state 2," i.e., the switch tyrosine corresponding to Tyr32 in H-Ras is close to the γ-phosphate of the triphosphate nucleotide, except for RalA (1u8y) where the switch I region is in the "open" conformation even in the GTP state, consistent with solution NMR studies. H-Ras GppNHp (5p21) is shown in *black* for comparison

γ-phosphate of a GTP would be located. The structure thus resembles a transition (or product) state, and indeed, the switch regions assume the canonical positions including the hydrogen bonds of the phosphate ion that mimicks the γ-phosphate of GTP to Thr39 and Tyr36 of switch I, and Gly64 of switch II (Thr35, Tyr32, and Gly60 in Ras).

RheB (Ras Homolog Enriched in Brain) proteins have low intrinsic GTPase activity and thus are mostly GTP-bound in the cell. The impaired hydrolysis can be attributed to the unusual conformation of the switch II region (Fig. 2.6) where the α-helix preceding switch II is unraveled, leading to displacement of the catalytic glutamine (Yu et al. 2005). Upon nucleotide exchange, switch II remains in the same, extended conformation, whereas switch I undergoes the canonical rearrangement. The same conformation is observed in the RheB-like protein (structure 3oes, unpublished). RalA-GppNHp (Ras-like) has an open switch I (1u8y, Fig. 2.6), corresponding to the NMR findings that RalA's equilibrium of open/closed is slightly shifted towards the open form (Liao et al. 2008).

The RGK family proteins [Rad (Ras associated with diabetes), GEM/Kir (gene overexpressed in skeletal muscle), Rem (Ras and Gem-related)] have a *bona fide* G domain with N- and C-terminal extensions (between 29 and 90 residues) and are characterized by the unusual DXWE/D motif that replaces the canonical "DxxG" motif at the G3 position immediately before the start of switch II (Fig.2.1). Although the members of the RGK family bind GTP and GDP, there are no indications of conformational changes upon nucleotide exchange. The tryptophan of the DXWE/D motif forces the switch II loop into a sharp turn so that it prevents switch I from assuming the canonical position close to the γ-phosphate. Consequently, the switch I region is disordered in all of the available structures. The

switch regions are generally not conserved within the RGK family, including the residues equivalent to Thr35, Tyr32, Gly60, and Gln61 in Ras (Splingard et al. 2007; Sasson et al. 2011). Rem1, Rad, and GEM are thought to regulate Ca++ channels via interactions of their β-subunit (Colicelli 2004). Gem proteins seem to have a generally lower nucleotide affinity (Splingard et al. 2007), probably due to the missing phenylalanine in the switch I region (Phe28 in Ras). This phenylalanine is conserved in the Ras, Rab, Rho, and Ran families, but, besides the RGK family, not in Sar and Arf proteins and in some Arls.

Rerg (Ras-related and Estrogen-Regulated Growth inhibitor, 2atv, unpublished) and RasL12 (also known as Ris or RasLC, 3c5c, unpublished) are related between each other (43 % sequence identity) and to the RGK family members (38 % sequence identity to Rem2). Both are crystallized in the GDP form and do not have the tryptophan of the DXWE/D motif, leading to positions of the switch regions that resemble the canonical conformations. In contrast to RasL12, Rerg does have the canonical residues Thr35/Gly60/Gln61 and has been shown to function as a *bona fide* molecular switch (Key et al. 2006).

2.10 Rab Family

The Rab proteins are structurally relatively homogeneous, especially in the GTP form: the structures of 44 chains from 28 (uncomplexed) structures covering 19 different Rab families superimpose very well (Fig. 2.7a). The only exceptions where the conserved glycine and threonine of the switch regions are not contacting the γ-phosphate oxygen are some chains of Rab21 (1z08 and 1yzu) where switch II is poorly ordered even in the active conformation [Fig. 2.7a (Eathiraj et al. 2005)]. Three insertions are noteworthy in Rab33, Rab26, and Ypt51, the first one in the loop preceding the C-terminal helix (G5 region), the latter two in the same loop that contains the Rho family insert helix (Figs. 2.7 and 2.1). The switch I tyrosine corresponding to Tyr32 in Ras is only partially conserved in Rab proteins (e.g., in Rab1, Rab35, and Ypt1), in some of them it is replaced by a phenylalanine (e.g., in Rab3, Rab8, Ran26) or by other amino acids. If it is a tyrosine, it does not always contact the γ-phosphate oxygens but shows an even greater variation in positions as compared to the genuine Ras family. The Rab-homology domain of RasEF (also called Rab45, 2p5s, unpublished) is mentioned here only because it has an α-helical insert remotely resembling the canonical Rho-family insert at the same position of the structure (Fig. 2.7b). The Rab3B-GDP-structure 3dz8 (Zhang et al. 2012) is the only Rab structure that features an extra β-strand in the switch I region (Fig. 2.7b) as well as a shift of the two "interswitch"-β-strands relative to the GTP form (interswitch toggle). These conformational changes are typical only for the GDP form of Arf- and Ran proteins (Fig. 2.1). Interestingly, the 86 % identical Rab3D does not show a β-strand conformation of the switch I region in its GDP form (2gf9), although practically all residues of the involved regions (strands β1, β2, β3 and the switch I region) are conserved between the two proteins. A closer look at the

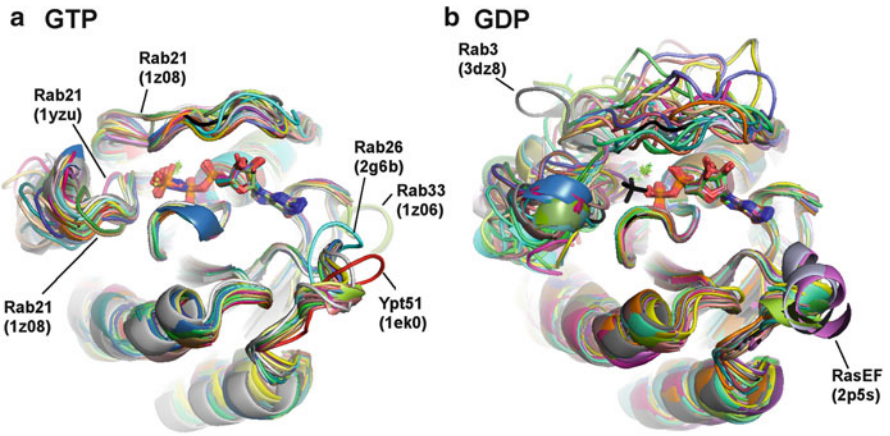

Fig. 2.7 Rab family GTP (**a**) and GDP forms (**b**). H-Ras GppNHp (5p21) is shown in *black* for comparison. Rab21 (1z08) shows canonical threonine/glycine contacts only in chains A and B, whereas chains C and D are distant from the γ-phosphate. Another crystal form of Rab21 (1yzu) has two chains with a distant and disordered switch II region, respectively, but shows a canonical position of the threonine. Rab3B (3dz9) is the only Rab-GDP structure with switch I forming the extra β-strand that is characteristic for Arf- and Ran proteins

structure 3dz8 reveals that there are additional residues (^{10}RENLYFQG^{17}) at the N-terminus of residues 18–190 of Rab3B, and those extra residues form a β-strand that interacts tightly with the unusual "extra" switch I β-strand of a symmetry-related molecule. This suggests that this conformation might not be physiologically relevant, but it is still surprising that a proper "interswitch toggle" can apparently be triggered quite easily in the GDP form of a Rab protein by the relatively weak packing forces in a crystal.

2.11 Rho Family

In contrast to the quite homogeneous Rab family structures, the uncomplexed Rho-family structures bound to GTP or its analogues show 7 structures out of 20 with an "open" switch I region (Fig. 2.8). The "closed" forms show the canonical conformation with the switch I tyrosine that is conserved in Rho family proteins (Tyr 32 in Ras) interacting with the γ-phosphate. The seven "open" structures can be divided into three groups according to the most likely reasons for the specific switch I conformation:

1. "Disturbed by disorder of neighboring region": The splice variant of Rac1b (1ryh) has a 19 amino acid insertion close to the end of the switch II region that is not visible in the electron density. This flexible insert might cause the adjacent switch II region to be disordered as well (Fiegen et al. 2004). The lack of stable

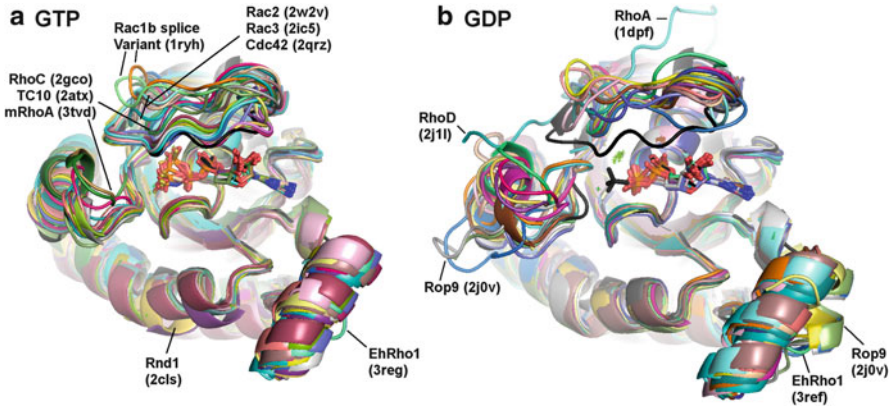

Fig. 2.8 Rho family GTP (**a**) and GDP (**b**) forms. H-Ras GppNHp (5p21) is shown in *black* for comparison. EhRho1 is Rho1 of *Entamoeba histolytica* that lacks the insert helix typical for the Rho family. In (**b**), the tilted insert helix of the Rop proteins is indicated (Rop9)

interactions of switch II with the switch I region might lead in turn to the destabilization of the switch I region. The affinity of the Rac1b splice variant for the nucleotide is drastically reduced by a factor of 57, and nucleotide hydrolysis is 30 fold slower as compared to Rac1b that lacks the 19 amino acid insertion (Fiegen et al. 2004), highlighting the importance of conformationally stable active site residues for the hydrolysis reaction. In contrast, the Rac1-GppNHp wild-type structure (1mh1) shows a "closed" switch I region although there are no obvious contacts to neighboring molecules in the crystal, suggesting that in Rac1 wild type the closed form probably also exists in solution.

2. "Probable packing artifacts combined with a mobile switch I region" [Rac2-GTP-γS (2w2v), Cdc42-GppCH2p (2qrz), Rac3-GppNHp (2ic5)]: These three protein structures show a wide open switch I whose threonine is completely detached from the γ-phosphate. Thus, Rac2-GTP-γS looks very similar to the Rac2-GDP structure (Bunney et al. 2009). The space groups of the latter two structures appear to be different, but the unit cell dimensions are very similar, as is the packing of the molecules, thereby providing a possible explanation for the similar conformations. The Cdc42-GppNHp structure also has switch I packing against a neighboring molecule, and it is concluded that the switch I region is most likely mobile in both nucleotide states (Phillips 2008), a finding that is also supported by NMR data (Feltham et al. 1997). Similarly, crystal contacts appear to stabilize the switch I region in the unpublished Rac3-GppNHp structure (2ic5).
3. "Partially open conformation of switch I" (mouse RhoA-GppNHp (3tvd), TC10-GppNHp (2atx), and RhoC-GTP-γS (2gco)): Those three structures show a position of their "open" switch I regions that is different from the proteins above (group 2), but very similar among each other, especially in the region

around the conserved threonine. In the canonical "closed" state the threonine contacts the magnesium ion via its hydroxy group. Here, it flips by 180° and now contacts the magnesium position with its carbonyl group. RhoA from mouse (3tvd, only one residue in the CAAX box differs from human RhoA (Jobichen et al. 2012)) is the only uncomplexed RhoA-wild-type-GTP-form structure available. Two other RhoA structures, one with GppNHp (1kmq) and one with GTP-γS (1a2b) both have "closed" switch I regions. Since mouse RhoA (3tvd) was crystallized at low pH (4.6) and as a dimer, in contrast to the other two RhoA structures (1a2b, 1kmq), the partially open switch I could be seen as an artifact of the crystallization conditions. However, the structure of TC10 [2atx, (Hemsath et al. 2005)], a close relative of Cdc42, has switch I in a very similar position to 3tvd. In this case the crystal packing shows no tight contacts that could hold the switch I regions in the closed state, and the switch I tyrosine packs only loosely against a neighboring molecule, suggesting that this "open" position of the switch I region is not induced by packing against neighboring molecules. This hypothesis is corroborated by RhoC that was crystallized with either GppNHp (2gco, "open") or GTP-γS (2gcp, "closed") in two different crystal forms (Dias and Cerione 2007). The "open" form of 2gco again showed the effector loop in a very similar conformation to 3tvd (mouse RhoA) and 2atx (TC10) and is not altered by any crystal contacts. It was speculated that this conformation might represent a "partially activated" state of Rho-family proteins (Dias and Cerione 2007) that is stabilized by the unique conserved phenylalanine of the Rho family switch I region (Phe39 in RhoA and RhoC). This phenylalanine also mediates hydrophobic contacts with effector molecules. The "partially activated" conformation has the potential to be a major form also in solution and would be one of the few examples where at least one of the "open" or "state 1" conformations of the switch I region shows a defined structure instead of being another crystallization artifact. This exemplifies how the availability of many crystal structures can help to distinguish between "real" intermediates of the open forms and crystal artifacts.

The short helix forming the typical insert of the Rho family is absent in the structures of Rho1 of *Entamoeba histolytica* (3reg, 3ref, 4dvg) where it is replaced by a loop that is only slightly longer than in Ras proteins (Fig.2.8). Otherwise, the position of the insert helix is remarkably conserved among Rho family proteins and not influenced by the type of bound nucleotide. Only the Rop proteins seem to be an exception with a shorter insert helix whose axis is rotated relative to the "canonical" position (Fig. 2.8b).

2.12 Arf/Sar Family

Characteristic for the Arf/Arl proteins is the formation of an extra β-strand in the switch I region in the GDP state, similar to the Ran proteins (Fig. 2.9). The sequence of this extra strand (^{42}IVTTIPTIGF^{51}) is conserved in Arf proteins, and

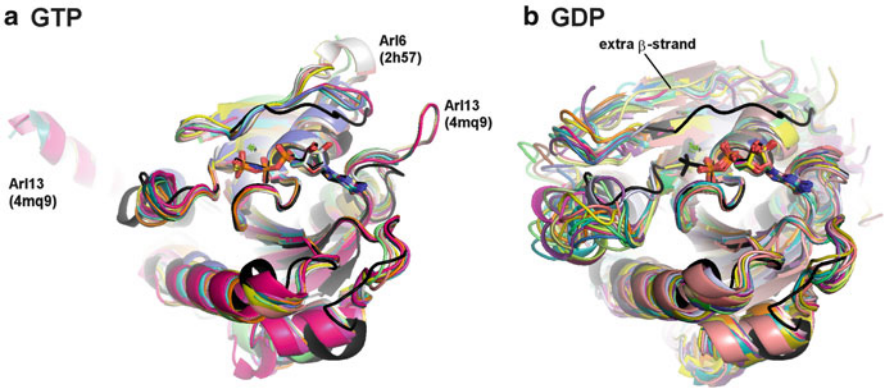

Fig. 2.9 Arf/Sar family GTP (**a**) and GDP forms (**b**). The Sar-GDP structures (2fmx, 1f6b, 2fa9, 2gao) and Arl10B-GDP (1zd9) are omitted for the sake of clarity. H-Ras GppNHp (5p21) is shown in *black* for comparison. All Arf/Arl structures lack the switch I tyrosine (Tyr32 in Ras)

Gly50 (equivalent to D38 in Ras) allows formation of the β-turn that is required for this conformation (Goldberg 1998). Indeed, this glycine is also conserved in Ran proteins. In Rho proteins, a phenylalanine (Phe39 in RhoA and RhoC) occupies this important position and is a determinant for switch I conformation and effector interaction as discussed in Sect. 2.10. Surprisingly, Arl10B-GDP (1zd9, unpublished) shows switch I in the "canonical" Ras position, i.e., without the extra β-strand, in contrast to two other Arl10-GDP structures (2h18 (Arl10B) and 2al7 (Arl10C), both unpublished), with a "normal" extra β-strand. All three structures have the conserved Gly50. Arl10B and Arl10C [also termed Arl8A and Arl8B (Kahn et al. 2006)] lack several canonical residues of other Arf/Arl GTPases, and it was even suggested to place them in a separate category (Neuwald 2010). However, the observed difference in the switch I position between the two Arl10B-GDP structures (2h18 and 1zd9) might again be a crystal packing effect: In 2h18, the N-terminus of a symmetry-related molecule blocks the "GTP" position of switch I, whereas in 1zd9, switch I might be stabilized in the "GTP" orientation by the neighboring molecules, accompanied by the canonical "interswitch toggle" of strands β2 and β3. Like in case of the Rab3B (3dz8) described above, Arl10B can apparently easily interconvert between the GTP and GDP conformations even with the same nucleotide (GDP) bound.

Arl6 and Arl13 also show some noncanonical features: Arl13 has a long C-terminal helix that is added to the G-domain with an unusual 90° kink [Fig. 2.9a, (Miertzschke et al. 2014)]. The catalytic glutamine is replaced by a glycine, explaining the lack of intrinsic GTP hydrolysis activity. It also has an insertion of five residues with unknown function after the SAK motif (TAK in Arl13) forming a loop (Fig. 2.9a) that seems to be relative stable since it lacks crystal contacts at least in two of the three monomers in the crystal. Arl6 (2h57, unpublished) has an insertion of two residues in the effector loop that does not interfere with the threonine assuming the canonical position (Fig. 2.9a).

Although Sar proteins are sometimes classified as a separate family, they are functionally and structurally closely related to Arf, with a similar retracted interswitch region in the GDP-bound form. They are not posttranslationally modified like Arf proteins, but, like the Arfs, they also have a N-terminal extension (albeit in a different orientation relative to the G domain) that confers interactions with membranes of the endoplasmatic reticulum via bulky hydrophobic amino acids in the so-called "STAR" motif (Huang et al. 2001). The N-terminus is important for the interaction with the Sec23/24 GAP and the cargo selection of the COP I coat. Amino acids 156–171 are an insertion relative to the Arf proteins that form a loop. The Sar1 switch I region lacks the conserved glycine 50 of the Arf/Arl proteins and, indeed, does not form the extra β-strand found in Arf and Ran proteins in the GDP conformation.

Switch I in the Sar1-GDP structures is in a position relatively similar to the "closed" position, but in different positions in each of the available structures as expected from the unattached threonine residue. The catalytic glutamine is replaced by a histidine but has a similar function in orienting the catalytic water molecule, similar to protein synthesis elongation factors (Huang et al. 2001).

2.13 Ran

The Ran family is characterized by a C-terminal helix extension (Fig. 2.1a) that is attached to the G domain in the GDP form (Fig. 2.10b) and detached in the GTP form (Fig. 2.10a). The switch I region in the "closed" form would clash with the stretch of residues preceding the C-terminal helix (arrow in Fig. 2.10b), causing the C-terminal helix to be dislocated (Vetter et al. 1999). As in case of the Arf proteins, the switch I region forms an additional β-strand in the GDP form (Fig. 2.10b), leaving the nucleotide exposed to the solvent. In complex with karyopherins that have a sufficiently large contact area to Ran proteins to be able to bind Ran even in the GDP form with significant affinities, the switch I region can be forced into the closed conformation [3ea5, (Forwood et al. 2008)]. The plasticity of the switches is highlighted by the structure of a Q69L mutant of canine Ran [3ran, (Stewart et al. 1998)] where conformational changes of up to 2 Å are observed (Fig. 2.10b). In the monoclinic crystal form (3ran), the switch II region in the conformation of the wild type would clash with a symmetry-related molecule which might be the cause of the altered conformation. The Ran-GTP complex structures appear quite homogeneous; the switch regions are relatively well defined. In contrast, helix α4 shows relatively large changes of the helix axis orientation compared to the GTP form. The area around helix 4 forms one of the main contacts with the α-solenoids of the karyopherins, and might thus be important for lowering the affinity to those effector proteins in the GDP form of Ran.

The structure of an orthologue of Ran from *Encephalitozoon cuniculi* (4djt, unpublished) has all GTP-interacting motifs including the catalytic glutamine

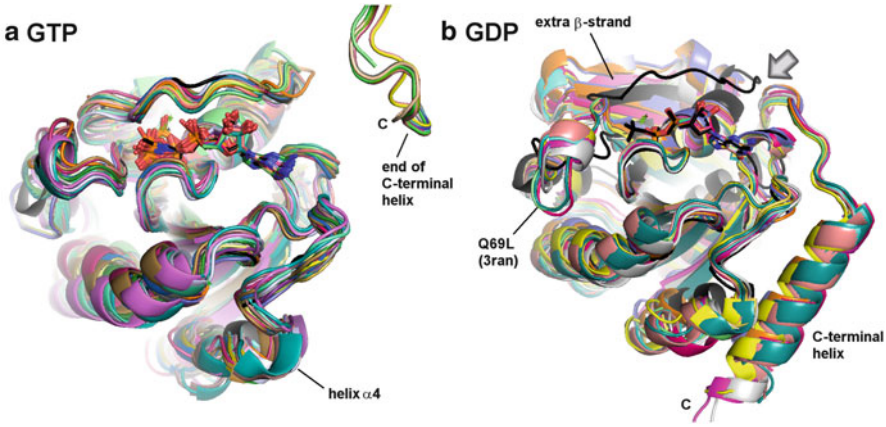

Fig. 2.10 Ran GTP (**a**) and GDP forms (**b**). The triphosphate structures are in complex with various Ran-binding proteins and karyopherins since there is no uncomplexed Ran GTP structure available. H-Ras GppNHp (5p21) is shown in *black* for comparison. The backbone of H-Ras-GppNHp in (B) emphasizes that the switch I region in the closed conformation would clash (indicated by an *arrow*) with the residues preceding the C-terminal helix, causing it to detach from the remainder of the G domain in the GTP conformation (**a**)

conserved. It was crystallized in the GDP-bound form. Interestingly, its C-terminus does not form a helix, but a random coil that is positioned roughly where the switch I region of the GTP form is located.

All Ran-GTP structures show the switch I Tyr39 (Tyr41 in yeast Ran) closed over the nucleotide, the hydroxy group contacting the γ-phosphate oxygens. The side chain of the catalytic glutamine (Gln69 in human and Gln71 in yeast Ran) shows again random rotamers except in the complex with RanGAP, indicating catalytically incompetent forms that are consistent with the slow or unmeasurable hydrolysis in the (non-GAP) complex structures.

2.14 Summary/Concluding Remarks

The G domain is a very versatile and evolutionary ancient structure whose dynamic switch regions can sense the nucleotide state and alter the outer shape of the molecule, allowing binding to specific effector and regulator proteins. The switch regions are in a delicate balance between a transiently stable GTP-bound form that often interconverts between an “open” and a “closed” form on a fast timescale, and a GDP form with even less well-defined switch regions (except for the Arf and Ran families). The dynamics of the switch regions influence the intrinsic hydrolysis rate by positioning the catalytic machinery more or less closely to the optimum position. Some of the subgroups of the Ras superfamily use additional secondary structure elements like N- and C-terminal helical extensions to achieve regulation of

transient membrane interaction (Arf/Sar) or enable the specific formation and dissociation of extremely high-affinity complexes to facilitate nuclear transport (Ran). Although the number of structures in the protein databank grows rapidly and more and more structures of G domains become available, the analysis especially of proteins with flexible regions has many pitfalls and should take into account that crystallization conditions, crystal packing, and freezing of the crystals for data collection can introduce artifacts into the structure.

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Chapter 3
GEFs and GAPs: Mechanisms and Structures

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Abstract Small G proteins (called small GTPases hereafter) regulate many aspects of the cell logistics by their ability to alternate between an inactive, GDP-bound form and an active, GTP-bound form [reviewed in Vetter and Wittinghofer (Science 294: 1299–304, 2001), Cherfils and Zeghouf (Nat Chem Biol 7: 493–495, 2011)]. The GDP/GTP switch is exquisitely controlled in space and time by regulators that work in opposite ways: guanine nucleotide exchange factors (GEFs) turn the molecular switch on by stimulating the dissociation of the tightly bound GDP nucleotide, and GTPase activating proteins (GAPs) turn it off by stimulating the intrinsically slow hydrolysis of GTP [reviewed in Bos et al. (Cell 129: 865–877, 2007), Cherfils and Zeghouf (Physiol Rev 93: 269–309, 2013)]. Each family of small GTPases has one or several associated GEFs and GAPs families, which combine in various ways to determine where, when and for how long each small GTPase is to be active. Over the last decade, the biochemical and structural mechanisms of most major GEFs and GAPs catalytic domains have been elucidated, illuminating their general workings and revealing unique features. GEFs and GAPs have themselves sophisticated mechanisms that regulate their activities, whose biochemical and structural analysis are key issues for future investigations. The mechanisms and regulation of GEFs and GAPs have been covered in a recent review by this author [Cherfils and Zeghouf (Physiol Rev 93: 269–309, 2013)], an overview of which is presented in this chapter and completed with studies that have appeared in the literature recently.

Keywords Small GTPase • GDP/GTP switch • GEF • GAP • Structure • Mechanism • Regulation

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Table 3.1 Major subfamilies of small GTPases and their associated GEFs and GAPs domains

Small GTPase family	GEF catalytic domain	GAP catalytic domain
Ras/Rap/Ral (36 members)	CDC25 (27 members)	RasGAP (≈12 members) RapGAP (≈10 members)
Rho/Rac/Cdc42 (22 members)	Dbl-homology (DH) (≈70 members) DOCK homology (DHR2) (11 members) PRONE (plant GEFs)	RhoGAP (70 members)
Rab (>60 members)	VPS9 (10 members) DENN (18 members) TRAPP complex Rabin8/Sec2 MSS4	TBC (>40 members)
Arf (5 members)	SEC7 (16 members)	ArfGAP (31 members)
Arl3		RP2
Sar1	Sec12	Sec23/Sec13
Ran	RCC1	RanGAP

The approximate number of members in each family in human is indicated [references in Cherfils and Zeghouf (2013)]

3.1 A Myriad of GEFs and GAPs

Each family of small GTPases has its own GEFs and GAPs families, which are characterized by the presence of a conserved catalytic domain with GEF or GAP activity and to which a variety of domains are appended that are involved in their regulation and membrane targeting. The best studied families of GEFs and GAPs are listed in Table 3.1. Other proteins have been proposed to act as GEFs and GAPs for subsets or individual small GTPases, which are awaiting in-depth biochemical and structural characterization [reviewed in Cherfils and Zeghouf (2013)]. Structural studies highlighted that there is an intriguing absence of evolutionary relationship between GEFs families or between GAPs families, suggesting that the small GTPase module originally did not function as a switch. This diversity also underscores the extraordinary fitness of the small GTPase structure to establish protein–protein interactions, a property that is encoded mostly within its nucleotide sensor regions (the switch 1 and switch 2 regions) (reviewed in Biou et al. (2010)]. A flipside of this multispecificity is that it makes small GTPases frequent targets for bacterial effectors with GEFs and GAPs functions, which are secreted by pathogens to take command of host cell pathways (reviewed in Cherfils and Zeghouf (2013) and references therein). It is also noteworthy that the number of GEFs and GAPs varies greatly between small GTPases families, including small GTPases that are still orphans of GEFs and/or GAPs, found mostly in the Arf-like family (see approximate numbers for the human genome in Table 3.1). It is likely that more regulators remain to be discovered, although it is also possible that these variations reflect unique functional determinants.

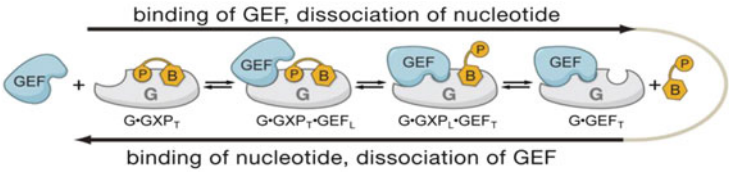

Fig. 3.1 GEF-stimulated GDP dissociation: the general mechanism. *T* tight interaction, *L* loose interaction. Reproduced from (Bos et al. 2007) with permission

3.2 General Principles of GEFs and GAPs Mechanisms

3.2.1 Mechanisms of GEF-Stimulated GDP/GTP Exchange

Small GTPases establish an intimate meshwork of interactions with GDP and its associated Mg^{2+} ion, which make the release of GDP a very slow process in general. The core function of GEFs is to facilitate the dissociation of GDP, which is achieved by a multistep reaction depicted in Fig. 3.1 [reviewed in Cherfils and Chardin (1999), Bos et al. (2007), Cherfils and Zeghouf (2013)]. The first step of the exchange reaction is the docking of the GEF onto the GDP-bound small GTPase, forming a low affinity ternary intermediate. Dissociation of GDP then converts this complex into a high affinity nucleotide-free complex. Finally, binding of GTP dissociates the GEF and leads to the formation of a high affinity small GTPase-GTP complex which is competent for recognition of effectors. Formation of a nucleotide-free high affinity complex is often considered a reference assay to identify candidate GEFs. Structures of nucleotide-free complexes and of a few nucleotide-bound ternary complexes have been solved over the last decade for representative members of most major eukaryotic GEF families, drawing a general picture of how GEFs work at the atomic level [reviewed in Cherfils and Zeghouf (2013) and references therein). All complexes feature a very large GTPase/GEF interface, in which the GEF clamps the switch 2 region and displaces the switch 1 region away from the nucleotide-binding site. These interactions concurrently facilitate GDP release by opening the nucleotide-binding site and make up for the tendency of nucleotide-free small GTPases to unfold. Apart from these general traits, the mechanisms whereby each GEF domain stimulates GDP dissociation vary considerably between families, even between GEFs whose substrates belong to the same small GTPase family. For example, Sec7 domain-containing ArfGEFs (Goldberg 1998) and VPS9 domain-containing RabGEFs (Delprato and Lambright 2007) insert an acidic residue into the phosphate-binding site that contributes repulsive interactions with the nucleotide phosphates; Cdc25 domain-containing RasGEFs (Boriack-Sjodin et al. 1998) and DH domain-containing RhoGEFs (Worthylake et al. 2000) remodel the switch 2 to hinder the Mg^{2+}-binding site, while DHR2 domain-containing RacGEFs (DOCK family) insert a hydrophobic residue to impair the Mg^{2+}-binding site (Yang et al. 2009).

Structures of nucleotide-bound intermediates provided a glimpse of the dynamics of GEF-stimulated GDP/GTP exchange reactions. Such structures have been captured for ArfGEFs (Renault et al. 2003; Aizel et al. 2013), for PRONE and DOCK RhoGEFs (Thomas et al. 2007; Yang et al. 2009), and for Vps9 and Rabin8 RabGEFs (Uejima et al. 2010; Guo et al. 2013). Possibly the most striking "3days-movie" of the exchange reaction was the one assembled from structures of GDP-bound (Renault et al. 2003) and nucleotide-free (Goldberg 1998) Arf/ArfGEFs intermediates. These series of intermediates, one of which was trapped by the drug Brefeldin A, revealed that Arf GTPases undergo large conformational changes and rotational movements with respect to the GEF domain, which couple recruitment of Arf to membranes to its activation by GTP. Such large positional changes or remodeling has not been observed for other small GTPases. In the DOCK9/Cdc42 structures, GDP dissociation is driven by a local disorder-to-order transition in a loop from the GEF (Yang et al. 2009). No conformational change is seen in the PRONE (Thomas et al. 2007) and VPS9 (Uejima et al. 2010) complexes, although the γ-phosphate-binding site is obstructed in all structures indicating that other structural intermediates likely form along the exchange reaction. Structures of nucleotide-free, GDP-bound and GTP-bound Rab8/Rabin8 intermediates uncovered still another variation on the theme, in which the GDP/GTP-binding site is fully available and loss in nucleotide affinity is due to conformational changes in the switch 1 that result in the loss of an interaction with the guanine base and obstruction of the Mg^{2+}-binding site (Guo et al. 2013). Overall, biochemical and structural studies emphasize the role of structural dynamics as a major component of GEF-stimulated nucleotide exchange. They also underpin the importance of taking mechanistic differences into account when interpreting the effects of mutations used in functional studies.

3.2.2 Mechanisms of GAP-Stimulated GTP Hydrolysis

The function of GAPs is to terminate small GTPase signaling in cells by stimulating their otherwise very slow intrinsic GTPase activity. Understanding the structural workings of GAPs has been made possible by the use of aluminium fluoride, which allows the formation of a stable GTPase/GAP complex in the presence of GDP by mimicking the transition state of phosphate hydrolysis (Scheffzek et al. 1997). Formation of such complex is often considered a reference assay to demonstrate that a protein is an actual GAP (Wittinghofer 1997). The crystal structures of representative small GTPase/GAP complexes from all major families have now been solved. As for GEFs, GAPs generally bind to the switch 1 and switch 2 regions, but unlike GEFs they do not induce large conformational changes in their cognate small GTPases. Structures of GAP/GTPases complexes revealed why small GTPases are not enzymes in their own right, and how GAPs turn them into efficient GTPases (reviewed in Cherfils and Zeghouf (2013) and references therein). They showed that efficient GTP hydrolysis requires a tandem of residues: one that

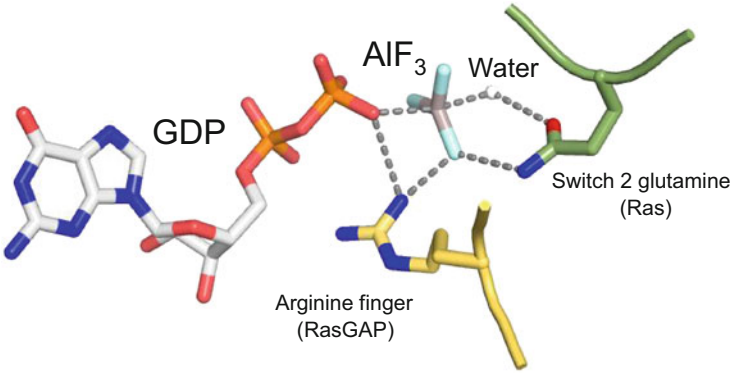

Fig. 3.2 Stimulation of GTP hydrolysis by GAPs. Close-up view of the Ras-GDP-AlF_3-RasGAP complex (Scheffzek et al. 1997)

stabilizes the partial negative charges that develop at the transition state and one that activates the nucleophilic water molecule. Figure 3.2 shows the example of the Ras-RasGAP complex (Scheffzek et al. 1997). In this complex, the GAP domain provides an "arginine finger" to stabilize partial negative charges, and it stabilizes a flexible glutamine from the switch 2 region of Ras to activate the nucleophilic water molecule. Remarkably, although the principle holds for all GAP mechanisms that have been characterized so far, not all GAPs use the GAP arginine finger/switch 2 glutamine tandem to hydrolyze GTP. For example, TBC domain-containing RabGAPs use a regular arginine finger but replace the switch 2 glutamine by a glutamine from the GAP domain despite the fact Rab GTPases carry a glutamine in their switch 2 (Pan et al. 2006); RanGAP uses a tyrosine from the switch 1 and the regular glutamine from the switch 2 (Seewald et al. 2002), and RapGAPs use a switch 1 tyrosine and an asparagine from the GAP (Scrima et al. 2008). These observations undescore that, as for GEFs, it is critical to take these family-specific mechanisms into account when devising tools for addressing biological functions and interpreting mutational data.

3.3 Mechanisms of Regulation of GEFs and GAPs

Over the last decade, it has become increasingly clear that GEFs and GAPs are themselves exquisitely regulated by mechanisms that involve non-catalytic domains appended in N- or C-terminus of their catalytic domains (reviewed in (Cherfils and Zeghouf 2013) and references therein). Regulation of GEFs and GAPs can be broadly sorted into two, nonexclusive, types of mechanisms: targeting and translocations to subcellular membranes and structural responses to molecular signals such as phosphorylations, signaling small molecules or lipids, or interacting proteins. Important insights into these mechanisms have been gained from

biochemical assays that reconstitute GEF and GAP reactions on membranes and from structural studies that investigate auto-regulations and conformational changes. Recent studies have begun to establish that these mechanisms can be interconnected in cascades and/or feedback loops, which are probably key for orchestrating physiological responses.

3.3.1 Regulation by Targeting to Subcellular Membranes

The output of activation and inactivation of any small GTPase is intimately linked to which membrane these reactions are taking place on. Accordingly, it is predictable that membranes play a key role in the functions of most GEFs and GAPs. ArfGEFs and ArfGAPs are probably the families in which this has been most investigated. The example of the ArfGEF BRAG2 illustrates the net gain in efficiency that is achieved by the mere co-localization of the small GTPase and its GEF on the same membrane (Aizel et al. 2013). BRAG2 contains a Sec7 and PH domains, which function as a constitutively active tandem in solution. Yet, co-localization of BRAG2 and Arf on membranes potentiates this high basal exchange efficiency by three orders of magnitude compared to the same reaction in solution. This effect probably arises from the increased probability of encounter between the small GTPase and the GEF (entropic effect) combined with optimization of their relative orientations (conformational effect). Conversely, failure of BRAG2 to be recruited to membranes (for instance by replacing anionic liposomes by uncharged liposomes) returned the GEF to its basal exchange activity.

Regulation by membranes can also operate by structural mechanisms in which elements from the GEF or the GAP block access to lipid- or membrane-binding sites. A representative example is that of the bacterial ArfGEF RalF, which contains a Sec7 domain that is auto-inhibited by a capping domain unrelated to known eukaryotic domains (Amor et al. 1994). Biochemical reconstitution of the GEF activity of RalF on artificial membranes revealed that it is activated by membranes by a factor of about 1,000 fold, and that the membrane-binding region is identical to the auto-inhibitory region (Folly-Klan et al. 2013). Activation by unmasking of membrane-binding determinants has also been uncovered for the RhoGAP β2-chimaerin. β2-chimaerin carries a C1 domain [which binds the lipid second messenger diacylglycerol (DAG)] which is appended to its RhoGAP domain. Structural studies showed that the DAG-binding site of the C1 domain is obstructed by an N-terminal peptide, and structure-based mutations predicted to impair this interaction resulted in increased downregulation of Rho GTPases in cells (Canagarajah et al. 2004). This underpins a mechanism of activation of β2-chimaerin by recruitment to DAG-containing membranes. In the examples above, large conformational changes are predicted to take place upon translocation of these regulators to membranes.

ArfGAP1, a Golgi-localized GAP that inactivates Arf GTPases on COP1-coated vesicles, is activated by translocation to membranes in a completely different way (Bigay et al. 2003). ArfGAP1 contains an atypical peptide, coined the ALPS motif,

which excludes it from flat membranes and recruits it to curved membranes by a disorder-to-helical conformational change, which ensures that Arf inactivation coincides with completion of vesicle coating (Bigay et al. 2003). This example underscores the importance for certain GEFs and GAPs to modulate their activities by sensing membrane curvature, notably in trafficking events [reviewed in Antonny (2011)].

3.3.2 Regulation by Auto-inhibition

Auto-inhibition by segments or domains that obstruct access to the GEF active site has been described in several GEF families (Table 3.2). Such inhibitory mechanisms require large conformational changes to convert the GEF from its auto-inhibited to its active conformation, which have been described at high resolution for only very few GEFs. A representative example of such large amplitude movements is that of the RapGEF EPAC, which has been captured in both inactive and cAMP-activated conformations (Rehmann et al. 2008; Rehmann et al. 2006) (Fig. 3.3). These structures showed that EPAC is auto-inhibited by a tandem of cAMP-binding domains that block access to the Rap-binding site. Binding of cAMP activates the GEF by stabilizing one of these domains away from the GEF active site through a very large movement with formation of an alternative intramolecular interaction. The case of DH-PH containing RhoGEFs illustrates that the same scaffold can be regulated by a variety of auto-inhibitory mechanisms (Table 3.2). In the RacGEF VAV, auto-inhibition of the DH domain is mediated by two layers of interactions involving an acidic peptide and a CH domain (Yu et al. 2010). These interactions are relieved by successive phosphorylations of tyrosines in the acidic motif, which likely facilitate the subsequent displacement of the CH domain. In another DH-PH containing GEF, ASEF, auto-inhibition is mediated by an SH3 domain, which is released by interaction with another protein, APC (Murayama et al. 2007; Mitin et al. 2007), while in p63RhoGEF, the DH domain is inhibited by its own PH domain and activated by interaction with activated $G_{\alpha q}$ (Lutz et al. 2007). A variety of auto-inhibitory mechanisms have also been characterized for Sec7 domain-containing ArfGEFs, including auto-inhibition of cytohesins by their atypical PH domain (see below), of large Golgi ArfGEFs by their C-terminal domains (Richardson et al. 2012) and of bacterial RalF proteins by their membrane-binding domain (see above), each with a distinct mechanism of activation. Similar auto-inhibitory mechanisms presumably also exist in GAP families, but are still poorly understood.

3.3.3 Regulation of GEFs by Feedback Loops

Feedback loops, in which GTP-bound small GTPases bind to their own GEFs and modulate their activities, are emerging as essential regulatory components.

Table 3.2 Mechanisms of GEF regulation: representative examples of auto-inhibitory mechanisms

Small GTPase	GEF	Auto-inhibitory domain	Activator	References
Rap	EPAC	cAMP-binding domains	cAMP	Rehmann et al. (2008), Rehmann et al. (2006)
Arf	Cytohesin	PH domain and flanking elements	Arf-GTP	Cohen et al. (2007), DiNitto et al. (2007), Stalder et al. (2011), Malaby et al. (2013)
Arf	Bacterial RalF	Membrane-binding domain	Translocation to membrane	Amor et al. (2005), Folly-Klan et al. (2013)
Rho	P63RhoGEF	PH domain	Gαq-GTP	Lutz et al. (2007)
Cdc42	ASEF	SH3 domain	APC	Murayama et al. (2007), Mitin et al. (2007)
Rac	VAV	Acidic peptide and CH domain	Sequential phosphorylations	Yu et al. (2010)

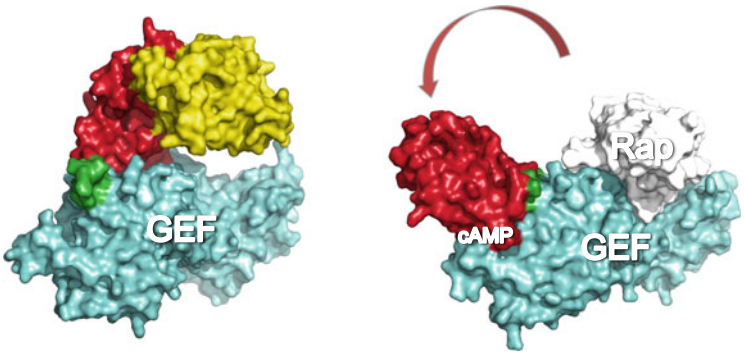

Fig. 3.3 Regulation of GEFs by auto-inhibition. Cartoons showing auto-inhibited (*left*) and cAMP-activated (*right*) structures of the RapGEF EPAC (Rehmann et al. 2008; Rehmann et al. 2006). The GEF domain is in *blue*, the cAMP-binding domains are in *red* and *yellow*, the hinge region is in *green*, and nucleotide-free Rap is in *gray*. The interfacial cAMP-binding site of cAMP is shown

Activation of a GEF by a positive feedback loop was first discovered in the RasGEF SOS, following the observation that SOS has an allosteric Ras/GTP-binding site for Ras-GTP located on its GEF domain opposite to its active site (Margarit et al. 2003). Binding of Ras-GTP amplifies the basal exchange rate on artificial membranes (Gureasko et al. 2008), notably by displacing membrane-binding domains and increasing their interactions with membranes (Sondermann

et al. 2004; Gureasko et al. 2010). Positive feedback loops have subsequently been characterized in Sec7-containing ArfGEFs [reviewed in Stalder and Antonny (2013), Richardson and Fromme (2012)] and in a RhoGEF (Medina et al. 2013). Arf-GTP binds to the PH domain of cytohesins and recruits them to membranes (Cohen et al. 2007). Structural studies showed that cytohesins are auto-inhibited by their PH domain (DiNitto et al. 2007) and that Arf-GTP binds to auto-inhibitory elements in a manner that is not compatible with this auto-inhibited conformation (Malaby et al. 2013), resulting in auto-inhibition release and a strong positive feedback effect (Stalder et al. 2011). Surprisingly, the lipid-binding pocket of the PH domain is not blocked in auto-inhibited cytohesins (DiNitto et al. 2007), suggesting that signaling lipids and Arf-GTP regulate cytohesins in coincidence. A feedback effect, although significantly smaller, has also been reported for the Golgi ArfGEF Sec7 (Richardson et al. 2012), whose structural basis remains to be elucidated. The DH-PH containing LBC RhoGEF has a binding site for Rho-GTP in its PH domain, but unlike in cytohesins the PH domain is not auto-inhibitory (Chen et al. 2010). Rho-GTP had no effect on nucleotide exchange in solution (Chen et al. 2010) but increased nucleotide exchange in the presence of phospholipid vesicles (Medina et al. 2013), suggesting that it may be involved in a positive feedback effect. Positive feedback loops with an ultrasensitive regulatory step enable bistable switches (Brandman and Meyer 2008), whose significance for these GTPases and GEFs is now to be investigated in vivo [reviewed in Stalder and Antonny (2013), Richardson and Fromme (2012)].

Recent studies showed examples of GEFs closely related to feedback regulated GEFs that are not themselves regulated by a feedback mechanism. It is the case for RasGRF1, which is structurally close to SOS but does not have an allosteric site for Ras-GTP and, accordingly, is not amplified by Ras-GTP (Iwig et al. 2013). Likewise, among Sec7-PH containing ArfGEFs, only cytohesins are regulated by a positive feedback loop and by auto-inhibition by the PH domain, while BRAG2 is not auto-inhibited and has no feedback control (Aizel et al. 2013), and EFA6 is under negative feedback regulation by Arf-GTP (our unpublished results). An appealing scenario would be one in which GEFs with different regulatory regimes combine to establish regulatory circuits. For example, a GEF regulated by a negative feedback loop may produce limited amounts of an activated small GTPase, which would remain dormant until mobilized to activate another GEF in coincidence with another signal (for instance a signaling lipid). It is possible that cascades of regulators, such as those that have been described for Rab or Ras GTPases, involve variations of these mechanisms of feedback regulations.

3.4 Concluding Remarks

Our understanding of the mechanisms of GEFs and GAPs has considerably expanded over the last decade, leaving few families that do not have at least one member whose structure and basic biochemical mechanism have been

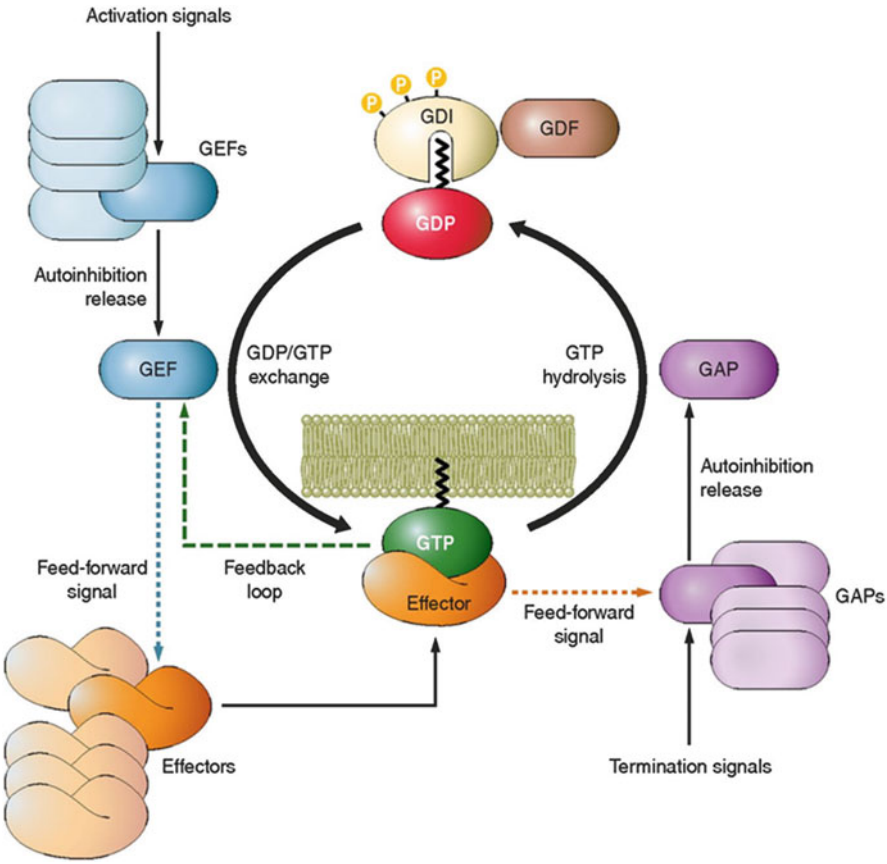

Fig. 3.4 Regulation of small GTPases by GEFs and GAPs: a growing complexity of mechanisms. Reproduced from (Cherfils and Zeghouf 2013) with permission

characterized in depth. Atypical GEFs and GAPs that do not to belong to major families now await refined characterization, and more GEFs and GAPs probably remain to be discovered. Understanding unique variations in the mechanisms of GEFs and GAPs is important to interpret the effects of mutations used to interrogate functions in cells and should remain important issues to investigate [reviewed in Cherfils and Zeghouf (2011)]. This was strikingly highlighted by a recent comparison of the mechanisms of nucleotide release by several families of RabGEFs, which uncovered that mutations of the switch 2 glutamine assumed to yield constitutive active Rab proteins resulted in fact in mutants that failed to be activated by a subset of their GEFs (Langemeyer et al. 2014). Recent studies underlined an expanding repertoire of GEF and GAP regulatory regimes, including cascade and feedback regulations and a complex interplay between membrane and regulators (Fig. 3.4). Understanding the biochemical and structural basis of these mechanisms and their in vivo significance will be key issues for future investigations. Such

integrated knowledge should be a source of inspiration to harness the complexity of GEFs and GAPs for therapeutic strategies in the many diseases where small GTPases, GEFs and GAPs, are impaired.

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Chapter 4
Bacterial Protein Toxins Acting on Small GTPases

Klaus Aktories and Gudula Schmidt

Abstract Numerous bacterial protein toxins and effectors target eukaryotic cells by covalent modification of low molecular mass GTP-binding proteins to manipulate their switch functions. Frequent targets are Rho, Ras, and Rab proteins which are modified by ADP-ribosylation, adenylylation, mono-*O*-glycosylation, deamidation, transglutamination, phosphocholination, and proteolytic cleavage. Thereby, the GTPases are activated or inactivated. Other bacterial effectors manipulate the cellular functions of small GTPases by mimicking endogenous regulators of the switch proteins. They act as guanine nucleotide exchange factors (GEFs) or GTPase-activating proteins (GAPs). The chapter describes the bacterial toxins and effectors and discusses the functional consequences of their actions.

Keywords Bacterial protein toxins • Bacterial effectors • Effector modification • ADP-ribosylation • Glycosylation • Deamidation • AMPylation • Adenylylation • Phosphocholination

4.1 Introduction

Small GTPases are frequent targets of bacterial toxins[1] and effectors [see recent reviews Visvikis et al. (2010), Lemonnier et al. (2007), Aktories (2011), Aktories and Barbieri (2005), Lemichez and Aktories (2013)] (Tables 4.1 and 4.2). As

[1] Here, **toxins** are designated as bacterial factors which are released from bacteria into the environment and then enter target cells independently of the pathogen. In contrast, bacterial **effectors** are introduced into host cells by an injection machinery of the bacteria as a result of direct contact of the pathogen with host cells.

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Table 4.1 Bacterial toxins and effectors that target small GTPases by covalent modifications

Bacterium	Toxin	Substrate	Modification	Functional consequence
C. botulinum	C3bot	RhoA, B, C	ADP-ribosylation	Inactivation
C. limosum	C3lim	RhoA, B, C	ADP-ribosylation	Inactivation
B. cereus	C3cer	RhoA, B, C	ADP-ribosylation	Inactivation
S. aureus	C3stau1	RhoA, B, C	ADP-ribosylation	Inactivation
S. aureus	C3stau2	RhoA, B, C, RhoE	ADP-ribosylation	Inactivation
S. aureus	C3stau3	RhoA, B, C	ADP-ribosylation	Inactivation
P. luminescens	TccC5	Rho, Rac, Cdc42	ADP-ribosylation	Activation
C. difficile	Toxin A	Rho, Rac, Cdc42	Glucosylation	Inactivation
C. difficile	Toxin B	Rho, Rac, Cdc42	Glucosylation	Inactivation
C. sordellii	LT	Rac, Ras, Ral, Rap	Glucosylation	Inactivation
C. sordellii	HT	Rho, Rac, Cdc42	Glucosylation	Inactivation
C. novyi	α-toxin	Rho, Rac, Cdc42		Inactivation
C. perfringens	TpeL	Ras, (Rac)	GlcNAcylation (Glucosylation)	Inactivation
P. asymbiotica	PaTox	Rho, Rac, Cdc42	GlcNAcylation	Inactivation
V. parahemol.	VopS	Rho, Rac, Cdc42	Adenylylation	Inactivation
H. somni	IbpA	Rho, Rac, Cdc42	Adenylylation	Inactivation
L. pneumophila	DrrA	Rab1	Adenylylation	Activation
L. pneumophila	SidD	Rab1, Rab35	De-Adenylylation	
L. pneumophila	AnkX	Rab1, Rab35	Phosphocholination	Inactivation
L. pneumophila	Lem3	Rab1, Rab35	De-Phosphocholination	
Y. enterocolitia	YopT	Rho, Rac, Cdc42	Proteolysis	Inactivation
P. luminescens	LopT	Rho, Rac	Proteolysis	Inactivation
E. coli	CNF1	Rho, Rac, Cdc42	Deamidation	Activation
E. coli	CNF2	Rho, Rac, Cdc42	Deamidation	Activation
E. coli	CNF3	Rho, Rac, Cdc42	Deamidation	Activation
Y. pseudotub.	CNFy	Rho, Rac	Deamidation	Activation
B. bronchiseptica	DNT	Rho, Rac, Cdc42	Transglutamination	Activation

outlined in other chapters in great detail, these switch proteins are involved in numerous cellular functions. Naturally, the GTPases are also involved in diverse defense mechanisms of the immune system and specifically in signaling and functioning of all types of immune cells. Targeting these GTPases by means of toxins and effectors is often an essential part of host–pathogen interactions, allowing the survival and proliferation of bacteria in a hostile environment. For example, GTPases are essential regulators for organization of the barrier functions of epithelial cell layers. They are essential for motile functions and adhesion of immune cells to reach their pathogen targets. They play pivotal roles in sensing and signaling of pathogen-associated molecular patterns (so-called PAMPS) and regulate inflammatory responses of the host organism. Moreover, they are essential for phagocytosis by monocytes and macrophages and control T-cell and B-cell activities.

To understand the modification and manipulation of GTPases by bacterial toxins and effectors, regulation of the GTPases is briefly described. For detailed information related chapters of this volume are recommended. Because many toxins act on

Table 4.2 Bacterial effectors that target small GTPase in a GEF- or GAP-like manner

Bacterium	Effector	Substrate	Activity
S. Typhimurium	SopE	Rac, Cdc42	GEF
S. Typhimurium	SopE2	Rac, Cdc42	GEF
B. pseudomallei	BopE	Rac, Cdc42	GEF
C. violaceum	CopE	Rac, Cdc42	GEF
S. flexerni	IpgB1	Rac, Cdc42	GEF
S. flexerni	IpgB2	RhoA, Rac, Cdc42	GEF
E. coli	Map	Cdc42	GEF
E. coli, *Citrob.*	EspM	RhoA	GEF
E. coli, *Citrob.*	EspT	Rho	GEF
S. Typhimurium	SifA	Rho?	GEF?
S. Typhimurium	SifB	Rho?	GEF?
S. Typhimurium	SptP	Rac, Cdc42	GAP
Y. pseudotub.	YopE	Rho, Rac, Cdc42	GAP
P. aeruginosa	ExoS	Rho, Rac, Cdc42	GAP
P. aeruginosa	ExoT	Rho, Rac, Cdc42	GAP
A. salmoncida	AexT	Rho, Rac, Cdc42	GAP
L. pneumophila	LepB	Rab1	GAP

small GTPases of the Rho protein family, regulation of these proteins are taken as an example. Rho proteins comprise a family of small GTP-binding proteins with ~20 family members (Hall 1993; Takai et al. 1993; Nobes and Hall 1994; Heasman and Ridley 2008; Burridge and Wennerberg 2004). Best studied are Rho, Rac, and Cdc42 isoforms. They are involved in regulation of the actin cytoskeleton. Classical studies showed that RhoA is involved in stress fiber formation, whereas Rac organizes membrane ruffling and lamellipodia formation and Cdc42 participates in filopodia formation (Hall 1994, 1998). Rho proteins are inactive in the GDP-bound state and gain signaling activity after GDP/GTP exchange, induced by guanine nucleotide exchange factors (GEFs) (Jaffe and Hall 2005). Numerous GEFs (>80 proteins) are known, which have specific and/or overlapping functions depending on cell type and functional context. In the GTP-bound state Rho proteins interact with Rho effectors (note that the term effectors should not be confused with the same term used for bacterial effectors) to transfer Rho signaling functions. The active state is terminated by hydrolysis of bound GTP due to inherent GTPase activity. This process is greatly enhanced by GTPase activating proteins (GAPs). Again numerous GAPs are known, which act in a cell type and function-dependent manner. Rho GTPases are also regulated by guanine nucleotide dissociation inhibitors (GDIs). GDIs (only three mammalian types are known) bind to Rho proteins and intensively interact with the C-terminally located isoprenyl moiety of the GTPases that is responsible for attachment of Rho GTPases to membranes. Thereby, GDI extracts Rho proteins from membranes and keeps them in the inactive GDP-bound form in the cytosol.

4.2 ADP-Ribosylating Toxins

ADP-ribosyltransferases transfer the ADP-ribose moiety of NAD^+ onto target proteins. Prototypes of this toxin family are diphtheria toxin (Honjo et al. 1968; Gill et al. 1969), cholera toxin (Cassel and Pfeuffer 1978; Moss and Vaughan 1977; Gill and Richardson 1980), and pertussis toxin (West et al. 1985; Ui 1984), which modify elongation factor 2 and heterotrimeric G proteins, respectively. In the late 1980s, the Rho-modifying *Clostridium botulinum* ADP-ribosyltransferase C3 was serendipitously discovered in the supernatant of *Clostridium botulinum* culture (Aktories et al. 1987), as a third bacterial ADP-ribosylating enzyme besides the botulinum neurotoxin BoNTC and the binary *C. botulinum* C2 toxin, which modifies G-actin (Aktories et al. 1986). Later it turned out that C3 ADP-ribosylates Rho proteins (Aktories et al. 1989; Chardin et al. 1989), a finding which was of major importance for the whole field of small GTPases.

4.2.1 C3-Like ADP-Ribosylating Toxins

C3 toxins are produced by various types of bacteria including clostridia (C3bot from *C. botulinum* and C3lim from *C. limosum*), *Bacillus cereus* (C3cer), and *Staphylococcus aureus*. At least two isoforms of *C. botulinum* C3 toxin (C3bot 1 and 2) and three isoforms of *S. aureus* C3 toxin (C3stau1, 2, and 3; also called EDIN-A, B, and C) have been described (Aktories et al. 1989; Rubin et al. 1988; Popoff et al. 1990; Inoue et al. 1991; Wilde et al. 2001; Just et al. 1992). The enzymes are small ~25 kDa proteins. The crystal structures of C3bot (Han et al. 1999), C3lim (Vogelsgesang et al. 2008), and C3stau2 (Evans et al. 2003) have been described in the presence and absence of NAD^+, showing a very similar architecture although C3bot and C3stau2 are only ~31 % identical. The structure is characterized by a mixed α/β fold with a β sandwich core, which forms a pocket for NAD^+ binding. The central pocket is bordered on one side by the ADP-ribosylation turn-turn (ARTT) loop, which is suggested to be involved in substrate recognition (Han and Tainer 2002). Moreover, it contains an invariant glutamate (referred to as the catalytic glutamate; e.g., C3bot ^{E174}), glutamine (C3bot ^{Q172}), and phenylalanine (C3bot ^{F169}) residue.

4.2.1.1 Substrates and Functional Consequences of Rho Protein ADP-Ribosylation

All C3 toxins modify RhoA, B, and C at asparagine41 (Sekine et al. 1989). RhoE is an additional substrate of *S. aureus* C3stau2 (Wilde et al. 2001). Rac proteins are only very poor in vitro substrates (Ridley and Hall 1992; Ridley et al. 1995; Just et al. 1992). The main effect of C3-induced ADP-ribosylation of RhoA is inhibition of its biological effects. In all cases studied so far, RhoA-dependent signaling is blocked by ADP-ribosylation at Asn41. What is the reason for inhibition of RhoA

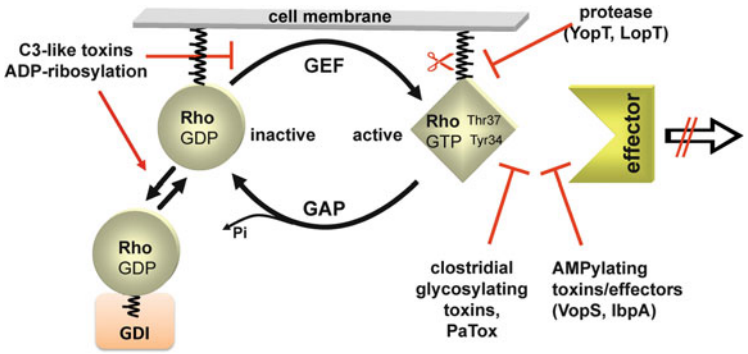

Fig. 4.1 Inhibition of Rho proteins by covalent modifications. Rho proteins are regulated by a GTPase cycle. They are inactive in the GDP-bound form and active after GDP/GTP exchange induced by guanine nucleotide exchange factors (GEFs). The active form is attached to membranes by C-terminal isoprenylation. The active form interacts with numerous effectors. The active state is terminated by GTP hydrolysis. This is facilitated by GTPase activating proteins (GAPs). Inactive Rho is extracted from membranes by guanine nucleotide dissociation inhibitors (GDIs). Rho proteins are inhibited by mono-*O*-glycosylation induced by clostridial glycosylating toxins. *C. difficile* toxins A and B and *C. sordellii* hemorrhagic toxin cause glucosylation of threonine 37/35 of Rho proteins. *C. sordellii* lethal toxin glucosylates Rac and Ras subfamily proteins at the same site. *C. novyi* α-toxin GlcNAcylates Rho protein at threonine 37/35. PaTox, a recently discovered toxin from *Photorhabdus asymbiotica* causes GlcNAcylation at tyrosine34/32 of Rho proteins. Rho proteins are adenylylated (also called AMPylation) at threonine 35/37 by the *Vibrio parahaemolyticus* effector VopS. The effector IbpA from *Histophilus somni* AMPylates Rho proteins at tyrosine 34/32. C3-like toxins ADP-ribosylate RhoA, B, and C in asparagine41. The modification inhibits GEF-induced action of the GTP-binding proteins and increases the affinity of the inactive Rho protein for GDI. YopT from *Yersinia* species and LopT from *Photorhabdus luminescens* inhibit Rho proteins by proteolytic cleavage before the C-terminal cysteine residue, which carries the isoprenyl moiety

function? ADP-ribosylation of RhoA at Asn41 has no effect on RhoA-effector interaction and RhoA nucleotide binding (GDP or GTPγS) is hardly affected. Also a slight increase (~2-fold) in basal GTP hydrolysis activity cannot explain the functional consequences of ADP-ribosylation (Sehr et al. 1998). More important is the inhibition of nucleotide exchange induced by GEFs (Barth et al. 1999) (Fig. 4.1). Finally, ADP-ribosylated Rho protein exhibits high affinity for GDI and is entrapped in a RhoA–GDI complex (Genth et al. 2003). Thus, both inhibition of GEF-induced nucleotide exchange and stabilization of the inactive Rho–GDI complex cause blockade of Rho signaling after ADP-ribosylation at Asn41 (Fig. 4.1).

Although ADP-ribosylation by C3 is highly specific for RhoA, B, and C, other small GTPases also interact with C3. It was shown that the small GTPase Ral, which is ~35 % identical with RhoA, inhibits the C3-induced ADP-ribosylation of Rho proteins. Both isoforms RalA and RalB, which are not substrates for C3-catalyzed ADP-ribosylation, bind with high affinity to C3 (K_D12–30 nM) (Wilde et al. 2002). Moreover, binding of C3, but not ADP-ribosylation, blocks the activation of phospholipase D by RalA. The precise interaction of C3 and RalA has been elucidated by crystal structure analysis of the RalA–C3 complex, thereby showing that mainly a

helix-loop-helix motif, covering helices α3 and α4 of C3, interacts with the switch-II region, helix α3, and the P-loop of Ral. The helix-loop-helix motif is clearly located outside the active site of C3. The crystal structure also explains the functional inhibition of Ral by C3 (observed in vitro) as a GDI-like effect, which blocks nucleotide exchange (Pautsch et al. 2005). Although the biological consequences of the interaction of C3 with Ral proteins are not clear, the high affinity of the interaction suggests in vivo relevance of the interaction as well.

4.2.1.2 The Role of C3 Toxins in Infection

C3 toxins consist only of the ADP-ribosyltransferase domain. Because exotoxins usually contain cell-binding and translocation domains, the C3 family members were often designated as C3 exoenzymes. Usually, high concentrations (e.g., >1 μg/ml) of C3 and long incubation times (≥24 h) are necessary to achieve cellular effects (Just et al. 1992; Wiegers et al. 1991), which is unusual for toxins. This suggested that cell entry occurs mainly by unspecific mechanisms. However, some cell types (e.g., macrophages) are intoxicated at a low concentration of C3 toxin (0.1 μg/ml) after a relatively short time (e.g., 3 h) (Fahrer et al. 2010). Moreover, C3 toxins from *S. aureus* may not need to be transported into target cells, because these bacteria are also intracellular pathogens. In line with this hypothesis it was shown that C3stau is released from host cell-invading *S. aureus* (Molinari et al. 2006).

C3 toxin was extremely instrumental for the elucidation of the role of Rho GTPases, because these agents were specific tools to inactivate RhoA, B, and C, allowing their physiological role as compared to Rac and Cdc42 to be elucidated. For this purpose C3 was initially microinjected (Paterson et al. 1990) or employed at very high toxin concentrations. Later fusion toxins were constructed for delivery of C3 into target cells. This was performed with diphtheria toxin (Aullo et al. 1993), *C. botulinum* C2 toxin (Barth et al. 1998), *C. perfringens* iota toxin (Marvaud et al. 2002), and the translocation component of anthrax toxin (Rolando et al. 2009). Moreover, it has been shown that short transport peptides (e.g., TAT and others) fused to C3 increase the cellular uptake of the toxin (Sauzeau et al. 2001; Park et al. 2003; Sahai and Olson 2006; Winton et al. 2002). In this respect it is notable that a fusion peptide of C3 toxin, which enters cells more easily, is in clinical trials as a drug BA-210 (trademarked as Cethrin®) for treatment of spinal cord injury, because it was shown that inhibition of RhoA enhances neuronal regeneration (Wahl et al. 2000) in the central nervous system (Lehmann et al. 1999).

4.2.2 ADP-Ribosylation of Rho Proteins by Photorhabdus Luminescens Tc Toxin

Recently it has been shown that a *Photorhabdus luminescens* Tc toxin ADP-ribosylates Rho proteins. In this case, however, toxin-catalyzed ADP-ribosylation of Rho proteins causes activation of the small GTPases.

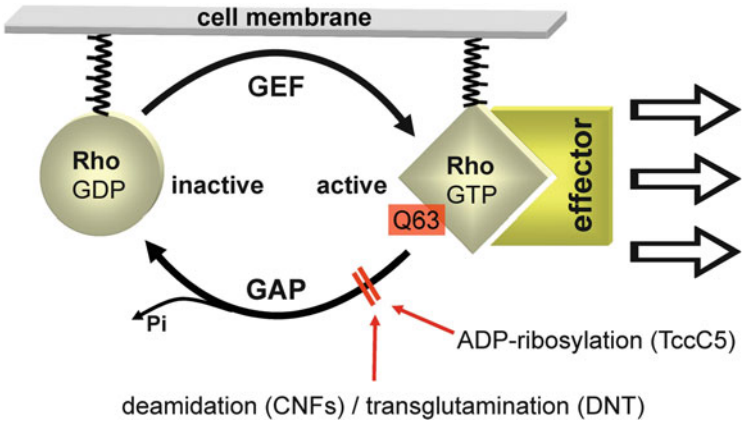

Fig. 4.2 Activation of Rho proteins by bacterial toxins. Cytotoxic necrotizing factors (CNFs) from *E. coli* and *Yersinia* species activate Rho proteins by deamidation of glutamine 61/63. This residue is involved in GTP hydrolysis. Deamidation of glutamine to glutamic acid inhibits GTP hydrolysis and thereby blocks the switch-off reaction. Dermonecrotic toxin (DNT) from *Bordetella* ssp. causes transglutamination of glutamine61/63 with the same consequence. The TccC5 isoform of the *Photorhabdus luminescens* tripartite Tc toxin ADP-ribosylates glutamine61/63. Also this modification inhibits the inactivation of Rho proteins

Tc is a toxin produced by entomopathogenic *Photorhabdus luminescens* bacteria. The organism lives in the gut of nematodes of the family *Heterorhabditidae*. The nematodes invade insect larvae, where they release the bacteria (Forst et al. 1997; Waterfield et al. 2009; Ciche 2007; Ciche et al. 2008). Subsequently the bacteria produce toxins to kill the larvae, thereby producing a source of nourishment for bacteria and nematodes. The most potent agents produced by the bacteria are Tc (toxin complex) toxins, which occur in several isoforms (Waterfield et al. 2009; Ffrench-Constant et al. 2003). *Photorhabdus* Tc toxins are essential for insecticidal activity and for a productive symbiosis with nematodes. The toxin complex is very large (>1.7 MDa) (Sheets et al. 2011; Gatsogiannis et al. 2013) and consists of the three components TcA, TcB, and TcC (Ffrench-Constant and Bowen 2000; Ffrench-Constant and Waterfield 2006; Waterfield et al. 2001). TcA (~285 kDa) is the cell-binding component and TcC (~112 kDa) the biologically active component. TcB (~170 kDa) acts as a linker between TcA and TcC (Gatsogiannis et al. 2013; Busby et al. 2013; Landsberg et al. 2011). Several TcC isoforms exist. Two of them (TccC3 and TccC5), which possess ADP-ribosyltransferase activity, have been analyzed recently. While TccC3 ADP-ribosylates actin, TccC5 modifies Rho proteins (Lang et al. 2010) (Fig. 4.2).

Interestingly, TccC5 ADP-ribosylates glutamine63 of RhoA and glutamine61 of Rac and Cdc42 (Lang et al. 2010). These glutamine residues are essential for the function of Rho proteins and play a pivotal role in the turn-off mechanism of the GTPase cycle of Rho proteins and most other small GTPases (Vetter and Wittinghofer 2001) (see below). Modification of glutamine61/63 blocks

endogenous and GAP-stimulated GTP hydrolysis of Rho proteins (Lang et al. 2010). Therefore, after TccC5-induced ADP-ribosylation Rho proteins are persistently activated. Although modified by the large ADP-ribose moiety, modified Rho proteins efficiently couple to their effectors. Thus, RhoA ADP-ribosylated at glutamine63 interacts with its effector rhotekin. Increase in RhoA–rhotekin interaction is observed in vitro with isolated proteins as well as after treatment of intact cells with the toxin. Treatment of insect- or mammalian target cells with the combination of the cell-binding component TcA, the linker TcB, and the ADP-ribosylating toxin component TccC5, results in pronounced formation of stress fibers (Lang et al. 2010). Induction of stress fiber formation is a typical feature of RhoA activation (Ridley and Hall 1992, 1994; Nobes and Hall 1995; Lim et al. 1996). Similar to RhoA, Rac and Cdc42 are activated by TccC5. In these cases, glutamine61 is ADP-ribosylated, the GTP hydrolysis is blocked, and thereby the interaction of Rac or Cdc42 with effectors (e.g., PAK-kinase) is enhanced. Typical cellular consequences of the activation of Rac and Cdc42 are membrane ruffling, lamellipodia, and filopodia formation, respectively (Ridley et al. 1992). However, TccC5 induces a dominant effect on stress fiber formation without major effects on lamellipodia or filopodia formation. The reason for this phenotype is not clear.

4.3 Glycosylating Toxins

4.3.1 Mono-O-Glycosylation of Rho and Ras Proteins by Clostridial Toxins

Rho and Ras proteins are substrates for modification by bacterial glycosylating toxins [for recent reviews see Jank and Aktories (2008), Schirmer and Aktories (2004), Just and Gerhard (2004), Belyi and Aktories (2010), Genth et al. (2008), Popoff and Geny (2011)]. Most important are clostridial glycosylating toxins, which are responsible for numerous diseases of animals and humans. Among these toxins are *Clostridium difficile* toxins A (TcdA) and B (TcdB), which are the responsible agents for antibiotics-associated diarrhea and pseudomembranous colitis (Bartlett et al. 1978; Voth and Ballard 2005; Kelly and LaMont 2008; Bartlett 2010; Schirmer and Aktories 2004; Just and Gerhard 2004). *Clostridium difficile* are anaerobic, spore-forming bacteria, which colonize and multiply when the normal gut flora is altered by antibiotic treatment. During recent years infections by these pathogens have become the most important nosocomial infections in developed countries responsible for hundreds of thousands cases with ~15,000 fatal outcomes each year only in the USA (http://www.cdc.gov/hai/eip/pdf/Cdiff-factsheet.pdf). Toxins A and/or B are crucial for *C. difficile* diseases (Kelly and LaMont 2008). Other members of this toxin family are *C. sordellii* lethal and hemorrhagic toxins, and *C. novyi* α-toxin, which are all involved in pathogenesis of gas gangrene

syndrome (Bette et al. 1991; Boriello and Aktories 2005; Popoff and Geny 2011). Moreover, *C. perfringens* toxin TpeL was recently recognized as a new member of this toxin family (Amimoto et al. 2007). All these toxins have a common architecture, consisting of several functional modules, and possess molecular masses between 200 and 308 kDa. An ABCD model has been suggested for the toxin structure (Jank and Aktories 2008). The glycosyltransferase domain (A-domain) is located at the N terminus (Hofmann et al. 1997), followed by a cysteine protease domain (C-domain) (Egerer et al. 2007). At the C terminus, most clostridial glycosylating toxins harbor a region of polypeptide repeats referred to as CROP (combined oligopeptide repeats) (Von Eichel-Streiber and Sauerborn 1990; Greco et al. 2006), which is suggested to be involved in receptor binding (B, binding domain) (Sauerborn et al. 1997). Between the CROP domain and the cysteine protease domain a region is located, which is responsible for the translocation of the toxin into target cells. This part was termed the delivery domain (D-domain). However, recent studies suggest that the D-domain consists of a translocation part and a part representing a second receptor-binding side (Genisyuerek et al. 2011).

4.3.1.1 Mode of Actions of Clostridial Glycosylating Toxins

Clostridial glycosylating toxins bind to unknown cell surface receptors and thereafter are taken up in a clathrin- and dynamin-dependent manner to reach an acidic endosomal compartment (Papatheodorou et al. 2010) (Fig. 4.4). At low pH, the toxins undergo a conformational change, insert into the vesicle membrane, and form pores (Barth et al. 2001; Qa'Dan et al. 2000; Genisyuerek et al. 2011). Subsequently, parts of the toxins (most likely the glycosyltransferase and cysteine protease domain) are translocated through the membrane into the cytosol (Pfeifer et al. 2003). Here, the toxin cysteine protease is activated by inositol hexakisphosphate (Shen et al. 2011; Reineke et al. 2007; Egerer et al. 2007, 2009; Guttenberg et al. 2011). The glycosyltransferase domain is released by autocleavage to target Rho/Ras proteins (Pfeifer et al. 2003; Jank and Aktories 2008).

Modification of Rho proteins by *C. difficile* toxins A and B has been studied most intensively. Both toxins mono-*O*-glucosylate Rho proteins, using UDP-glucose as a sugar donor (Just et al. 1995a, b). UDP-glucose serves also as the sugar donor for *C. sordellii* lethal toxin, which most efficiently modifies Rac and Ras proteins, including Ras, Ral, and Rap (Just et al. 1996; Popoff et al. 1996). *C. novyi* α-toxin causes GlcNAcylation of Rho proteins and uses UDP-GlcNAc (Selzer et al. 1996). TpeL toxin from *C. perfringens* modifies Ras proteins preferably by GlcNAcylation (Guttenberg et al. 2012). However, in vitro studies also show additional modification of Rac and usage of UDP-glucose as a sugar donor (Amimoto et al. 2007; Nagahama et al. 2010).

All abovementioned clostridial glycosyltransferases mono-*O*-glycosylate Rho/Ras proteins at threonine35/37. This threonine residue is highly conserved within the whole family of small GTPases. It participates in coordination of the divalent cation magnesium and the binding of GTP (Pai et al. 1990). The hydroxyl

group of the threonine residue binds to the γ-phosphate of GTP and is directed into the protein. This explains why the GTP-bound form of the GTPases is a poor substrate for glycosylation (Just et al. 1996).

Because threonine35/37 has a central role in the activity of the GTPases (Pai et al. 1990), its modification has numerous functional consequences: While GDP-binding is hardly affected by glucosylation of Rho proteins, the affinity of GTPγS is reduced (the dissociation rates of bound GTPγS for RhoA, Rac1, and Cdc42 increased about three-, four-, and sevenfold, respectively) (Sehr et al. 1998). Basal GTP hydrolysis by the Rho proteins is reduced (about fivefold) after glucosylation, and the proteins are GAP insensitive (Sehr et al. 1998). Glucosylated GTPases are no longer activated by GEFs (Barth et al. 1999). Remarkably, glucosylation inhibits the induction of the active conformation of Rho/Ras proteins even after binding of GTP (Vetter et al. 2000; Geyer et al. 2003). In addition, Rho proteins glucosylated at threonine35/37 do not cycle; they stick to membranes and are not released and sequestered by GDI in the cytosol (Genth et al. 1999). In summary, glucosylation results in functional inactivation of Rho/Ras proteins and blockade of their signaling functions (Fig. 4.1).

4.3.2 *Glycosylation of Rho Proteins by Photorhabdus Asymbiotica Toxin PaTox*

PaTox is a newly described toxin from *Photorhabdus asymbiotica*. This pathogen is highly related to *Photorhabdus luminescens* and shares symbiosis with nematodes and entomopathogenic activity (Gerrard et al. 2006). However, *P. asymbiotica* is also pathogenic to humans and induces epidermal and soft tissue ulcers (Gerrard et al. 2004; Peel et al. 1999). It does not produce the tripartite Tc toxin of *P. luminescens*, which was shown to activate Rho proteins by ADP-ribosylation. However, *P. asymbiotica* produces a unique large toxin of 2,957 amino acids called PaTox, which possesses glycosyltransferase activity targeting Rho proteins (Jank et al. 2013). The toxin was identified based on significant amino acid sequence similarity with Rho/Ras-modifying clostridial glycosyltransferases. This similarity, however, is limited to a small C-terminal glycosyltransferase domain of 334 amino acids.

In the presence of UDP-GlcNAc, full length PaTox or the transferase domain alone modifies ~25 kDa proteins in lysates of insect and mammalian cells. Further studies revealed that in vitro RhoA, B, C, Rac, and Cdc42 but not Ras or Rab subfamily proteins are modified. Surprisingly, it has emerged that Rho proteins previously glucosylated at threonine37 by *C. difficile* toxin B were still modified by PaTox. Subsequent mass spectrometric analysis showed that PaTox catalyzed the GlcNAcylation of tyrosine34 of RhoA and the equivalent residue tyrosine32 in Rac and Cdc42 (Jank et al. 2013) (Fig. 4.1). Thus, PaTox is the first known mono-*O*-glycosyltransferase which modifies Rho proteins at tyrosine residues.

4.3.2.1 Structure of the Glycosyltransferase Domain of PaTox

Crystal structure analysis of the complex of the glycosyltransferase domain of PaTox with UDP-GlcNAc at 1.8 Å revealed fundamental insights into the architecture of the transferase domain (Jank et al. 2013). The domain has a cone-like shape comprised by a globular catalytic head and a 3-helical extension. Altogether the enzyme is one of the smallest protein-glycosyltransferases known and belongs to the GT-A family of glycosyltransferases with a Rossmann-like fold of a central 6-stranded β-sheet sandwiched by α-helices on both sides (Lairson et al. 2008; Jank et al. 2013). The typical divalent cation is coordinated by the pyrophosphate of UDP and the carboxylate groups of the DxD motif. As in other GT-A-like enzymes, exchange of the DxD motif to NxN completely blocks glycosyltransferase activity of PaTox (Wiggins and Munro 1998; Busch et al. 1998). Amino acids involved in UDP-GlcNAc interaction are highly conserved and are found in *C. difficile* toxin B and *Legionella pneumophila* glycosyltransferase Lgt at similar positions (Lu et al. 2010). Interestingly, glycogenin, which causes auto-glycosylation at a tyrosine residue, exhibits next nearest structural homology with the PaTox domain besides clostridial toxins and Lgt.

4.3.2.2 Functional Consequences of Tyrosine Glycosylation of Rho Proteins

Tyrosine34 glycosylation of RhoA has no effect on nucleotide binding. However, it blocks the interaction of RhoA with its effector rhotekin. This is plausible because tyrosine-34 is located in the switch-I region of RhoA, which is involved in Rho-effector interaction. Similarly, Rac, gylcosylated by PaTox on tyrosine-32, is no longer able to interact with its effectors e.g., PAK. Moreover, the activation of Rho proteins by RhoGEFs (e.g., by LARG, PDZ RhoGEF, and p47-LBC GEF) is blocked by PaTox-induced GlcNAcylation of tyrosine32/34. Finally, tyrosine-GlcNAcylated RhoA can no longer interact with GAP proteins. Remarkably, GlcNAcylation of RhoA at tyrosine34 also reduces basal GTP hydrolyzing activity, suggesting a possible involvement of this residue in GTP hydrolysis (Jank et al. 2013).

Many effects and functional consequences of PaTox-induced GlcNAcylation of tyrosine32/34 are also observed with Rho proteins glucosylated at threonine35/37 by clostridial glucosylating toxins (Sehr et al. 1998). However, one major difference was observed. While clostridial toxins, e.g., *C. difficile* toxin B preferentially modifies Rho protein in its GDP-bound form, the opposite is true for modification of Rho proteins by PaTox (Jank et al. 2013). The latter toxin modifies GTP or GTPγS-bound RhoA much more efficiently than GDP-bound RhoA. This is explained by the conformational changes of RhoA that are induced by GTP-binding. Tyrosine34 is positioned almost in the middle of the switch-1 region, which undergoes dramatic conformational changes upon nucleotide binding (Rittinger et al. 1997). In the GTP-bound form of RhoA, tyrosine34 is readily accessible for modification by the toxin.

Surprisingly it has been found that PaTox alone can activate Rho proteins. A deamidase domain, located C-terminally of the glycosyltransferase domain, is able to activate heterotrimeric G proteins by deamidation in a similar manner to that seen for the *Pasteurella multocida* toxin (Orth and Aktories 2012; Orth et al. 2009). Subsequently, activation of heterotrimeric G proteins (most likely Gq) causes activation of RhoA and, for example, induces stress fiber formation (Jank et al. 2013). However, activation of RhoA by PaTox is only observed when the glycosylation domain is inactivated; otherwise GlcNAcylation is dominant and results in inactivation of RhoA.

4.4 Toxin-Induced Adenylylation and Phosphocholination

Small GTPases are also modified by toxin-induced attachment of AMP (Yarbrough et al. 2009; Kinch et al. 2009; Worby et al. 2009; Muller et al. 2010, 2014; Ham and Orth 2011). This reaction type is an adenylylation and was already described in the late sixties as modification by glutamine synthetase (Kingdon et al. 1967). Rediscovered as a molecular mechanism by which bacterial effectors modify target proteins, the reaction is now also called AMPylation (Yarbrough et al. 2009). Using ATP as a cosubstrate, the bacterial effector transfers AMP onto a Rho protein and pyrophosphate is released. So-called FIC domains (filamentation induced by cyclic AMP), which are characterized by a HXFXX(G/A)N(G/K)R consensus sequence, have been identified to be responsible for this type of posttranslational modification of Rho proteins (Xiao et al. 2010). While Fic domain like structures are very common within the prokaryote kingdom, they are structurally different from the glutamine synthetase-like type of transferases, which are DNA polymerase β-like transferases (Xu et al. 2010).

4.4.1 Adenylylation of Rho Proteins by Vibrio Parahemolyticus VopS and Histophilus Somni IbpA

Vibrio parahemolyticus VopS was the first Fic-domain containing bacterial effector identified to modify small GTPases by adenylylation (Yarbrough et al. 2009). The pathogen is a Gram-negative bacterium which is responsible for an increasing incidence of food-borne acute gastroenteritis (Newton et al. 2012). Its effector VopS is introduced into eukaryotic target cells by a type III secretion system, which depends on the direct contact of the pathogen with its host cell. VopS attaches AMP onto threonine35/37 of Rho proteins including RhoA, B, C, Rac, and Cdc42 (Yarbrough et al. 2009). The functional consequences are very similar to glycosylation of Rho proteins, resulting in inhibition of Rho signaling, with cytotoxic effects, disruption of the intestinal epithelium, and intestinal inflammation (Yarbrough et al. 2009; Ritchie et al. 2012).

Another bacterial pathogenic factor IbpA (immunoglobulin-binding protein A), which modifies Rho proteins by adenylylation is produced by *Histophilus somni*, a pathogen, which causes infections in livestock. IbpA is a ~350 kDa protein which adenylylates Rho proteins not at a threonine residue like VopS but at tyrosine32/34 of Rho proteins (Worby et al. 2009); thus, IbpA adenylylates the same residue of Rho GTPases as is GlcNAcylated by *P. asymbiotica* PaTox (Jank et al. 2013). The functional consequence is blockade of downstream Rho-signaling (Worby et al. 2009). Similar inhibiting effects on Rho signal pathways are caused by PaTox-induced GlcNAcylation and also by VopS-catalyzed adenylylation of threonine35/37. Interestingly, a human Fic domain-containing protein called HYPE has been shown to modify Rho proteins at tyrosine32/34 at least in vitro (Worby et al. 2009). However, the efficiency of Rho AMPylation by HYPE is less than that of IbpA (Mattoo et al. 2011). It is also notable that the in vitro cosubstrate specificity of the Fic domain like transferases is not very high; for example, VopS accepts GTP as a substrate for nucleotidylylation with the same efficiency as ATP (Mattoo et al. 2011).

4.4.2 Adenylylation and de-Adenylylation by Legionella Pneumophila Effectors

Rho proteins are not the only targets for bacterial effectors. Rab proteins, which play central roles in intracellular vesicle traffic, are AMPylated by *Legionella pneumophila*. This pathogen is the cause of the Legionnaires' disease, which is characterized by a severe and often fatal pneumonia in humans (Diederen 2008).

The life cycle of *Legionella pneumophila* starts with phagocytosis by host macrophages, where the pathogen reconstructs the phagosome into a replication vacuole (also called *Legionella* containing vacuole, LCV), thereby controlling the cellular metabolism and intracellular organelle traffic and organization (Isberg et al. 2009). To this end, *Legionella* translocates ~300 different effectors into the host cytosol via a type IV secretion system called Dot/Icm (Richards et al. 2013; Segal et al. 2005). Among these factors are also the *Legionella* glucosyltransferases Lgt1-3, which are related to clostridial Rho glucosylating toxins, but they inhibit protein synthesis by glucosylation of the GTPase elongation factor 1A (Belyi et al. 2006, 2008).

Legionella pneumophila produces various effectors, which target Rab proteins of the host. The effector DrrA (also called SidM), which recruits Rab1 to the LCV to generate a pool of membrane-associated Rab1, was shown to adenylylate the GTPase (Muller et al. 2010; Ham and Orth 2011; Goody and Itzen 2013). DrrA possesses Rab GEF activity (Murata et al. 2006; Machner and Isberg 2007) and its N-terminal part shares the DNA polymerase β-like nucleotidylyl transferase structure with glutamine synthetase (Muller et al. 2010). DrrA modifies Rab1b at tyrosine77, which is located in the switch II region of the GTPase. Modification

of Rab1 by attachment of AMP prevents GTPase stimulation by GAPs (including the *Legionella* GAP protein LepB) and locks Rab1 in its GTP-bound state (Muller et al. 2010). Thereby, Rab1 is trapped at the membrane of the LCV, which is essential for proliferation of the pathogen inside the host cell.

However, activated and AMPylated Rab1 is only transiently present at the LCV membrane, suggesting a further processing of AMPylated Rab1. Excitingly, it turned out that AMPylation-induced effects of Rab1 are reversed by a de-AMPylation (de-adenylylation) reaction, which is caused by the *Legionella pneumophila* effector SidD (Neunuebel et al. 2011; Tan and Luo 2011). SidD is a protein of 507 amino acids. Whereas the N-terminal part (residues 1–379) harbors the de-AMPylation activity, the C-terminal part is essential for in vivo biological activity (e.g., rescue of DrrA/SidM-induced rounding of COS1 cells) and responsible for the proper localization of SidD at the Golgi. Recently the crystal structure of SidD has been analyzed, showing resemblance to metal-dependent protein phosphatases (Chen et al. 2013). After SidD-catalyzed de-AMPylation, Rab1 can be inactivated by the *Legionella* GAP protein LepB. Rab35, which is involved in endosomal traffic as well, is AMPylated and also de-AMPylated by SidD (Neunuebel et al. 2011). Notably, AMPylated Rho proteins are not substrate for de-AMPylation by SidD, indicating the high specificity of the hydrolase for Rab1 and Rab35 (Chen et al. 2013).

4.4.3 Phosphocholination and de-Phosphocholination by Legionella Pneumophila Effectors

The *Legionella* effector protein AnkX, which induces Golgi fragmentation and prevents the traffic of the *Legionella*-containing vacuole to lysosomes, harbors a Fic domain, including the invariant catalytic histidine residue. Exchange of this histidine residue prevents Golgi fragmentation (Roy and Mukherjee 2009), suggesting an essential role of the Fic domain in the action of AnkX. In view of these findings, it was initially proposed that AnkX is also an AMPylating enzyme (Roy and Mukherjee 2009). However, it turned out that the Fic domain of AnkX induces the phosphocholination of Rab1 (Mukherjee et al. 2011; Tan et al. 2011; Goody et al. 2012). AnkX uses CDP-choline as a co-substrate to transfer phosphocholine onto serine76 of Rab1b and threonine76 of Rab35 of host cells (Mukherjee et al. 2011; Goody et al. 2012). Both GDP- and GTP-loaded Rabs are modified, but the GDP-bound form is preferred. Phosphocholination of Rabs inhibits their activation by GEFs (activation of Rab35 is stronger affected than that of Rab1) and, importantly, the interaction with RabGDI. Interaction with the *Legionella* Rab effector LidA is still possible. Inactivation of Rabs by GAPs is less strongly affected. Altogether, phosphocholination of Rab1 and Rab35 appears to result in a stable attachment of inactive Rabs to membranes, where they cannot be extracted by GDI (Goody et al. 2012). Excitingly, phosphocholination is also a reversible

modification. *Legionella* produces the Dot/Icm effector Lem-3 that is able to catalyze the dephosphocholination of modified Rab proteins (Tan et al. 2011; Goody et al. 2012).

4.5 Toxin-Induced Proteolysis

Yersinia species counteract the immune system of the host by injecting several effector molecules (*Yersinia* outer proteins, Yops) via a type III secretion system. Three human pathogenic *Yersinia* strains are known: *Y. pestis* is the agent of plague, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are food-borne pathogens, which cause acute and chronic gastrointestinal infections. Some of the injected Yops interfere with the function of Rho GTPases. The GAP-like protein YopE transiently inactivates Rho GTPases. The *Y. enterocolitica* effector YopT proteolytically cleaves off the membrane anchor of Rho proteins. YopT was discovered in 1998 by Iriarte and Cornelis as an effector protein present on the *Yersinia* virulence plasmid (pYV) (Iriarte and Cornelis 1998).

It was shown that YopT induces a redistribution of RhoA from membranes of *Yersinia*-infected cells towards the cytosol consistent with the YopT-induced disruption of the actin cytoskeleton resulting in cell rounding. This release occurred also with recombinant YopT and isoprenylated RhoA in artificial membranes (Sorg et al. 2001). For their correct localization and function, small GTPases with a C-terminal CaaX-box (C, cysteine; a, aliphatic residue, X, any residue) are posttranslationally modified (Zhang and Casey 1996). The CaaX-box cysteine is isoprenylated followed by the cleavage of the –aaX tripeptide and methylation of the prenylated cysteine (Otto et al. 1999; Winter-Vann and Casey 2005). YopT cleaves Rho proteins directly in front of the posttranslationally modified cysteine of Rho GTPases, thereby releasing the GTPases from the membrane. This leads to loss of function of the GTPases (Shao et al. 2002, 2003). The cleavage of Rho proteins is independent of their activation status but requires isoprenylation (Shao et al. 2003; Fueller and Schmidt 2008). Rac, which is released from membranes by YopT, is effectively translocated into the nucleus (Mohammadi and Isberg 2009). In vitro YopT acts on various Rho GTPases. However, RhoA appears to be the preferred substrate in HeLa cells and HUVEC (Aepfelbacher et al. 2003). For efficient translocation of the protease into the host cell, YopT requires a specific chaperone SycT (specific Yop chaperone T) (Trulzsch et al. 2004). SycT reveals a homo-dimer with a typical overall fold (Locher et al. 2005; Buttner et al. 2005). Amino acids 52–103 of YopT are bound to the SycT dimer revealing a chaperone-effector stoichiometry of 2:1 (Buttner et al. 2005).

YopT belongs to a growing clan of papain-like cysteine proteases which share a catalytic triad of Cys, His, and Asp and are inhibited by the protease inhibitor E64. These proteases encompass a new protein fold similar to papain but with differing substrate specificity. Like YopT, the homologous enzyme LopT produced by *Pseudomonas luminescens* targets Rho proteins in mammalian and insect cells

and releases Rho and Rac from membranes. Other examples are AvrPphB (Avirulence protein (Avr) from the plant pathogen *Pseudomonas syringae*) and NopT from *Rhizobium* sp. (Shao et al. 2002; Dowen et al. 2009). AvrPphB undergoes autocatalytic processing and subsequent cleavage of the serine/threonine kinase PBS1 in plant cells (Zhu et al. 2004). Several members of the YopT family of cysteine proteases have been identified by genome sequencing and the list will presumably grow (Zhu et al. 2004).

4.6 Rho Deamidating Toxins

Most known bacterial toxins inhibit the action of small GTPases. However, at least six different bacterial enzymes catalyze a modification of Rho proteins, leading to constitutive activation of the small GTPases. Interestingly, all of them modify the same amino acid, which is glutamine63 (RhoA sequence numbering). This glutamine is conserved between different members of the Ras superfamily of small GTPases. However, the toxins exclusively target Rho-proteins catalyzing different modifications. All Cytotoxic Necrotizing Factors (CNFs), which are *Escherichia coli* CNF1, CNF2, and CNF3, as well as the homologous *Yersinia pseudotuberculosis* CNFy, deamidate Gln63 of RhoA, thereby generating glutamate at this position. *Bordetella bronchiseptica* dermonecrotic toxin (DNT) catalyzes a transglutamination of this amino acid (Schmidt et al. 1999; Masuda et al. 2000). Transglutaminases catalyze the exchange of a free amine group (e.g., in the side chain of lysine) and the carbamoyl group of a glutamine leading to a cross-link of proteins (Folk 1980). However, in place of the protein-bound lysine, also small primary amines (e.g., putrescine or spermidine) can be attached (Schmidt et al. 2001; Masuda et al. 2000). As mentioned above, besides toxins from human pathogenic bacteria, the insecticidal Tc toxin (TccC5) from *Photorhabdus luminescens* also modifies Rho GTPases. It catalyzes the ADP-ribosylation of glutamine63 (Lang et al. 2010) (compare 2.2). All modifications lead to the blockage of GTP hydrolysis and, therefore, to constitutive activation of the Rho proteins targeted.

4.6.1 CNFs as Virulence Factors

Cytotoxic Necrotizing factors (CNF1-3) are crucial virulence factors for uropathogenic *Escherichia coli* (UPEC)-caused diseases such as urinary tract infections, meningitis, and soft tissue infections (Petkovsek et al. 2009). It is generally accepted that most UPEC strains live in the intestine and enter the urinary tract via the urethra. While acute urinary tract infections (UTI) can be treated with common antibiotics, the chronic recurring UTIs bear a risk of septicaemia due to the invasion of bacteria into the bloodstream. CNF1 is associated with several other

virulence factors including aerobactin, P fimbriae, and hemolysin with 98 % of cnf1 + strains being also positive for hly (Yamamoto et al. 1995). Recent studies show that CNF is not only associated with uropathogens. It is also associated with skin and soft tissue infections (Petkovsek et al. 2009) and neonatal meningitis (Foxman 2002). In fact CNF1 was found in bacteria isolated from meningitis affected children with the first report on CNF1 published in 1983 by Caprioli and coworkers (1983). Following the isolation and sequencing of the cnf1 gene, CNF1 was cloned and expressed as a highly purified recombinant protein, and CNF-deficient *E. coli* strains were generated (Falbo et al. 1993). Using these strains it was shown that the colonization and tissue damage of the urinary tract of mice and of rat prostate tissue were less pronounced when no CNF1 was produced as compared to the isogenic wild-type strains (Rippere-Lampe et al. 2001b). Additionally, it was proven that bacteria which produce CNF are able to cross the blood–brain barrier (Khan et al. 2002). Less is known about the role of *Yersinia pseudotuberculosis* CNFy. Recent data show that CNFy supports the delivery of other *Yersinia* effectors, the Yersinia outer proteins (Yops) (Schweer et al. 2013). These proteins are directly injected into the mammalian cell by type-III secretion, following a direct contact of the bacterium with the cell. Without doubt, effects of CNF on the immune system are of major importance for their role as virulence factors. Due to the strong activation of Rho proteins, cells treated with CNFs show a strong network of actin stress fibers, filopodia, and membrane ruffles [for review see Schmidt and Aktories (2000)]. Moreover, multinucleated giant cells are formed (de Rycke et al. 1996). Activation of Rho GTPases by CNF1 leads to strong spreading of fibroblasts and reduction of cell motility. However, deamidated, activated Rac is degraded by the proteasomal machinery (Lerm et al. 2002; Doye et al. 2002), which leads to an increase of the formerly blocked cellular motility (Doye et al. 2002). In other cells, for example, in 804G-bladder cells also CNF-activated RhoA is ubiquitinated and degraded by proteasomes (Doye et al. 2002). The exact temporal regulation of the Rho activation pattern seems to be crucial for the pathogenicity of the bacteria with CNFs being important pathogenicity factors of *E. coli* infections (Rippere-Lampe et al. 2001a) largely interfering with the innate and with the acquired immune system by switching Rho GTPases on (deamidation) and off (degradation).

4.6.2 Structure and Mode of Action of CNFs

CNFs are single chain toxins encompassing an N-terminal receptor-binding domain, a central region important for membrane translocation and a catalytic domain located at the C terminus. The structure of the catalytic domain shows a new fold with a catalytic Cys-His dyad stabilized by a valine (Schmidt et al. 1998; Buetow et al. 2001). The catalytic center represents a similar assembly to that of cysteine proteases, transglutaminases, and the *Pasteurella multocida* toxin (PMT), which in fact is also a deamidase targeting heterotrimeric G proteins (Orth and

Aktories 2012). All four described CNF family members are homologous, identical in length (1,014 amino acids), and with the catalytic amino acids cysteine 866 and histidine 881 at identical positions. CNF1 and CNF2 show the highest homology (85 % identity) between the toxin family members. Despite the high identity between all CNFs, the toxins differ in substrate preference. In contrast to the *E. coli* toxins, CNFy modifies RhoA but not Rac or Cdc42 at low toxin concentrations (Hoffmann et al. 2004; Stoll et al. 2009). However, Rac is also activated in CNFy-treated cells after a period of several hours, or when higher toxin concentrations are used (Stoll et al. 2009; Schweer et al. 2013).

4.6.3 Uptake and Receptor of CNF1

CNF1 is a typical single chain multimodular AB-toxin which is composed of an enzymatic domain and a structure mediating uptake into mammalian cells. CNFs are taken up by receptor-mediated endocytosis. The non-integrin laminin receptor p67 has been identified to mediate the uptake of CNF1. Its cytosolic precursor protein p37 interacts with the N-terminal receptor-binding domain of CNF1 (Chung et al. 2003). A second cell surface receptor mediates strong binding and uptake into mammalian cells: CNF1 binds to the basal cell adhesion molecule (BCAM) on the surface of cells (Piteau et al. 2014). BCAM physiologically interacts with laminin and mediates attachment of cells with the extracellular matrix. Cells which are deficient for BCAM are resistant to CNF1 intoxication. Interestingly, CNF1 binds to BCAM with a domain different from the p37-binding site, suggesting that both molecules may be involved in binding and/or uptake into mammalian cells (Piteau et al. 2014). Competition studies suggested that CNF1 and CNF2 bind to the same cellular receptors, whereas CNF3 and CNFY interact with a different one (Stoll et al. 2009). Upon binding to the receptor/s, CNFs are taken up by endocytosis and released into the cytosol. For this process, acidification of the endosome is necessary. Moreover, two hydrophobic regions in the center of CNF1 are involved in the translocation process (Lemichez et al. 1997). CNF1 secondary structure predictions of this region suggest the formation of a hairpin, which is formed by two alpha helices connected by a loop. Mutations in this loop region block membrane insertion and consequently translocation into the cytosol (Pei et al. 2001). However, the exact mechanism of translocation through the endosomal membrane is not yet known.

4.6.4 CNFs as Pharmacological Tools and Potential Drugs

CNFs are selective activators of Rho GTPases. Moreover, they encompass all they need to enter mammalian cells. Therefore, the toxins are effective tools for analyzing Rho-dependent signaling pathways or for influencing the activity of Rho

GTPases in the pursuit of therapeutic aims. One of the most interesting aspects in this regard is the potential treatment of neurodegenerative disorders, since Rho GTPases are involved in the formation of new synapses. Recent analysis of intracerebroventricular injection of very low amounts of CNF1 led to the enhancement of learning capability of mice and counteracted neuroinflammation in a murine model of Alzheimer's disease (Diana et al. 2007; Loizzo et al. 2013). Another potential use of CNFs may be their influence on apoptosis with the aim of developing a potential tumor therapy. CNF1 protects cells from apoptosis induced experimentally by detaching epithelial cells from surfaces (Fiorentini et al. 1998) or by UV light (Miraglia et al. 2007). In contrast, activation of RhoA by the *Yersinia* toxin CNFy is sufficient to stimulate the intrinsic apoptotic pathway in the prostate cancer cell line LNCaP (Augspach et al. 2013). For the potential use of any bacterial toxin within the human body however, targeted transport mechanisms are required which guarantee the selective uptake of the enzymes into specific cells and tissues.

4.7 Bacterial Regulatory Mimics

The abovementioned bacterial protein toxins and effectors affect signal pathways and activities of small GTPases by covalent modification. However, bacteria have developed many tools and mechanisms to manipulate the GTPase activities of their hosts by non-covalent modification, mimicking the role of the two general types of endogenous GTPase regulators, i.d. GAPs and GEFs (Fig. 4.3). Usually, these mimics are transferred into host cells by type III secretion or type IV secretion systems. In the following the effectors will be briefly discussed (Table 4.2).

4.7.1 Bacterial GEF-Like Effectors Acting on Host GTPases

The Rho proteins Rac and Cdc42 are activated by *Salmonella Typhimurium* SopE and SopE2, which act in a GEF-like manner (Orchard et al. 2012). Both effectors are injected by the type-III secretion system SPI-1 into target cells to initiate the uptake of *Salmonella*. Sops are helical proteins with a V-like structure (Buchwald et al. 2002). The catalytic part of SopE binds to Cdc42 between switch regions I and II, thereby changing the conformation of these functionally crucial regions. Especially the ^{166}GAGA^{169} loop between the two bundles of the V-like structure of the GAPs disturbs movement of both switch regions. Thereby it locks the GTPase in a conformation that prevents divalent cation binding and causes the release of GDP. Subsequent binding of GTP, which has a higher cellular concentration than GDP, reconstitutes a conformational change in the GTPase to yield the active form. Thus, the mode of action of Sops is very similar to eukaryotic GEFs (including destabilization of the divalent cation binding). However, they have a completely different

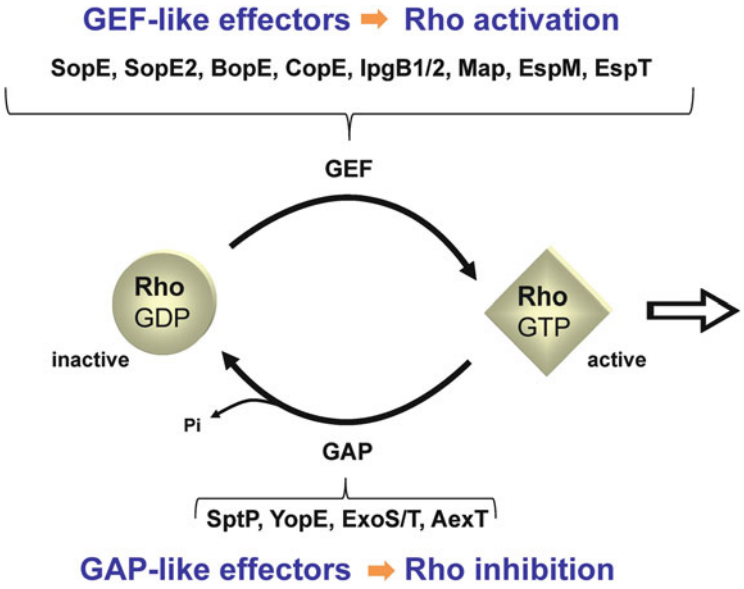

Fig. 4.3 Modulation of the Rho GTPase cycle by GEF- and GAP-like effectors. The activity state of Rho proteins is modulated by bacterial effectors, which act in a manner like guanine nucleotide exchange factors (GEFs), including *S. Typhimurium* SopE and SopE2 from, *Burkholderia pseudomallei* BopE, *Chromobacterium violaceum* CopE, *Shigella flexneri* IpgB1, 2, *E. coli* and *Citrobacter rodentium* Espm effectors, Map from *E. coli*, and EspT from *E. coli* and *C. rodentium*. In a manner like GAP-activating proteins (GAPs) act *S. Thyphimurium* effector SptP, *Yersinia* effector YopE, *Pseudomonas aeruginosa* effectors ExoS/T and AexT *from Aeromonas salmonicida*

structure. BopE, a RhoGEF effector protein from *Burkholderia pseudomallei* has a similar fold to that of SopE (Upadhyay et al. 2008). Another member of this family is CopE from *Chromobacterium violaceum*, which activates Rac and Cdc42 and is also involved in host cell invasion (Miki et al. 2011) (Table 4.2).

A second family of bacterial Rho GEFs are characterized by a WXXXE motif, which is absent in the SopE-like GEFs. Members of this bacterial effector family are IpgB1 and IpgB2 from *Shigella flexneri* (Ohya et al. 2005; Handa et al. 2007; Klink et al. 2010) and Map, EspM2, and EspT from *Escherichia coli* and *Citrobacter rhodentium* (Huang et al. 2009; Bulgin et al. 2009; Arbeloa et al. 2008, 2010). Structural studies showed that WXXXE motif proteins possess a V-like structure like SopE family proteins and apparently share the same mechanism to activate Rho proteins (Klink et al. 2010; Huang et al. 2009). SifA and SifB from *S. Typhimurium* also belong to the WXXXE motif family. The structure of SifA has been solved (Ohlson et al. 2008) revealing a SopE-like fold. However, so far a direct activation of Rho proteins was not shown for SifA and SifB.

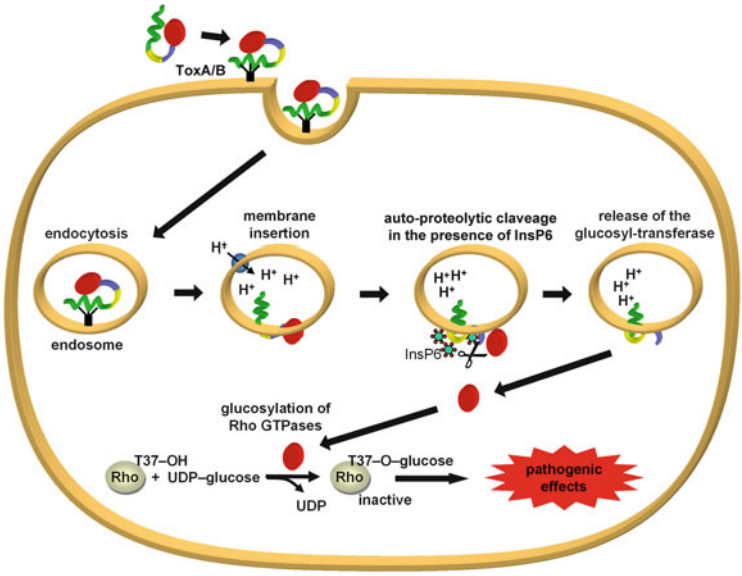

Fig. 4.4 Scheme of the uptake and action of *Clostridium difficile* toxins A and B. The toxins bind with their C-terminal part to cell surface receptors, which are not known so far. Thereafter, they are endocytosed. At low pH of endosomes, a conformational change of the toxins occurs and the toxins insert into the vesicle membrane and form a pore. At least part of the toxins is translocated into the cytosol through the pore. In the cytosol, the inherent cysteine protease of the toxins is activated by InsP6, resulting in auto-cleavage and release of the glucosyltransferase domain, which modifies Rho proteins by glucosylation at threonine35/37. Glucosylated Rho protein is inactive

4.7.2 Bacterial GAP-Like Effectors Acting on Host GTPases

A myriad of eukaryotic GAP proteins inactivate Rho/Ras proteins by increasing the rate of GTP hydrolysis. The main mechanism of these GAPs is to provide an arginine residue (also called an arginine finger) to complement the catalytic side of the small eukaryotic GTPases. Together with a highly conserved glutamine residue (e.g., Gln63 in RhoA, which is also targeted by activating toxins; see above), the arginine finger of the GAPs secures the proper positioning of GTP and of a water molecule to induce GTP hydrolysis (Vetter and Wittinghofer 2001). In analogy with eukaryotic GAPs, the bacterial effector SptP from *S. Typhimurium* provides an arginine finger for GTP hydrolysis of Rho family proteins (Fu and Galan 1998; Galan and Fu 2000). Interestingly, SptP, which consists of an amino-terminal Rho GAP domain and a C-terminal tyrosine phosphatase domain, is delivered into host cells by the SPI type-III secretion system, which also delivers Sops into target cells. Thus, to promote its cellular uptake, *Salmonella* activates and inactivates Rho proteins by mimicking eukaryotic bidirectional regulation. To coordinate Rac/Cdc42 activation and inactivation in a time- and spatially dependent

manner, *Salmonella* hijacks the host proteasome degradation pathway, resulting in rapid degradation of SopE as compared to SptP (Kubori and Galan 2003).

The *Yersinia pseudotuberculosis* effectors YopE (von Pawel-Rammingen et al. 2000), *Pseudomonas aeruginosa* exoenzymes ExoS and ExoT (Goehring et al. 1999; Krall et al. 2000), and AexT from *Aeromonas salmonicida* increase the rate of GTP hydrolysis of Rho family proteins by the same mechanism. Whereas YopE has a single-domain structure, ExoS and ExoT possess additional ADP-ribosyltransferase activities. ExoS ADP-ribosylates Ras, RalA, and Rab5, 9, and 11 (Deng and Barbieri 2008), ExoT modifies CRK proteins (Sun and Barbieri 2003) and AexT ADP-ribosylates actin (Fehr et al. 2007). Bacterial GAP-like effectors do not exclusively target Rho proteins. As already mentioned above, the type-IV secretion effector LepB from *L. pneumophila* possesses Rab GAP activity and interferes with vesicle traffic in the host cells (Ingmundson et al. 2007).

4.8 Conclusion

Pathogenic bacteria produce protein toxins with diverse activities to trigger the behavior of mammalian cells. One crucial task of these molecules is to inhibit the function of the immune system, including migration of macrophages and clonal B-cell expansion. Moreover, some of the toxins weaken the barrier function of epithelial cells. This allows the bacteria to enter host tissues. Many bacterial toxins act on Rho GTPases, which mainly govern the actin cytoskeleton most probably because the cytoskeleton plays an important role in the activity of cell-mediated immune responses. Inhibition as well as constitutive activation of Rho proteins destroys the physiological regulation and signaling of the GTPases. This suggests that inhibition of immune cell migration and epithelial barrier function is more important for bacterial virulence than growth inhibition. However, few toxins also modify proteins of the Ras and Rab subfamilies of small GTPases. Studies from recent years have shown that evolution provided bacteria with unexpected tools to manipulate the activities of small GTPases. Studies on bacterial toxins and effectors will allow us to exploit this arsenal for cell biology, pharmacology, and medicine.

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Chapter 5
Posttranslational Modifications of Small G Proteins

Bingying Zhou and Adrienne D. Cox

Abstract The numerous biological functions of Ras superfamily small GTPases are highly dependent upon specific posttranslational modifications that guide their subcellular localization and interaction with regulators and effectors. Canonical modifications of their carboxyl termini include prenylation by farnesyl or geranylgeranyl isoprenoid lipids (Ras, Rho, Rab families). These serve as important components of their membrane targeting motifs and promote membrane binding, analogously to the cotranslational amino-terminal myristoylation of Arf family proteins. Reversible carboxymethylation of the prenylated cysteines and reversible acylation by one or more nearby palmitates promote dynamic membrane interactions to complement the permanent lipid modifications. Small GTPases are also regulated in both normal and disease states by several dynamic non-lipid posttranslational modifications. For example, many Ras and Rho family members are phosphorylated in an isoform-specific manner, largely by a select group of serine/threonine kinases such as protein kinase Cα or protein kinase A. Such phosphorylation events, as well as other modifications such as nitrosylation, mono- and di-ubiquitination, peptidyl-prolyl isomerization, acetylation, and oxidation, typically alter small GTPase location and/or interaction with regulatory molecules. By contrast, several distinct E3 ligases posttranslationally regulate small GTPase abundance and function at distinct cellular sites by promoting polyubiquitination and subsequent proteasomal degradation. Finally, numerous pathogenic bacterial toxins disrupt or enhance small GTPase function by a wide variety of posttranslational modifications including ADP ribosylation for which the Arf proteins are

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named. Here we summarize the rapidly evolving understanding of this fascinating area of small G protein regulation.

Keywords Ras • Rho • Prenylation • Phosphorylation • Acylation • Palmitoylation

5.1 Introduction

Small G proteins of the Ras superfamily, otherwise known as small GTPases, are comprised of five main subfamilies: Ras, Rho, Rab, Arf, and Ran, that together control a vast variety of cellular functions. These processes have been summarized recently in numerous excellent reviews, among them (Alan and Lundquist 2013; Boulter et al. 2012; Cox and Der 2010; DeGeer and Lamarche-Vane 2013; Deretic 2013; Heasman and Ridley 2008; Johnson and Chen 2012; Karnoub and Weinberg 2008; Kashatus 2013; Loirand et al. 2013; Rauen 2013; Seixas et al. 2013; Shi et al. 2013; Thumkeo et al. 2013; White 2013). Acting as molecular switches, Ras superfamily proteins bind GTP in their active conformations and bind GDP at rest. At the most basic level, their cycling between GTP- and GDP-bound states is controlled by regulatory proteins that promote guanine nucleotide exchange (e.g., guanine nucleotide exchange factors, GEFs) to enhance the active, GTP-bound state or that promote GTP hydrolysis (e.g., GTPase accelerating proteins, GAPs) to return to the inactive, GDP-bound state. Many superfamily members are also regulated by chaperones such as guanine nucleotide dissociation inhibitors (GDIs) or Rab escort proteins (REPs). However, to achieve the required signaling specificity, Ras superfamily small GTPases are also modified posttranslationally to modulate their subcellular localizations, their interactions with positive and negative regulators, chaperones and downstream effector targets, and consequently their biological activities.

With the exception of Ran, which uniquely comprises its own family, each small GTPase family consists of multiple members, grouped by structure and function (Wennerberg et al. 2005). Within each family are closely related isoforms that maintain high sequence identity over the majority of their lengths, where the G domain includes all five conserved GTP-binding motifs (residues 1–168/169 in Ras numbering), and that differ primarily in the last 20 carboxyl-terminal amino acids which comprise their hypervariable domains (HVD), more properly termed hypervariable regions (HVR) (Fig. 5.1). The G domains fold to form hydrophilic regions, whereas the HVRs, which are highly unstructured and therefore often truncated for structural studies, are also the major contributors to membrane targeting. Indeed, the last 20 amino acids of Ras and Rho proteins are sufficient to accurately target heterologous proteins such as green fluorescent protein (GFP) to the same subcellular locations as the full-length endogenous GTPases. Importantly, these regions terminate in motifs that have been known for decades to dictate permanent lipid modifications to Ras, Rho, and Rab proteins that help to confer hydrophobicity for membrane interactions. Numerous dynamic posttranslational modifications have

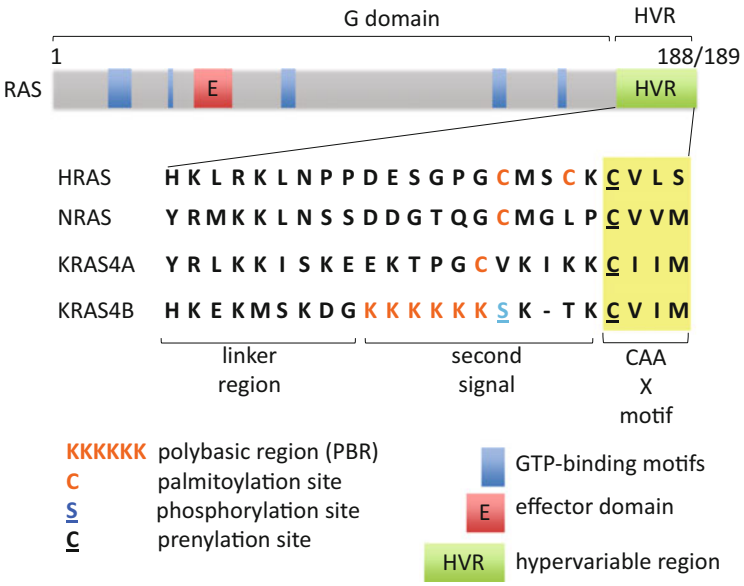

Fig. 5.1 Ras overall domain structure and hypervariable membrane targeting region. Small GTPases of the Ras, Rho, and Rab families consist of a structured globular G domain, and an unstructured hypervariable region (HVR) or "tail" (∼last 20 amino acids) that serves as the major membrane targeting region. The sequences in the C-terminal CAAX motif dictate prenylation and post-prenyl processing, whereas the elements upstream of the CAAX motif include "second signals" such as a polybasic region (PBR), cysteine acceptor sites for palmitoylation, and serine acceptor sites for phosphorylation. Different isoforms terminate in distinct combinations of second signals and CAAX motifs. The four Ras protein isoforms are shown as examples

been uncovered, as have permanent cotranslational lipid modifications to Arf proteins. Some modifications have been discovered by biochemical analyses of small GTPases obtained from cell lysates, others by the determination that some GTPases are substrates of toxins produced by bacterial pathogens, some by mass spectrometry, and still others by observing the consequences of enzymatic inhibitors. Even so, despite years of detailed study, the full story of how, where, and when these critical signaling proteins are modified, as well as a complete unraveling of the complex interactions between these modifications, has yet to be told. The trajectory of new discoveries makes clear that additional modifications and interactions are likely to be uncovered. This chapter summarizes our current understanding of the nature and consequences of posttranslational modifications of these crucial signaling proteins.

5.2 CAAX-Signaled Modifications of Membrane-Targeting Hypervariable Regions

5.2.1 Carboxyl-Terminal Isoprenylation: Farnesylation or Geranylgeranylation

All small GTPases are synthesized in the cytosol, but most act at least in part at multiple membrane sites including the plasma membrane and endomembranes of the secretory, endosomal, and lysosomal pathways. Very early on, it was recognized that the C terminus of Ras was required for membrane association and biological activity (Cox and Der 2010; Willumsen et al. 1984a, b). To promote membrane binding, nearly all Ras, Rho, and Rab superfamily small GTPases require permanent modification by the posttranslational addition of an isoprenoid lipid to each newly synthesized protein (Leung et al. 2006; Wright and Philips 2006). Isoprenoid groups are covalently attached by isoprenylating enzymes to specific terminal or near-terminal cysteines in the carboxyl-terminal HVRs, which are the key membrane-targeting regions of these small GTPases (Laude and Prior 2008). The HVRs are further composed of a "linker" domain (amino acids 166–∼179 in Ras numbering) (Laude and Prior 2008) and the remainder of the HVR (amino acids ∼179–189 in Ras numbering) that includes key sites for posttranslational modifications. The canonical Ras proteins are modified by a C15 farnesyl isoprenoid (Casey et al. 1989; Tamanoi et al. 1988) attached by farnesyltransferase (FTase) to the CAAX motifs found at their extreme carboxyl termini (in the CAAX motif, C = cysteine, A = aliphatic, and X = any amino acid; also known as a CAAX box) (Cox and Der 2010). FTase most prefers CAAX motifs where X = S or M (Kinsella et al. 1991b; Moores et al. 1991; Reid et al. 2004; Reiss et al. 1991). Most Rho proteins, along with many Ras-related members of the Ras family, also terminate in CAAX motifs and are modified by a longer, C20 geranylgeranyl isoprenoid (Adamson et al. 1992; Yoshida et al. 1991; Didsbury et al. 1990; Roberts et al. 2008) attached by geranylgeranyltransferase I (GGTase I), which prefers CAAX motifs where X = L (Finegold et al. 1991; Kinsella et al. 1991a, b; Reid et al. 2004; Yokoyama et al. 1991; Yoshida et al. 1991). Members of the Rab family terminate in CC, CXC, CCXX, CXXX, or CCXXX motifs, where they are singly or dually modified by geranylgeranyl groups (Farnsworth et al. 1994; Khosravi-Far et al. 1991, 1992; Kinsella and Maltese 1992) attached by GGTase II, also known as Rab GGTase (Seabra et al. 1992), when accompanied by a Rab escort protein (REP1/2) (Alexandrov et al. 1994; Andres et al. 1993; Wu et al. 2007). The prenylating enzymes reside in the cytosol and are thus the first class of modifying enzymes encountered by newly synthesized small GTPases on their way to their final destinations at membrane sites. These isoprenoid lipids are permanent, irreversible additions, removed only upon protein breakdown (Zhang et al. 1997). Like the (cotranslational) N-myristoylation of Arf family proteins that is required for their binding to membranes (Antonny et al. 1997; Haun et al. 1993; Randazzo

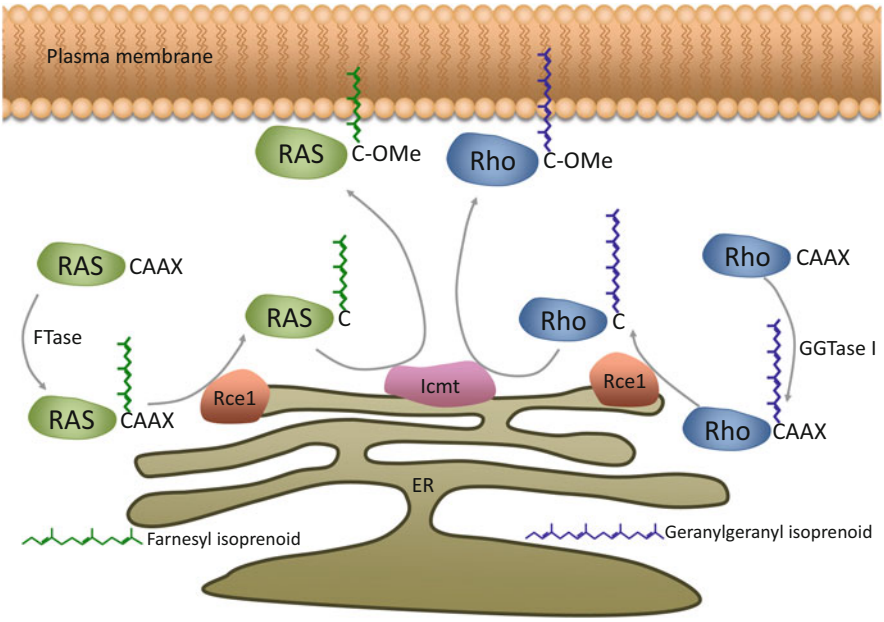

Fig. 5.2 CAAX-signaled processing steps promote transit from cytosol to membranes. Ras (prototypical farnesylated small GTPase) and Rho (prototypical geranylgeranylated small GTPase) undergo prenylation in the cytosol by FTase or GGTase I, respectively. The isoprenoid lipid modification confers sufficient membrane affinity to promote association with the endoplasmic reticulum, where they undergo Rce1-mediated proteolysis of the AAX residues and Icmt-mediated carboxymethylation of the now-terminal prenylated cysteine. Proteins such as K-Ras4B, that contain a polybasic region, traffic directly to the plasma membrane via an unknown mechanism. Proteins such as H-Ras and N-Ras, that are targeted to the plasma membrane by palmitoylation of one (N-Ras) or two (H-Ras) cysteine residues in their hypervariable domains, first transit to the Golgi for acylation by the addition of a C16 palmitoyl fatty acid

et al. 1995), the isoprenoids are necessary but not sufficient for full membrane binding of prenylated proteins, and serve as the foundation upon which other posttranslational modifications are built. Prenylation confers relatively weak but sufficient membrane affinity to promote the next processing steps, which are performed by membrane-resident enzymes in the endoplasmic reticulum (ER) (Choy et al. 1999) (Fig. 5.2).

5.2.2 Prenylation as a Modifier of Chaperone Interactions

Prenylation is an obligate step for proper membrane localization of the small GTPases so modified. Mutation of the prenylated cysteine(s) to serine and pharmacological inhibition of the prenyl transferases resulting in nonprenylated proteins indicate that these cannot attach correctly to membranes and do not signal properly.

Thus, a Cys186Ser (C186S, also known as a "SAAX") mutation in the CAAX motif of H-Ras or the cognate Cys185Ser (C185S) mutation in K-Ras4B result in cytosolic Ras proteins that do not trigger downstream events such as activation of the Ras–Raf–MEK–ERK kinase cascade and do not transform cells (Willumsen et al. 1984a, b); similarly, SAAX mutants of other CAAX-terminating proteins are typically cytosolic and nonfunctional (Roberts et al. 2008). If they also carry oncogenically activating mutations, SAAX mutants can even act as dominant negatives by interacting with effector proteins in a nonproductive manner. Hence, the prenyl transferases have been the target of numerous attempts to block small GTPase function for therapeutic benefit. In particular, because of the critical importance of oncogenically mutated Ras in cancer, extensive efforts were devoted to the development of FTase inhibitors (FTIs) for cancer treatment (Cox and Der 1997). These efforts were foiled by the discovery that, although FTIs could indeed inhibit FTase, the intended downstream targets oncogenic K-Ras and N-Ras escape functional inhibition by serving as alternative substrates for GGTase I in the presence of FTIs (Rowell et al. 1997; Whyte et al. 1997). Thus, FTIs can block only H-Ras and are not true "Ras inhibitors." Instead, FTIs have generated interest in the treatment of diseases dependent on other FTase substrates, such as Hutchinson–Gilford progeria (Gordon et al. 2012), a disease of rapid aging caused by the accumulation of defective farnesylated nuclear lamin A, and of tropical parasitic diseases (Buckner et al. 2012; Crowther et al. 2011; Gelb et al. 2006). The search for effective pharmacological inhibitors of Ras membrane binding has switched to competitors of farnesylated Ras binding to membrane chaperones such as galectins, e.g., salirasib, also known as farnesylthiosalicylic acid (FTS) (Elad et al. 1999; Paz et al. 2001), or phosphodiesterase-6delta (PDE6delta), e.g., deltarasin (Zimmermann et al. 2013); to inhibitors of K-Ras nanoclustering, e.g., fendiline (van der Hoeven et al. 2013); and to inhibitors of other steps modulated by enzymatic activities or protein:protein interactions. This is an active area of investigation (Cox et al. 2014).

5.2.3 *Post-prenyl Processing of CAAX and Related Motifs: Proteolysis and Carboxylmethylation*

Prenylation triggers further processing of the CAAX motif. Almost immediately following prenylation, the "AAX" or "XXX" amino acids C-terminal to the newly prenylated cysteines are proteolytically cleaved by the endoplasmic reticulum (ER)-integral metalloprotease "Ras-converting enzyme 1" (Rce1, which acts on more than just Ras). The now-terminal prenylated cysteines become substrates for methyl esterification by isoprenylcysteine carboxymethyltransferase (ICMT), which transfers a methyl group from the methyl donor *S*-adenosyl-L-methionine (SAM). For some small GTPases, such as K-Ras4B, these steps are sufficient to confer plasma membrane association (Fig. 5.2). Ras (Gutierrez et al. 1989), Rho

(Backlund 1997), and CAAX-terminating Rab proteins (Leung et al. 2007) all undergo this type of processing. Perhaps surprisingly for such a key modification, a single isoform handles all of the carboxylmethylation (Bergo et al. 2001). Both Rce1 and ICMT are resident in the ER (Dai et al. 1998; Schmidt et al. 1998); once processed by these enzymes, the newly prenylated and post-prenyl-processed GTPases then traffic to other membrane sites. Mutational analyses demonstrated that Ras proteins that are unable to be clipped and methylated (e.g., unfavorable A2 positions of the CAAX sequence, such as CVYS) are improperly localized and defective (Kato et al. 1992). These results indicate that post-prenyl processing is required at least for Ras. One study overexpressing GFP-tagged proteins in Rce1 or Icmt knockout mouse fibroblasts argued that Ras proteins but not Rho proteins require post-prenyl processing (Michaelson et al. 2005), but the story is clearly complicated (Roberts et al. 2008) and the criteria not yet fully defined.

Studies on the relative importance of Rce1 and ICMT for localization and function have come to surprising conclusions. Genetic ablation of *RCE1* or *ICMT* caused severe developmental defects, even when targeted only to specific tissues (Bergo et al. 2001, 2004) presumably due to their numerous key substrates. But in adult animals, loss of *ICMT*, as expected, ameliorated KRAS-driven myeloproliferative disorders (Wahlstrom et al. 2008), whereas loss of the former quite unexpectedly accelerated it (Wahlstrom et al. 2007). In cell culture studies using *ICMT* or *RCE1* knockout mouse embryo fibroblasts, some Rho proteins that tolerate homozygous loss of *RCE1* are severely impaired in their localization and ability to drive morphological or growth changes in the absence of ICMT (Roberts et al. 2008). A better understanding of which substrates contribute to localization of relevant chaperones or other interacting proteins might help to unravel this conundrum.

5.2.4 *Non-prenylated Versus Non-lipid-Modified Small GTPases*

The Rho family members Wrch-1 and Wrch-2/Chp are examples of small GTPases that are targeted to membranes by lipid modifications other than isoprenoids. Each of these proteins terminates in a CXX motif in which the cysteine is a site for palmitoylation (Berzat et al. 2005; Chenette et al. 2005) rather than a CXXX motif in which the cysteine is prenylated. Wrch-1, but not Wrch-2/Chp, harbors an additional cysteine immediately upstream of the CXX (CCXX), which provides a second site for palmitoylation and is crucial for its membrane binding and biological activities (Berzat et al. 2005). Palmitoylation is also essential for additional posttranslational modifications of Wrch-1 such as the crucial tyrosine phosphorylation of Tyr-254 near the CCXX motif (Alan et al. 2010; Brady et al. 2009). It is not understood what signals are utilized for membrane binding by the small

GTPases that lack any prenyl or other lipid group, yet each of the families includes at least one such member (Colicelli 2004).

5.3 "Second Signals" for Small GTPase Membrane Association: Palmitate and Polybasic Residues

The CAAX-signaled modifications increase lipophilicity and are required for membrane binding, but are not sufficient. It has been known for many years that at least one "second signal" is required to enhance membrane interactions initiated by prenylation of the CAAX motif, as first identified in prenylated Ras proteins (Hancock et al. 1991; Silvius et al. 2006). The "second signals" for small GTPases generally consist of either palmitoylatable cysteines or a stretch of polybasic residues (PBR) that promote electrostatic interactions with the negatively charged membrane phospholipids (Hancock et al. 1989, 1990, 1991). In addition, sequences surrounding the palmitoylated cysteines influence Ras location and function, forming a "third signal" (Willumsen et al. 1996). It is interesting that many closely related isoforms of these proteins differ most obviously by whether they contain a PBR, palmitoylatable cysteine(s), or both, in their HVRs. The evolutionary conservation of each of these second signals, and the frequent finding of both types in a given family, strongly suggest that they confer useful distinct properties that help to differentiate the functions of the family members that might otherwise be redundant.

5.3.1 Acylation by the Fatty Acid Palmitate

Addition of the fatty acid palmitate to one or more cysteines near the prenylated cysteine (Ras, Rho, Rab families) or the myristoylated glycine (Arf family) confers additional membrane affinity and helps to stabilize small GTPase interactions with membranes. Prenylation is obligatory for subsequent acylation, possibly because prenylation is required to retrieve the prenylated proteins from the cytosol onto Golgi membranes where the protein acyltransferases (PATs) reside (Swarthout et al. 2005) (Fig. 5.3). The yeast Saccharomyces cerevisiae is an exception, where a PBR is sufficient (Mitchell et al. 1994). In yeast, the Ras PAT is the multimeric complex of Erf2 and Erf4 (Lobo et al. 2002), whereas in mammalian cells, DHHC9 and GCP16 together create an S-acyltransferase PAT with selectivity for H-Ras and N-Ras (Swarthout et al. 2005). The same PAT acylates each of the two C-terminal cysteines on H-Ras (Cys-181 Cys-184); palmitoylation of specific sites can drive distinct consequences for H-Ras localization and signaling (Roy et al. 2005). Other DHHC PATs drive palmitoylation of non-Ras targets. Surprisingly, there is still no clear consensus sequence to define which cysteines are

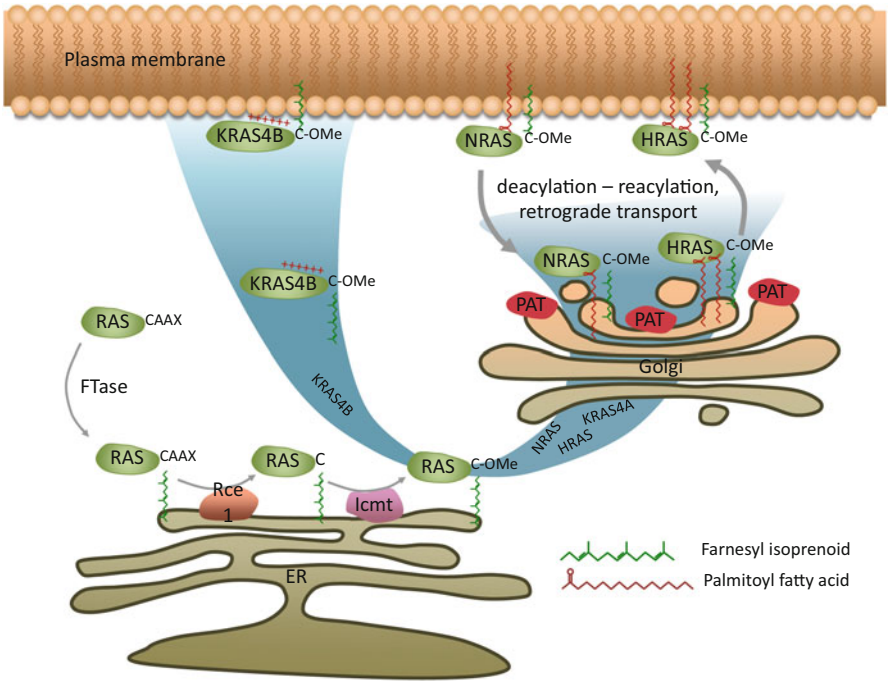

Fig. 5.3 The acylation/deacylation/reacylation cycle. Numerous small GTPases contain palmitoylatable cysteines that help to dynamically regulate their association with membranes. In this depiction, Ras proteins undergo palmitoylation by palmitoyl acyltransferases (PATs) that are resident on the Golgi. Depalmitoylation occurs within minutes, whether enzymatically by acyl protein thioesterase (APT1) or not, which promotes transient weak interactions with any endomembrane. Small GTPases that interact with the Golgi network become trapped there by palmitoylation, which allows them to enter the secretory system for transport to the plasma membrane, where the cycle begins again

substrates for palmitoylation (Linder and Jennings 2013), although improved proteomics technology has enabled great advances in detecting them. The dynamic nature of palmitoylation allows it to drive several important aspects of small GTPase function (Eisenberg et al. 2013).

5.3.2 Regulation of the Palmitoylation/Depalmitoylation Cycle

Palmitoylation/depalmitoylation creates a highly dynamic modification cycle (Fig. 5.3). First, the thioester bond is labile, and the half-life of palmitate on Ras proteins is measured in minutes (Baker et al. 2003; Buss and Sefton 1986; Magee et al. 1987). Second, because both palmitoylation and depalmitoylation are thought

to be critical for regulation of the location and activity of palmitoylated small GTPases, cycling between these states is also crucial (Goodwin et al. 2005; Rocks et al. 2005). In one current model, depalmitoylated Ras proteins redistribute randomly and transiently to all endomembranes until they are trapped by repalmitoylation at the Golgi, and are then able to traffic back to their plasma membrane locations through the secretory system (Rocks et al. 2010). Disruption of this cycle results in nonspecific accumulation of palmitoylated Ras proteins at any cellular membrane, impairing their signaling functions (Goodwin et al. 2005; Rocks et al. 2005). Depalmitoylation of H-Ras appears to be regulated in part by a peptidyl-prolyl isomerase activity acting on Pro-179 (Ahearn et al. 2011), whereas a more general depalmitoylation model includes one or more specific acylprotein thioesterase activities (e.g., APT1) (Duncan and Gilman 2002), although this is currently a confused and somewhat controversial area (Ahearn et al. 2012; Cox 2010). The acylation cycle has become an attractive target for drug discovery (Dekker et al. 2010; Xu et al. 2012), although further work will be required to fully understand the specificity of these agents.

Until very recently, little special attention has been paid to CAAX motifs that include a double cysteine (CCXX) and are modified by the CAAX prenyltransferases rather than by Rab GGTase. However, an intriguing recent study (Nishimura and Linder 2013) showed that such motifs can signal to either of two alternative fates for the same protein (1) the classical prenylation/proteolysis/methylation pathway and (2) a novel prenylation/palmitoylation pathway, where the second cysteine becomes palmitoylated rather than being cleaved off as part of the "AAX." Both geranylgeranylated small GTPases (RalA, RalB, and the brain form of Cdc42, bCdc42) and the farnesylated tyrosine phosphatase, PRL-3, were shown to undergo this alternative processing. When bCdc42 was both prenylated and palmitoylated, its interaction with RhoGDI was decreased, and accordingly it was enriched at plasma membrane sites. This dual lipidation mechanism may explain dynamic regulation of bCdc42 at dendritic spines (Kang et al. 2008).

5.3.3 Polybasic Residues and Other Sites Within the HVR

The first polybasic stretch of importance as a second membrane targeting signal in Ras proteins was that of the six contiguous lysines in K-Ras4B (Hancock et al. 1990), a run also found in the Ras-related protein Rap1A. Several other Ras family and Rho family proteins (notably Rac1) contain a similar PBR, sometimes of lysines and arginines mixed together, sometimes intermingled with other residues (Laude and Prior 2008). Since some small GTPases, such as K-Ras4B, are irreversibly prenylated and contain only permanent PBRs as their second membrane targeting signals, yet move dynamically within and among membranes (Prior and Hancock 2012; Silvius et al. 2006), other posttranslational modifications provide the ability to rapidly transition among different compartments.

5.4 C-Terminal Phosphorylation Is Isoform-Selective

5.4.1 Phosphoryation of Ras Family Proteins

PKC substrates: K-Ras4B, RalB *K-Ras4B:* First described biochemically as a response to phorbol-12-myristate-13-acetate (PMA) stimulation of protein kinase C (PKC) activity, phosphorylation of K-Ras4B was speculated to occur on Ser-181 (Ballester et al. 1987), a residue just upstream of the farnesylated CAAX motif, although neither farnesylation nor CAAX signals were yet recognized. Twenty years later, the fortuitous discovery of K-Ras4B translocation from the plasma membrane to internal membranes in response to transient PKC stimulation led to further investigation of K-Ras4B as a physiological substrate of PKC, to confirmation that Ser-181 was the physiologically relevant site of phosphorylation, and to the recognition that this modification converted K-Ras4B from a growth-promoting to a growth-suppressing protein (Bivona et al. 2006). The latter mechanism has been traced to the ability of phosphorylated K-Ras4B to interact with inositol trisphosphate receptors (InsP3) on the endoplasmic reticulum and thereby block Bcl-xL potentiation of the InsP3-regulated flux of calcium from ER to mitochondria (Sung et al. 2013). C-terminal phosphorylation of small GTPases frequently alters both binding to specific membranes and, consequently, specific effector utilization, thereby generating signaling diversity (Fig. 5.4).

An S181E phosphomimetic mutation was shown to perturb K-Ras4B nanoclustering in cholesterol-independent plasma membrane microdomains (Plowman et al. 2008), a distribution that is regulated by interaction with the galectin-3 scaffold (Shalom-Feuerstein et al. 2008). Not surprisingly, other researchers have shown that phosphorylation of Ser-181 is mutually exclusive with binding to calmodulin (Villalonga et al. 2002), which binds to the PBR through a calcium-regulated electrostatic interaction. However, they reached opposite conclusions regarding the consequences of phosphorylation at this site to K-Ras4B localization and function (Alvarez-Moya et al. 2010). The reasons for the discordant observations are unclear. The identity of the phosphatase(s) that dephosphorylate K-Ras4B at Ser-181 is also not currently known.

Although none of the other three Ras isoforms harbors a PKC consensus site, Ras-related family members are also substrates for similar modifications that modulate their subcellular locations and biological activities. Searching for other small GTPases that contain a PKC consensus site led to findings that a similar "farnesyl electrostatic switch" [by analogy to the myristoyl electrostatic switch (McLaughlin and Aderem 1995)] also operates in RhoE/Rnd3 (Madigan et al. 2009; Riento et al. 2005), as described further in Sect. 5.4.2. In addition, the Ras-related protein RalB also undergoes phosphorylation by PKCalpha.

RalB: The Ral branch of the Ras superfamily consists of RalA and RalB, which are ~80 % identical through their G domains but differ at their HVRs (Chardin and Tavitian 1986). These geranylgeranylated proteins both bind to the same effectors, including to the exocyst (Camonis and White 2005; Shipitsin and Feig 2004;

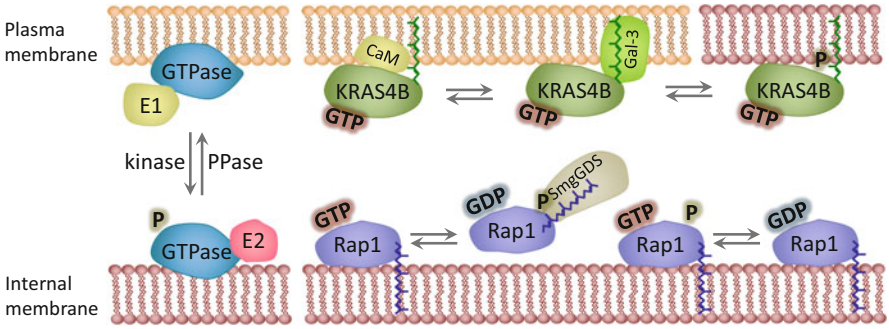

Fig. 5.4 C-terminal phosphorylation of small GTPases modulate their location, effector interaction, and activity. Phosphorylation of small GTPases within their membrane targeting regions (HVRs) modulate the specific membranes with which they interact; typically, HVR phosphorylation decreases plasma membrane interaction and promotes association with internal membranes, much as depalmitoylation does in the acylation cycle. Due to the availability of different effectors and regulators in each compartment, signaling activity and output specificity are also affected

Sugihara et al. 2002; van Dam and Robinson 2006; Rosse et al. 2006; Bodemann et al. 2011) but have different biological consequences (Lim et al. 2005; Shipitsin and Feig 2004). One way in which this can be accomplished is by differing subcellular locations and differing posttranslational modifications. Accordingly, only the RalB isoform contains a PKCalpha consensus site. Ser-198 of RalB is phosphorylated by PKCalpha, leading to its increased endosomal accumulation and decreased Sec5 interaction (Martin et al. 2012). Thus, PKCalpha modulates RalB-mediated vesicular trafficking. This modification is also important for RalB oncogenic function (Wang et al. 2010).

RalA—a substrate for Aurora A kinase: The RalA isoform does not contain a PKCalpha consensus site; instead, it contains an RSKL motif that is a consensus binding site for the serine/threonine kinase, Aurora A (Wu et al. 2005). RalA is normally localized to the plasma membrane and endosomes (Shipitsin and Feig 2004), from whence it also interacts with the exocyst, and causes cytoskeletal alterations. Phosphorylation of the C-terminal Ser-194 residue by the mitotic (and oncogenic) kinase Aurora A causes RalA translocation from the plasma membrane to internal membranes (Lim et al. 2010). Among these are mitochondrial membranes (Lim et al. 2010), where it brings its effector RalBP1 to regulate mitochondrial fission at mitosis (Kashatus et al. 2011), instead of regulating the exocyst. Ser-194 is also a consensus site for protein kinase A, and can be phosphorylated by PKA in vitro (Wang et al. 2010), raising the possibility that Aurora A and PKA compete for this site in vivo. Ser-194 can be dephosphorylated by the tumor suppressor phosphatase PP2A-Abeta (Sablina et al. 2007). The modification by distinct kinases of such closely related family members, that bind to and activate the same downstream effector molecules, helps to increase their signaling diversity.

PKA substrates: Rap1, RhoA *Rap1:* Among the first small GTPases to be identified as substrates for phosphorylation were Rap1A and Rap1B, targets of

cyclic AMP-dependent kinase, PKA. These geranylgeranylated Ras family members play prominent roles in cell adhesion, migration, and polarity, particularly in hematopoietic and neuronal cells (Boettner and Van Aelst 2009; Gloerich and Bos 2011; Bos 2005; Jeyaraj et al. 2011; McLeod et al. 2004; Pannekoek et al. 2009). Numerous biochemical investigations determined that Rap1A was phosphorylated by PKA on Ser180 (Hoshijima et al. 1988; Quilliam et al. 1991) and Rap1B on Ser179 (Hoshijima et al. 1988; Kawata et al. 1989; Lapetina et al. 1989; Siess et al. 1990), proximal to their CAAX sequences. Rap2 does not appear to be phosphorylated (Lerosey et al. 1991). It soon became clear that this phosphorylation event enhanced interactions of Rap1 with the cytosolic noncanonical GEF, SmgGDS (Beranger et al. 1991; Hata et al. 1991; Hiroyoshi et al. 1991). Numerous functional consequences were ascribed to the phosphorylation of these residues, including altered effector interactions and subcellular localization (Beranger et al. 1991; Nomura et al. 2004). In light of recent studies on SmgGDS, the early findings that phosphorylation of Ser-179 led to increased cytoplasmic association (Kawamura et al. 1991; Lapetina et al. 1989) are highly intriguing, but in an unexpected way.

These findings are in agreement with the general principle that C-terminal phosphorylation of small GTPases results in their translocation from plasma membrane sites to internal membranes, or off membranes altogether. However, there are two added twists: first, in addition to the well-characterized 558-residue SmgGDS, which escorts newly prenylated Rap1 to the plasma membrane, there is another splice variant of 607 residues (SmgGDS-607) that has recently been found to preferentially associate with nonprenylated Rap1 (and other geranylgeranylated small GTPases with polybasic domains, such as Rac1 and RhoA) and to facilitate their prenylation (Berg et al. 2010). Second, adenosine A2B receptors (A2BR) stimulates PKA to phosphorylate Rap1B before it is prenylated, thereby decreasing its interaction with SmgGDS-607 and delaying SmgGDS-607-facilitated prenylation, which in turn decreases Rap1B activity (Ntantie et al. 2013). Thus, increased adenosine signaling, such as that occurs in tumor cells, enhances cell scattering by impairing the cell:cell adhesion function of Rap1B (Ntantie et al. 2013). These findings have several important implications, among them being the possibility that downregulating A2BR could help to mitigate the pro-invasive phenotype of cancers with high levels of adenosine signaling. In addition, a similar mechanism could apply to the prenylation and trafficking of other small GTPases that also have phosphorylation sites in their polybasic domains and that interact with SmgGDS-607, such as K-Ras, RhoA, Cdc42, and Rnd3 (Berg et al. 2010). RhoA phosphorylation is described below.

5.4.2 Phosphorylation of Rho Proteins

RhoA The regulation of this canonical Rho family member by phosphorylation has been studied extensively and found to be highly complex. Mediated by PKA, PKG,

and SLK, each of which can phosphorylate RhoA on Ser-188 in the HVR (Dong et al. 1998; Ellerbroek et al. 2003; Forget et al. 2002; Guilluy et al. 2008; Lang et al. 1996; Nusser et al. 2006; Rolli-Derkinderen et al. 2005; Sauzeau et al. 2000) only two residues from the geranylgeranylated Cysteine-190, the consequences of this phosphorylation are generally to increase binding to RhoGDI (Ellerbroek et al. 2003; Forget et al. 2002; Tamma et al. 2003). Consistent with this, Ser-188 phosphorylation also extracts RhoA from the plasma membrane into the cytosol, sequestering it from effectors and downregulating its activity in various contexts, from stress fiber formation and cellular morphology (Dong et al. 1998; Ellerbroek et al. 2003; Lang et al. 1996) to vascular smooth muscle contraction (Guilluy et al. 2008; Rolli-Derkinderen et al. 2005; Sauzeau et al. 2000). The role of phosphorylation in RhoA/ROCK signaling in vascular smooth muscle pathology has come under increasing scrutiny (Loirand et al. 2006, 2013), with much attention paid to the possibility of their being useful therapeutic targets in cardiovascular disease. More recently, the Ser-188 residue has also been found to be a site for phosphorylation by AMP-activated kinase (AMPK) activity stimulated by estrogen (Gayard et al. 2011). As with the PKA, PKG, and SLK kinases, AMPK-mediated phosphorylation of Ser-188 also decreases RhoA activity in this context, a desirable effect in that it reduces vascular pathology, potentially contributing to the ability of estrogen to act as a vasoprotector (Gayard et al. 2011). How competition among these kinases for the Ser-188 site is managed is an interesting question of context-dependent spatiotemporal regulation.

Rnd3/RhoE Rnd3 is a substrate for both PKCa and ROCK. Rnd3 is an atypical Rho protein in several ways: it is an immediate-early protein, it is constitutively active by virtue of alternative residues at positions 12, 59, and 61, and it is a farnesylated Rho protein (Foster et al. 1996). C-terminal phosphorylation therefore represents a mechanism to dynamically control an otherwise always-active protein in a spatiotemporally regulatable manner. Indeed, Rnd3 has been shown to be regulated dynamically by PKCa- and ROCK I-mediated phosphorylation (Komander et al. 2008; Madigan et al. 2009; Riento et al. 2005). Although identifying the role of the Ser-240 site near the prenylated cysteine was extremely challenging (Riou et al. 2013), recently it has been shown that Rnd3 phosphorylation, together with farnesylation, creates a high-affinity binding site for the 14-3-3 scaffold; Rnd1 and Rnd2 also do this (Riou et al. 2013). Consistent with the consequences of prenyl/C-terminal phosphate modifications of other small GTPases (Alan et al. 2010; Bivona et al. 2006; Lim et al. 2010; Madigan et al. 2009; Martin et al. 2012), 14-3-3 binding to Rnd3 extracts it from the plasma membrane and impairs its functions, in this case the ability to cause cell rounding. This is of particular interest because of the constitutively active status of Rnd3, which is not subject to GTP/GDP cycling or to GEF/GAP regulation (Foster et al. 1996). 14-3-3 thus acts somewhat like a GDI for Rnd3. Although there is a groove in 14-3-3 that accepts the farnesyl group of Rnd3, it can also accept geranylgeranylated Rap1A (Riou et al. 2013), suggesting that other Ras family proteins may also be modulated by GDI-like chaperones, similarly to the galectins that shepherd K-Ras4B and H-Ras, and nucleolin that shepherds N-Ras.

Surprisingly, 14-3-3 does not appear to interact with farnesylated K-Ras, which shares with Rap1A the property of a polybasic domain in its HVR. Thus the criteria for interactions between 14-3-3 and prenylated small GTPases are only partially defined to date. Perhaps additional small GTPase scaffolding functions of 14-3-3 proteins are yet to be revealed.

Rac1 The Rac1 small GTPase is phosphorylated at Ser-71, a putative Akt site (Kwon et al. 2000). The consequences of this modification seem to be highly context-dependent. In one recent report, this modification resulted in polyubiquitination by the E3 ligase FBXL19 and subsequent proteasomal degradation (Zhao et al. 2013), which impaired cell migration. In another, it shifted the specificity of effector signaling, abrogating binding to Sra-1/WAVE and Pak while still allowing interaction with IQGAPs and MRCKalpha (Schwarz et al. 2012), resulting in a switch from lamellipodia to filopodia generation and in decreased cell spreading. Further, phosphorylation of this site partially protects Rac1 from glucosylation by *Clostridium difficile* toxin A (TcdA) (Schoentaube et al. 2009) and toxin B (TcdB) (Brandes et al. 2012), although whether it has the same consequences on Pak effector interaction is unclear (Schoentaube et al. 2009). In addition, Rac1 and Cdc42 are both phosphorylated by Src, on Tyr-64 in the switch II region. This causes increased activation of Rac1, with concomitant increased cell spreading (Chang et al. 2011). Cdc42 Y64 had been reported previously to be modified by Src phosphorylation downstream of EGF signaling (Tu et al. 2003), which affected its interaction with RhoGDI but not downstream effectors.

Wrch-1/RhoU Wrch-1, but not Wrch-2/Chp/RhoV, is also tyrosine phosphorylated by Src, but on a tyrosine residue (254) in the HVR (Alan et al. 2010). This causes translocation of active Wrch-1 from the plasma membrane to internal membranes, where its activity is downregulated and its interaction with its effectors is impaired (Alan et al. 2010). Accordingly, its functions in cell:cell junctional control, cystogenesis, and transformation are also impaired. As no other small GTPases possess a tyrosine in this context, Wrch-1 may be regulated uniquely.

Other Rho proteins CDK-5 phosphorylation of TC10/RhoQ on Thr197 just upstream of the polybasic domain is required for TC10 activation and association with lipid rafts upon insulin stimulation, but the specific mechanisms mediating these effects are unknown (Okada et al. 2008)

5.4.3 Phosphorylation of Rab Proteins

Rab4 is an early endosome-associated small GTPase that regulates the endocytosis of a widely varied group of targets including GLUT4, integrins, and angiotensin (Jones et al. 2006). It was the first Rab to be identified as modified by C-terminal phosphorylation, and is the isoform for which there is the most evidence for a functional importance of this type of modification. Rab4 is phosphorylated on Ser-196 by the mitotic kinase Cdc2 (van der Sluijs et al. 1992), which dissociates it from endosomes and causes cytoplasmic accumulation (Ayad et al. 1997) and

binding to the prolyl isomerase Pin-1 (Gerez et al. 2000). Collectively, these changes may promote the downregulation of endocytic transport that is required for efficient mitosis.

Other Rabs phosphorylated in vivo *Rab 11* is a substrate of both classical and novel PKC isoforms: PKCβII and PKCε phosphorylate it on Ser177 and may contribute to the ability of Rab11 to inhibit transferrin recycling (Pavarotti et al. 2012). Phosphorylation of yeast *Sec4p* on either N-terminal sites Ser8/Ser11 or C-terminal sites Ser201/Ser204 negatively regulates its ability to regulate polarized growth, perhaps because this modification interferes with Sec4p binding to the exocyst (Heger et al. 2011). Mammalian orthologs to Sec4p are Rab8 and Rab13, which may also be regulated similarly. *Rab24* is weakly geranylgeranylated due in part to its suboptimal CCXX motif (CCHH), and not much bound to Rab GDI (Erdman et al. 2000). It appears to function in autophagy (Klionsky et al. 2011) and in chromosome segregation and cytokinesis (Militello et al. 2013). Rab24 phosphorylation on Tyr-172 (Ding et al. 2003) was shown to be enhanced in the cytosol compared to membranes and abrogated by the Src inhibitor PP2. Whether Rab24 phosphorylation status at Tyr-172 regulates its functions in these processes would be interesting to determine.

Rabs phosphorylated in vitro Rab5 has three isoforms that may be differentially regulated by distinct kinase phosphorylation of the same Ser-123 site: in vitro, ERK1 but not ERK2 phosphorylates Rab5a, whereas Rab5b is preferentially phosphorylated by Cdc2 (Chiariello et al. 1999). Whether this preference plays out in vivo has not been determined. Finally, in platelets, thrombin activation stimulates phosphorylation of Rab3B, Rab6, and Rab8 (Karniguian et al. 1993), but the sites and consequences are unknown.

5.4.4 Arf/Arl Proteins and Their Regulators

Members of the Arf family do not appear to be regulated by direct phosphorylation, although their regulators are frequently so modified (Cherfils and Zeghouf 2013). Instead, Arfs and Arls are regulated conditionally by acetylation, palmitoylation, SUMOylation, and other modifications. For example, Arl8, which is not N-terminally myristoylated, has been reported to be N-terminally acetylated (Hofmann and Munro 2006), a modification that is required for its localization to lysosomes. On the other hand, the Joubert Syndrome-associated Arl13B, which is important for Hedgehog signaling (Caspary et al. 2007) and ciliary function (Caspary et al. 2007; Li and Hu 2011), is both palmitoylated (Cevik et al. 2010) and C-terminally SUMOylated by Ubc9 (Li et al. 2012). Failure of Arl13B to become SUMOylated results in impaired sensory functions of cilia (Li et al. 2012).

5.4.5 *Phosphorylation of Ran*

The Ran GTPase is crucial for nucleocytoplasmic shuttling, mitotic spindle formation, and nuclear envelope remodeling (Yokoyama and Gruss 2013), and has an increasingly appreciated role in ciliary transport (Li and Hu 2011; Lim et al. 2011). Although it has no known lipid modifications, in Xenopus Ran has been shown to be a direct target for Pak4-mediated phosphorylation at Ser-135 (Bompard et al. 2010). This phosphorylation, which occurs in a cell cycle-dependent manner, stabilizes Ran activity by preventing its interaction with the Ran GEF RCC1 and with RanGAP1, and colocalizes active Ran-GTP to specific sites where Pak4 is localized, thereby promoting the specificity of Ran interactions with components of the mitotic spindle apparatus.

5.5 Other Modifications

SUMOylation Rac1 is SUMOylated in its polybasic region, through the SUMO E3 PIAS3, in response to hepatocyte growth factor (HGF) signaling. This SUMOylation increases Rac1-GTP, which promotes cell scattering in response to HGF, and regulates cell migration, all of which enhance the invasive phenotype in tumor cells (Castillo-Lluva et al. 2010).

HACE-mediated polyubiquitination and degradation Recently, Rac1 has been shown to be polyubiquitinated by the HECT domain E3 ligase and tumor suppressor, HACE1 (Daugaard et al. 2013; Mettouchi and Lemichez 2012), which targets it for degradation by the proteasome. One consequence of this is limiting cell motility (Castillo-Lluva et al. 2013), in a manner that can reverse the enhanced motility induced by SUMOylated Rac1 (Castillo-Lluva et al. 2010). And, because HACE1 targets active Rac1 bound to the NADPH-oxidase complex, this creates a novel mechanism for downregulating Rac1-stimulated oxidants to improve cellular redox control.

SMURF1/2-mediated polyubiquitination and degradation Not all downregulation of RhoA activity mediated by phosphorylation of Ser-188 is attributable to enhanced interactions with RhoGDI. For example, in the absence of RhoGDI, when bacterial (i.e., nonprenylated) RhoA was phosphorylated in vitro, its binding to its effector ROCK was less efficient than if it was not phosphorylated (Dong et al. 1998). Another interesting consequence of phosphorylation of RhoA at Ser-188 is its potential to modulate Smurf1-mediated proteasomal degradation (Wang et al. 2003). RhoA signaling to its effectors can be downregulated by increased binding to RhoGDI, by decreased binding to GTP, or by decreased abundance at a particular site or overall. Spatially regulated modulation of the ability of the E3 ligase Smurf1 to ubiquitinate RhoA can therefore dramatically alter RhoA function. Localized increases or decreases in Smurf1 activity at the leading edge of cells or at cell:cell junctions significantly alters cell shape, cell

polarity, and tumor cell invasion (Sahai et al. 2007; Wang et al. 2003). They can also lead to pathological developmental defects in vascular integrity, such as occurs in cerebral cavernous malformations due to mutations in CCM2 that enhance Smurf1 degradation of RhoA (Crose et al. 2009). Conversely, competition with Smurf1 by the actin-associated protein synaptopodin in kidney podocytes decreases RhoA degradation, resulting in increased podocyte stress fiber formation and cell motility (Asanuma et al. 2006).

In neuronal development, both RhoA and Rap1B are targets of Smurfs (Schwamborn et al. 2007). In the case of Rap1B, the localization of Smurf2 and of inactive Rap1B assures that Rap1B becomes ubiquitinated and degraded everywhere except for a single axon, thereby promoting neuronal polarity.

F-box-mediated polyubiquitination and degradation The F-box E3 ligase FBXL19 downregulates Rac1 upon phosphorylation at Ser-71 (Zhao et al. 2013). Recently, another mechanism of RhoA degradation has been revealed: RhoA is also subject to ubiquitination by the same F-box E3 ligase (Wei et al. 2013), which in turn is modulated by yet another phosphorylation event, this one mediated by the ERK2 MAP kinase.

Mono- and di-ubiquitination Both H-Ras and K-Ras isoforms have recently been shown to undergo mono- and di-ubiquitination at low stoichiometry. H-Ras (Jura et al. 2006) and K-Ras (Sasaki et al. 2011) are ubiquitinated in a Rabex-5-dependent manner (Xu et al. 2010). Although the HVR is required for H-Ras to become ubiquitinated (Jura et al. 2006), mass spectrometry analyses have demonstrated that the acceptor sites (Lys-117, -147, -170) lie outside that region (Sasaki et al. 2011). Ubiquitinated H-Ras was enhanced at endosomal sites, where its signaling to the MAPK cascade was impaired (Jura et al. 2006). Thus, this form of modification can have a result similar to that of phosphorylation, in terms of driving small GTPases off the plasma membrane to internal membranes or to the cytosol and downregulating their signaling activities. Rabex-5 is both an E3 ligase for Ras and a GEF for Rab5, which sorts cargo for transport between the plasma membrane and endosomes (Aikawa and Lee 2013). It is recruited to endosomes by the Ras effector RIN1 (Xu et al. 2010) to form a feedback loop between active Ras-GTP that can bind its effector RIN1, and the turned-off Ras that has been ubiquitinated by Rabex-5 (Xu et al. 2010). The context of site-specific ubiquitination may be critical to its biological consequences. Ubiquitination of H-Ras at Lys-117 was reported to activate the GTPase by enhancing nucleotide exchange (Baker et al. 2013b) whereas ubiquitination of K-Ras at Lys-147 (a site shared with H-Ras) was reported to activate it (Sasaki et al. 2011) by impairing GAP-mediated downregulation (Baker et al. 2013a).

Redox-regulated modifiers: S-nitrosylation, S-glutathiolation, oxidation The Cys-118 residue that is highly conserved among Ras proteins is subject to S-nitrosylation upon exposure to nitric oxide, which promotes guanine nucleotide exchange and therefore an increase in active, GTP-bound Ras (Lander et al. 1995, 1996, 1997). Accordingly, an endothelial nitric oxide synthase (eNOS)-selective inhibitor, L-NAME, impaired the growth of KRAS-driven pancreatic cancer xenografts (Lampson et al. 2012), and ablation of eNOS in a KRAS-driven mouse model

of pancreatic cancer prolonged survival of tumor-bearing animals (Lim et al. 2008). Cys-118 is part of the NKXD motif, where the X residue can be cysteine. Other reactive cysteines (e.g., key residues subject to palmitoylation) may also be subject to S-nitrosylation and/or S-glutathiolation (Mallis et al. 2001) although the latter is unlikely to cause activation (Mitchell et al. 2013). Oxidation of the C-terminal palmitoylated cysteines in Ras, for example under conditions of metabolic stress, can lead to loss of membrane localization and Ras-dependent downstream signaling (Burgoyne et al. 2012); whether the ensuing cell death is specifically Ras-dependent has not been determined.

RhoA and Rac1 also have redox-sensitive cysteines, but at the opposite end of the protein from those in Ras. RhoA can also be activated by direct oxidation of Cys-20, in a manner that is incompletely understood (Aghajanian et al. 2009). Similarly, glutathiolation of Rac1 at Cys-18 can lead to increased nucleotide exchange (Hobbs et al. 2014); however, clearly some redox conditions are deleterious (Mitchell et al. 2013). It will be important to unravel the consequences of the range of physiological and pathological redox states to Rho GTPase modification and functions.

Acetylation In keeping with the theme of isoform differences in posttranslational modifications, K-Ras but not H-Ras or N-Ras has recently been shown to be acetylated at Lys-104 in a manner that decreases GEF-mediated nucleotide exchange and activation, and that modestly decreases K-Ras transforming ability (Yang et al. 2012). It is not known whether endogenous K-Ras4A and K-Ras4B are equally acetylated, or equally dependent on this modification. Conversely, the tubulin deacetylases HDAC6 and SIRT2 enhanced K-Ras transformation and their knockdown impaired it (Yang et al. 2013), suggesting that HDAC inhibitors could be therapeutically useful against K-Ras-driven cancers if their effects prove to be related specifically to K-Ras function. There are many ways in which such inhibitors could be relevant but indirect, including the fact that active FTase and HDAC6 are in a complex together on microtubules (Zhou et al. 2009). And, as indicated above in Sect. 5.4.4, Arl proteins are also acetylated, perhaps in lieu of a myristoyl-electrostatic switch.

Bacterial toxin-induced modifications: ADP-ribosylation, glucosylation, de-amidation, adenylylation, etc. Numerous classes of pathogenic bacteria act by elaborating toxins that mimic or block the activity of small GTPases, particularly of the Rho and Arf families. The founding modification is that of *Clostridium botulinum* C3 toxin-mediated ADP-ribosylation of the classical RhoA/B/C proteins at Asn-41, in the switch I effector interaction region, that also enhances binding to RhoGDI and thereby blocks nucleotide exchange and downstream function. Inactivation of Rho disrupts the actin cytoskeleton, with drastic effects on cell physiology. This toxin also acts on Ras to perturb nucleotide exchange. Conversely, the affinity of Rab proteins for RabGDI can be decreased by toxins that adenylylate them (Cheng et al. 2012; Oesterlin et al. 2012). Several other toxins directly modify Thr-35 of Ras and Rho family proteins, which is in the middle of the effector domain and required to coordinate the essential Mg++ ion. Still others assume conformations that do not modify small GTPases directly, but rather mimic their

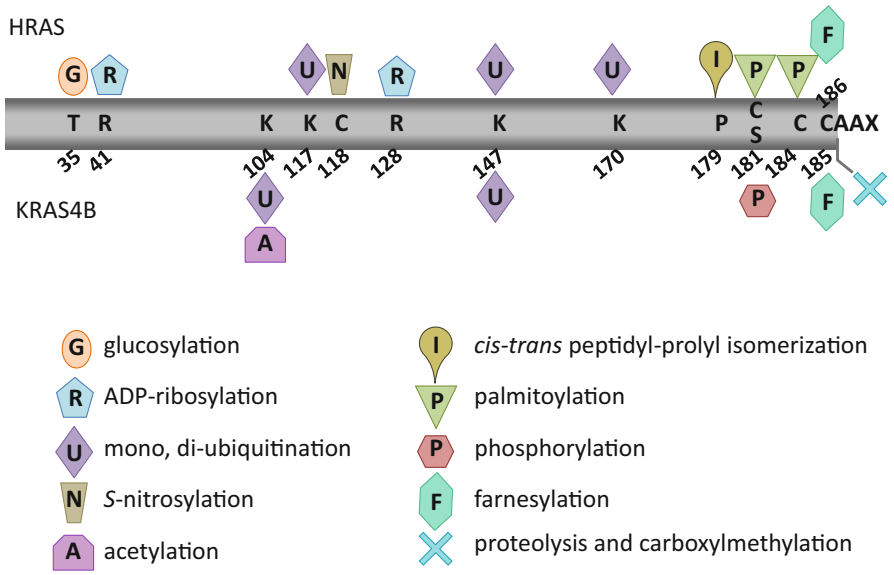

Fig. 5.5 Posttranslational modifications of small GTPases may be common or isoform-dependent. Using H-Ras and K-Ras4B as models for comparison, the diversity and distribution of permanent and dynamic posttranslational modifications across the protein structure are shown. Some modifications are common to both H-Ras and K-Ras, and occur on cognate residues in each isoform (e.g., farnesylation at Cys-186/185 of the CAAX motif; S-nitrosylation and oxidation of Cys-118); others are common to both isoforms but occur on different or only partially overlapping residues (e.g., mono/di-ubiquitination); still others are specific to one isoform [e.g., palmitoylation of cysteine residues (H-Ras only); phosphorylation of serine residues (K-Ras4B only); lysine acetylation (K-Ras4B only)]

regulators. Two excellent recent reviews (Aktories 2011; Lemichez and Aktories 2013) and Chap. 4 extensively summarize these features.

5.6 Conclusions

Functionally relevant posttranslational modifications of small GTPases can be grouped generally into those that regulate their location, their activation, and/or their abundance. Many of these are depicted in Fig. 5.5, using Ras as a paradigm.

Location-regulating modifications These include *prenylation* by farnesyl or geranylgeranyl lipids, a modification that is required for membrane binding and that may (Ras, Rho, some Rabs) or may not (other Rabs) be followed by proteolysis and carboxymethylation; *palmitoylation* and depalmitoylation of Ras, Rho, and Arf proteins to coordinate traffic between the plasma membrane, endomembrane structures, and especially the Golgi network; *peptidyl-prolyl cis-trans isomerization*

(H-Ras only) that contributes to the depalmitoylation cycle; *phosphorylation* (Ras, Rho, Rab, Ran) that promotes endomembrane localization.

Activation-regulating modifications These include S-*nitrosylation* and *oxidation* (Ras, Rho), *acetylation* (K-Ras, Arl13B), *ADP-ribosylation*, *glucosylation*, and several others induced by toxic bacteria.

Abundance-regulating modifications Two examples are *polyubiquitination* and *SUMOylation*, both of which lead to proteasomal degradation.

Finally, many of the regulators and effectors of small GTPases are also regulated similarly; this is particularly true for phosphorylation, and possibly for acetylation, of GEFs, GAPs, and chaperones. Together these posttranslational modifications, whether constitutive or regulated, contribute to the vast diversity, the tight specificity, and the precise spatiotemporal control demanded by small GTPase signaling and function.

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Part II
Ras Subfamily

Chapter 6
Targeting the Raf-MEK-ERK Mitogen-Activated Protein Kinase Cascade for the Treatment of *RAS* Mutant Cancers

Tikvah K. Hayes and Channing J. Der

Abstract The three *RAS* genes comprise the most frequently mutated oncogene family in human cancer; furthermore, substantial experimental evidence supports their key driver roles in cancer development and growth. Consequently, there has been considerable interest and effort in developing therapeutic approaches for blocking aberrant Ras function for cancer treatment. Despite over three decades of intensive effort, to date no effective anti-Ras therapeutic approaches have reached the clinic. Currently, the most promising direction involves inhibitors of Ras effector signaling, with the Raf-MEK-ERK mitogen-activated protein kinase cascade the most intensively pursued. Presently, there are at least 33 inhibitors of this pathway under clinical evaluation. In this chapter, we provide a summary of this key Ras effector signaling network and the efforts to target the Raf-MEK-ERK cascade for the treatment of *RAS* mutant cancers.

Keywords Ras • Raf • MEK • ERK • MAPK

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6.1 Introduction, Ras Proteins

Ras is a ~21 kDa small GTPase that functions as a molecular switch, regulating a number of important signal transduction cascades, resulting in changes to several crucial cellular processes including, but not limited to proliferation, apoptosis, autophagy/metabolism, vesicular trafficking, morphological changes, and gene expression (Cox and Der 2010). Ras proteins act as binary on–off switches cycling between active Ras-GTP and inactive Ras-GDP states (Fig. 6.1). Ras-GTP formation is regulated by Ras-selective guanine exchange factors (RasGEFs), while GTPase activating proteins (RasGAPs) accelerate the low intrinsic GTP hydrolysis of Ras.

The three human *RAS* genes encode four distinct isoforms: H-Ras, N-Ras, K-Ras4A, and K-Ras4B, where K-Ras4A and 4B are splice variants of exons 4A and 4B. Ras proteins share strong sequence identity in their N-terminal G domain (residues 1–164), but they diverge significantly in their C-terminal sequences, which is critical for membrane localization and subsequent activation.

6.2 RAS and Cancer

Data in COSMIC show that the *RAS* genes are mutated in 33 % of all cancers evaluated, making it the most frequently mutated oncogene family in cancer (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). There are three common sites for Ras mutational activation, residues G12, G13, and Q61, which together account for >95 % of identified mutations (Cox and Der 2010). Mutational activation at G12 and G13 interferes with the ability of Ras to be stimulated by GAPs, as any other residue aside from glycine creates steric hindrance (Scheffzek et al. 1997). However, mutational activation at Q61 disrupts the coordination of the water molecule necessary for GTP hydrolysis (Scheidig et al. 1999). Once mutated at G12, G13, or Q61 Ras becomes constitutively active, leading to aberrant activation of downstream effectors. Of the Ras isoforms, *KRAS* is the most frequently mutated accounting for ~85 % of all *RAS* mutations found in cancer (Cox and Der 2010).

There are substantial cell culture and mouse model analyses supporting the role of mutant Ras as a driver of cancer initiation and growth. Many transgenic mouse models have demonstrated that mutant Ras can initiate tumor growth (Colvin and Scarlett 2014). In particular, Jacks and colleagues made the key observation that somatic activation of endogenous mutant *KRAS*-induced lung tumor development (Johnson et al. 2001). Furthermore, Sasazuki and colleagues demonstrated that the continued expression of mutant Ras is required for maintenance of tumor growth by homologous recombination *KRAS* knockout studies in colon tumor cell line where loss of the mutant *KRAS* allele abolished tumorigenic growth in vivo (Shirasawa et al. 1993). Later, it was demonstrated that shRNA silencing of *KRAS* impaired the

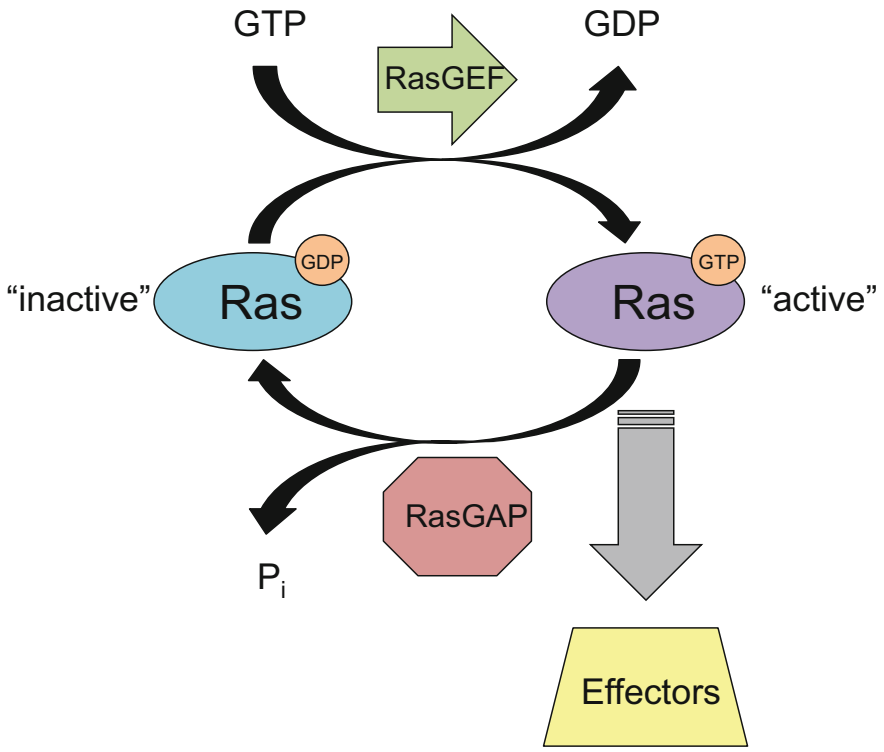

Fig. 6.1 Ras GDP-GTP cycle. Ras proteins act as molecular switches cycling between a GTP- (active state) and GDP- (inactive state) bound state, where Ras-GTP binds preferentially to downstream effectors. RasGEFs (e.g., Sos1) catalyze the release of nucleotide allowing Ras to bind GTP, while RasGAPs (p120 RasGAP, neurofibromin) increase the intrinsic hydrolysis rate of Ras proteins

tumorigenic growth of a *KRAS* mutant pancreatic cancer cell line (Brummelkamp et al. 2002). This observation was extended to mouse studies where DiPinho and colleagues used inducible H-Ras expression and showed reduction in melanoma tumor growth upon H-Ras inactivation (Chin et al. 1999). More recently, use of inducible mutant *Kras* G12D alleles was done to show that continued mutant *Kras* expression was required for the maintenance of pancreatic cancer in genetically engineered mouse models (Collins et al. 2012; Ying et al. 2012). Similarly, inducible RNA interference ablation of mutant *KRAS* expression in *KRAS* mutant human tumor cell line-induced xenograft tumors also demonstrated *KRAS* dependency in vivo (Hofmann et al. 2012; Lim and Counter 2005).

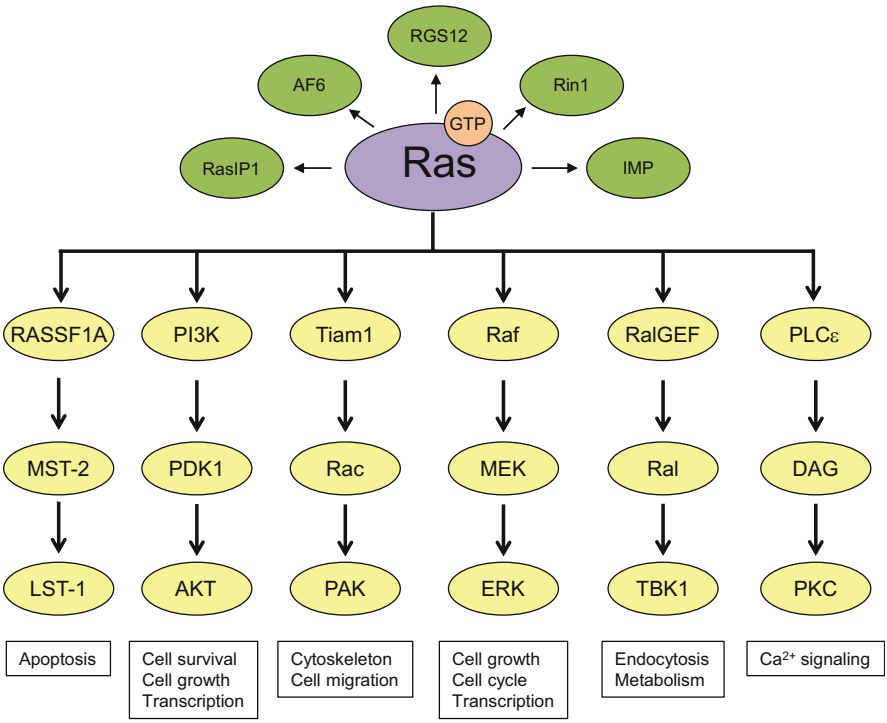

Fig. 6.2 Ras effector signaling. Ras-GTP binds preferentially to 11 catalytically-distinct classes of effectors. Cell culture and/or mouse model studies have implicated six classes in Ras-mediated tumor initiation, progression, and/or maintenance. This includes the p110 catalytic subunits of class I PI3Ks, GEFs for the Ral small GTPases (RalGEFs; RalGDS, Rgl, Rgl2, and Rgl3), the Tiam1 Rac small GTPase GEF (RacGEF), and PLC_{ε} whose functions are necessary for tumor growth. In contrast, RASSF1A family members are negative regulators and their expression is lost in cancer

6.3 Ras Effectors

There are at least 11 catalytically distinct classes of Ras effectors (Vigil et al. 2010). Ras effectors typically possess either a Ras binding (RBD) or Ras association (RA) domain that facilitates preferential binding to activated GTP-bound Ras. Of these effector classes, six have validated roles in Ras-mediated oncogenesis: Raf serine/threonine kinases, class I phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3K), GEFs for the Ral (RalGEFs) and Rac1 (Tiam1) small GTPases, phospholipase C epsilon, and RASSF1A (Downward 2003) (Fig. 6.2). Of these, Raf and PI3K are the best validated, in part, because of their frequent mutational activation in human cancer (Davies et al. 2002; Karakas et al. 2006). In addition to their mutational prevalence in cancer, both Raf and PI3K possess catalytic activities that represent tractable drug targets. Furthermore, downstream components of both Raf and PI3K signaling cascades have been targeted for drug discovery with some

success. Taken together these findings have placed Raf and PI3K at the forefront of drug discovery as the two key downstream effectors of Ras.

6.3.1 *Mitogen-Activated Protein Kinase Signaling*

The Raf-MEK-ERK three-tiered protein kinase cascade is one of three major Mitogen-Activated Protein Kinase (MAPK) kinase kinase (MAPKKK/MAP3K)-MAPK kinase (MAPKK/MAP2K)-MAPK modules involved in cytoplasmic signaling downstream of cell surface receptor signaling. The other two MAPKs, p38 and JNK, have less significant roles in oncogenesis and are not known to be directly activated downstream of Ras. Therefore, in this chapter, we focus on the Raf-MEK-ERK cascade in Ras signaling.

6.4 Raf-MEK-ERK Kinases

6.4.1 *Raf Serine/Threonine Kinases*

There are three distinct Raf (MAPKKK/MAP3K) isoforms: A-Raf, B-Raf, and Raf-1 (c-Raf). Like Ras isoforms, Raf isoforms share similar sequence identity and conserved domain topology (Fig. 6.3). The N-terminal region contains an RBD followed by a cysteine-rich domain (CRD/C1), while the C-terminal region contains the serine/threonine kinase domain. Generally, N-Ras, H-Ras, and K-Ras activate Raf-1 and B-Raf similarly, whereas A-Raf is only weakly activated by Ras (Rodriguez-Viciana et al. 2004). Ras-GTP binding to the RBD relieves the N-terminal auto-inhibitory activity and additionally recruits Raf to the plasma membrane, where additional phosphorylation events and interactions facilitate activation of Raf catalytic function (Fig. 6.4). How Ras causes activation of Raf is complex and still not completely understood, most studies have focused on Raf-1, the isoform found originally transduced and activated in the 3611-MSV oncogenic retrovirus that caused *r*apidly *a*ccelerated *f*ibrosarcomas (Matallanas et al. 2011).

***Raf-1* Activation** Inactive Raf-1 exists in a closed confirmation stabilized by the 14-3-3 complex interacting with residues S259 in the N-terminal region and S621 in the C-terminal region (Roskoski 2010). RTKs recruit the Sos1 RasGEF to the plasma membrane where it catalyzes the formation of Ras-GTP (Fig. 6.5). PP1 and PP2A phosphatases dephosphorylate Raf-1 at residue S259 permitting Ras-GTP to bind the Raf-1 RBD effectively resulting in an open yet inactive confirmation. Several known (PAK, CK2, Raf, Src, and Jak) and unknown kinases phosphorylate the C-terminal region of Raf-1 at residues S338 and Y341. Once activated, Raf proteins either homo- or heterodimerize, which is stabilized by either the 14-3-3 complex, KSR-1, or MLK-3, all well validated scaffolds.

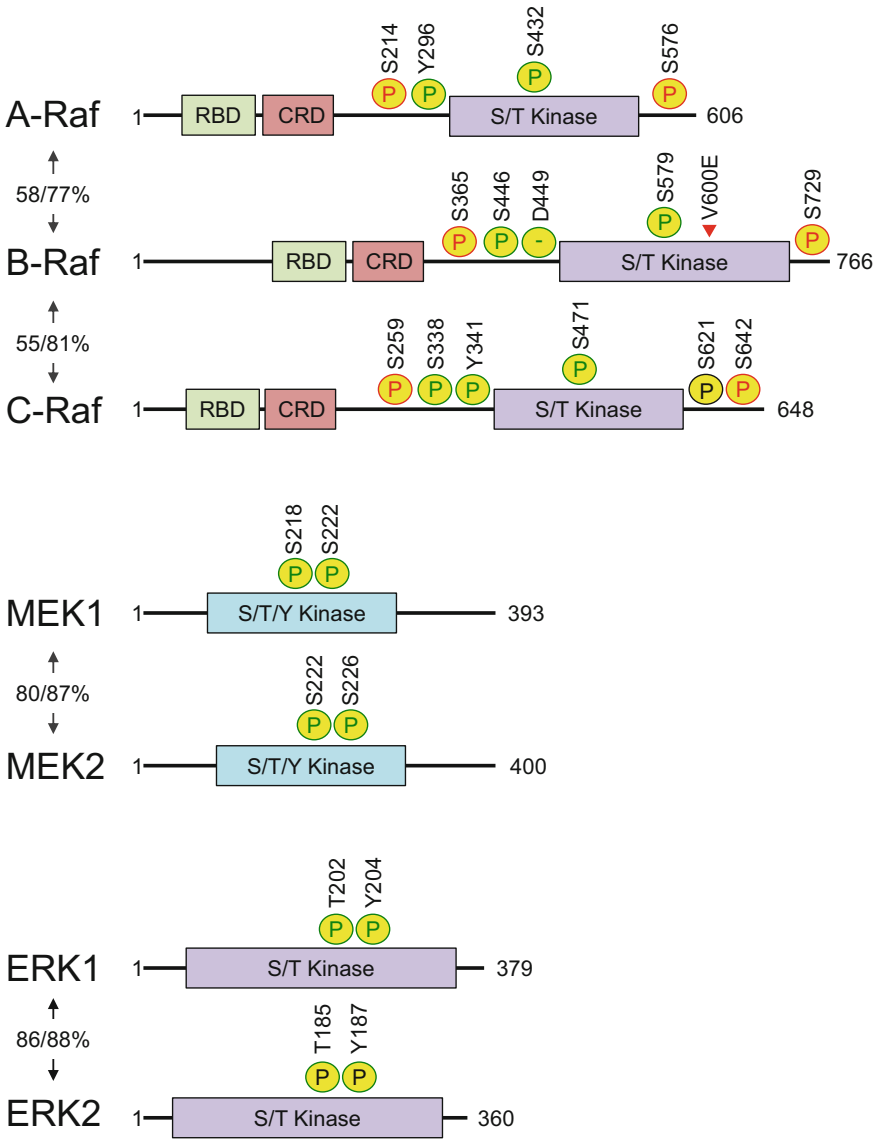

Fig. 6.3 Components of the Raf-MEK-ERK protein kinase cascade. There are structurally and functionally related isoforms at each level of the ERK MAPK pathway. Shown here are the human proteins, with domain structure determined in SMART. The degree of overall and kinase domain amino acid identity is indicated and is determined by Clustal/W analyses. The phosphorylation sites that regulate Raf kinase activity are complex and include both positive (*green*) and negative (*red*) phosphorylation events. We have not included all known phosphorylation sites and have included only the key sites. The negative regulatory sites are conserved in all Raf isoforms and serve as recognition sites for 14-3-3 binding and inhibition of Raf. Raf phosphorylation sites in MEK1/2 and MEK1/2 phosphorylation sites in ERK1/2 are indicated. The V600E amino acid substitution comprises ~80 % of cancer-associated activating mutations in B-Raf. *RBD* Ras-binding domain; *CRD* cysteine-rich domain; *S/T* serine/threonine, *S/T/Y* serine/threonine/tyrosine

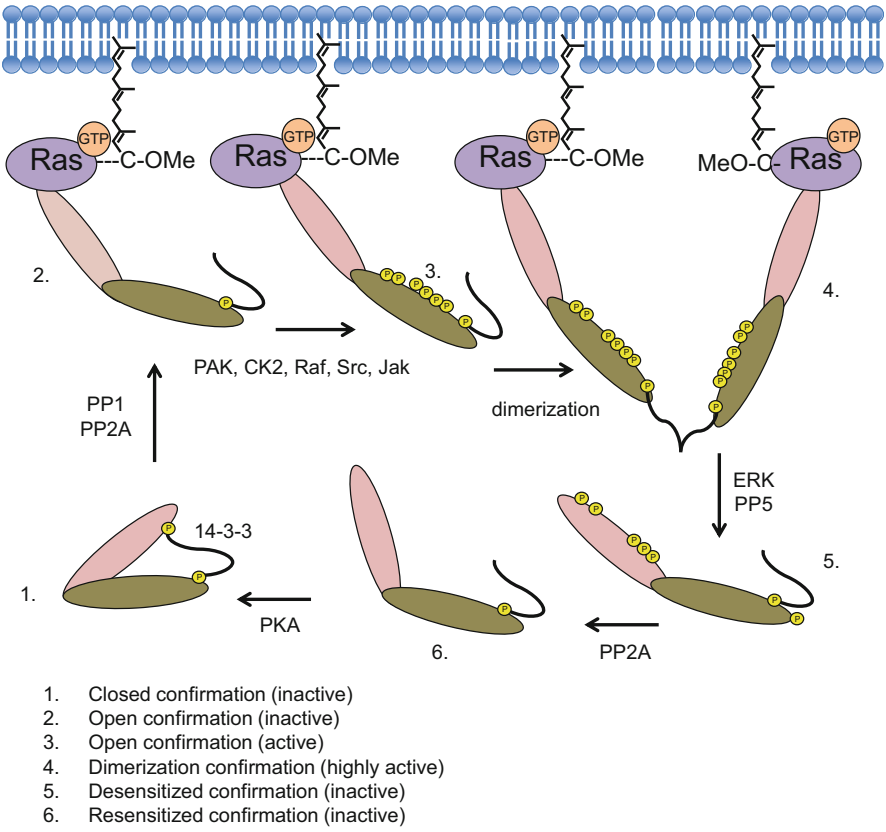

Fig. 6.4 Regulation of Raf dimerization and activation. Ras-GTP recruits Raf to the plasma membrane. Here PP1 or PP2A dephosphorylates Raf in its inhibitory domain. This event primes Raf interaction with Ras-GTP and promotes several important phosphorylation events. Once phosphorylated Raf proteins dimerize and are considered active. PP5 and PP2A dephosphorylate each Raf monomer leading to dissociation from the plasma membrane and monomerization. Finally, PKA phosphorylates Raf leading it to adopt a closed, "inactive," confirmation

Once Raf-1 is activated, it can phosphorylate its only known physiological downstream substrates, the closely related MEK1 and MEK2 dual specificity protein kinases. Raf kinases phosphorylate MEK1 and MEK2 at two sites (S218 and S222), which are located in the activation loop. Once active MEK1/2 phosphorylates ERK, which also has two distinct isoforms. As a consequence, the field has heavily relied on changes to levels of phosphorylated ERK1 and ERK2 to determine Ras-Raf signaling activation.

B-Raf Activation B-Raf activation is similar to Raf-1 activation, though several observations have suggested that B-Raf activation may require fewer components (Matallanas et al. 2011). Currently, the model for B-Raf activation is thought to require Ras and 14-3-3 complex interactions for activation. Unlike Raf-1, B-Raf

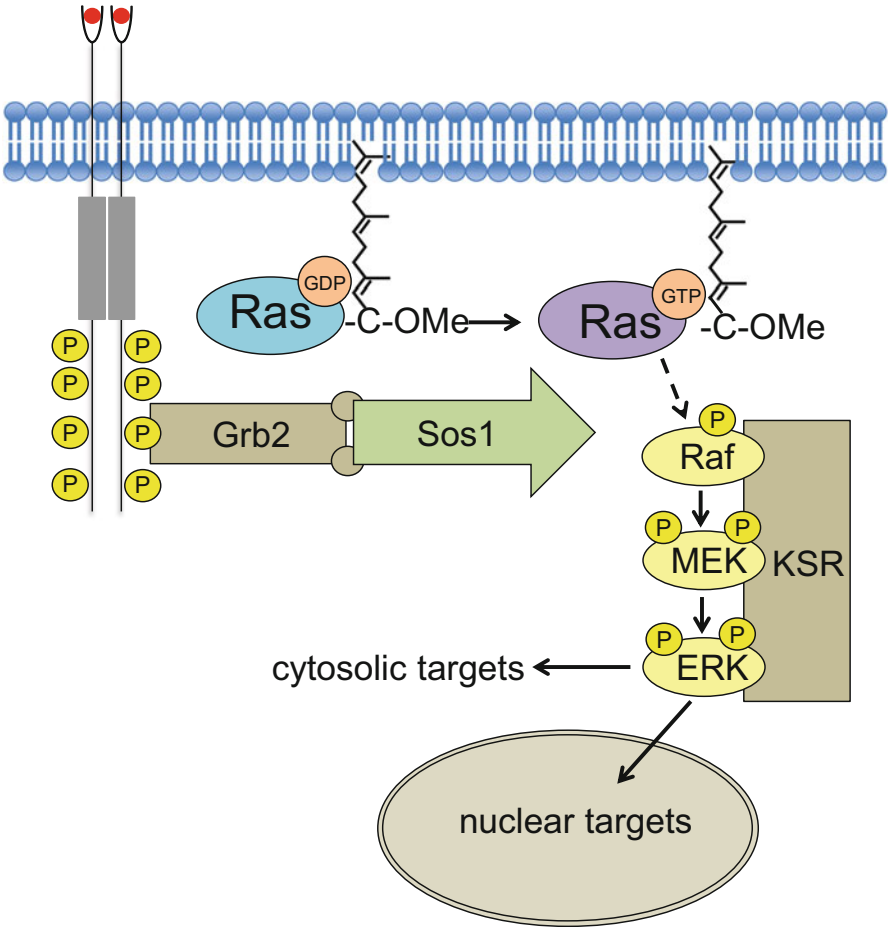

Fig. 6.5 Receptor tyrosine kinase-mediated activation of wild-type Ras. Wild-type Ras activation occurs when ligands (e.g., epidermal growth factor; EGF) stimulate activation of receptor tyrosine kinases (RTK; e.g., EGF receptor). Once stimulated, RTKs autophosphorylate tyrosine residues in the cytoplasmic domain, creating docking sites for Src homology 2 (SH2) domain-containing proteins (e.g., Grb2). The tandem SH2 domains of Grb2 interact with proline-rich sequences in the Sos1 RasGEF, promoting Sos1 translocation to the plasma membrane, leading to activation of membrane-associated Ras. Sos1-mediated formation of Ras-GTP then promotes Ras association with Raf, leading to activation of the ERK MAPK cascade. Shown here is KSR association with Raf, MEK, and ERK. KSR is but one of a number of scaffolding proteins that associate with one or more components of the three-tiered protein kinase cascade. Scaffolds modulate the composition of the pathway and additionally influence temporal and spatial activity of ERK signaling. Other ERK scaffolds include IQGAP1, MP1, Sef, and β-arrestin

contains a negatively charged N-terminal domain due to the presence of both aspartate at the position corresponding to Raf-1 residue Y341 (D448/449) and constitutive phosphorylation of B-Raf at residue S446, which corresponds to

Raf-1 S338. Together these residues promote the stabilization of the 3-dimensional catalytic domain. Like Raf-1, active B-Raf also phosphorylates the MEK1 and MEK2 kinases leading to ERK1 and ERK2 phosphorylation.

A-Raf Activation Of the Raf kinases, A-Raf is most poorly understood, as it is not mutated in cancer nor has it been identified as a resistance mechanism to any targeted therapies. A-Raf association with mutated Ras is weak as a consequence of the presence of a lysine at position 22, located within the A-Raf RBD (Matallanas et al. 2011). Consistent with this observation, A-Raf seems to be only weakly activated by Ras. Furthermore, there are several non-conserved acidic amino residues found in the N-terminal domain of A-Raf, most important being Y296, which if mutated results in a constitutively active kinase. This residue, in particular, is thought to stabilize the N-terminal domain interaction with the catalytic domain promoting a closed kinase confirmation. In the C-terminal domain, residue S432, located between the ATP-binding motif and the activation loop, is crucial for both MEK activation and A-Raf signaling. Unlike B-Raf, A-Raf contains a cluster of phosphorylation sites between residues 248 and 267, which, once activated, contributes to dissociation from the plasma membrane. Thus, A-Raf signaling has been found at several subcellular compartments, including the Golgi apparatus, endosomes, and mitochondria.

6.4.2 MEK Dual Specificity Kinases

MEK1 and MEK2 (MAP/ERK kinase; MAPKK/MAP2K) are highly related dual specificity kinases that catalyze the phosphorylation of both threonine and tyrosine residues in the TxY motif of their only known substrates, ERK1 and ERK2 (Roskoski 2012b) (Fig. 6.3). MEK1/2 structure can be split into three distinct functional domains: N-terminal domain, protein kinase domain, and a short C-terminal domain (Fischmann et al. 2009). The N-terminal region consists of an inhibitory domain, a nuclear export domain, and a domain that aids in the ability to bind the ERK kinases. The kinase domain comprises the majority of MEK1/2 structure and includes the activation segment and the proline-rich segment. Raf activates MEK1/2 by dual phosphorylation at tandem serine residues (Fig. 6.3). Two other known activators of MEK1/2 are the COT/Tpl2 and Mos serine/threonine kinases. Like Raf, Tpl2 and Mos were also identified originally as retroviral oncogenes and act as MAPKKK/MAP3Ks (Moloney 1966; Salmeron et al. 1996). The PAK1 serine/threonine kinase can phosphorylate and modulate MEK1 at S298, promoting Raf activation of MEK (Coles and Shaw 2002; Slack-Davis et al. 2003).

6.4.3 ERK Serine/Threonine Kinases

The only well-established MEK1 and MEK2 substrates are the highly related ERK1 and ERK2 serine/threonine kinases (Fig. 6.3). Phosphorylation of the ERK kinases is the most common readout for Ras activation of the Raf-MEK-ERK signaling cascade (Roskoski 2012a). Like many protein kinases ERK1 and ERK2 have short N-terminal and C-terminal domains, with the protein kinase domain making up the largest region. All known cellular activators of ERK1 and ERK2 lead to phosphorylation and activation of both kinases in parallel (Lefloch et al. 2009). To become active MEK1/2 phosphorylate ERK1 and ERK2 at residues T202 and Y204, starting with the tyrosine residue.

Whether ERK1 and ERK2 have unique biological functions has been under evaluation for the last decade. There is evidence that genetic ablation of *Erk2* but not *Erk1* causes embryonic lethality (Pages et al. 1999; Saba-El-Leil et al. 2003; Yao et al. 2003; Hatano et al. 2003). Furthermore, ERK2 but not ERK1 was necessary for H-Ras-induced epithelial-to-mesenchymal transformation in MCF-10A breast epithelial cells (Shin et al. 2010). RNAi silencing of either *ERK1* or *ERK2* impaired the growth of *BRAF* mutant melanoma cells (Qin et al. 2012). Together these observations suggest that ERK1 and ERK2 have distinct biological functions. Future studies will be needed to further characterize these distinct biological functions.

Unlike the highly restricted substrates of the Raf and MEK isoforms, the ERK1 and ERK2 (extracellular signal-regulated kinase) kinases are thought to share up to 200 substrates, in both nuclear and cytosolic compartments (Yoon and Seger 2006). Once activated ERK1 and ERK2 can phosphorylate cytoplasmic substrates and additionally translocate into the nucleus and phosphorylate nuclear substrates.

Multiple ERK substrates can contribute to the essential role of ERK in cancer growth. Key cytoplasmic substrates include the 90 kDa RSK (ribosomal S6 kinases) serine/threonine kinases (Romeo et al. 2012). RSK proteins (RSK1-4) are regulators of diverse cellular processes, including cell proliferation, survival, and motility. Other ERK substrates include the cytoplasmic MNK and nuclear MSK family kinases (Hauge and Frodin 2006; Hou et al. 2012). ERKs also phosphorylate many nuclear transcription factors that include Ets family transcription factors (e.g., Elk-1), Fos and Myc. ERK phosphorylation of Myc at S62 stabilizes Myc and prevents FBW7 E3 ligase-mediated proteasomal degradation (Farrell and Sears 2014).

6.5 Raf-MEK-ERK Target Validation in Ras Mutant Cancers

6.5.1 Raf is Necessary and Sufficient for Ras Transformation

The Raf-MEK-ERK signaling cascade has been rigorously validated as a necessary effector for Ras transformation (White et al. 1995; Khosravi-Far et al. 1996;

Khosravi-Far et al. 1995; Cuadrado et al. 1993). In early focus formation and clonogenic growth assays, several laboratories observed that dominant-negative mutants of Raf, MEK, or ERK effectively inhibited Ras-driven transformation (Cowley et al. 1994; Kolch et al. 1991; Schaap et al. 1993). Consistent with the importance of Raf-MEK-ERK signaling, it was also demonstrated that the Ras effector domain mutant T35G, which preferentially impairs Ras-Raf interaction relative to PI3K and RalGEF, inhibited H-Ras transforming capabilities in NIH 3T3 mouse fibroblasts (White et al. 1995). Expression of activated Raf-1 could overcome growth inhibition associated with loss of Ras or expression of Ras dominant-negative mutant S17N (Feig and Cooper 1988). Finally, genetic loss of all three *RAS* isoforms causes growth cessation of mouse embryo fibroblasts, and only activated Raf (and not PI3K and/or RalGEF) could rescue the growth defect of "Rasless" cells (Drosten et al. 2010). Activated MEK or ERK could also partially restore growth. Together these initial observations demonstrated that the Raf-MEK-ERK signal cascade was both downstream of Ras in mammalian cells and necessary for Ras transformation.

6.5.2 *In Vitro and In Vivo Raf Validation in Mutant Ras Cancers*

Raf has been validated extensively in human cancer cell lines as a target for therapies (Hingorani et al. 2003; Hoeflich et al. 2006; Sharma et al. 2005; Sumimoto et al. 2006; Karasarides et al. 2004). This is a direct consequence of its mutational activation as well as mutual exclusivity from Ras mutations in cancers (Davies et al. 2002; Karasarides et al. 2004; Rajagopalan et al. 2002; Sieben et al. 2004; Singer et al. 2003). The nonoverlapping occurrence of Raf and Ras mutations in some cancer types (e.g., melanoma, colorectal cancer) suggests that Raf is likely the most significant downstream effector in these Ras mutant cancers. This contrasts with activating mutations in *PIK3CA* (encodes p110 alpha) that can occur with *RAS* mutations. Consistent with this observation, several studies have validated the role of Raf downstream of mutant Ras in colorectal, pancreatic, and lung tumor cell lines (Campbell et al. 2007; Subramanian and Yamakawa 2012; Li et al. 2013). Together these data suggest that therapies for Ras mutant cancer treatments should be, at least partially, focused on targeting the Raf-MEK-ERK signaling cascade.

While RNAi use in cell culture is a strong tool for validating the importance of a cancer target, there are several caveats associated with genetic manipulation and its translation into actual cancer therapies. First, in vivo RNAi targeting is still under investigation and far from use as an effective therapy. As such, our most effective tools for cancer treatment are still targeted small molecule inhibitors. Inhibitors, generally, block catalytic function or prevent protein–protein activation; however, RNAi targets the entire protein for depletion, which is vastly distinct from catalytic or allosteric inhibition. Though RNAi is far from ideal, it still remains a powerful tool for targeted drug discovery.

Raf has also been sufficiently validated as an in vivo target for mutant Ras-driven cancer initiation and progression. The two-stage chemical carcinogenesis model, where a single treatment with the mutagen 7,12-dimethylbenz[*a*]anthracene (DMBA), followed by repeated applications of a pro-inflammatory phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), causes *Hras* Q61L-induced squamous cell carcinomas and has been widely used to assess the role of Ras effectors (e.g., Tiam1, RalGDS, PLCε, and p110α) in cancer formation (Slack-Davis et al. 2003; Malliri et al. 2002; Bai et al. 2004; Gupta et al. 2007). Using this mouse model, Baccarini and colleagues showed that conditional loss of one *c-Raf* allele in the epidermal tissue reduced the number of tumors as well as tumor size, with complete inhibition of tumor formation upon loss of both *c-Raf* alleles (Ehrenreiter et al. 2009). Extending this to *Kras*-driven cancers, two separate studies observed that *Craf* but not *Braf* deficiency impaired *Kras* G12D-induced lung tumor formation (Karreth et al. 2011; Blasco et al. 2011). However, in contrast to these studies, *Craf* was found to be dispensable for *Kras* G12D-induced pancreatic cancer formation (Eser et al. 2013). While a role for *Braf* was not addressed in this study, the finding by McMahon and colleagues that activated *Braf* V600E alone could phenocopy activated *Kras* G12D and induce pancreatic cancer formation suggests that different *RAF* isoforms may drive *KRAS*-driven cancer development in different tissues (Collisson et al. 2012).

The studies above provide validation that Raf is necessary for tumor initiation and progression. However, whether Raf plays a role in mutant Ras tumor maintenance remains partially answered. Counter and colleagues observed that ERK plays a role in tumor maintenance of Ras transformed cells, as an inducible dominant-negative MEK prevented continued tumor growth in a xenograft mouse model (Lim and Counter 2005). However, their key finding was that a membrane-targeted, activated, variant of p110α, not *c-Raf*, was sufficient to maintain tumorigenic growth of *KRAS* mutant human colon and pancreatic cancer cell lines when *KRAS* expression was ablated. This result suggests that PI3K rather than Raf inhibition will be required to block the *KRAS* mutant tumor growth.

Use of genetic knockout mouse models where effector function is ablated concurrently with *RAS* activation addresses the role of that effector in tumor initiation and progression but not maintenance. Additionally, genetic ablation of an effector, resulting in loss of protein expression, is not an accurate modeling of the consequences of pharmacologic inhibition of the catalytic function of the effector. With the development of potent and selective pharmacologic inhibitors of the Raf-MEK-ERK cascade, the limitations in these studies can be overcome. However, they still face the limitations of our current mouse models of cancer (Colvin and Scarlett 2014). Orthotopic tumors induced by implantation of human tumor cells into immunocompromised mice provide another model. However, with the obvious importance of the immune system in host response to tumor growth, these studies cannot evaluate drug response in the context of an intact immune response. Genetically engineered mouse models overcome these limitations. However, since tumor development is initiated by one or two genetic alterations, they are genetically less complex than bona fide human cancers.

6.6 Pharmacologic Inhibition of Raf-MEK-ERK Signaling in Mutant *RAS* Cancers

In this section we summarize the development and evaluation of pharmacologic inhibitors of Raf, MEK, and ERK for the therapeutic treatment of *RAS* mutant cancers (Fig. 6.4).

Raf Inhibitors Pharmacologic inhibitors of Raf have not been effective against *RAS* mutant cancers. Their ineffectiveness is due to the paradoxical activation rather than inactivation of ERK signaling (Fig. 6.5a). Studies in cell culture and mouse models determined that Raf inhibitor treatment caused the formation of B-Raf/C-Raf heterodimers that are dependent on activated Ras. Ras activation promotes Raf dimerization, primarily B-Raf/C-Raf heterodimers. In the Raf dimer, B-Raf is inhibited, but it then causes activation of the non-inhibited C-Raf molecule. Only at high inhibitor concentrations that cannot be achieved in the patient, both Raf molecules are inhibited and ERK signaling is blocked.

To date, the most successful Raf inhibitor is vemurafenib (ZELBORAF), which targets mutant $BRAF^{V600E}$ in melanoma (Flaherty et al. 2010). A second Raf inhibitor, dabrafenib (Tafinlar), was later approved for the same patient population. However, its success has been complicated by the reoccurrence of tumors harboring Ras mutations (Nazarian et al. 2010). In one study, vemurafenib inhibition caused accelerated ERK signaling resulting in aberrant growth and tumor formation in tissues, which harbored mutant *RAS*. Currently, efforts to develop Raf inhibitors that do not promote Raf dimerization or have more pan-Raf inhibitory activities, or inhibitors of Raf dimerization, are being pursued to overcome the limitation of first generation Raf inhibitors.

MEK Inhibitors MEK inhibitors have also shown limited to no antitumor efficacy in *RAS* mutant cancers. For example, Rosen and colleagues found that MEK inhibitor treatment was effective against *BRAF* but not *RAS* mutant human cancer cell lines (Solit et al. 2006; Daouti et al. 2010). MEK inhibition alone was not effective in a mouse model of *Kras*-driven lung cancer formation (Engelman et al. 2008). The ineffectiveness of MEK inhibition is attributed to the loss of ERK activation-induced feedback inhibitory mechanisms. Flux through the Raf-MEK-ERK cascade requires critical regulation, with high levels of activated ERK causing growth suppression; ERK activation induces feedback inhibition mechanisms that dampen upstream activators of the pathway (Fig. 6.5b). These mechanisms include ERK phosphorylation of Raf to dampen Ras activation of Raf (Dougherty et al. 2005). Other feedback mechanisms include ERK phosphorylation of Sos1 or the EGFR or transcription factor-mediated induction of gene expression of negative regulators such as DUSP protein phosphatases (Pratilas et al. 2009; Little et al. 2011; Wagle et al. 2014) or Sprouty (Roskoski 2010). In a recent unbiased approach to define mechanisms that drive resistance to MEK inhibition, Johnson and colleagues showed that MEK inhibition of *KRAS* mutant breast cancer cell lines resulted in the activation of multiple RTKs (Duncan et al. 2012). They

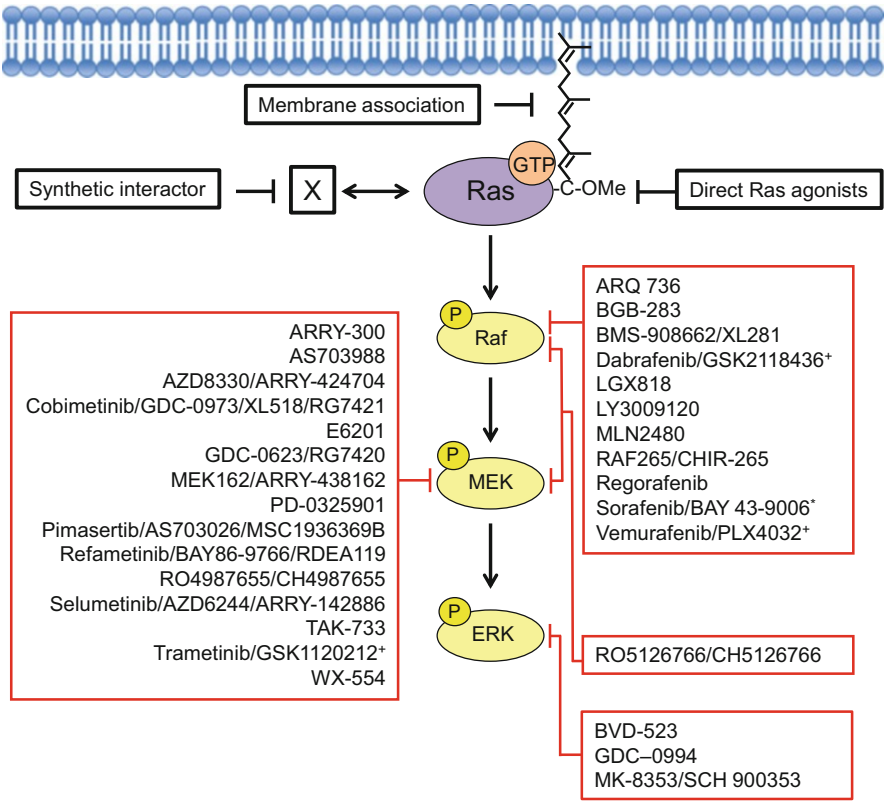

Fig. 6.6 Pharmacologic inhibitors of Raf-MEK-ERK under clinical evaluation. Compiled from ClinicalTrials.gov. Past and/or ongoing approaches for targeting Ras include direct Ras binders and inhibitors of Ras function and inhibition of Ras membrane association. Functional si/shRNA library screens have been applied to identify genes (x), that when silenced, impair the growth of *RAS* mutant but not wild-type tumor cell lines (aka synthetic lethal interactors of mutant Ras). *Asterisk* FDA approved for the treatment of renal cell, hepatocellular and thyroid carcinoma; *Plus* FDA approved for the treatment of *BRAF*-mutant melanoma

further showed that concurrent inhibition of RTK activation then enhanced the antitumor activity of MEK inhibition in vitro and in vivo.

One MEK1/2 inhibitor (trametinib/Mekinist) has been approved for use alone, or together with the Raf inhibitor dabrafenib, for *BRAF* mutant melanoma. Trametinib is an allosteric, non-ATP competitive inhibitor that prevents activated MEK1/2 phosphorylation of its substrates. There are at least 16 additional MEK1/2 inhibitors under clinical evaluation (Fig. 6.6), many also allosteric non-ATP competitive inhibitors.

ERK Inhibitors With the ineffectiveness of anti-Raf and –MEK therapies in *RAS* mutant cancers due largely to kinome reprogramming mechanisms that caused reactivation of ERK, it prompted studies to address whether inhibition of ERK directly may overcome these limitations. Recently, it was shown that an ERK1/2-selective inhibitor, SCH772984, was active in Raf- and MEK-resistant *BRAF* mutant melanoma in preclinical models (Morris et al. 2013). Additionally, another group identified another ERK inhibitor capable of overcoming resistance to MEK inhibitors (Hatzivassiliou et al. 2012). ERK inhibition represents a new approach to blocking an old pathway; however, the question remains whether ERK inhibition as a therapy will be successful in combating *RAS* mutant cancers or succumb to some of the limitations associated with Raf and MEK inhibition. Currently, three ERK inhibitors are under clinical evaluation (Fig. 6.6). MK-8353/SCH 900353 is an orally available analog of SCH772984.

6.7 Conclusion

Although Ras uses multiple effector pathways to mediate cancer growth, arguably the Raf-MEK-ERK cascade is the most critical and important driver of RAS-dependent cancer growth. The ineffectiveness of Raf or MEK inhibitors against RAS mutant cancers supports the critical role of the ERK MAPK cascade in cancer growth, as we have learned that cancer cells can adapt to the inhibitor-mediated loss of one specific component by dynamically changing the signaling properties to overcome that loss. This robustness is reflected in the multitude of *de novo* or acquired mechanisms of resistance seen with Raf and MEK inhibitors. Hence, the view of a simple linear pathway, where inhibition at the level of Raf or MEK should block ERK, is clearly not the case. Perhaps combinations of Raf, MEK, and ERK inhibitors will be advantageous over use of any one inhibitor alone, as inhibition of ERK signaling at multiple nodes will be required for effective and long-term blockage of this effector pathway. This concept is supported by the combined use of the Raf inhibitor dabrafenib and MEK inhibitor trametinib (Mekinist) for *BRAF* mutant melanomas (FDA approved in 2014), where there is greater antitumor efficacy and reduced toxicity than seen with the application of either inhibitor alone (Flaherty et al. 2012). Finally, even with effective combination inhibitor suppression of ERK signaling, effective inhibition of Ras will likely require concurrent inhibition of other Ras effector pathways (Fig. 6.7).

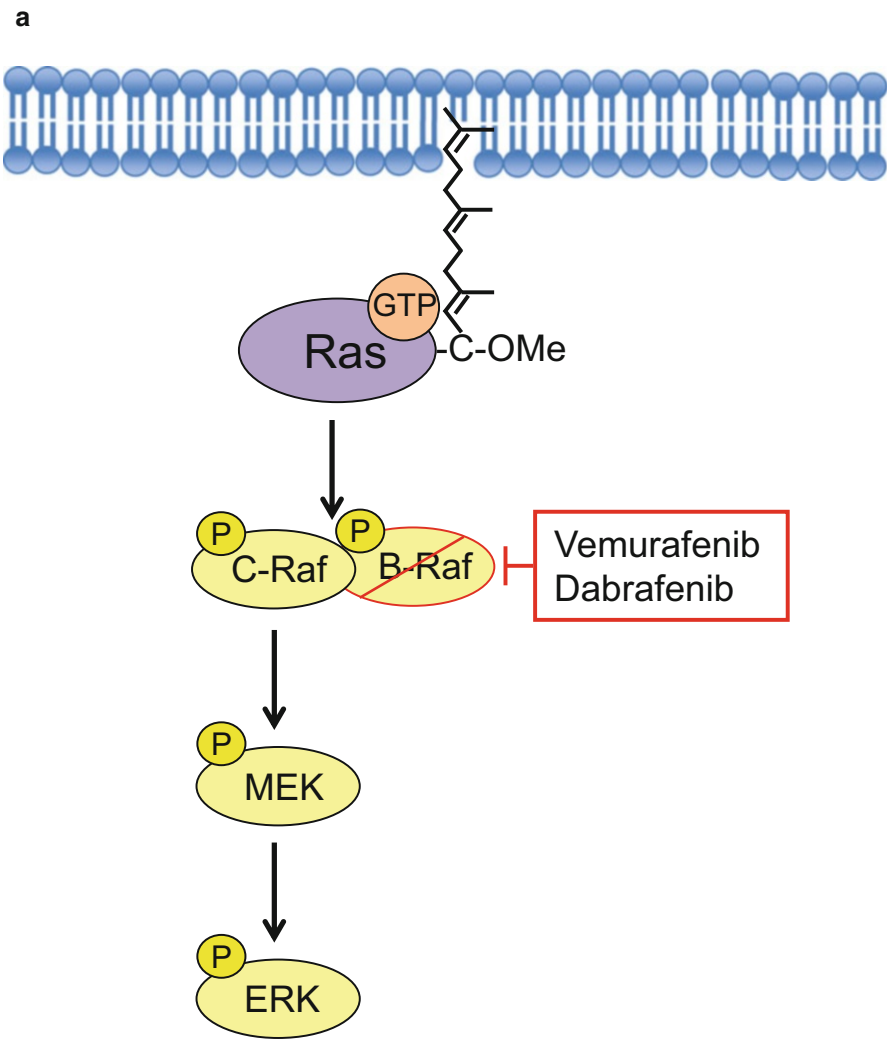

Fig. 6.7 (continued)

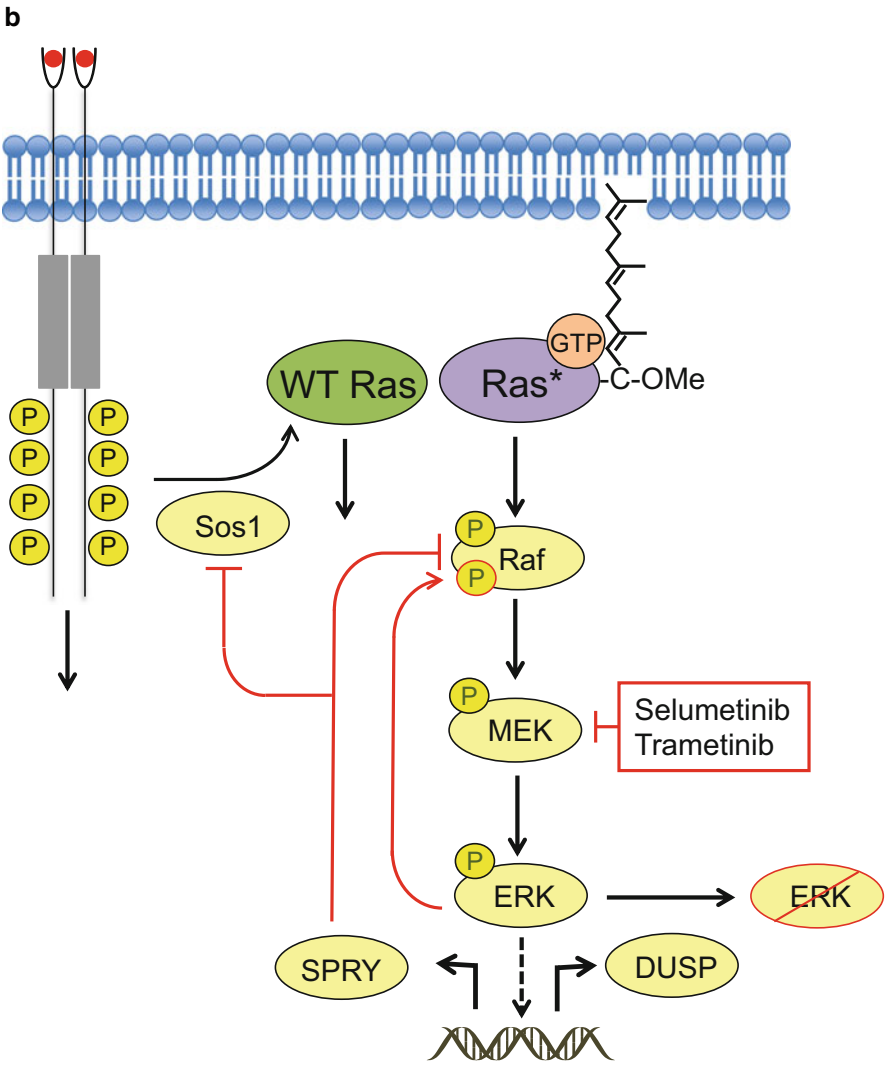

Fig. 6.7 Mechanisms of *RAS* mutant cancer cell resistance to Raf or MEK inhibitors. (**a**) *RAS* mutant cancer cells exhibit paradoxical activation rather than inactivation of ERK signaling. (**b**) *RAS* mutant cancer cells exhibit multiple mechanisms of resistance to MEK inhibition. The mechanisms that relate to RTK and Sos activation of Ras are not expected to be relevant for mutant Ras activity, since it is already persistently GTP-bound to the GAP defect. However, since there is evidence that wild-type Ras proteins support mutant Ras in cancer growth, these feedback mechanisms are then still important even in *RAS* mutant cancer cells

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Chapter 7
RAS Genes and Cancer

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Abstract The three *RAS* genes (*HRAS*, *KRAS*, and *NRAS*) comprise the most commonly mutated oncogene family in human cancer. *RAS* genes encode highly related small GTPases that are key regulators of cytoplasmic signaling networks that include the Raf-MEK-ERK mitogen-activated protein kinase cascade and the PI3K-Akt signaling cascade. There is increasing evidence that all *RAS* mutations are "not created equal" and that mutation specific therapies may be needed, that there will not be a "one size fits all" anti-Ras therapy. In this chapter, we summarize the frequency and nature of *RAS* mutations in human cancers, with a focus on the

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two cancers with the highest frequency of *RAS* mutations, pancreatic ductal adenocarcinoma (95 %), and colorectal (40 %) cancers.

Keywords Colorectal cancer • ERK • HRAS • KRAS • NRAS • Pancreatic cancer • PI3K • Raf

7.1 Introduction

In 1982, the *RAS* genes became the first mutated genes identified in human cancers (Cox and Der 2010). In this chapter we discuss the role of *RAS* gene mutations in cancer. In particular, we focus on the *RAS* gene most commonly mutated in human cancers, the *KRAS* oncogene (Prior et al. 2012). Although *KRAS* mutations are found in many cancer types, the highest frequencies are found in pancreatic ductal adenocarcinoma (PDAC) and colorectal carcinoma (CRC), ~95 % and ~50 %, respectively (Jones et al. 2008; Biankin et al. 2012; Wood et al. 2007; Bass et al. 2011; Seshagiri et al. 2012; Cancer Genome Atlas Network 2012) (Table 7.1). Before we discuss *RAS* genes and their protein products, the Ras proteins, we first provide a brief overview of the clinical treatment of these two cancers.

PDAC is the fourth leading cancer-related death in the United States (Siegel et al. 2013). Among 45,220 individuals diagnosed with PDAC in 2013, approximately 38,000 died of the disease (Siegel et al. 2013). While the mortality rate for most cancers is declining, PDAC is projected to become the second most common cause of cancer-related death by 2020. Given the vague clinical symptoms and the lack of effective screening methods, only 10–20 % of PDAC patients are candidates for curative resection at the time of diagnosis. Even with surgical resection followed by adjuvant therapy, the median overall survival of those patients is around 2 years (Oettle et al. 2007; Neoptolemos et al. 2010). Among the rest of the PDAC patients who are not candidates for resection, half of them have localized disease (borderline resectable and locally advanced disease) and half have metastatic disease at the time of diagnosis. Patients with localized disease are most often treated with chemotherapy with or without radiation, and their median overall survivals are around 15 months (Huguet et al. 2007). In patients with metastatic PDAC, the prognosis is extremely dismal. With the recent development of chemotherapy regimens such as FOLFIRINOX (Conroy et al. 2011) and gemcitabine plus nab-paclitaxel (Von Hoff et al. 2013), the survival of metastatic PDAC has moved beyond 6 months (Burris et al. 1997), but remains less than 1 year. At present, targeted therapies have not provided any meaningful clinical benefit for PDAC patients despite the approval of the EGFR inhibitor erlotinib (Moore et al. 2007). The poor prognosis associated with PDAC reflects an urgent need for novel drug development.

CRC is the third most common cancer overall and was the second most common cause of cancer in men and women in the United States (Siegel et al. 2013). In 2013, approximately 150,000 Americans were diagnosed with CRC, and about 50,000

Table 7.1 Frequency of *RAS* mutations in human cancers[a]

Cancer	% *KRAS*	% *NRAS*	% *HRAS*	% All *RAS*
Pancreatic ductal adenocarcinoma	92	1	0.5	94
Colorectal adenocarcinoma	45	7	0.0	52
Multiple myeloma	26	25	0.0	51
Lung adenocarcinoma	31	1	<1	32
Skin cutaneous melanoma	1	27	1	29
Uterine corpus endometrioid carcinoma	21	4	<1	25
Thyroid carcinoma	1	9	4	14
Stomach adenocarcinoma	11	1	0	12
Acute myeloid leukaemia	3	7	2	12
Bladder urothelial carcinoma	3	1	6	10

[a]Compiled from cBioPortal (http://www.cbioportal.org/public-portal/)

died from this disease (Siegel et al. 2013). Due to the implementation of screening colonoscopy, about 70–80 % of patients with CRC are able to undergo curative resection at the time of diagnosis. Patients with stage I disease are essentially cured with a 5-year survival rate greater than 95 % with surgery alone (Nivatvongs 2000). Adjuvant chemotherapy is indicated for high risk stage II and all stage III CRC (Andre et al. 2009), with neoadjuvant therapy given specifically to patients with rectal cancer. The 5-year survival rates for II and III colon cancer are 83 % and 72 % (Andre et al. 2009), respectively. Treatment for patients with metastatic CRC consists of systemic and targeted therapies. The median overall survival of patients with metastatic CRC has significantly improved over the last decade, from 6 months on supportive care up to 2 years with the development of newer chemotherapy agents oxaliplatin (de Gramont et al. 2000) and irinotecan (Douillard et al. 2000) and targeted therapy including EGFR inhibitors (Cunningham et al. 2004; Van Cutsem et al. 2007) and angiogenesis inhibitors (Grothey et al. 2013; Hurwitz et al. 2004; Van Cutsem et al. 2012). Furthermore, CRC has stepped into the era of personalized medicine. CRC tumors that harbor the *KRAS* mutation do not respond to EGFR inhibition (Amado et al. 2008; Lievre et al. 2008); therefore, wild-type *KRAS* is required prior to initiation of any EGFR inhibitor (Allegra et al. 2009). In light of the fact that many CRC patients still have a good performance status after progressing on all standard therapies, the need for novel second or third line therapies is urgently needed, and this is especially true for CRC with *KRAS* mutation.

7.2 *RAS* Genes and Ras Proteins

The three human *RAS* genes encode four highly related 188–189 amino acid 21 kDa small GTPases (Fig. 7.1). K-Ras4A and K-Ras4B are splice variants encoded by alternative exon 4 usage and differ primarily at their C-terminal sequences. The *RAS* genes comprise one of the most frequently mutated gene family in human cancer (Cox and Der 2010). In cancer and various developmental disorders

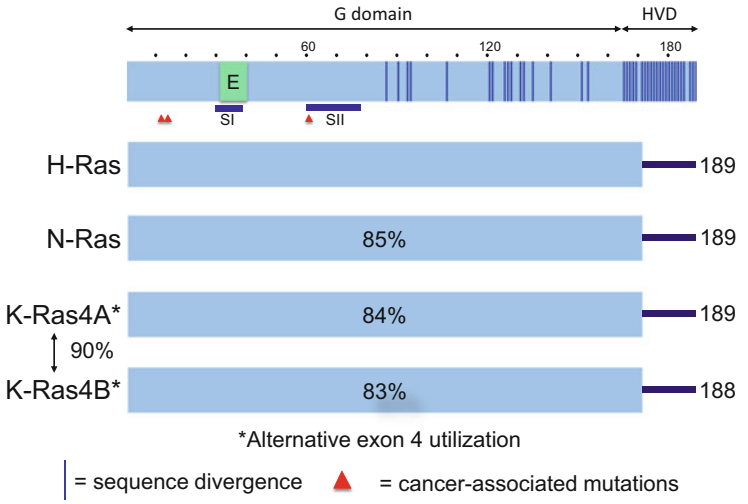

Fig. 7.1 Human Ras proteins. The three *RAS* genes encode four highly related 188/189 amino acid Ras proteins. The two highly related K-Ras 4A and 4B isoforms (90 % identical) arise from alternative gene splicing and utilization of alternative fourth exons 4A and 4B. The numbers indicate percent sequence identity with H-Ras (83–85 %). Residues 1–164 comprise the G domain that binds and hydrolyses GTP and includes the switch I (SI; aa 30–38) and II (SII; aa 60–76) sequences that change in conformation during GDP-GTP cycling. The core effector domain (E; residues 32–40) is essential for Ras-GTP binding to downstream effectors. The predominant missense mutations (~99 %) found in cancer result in single amino acid substitutions at residues 12, 13, or 61. Ras proteins diverge in their C-terminal sequences comprised of the hypervariable domain (HVD)

(referred to as Rasopathies), *RAS* genes harbor missense mutations that encode mutant proteins that are altered in their biochemical properties (Rauen 2013; Prior and Hancock 2012).

Ras proteins act as binary switches regulating a number of biological processes that include cellular proliferation, survival, and differentiation. Ras proteins share 90 % sequence identity in the G domain (1–164), which is important for guanine nucleotide binding and GTP hydrolysis, and interaction with regulators and effectors. In contrast, the C-terminal 24/25 residues exhibit significant sequence divergence (8 % amino acid identity) and is therefore referred to as the hypervariable region (HVR).

Ras activity is regulated by two distinct classes of regulatory proteins: Ras-selective guanine exchange factors (RasGEFs) and GTPases activating proteins (RasGAPs) (Fig. 7.2) (Vigil et al. 2010). RasGEFs (e.g., Sos1) accelerate the slow intrinsic guanine nucleotide exchange rate of Ras proteins. Since the intracellular concentration of GTP is 10-fold more abundant than GDP, this favors formation of the active Ras-GTP complex. Once bound to GTP, Ras is considered activated and can bind to a number of catalytically distinct downstream effectors to regulate a diversity of cytoplasmic signaling networks. In order to terminate Ras

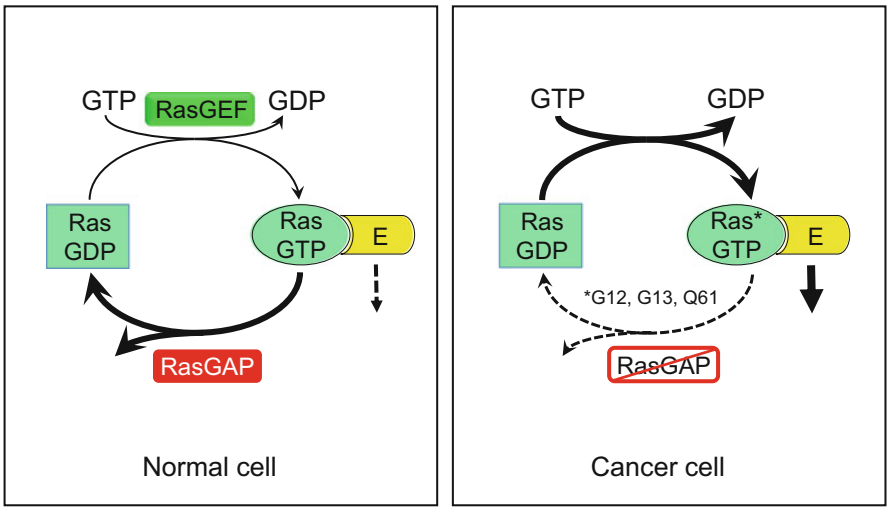

Fig. 7.2 Regulation of the Ras GDP-GTP cycle. Ras proteins act as molecular switches alternating between GTP- (active state) and GDP- (inactive state) bound states, where Ras-GTP binds preferentially to downstream effectors (E). There are two classes of regulatory proteins that regulate this cycling process: RasGEFs (guanine exchange factors) and RasGAPs (GTPase activating proteins). In resting cells, normal Ras is predominantly GDP-bound (~95 %). Upon growth factor stimulation and activation of RasGEF, rapid and transient GDP-GTP exchange is stimulated. RasGAP stimulation of the intrinsic GTPase activity and GTP hydrolysis restores the inactive Ras-GDP state. Mutant Ras proteins are impaired in their intrinsic and GAP-stimulated GTP hydrolysis activities, resulting in stimulus-independent, persistent Ras-GTP formation (~80 %)

signal transduction, RasGAPs (e.g., neurofibromin, p120 RasGAP) bind to Ras and accelerate its weak intrinsic GTP hydrolysis activity, returning Ras to the inactive GDP-bound form. However, when Ras proteins are mutated, they display altered intrinsic and GAP-stimulated GTPase activity, favoring a GTP-bound state and promotion of aberrant signal transduction.

The C-terminal sequence is crucial for Ras membrane association and subcellular localization (Fig. 7.3). The terminal four residues comprise the CAAX motif (C = cyteine, A = aliphatic amino acid, X = terminal amino acid), which signals for three sequential posttranslational modifications that increase hydrophobicity and promote membrane association (Ahearn et al. 2012). The sequences immediately upstream of the CAAX motif contain a second membrane targeting signal. For H-Ras, K-Ras4A, and N-Ras, cysteine residues signal for covalent addition of a palmitate fatty acid, whereas K-Ras4B has a polybasic stretch that serves a similar role. K-Ras4B additionally contains a serine residue (S181) that is phosphorylated by protein kinase C. This modification regulates a dynamic trafficking between the plasma and endomembranes. In addition to association with the plasma membrane, Ras proteins are also found in other cellular endomembrane compartments that include the Golgi, endoplasmic reticulum, mitochondria, and endosomes.

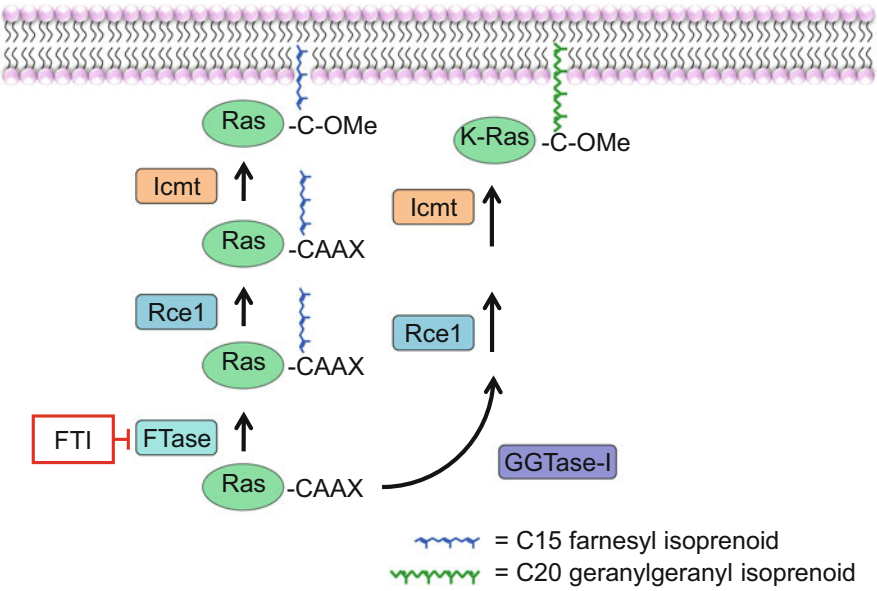

Fig. 7.3 Ras proteins and membrane association. Ras proteins are synthesized initially as cytosolic and inactive proteins. Within minutes, they undergo a series of posttranslational modifications signaled by the C-terminal CAAX motif. First, cytosolic farnesyltransferase (FTase) catalyzes covalent, irreversible addition of a C15 isoprenoid lipid to the cysteine residue of the C-terminal CAAX motif. This then allows Rce1-catalyzed proteolytic removal of the AAX residues and Icmt-catalyzed, reversible carboxylmethylation (-OMe) of the now terminal farnesylated cysteine. H-Ras is the only Ras isoform that is solely modified by FTase. Although normally also FTase substrates, when FTase activity is blocked by FTase inhibitor (FTI) treatment, K-Ras and N-Ras, can now be modified by geranylgeranyltransferase-I (GGTase-I)-catalyzed addition of a related C20 geranylgeranyl isoprenoid, resulting in membrane association

7.3 *RAS* Mutations Are Early Events in Cancer Development and Progression

Ras mutations are generally early events in cancer development and progression (Fig. 7.4). For pancreatic cancer, *KRAS* mutations are the initiating genetic event, followed by a progression of mutations in three tumor suppressor genes. For colorectal cancer, mutation of the APC tumor suppressor is the initiating event, followed by *KRAS* mutation.

The early onset of *RAS* mutations in cancer emphasizes their key role in promoting the initiation and progression of cancer. This role is supported by genetically engineered mouse model studies where tissue restricted *Kras* mutational activation initiates the early stages of cancer development (Hingorani et al. 2003). However, when coupled with additional mutations in tumor suppressors, *Kras*-initiated tumor formation was accelerated and advanced to invasive and metastatic disease. For example, *Kras* (G12D) activation alone induced ductal

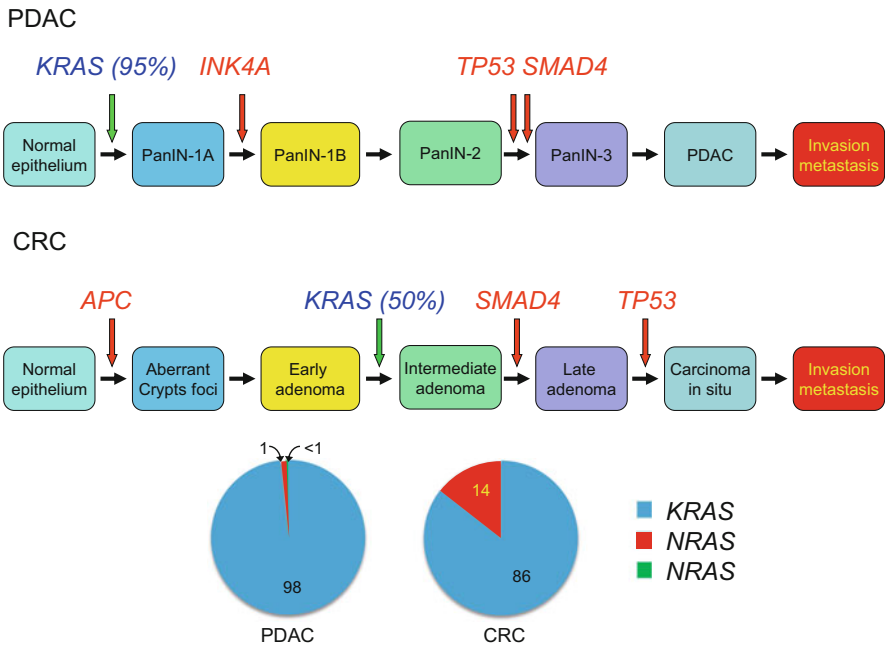

Fig. 7.4 PDAC and CRC genetic progression models. *KRAS* mutations occur early in cancer progression followed by loss of important tumor suppressors. *KRAS* mutation is the initiating step in PDAC development, followed by mutational loss of the *CDKN2A*, *TP53*, and *SMAD4* tumor suppressors. Mutation of the *APC* tumor suppressor gene is the initiating step in CRC development, followed by *KRAS* activation and mutational loss of the *SMAD4* and *TP53* tumor suppressor genes. *KRAS* is the *RAS* isoform predominantly mutated in PDAC (98 % of all *RAS* mutations) and CRC (86 %)

lesions that recapitulated the full spectrum of human pancreatic intraepithelial neoplasias (PanINs), the putative precursors to invasive pancreatic cancer (Hingorani et al. 2003). At low frequency, these lesions also progressed to invasive and metastatic adenocarcinomas. However, when combined with a mutation in the *Tp53* (R172H) tumor suppressor, rapid onset of invasive and widely metastatic carcinoma was seen (Hingorani et al. 2005). Decreased latency and acceleration of metastatic PDAC was seen when mutational activation of *Kras* (G12D) was coupled with the deletion of either of the other two key tumor suppressor lesions in this cancer (*CDKN2A*/*INK4A* and *SMAD4*) (Bardeesy et al. 2006a; Bardeesy et al. 2006b). Similarly, *Kras* (G12D) alone caused hyperplasia in the colonic epithelia but not neoplasia. However, when combined with deletion of the *APC* tumor suppressor gene, tumor formation occurred (Haigis et al. 2008).

Despite their clear role in tumor initiation and progression, continued expression of mutant *KRAS* is still required for maintenance of the primary and advanced metastatic cancer. For pancreatic cancer, this was first demonstrated by RNA interference suppression of mutant *KRAS* (G12V) expression in the *KRAS* mutant

Capan-1 human PDAC cell line, causing loss of anchorage-independent growth in vitro and impaired tumorigenic growth in vivo (Brummelkamp et al. 2002). Subsequently, it was shown that induction of RNAi suppression of *KRAS* in vivo impaired the growth of Capan-1 PDAC cell line xenograft tumors (Lim and Counter 2005). Subsequent studies showed that shRNA suppression of KRAS expression impaired the growth of other *KRAS* mutant PDAC cell lines (Singh et al. 2009). More recently, two groups independently showed that continued mutant *Kras* G12D expression was required for the maintenance of PDAC growth in *Kras* G12D/*Tp53* null mouse models of PDAC (Collins et al. 2012a; Ying et al. 2012; Collins et al. 2012b).

7.4 RAS Mutations in Human Cancers

The frequency of mutation of the three *RAS* genes varies significantly with *KRAS* is the most commonly mutated isoform. Mutation data available in COSMIC v68 (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) show that *KRAS* mutations were found in 29,557 of 139, 474 unique samples analyzed (21.2 %), followed by *NRAS*, found in 3,587 of 62,609 samples (5.7 %), with *HRAS* mutations relatively rare, found in 1,127 of 35,366 samples (3.2 %). The simple summation of these data is the basis for the ~30 % *RAS* mutation frequency found in all human cancers. Mutation frequencies from other databases (e.g., cBioPortal; http://www.chbioportal.org/public-portal/), representing a smaller dataset, although with more accurate data restricted to deep sequencing studies, come up with a reduced frequency. Since the cancers represented in each database are different, no one source provides a truly accurate determination of the frequency of *RAS* mutations in cancer.

There is preferential mutation of a specific isoform in different cancers, with near exclusive mutation of *KRAS* in CRC and PDAC. In contrast, *NRAS* is the predominant isoform mutated in melanoma and acute myelogenous leukemias, whereas *HRAS* is the predominant isoform mutated in bladder and head and neck squamous cell carcinomas. The majority of missense mutations found in *RAS* occur at G12, G13, and Q61. This pattern contrasts with *RAS* mutations found in developmental disorders. There are also *RAS* isoform distinct frequencies in these mutations, with Q61 mutations rare in *KRAS*, yet the predominant mutation seen in NRAS. Finally, the mutation spectrum found in *KRAS* varies widely in different cancers. For example, the G12C is the predominant mutation found in lung cancer; it is rarely seen in PDAC or CRC.

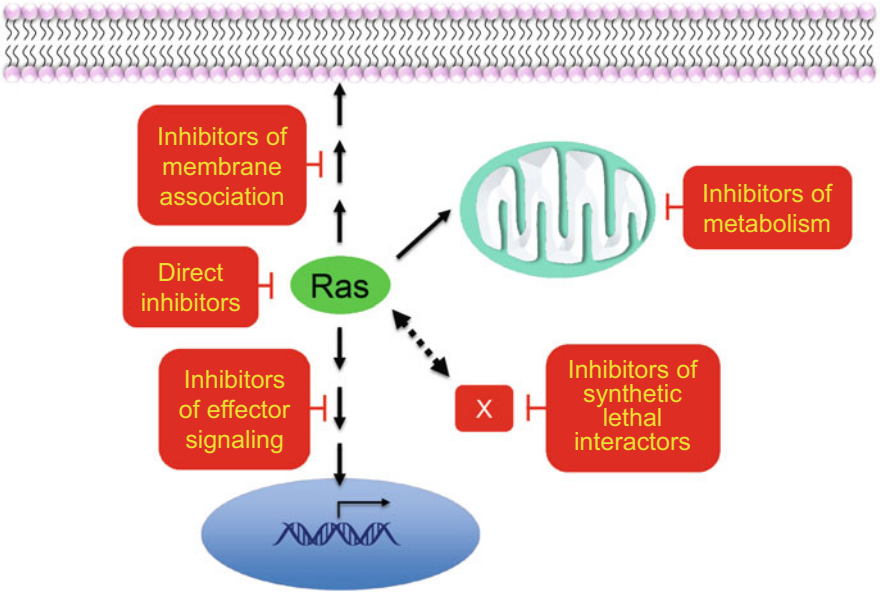

Fig. 7.5 Approaches for the development of anti-Ras drugs. Once considered impossible, recent studies have identified direct Ras-binding small molecules that alter Ras function. Approaches to inhibit Ras membrane association include the development of farnesyltransferase inhibitors. Inhibitors of Ras effector signaling, in particular the Raf-MEK-ERK protein kinase cascade comprise the most active direction of anti-Ras drug discovery. Unbiased functional siRNA/shRNA library screens have identified genes that when suppressed, cause growth suppression of *RAS* mutant but not *RAS* wild type tumor cells. The identified synthetic lethal interactors (X) typically involve components that have no direct association with Ras signaling. Recently, with evidence that mutant RAS causes altered glucose and glutamine metabolism, approaches to target metabolism have also been considered

7.5 Targeting *RAS* for Cancer Treatment

As indicated above, the current therapeutic options for PDAC and CRC are limited and ineffective. With the high frequency of *KRAS* mutations in these cancers and strong preclinical evidence that disruption of KRAS function will impair cancer growth, the development of effective anti-KRAS inhibitors has been actively pursued. However, despite more than three decades of intensive effort by the pharmaceutical industry and academia, to date, no effective therapeutic strategies have reached the clinic (Bryant et al. 2014; Stephen et al. 2014). In this section, we provide a summary of past and ongoing efforts to develop anti-Ras therapeutic strategies (Fig. 7.5).

As described above, the Ras C-terminal CAAX motif signals for posttranslational modifications that promote Ras membrane association. That mutation of the

cysteine residue to serine (SAAX) to prevent the addition of the farnesyl isoprenoid lipid or truncation of the AAX residues results in completely inactive Ras proteins supported the rationale to target farnesyltransferase as a therapeutic strategy (Berndt et al. 2011). Numerous companies successfully developed potent and selective farnesyltransferase inhibitors (FTIs), with two (tipifarnib and lonafarnib) advancing to Phase III clinical evaluation (Basso et al. 2006). Despite promising cell culture and mouse model observations with FTIs, FTIs failed to show any clinical benefit in pancreatic and colorectal cancer patients. Their failure was attributed to the earlier finding that the K-Ras and N-Ras proteins, when FTase activity is blocked, can then serve as a substrate for the related geranylgeranyltransferase-I enzyme (GGTase-I) and be modified by the related geranylgeranyl isoprenoid lipid. Since geranylgeranyl-modified Ras retains the ability to be membrane-associated and transforming, this bypass mechanism prevented the effectiveness of FTIs for *KRAS* mutant cancers.

Currently, alternative strategies to disrupt Ras membrane association are being considered. These include targeting the other two CAAX modification enzymes, ICMT and Rce1. Another approach are farnesyl lipid mimics, salirasib, that act apparently by competing for Ras membrane association (Bustinza-Linares et al. 2010). More recently, inhibitors of a chaperone protein, the prenyl-binding protein phosphodiesterase 6 delta that modulates Ras trafficking to the plasma membrane, have been described (Chandra et al. 2012; Zimmermann et al. 2013).

Currently the most aggressively pursued anti-Ras strategy involves inhibition of Ras downstream effector signaling. However, these efforts are complicated by the fact that Ras uses multiple effectors to promote cancer growth (Mitin et al. 2005). Of these effector pathways, the Raf (A-Raf, B-Raf, and C-Raf) and class I phosphatidylinositol-3 kinases (PI3K; p110α, γ, and δ) effector pathways have attracted the greatest interest (Nissan et al. 2013; Fritsch and Downward 2013), with multiple inhibitors of components of each pathway currently under clinical evaluation. That these effector pathways have driver functions in *KRAS*-dependent cancer growth is supported by their frequent mutational activation in cancer: *BRAF* (20 %) and *PIK3CA* (encodes p110α; 12 %) (COSMIC). However, when applied as monotherapies, these inhibitors have shown limited to no clinical activity in *RAS* mutant cancers. There are numerous ongoing clinical trials evaluating whether concurrent inhibition of Raf and PI3K effector signaling will be more effective (http://clinicaltrials.gov/).

An approach once thought impossible involves direct inhibition of mutant Ras. Initial efforts to disrupt GTP binding were not successful, due to the picomolar affinity of GTP binding to Ras. This contrasts with the low micromolar-binding affinity of ATP to protein kinases, where effective ATP-competitive protein kinase inhibitors have been developed successfully. Similarly, efforts to identify small molecules that can act as a GAP for mutant Ras proteins did not succeed. Recently, small molecules that directly bind Ras and perturb either RasGEF activation or effector binding have been described (Sun et al. 2012; Maurer et al. 2012; Ostrem et al. 2013). Whether these early stage Ras binders can be advanced to more potent

and selective Ras-binding molecules and whether they can effectively block the critical functions of Ras to have a clinical consequence remains to be determined.

Other directions considered for anti-Ras drug discovery include targeting the metabolic changes in glucose and glutamine metabolism found in *RAS* mutant cancers (Ahearn et al. 2012; Sun et al. 2012). RNAi targeting of RAS gene expression is also being pursued. Here, whether these can be effectively delivered to the cancer, and whether sufficient suppression of *RAS* gene expression can be achieved, are the current uncertainties in these directions. Finally, unbiased RNA interference screening has been applied to search for synthetic lethal interactors of mutant *RAS*. However, these studies have been hampered by the lack of reproducibility in the findings (Luo et al. 2012; Weiwer et al. 2012).

7.6 Concluding Comment and Future Directions

In the mid-2000s, when the development of FTIs failed to achieve effective inhibition of the Ras isoforms most commonly mutated in human cancers, a diminished interest in developing anti-Ras drugs set in. With the decreasing cost of DNA sequencing came exome-wide sequencing of many human cancers, with the goal of unearthing more promising targets for anticancer drug discovery. However, these studies did not identify new genetic alteration found in the majority of PDAC or CRC cancers, and instead, verified that *KRAS* is the most commonly mutated oncogene in these cancers. With this realization, the National Cancer Institute initiated the Ras Project in 2013, with the goal of accelerating efforts to develop the elusive anti-Ras drugs. With lessons learned from past failures, with new technologies and approaches, there is strong optimism that the long elusive holy grail of cancer research may at long last finally be achieved.

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Chapter 8
The Spatial Organization of Ras Signaling

Björn Papke, Malte Schmick, Nachiket Vartak, and Philippe I.H. Bastiaens

Abstract Ras or "Rat sarcoma" is a central node in signal transduction networks that includes a range of oncogenic proteins in its family. These small guanine nucleotide-binding proteins transmit signals from lipid membranes in the cell with which they interact by an acquired affinity through posttranslational modifications at their C-terminal hypervariable region (HVR). Ras bound to the plasma membrane can be switched to the active, GTP-bound state by guanine nucleotide exchange factors (GEFs) that interact with activated growth factor receptors. Signals are then transmitted by the activation of effector proteins through spatial dimensionality reduction from a 3D cytosolic volume to a 2D plasma membrane surface. The enrichment of Ras at the plasma membrane is therefore an important parameter that determines Ras signaling output. Based on the finding that GTPases of the Ras family use farnesyl-binding chaperones to maintain their spatial organization, we discuss the molecular components and opposed mechanisms of directional flux and diffusional randomization that partition Ras proteins on membranes. The pharmacological modulation of these spatially organizing systems can be exploited to affect oncogenic Ras signaling in cancer cells.

Keywords Ras mediated signaling • Ras spatial cycles • Dimensionality reduction • Ras signal transduction • Spatial organization of Ras

8.1 Ras Signaling from the Plasma Membrane

The G-domain of 21 kDa Ras proteins that binds the guanine nucleotides has no preference for binding guanosine triphosphate (GTP) above guanosine diphosphate (GDP). It is an inefficient GTPase requiring interaction with catalytic residues

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provided by GTPase activating proteins (GAPs) for hydrolyzing GTP to GDP. The exchange of GDP for GTP in the next catalytic turnover requires interaction with guanine nucleotide exchange factors (GEFs), which expose the guanine nucleotide-binding pocket for effective exchange by the ~10-fold surplus of cytoplasmic GTP with respect to GDP (Bos et al. 2007). In the GTP state, the conformation of two so-called switch regions in Ras enables the interaction with downstream effectors that harbor Ras binding (RB) or Ras association (RA) domains (Nassar et al. 1995; Ponting and Benjamin 1996). As a peripheral membrane protein, Ras is therefore a switchable recruitment factor that actuates a dimensionality reduction of the space in which cytosolic effectors can diffuse (Fig. 8.1a). This enhances their effective, local concentration and thereby their reactivity (Adam and Delbruck 1968; Kholodenko et al. 2000). However, membrane association also slows down diffusion with respect to the cytosol, which counteracts reaction efficiency. Daniel Axelrod has considered this dilemma in light of ligands binding to receptors at the cell surface (Axelrod and Wang 1994), but his theory also applies to protein molecules associated to membranes in the intracellular space. This theory can be condensed to compare the reaction rates of protein interactions occurring in the cytosol or at the membrane interface within the dimensions of a typical cell (Zamir et al. 2013). According to the law of mass action, the kinetics of a signaling reaction is determined by the product of the rate of random collisions and the probability (X) that a collision will lead to binding. The reaction rate (F) is thus factorized by the concentration of the reactants and the rate constant that encapsulates the product of the summed diffusion speed (D) of the reactants and X. The probability that particles of radius r_a are in colliding distance is proportional to their space occupancy. This factor represents the dimension of the reacting particle and is simplified in 2D by the circumference ($2\pi r_a$) and in 3D by the surface (proportional to $\pi {r_a}^2$) resulting in a $r_a/2$-fold larger space occupancy. The reaction rate (F) is therefore proportional to the product of the concentration of a protein, its diffusion constant, its space occupancy, and the probability that a collision will lead to binding. Let's consider the simple example of a protein at a certain copy number ρ that reacts with itself to form dimers in the cytoplasm of a spherical cell of radius R. Its cytoplasmic concentration will be $\rho/(4/3\pi R^3)$. If, however, all this protein is associated with the plasma membrane of this spherical cell, its concentration will increase R/3-fold to $\rho/(4\pi R^2)$. The ratio of the dimerization reaction speed at the membrane (F_2) and the cytoplasm (F_3) is then:

$$F_2/F_3 = (X_2/X_3)(D_2/D_3)(R/3)(2/r_a)$$

For a hypothetical cell with a radius of 10 μm, the association rate of a protein of radius 3.3 nm would be at least 200-fold higher after recruitment to the plasma membrane than in the cytosol. This estimation includes the tenfold lower diffusion constant for lateral membrane diffusion compared to cytosolic diffusion and is an underestimation due to the reduction in the rotational degrees of freedom of the protein upon membrane binding that in general causes the first factor (X_2/X_3) to be greater than 1. This means that the concentration effect of membrane recruitment

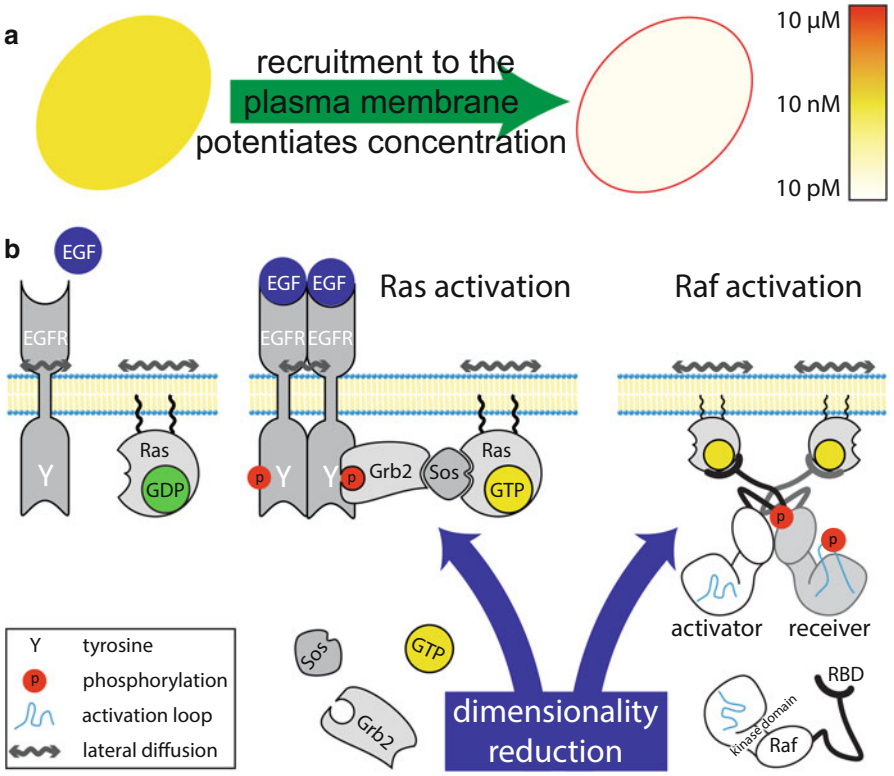

Fig. 8.1 Dimensionality reduction in Ras-mediated signaling. (**a**) By reducing the diffusible space of a 3D-cytosolic protein to the 2D-plasma membrane, its local concentration increases by at least three orders of magnitude. (**b**) Prior to binding of EGF ligand to its receptor, EGFR, most Ras molecules are in a GDP-bound state. Phosphorylation of tyrosine residues after ligand-induced dimerization of EGFR is recognized by Grb2 that sequesters the Ras-GEF Sos and thereby generates a high fraction of Ras-GTP at the plasma membrane. In turn, Ras-GTP binds the RBD of Raf and the resulting increased concentration of Raf at the plasma membrane actuates Raf cis-auto-phosphorylation in the receiver kinase that is allosterically activated by asymmetric dimerization with the activator kinase. Both the activation of Ras via the interaction with Sos as well as the autocatalytic activation of Raf by dimerization are actuated by dimensionality reduction

dominates the reaction rates, which are increased by more than one order of magnitude in cells with a radius larger than 0.5 μm.

How does this biophysical concept of dimensionality reduction apply to Ras-mediated signaling? As an example of growth factor-induced Ras signaling, we will consider the activation of epidermal growth factor receptor (EGFR), which dimerizes in an asymmetric head-to-tail configuration after EGF binding (Jura et al. 2011; Zhang et al. 2006). In this allosteric activation mechanism, the activator kinase domain stabilizes the active conformation of the receiver kinase that phosphorylates in *trans* the C-terminal tail of the activator. The phosphorylated tyrosine residues recruit the cytosolic guanine nucleotide exchange factor (GEF) Son of

sevenless (Sos) via the adapter protein Grb2 (growth factor receptor-bound protein 2) to the cytoplasmic side of the receptor (Fig. 8.1b). This represents the first instance of dimensionality reduction in this signaling cascade. The enhanced reactivity of the Sos–Ras-complex results in an effective GDP/GTP exchange reaction that is further amplified by the interaction of RasGTP with an allosteric site on Sos (Boykevisch et al. 2006; Margarit et al. 2003). This secondary form of Sos-recruitment to the plasma membrane is another example of dimensionality reduction. Although the accessibility of the allosteric site exhibits another level of regulation via the interaction of the histone and PH domains on Sos with lipids on the plasma membrane (Gureasko et al. 2010; Zhao et al. 2007), it clearly constitutes a node for positive feedback regulation of Ras activity. Such a system can generate an all-or-none (bistable) response dependent on the strength of the feedback connection (Sabouri-Ghomi et al. 2008; Tyson et al. 2003). Because the strength of the feedback on Sos is given by the local Ras concentration on the plasma membrane, the reaction systems that maintain Ras on the plasma membrane will also affect the response of Ras to growth factors. This could have major implications for oncogenic Ras signaling, where we need to consider another layer of Ras signal output regulation that is dependent on its localization at the plasma membrane. For this, it is important to realize that oncogenic mutations occur mostly on a single allele. This implies that an oncogene product such as RasG12V can coexist with wild-type variants of the different Ras isoforms in the cell, even if a wild-type allele is lost due to genetic instability. If the activity of the oncogene product and wild-type Ras would be completely uncoupled, the oncogene product would cause a constitutive offset in the activity state of downstream signaling molecules such as extracellular signal-regulated kinases (Erk). Some of the gene-expression machinery behaves in accordance with Weber's law (Ferrell 2009), not responding to absolute levels of signaling activity but to fold-changes in activity relative to the background (as, for example, shown for mitogen-activated protein kinase (MAPK) and "wingless and Int-1" (Wnt) signaling: Cohen-Saidon et al. 2009; Goentoro and Kirschner 2009). Therefore, the wild-type Ras population might still provide the switchable activity that transmits changes in the composition of the extracellular milieu to the gene expression machinery and thereby controls the phenotype of the cell. However, the guanine nucleotide-binding state of oncogenic and wild-type Ras are coupled via positive feedbacks as described above for the RasGEF, Sos. The activation of Sos, and thus Ras, via this feedback mechanism is dependent on the localization of active Ras at the plasma membrane. Oncogenic RasG12V-GTP can thus activate the exchange activity of Sos at the plasma membrane and thereby switch the wild-type Ras population to the active GTP-bound state. This activation would depend on the dose of oncogenic RasG12V that resides at the plasma membrane. A high dose of oncogenic RasG12V at the plasma membrane might thus lead to fully activated wild-type RasGTP, resulting in cell senescence due to strong amplitude-mediated survival-, but absence of fold-changes in proliferative signals. When the amount of oncogenic and wild-type Ras are comparable at the plasma membrane (oncogenic mutation on one allele, wild-type allele not lost), the feedback coupling to Sos might fall below a threshold whereby the remaining wild-

type Ras population could re-acquire its GEF-regulated switching ability that is dependent on growth factor receptor activation. However, the interaction of oncogenic Ras with the allosteric site on Sos will tend to lower the growth factor dose at which the wild-type Ras will switch to its active state. This putative scenario will lead to a cell that exhibits a proliferative response at low growth factor dose and at the same time has strong intrinsic survival signals. Could this situation in which oncogenic Ras coexists with wild-type Ras already represent a transformed phenotype?

The guanine nucleotide exchange mechanisms described above result in a structural change of the switch I and II regions of Ras, thereby facilitating the interaction of Ras with cytosolic effectors like the Serine/threonine specific rapidly accelerated fibrosarcoma (Raf) kinase. The concentration of Raf in the cytosol of a non-stimulated cell is in the lower nanomolar range (Fujioka et al. 2006) and any spurious phosphorylation of Raf in its activation loop (AL) in the cytosol is likely counteracted by phosphatases that operate at low K_M (Zhao and Zhang 2001). BRaf is activated in asymmetric dimers that contain an activator kinase that allosterically induces cis-autophosphorylation in the AL of the receiver kinase (Hu et al. 2013). The interaction of BRaf with Ras enhances the BRaf-dimerization reaction (Nan et al. 2013) by dimensionality reduction at the plasma membrane (Fig. 8.1b). Furthermore, the phosphorylation of CRaf in the N-terminal acidic (NtA) motif by membrane-associated kinases such as Src (Fabian et al. 1993) or protein kinase C (Kolch et al. 1993) allow it to assume activator functionality in asymmetric dimers (Chong et al. 2003). Protein kinase C (PKC) is itself recruited to the plasma membrane by the lipid second messenger diacylglycerol that is generated from phosphatidyl-inositol-bisphosphate by the recruitment of phospholipase C γ to phosphotyrosines on the activated growth factor receptor. Both CRaf and one of its activating kinases are thus translocated and concentrated on the plasma membrane, thereby largely increasing the kinetics of CRaf NtA phosphorylation by PKC and enabling the propagation of signal via Ras and its downstream MAPK pathway. Although this model is highly oversimplified, it indicates the main aspect of Ras function as a reactivity-enhancing, switchable recruitment factor. Therefore, the amount of Ras at the plasma membrane has a large impact on its activation by the interaction with GEFs, as well as on its signaling output by enhancing the reactivity of effectors at the plasma membrane.

8.2 Farnesylated Ras Partitions to the Extensive Endomembranes at Equilibrium

The interaction of Ras with the plasma membrane depends on a variety of post-translational modifications (PTMs) at its C-terminal hyper variable region (HVR). *Irreversible* prenylation via a thioether bond at the C-terminal cysteine include the addition of a 15-C farnesyl chain as an important prerequisite for these proteins to

form weak associations with membranes (Hougland and Fierke 2009). Some Ras family proteins—typically but not exclusively H/NRas—undergo *reversible* S-palmitoylation via a thioester bond on cysteines at the C terminus. 25 members of DHHC-cysteine-rich domain proteins are encoded in the human genome and catalyze this palmitoyl transferase reaction with broad substrate specificity (Hou et al. 2009; Raymond et al. 2007). Palmitoylation further increases the hydrophobicity of these farnesylated Ras molecules and hence stabilizes the interaction with any membrane in the cell. In contrast, KRas4B has a polybasic lysine stretch in its HVR that enables electrostatic interactions with negatively charged phospholipids, such as phosphatidylserine, that is supposed to stabilize its binding to the plasma membrane (Hancock et al. 1990).

However, most cells contain extensive endomembrane systems. At thermodynamic equilibrium the partitioning of lipidated Ras over membranes is determined by the relative ratio of the on- (k_{on}) and off- (k_{off}) rate constants of membrane binding. Because the capacity of a membrane to bind Ras is proportional to its surface area (S), this partitioning is also proportional to the ratio of the membrane surfaces:

$$\left[\mathrm{Ras}^{\mathrm{PM}}\right]/\left[\mathrm{Ras}^{\mathrm{EM}}\right] = \left({k_{\mathrm{on}}}^{\mathrm{PM}}/{k_{\mathrm{on}}}^{\mathrm{EM}}\right)\left({k_{\mathrm{off}}}^{\mathrm{EM}}/{k_{\mathrm{off}}}^{\mathrm{PM}}\right)\left(S^{\mathrm{PM}}/S^{\mathrm{EM}}\right),$$

where the superscripts PM and EM denote plasma membrane and endomembrane, respectively. Even though the dissociation rate constant of KRas is different for endomembranes and the plasma membrane due to electrostatic interactions with the negatively charged plasma membrane, lipidated, solely farnesylated HRas that lacks the polybasic stretch is likely to interact equally well with all cellular membranes. Therefore, its partitioning to membranes is determined by the relative surface ratios of the plasma membrane to endomembranes. The following thought experiment addresses the question, how the plasma membrane surface relates to the endomembrane surfaces. Consider a cell with a 10 μm radius and a uniform population of vesicles with a radius of 20 nm. 250,000 of these vesicles must fuse to regenerate the plasma membrane of this 10 μm cell. However, 250,000 vesicles only occupy 0.2 % of the enclosed volume of this cell, which is far below the volume typically occupied by endomembranes in the cytoplasm (Terasaki et al. 2013). Estimating the contributions of nucleus, cytosol, and proteins as 60 % of the cell's total volume, if one fills the remaining 40 % of the cell with 20 nm vesicles (a total of 50,000,000 vesicles), this "foam" has a surface that is 200 times as big as the plasma membrane surface. Fusing 50,000,000 vesicles into a singular compartment could never result in a spherical shape with a 200 times bigger surface enclosed in a spherical plasma membrane of 10 μm radius, but creates a highly folded compartment with almost fractal structure and a complicated topology to accommodate so little lumen in such a large surface (Fig. 8.2).

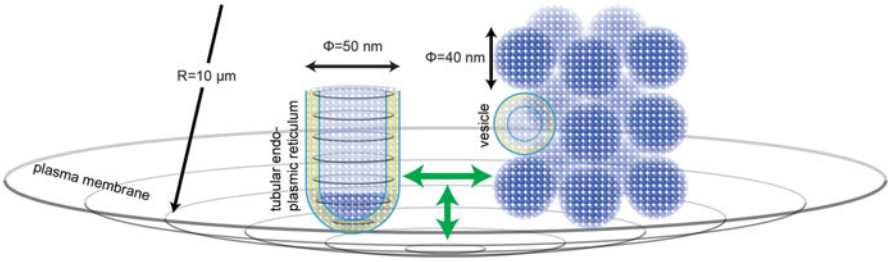

Fig. 8.2 How to rearrange the membrane surface of a 10 µm spherical cell. To reconstitute the surface area of a spherical cell with 10 µm radius, 250,000 vesicles with 20 nm radius suffice. Arranged in a rectangular grid 20 × 25 of touching vesicles, a stack of 500 such layers (20 × 25 × 500 = 250,000) would span the 20 µm diameter of the sphere. Fusing these 250,000 vesicles into tubular structures of a diameter of 50 nm typically observed in the tubular endoplasmic reticulum, only 20 × 20 tubes of 20 µm length would have the same surface as the plasma membrane of the cell. Arranged in circular sheets of 50 nm thickness with the same 10 µm radius as the whole cell, two such bilayer sheets suffice to comprise the cellular surface. Electron microscopy experiments indicate that intracellular endoplasmic reticulum membrane sheets are densely packed and extensive structures (Terasaki et al. 2013). It is therefore not surprising that the total endomembrane surface area can easily be two orders of magnitude larger than the plasma membrane

8.3 Ras Is Dynamically Maintained at the Plasma Membrane

At a plasma membrane to endomembrane surface ratio of 1:200, thermodynamic equilibrium would result in an extensive partitioning of farnesylated Ras to endomembranes. Processes like endocytosis enhance this entropic tendency. While KRas can more easily dissociate from endomembranes that lack the negative surface charge of the plasma membrane, the strong hydrophobic interaction of palmitoylated Ras with membranes is weakened by diffusely localized acyl protein thioesterases (APTs). These activities remove the S-palmitoylation from Ras proteins but leave the farnesyl moiety (Dekker et al. 2010). Depalmitoylation and absence of electrostatic interaction destabilize membrane association and thereby increase the cytosolic fraction of Ras, to speed up equilibration to all membranes. This is further enhanced by the passive sequestration of soluble farnesylated Ras by a guanine nucleotide dissociation inhibitor (GDI)-like solubilization factor (GSF) called phosphodiesterase 6 delta subunit (PDEδ), which facilitates Ras diffusion in the cytoplasm (Chandra et al. 2012; Hanzal-Bayer et al. 2002; Ismail et al. 2011). Counter intuitively, RNAi-based knockdown of PDEδ results in the loss of plasma membrane localization of both palmitoylated as well as polybasic stretch containing Ras isoforms. As a consequence, such enrichment at the plasma membrane must be an out-of-equilibrium phenomenon. Indeed, binding of the GTP-bound form of the Arf-like 2 (Arl2) GTPase to PDEδ was shown to allosterically expel farnesylated proteins from the binding pocket of PDEδ (Ismail et al. 2011), and knockdown of Arl2 results in complete solubilization of KRas in

Hela cells (Schmick et al. 2014). This localized displacement of Ras from PDEδ manifests as an enrichment of farnesylated Ras proteins on perinuclear membranes, where the GTP-bound, active form of the GTPase Arl2 exerts its allosteric influence on PDEδ unloading. How is Ras then concentrated on the plasma membrane? In this perinuclear region, two different compartments act as collectors of unloaded KRas or depalmitoylated H/N-Ras. As a compartment with negatively charged membrane surfaces (Chen et al. 2010), the recycling endosome (RE) is capable of trapping KRas with its polybasic HVR stretch (Schmick et al. 2014). On the other hand, palmitoylation by palmitoyl transferases (PATs), which are localized to the cytoplasmic face of the Golgi apparatus, increases N/H-Ras membrane affinity by more than 100-fold as compared to only farnesylated Ras. For N/H-Ras, posttranslational palmitoylation of depalmitoylated Ras thus provides the means of trapping these molecules on the Golgi. From both compartments—the recycling endosome and the Golgi apparatus—vesicular traffic transports this high concentration of trapped Ras proteins (back) to the plasma membrane, thereby closing the Ras spatial cycles (Rocks et al. 2005, 2010; Vartak and Bastiaens 2010; Schmick et al. 2014).

Interestingly, the mammalian target of rapamycine (mTor) signaling activator Ras homolog enriched in brain (Rheb) (Guertin and Sabatini 2007) is a farnesylated member of the Ras superfamily that lacks both palmitoylatable cysteines and a polybasic stretch in the HVR. The Arl2-mediated release of Rheb from PDEδ therefore results in an out-of-equilibrium enrichment on perinuclear membranes (Chandra et al. 2012). This enrichment on perinuclear membranes could well improve its interaction with its downstream mTor-signaling targets on perinuclear lysosomes (Chandra et al. 2012; Sancak et al. 2010). The PDEδ/Arl2 system therefore serves several clients in the farnesylated Ras family to maintain their spatial organization, which are however distinct by additional features in the HVR (Fig. 8.3).

8.4 Ras Spatial Cycles Shape Its Signaling Response

The plasma membrane localization of Ras isoforms is maintained by constitutive cycles independent of their activity state. In response to an extracellular signaling event, the cyclic nature of these spatially organizing mechanisms resurfaces to propagate Ras signals through the cytosol to compartments other than the plasma membrane. Consequently, the relatively short 5–10 min response pulse of Ras activity at the plasma membrane following growth factor stimulation can be detected at the Golgi as a time-dilated echo that lasts up to 30 min (Lorentzen et al. 2010). Signal-pulse broadening and loss of amplitude are a result of the intervening diffusive processes of Ras on its journey from the plasma membrane to the Golgi apparatus, imparting a significant temporal dimension to the flow of information between cellular compartments. The mammalian Golgi membrane contains a Ras-specific scaffold protein Sef, several Ras effectors such as RasIP-1

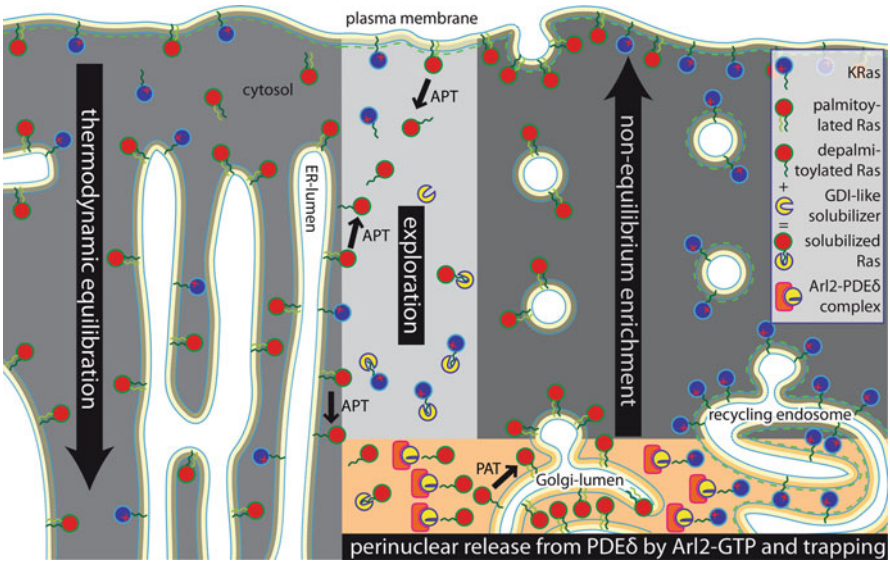

Fig. 8.3 The spatial organization of Ras isoforms. The localization of palmitoylatable Ras isoforms and KRas arise from analogous, energy-driven cyclic mechanisms. Enrichment at the plasma membrane is countered by spontaneous dissociation and endocytosis, which equilibrates Ras to the extensive endomembrane system. There, the lack of negative surface charge and the cytosolic, homogeneous depalmitoylation activity of APTs increase the cytosolic fractions of both KRas and depalmitoylated H/N-Ras. These soluble Ras fractions are sequestered by PDEδ to be unloaded in an Arl2-GTP-dependent manner in the perinuclear area. Here, Golgi-localized PAT activity and the negatively charged recycling endosome can trap the respective Ras isoforms to be transported back to the plasma membrane by vesicular transport

and modulators of the MAPK, but no specific GEF for Ras proteins in most cell types except lymphocytes (Philips 2004). Thus, propagation of Ras signaling activity to the Golgi apparatus is facilitated by the acylation cycle in the role of a carrier wave (Lorentzen et al. 2010). KRas signaling is also coupled to the reaction systems that maintain its spatial organization. For example, downstream signaling of KRas through PI3K and calcium (Alvarez-Moya et al. 2010; Wee et al. 2009) could lead to the recruitment of PKC to the plasma membrane where it phosphorylates a serine residue (S181) on KRas within the polybasic stretch (Bivona et al. 2006). The negatively charged phosphate group partly neutralizes the positive charge of the polybasic stretch, thereby diminishing the electrostatic force that retains KRas on the plasma membrane. This causes KRas to redistribute over cellular membranes. This "electrostatic switch" exemplifies a negative feedback, since active but phosphorylated KRas is no longer enriched on the plasma membrane and cannot efficiently activate effectors for downstream signaling at the plasma membrane. Furthermore, the relocation of active KRas to mitochondrial membranes has been reported to promote apoptosis via Bcl-XL (Bivona et al. 2006). The dynamic mechanisms that maintain KRas localization are

therefore also used to propagate KRas signals to compartments other than the plasma membrane. Here, it would be of interest to investigate whether the recycling endosome as the perinuclear trapping compartment is also a passive receiver of KRas signals from the plasma membrane, analogous to the Golgi for H/N-Ras.

8.5 Targeting the Molecular Systems That Maintain the Spatial Organization of Ras

As described in the first section, the high concentration of Ras at the plasma membrane is essential to enrich Ras effectors (such as Raf) in response to growth factor binding to receptors. When oncogenic mutations occur in Ras, effector binding becomes uncoupled from growth-factor-mediated activation of receptors, leading to accumulation of effectors on the plasma membrane and thereby constitutive signaling from the oncogenic Ras molecules. Because the dose of (oncogenic) Ras at the plasma membrane has such a dramatic effect on effector reactivity and signaling output, countering the molecular systems that maintain the out-of-equilibrium Ras concentration on the plasma membrane are promising approaches to affect oncogenic signaling.

The first attempts to affect the spatial organization of Ras was by pharmacological inhibition of farnesyltransferases (FTs) to affect plasma membrane localization by the loss of the farnesyl to the cysteine of the C-terminal CAAX box. The inhibition of FTs reversed oncogenic HRas transformed cells and resulted in apoptosis or cell cycle arrest in cancer cell models (Berndt et al. 2011). Furthermore, preclinical trials using farnesyltransferase inhibitors (FTIs) demonstrated selective killing of cancer cells in vitro and in mouse models. An unexpected result was that clinical trials of FTIs did not show any significant decrease of tumor growth or higher survival rates in cancer patients. Further investigations revealed that cancer cells were able to bypass the inhibition of FTs by the compensating activity of geranylgeranyl transferases (GGTs), which reinstated Ras localization at the plasma membrane (Whyte et al. 1997). The administration of the first generation of GGT inhibitors (GGTI) showed more toxic side effects than FTIs leading to the conclusion that there either are more geranylgeranylated proteins or that some of the geranylgeranylated proteins have a high impact on cell survival. Combinational therapy with FTIs and GGTIs in a tumor mouse model indeed resulted in lethality within the first two weeks (Lobell et al. 2001).

Another approach that targets the plasma membrane enrichment of Ras is to disturb its C-terminal methylation by the inhibition of isoprenyl cysteine carboxyl methyltransferases (ICMT). C-terminal methylation negates the negative charge of the carboxylic acid on the cysteine and therefore reduces electrostatic repulsion from the negatively charged plasma membrane. The first approach was to mimic the Ras C-terminal farnesylated cysteine to compete with Ras for the active site of ICMTs. Marciano et al. (1995) designed a farnesyl mimic, Farnesylthiosalicylic acid (FTS, Salirasib), that is not a substrate of ICMTs, which reduced the plasma

membrane localization of Ras resulting in impaired Ras-dependent cell growth. Further investigations showed that FTS also competed with Ras binding via its farnesyl tail to escort proteins such as Galectins that are involved in regulating Ras clustering at the plasma membrane (Rotblat et al. 2008).

As was explained above, the plasma membrane localization of both palmitoylated H/N-Ras as well as the polybasic stretch containing KRas is maintained by energy-driven reaction-diffusion cycles, which have common mechanistic principles as well as protein elements. The common protein elements in the spatial organization of farnesylated Ras proteins are the GSF PDEδ and its allosteric regulator Arl2. The features of the HVR, like the polybasic stretch or palmitoylatable cysteines, or the lack of both, dictate the interaction of mislocalized Ras variants with the trapping compartment in the perinuclear area to reinstate plasma membrane localization by vesicular transport.

Ubiquitous depalmitoylation plays a crucial role in the maintenance of palmitoylated Ras localization and the inhibition of thioesterase activity is not lethal to cells (Dekker et al. 2010). Based on these premises, the thioesterase inhibitor Palmostatin B was recently developed and was shown to inhibit the Ras depalmitoylating enzyme APT1/2 (Dekker et al. 2010; Vartak et al. 2014). Most strikingly, treatment of cells with Palmostatin B leads to a random distribution of fully palmitoylated Ras over all membrane systems in the cell. The net result of thioesterase inhibition is the reduction of Ras at the plasma membrane and thereby the envisioned effect of the reduction of its interaction with effectors. On the phenotypic level, thioesterase inhibition by Palmostatin B caused a partial reversion of a tumor-like phenotype to a normal phenotype in HRasG12V transformed MDCK-f3 cells. Despite that new, more specific APT inhibitors have been developed (Adibekian et al. 2012), thioesterase inhibition is still in its infancy as an approach to affect oncogenic Ras signaling in cancer cells (Xu et al. 2012) and much research needs to be done to prove its efficacy in tumor models that bear oncogenic N- or HRas.

The universal functionality that maintains the spatial organization of farnesylated Ras proteins by enhancing their diffusion in the cytoplasm is the GSF PDEδ. The importance of PDEδ in maintaining the spatial organization of Ras proteins is apparent from the loss of the plasma membrane partitioning of both palmitoylated H/NRas as well as polybasic stretch containing KRas in cells in which PDEδ had been knocked down by RNA interference (Chandra et al. 2012). The loss of the spatial organization of Ras was paralleled by a reduction in EGF-induced Erk activity, and ectopic expression of PDEδ showed a several fold increase in EGF-induced Erk activity. Reinstating PDEδ by ectopic expression in human hepatocarcinoma cells that do express PDEδ at very low levels also reinstates the plasma membrane enrichment of Ras as well as EGF-induced Erk signaling. These experiments clearly demonstrate that effective coupling of Ras to its effectors indeed occurs at the plasma membrane. Evidence that PDEδ also affects oncogenic Ras signaling by maintaining its spatial distribution came from PDEδ knockdown experiments in both HRasG12V transformed fibroblasts and KRasG12D knock-in models for pancreatic adenocarcinoma cells (Chandra et al. 2012; Zimmermann et al. 2013). PDEδ downregulation resulted in a

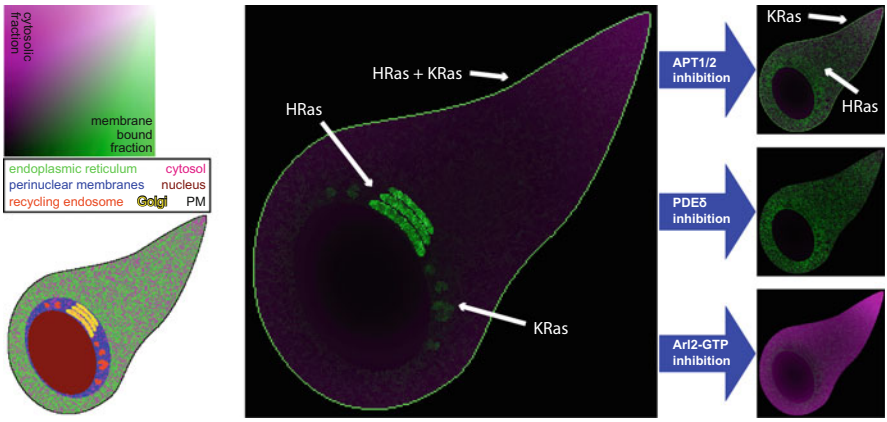

Fig. 8.4 Modulating Ras localization mechanisms. A reaction-diffusion simulation illustrates the effect of changing aspects of the Ras-localization mechanisms. In the unperturbed steady state, HRas and KRas are enriched at the plasma membrane, while HRas also exhibits a significant Golgi localization. Palmostatin B-mediated APT1/2 inhibition interferes with depalmitoylation, resulting in a redistribution of palmitoylated HRas to all membranes, while KRas stays at the plasma membrane. Competitive inhibition of the common GSF, PDEδ, redistributes both Ras isoforms to the extensive endomembranes, while disrupting the Arl2-GTP-mediated release of Ras from PDEδ results in solubilized Ras

randomized distribution of endogenous oncogenic Ras and strongly reduced cell proliferation/survival as observed by clonogenic assays. The reduction in proliferation was paralleled by a reduced Erk phosphorylation within 72 h of PDEδ knock down. Erk activity down-modulation was lost after longer times of PDEδ knockdown, possibly reflecting a selection process in the clonogenic assay that overcomes the lack of PDEδ-mediated Ras signaling from the plasma membrane by switching to a Ras-independent signaling pathway. Despite the general problem that genetically instable cancer cells can become resistant to therapy by selection processes under chemotherapeutic pressure, the approach of inhibiting the PDEδ–Ras interaction to affect the spatial organization of Ras has another boon to it that makes its pursuit worthwhile. Similar to thioesterase inhibition, the inhibition of PDEδ–Ras interaction will not completely remove Ras from the plasma membrane and the residual wild-type Ras at the plasma membrane in "normal" cells could still respond to growth factors and thereby maintain their viability. This is reflected by the fairly normal development of PDEδ knockout mice that have 20–30 % less body weight, apart from the retinal degeneration that occurs because of the role of PDEδ in transporting G-protein-coupled receptor kinase 1 (GRK1) and the catalytic subunit of PDE6 to the outer segment of photoreceptors (Zhang et al. 2007). PDEδ is a target with two sites for which small molecules could be developed. The farnesyl-binding pocket offers a site for competitive inhibition of Ras binding and the allosteric Arl2/3 site offers the possibility of locking PDEδ in either the closed or open conformation that will interfere with the delivery of Ras at the right membrane trap (Fig. 8.4).

The small molecule deltarasin was designed to block the farnesyl-binding pocket of the solubilization factor PDEδ and thereby compete against the binding of the farnesyl moiety of Ras proteins (Zimmermann et al. 2013). The inhibition of the Ras–PDEδ interaction with deltarasin resulted in relocalization of Ras to endomembranes. Deltarasin was cytotoxic to human pancreatic ductal adenocarcinoma (hPDAC) cell lines that were dependent on oncogenic Ras signaling whereas it exhibited only slight cytostatic effects on KRas-independent hPDAC cells. These cytotoxic and cytostatic effects on hPDAC cells were also found by an inducible knockdown of PDEδ using a doxycycline inducible anti-PDEδ shRNA expression system. A xenograft murine model bearing subcutaneous injected Ras-dependent hPDAC cells showed a pronounced cytostatic effect after intraperitoneal injection of deltarasin. Because of its importance in the localization of all farnesylated Ras proteins, inhibition of the binding capacity of PDEδ therefore represents a promising avenue of research to affect oncogenic Ras signaling.

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Chapter 9
Ras Nanoclusters

Yong Zhou and John F. Hancock

Abstract Ras proteins are lipid-anchored proteins that must be localized to cellular membranes, predominantly the plasma membrane, in order to function in signal transduction circuits. Recent work has shown that in addition to membrane localization, a complex nanoscale spatial organization is also required for Ras biological activity. Here we review the spatial organization of Ras proteins on the plasma membrane and discuss the molecular mechanisms that drive and potentially regulate this complex and dynamic nanoscale distribution.

Keywords Ras proteins • Ras Nanoclusters • Lipid composition • Phosphatidylserine • Cholesterol • Ras Nanoclusters inhibitors • RAF Inhibitors

9.1 Introduction

Ras GTPases are molecular switches that regulate critical cell functions including cell growth, proliferation, and differentiation (Hancock 2003; Mor and Philips 2006). For biological activity Ras must be localized to the inner leaflet of the plasma membrane. Membrane localization requires the attachment of a C-terminal lipid anchor, which is achieved through a set of sequential enzymatic reactions. The three ubiquitously expressed Ras isoforms, H-, N-, and K-Ras, are synthesized in the cytosol. Each comprises a near identical G-domain (amino acids 1–165), which binds guanine nucleotides and interacts with effectors and exchange factors and a highly divergent C-terminal hypervariable region (HVR amino acids 165–188/9) that ends in a conserved CAAX motif (Prior and Hancock 2001, 2012). Posttranslational processing is initiated by protein farnesyl transferase that adds a farnesyl group to the cysteine residue of the CAAX motif (Willumsen et al. 1984;

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Casey et al. 1989; Gutierrez et al. 1989; Hancock et al. 1989). The farnesylated CAAX motif directs Ras to the cytosolic surface of the endoplasmic reticulum, where it is further modified by Rce1 (Ras and a-factor converting enzyme), to remove the AAX tripeptide (Boyartchuk et al. 1997; Kim et al. 1999; Otto et al. 1999), and by ICMT (isoprenylcysteine carboxyl methyltransferase), which methyl esterifies the α-carboxyl group of the now C-terminal farnesylated cysteine (Hrycyna et al. 1991; Dai et al. 1998). N- and H-Ras are further lipid modified by endomembrane localized palmitoyl transferases, which add palmitate to one or two cysteine residues in the cognate HVR. N- and H-Ras then traffic to the plasma membrane via the classic secretory pathway through Golgi apparatus (Choy et al. 1999; Apolloni et al. 2000). In contrast a polybasic sequence in the K-Ras HVR, operating in concert with the processed CAAX motif, targets K-Ras to the plasma membrane directly from ER via a poorly characterized mechanism (Hancock et al. 1990, 1991). Recent work however has implicated two prenyl-binding chaperone proteins as regulators of K-Ras plasma membrane interactions. PDEδ and PRA1 bind the prenylated C terminus of multiple small GTPases, including K-Ras, and enhance the dissociation rate of K-Ras from the plasma membrane (Bhagatji et al. 2010). Specifically, PDEδ may maintain the fidelity of K-Ras plasma membrane targeting by increasing the diffusion rate of soluble, prenylated K-Ras (Bhagatji et al. 2010; Ismail et al. 2011; Chandra et al. 2012; Philips 2012). Indeed small molecules that block the interaction of PDEδ with K-Ras significantly mislocalize K-Ras from the plasma membrane and consequently inhibit K-Ras signal transmission (Zimmermann et al. 2013).

9.2 Nanocluster Organization on the Plasma Membrane

On the plasma membrane, a fraction of Ras spatially segregates into domains, termed nanoclusters with a radius of ~9 nm (Hancock and Parton 2005) (Fig. 9.1). The characterization of Ras nanoclusters has been quantified extensively using electron microscopy (EM) combined with spatial mapping. Intact plasma membrane sheets prepared from cells expressing GFP-tagged Ras are attached to EM grids, labeled with gold nanoparticles coupled to anti-GFP antibody and imaged using EM. The gold particle spatial point pattern distribution can then be mathematically analyzed using Ripley's K-function, a statistical analysis widely used in biology, ecology, astrophysics, and social sciences to quantify the spatial distribution of structures or events in a given area. The simplest use of spatial analysis is to determine whether events are randomly arrayed versus clustered or distributed and if so on what length scales (Ripley 1977; Diggle 1979; Diggle et al. 2000; Prior et al. 2003; Plowman et al. 2005; Perry et al. 2006; Kiskowski et al. 2009). EM-spatial mapping analysis shows that Ras proteins distribute in two populations on the plasma membrane: approximately ~44 % of Ras proteins segregate into nanoclusters, while the remaining Ras molecules exist as monomers. Each nanocluster contains ~6–7 Ras proteins. Both Ras nanoclusters and Ras monomers are randomly arrayed on the plasma membrane and are not associated

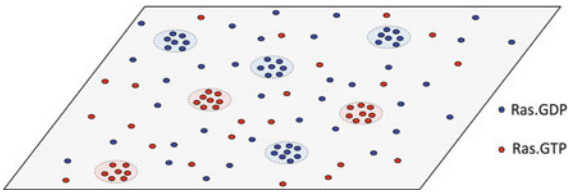

Fig. 9.1 Ras proteins form spatially segregated and functionally distinct nanoclusters on the plasma membrane. Ras proteins distribute heterogeneously on the plasma membrane into two populations: mobile monomers (~56 %) and immobile clusters (~44 %). The fraction of Ras proteins in nanoclusters, the clustered fraction is independent of expression level. A typical Ras nanocluster has a diameter of 12–20 nm and contains ~6–7 Ras proteins. The average lifetime of a Ras cluster is between 0.1 and 1 s. Ras isoforms segregate into isoform- and guanine nucleotide-specific nanoclusters (see Fig. 9.2)

with any EM-visible ultrastructure (Fig. 9.1). Single particle tracking experiments of GFP-Ras on the basolateral plasma membrane yield estimates of Ras nanocluster lifetimes in the range of ~0.1–1 s (Murakoshi et al. 2004; Plowman et al. 2005). Cholesterol-dependent H-Ras.GDP clusters have the shortest lifetime of <0.1 s while H-Ras.GTP and K-Ras.GTP nanoclusters have longer lifetimes of 0.5–1 s (Murakoshi et al. 2004; Hancock and Parton 2005). From these and other key experiments, a picture emerges of a highly dynamic spatial system where collision between freely diffusing Ras monomers forms transient, immobile nanoclusters randomly over the surface of the inner plasma membrane, which then disassemble back into freely diffusing monomers.

The spatiotemporal dynamics and nanoscale organization of the Ras nanocluster system are very similar to glycosylphosphatidylinositol (GPI)-anchored proteins on the outer leaflet of the plasma membrane that also coexist in a combination of immobile transient nanoclusters constantly exchanging with a larger population of freely diffusing monomers (Sharma et al. 2004). For both Ras and GPI-anchored proteins the fraction of proteins arrayed in nanoclusters, the clustered fraction, is insensitive to plasma membrane expression level, i.e., the system is actively held in a nonequilibrium state. In the case of the GPI-anchored proteins this nanoclustering system property is closely linked to actin trundling in the cortical actin cytoskeleton (Johannes and Mayor 2010; Chaudhuri et al. 2011; Gowrishankar et al. 2012). Like GPI-anchored proteins, Ras proteins bind to the plasma membrane primarily via their lipid anchors. In this context the lateral segregation of Ras proteins is a function of aggregate interactions of plasma membrane components with the different lipid anchors, but is also determined by polar residues in the flanking HVR and the G-domains (Prior et al. 2003; Rotblat et al. 2004; Plowman et al. 2005, 2008; Roy et al. 2005; Abankwa et al. 2007, 2008a; Gorfe et al. 2007b; Zhou et al. 2012). Generally, the fully saturated palmitoyl chains of H-Ras and N-Ras allow these isoforms to form nanoclusters with more tightly ordered membrane components, such as cholesterol, sphingolipids, and membrane phospholipids with fully saturated chains. In contrast the highly branched and poly-unsaturated farnesyl chains and polybasic sequence in the HVR of K-Ras favor

interaction with more polar lipids of the plasma membrane (Janosi et al. 2012; Li et al. 2012). These predictable biophysical principles of engagement with a lipid bilayer are however differentially utilized by the different isoforms.

9.3 Ras Nanocluster Composition

9.3.1 Isoform Specific and GTP-Dependent Lateral Segregation

A combination of EM spatial mapping methods, different FRET techniques, computational modeling, and molecular dynamic simulations (MD) have been used to investigate the structure and function of Ras nanoclusters (Prior et al. 2003; Plowman and Hancock 2005; Gorfe et al. 2007a; Abankwa et al. 2008b, 2010; Plowman et al. 2008; Janosi et al. 2012). A synthesis of these diverse data sets shows that H-, N-, and K-Ras assemble into spatially nonoverlapping nanoclusters, with each isoform exhibiting GTP-dependent lateral segregation into spatially nonoverlapping GDP- and GTP nanoclusters (Fig. 9.1). These different nanoclusters display different levels of sensitivity to plasma membrane perturbation, such as cholesterol-depletion, actin cytoskeleton disruption, and intercalation of biological amphiphiles NSAIDs and bile acids (Prior et al. 2003; Plowman et al. 2005; Zhou et al. 2012, 2013, 2014). H-Ras.GDP forms cholesterol-dependent nanoclusters that are markedly disrupted by cholesterol-depletion via methyl-β-cyclodextrin (MβCD) (Prior et al. 2003) and partially disrupted in cells treated with latrunculin to deplete actin (Plowman et al. 2005). H-Ras.GTP nanoclusters in contrast are insensitive to MβCD and latrunculin treatment, demonstrating that H-Ras.GTP forms cholesterol-independent, actin-independent clusters (Prior et al. 2003; Plowman et al. 2005). N-Ras.GDP nanoclusters are insensitive to cholesterol depletion while N-Ras.GTP clusters are highly sensitive to MβCD treatment (Roy et al. 2005), thus the cholesterol dependence of the guanine nucleotide-bound states of N-Ras nanoclusters is the reverse of H-Ras. Both GDP- and GTP-bound K-Ras assemble into nanoclusters that are insensitive to MβCD but which are perturbed by latrunculin treatment. K-Ras.GTP and K-Ras.GDP nanoclusters although spatially nonoverlapping are thus both actin-dependent but cholesterol-independent structures (Prior et al. 2003; Plowman et al. 2005).

9.3.2 Acidic Lipid Content

Apart from cholesterol the lipid composition of the various Ras nanoclusters is also vastly different (Fig. 9.2). In a systematic study, EM-bivariate spatial mapping with GFP-tagged lipid-binding domains, such as PH domains and C2 domains, and

RFP-tagged Ras proteins was used to characterize the lipid content of different Ras nanoclusters (Zhou et al. 2014). Phosphatidic acid (PA), labeled by GFP-Spo20, co-clustered with K-Ras.GTP and H-Ras.GTP nanoclusters, but not H-Ras.GDP nanoclusters, suggesting that PA is mainly found in K-Ras.GTP and to a lesser extent H-Ras.GTP nanoclusters, but not at all in H-Ras.GDP clusters. Phosphatidylinositol 4,5-biphosphate (PIP_2), labeled with GFP-PH-PLCδ, and phosphatidylinositol 3-phosphate (PI_3P), labeled with GFP-PH-FYVE, are both highly enriched in H-Ras.GDP nanoclusters, but not in H-Ras.GTP or K-Ras.GTP nanoclusters. A detectable but low level of phosphatidylinositol 4-phosphate (PI_4P), labeled with GFP-FAPP1, was found in all Ras nanoclusters examined. Of particular note phosphatidylserine (PS), labeled with GFP-LactC2, co-clustered with all Ras nanodomains tested (Fig. 9.2). However, as we will discuss later, although PS is a common constituent of all Ras nanoclusters investigated, PS is of variable importance to the structural integrity of different Ras nanoclusters.

9.3.3 Protein Scaffolds

In addition to lipids and the actin cytoskeleton, protein scaffolds also participate in facilitating or enhancing Ras nanoclustering. EM-spatial mapping experiments and FLIM-FRET experiments demonstrate that over-expression of Galectin-1 (Gal-1) enhances nanoclustering of H-Ras.GTP (Prior et al. 2003). Gal-1 co-localizes extensively with H-Ras.GTP on the plasma membrane (Fig. 9.2) and acute activation of H-Ras significantly enhances Gal-1 localization and clustering on the plasma membrane (Belanis et al. 2008). Together the evidence suggests that Gal-1 is a functional component of H-Ras.GTP nanoclusters and Gal-1 levels may therefore modulate H-Ras signaling activity. Conversely galectin-3 (Gal-3) associates extensively with K-Ras.GTP nanoclusters (Shalom-Feuerstein et al. 2008) (Fig. 9.2). EM-spatial mapping and FLIM-FRET experiments demonstrate that ectopic expression of Gal-3 enhances, whereas suppressing Gal-3 expression reduces K-Ras.GTP nanoclustering (Shalom-Feuerstein et al. 2008). Taken together these experiments suggest that the cytosolic level of Gal-3 contributes to setting the K-Ras.GTP clustered fraction (Shalom-Feuerstein et al. 2008). In support of this analysis a computational model of K-Ras Gal-3 interactions on the plasma membrane was able to reproduce the nonequilibrium distribution of K-Ras nanoclusters and monomers and show that the K-Ras clustered fraction is indeed a function of the cytosolic concentration of Gal-3 (Tian et al. 2010).

Other identified protein scaffolds for K-Ras nanoclusters are nucleophosmin (NPM) and nucleolin (Fig. 9.2). Although NPM and nucleolin are multifunctional phosphoproteins oscillating between nucleolus and cytoplasm, proteomic screening discovered that they can interact with K-Ras, but not H-Ras, on the plasma membrane (Inder et al. 2009). And mapped the N-terminal region of NPM as associating with the polybasic domain of K-Ras (Inder et al. 2009). Increasing the concentration of cytosolic NPM or nucleolin markedly enhances K-Ras

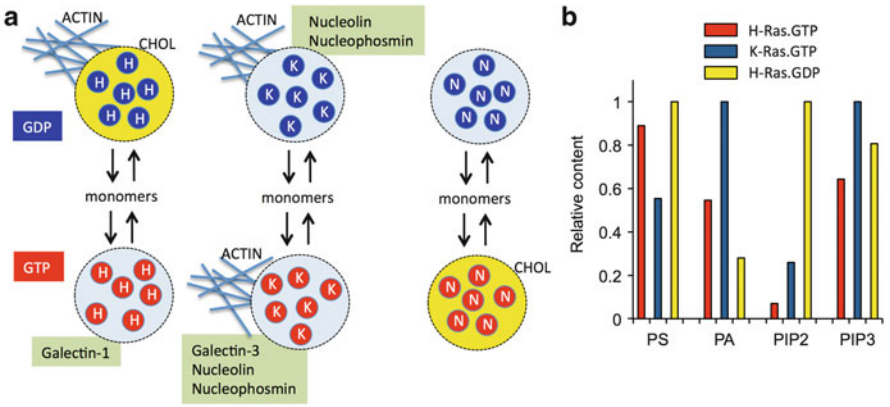

Fig. 9.2 Ras isoforms occupy spatially nonoverlapping nanoclusters. (**a**) H-, N-, and K-Ras isoforms exchange between monomers and clusters as in Fig 9.1. Each isoform however segregates into spatially distinct, nonoverlapping GDP- and GTP clusters. Each Ras nanocluster has a distinct proteolipid content that exhibits differential dependence on the actin cytoskeleton and cholesterol. The different nanocluster compositions specify recruitment of different sets of effectors, differential activation of signaling pathways, and thus underlay biological differences between the three Ras isoforms. (**b**) Characterization of the relative lipid composition of three different Ras nanoclusters

nanoclustering and stabilizes K-Ras localization to the plasma membrane (Inder et al. 2009) (Fig. 9.2).

9.4 Towards a Mechanism of Ras Nanocluster Formation

9.4.1 Lipid Anchors

The differential lateral segregation of Ras proteins on the plasma membrane is determined by multiple factors, including the lipid anchor, the HVR, and the G-domain. In addition to the C-terminal farnesyl-cysteine methyl ester shared by all three fully processed isoforms, Ras proteins need a second signal to localize to the plasma membrane. H-Ras has two palmitoyl chains located at cysteine (Cys) 181 and cysteine 184. These palmitoyl chains have distinct roles in regulating H-Ras localization and nanoclustering. Confocal imaging demonstrates that mono-palmitoylation on Cys181 (using a point mutant H-RasC184S) is sufficient to localize H-RasC184S to the plasma membrane, whereas mono-palmitoylation on Cys184 (using point mutant H-RasC181S) results in significant accumulation of H-RasC181S in the Golgi apparatus (Goodwin et al. 2005; Rocks et al. 2005; Roy et al. 2005). EM-spatial mapping experiments further reveal that mono-palmitoylation on Cys181 (mutant H-RasG12V C184S) missorts H-Ras.C184S.

GTP to cholesterol-dependent clusters that are sensitive to MβCD (Roy et al. 2005). Since dual-palmitoylated H-Ras.GTP localizes to cholesterol-independent domains, this data suggests that the palmitoyl chain on Cys181 is critical for correct H-Ras.GTP nanocluster assembly. Interestingly, the cholesterol-dependent clustering of mono-palmitoylated H-Ras.C184S.GTP is a phenocopy of N-Ras.GTP, which is also mono-palmitoylated on Cys181 (Prior et al. 2003; Roy et al. 2005). In contrast, nanoclusters of H-Ras.GTP mono-palmitoylated on Cys184 (mutant H-RasG12V C181S) maintain the insensitivity to MβCD treatment, similar to dual palmitoylated H-Ras.GTP (Roy et al. 2005), suggesting that palmitoylation on Cys184 is a key determinant of accurate H-Ras.GTP nanocluster formation. The C-terminal polybasic domain operating in concert with the farnesyl lipid anchor is essential for K-Ras localization to the plasma membrane (Hancock et al. 1990, 1991) and is sufficient for K-Ras nanoclustering (Prior et al. 2003). The high polarity of the K-Ras polybasic domain dictates that K-Ras favors a more polar, fluid, cholesterol-poor lipid environment.

9.4.2 HVR and G-Domain Conformational Orientation

The orientation of the G-domain with respect to the membrane normal is a novel codec for Ras membrane organization and isoform-specific signaling (Abankwa et al. 2007, 2008a). Results from molecular dynamics, FRET imaging, signaling, and EM data shows that the Ras G-domain undergoes nucleotide-dependent changes in orientation (Fig. 9.3). GTP loading triggers structural rearrangements in switch I and II that are transmitted through a network of salt bridges involving D47 and E49 in the β2–β3 loop, R161 and R164 in helix-α5 that ultimately release R169, and K170 in the HVR from membrane binding to allow, in the case of H-Ras, a ~100° rotation of the G-domain (Abankwa et al. 2007, 2008a). After rotation, R128 and R135 in helix-α4 now interact with membrane lipids and stabilize the new orientation. The coupling mechanism that transmits G-domain conformational changes was termed switch III, and the charged residues in helix-α4 or the proximal HVR that engage in mutually exclusive interactions with the lipid bilayer are called the switched elements (Abankwa et al. 2007, 2008a). The correct orientation of the H-Ras G-domain is critical for effector and scaffold interactions (Abankwa et al. 2008a, 2010; Guzman et al. 2014).

A similar switch III mechanism operates in N-Ras and K-Ras (Fig. 9.4), but each isoform has a different G-domain orientation that is optimal for binding to CRAF (Abankwa et al. 2008a, 2010). In consequence, mutations in helix-α4 that enhance CRAF and PI3K binding by H-Ras.GTP by stabilizing helix-α4 interactions with the membrane have precisely the opposite effect on CRAF and PI3K binding by K-Ras.GTP (Abankwa et al. 2008a, 2010). G-domain orientation is therefore a novel codec for regulating effector interactions. This orientation-based code is in good agreement with the various angularities noted previously for different Ras–effector complexes (Pacold et al. 2000; Herrmann 2003). It is worth emphasizing

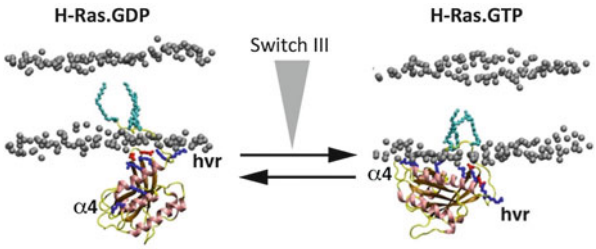

Fig. 9.3 MD atomistic snapshots of the GDP- and GTP orientations of H-Ras on a lipid bilayer, charged residues on the switch regions, helix-α4 or the HVR stabilize the alternate orientations

that the key residues in the switched elements are basic, thus electrostatic interactions with anionic lipids are as important for H- and N-Ras plasma membrane interactions as for K-Ras. Importantly, other investigators working with N-Ras and K-Ras in model membrane systems have now confirmed the role of the membrane in constraining Ras G-domain conformations (Kapoor et al. 2012a, b), and recently, mutations that activate Ras by subverting switch III have been identified in Noonan syndrome (Cirstea et al. 2010).

This balance model rationalizes the key contributions of the HVR to H-Ras lateral segregation identified in earlier functional studies (Jaumot et al. 2002; Rotblat et al. 2004). The model identifies the role of specific charged residues in the HVR and the need for a certain HVR length in order for the G-domain balance to operate. Implicit in this correlation between G-domain orientation and signaling function is the consecutive hypothesis that G-domain orientation is also fundamental to GTP-dependent lateral segregation, i.e., the different GTP and GDP orientations allow H-Ras to form different types of nanocluster. In addition to the different plasma membrane contacts of the two switch elements in each orientation that may directly enable different lipid sorting to occur, detailed MD simulations provide clues to an additional mechanism (Gorfe et al. 2007b). The anchor peptide of H-Ras.GDP is inserted more deeply among the polar lipid head groups of the bilayer than in H-Ras.GTP, and the order parameters of the two palmitate chains are higher in H-Ras.GDP than H-Ras.GTP (Gorfe et al. 2007b). The more ordered, extended palmitate conformations in H-Ras.GDP would be expected to favor interaction with liquid ordered, L_o, lipids and cholesterol and thus to preferentially assemble into lipid raft like assemblies.

Collecting these ideas together isoform-specific and GTP-dependent lateral segregation originates from a number of factors. Firstly, the lipid anchors on H-, N-, and K-Ras are different. Since lipid immiscibility within the plasma membrane must contribute to lateral segregation, mono-palmitoylated N-Ras, di-palmitoylated H-Ras, and polybasic K-Ras will necessarily favor different lipid environments (Janosi et al. 2012; Li et al. 2012), thus giving rise to nonoverlapping spatial distributions. Additionally, activation-dependent differences in G-domain orientation allow for different lipid sorting by anchor flanking sequences and changes also to the extent or nature of anchor engagement with the lipid bilayer (Fig. 9.4). These have been best characterized for H-Ras, but also operate in N-Ras and K-Ras (Abankwa et al. 2010). Indeed the G-domain orientations of K-Ras, N-Ras, and

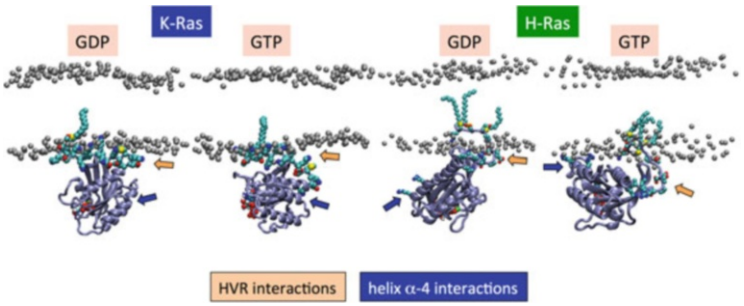

Fig. 9.4 H-, N-, and K-Ras have different G-domain orientations with respect to the plasma membrane. The G-domain of H-Ras exhibits ~120° reorientation between the GDP- and GTP bound states, which are stabilized by interactions of the HVR or helix α4. The orientation changes of K-Ras.GDP and K-Ras.GTP are more subtle and are mediated largely through their HVR. Different orientations of the Ras isoforms lead to different sequences in contact with the plasma membrane and a different capacity for lipid sorting and nano-assembly

H-Ras are all strikingly different and behave differently in response to GTP loading (Abankwa et al. 2010; Janosi and Gorfe 2010; Lukman et al. 2010), identifying the fundamental molecular mechanisms that drive Ras lateral segregation.

9.4.3 Influence of Lipid Content in the Plasma Membrane

Recent studies have shown that the lateral distribution of various plasma membrane lipids has significant effects on the efficient lateral segregation of Ras nanoclusters (Ariotti et al. 2014; Zhou et al. 2014) (Fig. 9.2). As discussed earlier, PS is a structural component of K-Ras nanoclusters. PSA3 cells lack the PS synthase (PSS1), which renders the cells ethanolamine auxotrophs (Lee et al. 2012), in consequence this allows dose-dependent manipulation of the PS content of the plasma membrane of PSA3 cells by controlling the ethanolamine content of the growth medium (Lee et al. 2012; Zhou et al. 2014). In PSA3 cells EM co-clustering and FLIM-FRET experiments show that efficient lateral spatial segregation of H-Ras.GTP from K-Ras.GTP only occurs over a narrow range of plasma membrane PS levels (Zhou et al. 2014). At PS levels above or below the optimal level, H-Ras.GTP and K-Ras.GTP molecules mix extensively and form heterotypic, mixed nanoclusters comprising H-Ras and K-Ras proteins. Cholesterol content also modulates lateral segregation. EM and FLIM-FRET experiments show that depleting plasma membrane cholesterol by MβCD leads to extensive mixing of H-Ras.GTP and H-Ras.GDP (Ariotti et al. 2014), indicating that the GTP-dependent lateral segregation of H-Ras is also highly sensitive to cholesterol content in the plasma membrane (Ariotti et al. 2014). This mixing of H-Ras.GTP and H-Ras.GDP attenuates H-Ras signal transmission (Ariotti et al. 2014) and also occurs in cells depleted of caveolae (Roy et al. 1999; Ariotti et al. 2014). Interestingly, the ability

of PS and cholesterol to non-linearly influence Ras isoform segregation mimics non-linear lipid demixing in synthetic model liposomes (Veatch and Keller 2002, 2003; Baumgart et al. 2003), suggesting conserved lipid biophysics.

Perturbations of not only lipid content but also lipid distributions in the plasma membrane can disrupt Ras lateral segregation. The majority of PS in the plasma membrane (~60 %) is immobile (Kay et al. 2012), and thus unlikely to be available to participate in Ras nanoclustering. However the stability and integrity of K-Ras nanoclusters are highly sensitive to changes in the PS mobile pools of the plasma membrane (Zhou et al. 2014). Thus increasing the PS mobile pool by disrupting actin, which does not alter the total PS level in the plasma membrane, leads to significant mixing of H-Ras.GTP and K-Ras.GTP (Zhou et al. 2014). A phenocopy of the effect of increasing the total PS level of the plasma membrane is discussed earlier. Interestingly, at normal plasma membrane PS levels, H-Ras.GTP competes with K-Ras.GTP for a mobile pool of PS and suppresses K-Ras.GTP nanoclustering. Activated H-Ras thus remotely regulates the formation of spatially segregated K-Ras.GTP clusters through the intermediary of PS spatiotemporal dynamics (Zhou et al. 2014), a phenomenon known as spatial cross talk. Together these data suggest that the spatial segregation between Ras isoforms is a highly dynamic process and lipid distribution and content in the plasma membrane play a critical role in the efficient spatial segregation of Ras isoforms.

9.5 Ras Dimerization

In addition to assembling into nanoclusters comprised of multiple Ras molecules together with well-defined, transient lipid assemblies, recent studies indicate that Ras proteins also dimerize (Guldenhaupt et al. 2012). Dichroic attenuated total reflectance-Fourier transform infrared (ATR-FTIR) measurements, coupled with molecular-mechanics (MM) simulations using purified N-Ras on a synthetic model bilayer, show that N-Ras G-domain orients almost perpendicular to the bilayer plane (Guldenhaupt et al. 2012), an orientation that is significantly stabilized by dimer formation. Interestingly a similar orientation for N-Ras in intact cells was suggested by the experiments of Abankwa et al (Abankwa et al. 2010). The dimerization interface located of N-Ras appears to involve helices α4/α5 and the β2-β3 loop (Guldenhaupt et al. 2012), thus in this system the dimerization is driven by G-domain interactions. Conversely, MD simulations of multiple copies of H-Ras on a phase separating lipid bilayer also shows that the minimal membrane-anchoring domain of H-Ras, tH (C-terminal amino acids 180–189), spontaneously forms nanoclusters with an average stoichiometry of ~6 molecules per cluster, emulating the situation in intact cells (Plowman et al. 2005), but on a background of a higher frequency of dimers than would be expected purely from random collisions, suggesting some intrinsic stability of anchor dimers (Janosi et al. 2012). In this case however the dimerization surface must involve the anchor peptide or anchor lipids not the (absent) G-domain. RAF also forms dimers on the

plasma membrane (Nan et al. 2013). Photo-activated localization microscopy (PALM) combined with spatial mapping shows that the presence of K-Ras.GTP induces dimerization of CRAF on the plasma membrane (Nan et al. 2013), suggesting that K-Ras dimers may also exist on the PM. It therefore seems likely that Ras dimers may be the building blocks for the higher order multimeric structures that become nanoclusters.

9.6 Ras Nanoclusters and Signal Transmission

9.6.1 Effector Binding

Ras nanoclusters are essential for MAPK signal transmission. Ras.GTP nanoclusters are the predominant sites for recruitment of RAF, MEK, and ERK to the plasma membrane. The latter two kinases are co-recruited on the scaffold protein KSR (Ory et al. 2003; McKay et al. 2009). In consequence activation of the RAF/MEK/MAPK cascade is spatially restricted to Ras.GTP nanoclusters (Harding et al. 2005; Tian et al. 2007; Harding and Hancock 2008a, b; Plowman et al. 2008; Kholodenko et al. 2010). Intriguing emergent properties flow as a result of the spatiotemporal dynamics of Ras nanocluster formation and disassembly being imposed on plasma membrane activation of the MAPK cascade. These will be discussed later. There is also direct correlation between efficient RAF and PI3K binding and correct Ras G-domain orientation providing a further link between effector recruitment and activation and hence Ras nanoclustering (Abankwa et al. 2008b, 2010).

Many Ras effectors have specific lipid-binding domains that mediate localization to the plasma membrane and subsequent activation. For example, RAF has distinct PS- and PA-binding domains and binding to PS and PA is critical for RAF activation (Ghosh et al. 1996, 2003; McPherson et al. 1999). Thus, Ras nanoclusters are recruitment sites, where all the necessary cofactors for activating RAF, including Ras and various signaling lipids, are assembled to facilitate effector activation. Indeed, FLIM-FRET experiments show that, while the isolated CRAF RBD, which does not have any lipid-binding domain, binds to H-Ras.GTP and K-Ras.GTP with similar efficacy, addition of a PS-binding cysteine-rich domain (CRD) markedly enhances the binding specificity of RBD-CRD to K-Ras.GTP (Abankwa et al. 2010). This result suggests that isoform specificity of signal transmission originates from interactions of RAF with the cohort of lipids that are selectively assembled in the different Ras.GTP nanoclusters. Supporting this idea is the observation that the PA content of K-Ras.GTP nanoclusters is greater than that of H-Ras.GTP nanoclusters; PA is a key cofactor for RAF activation and K-Ras is a much more potent activator of RAF/MAPK signaling than is H-Ras (Yan et al. 1998; Voice et al. 1999).

Further illustration that isoform-specific effector activation depends on nanocluster lipid environment comes from an analysis of CRAF targeted to the plasma membrane by the membrane-anchoring domains of H-, N-, or K-Ras. Although all CRAF fusion proteins are localized to the plasma membrane, CRAF activation and MAPK downstream signaling output is markedly different for each anchor (Inder et al. 2008). CRAF targeted by the minimal membrane anchor of H-Ras (-tH), which directs CRAF to cholesterol-dependent nanodomains, is inactive (Inder et al. 2008). Whereas CRAF targeted by the minimal membrane anchor of K-Ras (-tK) or the full length HVR of K-Ras (-CTK) to cholesterol-independent domains is highly active, and CRAF targeted by the full length HVR of H-Ras (-CTH) is active but much less so than CRAF-CTK (Inder et al. 2008).

9.6.2 Nanoclusters Act as Signal Nanoswitches

The spatiotemporal dynamics of the Ras nanocluster system imposes important emergent properties on RAF/MAPK activation. To reiterate some of these key features: approximately 40 % of K-Ras.GTP proteins are organized into nanoclusters (Hansen et al. 2003; Plowman and Hancock 2005). The lifetime of the clusters is short, <1 s so there is a dynamic flux with the 60 % of non-clustered K-Ras proteins that are arrayed as monomers (Murakoshi et al. 2004; Hancock 2005; Plowman and Hancock 2005). This distribution is also actively maintained in a nonequilibrium state such that the ratio of K-Ras.GTP proteins in nanoclusters to K-Ras.GTP proteins diffusing as monomers remains constant over a multi-log range of K-Ras.GTP levels (Plowman and Hancock 2005). This constant K-Ras.GTP clustered fraction (ϕ) therefore results in a linear relationship between K-Ras.GTP levels and number of K-Ras.GTP nanoclusters generated on the plasma membrane (Brauchi et al. 2007) (Fig. 9.5). Next, assembling the RAF/MEK/MAPK module exclusively in nanoclusters renders the biochemistry switch-like (Harding et al. 2005; Abankwa et al. 2010). Each nanocluster therefore operates as a transient, low threshold digital switch that dumps a fixed quantum of ERKpp into the cytosol (Brauchi et al. 2007; Harding and Hancock 2008b; Kholodenko et al. 2010), with ERKpp output limited by nanocluster lifetime. Although the biochemistry is digital the total ERKpp system response is analog because the number of nanoswitches is a linear function of the input parameter, for example, EGF (Brauchi et al. 2007; Harding and Hancock 2008b). This spatiotemporal organization of K-Ras allows the plasma membrane to operate as an analog-digital-analog (ADA) signal converter that transduces the strength of an epidermal growth factor (EGF) signal into a corresponding level of cytosolic activated ERKpp with high fidelity (Brauchi et al. 2007; Tian et al. 2007; Harding and Hancock 2008b; Kholodenko et al. 2010). A critical feature of the plasma membrane ADA converter is that the gain of the Ras.GTP to ERKpp signal response is set by ϕ (Fig. 9.5). Computation and experimentation show that as ϕ is reduced, the ERKpp signal response to EGF is decreased, even though the total amount of Ras.GTP generated

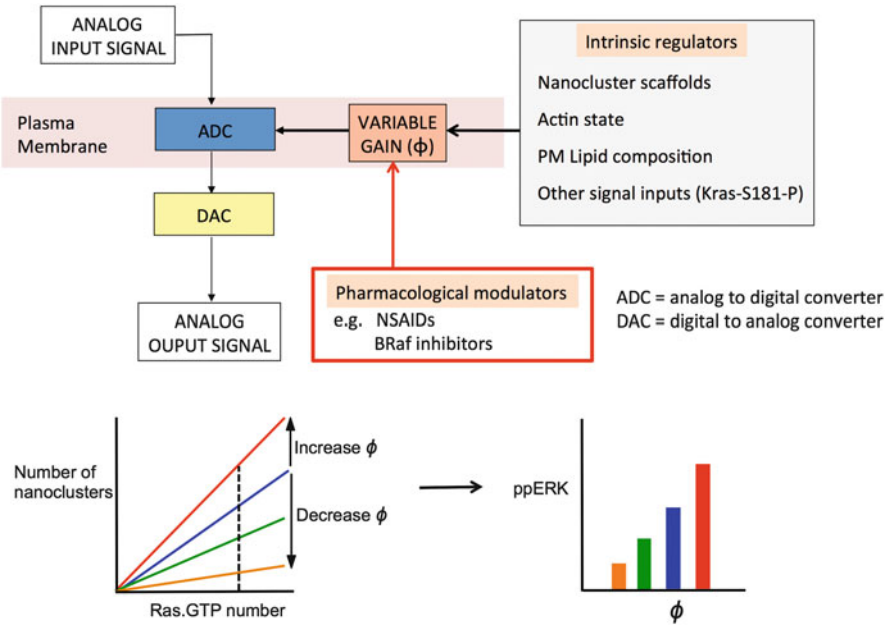

Fig. 9.5 The plasma membrane operates as an analog-digital converter (ADC) that digitizes signal input (e.g., EGF) by forming Ras nanoclusters. Each Ras nanocluster outputs a fixed quantum of ERKpp. The cytosol acts as a digital-analog converter DAC by summing the ERKpp outputs. The clustered fraction (ϕ) determines the number of nanoclusters formed from a given Ras.GTP level (*left graph*); therefore, at any specific Ras.GTP number (e.g., the *dotted vertical line*) a change in ϕ results in a change in the number of nanoclusters formed and a corresponding change in ERKpp output (*right graph*). ϕ can be regulated by intrinsic cell mechanisms and also pharmacologically

by each dose of EGF is unchanged (Brauchi et al. 2007; Tian et al. 2007; Harding and Hancock 2008b; Kholodenko et al. 2010). Importantly, we see exactly the same effect when ϕ is reduced in cells expressing oncogenic mutant Ras, although the cell has an elevated amount of Ras.GTP reducing ϕ abrogates Ras signal output (Plowman and Hancock 2005) (Fig. 9.5). As already discussed there are multiple intrinsic cellular inputs that set the value of ϕ (Fig. 9.5). These include: the actin cytoskeleton (Sharma et al. 2004; Plowman et al. 2005; Goswami et al. 2008; Chaudhuri et al. 2011), specific nanocluster scaffolds such as Gal3 and nucleophosmin (NPM) (Elad-Sfadia et al. 2004; Plowman et al. 2008; Shalom-Feuerstein et al. 2008; Inder et al. 2009, 2010; Tian et al. 2010), and caveolin-1 (Cav1) (Nicolau et al. 2006; Hill et al. 2008; Bastiani et al. 2009; Ariotti et al. 2014).

9.7 Perturbation of Ras Nanoclusters Leads to Altered MAPK Signaling

The critical role of Ras nanoclusters in Ras signal transmission suggests that pharmacological targeting of nanoclustering maybe a viable therapeutic strategy to block aberrant Ras signal transmission in human tumors. Some recent advances in this area have been reported.

9.7.1 Drugs Targeting PS Distribution

Ras nanocluster formation and stability depend heavily on interactions between Ras structure and lipids in the plasma membrane. Thus, lipid environment within the plasma membrane is crucial to the stability of Ras nanoclusters. Agents that alter lipid level and/or distribution in the plasma membrane change Ras nanoclustering and consequentially modify MAPK signal output. As a structural component of K-Ras nanoclusters, PS contributes significantly to the stability and dynamics of K-Ras clusters and hence to K-Ras-dependent MAPK signaling (Cho et al. 2012b; van der Hoeven et al. 2013; Zhou et al. 2014). By using high-content screening assays, fendiline and staurosporines were found to reduce plasma membrane PS content and perturb plasma membrane PS organization (Cho et al. 2012b; van der Hoeven et al. 2013). The molecular mechanisms are unrelated to the known pharmacology of fendiline as an L-type calcium channel blocker and the staurosporines as protein kinase C inhibitors. However, the effects of these compounds on PS distribution cause mislocalization of K-Ras.GTP from the plasma membrane and impaired nanoclustering of the K-Ras.GTP that remains on the plasma membrane (Cho et al. 2012b; van der Hoeven et al. 2013). The compounds have minimal effect on H-Ras.GTP plasma membrane localization or nanoclustering (Cho et al. 2012b; van der Hoeven et al. 2013) consistent with PS not being required for H-Ras nanocluster stability (Zhou et al. 2014). Fendiline and staurosporines both inhibit K-Ras dependent MAPK activation and block the proliferation of K-Ras transformed cancer cell lines (Cho et al. 2012b; van der Hoeven et al. 2013).

9.7.2 Biologically Active Amphiphilic Agents Partition into the Plasma Membrane and Interfere with Membrane Immiscibility

In addition to gross alteration in lipid levels in the plasma membrane, partitioning of amphiphilic agents into the plasma membrane can change lipid-lipid interactions, alter lipid immiscibility and ultimately perturb Ras nanoclustering and

signaling. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, indomethacin, naproxen and salicylate, and bile acids are biologically active amphiphiles that strongly associate with lipids and affect membrane properties (Hofmann and Small 1967; Lichtenberger 1995; Lichtenberger et al. 1995, 2006; Zhou and Raphael 2005; Zhou et al. 2009). For example, salicylate partitions into membranes, decreases bending rigidity, and enhances the formation of spontaneous pores (Zhou and Raphael 2005). Indomethacin, a highly potent NSAID, enhances phase separation and immiscibility in bilayers containing multiple lipid constituents (Zhou et al. 2010a, b). Indomethacin specifically stabilizes tightly packed lipids, such as fully saturated phosphatidylcholine (PC) and cholesterol whilst having little effect on the more fluid, loosely packed lipids (Zhou et al. 2010a, b). Subsequently, a study using EM-spatial mapping and FLIM-FRET shows that NSAIDs, including indomethacin, aspirin, ibuprofen, naproxen, and salicylate, stabilize cholesterol-dependent Ras nanoclusters such as H-Ras.GDP and N-Ras.GTP, in live cells, consistent with the findings in synthetic model bilayers (Zhou et al. 2012). On the other hand, NSAIDs have little effect on the cholesterol-independent K-Ras.GDP or K-Ras.GTP clusters, again consistent with the model liposome studies (Zhou et al. 2012). NSAIDs however induce mixing of originally spatially nonoverlapping cholesterol-dependent and cholesterol-independent clusters, such as H-Ras.GDP with either H-Ras.GTP or K-Ras.GTP. This NSAID-induced formation of heterotypic clusters composed of active Ras.GTP and GDP-bound Ras isoforms significantly compromises the ability of GTP-bound Ras to recruit RAF and thereby attenuates MAPK signaling (Zhou et al. 2012). NSAIDs, especially aspirin, are highly effective chemopreventive agents (Rothwell et al. 2007, 2010; Liao et al. 2012a, b; German et al. 2013). Furthermore, aspirin has been found to be especially effective in treating patients with PIK3CA mutations (Liao et al. 2012a, b; German et al. 2013). As PI3 kinases associate extensively with PIP_2, which preferentially localizes in cholesterol-enriched domains, the finding that NSAIDs specifically target cholesterol-dependent nanoclusters is potentially consistent with the clinical data. Traditionally, the biological effects of NSAIDs have been attributed to their ability to inhibit cyclooxygenase (COX) (Vane 1994). However, the ability of NSAIDs to alter Ras nanoclustering and MAPK signaling is completely independent of COX and depends exclusively on their ability to associate with lipid membranes (Zhou et al. 2012). The chemopreventive activity of NSAIDs may in turn be related in part to their effects on Ras nanoclustering.

Bile acids are biological detergents and are critical components in the mammalian digestive system (Hofmann and Small 1967). Bile acids behave as signaling molecules, in addition to aiding digestion of fat-soluble molecules (Hylemon et al. 2009). The molecular mechanism(s) for these biological effects of bile acids is still largely unknown. In phase separating giant plasma membrane derived vesicles (GPMVs) labeled with lipid probes that preferentially partition into either highly ordered cholesterol-enriched domains or highly fluid cholesterol-poor domains, the bile acid deoxycholic acid (DCA) preferentially partitions into and increases the polarity of the fluid, disordered phase (Zhou et al. 2013). DCA thereby

enhances phase separation and immiscibility in the GPMVs. Consistent with the findings in GPMVs, DCA selectively stabilizes K-Ras.GTP nanoclusters in intact cells, without having any effect on H-Ras nanoclusters (Zhou et al. 2013). DCA enhances MEKpp and ERKpp levels while having minimal effect on pAKT. Thus, while NSAIDs mainly decrease MAPK signaling and are chemopreventive, bile acids enhance MAPK signaling and are mostly carcinogenic. Their differential effects on Ras nanoclustering may underpin this distinct pharmacology.

9.7.3 RAF Inhibitors

Inhibitors of Ras effectors, such as RAF, also alter Ras nanoclustering. EM-spatial mapping, FLIM-FRET, and single particle tracking experiments show that BRAF inhibitors, such as SB590885 and sorafenib, markedly enhance nanoclustering of K-Ras.GTP and N-Ras.GTP while having little effect on H-Ras.GTP (Cho et al. 2012a). Over-expression of kinase-dead BRAF has a similar phenotype. The molecular mechanism involves inhibitor-induced homo-dimerization of BRAF and/or the inhibitor-induced heterodimerization of BRAF and CRAF (Hatzivassiliou et al. 2010; Heidorn et al. 2010; Poulikakos et al. 2010). The formation of stable RAF dimers with two RBDs generates a Ras cross-linker in treated cells that drives Ras dimerization and thereby enhances K-Ras.GTP and N-Ras.GTP nanoclustering (Cho et al. 2012a; Nan et al. 2013).

9.8 Conclusions and Perspective

Ras proteins form spatially segregated, dynamic, nanoclusters on the plasma membrane. The composition of nanoclusters is complex, including lipids and other membrane proteins and exhibiting variable requirements for the actin cytoskeleton. Ras isoforms form distinct nanoclusters that are dependent on the C-terminal lipid anchor, flanking protein sequences, guanine nucleotide-binding state, and G-domain orientation. The efficient lateral segregation of Ras proteins on the plasma membrane is essential for Ras signal transmission. Since generic biophysical interactions with lipids are a major contributor to Ras nanocluster formation, lateral segregation into nanoclusters may be a general feature of other lipid-anchored proteins. This possibility remains to be explored. Given the importance of nanoclustering to cellular signal transduction, targeting plasma membrane heterogeneity and nanoclustering is an attractive, viable new strategy for anti-Ras drug design.

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Chapter 10
Mouse Models of RAS-Induced Tumors and Developmental Disorders

Carmen Guerra and Mariano Barbacid

Abstract RAS oncogenes have been implicated in about one quarter of all human tumors including some of the cancers with worse prognosis such as lung adenocarcinoma, colorectal carcinoma, pancreatic ductal adenocarcinoma, and metastatic melanoma. In spite of the significant amount of knowledge accumulated over the last three decades regarding the molecular mechanisms by which RAS oncogenes induce malignant transformation, to date there are no efficacious therapies to selectively treat tumors carrying RAS mutations. One of the shortcomings in RAS research has been the lack of suitable experimental systems to study how RAS oncogenes induce cancer in an in vivo setting. The advent of sophisticated gene-targeting technologies are now making it possible to design mouse models of cancer that faithfully recapitulate the anatomo-pathological changes characteristic of those human tumors induced by RAS oncogenes. More recently, germline mutations in the three RAS loci have been found to be responsible for a series of developmental disorders known as RASopathies. Modeling these syndromes in mice should also help to understand the molecular events responsible for the developmental defects present in these human patients. This chapter summarizes those genetically engineered mouse models more frequently utilized to study RAS-induced tumors and developmental defects in an experimental setting. These mouse models should provide valuable experimental tools to identify molecular targets whose inhibition may open therapeutic avenues in the clinic, in a not too distant future.

Keywords Ras oncogenes • Cancer mouse models • Genetically engineered mouse models • Rasopathies

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10.1 Introduction

Arguably, the RAS family of proteins, H-RAS, N-RAS, K-RAS4A, and K-RAS4B, has been one of the most studied group of proteins in biology for the last three decades. Their prominence is due, in part, to their key role in controlling cell proliferation and differentiation by mediating mitogenic signaling from extracellular cues to the nuclear machinery as well as to their status as the representative group of more than 150 small G proteins involved in a diverse spectrum of cellular functions. Moreover, the RAS proteins are involved in a significant number of human cancers including those with worse prognosis such as adenocarcinomas of the lung, colon, and pancreas. More recently, RAS proteins have received additional attention due to their involvement in a series of rare developmental diseases, known as RASopathies.

Somatic mutations in RAS proteins have been identified in about ~30 % of human cancers (the COSMIC database; http://www.sanger.ac.uk/genetics/CGP/cosmic). Mutations affecting the K-RAS4A and K-RAS4B proteins are the most prevalent overall and are primarily involved in epithelial malignancies. In contrast, N-RAS mutations predominate in melanoma and hematopoietic tumors while H-RAS mutations are relatively rare (the COSMIC database; http://www.sanger.ac.uk/genetics/CGP/cosmic). Likewise, germline mutations in each of the three *RAS* genes induce similar, albeit distinct developmental disorders including Costello syndrome (CS) (H-*RAS*), Noonan syndrome (NS) (K-*RAS* and N-*RAS*), and Cardio-facio-cutaneous syndrome (CFC) (K-*RAS*) (reviewed in Rauen 2013). The molecular basis for the distinct incidence and differential involvement in various tumor types and/or developmental disorders are still unknown. It is possible that these differences are primarily due to the different patterns of expression of each of the three *RAS* loci. However, it is also possible that each RAS protein may have different signaling properties in different cellular contexts.

In an attempt to interrogate the precise mechanisms by which misregulation of RAS signaling induces cancer, investigators decided almost three decades ago to develop animal models of RAS-induced tumors. In the spring of 87, Brinster, Palmiter, and coworkers described that ectopic expression of a human H-*RAS* oncogene in acinar cells under the control of the Elastase promoter induced massive damage in the fetal pancreas (Quaife et al. 1987). Only mosaic transgenic mice survived to adulthood and developed pancreatic tumors. In the same year, Leder and coworkers reported the generation of another transgenic strain, later known as the "OncoMouse®," in which the retroviral v-H-*Ras* oncogene was ectopically expressed in breast tissue under the control of the MMTV promoter (Sinn et al. 1987). Unlike the Elastase-H-*RAS* transgenic animals, these mice were normal at birth. Yet, they developed malignant tumors, mainly adenocarcinomas of the mammary gland between 6 and 12 months of age (Sinn et al. 1987).

Additional mouse models expressing transgenes carrying RAS oncogenes were developed over the next decade. However, the value of these models to study human neoplasia was thwarted by two technical limitations. The transgenic RAS

oncogenes were not expressed from their endogenous promoter leading, in most cases, to aberrant levels of expression. Moreover, in some instances they did not generate the same tumor types in which they appeared mutated in human cancer. These considerations, not particularly obvious in those days, led to some misleading conclusions. One of the most notable was the inhibition of those mammary tumors present in the MMTV-v-H-*Ras* transgenic mice by Farnesyl Transferase inhibitors, a result that led to unfounded optimism regarding the potential use of these inhibitors in the clinic (Kohl et al. 1995). These results, along with those generated by using standard xenograft tumor models driven by human tumor cell lines in immuno-compromised mice, led some investigators to postulate that mouse tumors were not adequate models for human cancer.

The advent of genetic engineering by homologous recombination in embryonic stem cells opened the door to a new generation of mouse tumor models driven by mutations introduced in the endogenous loci, hence preserving all the regulatory properties of these cancer genes. Combination of these strains of genetically engineered mice (GEM) with strains carrying inducible recombinases has allowed investigators to express mutated oncogenes or inactivate tumor suppressors in a temporally (i.e., adult mice) and spatially (specific cell types or organs) controlled manner. These technologies have enormously facilitated the development of animal models that closely recapitulate the natural history of human tumors as well as other pathologies. Below is a brief overview of the most commonly used GEM models for RAS driven cancers and developmental disorders. A summary of these models appears in Table 10.1.

10.2 Mouse Models of K-Ras Driven Lung Adenocarcinoma

K-*RAS* oncogenes have been implicated in about one quarter of all human lung adenocarcinomas and are associated with the worse prognosis among this tumor type. The first GEM model aimed at recapitulating human lung tumors in mice was generated by Tuveson, Jacks, and coworkers (Johnson et al. 2001). This model, known as the K-Ras^{LA2} strain, was somewhat atypical since generation of a functional K-*Ras* oncogene relied on a stochastic recombinational event that could not be controlled experimentally (Johnson et al. 2001). Shortly thereafter, these investigators produced a fully controllable mouse model, the K-$Ras^{+/LSLG12D}$ strain that has become the "gold standard" for most K-*Ras* oncogene driven animal tumor models (Jackson et al. 2001). These mice carry an endogenous K-Ras^{G12D} allele whose expression is controlled by Cre-mediated recombination of a lox-STOP-lox (LSL) cassette inserted in the first intron of the K-*Ras* locus to prevent undesired expression of the K-$Ras^{LSLG12D}$ allele. Elimination of the LSL cassette by the Cre recombinase is most often carried out by intranasal instillation or tracheal infection with adenoviral particles expressing this bacterial recombinase (Adeno-Cre vectors). Infected K-$Ras^{+/LSLG12D}$ mice typically develop hyperplastic

Table 10.1 Summary of genetically engineered models for RAS-induced tumors and developmental disorders

Lung adenocarcinoma	**Activating strategy**	**Phenotype**	**References**
K-*Ras*^{LA2}	Spontaneous recombination of K-*Ras*^{G12D}	Adenomas and adenocarcinomas	Johnson et al. (2001)
K-*Ras*$^{+/LSLG12D}$ and K-*Ras*$^{+/LSLG12Vgeo}$	Intranasal or tracheal Adeno-Cre infection	Adenomas and adenocarcinomas	Jackson et al. (2001)
K-*Ras*$^{+/LSLG12Vgeo}$; *RERTn*$^{+/ert}$	4OHT injection/Tam diet[a]	Adenomas and adenocarcinomas	Guerra et al. (2003)
Tet-O-K*Ras*^{G12D}	Cross to CCSP-rtTA transgenic + Dox[a]	Adenomas and adenocarcinomas	Fisher et al. (2001)
K-*Ras*$^{+/LSLG12Vgeo}$	Cross to *RERTn*$^{+/ert}$ mice + Tam	Adenomas and adenocarcinomas	Guerra et al. (2003)
Pancreatic ductal adenocarcinoma	**Activating strategy**	**Phenotype**	**References**
K-*Ras*$^{+/LSLG12D}$	Cross to Pdx1-Cre or Ptf1a/P48-Cre transgenics	PanIN and PDAC with long latency	Hingorani et al. (2003)
K-*Ras*$^{+/LSLG12Vgeo}$ (embryonic activation)	Cross to Elastase-tTA;Tet-O-Cre transgenics	PanIN and PDAC with long latency	Guerra et al. (2007)
K-*Ras*$^{+/LSLG12Vgeo}$ (adult activation)	Cross to Elastase-tTA;Tet-O-Cre transgenics + Dox + Cer[a]	PanIN and PDAC with long latency	Guerra et al. (2007)
Hematological tumors	**Activating strategy**	**Phenotype**	**References**
K-*Ras*$^{+/LSLG12D}$	Cross to Mx1-Cre	Aggressive myeloproliferative neoplasia (MPN)	Braun et al. (2004)
N-*Ras*$^{+/LSLG12D}$	Cross to Mx1-Cre	Indolent MPN and myelodysplastic syndrome	Wang et al. (2010)
Intestinal and colonic adenocarcinomas	**Activating strategy**	**Phenotype**	**References**
Villin-K-*Ras*^{G12V}	N/A[b]	Adenomas and invasive adenocarcinomas	Janssen et al. (2002)
K-*Ras*$^{+/LSLG12Vgeo}$; *Apc*$^{lox/lox}$	Ah-Cre + β–napthoflavone	Intestinal adenomas and adenocarcinomas	Sansom et al. (2006)
K-*Ras*$^{+/LSLG12D}$; *Apc*$^{+/2lox14}$	Fabpl-Cre	Colonic adenomas and adenocarcinomas	Haigis et al. (2008)
K-*Ras*$^{+/LSLG12D}$; *Apc*$^{lox/lox}$	Adeno-Cre infection in colonic epithelium	Colonic adenomas and adenocarcinomas	Hung et al. (2010)

K-*Ras*$^{+/LSLG12D}$; *Tgfbr2*$^{lox/lox}$	Villin-Cre	Intestinal and colonic adenomas and adenocarcinomas	Trobridge et al. (2009)
K-*Ras*$^{+/LSLG12D}$; *p16Ink4a/p19Arf*$^{-/-}$	*Villin-Cre*	Serrated lesions and malignant spindle cell tumors	Bennecke et al. (2010)
K-*Ras*$^{+/LSLG12D}$; *Pten*$^{lox/lox}$	*Villin-CreERT2* + Tam	Serrated adenomas and metastatic carcinoma	Davies et al. (2014)
Ovarian cancer	**Genetic strategy**	**Phenotype**	**References**
K-*Ras*$^{+/LSLG12D}$; *Pten*$^{lox/lox}$	Adeno-Cre infection in the bursal cavity	Endometrial adenocarcinoma	Dinulescu et al. (2005)
RASopathies	**Genetic strategy**	**Phenotype**	**References**
K-*Ras*$^{+/V14I}$ and K-*Ras*$^{V14I/V14I}$	Germline	Noonan Syndrome	Hernández-Porras et al. submitted
H-*Ras*$^{+/G12Vgeo}$ and H-*Ras*$^{G12Vgeo/G12Vgeo}$	Germline	Costello Syndrome	Schuhmacher et al. (2008)
H-*Ras*$^{+/G12V}$	Germline	Costello Syndrome	Chen et al. (2009)

Mutant alleles from additional loci (*Apc, Pten, Tgfbr2, p16Ink4a/p19Arf*, etc.) have been included only in those models in which expression of a mutated *Ras* gene was not sufficient to induce neoplastic lesions

Variants of the models indicated in this Table carrying additional mutations such as *null* or conditional alleles of *p53*, *Smad4,* etc. have not been included for simplicity (see text for details)

[a]*4OHT* 4-hydroxytamoxifen, *Tam* Tamoxifen, *Dox* Doxycycline, and Cer, Cerulein

[b]Not applicable (N/A)

lesions within 4 weeks, with benign adenomas appearing shortly thereafter. Full-blown adenocarcinomas take a few months depending on the Adeno-Cre titer. Yet, these mice develop few high-grade invasive tumors that most often do not metastasize (Jackson et al. 2001). Preliminary results using exonic deep-sequencing indicate that these tumors accumulate very few additional mutations (T. Jacks, personal communication) suggesting that expression of the K-*Ras*^{G12D} oncogene might be sufficient for tumor development.

Similar results have been obtained with a related strain carrying a conditional K-*Ras*^{LSLG12Vgeo} allele (our own unpublished observations). This strain was generated using the same strategy utilized by Tuveson, Jacks, and coworkers (Guerra et al. 2003). In addition, the K-*Ras*^{LSLG12Vgeo} allele carries a beta-Geo bacterial fusion protein endowed with beta-galactosidase activity preceded by an internal ribosomal entry site (IRES). This strategy allows co-expression in a bicistronic fashion of the K-Ras^{G12V} oncoprotein and the beta-Geo marker upon Cre-mediated recombination (Guerra et al. 2003). As a consequence, this mouse model makes it possible to identify K-Ras^{G12V} expressing cells at the single cell level in normal as well as tumor tissue. This design has been very instrumental to identify those cells and tissues susceptible to transformation by K-*Ras* oncogenes. K-*Ras*$^{+/LSLG12Vgeo}$ mice were crossed to the *RERTn*$^{+/ert}$ knock-in strain. This tool strain expresses the inducible CreERT2 recombinase ubiquitously under the control of the locus encoding the large subunit of RNA polymerase II following a bicistronic strategy (Guerra et al. 2003). Exposure of K-*Ras*$^{+/LSLG12Vgeo}$;*RERTn*$^{+/ert}$ mice to 4OHT or to a tamoxifen diet results in widespread expression of the resident K-*Ras*^{G12V} oncogene in multiple tissues and cell types. Yet, these mice only develop overt lung tumors with an incidence and latency similar to those observed in K-*Ras*$^{+/LSLG12Vgeo}$ mice infected with Adeno-Cre particles (Guerra et al. 2003).

Induction of more aggressive lung adenocarcinomas requires the addition of other oncogenic mutations to these basic GEM tumor models. For instance crosses with mice carrying either germline or conditional mutations in the *p53* and the *p16INK4a*/*p19Arf* tumor suppressors significantly accelerate tumor development (Jackson et al. 2005; Ji et al. 2007). Interestingly, expression of endogenous K-*Ras*^{G12D} oncogene with concomitant loss of the tumor suppressor *Lkb1*, a combination often found in human lung adenocarcinomas (Ji et al. 2007), strongly accelerates lung tumor development generating tumors with more malignant and diverse phenotypic characteristics, including squamous cell carcinoma and large cell carcinomas (Ji et al. 2007). Moreover, these mice display a higher incidence of metastasis than K-*Ras*$^{+/LSLG12D}$ mice lacking *p53* or *p16INK4a*/*p19Arf* tumor suppressors (Ji et al. 2007). More recently, it has been reported that activation of Wnt/beta-Catenin signaling also leads to more aggressive K-*Ras*^{G12D} driven lung tumors (Pacheco-Pinedo et al. 2011). Yet, it is not clear whether activation of Wnt/beta-Catenin pathway plays a significant role in the development of human lung adenocarcinomas.

Other investigators have opted to use transgenic technologies to develop mouse models of K-*Ras* driven lung adenocarcinoma. For instance, Berns and coworkers designed a transgenic strain that carries a K-*Ras*^{G12V} oncogene driven by a strong

beta-Actin promoter (Meuwissen et al. 2001). Expression of the oncogenic protein is also dependent on Cre-mediated recombination of GFP-polyA sequences flanked by loxP sites strategically placed between the beta-Actin promoter and the K-*Ras* sequences. Tumor development in this strain follows similar kinetics as the Tuveson/Jacks model. In addition, these mice develop metastasis to lymph nodes and kidneys (Meuwissen et al. 2001). In another transgenic model, Varmus and coworkers inserted a K-*Ras4B*^{G12D} cDNA under the control of the Tet operon in the genome of a transgenic strain that expresses the reverse tetracycline trans-activator protein (rtTA) under the control of the CCSP promoter, a gene encoding a secretory protein primarily expressed in type II alveolar cells (Fisher et al. 2001). This mouse tumor model has the advantage to allow the possibility to turn on and off expression of the K-*Ras* oncogene by adding doxycycline to their drinking water. These mice develop hyperplasias that progress to adenomas and adenocarcinomas with kinetics similar to those observed in the K-*Ras*$^{+/LSLG12D}$ and K-*Ras*$^{+/LSLG12Vgeo}$ models infected with high titer Adeno-Cre particles. Removal of doxycycline leads to the rapid disappearance of these tumors by apoptotic mechanisms, thus demonstrating that expression of the K-*Ras* oncogene is essential for tumor maintenance and progression (Fisher et al. 2001). Addition of mutations in the *p53* and *p16Ink4a*/*p19Arf* tumor suppressors results in significant acceleration of tumor development. Yet, doxycycline withdrawal also leads to the rapid induction of apoptosis and tumor regression in spite of the absence of functional *p53* and *p16Ink4a*/*p19Arf* tumor suppressors. Thus indicating that these tumors are primarily driven by the K-*Ras*4B^{G12D} transgene.

10.3 Genetically Engineered Mouse Models of Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal types of cancer for which there are no effective therapies available (Hidalgo 2010). The most frequent precursor lesions of this tumor type are pancreatic intraepithelial neoplasias (PanINs) (Maitra and Hruban 2008). Low grade PanINs already carry K-*RAS* mutations, along with loss or inactivation of the *P16INK4a* tumor suppressor (Kanda et al. 2012). These lesions further progress to high-grade PanINs and invasive PDAC tumors upon accumulation of additional mutational events, mainly involving inactivation of other tumor suppressors such as *P53*, *SMAD4*, or *BRCA2* (Maitra and Hruban 2008).

Early attempts to model this disease in mice involved the generation of transgenic mice carrying an oncogenic K-*Ras*^{G12V} transgene linked to the Cytokeratin 19 promoter, a strategy that leads to efficient expression of the oncogene in ductal cells (Brembeck et al. 2003). These mice develop periductal lymphocytic infiltration but not PanIN lesions or PDAC tumors, thus suggesting that PDAC may not originate from direct transformation of ductal cells (Brembeck et al. 2003). Other

investigators expressed a similar K-*Ras*^{G12D} transgene in acinar cells, a strategy reminiscent of the early attempts to study RAS function in mouse models (Quaife et al. 1987). As in this early study, most mice did not survive embryonic or postnatal development (Grippo et al. 2003). Moreover, those founder mice that reached adulthood develop acinar to ductal metaplasia but not PanIN lesions (Grippo et al. 2003). A third transgenic model involves expression of TGF-alpha, a ligand for the EGF receptor and hence an indirect activator of Ras signaling, under the control of the Elastase promoter (Wagner et al. 2001). About half of the transgenic mice display differentiation of the acinar to ductal-like cells and development of tubular pancreatic tumors after long latency (Wagner et al. 2001). Inactivation of the p53 tumor suppressor results in accelerated tumor development. However, these tumors do not progress through the PanIN preneoplastic lesions characteristic of human PDAC (Wagner et al. 2001).

The first mouse model to faithfully reproduce the natural history of human PDAC involved expression of the endogenous K-*Ras*^{G12D} oncogene in all pancreatic lineages during early embryonic development by crossing the K-*Ras*$^{+/LSLG12D}$ strain described above with transgenic mice expressing the Cre recombinase under the control of the Pdx1 or the Ptf1a/P48 pancreatic specific promoters (Hingorani et al. 2003). Both compound strains develop, with complete penetrance, the full spectrum of PanIN lesions and PDAC tumors histologically indistinguishable from those present in human patients (Hingorani et al. 2003). Additional mutations present in human tumors, such as inactivation or deletion of tumor suppressors genes including *p16Ink4a*/*p19Arf*, *p53*, *Lkb1*, *p21*^{Cip1}, or *Smad4* significantly accelerate progression of PanIN lesions to invasive PDAC and induce metastasis in a significant percentage of cases (Aguirre et al. 2003; Bardeesy et al. 2006; Hingorani et al. 2005; Ijichi et al. 2006; Morton et al. 2010).

A second GEM tumor model that also recapitulates human PDAC was generated by expressing an endogenous K-*Ras*^{G12V} oncogene in pancreatic acinar cells during late embryonic development (Guerra et al. 2007). This model was generated by crossing mice carrying the K-*Ras*^{G12Vgeo} allele described above with double transgenic mice that express the Cre recombinase driven by the Tet promoter (Tet-O-Cre transgene). Expression of the Cre recombinase is controlled by the tetracycline trans-activator protein (tTA) driven by an Elastase promoter in a separate transgene (Elas-tTA) (Guerra et al. 2007). This Tet-off strategy allows expression of the K-Ras^{G12V} oncogene, along with the beta-Geo marker, in acinar cells in the absence of doxycycline. K-Ras^{G12V} expression starts during late embryonic development (E16.5), the time at which Elastase expression is turned on in acinar cells. In spite of the more limited range of K-*Ras* oncogene expression, this strain develops PanIN lesions as well as full-blown PDAC tumors with similar latencies and penetrance as the K-*Ras*^{LSLG12D};Pdx1-Cre or K-*Ras*^{LSLG12D};Ptf1a/P48-Cre mice that expressed the endogenous K-*Ras*^{G12D} allele in all pancreatic lineages (Hingorani et al. 2003; Guerra et al. 2007). Hence, these observations strongly suggest that the cell of origin of K-*Ras* driven PDAC is likely to be an acinar cell or an acinar cell precursor rather than a cell of ductal lineages.

Other GEM tumor models that target endogenous K-*Ras* oncogenes to the acinar cells using other Cre transgenes such as Elastase-Cre, Mist1-Cre, and Nestin-Cre (Carriere et al. 2007; De La O. 2008; Habbe et al. 2008) also point to the acinar cells as the cell of origin of PDAC tumors. It is important to notice that in spite of the wide expression of the endogenous K-*Ras* oncogene, these strains develop a relatively low number of PanIN lesions and even a lower number of PDAC tumors. Thus, indicating that the presence of oncogenic K-*Ras* is not sufficient to transform acinar or other pancreatic cells, suggesting that additional genetic or environmental factors might be required. Alternatively, it is possible that only a limited subpopulation of Elastase-expressing cells, such as acinar precursors or stem cells, are susceptible to K-Ras oncogene-induced transformation. As in the K-$Ras^{LSLG12D}$; Pdx1-Cre or K-$Ras^{LSLG12D}$;Ptf1a/P48-Cre models, the K-$Ras^{+/LSLG12Vgeo}$;Elas-tTA;Tet-O-Cre strain also shows complete tumor penetrance and reduced survival upon inactivation of the *p53* and *p16Ink4a*/*p19Arf* tumor suppressors (Guerra et al. 2011).

The K-$Ras^{+/LSLG12Vgeo}$;Elas-tTA;Tet-O-Cre model offers the possibility to turn on K-Ras^{G12V} oncogene expression in adult mice (Guerra et al. 2007). In this model, expression of the resident K-Ras^{G12V} oncogene is prevented by providing doxycycline in the drinking water to the pregnant mothers and to the pups until the desired age. Withdrawal of doxycycline results in the rapid expression of the K-Ras^{G12V} oncogene (Guerra et al. 2007). Surprisingly, expression of K-Ras^{G12V} in the acinar comparment of adult mice (8 weeks of age or older) fails to induce pancreatic lesions including benign acinar to ductal metaplasia and low grade PanINs (Guerra et al. 2007). Moreover, ablation of *p53* or *p16Ink4a*/*p19Arf* tumor suppressors in the very same pancreatic cells that express the K-Ras^{G12V} oncogene also failed to induce any lesions. These observations indicate that mouse adult acinar cells are resistant to transformation by some of the most common mutations involved in the development of human as well as mouse tumors (Guerra et al. 2011).

This apparent conundrum was partially resolved when Guerra, Barbacid, and coworkers submitted these adult K-$Ras^{+/LSLG12Vgeo}$;Elas-tTA;Tet-O-Cre mice to chronic exposure of low doses of carulein, a decapeptide analog of the pancreatic secretagogue cholecystokinin commonly used to induce experimental pancreatitis (Guerra et al., 207). This chronic treatment induces atrophic acini and mild panlobular lesions characteristic of chronic pancreatitis regardless of the genotype of the mice (Guerra et al. 2007). Moreover, this treatment also elicits a significant inflammatory response consisting of T cells and macrophages associated with B cells. However, only those mice expressing the K-Ras^{G12V} oncogene upon doxycycline withdrawal develop acinar to ductal cell metaplasia as well as focal and diffuse low grade PanINs. By 1 year of age all K-$Ras^{+/LSLG12Vgeo}$;Elas-tTA;Tet-O-Cre mice exposed to carulein develop low and high grade PanINs and about a third of them carry invasive PDAC tumors, an incidence and latency similar to that observed in the other PDAC GEM tumor models described above (Guerra et al. 2007). Adult K-Ras^{G12V} expressing mice do not require suffering chronic pancreatitis to develop PanIN lesions or PDAC. Indeed, limited bouts of

pancreatitis also contribute to tumor development. Mice exposed to carulein for just 3 months develop PanIN lesions and PDAC tumors with the same incidence and latency than those exposed to chronic treatment (Guerra et al. 2011). Even shorter treatments (e.g., 1 month) are also sufficient to induce PDAC development albeit with longer latencies. No PanIN lesions or PDAC tumors develop in mice that do not express the resident K-Ras^{G12V} oncogene. Similar treatments of adult mice that also harbor mutations in the *p53* or *p16Ink4a*/*p19Arf* tumor suppressors result in significantly faster tumor development. Indeed, all mice treated with carulein for 3 months succumb to PDAC tumors before 1 year of age (Guerra et al. 2011).

Whether human patients also require suffering some sort of pancreatic damage, along with sporadic K-*RAS* mutations, in order to develop PanIN lesions and, more importantly, invasive PDAC tumors is unknown. However, it is well documented that chronic pancreatitis is one of the highest risk factors for the development of PDAC in humans (Lowenfels et al. 1993; Malka et al. 2002). Another condition that also increases the risk of developing pancreatic cancer is obesity. Exposure of K-Ras^{LSLG12D}; Ptf1a/P48-Cre mice to a high fat diet (HFD) results in increased development of high grade PanIN lesions (Khasawneh et al. 2009). Interestingly, this effect on tumor promotion appears to be mediated by an HFD-induced inflammatory response rather than by alterations in insulin metabolism (Khasawneh et al. 2009). More recent studies using a related GEM tumor model in which expression of the K-*Ras*^{G12D} allele is controlled by a transgene in which the Elastase promoter drives expression of an inducible Cre recombinase (CreERT2) have reported that increased formation of PanIN lesions and PDAC tumors in mice exposed to a high fat diet is mediated by COX-2, a well-known mediator of inflammatory processes (Philip et al. 2013). Thus, alterations of normal pancreatic homeostasis by insults that induce an inflammatory response might play a critical role in the development of K-*Ras* driven PDAC tumors in human patients.

Two new GEM tumor models for PDAC that allow the reversible expression of the K-Ras^{G12D} oncoprotein have been recently generated (Collins et al. 2012a; Ying et al. 2012). In one of these models, expression of the oncogenic K-Ras^{G12D} protein is directly driven by the Tet promoter via a Tet-O-K-*Ras*^{G12D} transgene (Collins et al. 2012a). Activation of this transgene is mediated by the reverse tetracycline trans-activator protein rtTA inserted within the ubiquitously expressed Rosa26 locus and preceded by a LSL cassette. To ensure that the rtTA protein is selectively expressed in pancreatic tissue, this model also carries a Ptf1a/P48-Cre transgene. This strategy allows controlled expression of the K-Ras^{G12D} protein by adding (K-Ras^{G12D} on) or removing (K-Ras^{G12D} off) doxycycline from the drinking water (Collins et al. 2012a). These mice, when combined with a mutant allele of *p53*, *p53*^{R172H}, and exposed to a short bout of acute pancreatitis, develop aggressive PDAC tumors and some metastatic lesions. These tumors as well as the metastatic lesions rapidly disappear in the absence of doxycycline (Collins et al. 2012a, b). Thus, illustrating that tumor maintenance requires continuous expression of the K-Ras^{G12D} oncoprotein regardless of other oncogenic insults. The desmoplastic stroma characteristic of PDAC tumors also disappeared upon doxycycline withdrawal indicating its dependence on tumor tissue. Interestingly, addition of

doxycycline resulted in the rapid recurrence of the primary tumor mass, suggesting that some tumor cells can survive inactivation of the Tet-O-K-*Ras*^{G12D} transgene and are able to resume rapid growth upon K-*Ras*^{G12D} re-expression. The second inducible PDAC model uses basically the same strategy except for the insertion of a LSL cassette within the K-*Ras*^{G12D} transgene (Tet-O-LSL-K-*Ras*^{G12D}) to ensure a more strict dependence of K-Ras^{G12D} expression on Cre-mediated recombination (Ying et al. 2012). In addition, these authors utilize a *p53 null* allele to potentiate tumor development. As with the previously described model, doxycycline withdrawal also results in rapid tumor disappearance (Ying et al. 2012).

10.4 Mouse Models of Colon Cancer

For reasons that are not fully understood, the incidence of gastrointestinal (GI) adenocarcinomas is different in mice and humans. Whereas in humans most of these tumors appear in the colon and rectum, in mice preferentially originate in the small intestine. Although the structure of the intestinal and colonic crypts is significantly different, the underlying mechanisms of tumor development appear to be similar. Hence, mouse intestinal tumors appear to be an adequate experimental instrument to model human colorectal cancer.

Early studies using mice expressing a Villin-K-*Ras*^{G12V} transgene in the intestinal epithelium resulted in the generation of multiple intestinal lesions, ranging from aberrant crypt foci to invasive adenocarcinomas in more than 80 % of the transgenic animals (Janssen et al. 2002). However, these results are likely to be a direct consequence of the unusually elevated levels of K-Ras^{G12V} expression in these transgenic mice. Indeed, expression of an endogenous K-*Ras*^{G12V} oncogene driven from its own promoter in intestinal and colonic crypts using the K-*Ras*$^{+/LSLG12Vgeo}$;*RERTn*$^{+/ert}$ model described above has no phenotypic consequences (Guerra et al. 2003). Similar results were obtained using the K-*Ras*$^{+/LSLG12Vgeo}$ strain crossed to a transgenic mouse that expresses the bacterial Cre recombinase under the control of a Cytochrome p450 promoter (Ah-Cre) whose expression can be induced by beta-naphthoflavone (Sansom et al. 2006). Exposure of K-*Ras*$^{+/LSLG12Vgeo}$;Ah-Cre mice to this agonist results in widespread expression of the endogenous K-*Ras*^{G12V} oncogene throughout the entire GI track without inducing any significant alterations (Sansom et al. 2006). Analysis of these mice 8 months after removing the beta-naphthoflavone revealed the presence of K-Ras^{G12V} expressing crypts (based on the detection of the surrogate beta-Geo marker). Thus illustrating that expression of a resident K-*Ras*^{G12V} oncogene did not affect the generation of normal crypts from putative intestinal stem cells (unpublished observations). Other investigators, however, have reported that expression of an endogenous K-*Ras*^{G12D} oncogene in the distal intestinal epithelia using the K-*Ras*$^{+/LSLG12D}$ mice described above crossed to a Fabpl-Cre transgenic strain results in widespread hyperplasia throughout the colonic epithelium (Haigis et al. 2008). More recent studies expressing a K-*Ras*^{G12D} oncogene in Lgr5+ stem cells result

in expansion of these cells leading to competition with normal crypts (Snippert et al. 2014). Yet, neither of these mice developed tumors including benign adenomas (Haigis et al. 2008; Snippert et al. 2014). These results, taken together, indicate that expression of an endogenous K-*Ras* oncogene is not sufficient to initiate intestinal or colonic neoplasias.

However, expression of a resident K-*Ras* oncogene, regardless of the activating mutation (K-Ras^{G12D} or K-Ras^{G12V}) in colonic crypts defective for the tumor suppressor Apc, convert the benign adenomas induced by *Apc* loss into malignant adenocarcinomas (Sansom et al. 2006). Additional mutations such as inactivation or loss of the *p53* tumor suppressor further enhance the development of these intestinal adenocarcinomas (Martin et al. 2013). These observations confirm the classical tumor progression model proposed by Vogelstein and coworkers for the development of human colorectal cancers (Fearon and Vogelstein 1990). Interestingly, concomitant activation of K-Ras^{G12D} oncogene expression and loss of *Apc* driven by crossing the K-$Ras^{+/LSLG12D}$;$Apc^{+/2lox14}$ strain to transgenic Fapbl-Cre mice led to the generation of colonic adenomas and adenocarcinomas instead of intestinal tumors (Haigis et al. 2008). Finally, exposure of the distal colon of K-$Ras^{+/LSLG12D}$;$Apc^{lox/lox}$ mice to mechanical abrasion followed by infection with Adeno-Cre particles led to the rapid development of tumors of which a third were adenocarcinomas (Hung et al. 2010). Perhaps more importantly, 20 % of these mice develop liver metastasis within 6 months after Adeno-Cre treatment (Hung et al. 2010). Finally, expression of an endogenous N-Ras^{G12D} oncogene in the GI track of N-$Ras^{+/LSLG12D}$ mice do not produce intestinal hyperplasia or cooperate with loss of *Apc*, thus suggesting a different role for K-*Ras* and N-*Ras* oncogenes in the development of GI tumors (Haigis et al. 2008).

Mutations in other tumor suppressors also cooperated with K-*Ras* oncogenes in the induction of GI tumors. For instance, combined expression of K-Ras^{G12D} with loss of the *Tgfbeta receptor-2* gene in a conditional GEM tumor model dependent on a Villin-Cre transgene (K-$Ras^{+/LSLG12D}$;$Tgfbr2^{lox/lox}$;Villin-Cre strain) results in development of intestinal tumors in approximately 70 % of mice by 22 weeks of age (Trobridge et al. 2009). These tumors are primarily invasive adenocarcinomas and are evenly distributed between the small and large intestine. Moreover, approximately 15 % of these mice develop grossly visible metastases in regional lymph nodes or lung (Trobridge et al. 2009). Expression of the same endogenous K-Ras^{G12D} oncogene also cooperates with loss of the *p16Ink4a*/*p19Arf* tumor suppressor. K-$Ras^{+/LSLG12D}$;$p16Ink4a/p19Arf^{-/-}$ mice develop serrated lesions and malignant spindle cell tumors (Bennecke et al. 2010). Likewise, combined expression of this resident K-Ras^{G12D} oncogene with intestinal-specific deletion of the *Pten* tumor suppressor leads to perturbed homeostasis of the intestinal epithelium and the development of hyperplastic polyps, dysplastic sessile serrated adenomas, and metastasizing adenocarcinomas with serrated features (Davies et al. 2014).

10.5 A Mouse Model for Endometroid Ovarian Adenocarcinoma

The most frequent mutation identified in human ovarian carcinoma is loss or inactivation of the *p53* tumor suppressor, an event that occurs in over 55 % of all ovarian tumor types (the COSMIC database; http://www.sanger.ac.uk/genetics/CGP/cosmic). K-*RAS* mutations have been identified in a more limited percentage (12 % of all ovarian tumors) with varying incidences in mucinous, serous, or endometroid adenocarcinomas (the COSMIC database; http://www.sanger.ac.uk/genetics/CGP/cosmic). To date, the only GEM tumor model for ovarian carcinoma involving a K-*Ras* oncogene was generated by injecting Adeno-Cre vectors in the ovarian surface epithelium of K-$Ras^{+/LSLG12D}$ mice. Expression of the endogenous K-Ras^{G12D} oncogene in these cells induces benign epithelial lesions with endometroid glandular morphology (Dinulescu et al. 2005). About half of these mice also develop peritoneal endometriosis possibly originated by Adeno-Cre infection of uterine or tubal cells (Dinulescu et al. 2005). Yet, induction of endometroid ovarian adenocarcinomas requires additional mutations such as inactivation of the *Pten* tumor suppressor (K-$Ras^{+/LSLG12D}$;$Pten^{lox/lox}$ strain). The primary tumors that develop in this strain are located in the ovary and do not affect the uterus or the oviduct. Interestingly, Adeno-Cre infection of $Pten^{lox/lox}$ mice does not result in any histopathological alteration, thus suggesting that K-*Ras* is also the driver oncogene in these tumors (Dinulescu et al. 2005).

10.6 Mouse Models of RAS Oncogene-Induced Hematological Malignancies

Hematological cancers, including acute myeloid leukemia and myelodisplastic syndrome, chronic myelomonocytic leukemia, and acute lymphoblastic leukemia carry mutated N-*RAS* oncogenes with incidences varying between 10 and 15 %. The K-*RAS* oncogene is also activated in these malignancies, albeit with lower frequencies (4 to 5 %) (the COSMIC database; http://www.sanger.ac.uk/genetics/CGP/cosmic). In mice, activation of Ras signaling in the hematopoietic lineage, either by inactivating the *Nf1* tumor suppressor or by expressing an endogenous K-Ras^{G12D} allele in cells of hematologic lineage in the K-$Ras^{+/LSLG12D}$;Mx1-Cre strain, is sufficient to induce a fatal myeloproliferative disorder (MPD) similar to that observed in human patients (Braun et al. 2004; Chan et al. 2004). Interestingly, expression of the N-Ras^{G12D} oncogene in a similar GEM model (N-$Ras^{+/LSLG12D}$; Mx1-Cre strain) only results in a mild and variable myeloid phenotype (Li et al. 2011; Wang et al. 2010). Expression of the N-Ras^{G12D} oncogene in homozygosity (N-$Ras^{LSLG12D/LSLG12D}$;Mx1-Cre strain) results in a more aggressive myeloproliferative malignancy (Wang et al. 2011; Xu et al. 2012). These results suggest that different Ras isoforms and/or their expression levels affect the severity

of myeloproliferative disorders. These GEM tumor models have also been utilized to identify other mutations that cooperate with hyperactive Ras signaling in leukemogenesis by using retroviral insertional mutagenesis (Dail et al. 2010; Li et al. 2011). Somatic N-*RAS* and K-*RAS* mutations have also been identified in 25–30 % of patients with juvenile myelomonocytic leukemia (JMML) (Loh 2011). Interestingly, JMML is an alteration characteristic of a group of congenic syndromes originated by constitutive hyperactive Ras signaling, known as RASopathies (Rauen 2013; Schubbert et al. 2007) (see below).

10.7 N-*RAS* and Malignant Melanoma

Activation of the Ras signaling pathway is one of the primary causes of malignant melanoma. Although the most frequently mutated oncogene in this tumor type is B-*RAF*, the N-*RAS* oncogene has been identified in 15–20 % of the cases. H-*RAS* and K-*RAS* mutations have also been occasionally reported (about 1 % incidence) (the COSMIC database; http://www.sanger.ac.uk/genetics/CGP/cosmic). Interestingly, the first mouse model of RAS-induced malignant melanoma was a transgenic strain that selectively expresses a mutant H-Ras^{G12V} oncogene in melanocytes driven by the Tyrosinase promoter (*Tyr*). These mice display melanocytic hyperplasia with intense skin pigmentation that progress into skin melanoma with metastasis formation in lymph nodes and lung after treatment with carcinogens (Broome Powell et al. 1999). Crosses of this transgenic strain with mice deficient in the *p53* or *p16Ink4a*/*p19Arf* tumor suppressors resulted in the development of highly vascularized amelanotic melanomas that resembled human nodular melanomas (Chin et al. 1997). No metastasis can be observed in these mice. A similar strain carrying a *Tyr*-N-Ras^{Q61K} transgene was subsequently developed (Ackermann et al. 2005). These mice show hyperpigmented skin and develop cutaneous metastasizing melanoma at 6 months of age, but only when crossed to *p16Ink4a null* mice. Primary melanoma tumors are melanotic, multifocal, and microinvade the epidermis or epithelium of hair follicles. Moreover, they disseminate to lymph nodes, lung, and liver. In related studies, Delmas et al. (2007) showed that expression of a stabilized beta-Catenin isoform also cooperate with the *Tyr*-N-Ras^{Q61K} transgene in melanoma development by a mechanism involving silencing of the *p16Ink4a* promoter.

10.8 Mouse Models for RAS-Induced Developmental Syndromes

Mutations in *RAS* genes have been directly implicated in various developmental syndromes, now known as RASopathies, including Costello Syndrome (CS) (H-*RAS*), Noonan Syndrome (NS) (K-*RAS* and N-*RAS*), and Cardio-facio-

cutaneous syndrome (CFC) (K-*RAS*) (Rauen 2013). Whereas the mutations identified in the K-*RAS* locus are distinct from those present in human tumors, most mutations present in CS patients involve mutations in codons 12 and 13 coinciding with those previously observed in human tumors, including the H-RAS^{G12V} mutation. Thus, indicating that whereas oncogenic K-*RAS* mutations are likely to be embryonic lethal in humans (as they are in mice), oncogenic H-*RAS* mutations are well tolerated during human embryonic and postnatal development.

10.8.1 Mouse Models of Costello Syndrome

CS is a rare developmental disorder (about 300 known cases) (Gripp and Lin 2012) that results in multiple anomalies, including prenatal overgrowth followed by postnatal feeding difficulties and severe failure to thrive, short stature, distinctive coarse facial features, cardiac defects, musculoskeletal and ectodermal abnormalities, and neurocognitive delay (Rauen 2013). CS patients have an increased risk of developing neoplasias, including rhabdomyosarcomas, neuroblastomas, and bladder cancer. These solid tumors affect approximately to 15 % of CS patients. CS is caused by germline missense mutations in the H-*RAS* locus (Aoki et al. 2005). The distribution frequency of mutations revealed that more than 80 % of individuals with CS have a G12S substitution, followed by G12A (9 %) and G13D (1.4 %) (Gripp and Lin 2012).

Two independent GEM models involving expression of an endogenous H-Ras^{G12V} allele in the germline have been generated (Chen et al. 2009; Schuhmacher et al. 2008). Schuhmacher and colleagues described that germline expression of the oncogenic H-Ras^{G12V} allele phenocopy many of the abnormalities observed in CS patients, including facial dysmorphia and cardiomyopathies. These mice also display alterations in the homeostasis of the cardiovascular system, including development of systemic hypertension, extensive vascular remodeling, and fibrosis in heart and kidneys (Schuhmacher et al. 2008). This phenotype is age dependent and is a consequence of the abnormal upregulation of the renin-Ang II system, a potential reason for the sudden death described in these patients (Estep et al. 2006). Homozygous H-Ras^{G12V} animals are also viable and display similar, albeit more robust phenotypes (Schuhmacher et al. 2008). These mice also exhibit hyperplasia of the mammary gland but development of tumors is rare. In 2009, Chen and colleagues described the development of a second GEM model expressing the same H-Ras^{G12V} mutation. In this case, however, mice display high perinatal mortality, abnormal cranial dimension, defective dental ameloblast, and nasal septal deviation. Moreover, they develop papillomas and angiosarcomas (Chen et al. 2009). The phenotypic differences between these GEM models carrying the same mutation might be explained by gene modifiers due to genetic background differences and might serve to illustrate the different phenotypes observed in CS patients (Lin et al. 2008).

10.8.2 A Mouse Model of K-RAS Driven Noonan Syndrome

NS is one of the most common RASopathies with an estimated incidence of 1:1,000 to 1:2,500 live births, but mild cases may be even more common (Mendez and Opitz 1985). The typical signs of NS include typical facial feature, chest and spinal deformities, short stature, characteristic heart defects, and learning disabilities with mild mental retardation (Tartaglia and Gelb 2005). NS is caused by germline mutations in a variety of genes including *PTPN11*, *SOS1*, K-*RAS*, N-*RAS*, *RAF1*, B-*RAF*, *SHOC2*, *CBL,* and *RIT1*. Germline mutations in K-*RAS* usually correlate with more severe symptoms (Nava et al. 2007; Schubbert et al. 2006). Interestingly, the K-*RAS* activating mutations are widespread along the protein affecting the P-loop (V14I), Switch I (P34L and P34Q) and II (T58I and G60S) domains, and intermediate regions of the G domain (Q22R, M72L, N26I, and N116S). These mutations convey different biochemical properties to the mutated K-Ras isoforms. For instance, the K-RAS^{V14I} mutant shows a dramatic increase, both in intrinsic and GEF-catalyzed nucleotide Exchange, a property likely to account for its accumulation in the GTP-bound state and increased downstream signaling (Gremer et al. 2011). Other mutants such as KRASP34L, KRASP34R, and KRASG60R are characterized by a defective GAP sensitivity and a strongly reduced interaction with effectors. Overall, all the K-RAS mutants involved in NS display lower levels of signaling than the oncogenic K-RAS^{G12V} isoform (Gremer et al. 2011).

We have recently developed a mouse model for NS induced by an endogenous K-*Ras*^{V14I} allele, the most frequent K-*RAS* mutation in NS patients following the same conditional strategy previously used to generate the K-*Ras*^{G12D} and K-*Ras*^{G12V} alleles. K-*Ras*^{V14I} mice display multiple NS-associated developmental defects such as growth delay, craniofacial dysmorphia, cardiac defects, and hematologic abnormalities including fatal myeloproliferative disorder (MPD) that closely recapitulates JMML. These mice have increased predisposition to tumor development other than MPD and cooperate with tumor suppressors such as *p16Ink4a*/*p19Arf* and *p53* (Hernández-Porras et al., submitted for publication). To date, no GEM model for K-*RAS*-induced CFC syndrome has been generated.

10.9 Conclusions

The advent of recombinant engineering techniques has made possible the development of a new generation of mouse tumor models that closely recapitulate the natural history of many human malignancies. These GEM tumor models should serve to better understand the molecular mechanisms underlying the different steps involved in tumor development as well as in their metastatic spreading. In addition, they should serve as reliable tools for preclinical testing of novel therapeutic strategies. To date, treatment of K-Ras driven lung and pancreatic adenocarcinomas with standard chemotherapy protocols have yielded very similar results as those

obtained in human tumors (Chin et al. 2013). Work developing within the next few years will hopefully demonstrate that these GEM tumor models are going to be much more predictable than the classical tumor xenograft models still used in preclinical testing by the pharmaceutical industry. If so, GEM models should become the gold standard for preclinical testing of future anticancer therapies.

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Chapter 11
Rap Signaling

Willem-Jan Pannekoek and Johannes L. Bos

Abstract The Rap proteins comprise a subfamily of the Ras-like small G-proteins, most closely related to Ras, which were originally found to antagonize Ras-induced cell transformation. Rap transduces extracellular stimuli to a variety of different processes, amongst others cell–matrix adhesion, cell–cell adhesion, and actin dynamics. Here, we present an overview of the mechanisms that regulate activation, including guanine nucleotide exchange factors and GTPase activating proteins responsible for the regulation. We discuss the various biological functions that are controlled by Rap proteins and the molecular mechanism used by Rap1 to induce these responses.

Keywords Rap1 • Rap2

11.1 Rap Proteins

Rap proteins were first identified by Pizon and coworkers in a search for Ras-related genes in a human cDNA library (Pizon et al. 1988). Five Rap isoforms exist in humans: Rap1A, 1B, 2A, 2B, and 2C, which share approximately 50 % sequence identity with Ras and as such are most closely related to the classical Ras proteins. The Rap1 isoforms are identical in "the business end" of the molecule (Fig. 11.1, termed "switch 1" and "switch 2"), i.e., the region that changes conformation upon binding to GDP or GTP and that interacts with guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and effectors (Bos et al. 2007). Within this region, one amino acid difference exists between the Rap2 isoforms. Six amino acids within this region are different between Rap1 and Rap2, an important one is

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on position 39 (S in Rap1, F in Rap2), as this residue allows specific GEFs and effectors to bind to Rap2 (Miertzschke et al. 2007; Nonaka et al. 2008; Yaman et al. 2009). Most differences between the Rap isoforms reside within the C-terminal hypervariable region (HVR). This region determines the subcellular localization of the protein in three ways (Prior and Hancock 2012): (1) the last four residues constitute the CAAX-motif (C = cysteine, A = aliphatic, X = any amino acid). This CAAX-motif is recognized by either a farnesyltransferase or a geranylgeranyltransferase, which covalently attaches an isoprenoid (either farnesyl or geranylgeranyl) to the cysteine. These lipid modifications insert into lipid bilayers to target the protein to cell membranes. Rap1A, Rap1B, and Rap2B are geranylgeranylated, whereas Rap2A and Rap2C are farnesylated. (2) Rap1 proteins have a poly-basic region in the HVR that aid to membrane targeting. For instance, Rap1A contains two clusters of three lysine residues within the HVR, in which it differs subtly with Rap1B (Fig. 11.1). (3) In contrast, Rap2 proteins contain two cysteine residues that are palmityolated. Palmitoylation is dynamic and a driving force in recycling of small GTPases (Rocks et al. 2010). Although Rap proteins are found in the plasma membrane, intracellular positions have been identified as well (Berger et al. 1994; Hisata et al. 2007; Mochizuki et al. 2001).

Most research has focused on Rap1 as overexpression of Rap1, but not Rap2, suppresses Ras-induced cell transformation. This was shown in an elegant genetic screen by Noda and coworkers in 1989 (Kitayama et al. 1989). cDNAs were introduced into Ras transformed cells and selected for flat revertants. One of the hits was Kirsten Ras revertant 1 (Krev-1), identical to Rap1A. As Rap1 and Ras are very similar in their effector-binding region and effector proteins binding to Rap1 in pull-down assays invariably bind Ras as well, it was hypothesized that Rap interferes in Ras signaling by functioning as a decoy that traps Ras effectors in a nonfunctional compartment. Indeed, it was reported that Rap1 binds to and inhibits Raf1 (Cook et al. 1993; Nassar et al. 1995). In contrast, Rap1 was reported to be the dominant activator of B-Raf to positively regulate the ERK pathway (Vossler et al. 1997). Both these results were questioned by other groups, and the prevailing model is that the effect of Rap1 on the morphology of Ras-induced transformed cells is due to the ability of Rap1 to increase cell adhesion (Bos 2005). It should be noted that there is indeed evidence that Rap1 can influence the Raf/B-Raf-MEK-ERK pathway; however, whether this is a direct effect due to an interaction with Raf or B-Raf, or indirectly, for instance, due to the regulation of integrins, is still a matter of debate. Our own results point to an indirect effect (Zwartkruis et al. 1998).

Despite their similarities, Ras and Rap were early inventions in evolution (van Dam et al. 2011). Both proteins were already present in the last eukaryotic common ancestor. Rap1 and Rap2 deviate later during evolution. The presence of Rap in unicellular organisms further stresses a role of Rap proteins in the regulation of fundamental cellular events. Indeed, studies in yeast were critical in understanding the overall function of Rap1 proteins. The Rap1 ortholog in budding yeast, Bud1/Rsr1, is involved in bud site selection (Park et al. 1993; Chant et al. 1991). Spatial landmarks determine the position of a future bud adjacent to a budscar of a previous bud. Bud1 together with its GEF, Bud5, and GAP, Bud2, recognize the landmark

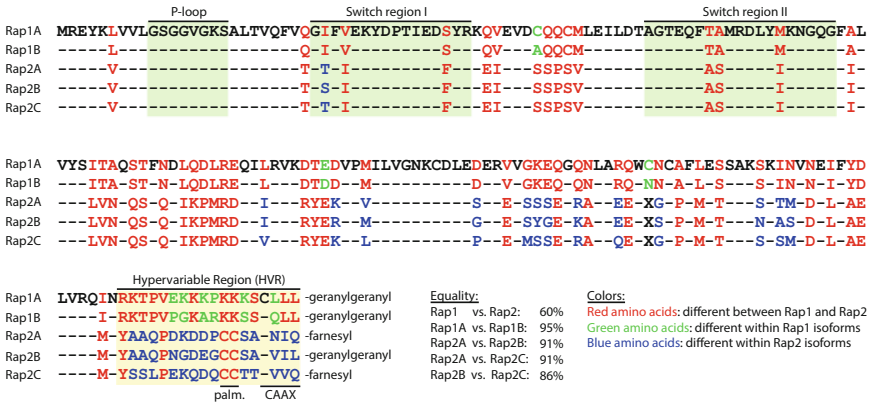

Fig. 11.1 Sequence alignment of the five Rap proteins. The amino acid sequence of Rap1A is indicated. *Dashes* indicate identical amino acids in the other isoforms. Red amino acids are different between Rap1 and Rap2. Green amino acids are different between Rap1 isoforms, whereas blue amino acids are different between Rap2 isoforms. *Green boxes* indicate the P-loop (important for nucleotide binding) and the Switch regions (important for GEF and effector binding). The *yellow box* indicates the hypervariable region (HVR) (important for localization), which includes the palmitoylatable cysteines and the CAAX-motif

and pass the signal on to recruit the actin cytoskeleton. This generated the hypothesis that Rap proteins are involved in the recognition of spatial cues to direct actin driven processes.

Also studies in Drosophila helped in understanding the function of Rap proteins. Disruption of Rap1 results in a dorsal closure defect suggesting an effect on cell migration (Asha et al. 1999). If the maternal contribution of Rap1 is disrupted as well, an early lethal effect is observed due to a failure of migration of the pole cells (early germ cells) during embryogenesis. Disruption of Rap1 in the wing results in a defect in DE-cadherin mediated cell-cell junctions (Knox and Brown 2002). Together, these studies point already to a function of Rap proteins in cell adhesion, migration, and the actin cytoskeleton. This is supported by the phenotype of Rap1 knockout mice. Rap1A−/− mice are viable and fertile, but cells isolated from these mice show defective integrin activation (Duchniewicz et al. 2006). Rap1B−/− mice show 85 % embryonic lethality due to excessive bleeding, probably attributable to weakening of the vessel wall (Chrzanowska-Wodnicka et al. 2005, 2008). By now, regulation of cell-cell adhesion, cell-matrix adhesion and actin dynamics confine the textbook functions of the Rap proteins in mammalian cells, the analysis of which has been performed along three main lines: the analysis of the molecular mechanism of Rap1 activation and inactivation, the analysis of the biological responses and the identification of the molecular mechanism, including the identification of the critical effects of Rap proteins. We will present the highlights of these studies.

11.2 Rap Activity Regulation

The first indication that Rap1 acts as a molecular switch came from work of Altschuler and coworkers who showed that cAMP activates Rap1 (Altschuler et al. 1995). They were using a classical labeling experiment, in which cells transfected with epitope-tagged Rap1 were labeled with ^{32}P orthophosphate. After immunoprecipitation of Rap1, the ratio GDP/GTP was determined by thin layer chromatography. Activation measurements became much easier by the introduction of the pull-down assay by Franke and coworkers (1997), which employs the Rap1-binding domain of RalGDS as a probe to precipitate Rap1GTP, followed by western blotting. Using this technology it was shown that Rap1 is activated by a large variety of stimuli, suggesting that multiple GEFs or GAPs are involved in the regulation of Rap1 (Gloerich and Bos 2011). Indeed, many of these regulatory proteins were identified and molecular details on their regulation elucidated.

11.2.1 RapGEFs

GEFs activate small G-proteins by decreasing their affinity for nucleotides (Bos et al. 2007). As a result, GDP is replaced by the more abundant GTP. The catalytic domain of RapGEFs is the CDC25 Homology Domain (CDC25-HD), flanked by a stabilizing Ras Exchange Motif (REM). In addition, these GEFs have usually several other domains (Fig. 11.2) or posttranslational modifications that respond to extracellular signals and regulate either activity, localization, or both.

11.2.1.1 C3G

C3G (Crk SH3 domain-binding GEF) (RapGEF1) was the first RapGEF identified by Tanaka et al. (1994) and Matsuda et al. (1994), based on its possession of a for RasGEFs characteristic REM-CDC25-HD region and its binding to the SH3 domain of the adaptor protein Crk through a proline-rich sequence. The predominant target of C3G is Rap1. C3G is ubiquitously expressed and C3G knockout mice are early embryonic lethal indicating the importance of this GEF (Ohba et al. 2001). C3G confers an auto-inhibited conformation, as its activity can be greatly enhanced by removal of the N-terminal half (Ichiba et al. 1999). In cells, release of autoinhibition is conferred by phosphorylation of Y504 by kinases of the Src family, which in their turn are activated by a variety of stimuli.

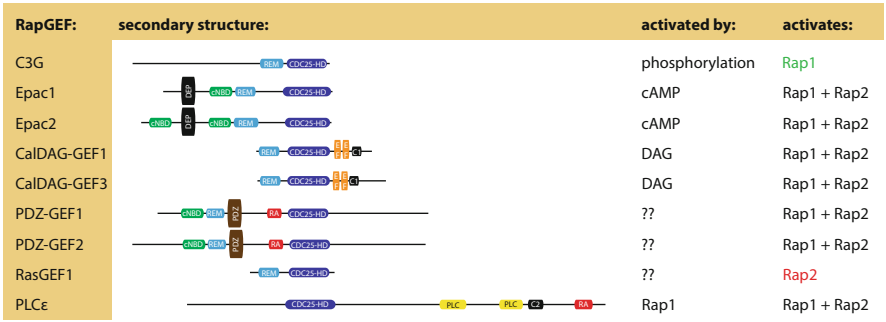

Fig. 11.2 The RapGEF family. Domain architecture of the RapGEFs is shown, in addition to their prototypical activating signal and Rap isoform preference

11.2.1.2 Epacs

A real surprise was the discovery of Epac proteins by De Rooij et al. and Kawasaki et al. in 1998 (de Rooij et al. 1998; Kawasaki et al. 1998a), as these proteins turn out to be regulated by direct cAMP binding. At that time it was commonly accepted that protein kinase A (PKA) and some ion channels were the only targets of cAMP. The Epac proteins, Epac1 (RapGEF3) and Epac2 (RapGEF4), are characterized by the presence of either one (Epac1) or two (Epac2) cyclic nucleotide-binding domains. Furthermore, both isoforms contain a DEP domain, the catalytic REM-CDC25-HD tandem, and an RA domain (Gloerich and Bos 2010). Expression of both Epac1 and Epac2 is highly variable between cell types, with Epac1 amongst others high in endothelial cells, kidney, heart, brain, adipose tissue, and ovary and Epac2 in brain, adrenal glands and beta cells of the pancreas (Kawasaki et al. 1998a). Epac1 and Epac2 single and double knockout mice are viable with mild defects (Pereira et al. 2013), suggesting a modulatory role rather than a critical role in Rap1 regulation.

Structure analysis of both active and inactive Epac2 revealed that Epac is normally in a closed inactive conformation, in which the N-terminal (regulatory) region occludes the Rap-binding site (Rehmann et al. 2006). Upon binding of cAMP, a massive conformational change allows Rap to reach the catalytic helix (Rehmann et al. 2008). Interestingly, in addition to allosteric activation, cAMP induces the translocation of Epac1, but not Epac2, to the plasma membrane (Ponsioen et al. 2009). Recently, the molecular mechanism of this translocation was eluded. Binding of cAMP induces a conformation change in the DEP domain (Li et al. 2011), allowing it to bind to phosphatidic acid, which is enriched in the plasma membrane (Consonni et al. 2012). Hence, cAMP controls both Epac1 activity as well as plasma membrane localization. A diverse set of proteins binds Epac1 resulting in a variety of different membrane localizations. These include the ERM proteins (Ezrin, Radixin, Moesin), RanBP2 and β-arrestin (Gloerich et al. 2010, 2011; Mangmool et al. 2010). In all cases cAMP remains required for activation.

11.2.1.3 RasGRPs (CalDAG-GEFs)

RasGRP was first identified as a RasGEF having, in addition to the REM-CDC25-HD module, (calcium-binding) EF hands and a (DAG-binding) C1 domain (Ebinu et al. 1998). Subsequently, additional members of the family were found that were specific for Rap proteins, or have dual specificity (Kawasaki et al. 1998b; Yamashita et al. 2000). At that time these were renamed in CalDAG-GEFs, but the official name remains RasGRP for all protein members. Both DAG and calcium are under control of phospholipase C (PLC), which converts the lipid PI-4,5P2 to DAG and IP3, the latter being an inducer of calcium release from the endoplasmic reticulum. Structural data show that RasGRP forms dimers in which the catalytic core is shielded. Binding of calcium and DAG overcomes this autoinhibition (Iwig et al. 2013). Furthermore, DAG employs additional control on RasGRPs. First, it enhances the targeting of RasGRP to cell membranes (Ebinu et al. 1998). Second, it activates PKC, which phosphorylates RasGRP (Zheng et al. 2005). This phosphorylation event is important for RasGRP activity status, but not its localization, suggesting DAG controls both RasGRP activity as well as localization. Here, it is important to note some intriguing differences between the RasGRPs. RasGRP2 does not directly bind DAG and therefore escapes DAG-mediated control on its localization (Johnson et al. 2007). Hence, RasGRP2 is only subjected to DAG-PKC-mediated activation. Furthermore, the RasGRPs display specificity in the activation of substrates. RasGRP2 shows substrate specificity towards the Raps and does not activate other Ras-like G-proteins. Conversely, RasGRP1 does not activate Raps but does activate Ras isoforms (Kawasaki et al. 1998b). RasGRP3 promiscuously activates both Rap and Ras isoforms (Yamashita et al. 2000). Hence, RasGRP2 and 3 are of importance for Rap. Both these isoforms are under control of DAG, albeit to different extents. We have kept the CalDAG-GEF nomenclature (RasGRP2 is CalDAG-GEF1 and RasGRP3 CalDAG-GEF3) for these two RapGEFs.

11.2.1.4 PDZ-GEFs

PDZ-GEF1 (RapGEF2) and PDZ-GEF2 (RapGEF6) are two RapGEFs that at first notice appear highly similar to the Epacs. PDZ-GEFs contain the REM-CDC25-HD tandem and an RA domain in their C-terminal half and two cyclic nucleotide-binding domains and a PDZ domain in the N-terminal half (de Rooij et al. 1999). However, the cyclic nucleotide-binding domains lack critical residues for cAMP or cGMP binding, and indeed cyclic nucleotides cannot activate PDZ-GEF in vitro (Kuiperij et al. 2003; Pham et al. 2000). Although PDZ-GEFs exhibit an autoinhibited state (de Rooij et al. 1999), the mechanism of its relieve is currently unclear. Additional control of PDZ-GEF can be conferred by interacting scaffolding proteins, phosphorylation, and degradation (Letschka et al. 2008; Magliozzi et al. 2013; Sakurai et al. 2006). Furthermore, active G-proteins can bind to the RA

domain present in PDZ-GEF. The RA domain of PDZ-GEF1 binds active Rap1 (Liao et al. 2001), whereas the RA domain of PDZ-GEF2 binds active M-Ras (Yoshikawa et al. 2007). Recently, PDZ-GEF was found to interact with phosphatidic acid in the apical membrane of intestinal epithelial cells. This interaction resulted in the activation of Rap2A (Gloerich et al. 2012).

11.2.1.5 RasGEF1s

The RasGEF1 proteins RasGEF1A, B, and C comprise a relatively uncharacterized family of RapGEFs (Yaman et al. 2009). They contain the typical REM-CDC25-HD tandem, but other designated domains or regulatory sequences are lacking. So far experimental data on the RasGEFs have been restricted to in vitro measurements to test their activity towards various G-proteins. This revealed that the RasGEFs confer substrate specificity towards the Rap2 proteins (Yaman et al. 2009). In line with this, depletion of RasGEF1C enhances the barrier function of endothelial monolayers, similar to depletion of Rap2 (Pannekoek et al. 2013).

11.2.1.6 PLCε

PLCε is an atypical RapGEF in the sense that it bears multiple catalytically active domains (Jin et al. 2001; Lopez et al. 2001; Song et al. 2001). The N-terminus contains the REM-CDC25-HD module to catalyze Rap activation. The CDC25-HD is flanked by a PLC domain, which processes phospholipids. Furthermore, PLCε also contains an RA domain that can bind active Rap1 (Jin et al. 2001). Thus, PLCε induces Rap1 activation at places where Rap1 activity is already present, establishing a positive feedback system to enhance Rap activation.

11.2.2 RapGAPs

Rap inactivation by GTP hydrolysis occurs as one of the phosphates of GTP (gamma-phosphate) reacts with H_2O to GDP and Pi (Bos et al. 2007). However, charges around the G-protein-bound GTP do not allow the correct orientation and polarization of the attacking H_2O molecule, resulting in a very slow intrinsic GTPase activity of small G-proteins. GAPs generally function to coordinate the attacking H_2O and neutralize the negative charge of the gamma-phosphate, thereby greatly enhancing the efficiency of the H_2O attack on the gamma-phosphate (Bos et al. 2007).

It should be noted that inactivation is an essential step in the dynamic control of small GTPases. For instance, Rap1 enhances migration of Dictyostelium by inducing attachment to matrix. Efficient migration requires the formation of attachment at the leading front and release of cell attachment at the rear end. The latter is

ensured by GAP-mediated inactivation of Rap1 (Jeon et al. 2007). Similarly, extravasation of tumor cells is blocked by both constitutive Rap1 activation and RapGAP overexpression, as these conditions confer either too strong or too weak adhesion to migrate through the vessel wall (Freeman et al. 2010).

11.2.2.1 RapGAPs

The function of Rap1GAP is still rather elusive. Rap1GAP, comprising several splice variants, contains a GAP domain that catalyzes GTP hydrolysis on Rap proteins and a GoLoco domain. The GoLoco domain was found to interact with Gα subunits of hetero-trimeric G-proteins as well as 14-3-3 (Jordan et al. 2005; Meng et al. 1999; Willard et al. 2007), but the precise function of this domain is still unclear as interactions were reported to be activating and inhibitory. Phosphorylation by cGMP-dependent kinases may activate Rap1GAP.

11.2.2.2 Spa1 and the SPARs

Spa1 and the related GAPs SPAR1, 2, and 3 are RapGAPs characterized by the presence of a C-terminal PDZ domain. PDZ domains are notorious protein–protein interaction domains that regulate spatial distribution of signaling. As such, Spa1 and the SPARs are also found to locate to various complexes under control of their PDZ domains. For instance, SPAR is localized to Ephrin receptors to induce Rap1-mediated de-attachment by Ephrins (Richter et al. 2007), and to dendritic spines to regulate spine morphology (Pak et al. 2001). Apart from localization, the SPARs are also regulated by protein degradation via multiple pathways, all of which function to manage sustained activity of Rap1 (Gloerich and Bos 2011).

11.2.2.3 Plexins

Plexins are cell surface receptors for the semaphorin family of guidance cues. Their cytosolic tail harbors a GAP domain that catalyzes inactivation of Rap G-proteins. When not bound to semaphorins, Plexins exist as monomers, the GAP domain of which is auto-inhibited. Plexins dimerize upon ligand binding, which induces release of auto-inhibition. Therefore, semaphorin molecules act as repulsive guidance cues by inducing Plexin-mediated Rap1 inactivation (Wang et al. 2012).

11.2.2.4 GAP1 Proteins and SynGAP

Analogous to the CalDAG-GEFs, which display dual specificity towards Ras and Rap, RasGAPs of the GAP1 family inactivate both Ras and Rap (Kupzig et al. 2006, 2009). These GAPs, termed RASA2 ($GAP1^{m}$), RASA3 ($GAP1^{IP4BP}$), RASA4

(CAPRI), and RASAL1, contain a central GAP domain which is flanked by two C2 domains at the N terminus and a PH domain at the C terminus (King et al. 2013). The dual specificity towards both Ras and Rap is surprising as RasGAPs and RapGAPs generally utilize different mechanisms: RasGAPs provide an arginine to reposition Gln61 in Ras to allow correct polarization of the attacking H_2O. Rap proteins lack Gln61, but its function can be fulfilled by an asparagine that is provided by RapGAPs (Daumke et al. 2004). The dual specificity GAPs in essence function as RasGAPs by providing the arginine residue to facilitate Ras inactivation. In addition to that, dual specificity GAPs contain sequences outside the GAP domain that induce conformational changes in Rap that allow its Gln63 to aid in correct positioning of the attacking H_2O, thereby allowing these GAPs to also catalyze Rap inactivation (Sot et al. 2010).

11.3 Rap Functions

Initially, the function of Rap1 in mammalian cells was studied by the introduction of a constitutively active Rap1 (RapV12), by the introduction of a dominant negative Rap1 (RapN17) or the introduction of a RapGAP. For instance, a key finding in elucidating Rap1 functioning was made in 1999, when overexpression of the RapGAP Spa1 was shown to prevent attachment of HeLa cells (Tsukamoto et al. 1999). Indeed subsequent analysis revealed that Rap1 is a key mediator of integrin-mediated cell adhesion and of cell–cell junction formation (Boettner and Van Aelst 2009; Pannekoek et al. 2009; Raaijmakers and Bos 2009). Many subsequent studies on the function of Rap1 were greatly helped by the development of an Epac-selective cAMP analogue, 8-pCPT-2′O-Me-cAMP (007) (Enserink et al. 2002). Using this analogue endogenous Epac and subsequently Rap proteins were selectively activated in cells, and biological responses could be identified and analyzed. As such, the Rap proteins have now been solidly implicated in regulating endothelial barrier function, epithelial cell–cell adhesion, homing of circulatory cells, neurite outgrowth, induction of cell polarity, and cardiac contraction. To this end, Rap controls the activity or localization effector proteins by binding to their RA, RBD, or B41/ERM domain, which are structurally similar domains (Kiel and Serrano 2006). The effectors of the Rap proteins can be classified in different groups: inducers of cell–matrix adhesion (RAPL, RIAM), inhibitors of Rho-mediated contraction (KRIT1, Radil, Rasip1, ARAP1, ARAP3, RA-RhoGAP) and activators of actin remodeling (TIAM1, Vav2, TNIK, NIK, MINK) (Fig. 11.3). Furthermore, the cell–cell adhesion scaffold AF6 and the lipid modifier (and RapGEF) PLCε are controlled by Rap1. We will discuss these effectors in light of the physiological effects of Rap1 (see Fig. 11.4 for an overview picture).

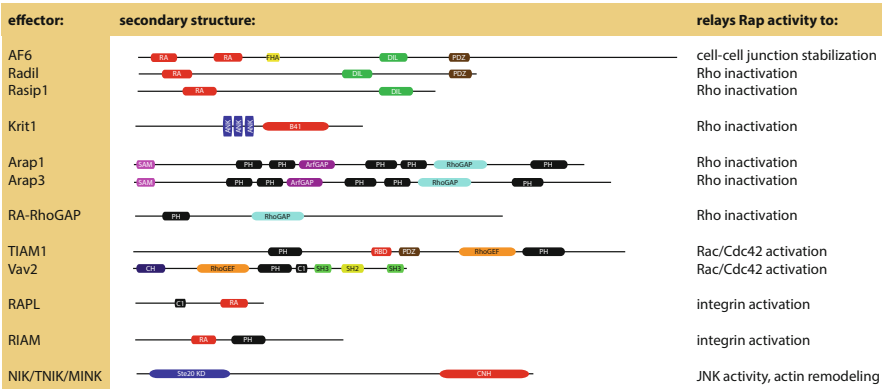

Fig. 11.3 Rap effectors. Domain architecture of Rap effector proteins is shown, in addition their Rap-induced function

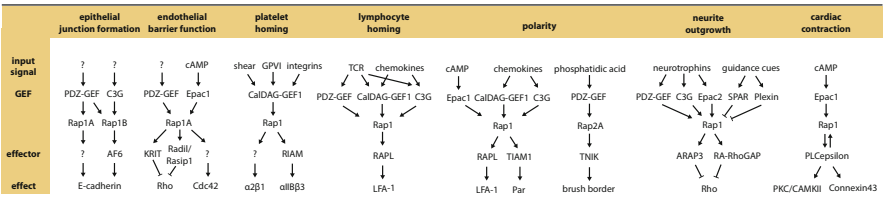

Fig. 11.4 Overview of Rap functions in physiological settings. Picture shows the core Rap signaling pathways in the physiological settings Rap functions in, indicating the known input signals, GEFs, Rap isoforms, and effector proteins. See text for details

11.3.1 Endothelial Barrier Function

The endothelium is the inner lining of the vascular system. Its main function is to form a barrier between the blood and the tissues underlying the vasculature. The tightness of this barrier should be very dynamically regulated, as controlled passage of fluid, solutes, and even circulating cells should be allowed upon request of the tissue (Komarova and Malik 2010). Rap1 activation enhances the barrier function of the endothelium (Pannekoek et al. 2014). To this end, barrier tightening agents increase cAMP levels, which activate both Epac1 and PKA to enhance barrier function (Lorenowicz et al. 2008). Also in the absence of cAMP moderate levels of active Rap1 are maintained to ensure a certain amount of basal barrier function. This effect is controlled by PDZ-GEF (Pannekoek et al. 2011). PDZ-GEF1 is the main isoform, which is supported by the defective cell–cell adhesion and concomitant yolk sac vasculogenesis observed in PDZ-GEF1−/− mice (Kanemura et al. 2009; Wei et al. 2007). Furthermore, Rap1 is activated when hyperpermeability is induced to prevent excessive leakage and ensure rapid reestablishment of the barrier (Birukova et al. 2013). Recent advances suggest that Rap1 controls

endothelial barrier by regulating tension on the actin cytoskeleton. To this end, various pathways have suggested, some of which may act in concert. Diminished tension on radial stress fibers is conferred by the Rap1 effectors KRIT1 (Glading et al. 2007; Stockton et al. 2010) or Rasip1/Radil (Post et al. 2013; Wilson et al. 2013; Xu et al. 2009). How KRIT1 regulates tension remains to be elucidated. Rasip1 and Radil control the RhoGAP ArhGAP29 to inhibit the tension pathway (Post et al. 2013; Xu et al. 2011). In contrast, Rap1 also induces tension in the actin cytoskeleton that runs along the cell–cell contact, a process mediated by Cdc42 (Ando et al. 2013). Hence, Rap1 simultaneously increases and decreases cytoskeletal tension depending on where the actin bundle is located. Together this renders cell–cell junction tightening. Interestingly, the Rap1-Rasip1/Radil-ArhGAP29 pathway also directs cell spreading, both in endothelial and in epithelial cells, due to the abovementioned relaxation of stress fibers (Post et al. 2013).

11.3.2 Epithelial Cell–Cell Adhesion

Similar to the endothelium, the epithelium is a cell layer that confers a barrier function to protect underlying tissue. The main difference between the two is that the epithelium is avascular, as the epithelium lines body cavities. Its barrier function depends on its location and can be very tight (e.g., skin) but also less tight (e.g., kidney). Rap1 is important for epithelial cell–cell adhesion. However, in contrast the endothelium, Rap1 effects on epithelial monolayers are only observed upon challenging of the monolayer: Rap1GAP overexpression prevents de novo formation of cell–cell junctions, but does not affect mature cell–cell junctions (Hogan et al. 2004). Similarly, effects of Rap1 siRNA could only be observed after replating the monolayer (Dube et al. 2008). Hence, Rap1 is required for epithelial cell–cell adhesion either during development or when the monolayer is challenged, but not for maintenance of cell–cell adhesion. To this end, Rap1 is activated by either C3G or PDZ-GEF2. Overexpression of dominant negative C3G prevents de novo junction formation, similar to Rap1GAP overexpression (Hogan et al. 2004). Here, the junctions lack E-cadherin, suggesting C3G/Rap1 functions in the recruitment of E-cadherin during junction formation. Depletion of PDZ-GEF2 has a milder effect: junctional E-cadherin levels are 80 % of their control counterparts and the junction appears zipper-like, indicative of high tension (Dube et al. 2008). Interestingly, these PDZ-GEF2 effects are differentially phenocopied by depletion of Rap1 isoforms: Rap1A depletion induces the zipper-like morphology, but does not affect the E-cadherin levels. The reverse goes for depletion of Rap1B, which affects E-cadherin levels but not junction morphology. It remains to be determined how these effects are controlled by Rap1. The junctional scaffold AF6 and the small G-protein Cdc42 have been suggested to control cell–cell adhesion downstream of Rap1, specifically considering E-cadherin levels (Fukuyama et al. 2005; Hogan et al. 2004; Hoshino et al. 2005). How these effectors cooperate and how the zipper phenotype is conferred remains to be elucidated.

11.3.3 Adhesion of Circulatory Cells

Circulatory cells like lymphocytes and platelets are non-adherent but can be rapidly induced to adhere when the body requires an immune response (lymphocytes) or blood clot formation (platelets) (Varga-Szabo et al. 2008; von Andrian and Mackay 2000). This adhesion relies on multistep adhesion cascades, in which activation of Rap1 ensures strong integrin-mediated adhesion to extracellular matrix. For platelets it has been shown that weak adhesion or prothrombotic factors impinge on CalDAG-GEF1 to induce activation of Rap1 and concomitant strong, integrin-mediated adhesion (Bernardi et al. 2006; Stefanini et al. 2009). Indeed, CalDAG-GEF1−/− mice display defective integrin-dependent platelet aggregation (Crittenden et al. 2004). Using model cell lines it is suggested that active Rap1 at the plasma membrane functions by recruiting RIAM, which induces the high affinity conformation of αIIbβ3 integrins via its binding protein Talin (Han et al. 2006; Lee et al. 2009). As a result, the platelets will form a meshwork with soluble fibrinogen fibers to form the blood clot. Additional control on this process is ensured by Rap1GAP. Nitric oxide prevents blood clot formation and it can do so by inducing phosphorylation of the platelet-specific isoform of Rap1GAP, thereby restricting Rap1 activation and concomitant integrin activation (Danielewski et al. 2005; Schultess et al. 2005).

Just as platelets, lymphocytes can be induced to adhere via a multistep adhesion cascade, thereby allowing lymphocyte adhesion at secondary lymphoid organs, where they screen for antigen, but also to allow strong adhesion between lymphocyte and an antigen presenting cell (APC). The latter relies on engagement of the T-cell receptor (TCR), which induces Rap1 activation via the GEFs C3G, CalDAG-GEF, and PDZ-GEF (Boussiotis et al. 1997; Katagiri et al. 2004b; Letschka et al. 2008). Why and how these three GEFs cooperate is unknown. Next, activated Rap1 engages with its effector RAPL to induce both clustering and activation of the LFA-1 integrin, thereby securing strong adhesion between lymphocyte and APC (Katagiri et al. 2003, 2004a).

Much less is known about adhesion of lymphocytes at secondary lymphoid organs. Here, reversible adhesion of lymphocytes is induced by chemokines, which impinge on CalDAG-GEF1 (Ghandour et al. 2007). However, the requirement of CalDAG-GEF1 for lymphocyte adhesion appears restricted to certain integrins, so other GEFs might contribute to regulate other integrins. The same holds for events downstream of Rap1: RAPL is required for stable arrest of lymphocytes. However, RAPL is not required for initial arrest, whereas Rap1 is (Ebisuno et al. 2010). Hence, other Rap1 effectors are at play here as well. RIAM would be the prime candidate, as it controls integrin activation downstream of Rap1. Indeed, RIAM depletion inhibits lymphocyte adhesion (Lafuente et al. 2004). However, as opposed to its function in platelet adhesion, RIAM does not require its Talin-binding domain to induce lymphocyte adhesion (Menasche et al. 2007). Instead, the central RA and PH domain are sufficient. These domains bind the adaptor protein SKAP1, which, together with its binding partners ADAP and

SLP-76, is required for Rap1 translocation (Kliche et al. 2006; Patzak et al. 2010). Furthermore, RIAM has been suggested to induce Rap1 activation by CalDAG-GEF1 by mobilizing intracellular calcium (Patsoukis et al. 2009). Apparently, the function of RIAM in Rap1 signaling may be more complex than solely being an effector in integrin signaling.

11.3.4 Neurological Functions

Rap1 is implicated in two aspects of neuronal functioning. First, Rap1 controls the extension of neurites, which will become the axon and dendrites (Anneren et al. 2000). Second, once a neurite establishes a synaptic contact, Rap1 controls synaptic transmission (Imamura et al. 2003). Little mechanistic data are known about the latter. However, Rap1 functioning in neurite extension is better understood. Activation of Rap1 is conferred by different inputs, which impinge on Epac2, C3G, and PDZ-GEF1 (Hisata et al. 2007; Liu et al. 2008; Radha et al. 2008). Intriguingly, the latter two seem to cooperate in maintaining Rap1 activity upon neurotrophin receptor stimulation by activating Rap1 at early endosomes (C3G) and late endosomes (PDZ-GEF) (Hisata et al. 2007). Guidance cues that either attract or repel the extending neurite also impinge on Rap1 activity, most notably via RapGAPs: Ephrins induce retraction by recruiting SPAR to the activated receptor (Richter et al. 2007), whereas Semaphorins bind to and induce dimerization of Plexins, thereby activating the GAP domain in the cytosolic tail of the Plexins (Wang et al. 2012). The mode by which Rap1 mediates neurite extension is clear-cut: it prevents retraction by inactivating Rho-mediated contraction. RA-RhoGAP and ARAP3 are effectors that harbor RhoGAP activity, thereby directly relaying Rap1 activation towards Rho inactivation (Jeon et al. 2010a, b; Yamada et al. 2005). In a different setting, the sequential activation of Rap1B and CDC42 determines the fate of an axon. This fate is regulated by selective protection for degradation of Rap1B in the future axon (Schwamborn et al. 2007; Schwamborn and Puschel 2004).

11.3.5 Polarity

Polarity occurs when a polarization cue induces an uneven distribution of proteins over the cell. By inducing cell adhesion to matrix or other cells, Rap can facilitate polarization. However, Rap can also directly induce polarization. Front-rear polarity induced by Rap1 is observed in migrating lymphocytes (Shimonaka et al. 2003) and neutrophils (Carbo et al. 2010) and apical-basal polarity induction by Rap1 is seen in hepatocytes during canalicular network formation (Fu et al. 2011) and in the endothelium upon VE-cadherin engagement (Lampugnani et al. 2010). In terms of mechanisms, the contribution of Rap2 to apical-basal polarity of the intestinal

epithelium is best understood (Gloerich et al. 2012). Here, induction of the polarity kinase Lkb1 induces translocation of PDZ-GEF to the apical membrane by increasing the membrane levels of phosphatidic acid, which functions as a direct anchor for PDZ-GEF. PDZ-GEF activates Rap2A, which induces translocation of its effector TNIK, its target MST4, and the MST4 substrate Ezrin. Next, activated Ezrin induces actin remodeling that results in the formation of a brush border. As such, a designated Rap2 signaling pathway couples induction of polarity to the polarized distribution of the actin cytoskeleton (Gloerich et al. 2012).

11.3.6 Cardiac Contraction

Cardiac myocytes are specialized muscle cells, the contraction of which functions to pump blood within the heart ventricles into the circulation. Contraction of cardiac myocytes relies on the electrically evoked release of calcium from the sarcoplasmatic reticulum. This release of calcium can be enhanced by cAMP via Epac1/Rap1. Activated Rap1 binds to the RA domain of PLCε, which induces the generation of DAG and IP3. These second messengers activate PKC to induce CaMKII-mediated phosphorylation of the ryanodine receptor, thereby inducing calcium release from the sarcoplasmic reticulum (Oestreich et al. 2009). As mentioned above, PLCε also harbors a CDC25-HD that can activate Rap1. This domain is also required for cardiac contraction. Here, Rap1 activated PLCε establishes a positive feedback loop to sustain Rap1 activity and cardiac contraction (Oestreich et al. 2009; Pereira et al. 2007).

Apart from directly inducing signaling to enhance excitation-contraction coupling, Rap1 also facilitates coordinated contraction of neighboring cells. This process is regulated by gap junctions, which allow the excitatory signal to be transduced to neighboring cells. The formation of gap junctions is facilitated by the presence of adherens junctions, which are induced by Epac1/Rap1 (Somekawa et al. 2005). Indeed, Rap1 increases cell–cell contact levels of Connexin43, which is the main constituent of gap junctions in cardiac myocytes (Somekawa et al. 2005). Furthermore, the aforementioned pathway Epac1/Rap1/PLCepsilon/PKC also increases phosphorylation of Connexin43, thereby further contributing to coordinated contraction (Duquesnes et al. 2010).

11.4 Concluding Remarks

The Rap1 protein, which originally was identified based on its capacity to revert Ras-induced cell transformation, is now known as a Ras-like G-protein that functions via its own independent signaling pathways. Rap1 is regulated by GEFs and GAP that are spatially localized in the cell and respond to a variety of extracellular stimuli. Molecular details of several downstream pathways have been revealed and

the emerging picture is that Rap proteins are molecular switches that in most pathways, but not exclusively, impinge on processes that are linked to the actin cytoskeleton. Indeed, Rap1 modulates Rho GTPases, like Rho, Rac, and Cdc42, as well as adhesion molecules, like integrins and cadherins, which are connected to the actin cytoskeleton. How are Rap1 proteins directing all these different functions? One could envision two models. The first is that depending on the cellular context and the activating signal, Rap1 mediates a localized event that results in a single response. The second is that activation of Rap1 results directly in a pleotropic effect, with multiple endpoints. The activation of Epac by the selective agonist 007 frequently results in pleiotropic effects (increased adhesion, tightening of junctions, inhibition of migration), suggesting that multiple pathways are activated. Indeed, the Radil/Rasip1-ArhGAP29 pathway mediates Epac1-induced cell spreading, but not Epac1-induced integrin-mediated adhesion in the same cell. In endothelial cells, Epac1 activation simultaneously inhibits Rho and activates CDC42, most likely through different pathways. In contrast to this multifaceted pathway, Rap2 employs a straightforward linear pathway to control intestinal epithelial cell brush border formation. Most likely, Rap proteins serve diverse roles in signaling, one as an upstream controller of diverse (actin-driven) processes, like the dynamic regulation of cell–cell and cell–matrix adhesion, and one that is more restricted to one directional event, like possibly the formation of brush borders, the determination of axon fate, and cardiac contraction.

In the past two decades we have obtained a fairly good picture on the function of Rap proteins and the molecular mechanism of several processes. It turned out to be one of the best model systems to study spatial and temporal control of signaling, with GEFs that are regulated by second messengers and that translocate to the site of action, with effectors that are spatially localized and activated after Rap activation, and with protein domains that change conformation to interact with spatial cues. However, much remains to be explored and many questions remain. For instance, relatively simple, why is Rap2A uniquely involved in brush border formation, whereas the other Rap proteins are expressed? More complicated is the integration of all Rap signaling events amongst each other and with other signaling events, which requires a systems biology approach. With 007 as a unique single trigger, and high-end single cell analyses, these latter studies can be done. In addition, the Rap signaling pathway controls many processes that are underlying disease, like endothelial cell leakage, cardiac contraction, blood cell, and platelet adhesion. Our knowledge of the Rap1 signaling pathway requires translation into the clinic. In our opinion selective activation of the Rap1 signaling pathway may be more beneficial than inhibition, e.g., to inhibit vessel leakage. 007 can be a lead compound, but specific activators for PDZ-GEF may be worth to develop.

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Chapter 12
The Coordinated Biology and Signaling Partners of Ral G-Proteins

Brian O. Bodemann and Michael A. White

Abstract The Ras-like (Ral) guanyl nucleotide-binding proteins, RALA and RALB, are highly similar proteins, which occupy sometimes overlapping, convergent, or divergent roles in regulating distinct biological processes. As downstream signaling partners of oncogenic Ras, these two proteins have been described to be hyper-activated in tumors to support aberrant biology during oncogenic transformation. To regulate a varied collection of normal and oncogenic biological processes, Ral G-proteins engage with six upstream RalGEF proteins, two upstream RalGAP complexes, and at least five distinct downstream effector pathways. Further specification of Ral signaling activity is ascribed to distinct posttranslational modifications of RALA, RALB, their upstream regulators, and their effectors. Emerging signaling paradigms within Ral signaling networks provide important insight into the signaling architectures exhibited by not only Ral G-proteins but also the wider range of Ras superfamily small G-proteins as well.

Keywords RalA • RalB • Sec5 • Exo84 • RalBP1 • Exocyst • Ras family G-proteins • RalGEF • RalGAP • Autophagy • Innate immunity • Oncogenes

12.1 Introduction

The Ras-like (Ral) guanyl nucleotide-binding proteins, RALA and RALB, were first identified decades ago by Pierre Chardin who isolated their cognate genes from a hybridization screen of B-lymphocyte cDNAs using degenerate probes containing highly conserved Ras sequences (Chardin and Tavitian 1986). Sharing 82 % identity at the amino acid level, we now know that RALA and RALB complete the Ral branch of the over 170-strong Ras family G-protein tree (Colicelli 2004). Study of

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Ral G-proteins gained momentum after recognition that a class of Ral-specific guanyl nucleotide exchange factors are direct effectors of oncogenic Ras. Importantly, both loss-of-function and gain-of-function studies identified Ral activation as a proximal consequence of Ras expression that supported Ras-induced oncogenic transformation in both cell culture and mouse model systems (Camonis and White 2005; Feig 2003; Feig et al. 1996; Hamad et al. 2002; Lim et al. 2006; Peschard et al. 2012; Rangarajan et al. 2004; Ward et al. 2001). Ral G-proteins have been implicated in the distinct but possibly interconnected processes of cell proliferation, motility, protein sorting, and maintenance of cellular architecture and energy balance (Bodemann et al. 2011; Bodemann and White 2008; Camonis and White 2005; Feig 2003; Martin et al. 2013). Here, we will provide an overview of the molecular architecture of Ral signaling networks and their importance in oncogenic transformation and explore emerging paradigms within the stimulus response networks regulated by Ral G-proteins.

12.2 Ral G-Protein Signaling Partners

RALA and RALB utilize an almost entirely shared set of direct interactions to regulate signal-evoked response networks. Upstream regulators alter the signaling potential or preference of the G-protein to engage downstream effectors, which mediate the signaling response to Ral G-protein activation.

12.2.1 Upstream Regulators of Ral G-Proteins

The signaling potential of Ral G-proteins is chiefly controlled by the status of its bound guanyl nucleotide (Fig. 12.1). When bound to guanosine 5′-diphosphate (GDP), Ral proteins adopt a conformation that prevents binding to its downstream effectors. Conversely, effector binding is increased by orders of magnitude when the G-protein binds to guanosine 5′-triphosphate (GTP). Upstream regulators of Ral G-proteins can be divided into three groups:

- Guanyl nucleotide exchange factors (GEFs)
- GTPase-activating proteins (GAPs)
- GTP-independent regulators

12.2.1.1 Ral Guanyl Nucleotide Exchange Factors

The Ral G-proteins, similar to most other Ras superfamily small G-proteins, have a group of dedicated enzymes that share the capability to catalyze the exchange of GDP for GTP. This activity is ascribed to a “CDC25 homology” domain, which

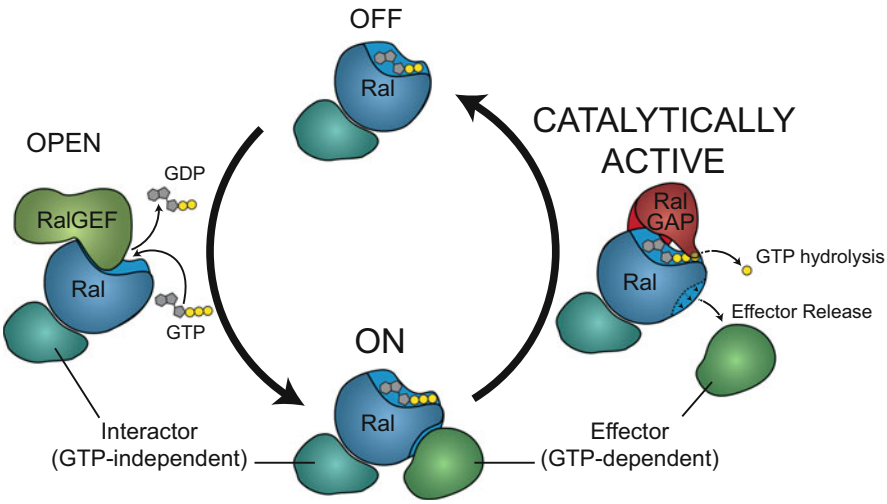

Fig. 12.1 The Ral GTPase cycle. Ral G-proteins are molecular switches that cycle between a GDP-bound "off" state and a GTP-bound "on" state. RalGEFs promote the GTP-bound "on" state by promoting an open confirmation in guanyl nucleotide binding pocket, which facilitates the exchange of GDP for more abundant GTP. RalGAPs facilitate a catalytically active state, which increases the rate of GTP to GDP hydrolysis to promote the "off" state. Typically, downstream effectors bind to Ral G-proteins only in the "on" state. Finally, GTP-independent interactors associate with Ral G-proteins regardless of the guanyl nucleotide bound

transiently and directly reduces Ral G-protein affinity for guanyl nucleotides, therefore allowing the exchange of GDP for the considerably more abundant GTP. Structural features of the known Ral Guanyl Nucleotide Exchange Factors (RalGEFs) allow them to be parsed into two distinct families based on the presence of a Ras-association (RA) domain that links Ras activation to Ral activation—RALGDS, RGL1, RGL2/RLF, and RGL3—or the presence of a pleckstrin homology domain that ostensibly allows signaling activation of Ral G-proteins through yet to be described Ras-independent mechanisms—RALGPS1A/B and RALGPS2 (Wolthuis et al. 1997; Rebhun et al. 2000; Linnemann et al. 2002; Colicelli 2004; Bodemann and White 2008; Vigil et al. 2010). One can imagine that the diversity of RalGEFs help to specify Ral G-protein function; however, distinct pairs of RalGEFs were described to work collaboratively during cytokinesis—RALGDS, RALGPS2, and RALA were required for cleavage furrow formation; and RGL1, RALGPS1, and RALB were required for midbody abscission (Cascone et al. 2008).

12.2.1.2 Ral GTPase-Activating Proteins

Two distinct Ral GTPase-Activating Protein (RalGAP) complexes, RalGAP1 and RalGAP2, oppose the effects of RalGEFs on Ral G-proteins. RalGAP complexes increase the catalytic activity of Ral G-proteins by contributing a critical histidine

residue to the active site of the GTPase, which facilitates the inactivation of downstream signaling through GTP to GDP hydrolysis. Each complex consists of one of two catalytic alpha subunits, RALGAPA1 or RALGAPA2, paired with a single regulatory beta subunit, RALGAPB (Shirakawa et al. 2009; Chen et al. 2011). Loss of both RalGAP complexes through shRNA depletion of RALGAPB is reported to release restraint on a RALB–SEC5–mTORC1-mediated growth pathway (Martin et al. 2013). RALGAPA2 is a critical mediator of insulin-stimulated GLUT4 exocytosis (Chen et al. 2011), which will be detailed later in Sect. 12.4.2.

12.2.1.3 GTP-Independent Regulators of Ral G-Protein Function

In addition to RalGEF/RalGAP regulation of the guanyl nucleotide status of Ral G-proteins, numerous posttranslational modifications have been reported to alter the function of Ral G-proteins. The phosphorylation of RALA at serine-194 by Aurora A triggered re-localization of RALA to mitochondria during mitosis, where RALA engaged RALBP1 to promote the fission and distribution of mitochondria to daughter cells (Kashatus et al. 2011). Similarly, phosphorylation of RALB at serine-198 by protein kinase C alpha (PKC_{α}) resulted in both an increase in GTP loading of RALB and its localization to endomembranes to support vesicle trafficking (Martin et al. 2012). In addition, RALA and RALB were both reported to exhibit altered functional characteristics when modified by mono-ubiquitylation (Neyraud et al. 2012; Simicek et al. 2013), and this will be discussed further in Sect. 12.5.2.

12.2.2 Downstream Effectors of Ral G-Protein Function

Downstream effectors of Ral G-proteins require direct interaction with Ral for their signaling activities. These interactions can be dependent or independent of the identity of guanyl nucleotide bound to the G-protein. Canonically, GTP-loaded Ral G-proteins adopt a conformation that facilitates direct interaction at effector binding loops within the core GTPase domain. Conversely, a GTP-independent effector binds outside of the core GTPase domain but still requires Ral binding for their signaling activity. There are four well-annotated Ral–effector pathways:

- RALA binding protein 1 (RALBP1)
- ZO-1-associated nucleic acid-binding protein (ZONAB)
- Exocyst complex subunits (SEC5 and EXO84)
- Phospholipase D1 (PLD1)

12.2.2.1 RALA Binding Protein 1

In addition to its role in mitochondrial fission mentioned earlier, the GTP-dependent effector, RALBP1, also mediates Ral G-protein involvement in clathrin-mediated endocytosis through direct interactions between RALBP1 and the AP2 clathrin adaptor complex (Jullien-Flores et al. 2000). RALBP1 has also been described to modulate cell cycle progression by cytoplasmic sequestration of cyclin-dependent kinase inhibitor, p27 (Kfir et al. 2005; Tazat et al. 2013).

12.2.2.2 ZO-1-Associated Nucleic Acid-Binding Protein

ZONAB is a Y-box transcription factor that is reported to directly repress mitogen-stimulated gene expression programs. Interaction with GTP-bound Ral G-proteins restrains ZONAB at the plasma membrane, which relieves repression of immediate early gene promoters and increases responsiveness of cell cycle progression to mitogen stimulation (Frankel et al. 2005).

12.2.2.3 Exocyst Complex Subunits (SEC5 and EXO84)

The exocyst is a hetero-octameric protein complex engaged in vesicular trafficking, dynamic membrane assembly, and the assembly and activation of adaptive signaling cascades (Moskalenko et al. 2002; Rosse et al. 2006). GTP-loaded Ral G-proteins directly engage the exocyst through two distinct subunits, SEC5 and EXO84 (Moskalenko et al. 2002, 2003). These physical interactions collectively mobilize exocyst assembly for engagement of the full hetero-octameric complex as well as distinct exocyst subcomplexes. The Ral G-protein-dependent assembly of the full exocyst holocomplex is required for distinct mobilization and tethering events required for organization of polarized membrane domains and selective signal-dependent secretory events (Guo et al. 2000; Moskalenko et al. 2002; Hsu et al. 2004; He and Guo 2009). A RALB–SEC5 subcomplex promotes activation of TANK-binding kinase 1 (TBK1) to help engage the host defense response (Chien et al. 2006). Distinct SEC5- and EXO84-dependent subcomplexes mediate the cellular response to nutrient availability (Bodemann et al. 2011), which will be described in Sect. 12.5.1.

12.2.2.4 Phospholipase D1

PLD1 is a GTP-independent effector of Ral G-proteins, which binds to a short amino-terminal stretch of amino acids preceding the core GTPase domain. Ral G-proteins and ADP-ribosylation factor 1 (ARF1) synergistically activate PLD1 through distinct binding sites (Kim et al. 1998; Luo et al. 1998). RALA and PLD1

are reported to regulate signal-dependent exocytic and endocytic events (Shen et al. 2001; Vitale et al. 2005).

12.3 Ral G-Proteins in Cancer

The role of RALA and RALB in human cancer is highlighted by their place as downstream targets of Ras G-proteins, which are frequently mutated in human cancer. Ras promotes Ral activation through direct interaction with RA domain containing RalGEFs. In this section, we will examine how Ral G-proteins and their downstream signaling pathways are key mediators of oncogenic transformation with particular focus on their supporting role in oncogenic Ras mutation.

12.3.1 Strong Support: Ral G-Proteins in Oncogenic Ras Driven Malignancies

Ras effector mutants selectively uncoupled from RalGEFs and PI3Ks (RasT35S), RAF kinases and PI3Ks (RasE37G), or RalGEFs and RAF kinases (RasY40C) have been broadly employed in gain-of-function studies to evaluate the relative contributions of RAF, RalGEF, and PI3K activation to oncogenic Ras-induced tumorigenic transformation (White et al. 1995; Joneson et al. 1996; Camonis and White 2005). Expression of these variants in telomerase-immortalized human cell models, derived from normal tissues, revealed context-selective requirements for Ras effector pathway activation (Hamad et al. 2002). The combined effects of all three effector arms were required to support xenograft tumor formation in mammary epithelial cells (Rangarajan et al. 2004). Conversely, RalGEF activation together with PI3K activation or RAF activation was sufficient to transform human kidney epithelia or human fibroblasts, respectively (Rangarajan et al. 2004). Together, these results suggest a unanimous dependence on Ral pathway activation for oncogenic Ras-induced tumorigenic transformation. Furthermore, selective activation of the Ral G-proteins by Ras was also shown to be sufficient to promote bone metastasis in a xenograft model of prostate cancer (Yin et al. 2007), to mediate RAF/MAPK-independent inhibition of skeletal muscle differentiation by Ras (Ramocki et al. 1998), and to mimic defective myeloid differentiation characteristic of elevated Ras signaling in acute myeloid leukemia (Omidvar et al. 2006).

Loss-of-function studies have also revealed comprehensive participation of RALA and RALB signaling in the maintenance of tumorigenic phenotypes. RNAi-mediated evaluation of the distinct contributions of RALA and RALB to cancer cell viability revealed that RALA expression was necessary for anchorage-independent proliferation of transformed cells, while RALB expression was necessary for cancer cell survival (Chien and White 2003). The significance of these

phenomena to tumorigenicity was established with xenograft models of pancreatic cancer where Ral signaling is required for tumor metastasis (Lim et al. 2006). Subsequent studies have brought forth similar observations in other disease models together with directly implicating the Ral effector proteins RALBP1, SEC5, and EXO84 (Lim et al. 2005; Oxford et al. 2007; Issaq et al. 2010; Mishra et al. 2010; Zipfel et al. 2010; Martin et al. 2011).

An elegant "multi-hit" transgenic model employed stochastic expression of Ras effector mutants in lung epithelia to probe the relative requirements of RAF, RalGEF, and PI3K pathway activation in support of oncogenic K-Ras-induced lung adenocarcinoma (Musteanu et al. 2012). This approach employed a transgene carrying each of the three Ras effector mutants in an inverted orientation with respect to its promoters and flanked by heterotypic FRT sites. Following Cre induction of randomized expression of the effector-selective Ras mutants from the transgene in the lung, self-selected tumors were isolated to examine the frequency of co-occurrence of Ras effector mutant expression within the tumors. The majority—over 85 % of examined lesions—expressed all three effector mutants, further highlighting a compulsory collaborative role for RalGEF–Ral pathway activation in this model. Genetic ablation of RALA or RALB is possible in the laboratory mouse; however, compound knockout of RALA and RALB is embryonic lethal, which indicates that Ral G-protein signaling is necessary for development (Peschard et al. 2012). When conditional RALA and RALB alleles were evaluated in a mouse model of oncogenic K-Ras-induced lung cancer, only compound inactivation of RALA and RALB significantly reduced tumor burden, which clearly establishes that K-Ras lung tumors strongly depend on Ral G-protein signaling (Peschard et al. 2012).

12.3.2 Breaking Bad: Ral Effector Pathways hijacked for Tumorigenesis

The Ral G-proteins coordinate numerous tightly regulated signal response networks that facilitate a diverse array of biological processes. Aberrant activation of these signaling routines may be critical to the support of the tumorigenic platform by Ral G-proteins. This notion was first elaborated by the RALB-specific contribution to cancer cell survival through activation of TANK-binding kinase 1 (TBK1). This kinase is a central node in a response network required to activate host defense gene expression in the face of a virally compromised environment (Beutler 2004; Buss et al. 2004; Fitzgerald et al. 2003; Hiscott 2004; Kato et al. 2006; Kawai et al. 2005; Lee et al. 2006; McWhirter et al. 2004, 2005; Sharma et al. 2003). Through a process that is tethered to the exocyst, but perhaps independent of the established exocytic function of the exocyst, the RALB–SEC5 effector complex directly assembles with and activates a TBK1–AKT complex in response to viral exposure (Chien et al. 2006; Ou et al. 2011). This pathway is dispensable for the survival of

normal cells in culture; however, chronic RALB activation constitutively engages this signaling pathway in a variety of cancer cells, to restrict induction of apoptotic programs that are normally triggered in the context of oncogenic stress (Chien et al. 2006; Ou et al. 2011). Given that AKT is described to suppress RalGAP2 inhibition of RALA and RALB, it is conceivable that this pathway may further amplify both RALA and RALB activity during oncogenic transformation (Chen et al. 2011). The functional connection between the RALB–SEC5–TBK1–AKT activation complex and tumor cell survival suggests that oncogenic transformation can misappropriate normal cell-autonomous host defense signaling to deflect the activation of cell-death checkpoints. This relationship sheds light on a novel aspect of the aberrant cell regulatory programs supporting malignant transformation, and suggests that proteins like TBK1 might be conceptually idyllic candidate targets for the development of drugs with sizeable therapeutic windows.

This deviant turn in Ral-dependent biology during malignant transformation is not limited to RALB, as normal RALA-dependent signal response pathways are hijacked as well. Cell division is a tightly regulated process, which is dependent on the presence of numerous signals including appropriate substrate attachment. When detached from substrate, loss of integrin-mediated adhesion initiates caveolin-dependent internalization of cholesterol-rich and sphingolipid-rich lipid raft microdomains to recycling endosomes (del Pozo et al. 2004, 2005). These lipid rafts microdomains serve as signaling platforms, and their removal from the plasma membrane after substrate detachment is known to suppress growth signaling (del Pozo et al. 2004, 2005). Upon re-adhesion, RALA, through the exocyst complex, mediates integrin-dependent lipid raft exocytosis, and constitutively active RALA restores lipid raft exocytosis during detached conditions to promote anchorage-independent growth signaling (Balasubramanian et al. 2010). These examples of abnormal engagement of normal Ral-dependent signal response pathways are unlikely to be isolated events, and future work will likely unearth further contributions to tumorigenesis.

12.4 Ral G-Proteins in Action: Insulin-Stimulated GLUT4 Exocytosis

Insulin induces a rapid increase in the uptake of glucose in adipose and striated muscle cells through the regulated display of the glucose transporter type 4 (GLUT4) (Watson et al. 2004; Huang and Czech 2007). In the fasted, low insulin state, GLUT4 is sequestered at intracellular vesicles, and upon insulin exposure, GLUT4-containing vesicles translocate to and integrate with the plasma membrane to facilitate rapid glucose uptake (Watson et al. 2004; Huang and Czech 2007). The exocyst complex plays a crucial role in tethering GLUT4 vesicles during GLUT4 (Inoue et al. 2003, 2006). In addition, RALA is an essential regulator of this process (Chen et al. 2007), and these research works provide an outstanding exposition of

both canonical and emerging signaling paradigms within Ral G-protein signaling networks:

- **Localization Dynamics:** Ral association with cytoskeletal motor proteins can alter the localization of Ral, its effector exocyst complex, and associated cargo vesicles.
- **Regulation Dynamics:** Relief of inhibition by RalGAP proteins can serve as a positive instructive signal to drive Ral signaling.
- **Effector Dynamics:** Ral association with effectors is regulated not only by guanyl nucleotide status but also posttranslational modification of its effectors.

12.4.1 Localization Dynamics: RALA Utilizes GTP-Dependent and GTP-Independent Interactions to Drive GLUT4 Delivery and Tethering

Insulin-stimulated GLUT4 exocytosis requires the exocyst complex, which is known to associate with multiple small G-proteins; however, only RALA but not ARF6 or RAB11 co-precipitated with the exocyst proteins during insulin stimulation (Chen et al. 2007). Furthermore, knockdown of RALA and the Ral effectors and exocyst subunits, SEC5 and EXO84, disrupted insulin-stimulated GLUT4 exocytosis and glucose uptake in 3T3-L1 adipocytes (Chen et al. 2007). Mass spectrometry analyses of RALA immunoprecipitates identified the unconventional myosin motor protein, MYO1C (Chen et al. 2007), which was previously described to associate with and transport GLUT4-containing vesicles (Bose et al. 2002; Huang et al. 2005). The interaction of RALA and MYO1C was confirmed in vivo and in vitro and mapped to the IQ calmodulin-binding motif repeats within MYO1C (Chen et al. 2007). Calmodulin appears to regulate this process as calcium and Calmodulin were required for RALA–MYO1C interaction in vitro, the Calmodulin inhibitor trifluoperazine disrupted RALA–MYO1C association, and siRNA knockdown of Calmodulin or trifluoperazine treatment both disrupted insulin-stimulated GLUT4 exocytosis (Chen et al. 2007). Importantly, the interaction of MYO1C is independent of RALA guanyl nucleotide status, which suggests that RALA is free to form GTP-dependent interactions with its effector proteins, SEC5 and EXO84, when bound to MYO1C (Chen et al. 2007). Consistent with this model, exocyst proteins were found in cross-linked MYO1C immunoprecipitates and insulin-stimulated glucose uptake was synergistically disrupted when dominant negative forms of SEC5 and MYO1C were co-expressed in cells simultaneously (Chen et al. 2007). These findings add significant understanding to Ral G-protein signaling architecture as they illustrate the important and unique contributions of both GTP-dependent and GTP-independent Ral interactors.

Traditionally, Ral G-proteins are thought to contribute locality to their dependent signaling processes by associating with membranes through their unstructured, geranylgeranylated C terminus. This role is once again exhibited in RALA-

regulated GLUT4 exocytosis as RALA assembles its effector, the exocyst complex, on GLUT4-containing vesicles; however, the interaction of MYO1C with RALA suggests an additional important role for RALA in this process. By assembling with MYO1C upon insulin stimulation, RALA effectively becomes a mobile signaling tether for its dependent effector complex, the exocyst, and provides a means for delivering both GLUT4-containing vesicles and the exocyst complex necessary for its tethering to the plasma membrane. Once delivered to the plasma membrane the exocyst complex has been described to associate with additional G-proteins, such as TC10 for tethering and RAB11 for vesicle trafficking/recycling (Inoue et al. 2003, 2006; Wu et al. 2005; Zhang et al. 2004). The exact sequence and consequence of each G-protein–exocyst interaction during this process remains to be elucidated; however, through direct interactions with MYO1C and the exocyst complex, RALA is critical for both the mobilization and tethering of GLUT4-containing vesicles during insulin stimulation.

12.4.2 Regulation Dynamics: Relief of RalGAP2 Inhibition Serves as a Positive, Instructive Signal for RALA-Mediated GLUT4 Exocytosis

Activation of the PI3K–AKT pathway has been demonstrated to play a pivotal role in regulating the trafficking of GLUT4 vesicles to the plasma membrane during insulin treatment (Capilla et al. 2007; Sakamoto and Holman 2008; Whiteman et al. 2002). During insulin treatment of mouse 3T3-L1 adipocytes, the catalytic subunit, RALGAPA2, of the RalGAP2 complex was phosphorylated on serine-486, serine-696, and threonine-715 (Chen et al. 2011). Phosphorylation of these sites was blunted when AKT2 was depleted by siRNA or inhibited with an Akt-1/2 selective inhibitor (Chen et al. 2011). Inhibition of the PI3K–Akt pathway with wortmannin increased association of RALGAPA2 with a RALA(72L) mutant, which stabilizes RalGAP interactions (Chen et al. 2011). Thus, the association of the RalGAP2 complex with and consequently the inhibition of RALA by this complex is likely regulated by AKT-mediated phosphorylation of RALGAPA2 (Chen et al. 2011). Consistently, RALA activity was increased significantly in both baseline and insulin-stimulated conditions when RALGAPA2 was depleted by siRNA (Chen et al. 2011). RalGAP2 is likely only part of the story as insulin significantly increased RALA activity even when RALGAPA2 was depleted, which suggests that insulin may also regulate an unspecified RalGEF protein during this process as well (Chen et al. 2011).

Relief of RalGAP inhibition likely has an important temporal impact on Ral G-protein signaling. The dissociation constant for RALA–Effector complexes is significantly smaller when GTP-bound (RALA-GTP–SEC5: 10 nM) compared to GDP-bound (RALA-GDP–SEC5: 1,400 nM) (Jin et al. 2005); therefore, the association dynamics of Ral–Effector complexes will be largely determined by the rate

of Ral-GTP to Ral-GDP hydrolysis. Given the transient nature of Ral/RalGAP interaction, a RalGEF may still gain access to promote Ral GTP-loading and promote Ral–Effector complexes; therefore, concurrent activation of both RalGAP and RalGEF proteins would trigger transient Ral–Effector complexes—a favorable architecture for complex assembly and activation but not mobilization. Conversely, in the absence of RalGAP activity, a RalGEF can activate Ral to the GTP-bound state and promote long-lived Ral–Effector complexes as the intrinsic GTP hydrolysis rate of Ral G-proteins is exceeding slow. Thus, while either decreasing RalGAP activity or increasing RalGEF activity would both shift the overall balance of a given Ral–Effector complex, only inhibiting RalGAP activity is likely to provide the temporal stability necessary for Ral-dependent mobilization of effector complexes. During insulin-stimulated GLUT4 exocytosis, RALA serves as a molecular bridge between the MYO1C cytoskeletal motor protein, the exocyst tether complex, and GLUT4-containing vesicles; therefore, if Ral–SEC5 association was disrupted while the GLUT4-containing vesicle was en route to the plasma membrane, the vesicle might lose key exocyst factors necessary for its molecular addressing, docking, and fusion at the plasma membrane. Thus, relief of RalGAP2–RALA inhibition provides a noticeably well-matched regulatory mechanism for RALA during insulin-mediated GLUT4 exocytosis.

Activation of small G-proteins via AKT-mediated inhibition of their GAP complexes appears to be a familiar mechanism in the cellular response to feeding. In addition to the RalGAP2–RALA pathway described above, the TBC1 domain family member 4 (TBC1D4) RabGAP is also inactivated by Akt phosphorylation which frees RAB8, RAB10, and RAB14 to fulfill complementary roles in GLUT4 trafficking to the plasma membrane (Miinea et al. 2005; Sano et al. 2003, 2007). In addition, the tuberous sclerosis 1/2 (TSC1/2) tumor suppressor complex is a RhebGAP inhibited by multiple AKT phosphorylation events, which allows the G-protein, RHEB, to promote the activation of the key cell growth regulator, mTORC1, in response to insulin and amino acid stimulation (Tee et al. 2003). Evidence of RalGAPs as key mediators of growth signaling is mounting as it was recently reported that a RalGAP-regulated RALB–SEC5–mTORC1 pathway provides additional regulation of mTORC1 in mammalian cells, and in *Caenorhabditis elegans*, RalGAP replaces TSC1/2, which is unconserved, in regulating both RHEB- and RALA/B-dependent growth pathways (Martin et al. 2013).

12.4.3 Effector Dynamics: RALA–SEC5 Dissociation Is Regulated by a GTP-Hydrolysis Independent Mechanism

Upon RALA-dependent delivery to the plasma membrane, exocyst-associated GLUT4 vesicles are tethered to the plasma membrane for SNARE-dependent fusion with the plasma membrane. Recent findings have suggested that disruption

of RALA–SEC5 binding is critical for GLUT4 vesicle fusion to proceed. Conventionally, G-proteins are thought of as "switches" which are "turned off" by GTP to GDP hydrolysis catalyzed by GAP proteins. In this process, GTP hydrolysis on RALA is unlikely to play a significant role as insulin triggers RalGAP inhibition, which would be required for hydrolysis. RALA–SEC5 dissociation is instead regulated by PKC-catalyzed phosphorylation of SEC5 within its Ral binding domain. Mutation of this site, serine-89, to alanine in order to mimic the unphosphorylated state led to an accumulation of RALA–SEC5 complexes while, conversely, the phosphomimetic serine to glutamic acid mutation disrupted association with endogenous or constitutively active RALA(23V). Expression of either SEC5 serine-89 mutant perturbed insulin-stimulated fusion of GLUT4 vesicles. Thus, dynamic regulation of this site is required for successful GLUT4 delivery and fusion, which is consistent with a model where RALA binds to SEC5 with unphosphorylated serine-89 and releases SEC5 once the site has undergone phosphorylation by PKC. Multiple PKC isozymes were shown to phosphorylate this site specifically in vitro and inhibition of PKC with Bisindolylmaleimide I or the cell-permeable calcium chelator, BAPTA-AM, blocked serine-89 phosphorylation and insulin-stimulated GLUT4 exocytosis. Interestingly, appropriate regulation of this phosphorylation site was shown to be important for other processes as well, including cytokinesis, exocytosis of Transferrin receptor, and zebrafish embryogenesis, which advocates an important role of this modification as a GTP hydrolysis-independent regulator of Ral–Exocyst function.

12.4.4 A Coordinated Signaling Architecture Facilitates GLUT4 Exocytosis

Each of these signaling paradigms contributes to a coordinated signal response architecture tuned to achieve a successful biological outcome—insulin-mediated GLUT4 vesicle delivery (Fig. 12.2). First, Calcium/Calmodulin promotes the association of RALA with MYO1C, a molecular motor protein, in a GTP-independent fashion that allows RALA to accommodate an additional GTP-dependent interactor. Second, RALA recruits the exocyst tethering complex to GLUT4-containing vesicular cargo through a GTP-dependent association with SEC5. Importantly, RALA and SEC5 must maintain their association to facilitate a continued assembly of the cargo (GLUT4-containing vesicles), the mode of transit (MYO1C), and the molecular addressing system (exocyst). Third, this continued association is achieved, in part, through inactivation of RalGAP2 by PI3K–AKT pathway phosphorylation of RALGAPA2. Of note, RalGAP inactivation removes a canonical regulatory element from the network, such that RALA can deliver and tether GLUT4 vesicles to the cytosolic side of the plasma membrane, but RALA cannot release the vesicle for fusion by "letting go" of SEC5 through GTP hydrolysis. PKC phosphorylation of SEC5 provides an elegant biochemical solution for

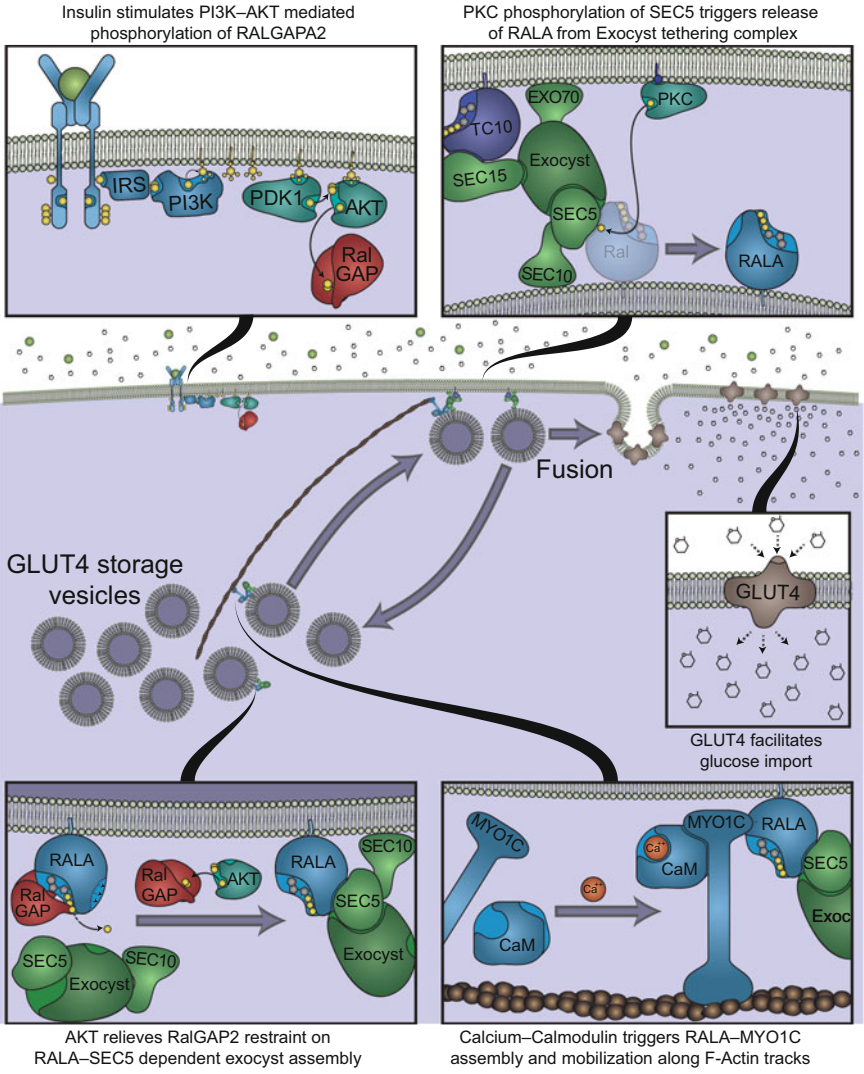

Fig. 12.2 RALA signaling during insulin-stimulated GLUT4 exocytosis. Insulin stimulates activation of the PI3K–AKT kinase pathway, which inactivates the RalGAP2 complex. This triggers SEC5 assembly with RALA on GLUT4 storage vesicles. Insulin stimulates Calcium–Calmodulin-dependent assembly of RALA with MYO1C, which facilitates transport of RALA and the partially assembled exocyst complex along F-actin tracks towards the plasma membrane. After exocyst-dependent tethering of the GLUT4 storage vesicle to the plasma membrane, RALA–SEC5 association is disrupted by PKC phosphorylation of SEC5. This allows GLUT4 vesicles to proceed with plasma membrane integration and, ultimately, for glucose import into the cell

the problem of disrupting RALA–SEC5 association. Of final note, the regulatory kinases mentioned, AKT and PKC, achieve full activation when associated with the membrane phospholipids, PIP_3 and DAG, respectively; thus, the signals regulating this network are spatially restricted to areas of active membrane modification allowing GLUT4 vesicles to be delivered, tethered, released, and integrated right at the very serum-facing surface where insulin liganded to the insulin receptor.

12.5 Ral G-Proteins in Action: Nutrient-Mediated Macroautophagy Regulation

Macroautophagy (commonly and herein referred to as autophagy) plays an important role in tissue homeostasis, in cellular adaptation to nutrient withdrawal, and in the removal of dysfunctional organelles and intracellular pathogens. The de novo generation of the double-membrane autophagosome requires responsiveness to inductive signals that specify location, contents, and duration. Amongst the earliest of inductive signals is the dephosphorylation of inhibitory mTORC1-dependent sites on the ULK1–Atg13–FIP200 induction complex and AMBRA1 of the AMBRA1–BECLIN1–ATG14L–VPS34 vesicle nucleation complex (Hosokawa et al. 2009; Nazio et al. 2013). This releases AMBRA1 to recruit TRAF6, which supports ULK1 ubiquitylation by lysine-63-linked chains (Nazio et al. 2013). This modification of ULK1 promotes its stabilization, self-association, and function (Nazio et al. 2013). Active ULK1 kinase phosphorylates AMBRA1 in a feed-forward loop, which releases AMBRA1–BECLIN1–ATG14L–VPS34 complex from an inhibitory association with dynein on microtubules (Di Bartolomeo et al. 2010). This frees the AMBRA1–BECLIN1–ATG14L–VPS34 complex to be trafficked to the site of autophagosome nucleation, where VPS34 coats the site with phosphatidylinositol-3-phosphate (PI-3-P), which serves as a recruitment signal for ATG16–ATG5/ATG12 component of the isolation membrane elongation machinery (Romanov et al. 2012; Suzuki et al. 2001). Two ubiquitin-like molecules, ATG12 and LC3, undergo conjugation to ATG5 and phosphatidylethanolamine respectively to advance autophagosome formation (Mizushima et al. 1998a, b). The lipidated form of LC3, LC3-II, decorates the inner and outer surfaces of the autophagosomes and serves as a discrete marker of autophagosomes (Kabeya et al. 2000, 2004). Dynamic membrane events and coordinated signaling events climax in the formation of a double-membrane autophagosome. The ultimate destination of the autophagosome is fusion with a lysosome, which facilitates the turnover of engulfed materials by lysosomal/vacuolar acid hydrolases and lipases. RALB plays a central role in assembling, activating, and mobilizing distinct exocyst subcomplexes with key members of the autophagy induction and nucleation machines to promote autophagy during nutrient withdrawal (Bodemann et al. 2011). These studies offer insight into emerging signaling paradigms within Ral G-protein signaling networks:

- **Effector Dynamics:** Ral can assemble with and activate distinct exocyst subcomplexes in response to distinct upstream signals.
- **Regulation Dynamics:** Ral ubiquitylation status regulates its availability to specific downstream exocyst effector pathways.

12.5.1 Effector Dynamics: RALB Engages Distinct Exocyst Subcomplexes to Mediate the Cellular Response to Nutrient Availability

Productive galvanization of autophagy machinery during nutrient deprivation requires the assembly, activation, and mobilization of numerous signaling machines. A screen for exocyst interacting proteins uncovered exocyst subunit interactions with the ULK1–Atg13–FIP200 autophagy induction complex. Knockdown of RALB but not RALA with siRNA significantly perturbed nutrient starvation induced autophagy, and the GTP loading of RALB but not RALA was increased during nutrient deprivation. Overexpression of constitutively active G-protein-deficient mutant, RALB(23V), increased the frequency of accumulation of markers of vesicle nucleation, vesicle elongation, and autophagy during unfavorable nutrient-rich conditions. Co-expression of a kinase-dead form of ULK1 blocked RALB(23V)-induced autophagosome accumulation; therefore, RALB activation is sufficient to engage autophagy during unfavorable conditions but requires ULK1 kinase activity to do so (Bodemann et al. 2011). RALB co-localized with the same markers of vesicle nucleation, vesicle elongation, and autophagy during nutrient restriction (Bodemann et al. 2011). Taken together, these results suggest RALB to be a spatially restricted activation signal for the engagement of autophagosome formation—a finding that has been corroborated in additional cell systems (Shi et al. 2012; Simicek et al. 2013).

Further candidate-based interaction studies revealed that BECLIN1, a key regulator of vesicle nucleation during autophagy, also associated with the exocyst subunits and Ral effector proteins, SEC5 and EXO84. Expression of RALB(23V) was sufficient to drive assembly of BECLIN1 and ULK1 with both EXO84 and SEC5. Conversely, BECLIN1 and ULK1 association with SEC5 and EXO84 was prevented when endogenous Ral–effector interactions were blocked by expressing the minimal Ral binding domain of RALBP1, and similar findings were observed for BECLIN1 interacting proteins ATG14L and the lipid kinase Vps34. Depletion of individual exocyst components with siRNA knockdown revealed a subset of SEC3, SEC8, EXO70, and EXO84 to be required for nutrient deprivation induced autophagy (Bodemann et al. 2011). Furthermore, nutrient availability was found to regulate the RALB engagement of either SEC5 or EXO84, under nutrient-rich or poor conditions, respectively (Bodemann et al. 2011). Together, these results suggest the presence of distinct nutrient-regulated exocyst subcomplexes (Fig. 12.3).

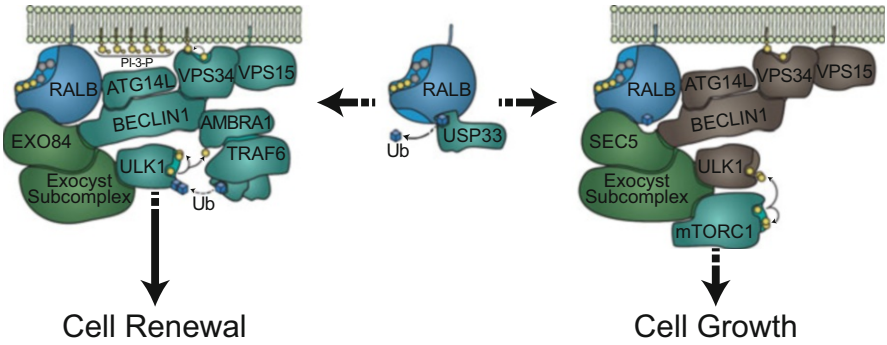

Fig. 12.3 UPS33 mediates RALB effector choice for cell growth or renewal. RALB engages EXO84 to stimulate the assembly of ULK1- and BECLIN1-dependent signaling complexes and promotes the activation of ULK1, VPS34, and autophagosome initiation. Conversely, RALB engages SEC5 to stimulate the assembly of ULK1- and BECLIN1-dependent signaling complexes to facilitate their inactivation by mTORC1. UPS33 mediates the choice between these two RALB complexes through de-ubiquitylation of RALB, which promotes RALB–EXO84 assembly

12.5.1.1 EXO84 Subcomplex

Though both EXO84 and SEC5 associated with autophagy regulatory components, RALB, ULK1, BECLIN1, and Vps34 were found to preferentially associate with EXO84 during nutrient restriction (Bodemann et al. 2011). Effector selective mutant forms of RALB provided further evidence that direct RALB–EXO84 binding promoted the assembly and binding of regulators of autophagy induction and vesicle nucleation with EXO84 (Bodemann et al. 2011). EXO84 knockdown prevented association of ULK1 with BECLIN1, and ULK1 co-precipitated by EXO84 was kinase active (Bodemann et al. 2011). By bringing active ULK1 into complex with BECLIN1, EXO84 likely facilitates AMBRA1 phosphorylation by ULK1, TRAF6 recruitment, and displacement of the AMBRA1–BECLIN1–ATG14L–VPS34 from microtubules to membrane surfaces allowing VPS34 to promote PI-3-P production. In support of this hypothesis, an EXO84-coupled mutant RALB(38R) was sufficient to drive the accumulation of markers of vesicle nucleation and autophagy (Bodemann et al. 2011).

12.5.1.2 SEC5 Subcomplex

RALB was found to preferentially associate with SEC5 under nutrient-rich conditions (Bodemann et al. 2011). Effector selective mutants showed that RALB–SEC5 binding was required to promote assembly binding of regulators of autophagy induction and vesicle nucleation with SEC5 (Bodemann et al. 2011). In contrast to EXO84, ULK1 co-precipitated by SEC5 lacked kinase activity necessary to drive autophagy induction, and the SEC5-coupled mutant RALB(48W) failed to promote the accumulation of markers of vesicle nucleation and autophagy (Bodemann

et al. 2011). Finally, SEC5 but not EXO84 co-precipitated components of mTORC1 (Bodemann et al. 2011), and recent findings have revealed that a RALB–SEC5-dependent pathway promotes mTORC1 activation (Martin et al. 2013). Thus, a RALB–SEC5 exocyst subcomplex promotes cell growth through inhibition of autophagy and activation of mTORC1.

In conclusion, RALB is a central mediator of two opposed signaling kinases, ULK1 and mTORC1, which mediate opposed biological processes, cell renewal and cell growth, respectively. In the final section, we will discuss how a posttranslational modification of RALB discriminates between exocyst effector proteins to engage distinct exocyst effector subcomplex-dependent signaling pathways.

12.5.2 Regulation Dynamics: RALB Lysine-47 Ubiquitylation Status Mediates Exocyst Effector Selection

A screen for Ral G-protein interacting proteins uncovered the ubiquitin-specific protease, USP33, as an interactor (Simicek et al. 2013). Furthermore, it was identified that RALA and RALB are ubiquitylated on multiple sites including lysine-47 (Simicek et al. 2013). Mutational analysis revealed the lysine-47 ubiquitylation event on RALB to be regulated by USP33 (Simicek et al. 2013). RALB-GTP levels were unaffected by USP33 expression; however, USP33 expression regulated RALB–Exocyst subcomplex engagement as overexpression of UPS33 promoted RALB–EXO84 association while USP33 shRNA knockdown promoted RALB–SEC5 association (Simicek et al. 2013). Structural analysis of the distinct RALB binding interfaces of EXO84 and SEC5 supports a model where RALB lysine-47 ubiquitylation is permissive for SEC5 but not EXO84 binding (Simicek et al. 2013). Activation of innate immune signaling with dsRNA, which is known to activate RALB–SEC5–TBK1 pathway, increased the amount of lysine-47 ubiquitylated RALB and increased the accumulation of RALB–SEC5–TBK1 in complex (Simicek et al. 2013). Consistent with the previous finding that nutrient deprivation increases RALB–EXO84 complex formation, it was found that nutrient deprivation decreased the amount of lysine-47 ubiquitylated RALB and increased accumulation of RALB–EXO84 complex (Simicek et al. 2013). USP33 depletion with shRNA increased SEC5–BECLIN1 complex formation and decreased autophagy levels (Simicek et al. 2013). Conversely, USP33 overexpression increased EXO84–BECLIN1 complex and increased autophagy levels (Simicek et al. 2013). Thus, lysine-47 ubiquitylation of RALB is a key determinant of selective RALB–effector pathway engagement (Fig. 12.3).

Ubiquitylation may also play a key regulatory role in RALA-dependent signaling as well. RALA is described to promote lipid raft exocytosis in detached cell cultures to maintain proliferative signaling (Balasubramanian et al. 2010). Under detached conditions it was found that RALA exhibited a twofold increase in

ubiquitylation (Neyraud et al. 2012). Disruption of lipid raft endocytosis through chemical inhibition of Dynamin-2 or knockdown of Caveolin-1 decreased RALA ubiquitylation, and a RALA-Ubiquitin fusion protein suppressed lipid raft internalization. These observations support a scenario where ubiquitylated RALA promotes lipid raft exocytosis, while the de-ubiquitylated form promotes lipid raft endocytosis (Neyraud et al. 2012). Thus, ubiquitylation of RALA likely plays an important role shaping its signaling output as well.

12.6 Concluding Remarks

Ral G-proteins are key mediators of numerous biological processes. The highly related isoforms, RALA and RALB, have been described to have opposing, cooperative, and independent roles in regulating signal response networks. The individual functions of RALA and RALB can be further subdivided by ubiquitylation or phosphorylation of the Ral G-protein, which allows for a bewildering array of biology to be regulated by these two nearly identical proteins. An ever-growing field of dedicated researchers studying these G-proteins has yielded credible insight into the molecular paradigms that fine-tune each Ral G-protein to purpose. Despite the many checks and balances that facilitate the orchestral execution of Ral-dependent biology, these finely tuned signal instruments are too easily made to play the chaotic din of oncogenic transformation.

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Chapter 13
Structure and Function of the mTOR Activator Rheb

Christopher B. Marshall, Mohammad T. Mazhab-Jafari, Vuk Stambolic, and Mitsuhiko Ikura

Abstract Ras homologue enriched in brain (Rheb) is a well-known activator of the mammalian target of rapamycin (mTOR) protein kinase. It is a highly conserved small GTPase protein that is ubiquitously expressed from yeast to mammals. Rheb is most similar to Rap and Ras GTPases; however, it bears amino acid substitutions to key conserved residues in the G1 box. Rheb possesses a C-terminal CaaX box motif that is modified by attachment of a farnesyl isoprenoid moiety that mediates localization to cellular endomembranes. Rheb has low intrinsic GTPase activity and exists in a highly activated state in cells relative to many other GTPases. Structures of Rheb revealed that the side chain of the canonical catalytic residue (Gln64, which corresponds to Ras Gln61) is buried in a hydrophobic pocket where it is not available for catalysis, and mutation of this residue has minimal effect on intrinsic GTP hydrolysis. Further, an interaction between Tyr35 and the nucleotide inhibits GTP hydrolysis, and mutation of this residue resulted in a tenfold increase in the intrinsic GTPase activity of Rheb. The protein product of the Tsc2 gene (hamartin) was identified as a GTPase activating protein (GAP) that inactivates Rheb-GTP by promoting GTP hydrolysis through an "asparagine thumb" mechanism similar to that of its homolog Rap1GAP. The existence and identity of a guanine nucleotide exchange factor (GEF) for Rheb remain unresolved. In yeasts, Rheb plays a role in regulating arginine uptake. In multicellular organisms from flies to mammals, activation of the (m)TOR signaling pathway by Rheb promotes protein synthesis, cell growth, and proliferation, although there is some debate about whether this occurs through a direct interaction or an indirect mechanism. mTOR is frequently hyperactivated in a wide variety of human cancers, and mTOR inhibitors have been approved for treatment of certain cancers. Rheb expression and activation are

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elevated in some tumours, and are implicated in carcinogenesis. Rheb has been reported to interact with a number of proteins, which implicate it in the regulation of additional cellular functions including apoptosis and autophagy. Rheb is essential for development, and its knockout in mice results in an embryonic lethal phenotype. This chapter will describe the structure and function of Rheb, its role in mTOR signaling and noncanonical functions, as well as its physiological importance in health and disease.

Keywords Rheb • Target of rapamycin (TOR) • Tuberous sclerosis complex (TSC) • Small GTPase protein • Farnesylation • Mutation • Protein structure/function

13.1 Identification of the Small GTPase Rheb

Ras homologue enriched in brain (Rheb) was initially identified using a differential cloning technique to identify genes induced in rat brain neurons by synaptic activity. In hippocampal granule cells, seizures and NMDA-dependent synaptic activity induce expression of Rheb mRNA, which suggested it may play a role in long-term potentiation (Yamagata et al. 1994). Rheb mRNA was found to be expressed at high levels in the rat cortex, and was also present in many peripheral tissues (Yamagata et al. 1994), and is ubiquitously expressed in humans with high levels in skeletal and cardiac muscle (Gromov et al. 1995).

Sequence analysis identified Rheb as a member of the Ras subfamily of small GTP-binding proteins with the highest similarity to Rap2 (37 % identical) and H-Ras (34 % identical) GTPases (Yamagata et al. 1994) (Fig. 13.1). Human Rheb shares 90 % sequence identity with rat Rheb at the cDNA level, but different expression patterns have been reported in the two species (Gromov et al. 1995). Small GTPase proteins serve as switch-like proteins in cellular signaling pathways whereby their GTP-bound state adopts an activated confirmation that interacts with and stimulates the biological activity of diverse effector proteins, whereas GTP hydrolysis results in a GDP-bound conformation that is generally inactive (Wittinghofer and Vetter 2011). GTP hydrolysis is catalyzed by GTPase activating proteins (GAPs), and Rheb-GDP can be reactivated by exchange of GDP for a new molecule of GTP, which is stimulated by guanine nucleotide exchange factors (GEFs). This "GTPase cycle" is discussed in more detail in another chapter of this book (GEFs and GAPs).

13.1.1 Insights from the Sequence of Rheb

Rheb is highly conserved and ubiquitously expressed from yeast to slime mold, fungi, fruit fly, zebra fish, and mammals (Yamagata et al. 1994; Reuther and Der 2000; Tabancay et al. 2003).

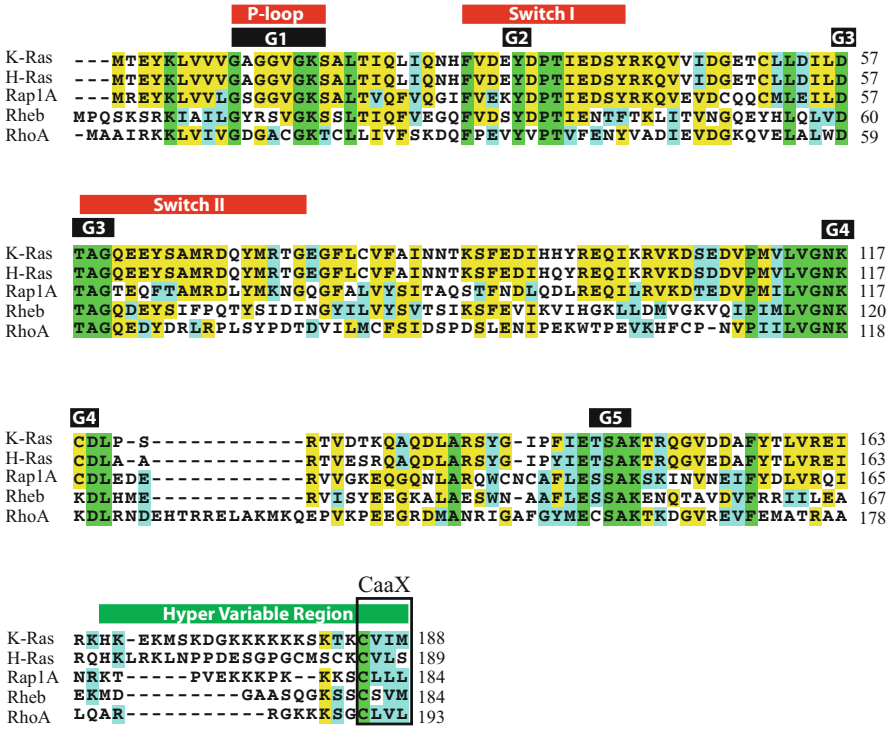

Fig. 13.1 Sequence alignment of Rheb with other small GTPase proteins. Alignment of Rheb with its close homologues K-Ras, H-Ras, and Rap1A, as well as the more distant homologue RhoA, from another subfamily of the Ras superfamily. Completely conserved amino acids are highlighted in *green*, those conserved in more than half of the alignment are shown in *yellow*, and semi-conserved amino acids in *cyan*. The position of the G1–G5 boxes are indicated with *solid bars* and the P-loop, switch I, and switch II regions are indicated in *red*. The position of the hyper variable region (HVR) is indicated with a *green bar*, and the CaaX box is highlighted with a *rectangle*

Rheb encodes a 184-a.a. small GTPase protein that possesses five G-box motifs (G1–G5) conserved amongst small GTPases (Fig. 13.1), which mediate nucleotide binding and hydrolysis, as well as interactions with effectors and regulators. In small GTPases, the G1 box forms the P-loop, which is responsible for phosphate binding, the G2 and G3 boxes form switch I and II, respectively, which interact with effector proteins, as well as the GAPs and GEFs that regulate the activation of small GTPase proteins (Wittinghofer and Vetter 2011). The G4 and G5 boxes bind the nucleotide base with G4 determining specificity for guanine.

Substitutions to conserved residues in the G1 box (relative to Ras, the founding member of the family) were immediately recognized as potentially important to Rheb function. Whereas two Gly residues (encoded by codons 12 and 13 in Ras) are highly conserved amongst Ras-subfamily members; these positions are substituted in Rheb (Arg-15 and Ser-15) (Yamagata et al. 1994) (Fig. 13.1). In Ras, mutations

that introduce any side chain into these positions impair both intrinsic and GAP-mediated GTP hydrolysis, thus generating hyperactivated Ras variants, and codons 12 and 13 of Ras isoforms are frequently mutated in cancers (Seeburg et al. 1984; Barbacid 1987; Cales et al. 1988; John et al. 1988; Krengel et al. 1990; Maruta et al. 1991; Gideon et al. 1992; Scheffzek et al. 1997; Ahmadian et al. 1999; Smith et al. 2013). The significance of these G1-box substitutions in Rheb is discussed in Sect. 13.3.

The sequence of Rheb also revealed the presence of a C-terminal CaaX box (C = Cys, a = an aliphatic residue, and X = the C-terminal residue) (Yamagata et al. 1994), a motif recognized by prenyl transferases for attachment of an isoprenoid moiety (Wright and Philips 2006). The importance of this posttranslational modification to the cellular functions of Rheb will be discussed in Sect. 13.4.

The human Rheb gene was localized to chromosome 7q36 and a second Rheb isoform [Rheb2 or Rheb-like 1 (RhebL1)], also ubiquitously expressed, was localized to chromosome 12q13.12 (Gromov et al. 1995; Mizuki et al. 1996; Patel et al. 2003; Tabancay et al. 2003; Yuan et al. 2005). Another Rheb gene mapping to chromosome 10q11 is considered a pseudogene (Rheb pseudogene 1 or RHEBP1).

13.1.2 Identification of the Function of Rheb

Rheb was initially reported to antagonize Ras signaling in mammalian cells (Clark et al. 1997) through interactions with Raf-1 kinases (Yee and Worley 1997; Im et al. 2002); however, a series of several exciting independent reports published in 2003 converged on defining an important novel role for this small GTPase. Studies based on diverse approaches including genetics, biochemistry, and cell biology involving organisms from *Drosophila* to mammals revealed remarkably consistent evidence that Rheb is a key regulator of the (mammalian) target of rapamycin ((m) TOR) signaling pathway (Kwiatkowski 2003; Manning and Cantley 2003; Li et al. 2004a (TiBS)). This finding was of great interest because mTOR is considered the "master regulator" of cell growth and cell cycle progression in response to availability of nutrients and growth factors, and its deregulation had been implicated in carcinogenesis (Bjornsti and Houghton 2004; Fingar and Blenis 2004). Simultaneous identification of Rheb-GTP as an mTOR activator and TSC2 as a GAP that inactivates Rheb-GTP by promoting GTP hydrolysis provided a missing link between mTOR and TSC2 (Fig. 13.2). TSC1/2 were already known to couple insulin signaling to TOR activation; however, the mechanism had been elusive. The role of Rheb in mTOR signaling is discussed in more detail in Sect. 13.2.

As the "switch" that transduces TSC1/2 signaling, Rheb plays an important role in the pathology of tuberous sclerosis, a benign tumour syndrome associated with inactivating mutations of TSC1 or TSC2 and hyperactivation of the mTOR pathway. As an activator of the master growth regulator mTOR, Rheb itself has been implicated in tumorigenesis. Rheb is overexpressed and hyperactivated in some

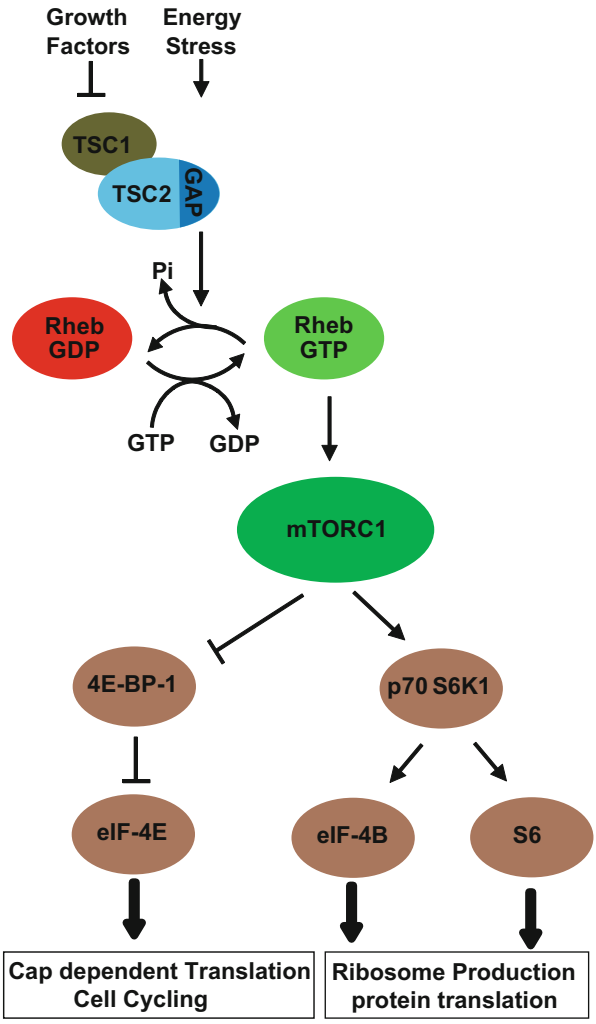

Fig. 13.2 Schematic illustration of Rheb in the mTOR signaling pathway. Rheb-GTP stimulates the kinase activity of mammalian target of rapamycin complex 1 (mTORC1), which phosphorylates substrates including 4EBP1 and p70S6K1 that promote protein biosynthesis and cell cycle progression. The GAP activity of the tuberous sclerosis complex (TSC1/2) catalyzes GTP hydrolysis to inactivate Rheb. TSC1/2 is regulated in response to the availability of growth factors and energy stress

cancers, its overexpression stimulates proliferation, and constitutively activated mutants can induce oncogenic transformation while knockdown of Rheb results in cell-cycle arrest (see Sect. 13.7).

In addition to an established role in mTOR signaling, Rheb has been reported to interact with a wide variety of proteins and is implicated in regulating diverse cellular functions including apoptosis and autophagy (discussed in Sect. 13.7.3). Rheb plays an essential role in development, and its knockout results in embryonic lethality in mice, however roles for Rheb in several organs have been determined using tissue-specific depletion or overexpression of Rheb (see Sect. 13.7).

13.2 A Role for Rheb in the TSC–mTOR Signaling Axis

In a relatively short period of time spanning 2003–2004, multiple lines of evidence from several independent labs led to a breakthrough in understanding the function of Rheb as a regulator of the (m)TOR signaling pathway. First, genetic screens in *Drosophila* identified Rheb as a gene whose overexpression promotes cell growth through TOR signaling (Saucedo et al. 2003; Stocker et al. 2003). Rheb overexpression accelerated passage through G1-S phase in mitotic tissues whereas its mutation impaired larval growth. Genetic analyses placed Rheb in the insulin signalling pathway downstream of Tsc1–Tsc2 and upstream of TOR (Fig. 13.2), as a driver of cell growth (Patel et al. 2003; Saucedo et al. 2003; Stocker et al. 2003). TSC2, which was known to play a role in coupling insulin signaling to TOR through a previously undefined mechanism, contains a domain homologous to Rap1GAP (Maheshwar et al. 1997), suggesting the possibility that it may function through GAP activity for Rheb.

In parallel to the fly studies, several studies in mammalian cells subsequently demonstrated that mammalian TOR (mTOR) is likewise activated by Rheb (Castro et al. 2003; Garami et al. 2003; Inoki et al. 2003a; Tee et al. 2003). Rheb overexpression leads to an increase in the phosphorylation of mTOR substrates S6K1 (Castro et al. 2003; Garami et al. 2003) and 4E-BP1 (Inoki et al. 2003a; Tee et al. 2003), even in the absence of growth factors. Together, these studies also confirmed the role of TSC1/2 as an upstream regulator of Rheb in mammalian cells through its GAP activity. Overexpression of TSC1/2 decreases the state of activation of Rheb (i.e., reduces the ratio of GTP/GDP bound to Rheb) (Castro et al. 2003; Garami et al. 2003; Inoki et al. 2003a), whereas overexpression of TSC2 bearing a disease-associated mutation of the putative catalytic residue (N1643K) did not affect Rheb activation (Garami et al. 2003). Consistently, TSC2-null MEFs showed elevated Rheb activation relative to wild-type MEFs (Garami et al. 2003). These in vivo studies of Rheb activation used thin-layer chromatography to assay the ratio of ^{32}P-GTP/GDP associated with immunoprecipitated Rheb following treatment of cells with [^{32}P]-orthophospate (Castro et al. 2005). While the activation of several small GTPases can be assayed by pulldown with immobilized effector domains [e.g., Ras-binding domains (RBDs)] that specifically interact with GTP-loaded GTPases (de Rooij and Bos 1997; Ren et al. 1999; Stofega et al. 2006), currently no analogous assay for Rheb activation is available. Finally, immunoprecipitated TSC1/2 was demonstrated to possess GAP activity for Rheb in vitro (Inoki et al. 2003a; Tee et al. 2003), and the presence of both TSC1 and TSC2 proteins was shown to be required for full GAP activity (Tee et al. 2003). *Drosophila* Rheb was also shown to be a direct target of TSC1/2 GAP activity in vivo and in vitro (Zhang et al. 2003). Finally, the isolated recombinant TSC2 GAP domain was shown to accelerate the GTP hydrolysis rate of Rheb in vitro, although the catalytic activity of the isolated TSC2 GAP domain is lower than that of RasGAPs, as much higher concentrations are required to accelerate GTP hydrolysis (Scrima et al. 2008; Marshall et al. 2009). Rheb has low intrinsic GTPase activity and exists in a highly

activated state in cells relative to many other GTPases including Ras (Im et al. 2002; Castro et al. 2003; Garami et al. 2003; Inoki et al. 2003a; Tee et al. 2003; Zhang et al. 2003). This suggests the importance of GAP activity in controlling Rheb signaling, and has led to speculation that Rheb may not require a GEF (discussed in Sect. 13.6).

The TSC1 and TSC2 genes were identified as genetic loci mutated in tuberous sclerosis, an autosomal dominant syndrome characterized by numerous benign tumours (hamartomas), renal angiomyolipomas, and pulmonary lymphangioleiomyomatosis (Crino et al. 2006; Huang and Manning 2008). TSC1 (hamartin) and TSC2 (tuberin) associate to form a heterodimer (van Slegtenhorst et al. 1998) in which TSC1 stabilizes TSC2 (Hodges et al. 2001; Nellist et al. 2001), which contains the GAP domain (Benvenuto et al. 2000; Chong-Kopera et al. 2006). TSC1/2 is regulated by phosphorylation in response to cytokines and growth factors, energy depletion, and hypoxia (Dan et al. 2002; Inoki et al. 2002, 2003b, 2006; Manning et al. 2002; Astrinidis et al. 2003; Brugarolas et al. 2004; Roux et al. 2004; Ma et al. 2005; Lee et al. 2007). The details of TSC1/2 regulation are beyond the scope of this chapter, but have been extensively reviewed (Kwiatkowski 2003; Astrinidis and Henske 2005; Kwiatkowski and Manning 2005; Huang and Manning 2008).

The catalytic mechanism by which TSC2 stimulates Rheb GTP hydrolysis is distinct from that of the RasGAPs but similar to that of its homolog Rap1GAP, and sensitivity of Rheb to TSC2 GAP activity requires some of the unique Rheb substitutions (e.g., Arg15) (Li et al. 2004b). Details of the Rheb intrinsic and TSC2 GAP catalytic mechanisms will be discussed in Sect. 13.3.

Interestingly, a binding site for calmodulin (CaM) has been mapped immediately C-terminal to the TSC2 GAP domain (residues 1740–1755) (Noonan et al. 2002). This CaM-binding site also interacts with the estrogen receptor alpha, and truncation or mutation of the CaM-binding site suppressed steroid ligand-induced transcription (York et al. 2005) and disrupts a nuclear localization sequence (York et al. 2006). However, it has not been reported whether CaM binding to this site affects GAP activity of TSC2. This helix is not highly conserved between TSC2 and Rap1GAP, which lacks a recognizable CaM-binding site, but two conserved Arg residues in the corresponding RapGAP helix interact with Rap and their mutation impairs GAP activity (Chakrabarti et al. 2007). We propose that CaM binding to the TSC2 helix would mask this region, obscuring residues important for Rheb binding as well as sterically hindering docking of Rheb to TSC2.

It was recently suggested that there is a third subunit of the TSC1–TSC2 complex. Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7 (TBC1D7) is required for proper association of TSC1 with TSC2 and its knockdown results in increased mTOR signaling and enhanced cell growth (Dibble et al. 2012). TBC domains in other proteins have GAP activity for Rab GTPases, but TBC1D7 has an unconventional TBC domain that lacks conserved motifs required for GAP activity. It remains unknown whether the TBC domain is directly involved in interactions with Rheb.

GTP-bound (activated) Rheb stimulates the protein kinase activity of mTOR complex 1 (mTORC1), a rapamycin-sensitive complex comprised of mTOR, mLST8, and raptor, but does not activate the rapamycin-insensitive mTORC2, which contains rictor rather than raptor (Sabatini 2006). Rheb stimulates phosphorylation of mTORC1 substrates including translational initiation factors eIF4G, 4E-BPs, relieving the inhibition of eIF4B, thereby activating cap-dependent translation of proteins (e.g., cyclin D1, and c-Myc) required for cell cycle progression (Hay and Sonenberg 2004). In addition, mTORC1 phosphorylation of S6 kinase allows the translation of ribosomal proteins, thus promoting protein production and cell growth. However the detailed mechanism by which Rheb activates mTORC1 kinase activity remains elusive. Many reports suggest that mTOR is activated through a direct interaction with Rheb, and it has been proposed that Rheb enhances recruitment of mTORC1 substrate proteins. Immunoprecipitated mTORC1 exhibits low kinase activity against 4E-BP1; however, this can be dramatically increased by recombinant Rheb-GTP, which promotes binding of 4E-BP1 to mTORC1 (Sato et al. 2009). Others have proposed that Rheb activates mTORC1 in an indirect manner, for example, by removing an inhibitory protein (Bai et al. 2007) or stimulating production of a second messenger (Sun et al. 2008) (see Sect. 13.6).

13.3 Rheb Structure and Function

Although Rheb shares 34 % sequence identity with H-Ras, including most of the conserved G1–G5 box motifs, two key variations were immediately apparent in its sequence (Fig. 13.1). In Ras, codons 12 and 13 encode glycine residues that are well conserved in the Ras subfamily of small GTPases. Mutations of these residues produce oncogenic Ras variants that are constitutively GTP bound (Prior et al. 2012). Mutation of Gly12, which occurs frequently in H-Ras in various tumor types, causes steric interference with the catalytic residue Gln61, thus distorting the catalytic machinery and impairing intrinsic GTP hydrolysis (Krengel et al. 1990). More importantly, any side chain introduced by mutation of Gly12 would also clash with the catalytic arginine "finger" of the RasGAPs, thus rendering these mutants resistant to GAP-catalyzed inactivation (Scheffzek et al. 1997; Gremer et al. 2008). Gln61 is located at the N terminus of switch II, where it stimulates GTP hydrolysis by activating a hydrolytic water molecule (H_2O^{cat}) positioned in-line with the γ-phosphate. The presence of any side chain at residue 12 would be predicted to distort the catalytic site, and all mutations of codon 12 were shown to impair GTP hydrolysis. Mutations of Gly13 occur in K-Ras in colon cancer (Prior et al. 2012), and the G13D mutation modestly impairs GTP hydrolysis, but profoundly accelerates nucleotide exchange (Smith et al. 2013). In Rheb, codons 15 and 16, which correspond to Ras 12 and 13, encode arginine and serine residues, respectively (Fig. 13.1). These substitutions suggested a mechanism for the slow GTP hydrolysis activity of Rheb in vitro and high activation state Rheb in the cell (~20 % bound to GTP) (Yamagata et al. 1994; Im et al. 2002; Li

et al. 2004b; Marshall et al. 2009). However an R15G substitution was not sufficient to enhance GTPase activity (Yamagata et al. 1994; Im et al. 2002; Li et al. 2004b), and when studied by an in vitro NMR-based assay, the R15G mutation was found to decrease intrinsic GTPase activity (Marshall et al. 2009).

To study the GTPase and GAP activity of Rheb and TSC2, a highly quantitative, NMR-based, real-time GTPase assay was developed (Marshall et al. 2009). This technique observes ^{15}N Rheb, taking advantage of structural differences between the GTP- and GDP-bound forms of the protein, which produce distinct crosspeaks in the ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) spectra. The kinetics of nucleotide hydrolysis can be determined by monitoring these peak heights over time. The unambiguous readout of this method makes it possible to precisely measure GAP activity of endogenous or overexpressed TSC1/2 from extracts of mammalian cells (Marshall et al. 2012), enabling studies of this large protein complex, which cannot be recombinantly expressed in bacteria. This new methodology has also been successfully applied to RAS (Smith and Ikura 2014; Smith et al. 2013) and RhoA (Gasmi-Seabrook et al. 2010; Meiri et al. 2012), and has proven to be extremely powerful for characterizing the function of small GTPases and their GAPs and GEFs.

An understanding of why GTP is not efficiently hydrolyzed by Rheb was not revealed by the primary sequence, but required the determination of a high resolution structure. The structural basis for the low GTPase activity of Rheb was revealed by crystal structures reported in 2005, including the inactive GDP-bound conformation (PDB: 1XTQ) as well as the activated conformation bound to GTP (PDB: 1XTS) or the non-hydrolyzable GTP analog guanosine 5-[β,γ-imido]triphosphate (GMPPNP) (PDB: 1XTR) (Fig. 13.3) (Yu et al. 2005). The overall fold was very similar to that of other members of the Ras subfamily (1.17 Å RMSD for Rheb-GMPPNP versus H-RasGMPPNP) (Fig. 13.4), comprising a six stranded β-sheet and five α-helices forming a globular protein. However, the structure of the activated conformation diverges from that of H-Ras in the functionally important switch I and II regions (Fig. 13.5). Rheb switch I forms a lid that covers the nucleotide phosphate groups and creates a pore to the solvent, which is occupied by the hydrolytic water molecule (H_2O^{cat}). This lid is stabilized by H bonding between the hydroxyl group of the conserved Tyr35 in switch I and the γ-phosphate, whereas the corresponding tyrosine of H-Ras (Tyr32) does not interact with the γ-phosphate and rather assumes an open conformation that leaves the nucleotide phosphate groups exposed to solvent. The significance of the orientation of Rheb Tyr35 is discussed below. The conformation of Rheb switch II also differs from that of Ras. The switch II region of H-Ras contains a 10-residue alpha helix and is relatively detached from the globular domain, whereas Rheb switch II is less structured with a shorter helix, but forms more points of contact with the G-domain (Fig. 13.5). The result is the displacement away from the nucleotide binding site of Rheb switch II residues downstream of the conserved $D_{60}xxG_{63}$ motif (G3-box) that coordinates Mg^{2+}, H_2O^{cat}, and the γ-phosphate. The side chain of Gln64 (which corresponds to H-Ras Gln61) is flipped away from the nucleotide binding site (Fig. 13.5) and

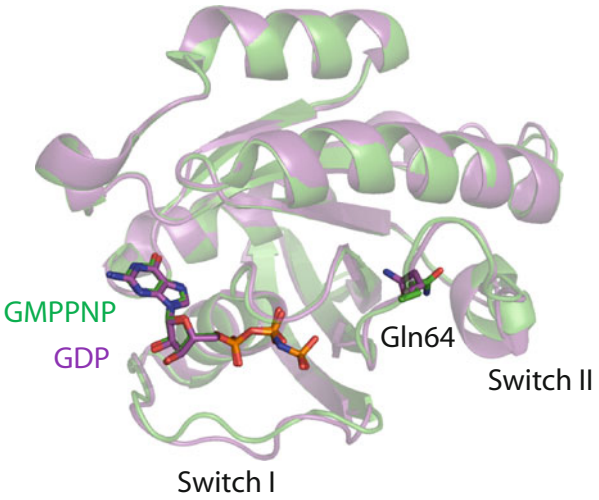

Fig. 13.3 Structural overlay of the activated and inactive conformations of Rheb. Crystal structures of the inactive conformation of Rheb bound to GDP (*magenta*, PDB ID: 1XTQ) versus activated Rheb-GMPPNP (*green*, PDB ID: 1XTR). Switch I and II and Gln64 are indicated

buried in a hydrophobic pocket formed by Leu12, Phe70, Pro71, Tyr74, and Ile99 where it is not available to participate in catalysis. This conformation of switch II is stabilized by interactions between Glu66 in switch II and Lys91 and Lys102 in the α3 helix. Thus, the structure indicated that while the canonical catalytic glutamine residue is conserved in the Rheb sequence, its divergent structure renders it non-catalytic, consistent with the minimal impact of its mutation on intrinsic GTP hydrolysis (i.e., Q64L) (Inoki et al. 2003a; Li et al. 2004b; Marshall et al. 2009). Interestingly, in another close homolog of Rheb, Rap1, this glutamine residue is substituted by threonine, which is also non-catalytic; thus Rap1 also exhibits very poor GTPase activity (Chakrabarti et al. 2007).

The resonances of the backbone of the GTPase Rheb in its GDP-and GMPPNP-bound forms were assigned by NMR (Berghaus et al. 2007; Marshall et al. 2009) and an NMR structure of Rheb-GDP was solved (Karassek et al. 2010). The conformation of switch II in the NMR structure differs slightly from the crystal structure, and is less defined suggesting that switch II exists in multiple conformations.

13.3.1 Autoinhibition by Y35 and Noncanonical Catalytic Mechanism

Although the unusual structure of switch II renders Rheb Gln64 non-catalytic, our work has revealed that this small GTPase is not simply a "defective" Ras homolog that lost the ability to hydrolyze GTP. We revealed that Rheb has a latent capacity for robust GTPase activity, as a single mutation can activate its intrinsic GTPase activity to a level approaching that of Ras. Mutation of Tyr 35 to Ala increased the

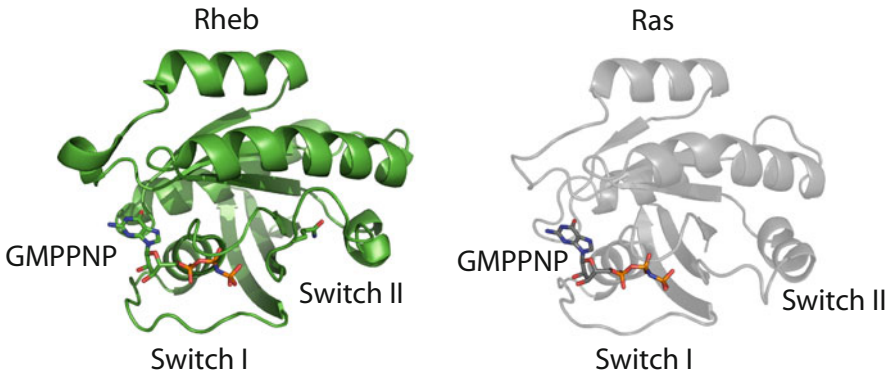

Fig. 13.4 Structures of the activated conformations of Rheb and Ras. *Left panel*, crystal structure of the activated conformation of Rheb bound to GMPPNP (*green*, PDB ID: 1XTR). *Right panel*, crystal structure of the activated conformation of K-Ras-GMPPNP (*gray*, PDB ID: 5P21)

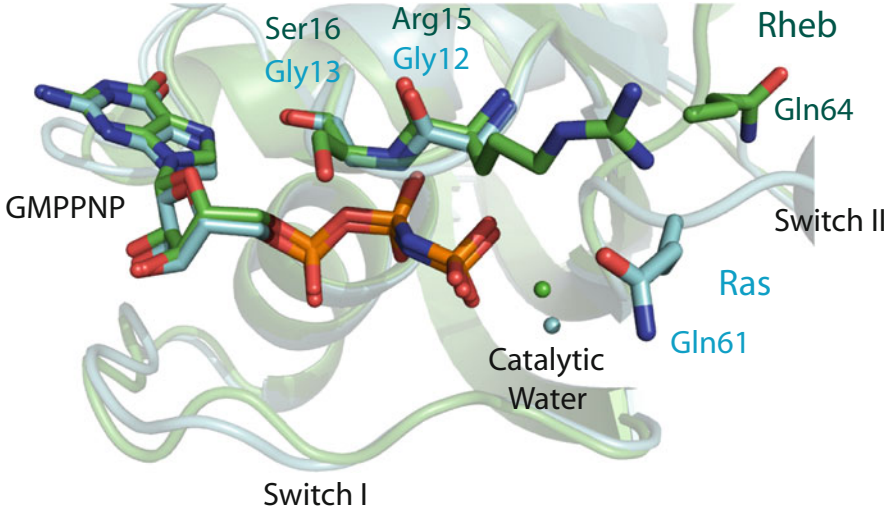

Fig. 13.5 Structural overlay of the nucleotide-binding site of the activated conformations of Rheb and Ras. Crystal structures of the activated conformation of Rheb bound to GMPPNP (*green*, PDB ID: 1XTR) versus activated K-Ras-GMPPNP (*cyan*, PDB ID: 5P21). Switch I and II are indicated along with key residues that are substituted or structurally divergent between the two proteins

intrinsic GTPase activity of Rheb by a remarkable tenfold, while its mutation to Phe increased activity fivefold (Mazhab-Jafari et al. 2012), whereas mutation of the corresponding Y32 in Ras had a negligible effect (Yamasaki et al. 1994). Thus the unusual switch I conformation and interaction between Tyr35 and the γ-phosphate appears to autoinhibit GTP hydrolysis by stabilizing the catalytic machinery in a nonproductive orientation. The accelerated catalysis of the Y35A mutant is

independent of Q64 as mutation of this residue does not impair the GTPase activity in the Y35A, Q64L double mutant; however, mutation of D65 reduces GTPase activity of the Y35 mutant by 60 %, suggesting D65 is involved in a noncanonical catalytic mechanism (Mazhab-Jafari et al. 2012). Fluorescent 2′(3′)-*O*-(*N*-Methylanthraniloyl)-tagged GTP (mantGTP) is hydrolyzed by Rheb tenfold faster than native GTP but mantGTP-bound Rheb is resistant to the GAP activity of TSC2, whereas these effects were not seen with Ras or RhoA (Mazhab-Jafari et al. 2010). The predicted position of the mant fluorophore suggested that it may disrupt the autoinhibitory conformation of Y35.

13.3.2 Structure Guided Mutations of Gly63

Point mutations of the catalytic glutamine residue are widely used to engineer activated GTPase mutants as probes of signaling pathways (Der et al. 1986). Because Gln64 is non-catalytic in Rheb, its mutation has no impact on intrinsic hydrolysis in vitro, although it does slightly reduce its sensitivity to TSC2 GAP-mediated hydrolysis (Li et al. 2004b; Marshall et al. 2009), thus increasing GTP loading in cells (Inoki et al. 2003a; Li et al. 2004b). A constitutively activated Rheb mutant with more profound impairment of both intrinsic and GAP-catalyzed GTP hydrolysis may serve as a stronger gain-of-function variant in signaling studies. Further, one-quarter of small GTPases bear substitutions of the catalytic glutamine; thus, alternate engineering strategies are required to engineer activated mutants. As discussed above, the positions equivalent to Ras codons Gly12 and Gly13 are already substituted in Rheb, thus mutations analogous to the Ras oncogenic mutations at these positions are not applicable to Rheb. Therefore, the structure of Rheb was used to design mutations of the ultra-conserved glycine in the G3-box DxxG motif (Gly63 in Rheb). This glycine is 93 % identical amongst small GTPases and its backbone amide plays a conserved role in coordinating the hydrolytic water molecule (H_2O^{cat}) and the γ-phosphate (Sprang 1997). The introduction of an Ala side chain selectively perturbed binding of the H_2O^{cat} thus impairing GTP hydrolysis and yielding an activated variant, whereas the larger side chain of valine blocked the γ-phosphate yielding a constitutively inactive mutant that can only accommodate GDP (Mazhab-Jafari et al. 2014).

13.3.3 Structural Aspects of TSC2GAP Activity

The structure of the TSC2GAP domain remains to be determined; however in its absence, much insight has been derived from the structure of its homolog Rap1GAP and its complex with Rap1 (Daumke et al. 2004; Scrima et al. 2008) and biochemical and biophysical studies of the Rap1GAP-catalyzed reaction (Chakrabarti et al. 2007). Rap1GAP works through a mechanism distinct from the RasGAP-

catalyzed reaction in which RasGAP provides a positively charged arginine "finger" that functions in concert with the catalytic glutamine of Ras to stabilize the transition state (Bos et al. 2007) (discussed in another chapter of this book, GEFs and GAPs). In contrast, Rap1GAP provides an asparagine residue called the asparagine "thumb" that takes the place of the cis-glutamine of Ras that is not present in Rap1 (Scrima et al. 2008).

TSC2 appears to catalyze Rheb GTP hydrolysis through a mechanism similar to Rap1GAP that is independent of Rheb Q64. The putative TSC2 asparagine thumb Asn1643 has been found mutated to Lys, Ile, or His in tuberous sclerosis patients (Maheshwar et al. 1997; Au et al. 1998; Jones et al. 1999; Dabora et al. 2001), deregulating signaling through Rheb (Zhang et al. 2003; Li et al. 2004b) and eliminating GAP activity in vitro (Marshall et al. 2009). Several other tuberous sclerosis-associated mutations in the TSC2 GAP domain also eliminate GAP activity, and are thought to disrupt the Rheb:TSC2 interaction rather than impair catalysis, although this has not been demonstrated due to the extremely transient nature of the interaction (Marshall et al. 2009). The R15G Rheb mutation reduced its sensitivity to TSC2 GAP activity by 95 % (Marshall et al. 2009), in contrast with Ras where mutation of Gly12 eliminates sensitivity to RasGAPs, consistent with a different catalytic mechanism.

13.3.4 Conformational Equilibrium in Rheb

^{31}P NMR studies of the activated form of Ras (i.e., bound to GTP or GTP analogs) have detected the presence of two slowly interconverting conformational states that produce distinct ^{31}P NMR signals (at 5 °C) (Geyer et al. 1996; Spoerner et al. 2001, 2007, 2010; Kalbitzer et al. 2009). Interaction with effector proteins shifts the equilibrium towards the major species, state 2, suggesting that this state is the conformation competent to signal (Geyer et al. 1996; Spoerner et al. 2001, 2004, 2005, 2007; Liao et al. 2008), whereas the minor conformer (state 1) exhibits significantly reduced affinity for effectors (Geyer et al. 1996; Gronwald et al. 2001; Spoerner et al. 2001; Kalbitzer et al. 2009). ^{31}P NMR studies have not been reported for Rheb, but similar conformational dynamics have been observed in some other GTPases (Geyer et al. 1999; Liao et al. 2008). Chemical Exchange Saturation Transfer (CEST) is a complementary approach to examine conformational exchange by NMR observations of the protein rather than the nucleotide. CEST experiments revealed that equilibrium conformational exchange is also present in Rheb, and that Rheb bound to the non-hydrolyzable analog GMPPNP exhibits accelerated exchange and an increased population of the minor conformer, relative to native GTP (Long et al. 2013). This is consistent with ^{31}P NMR studies of Ras, which observed that Ras-GMPPNP exhibits a higher population of state 1 than Ras-GTP (Spoerner et al. 2007, 2010).

13.4 Posttranslational Modification and Localization of Rheb

Similar to most other Ras subfamily small GTPases, the C terminus of Rheb contains a sequence motif that signals for attachment of an isoprenoid moiety that targets the protein to cellular membranes. This motif is referred to as a CaaX box, which is comprised of a Cys residue followed by two aliphatic residues and the C-terminal residue. Depending on the nature of the lipid chain that is attached to the C terminus (C15 farnesyl isoprenoid or C20 geranyl isoprenoid), as well as additional targeting signals directly N-terminal to the farnesylated cysteine in certain GTPases, CaaX-containing Ras family GTPases display variable affinity for intracellular or plasma membranes (Eisenberg and Henis 2008). The sequence of the Rheb CaaX box (CSVM) specifies farnesylation and Rheb was shown to be farnesylated in cells (Clark et al. 1997). Following posttranslational modification by farnesylation, the CaaX box is further processed by Ras converting enzyme (*Rce*), a peptidase that cleaves the aaX tripeptide, followed by carboxymethylation of the C-terminal cysteine by isoprenylcysteine carboxyl methyltransferase (*Icmt*) (Seabra 1998; Wright and Philips 2006).

The CaaX box is linked to the C-terminal helix of the GTPase domain (α-5) by a flexible region referred to as the hypervariable region (HVR) because this sequence is poorly conserved, even between closely related small GTPases. Like K-Ras, the Rheb HVR lacks additional lipidation sites, whereas N-Ras and H-Ras contain one and two additional Cys residues, respectively, that are reversibly palmitoylated (Rocks et al. 2005). Rheb also lacks the Lys-rich region that promotes localization of K-Ras to the plasma membrane through electrostatic interactions with anionic lipids enriched in this membrane (Hancock et al. 1990). In the absence of palmitoylation or polybasic motifs to target it to the plasma membrane, modified Rheb remains associated with intracellular membrane compartments such as the ER, Golgi apparatus, and lysosomes (Takahashi et al. 2005; Buerger et al. 2006).

Farnesylation of Rheb is critical for its ability to activate mTOR signaling, as it has been widely reported that mutation of the CaaX box farnesylation site (e.g., Cys181Ser) or general inhibition of farnesylation by farnesyltransferase inhibitors (FTIs) reduces the ability of Rheb to stimulate mTORC1 activity in cells (Castro et al. 2003; Tee et al. 2003; Li et al. 2004b; Buerger et al. 2006; Finlay et al. 2007). Thus it has been suggested that inhibition of farnesylation of Rheb may be responsible in part for the biological activity of farnesyl transferase inhibitors that were originally developed to target oncogenic Ras mutants (Castro et al. 2003; Mavrakis et al. 2008).

To study C-terminally processed Rheb, farnesylated, carboxymethylated Rheb was produced in a semi-synthetic manner combining solid-phase synthesis of a HVR lipopeptide with expressed protein ligation to the GTPase domain (Chen et al. 2010). This construct was used to study Rheb's interaction with the delta subunit of cGMP 3′,5′-cyclic phosphodiesterase (PDEδ), which is proposed to solubilize farnesylated Rheb by sequestering the hydrophobic farnesyl moiety to

facilitate transport between membranes (Ismail et al. 2011) (details discussed in Sect. 13.6).

How does the GTPase domain of Rheb behave when tethered to a lipid bilayer through the flexible HVR? Fluorescence studies and molecular dynamic (MD) simulations have been used to model interactions between membrane-anchored Ras and a bilayer membrane (Gorfe et al. 2007a, b; Abankwa et al. 2008a, b). Recently NMR methods were used to make direct observations of membrane-tethered Rheb (Mazhab-Jafari et al. 2013). Farnesylated Rheb was mimicked by using maleimide chemistry to link the CaaX-box Cys181 to a modified lipid (Gureasko et al. 2008) preassembled in nanodiscs, which are lipoprotein complexes comprised of a lipid bilayer encapsulated by engineered Apo-lipoprotein A (Denisov et al. 2004). The GTPase domain interacts transiently with the bilayer surface with two preferred orientations, where GDP- versus GTP-bound Rheb favor distinct orientations (Mazhab-Jafari et al. 2013). Rheb activation shifts the equilibrium towards an orientation in which the switch regions may be more accessible for interactions with effector proteins. Although the nucleotide-binding site is exposed in both orientations, membrane conjugation reduced the rate of intrinsic nucleotide exchange, suggesting that membrane association may allosterically affect protein dynamics (Mazhab-Jafari et al. 2013).

13.5 Activating and Inactivating Rheb Mutations

Constitutively activated GTPase mutants, together with inactive variants, are indispensible research tools for probing the function of GTPases, and dissecting the signaling pathways they regulate. Mutations that impair GTP hydrolysis generate constitutively activated small GTPase proteins that bind effector proteins and stimulate signaling pathways more potently than wild-type GTPases. Constitutively activated mutants may also help to identify GAPs, which may form more stable complexes in the absence of hydrolysis. Mutations that disrupt nucleotide binding generate inactive variants that may further act as dominant negative variants by forming stable nucleotide-free complexes with GEFs, thus blocking the activation of the wild-type GTPase.

Since the discovery of Rheb, a number of activating and inactivating mutations have been reported and characterized (Table 13.1). Initially, constitutively active and dominant negative Rheb mutations Q64L and S20N were designed by analogy to known Ras mutations (Clark et al. 1997). The subsequent finding that mutation of the non-catalytic residue Q64 has little impact on GTPase activity (see Sect. 13.3) suggested that alternate mutants were needed.

A dominant negative mutant (D60V) was identified in *S. pombe* Rheb by screening a mutagenized library for mutations that inhibit growth (Tabancay et al. 2003). Further site-directed mutagenesis studies of this site revealed that D60V and D60I exhibited preferential binding of GDP, whereas D60K failed to

Table 13.1 Gain and loss of function Rheb mutations and their effects on Rheb function

Rheb mutation	Mutant phenotype	Enzymatic property	References
S16H/N	Gain of function	GAP resistant	J Biol Chem. (2006) **281,** 19793
V17A/G	Gain of function	Lower GDP affinity	Mol Microbiol. (2005) **58,** 1074
V17A/D	Gain of function	Not determined	Genetics (2009) **183,** 517
S20N	Loss of function	Nucleotide deficient	J Biol Chem (1997) **272,** 10608
S21G/I	Gain of function	Lower GDP affinity	Mol Microbiol. (2005) **58,** 1074
Y35A	Gain and loss of funtion	Accelerated intrinsic hydrolysis but GAP resistant	Structure. (2012) **20,** 1528
T38M	Loss of function	Impaired mTORC1 communication	FEBS Letters. (2005) **579,** 4763
I39K	Loss of funciton	Impaired mTORC1 communication	Curr Biol (2005) **15,** 702-13
N41A	Loss of funciton	Impaired mTORC1 communication	FEBS Letters. (2005) **579,** 4764
Q52R/I76F	Gain of function	Not determined	Genetics (2009) **183,** 517
Y54A	Loss of funtion	Partial guanine nucleotide binding	FEBS Letters. (2005) **579,** 4764
L56A	Loss of funtion	Partial guanine nucleotide binding	FEBS Letters. (2005) **579,** 4764
D60V/K/I	Loss of function	No GTP binding	J Biol Chem. (2003) **278,** 39921
G63A	Gain of function	Impaired intrinsic hydrolysis and GAP resistant	J Biol Chem. (2014), **289,** 12195, Mazhab-Jafari et al
G63V	Not determined	Unable to bind GTP	J Biol Chem. (2014), **289,** 12195, Mazhab-Jafari et al
Q64L	Gain of function	Partially GAP resistant	J Biol Chem (1997) 272, 10608
D65A	Gain and Loss of funtion	Impaired hydrolysis and mTORC1 communication	Structure. (2012) **20,** 1528
Y67A/ I69A	Loss of function	Impaired mTORC1 communication	J Biol Chem. (2007) **282,** 18542
I76A/ D77A	Loss of function	Impaired mTORC1 communication	J Biol Chem. (2007) **282,** 18542
K120R	Gain of function	No GDP binding	Mol Microbiol. (2005) **58,** 1074
N153T/S	Gain of function	Lower GDP affinity	Mol Microbiol. (2005) **58,** 1072
C181S	Loss of funtion	Not farnesylated	Biochem Biophys Res Commun (2006) **344,** 869
M184L	FTI resistant	Geranylgeranylated	Genes Dev (2008) **22,** 2178-88

bind nucleotide. In mammalian cells, expression of Rheb D60K inhibited mTOR signaling and blocked sensitivity to nutrients or serum (Tabancay et al. 2003).

The same group later identified activating mutations of *S. pombe* Rheb. The single amino acid changes V17A/G, S21G/I, K120R, or N153T/S resulted in increased GTP loading due to decreased GTPase activities and/or decreased binding of GDP (Urano et al. 2005). Interestingly, the V17A mutation arose independently in a screen for constitutively activated *S. pombe* Rheb mutants based on resistance to the toxic arginine analog canavanine (Murai et al. 2009). This screen

also identified V17D and Q52R/I76F as constitutively activated mutants (Murai et al. 2009).

Rheb S16H was identified as a gain-of-function mutant that is resistant to TSC2 GAP activity, exhibits increased GTP loading, and activates mTOR signaling more strongly than wild-type Rheb (Yan et al. 2006).

Tee et al. identified critical residues in Rheb required for mTOR activation. Rheb mutations in the switch I region (T38M N41A) disrupted interaction with mTOR, while mutations in the region between switches I and II known as the constitutive effector (Ec) region (Y54A and L56A) partially disrupted nucleotide binding (Tee et al. 2005).

Long et al. found that Rheb binding to mTOR is impaired by an I39K mutation in switch I (Long et al. 2005). They subsequently performed an extensive alanine scanning mutagenesis study of surface exposed residues in Rheb to identify regions required for activation of TOR. Surprisingly two double mutations in switch 2 (Y67A/I69A and I76A/D77A) each severely impaired TOR activation, whereas extensive mutation of switch 1 and other surface residues was tolerated (Long et al. 2007). The switch 2 mutations did not affect expression or GTP binding, and impaired mTOR activation without affecting the interaction between Rheb and mTOR. This led the authors to propose that a Rheb switch 2-dependent interaction with another factor may be required for Rheb signaling to mTOR in vivo (Long et al. 2007).

More recently, the high resolution crystal structure of Rheb has been used to rationally design mutations to perturb Rheb function. As discussed in more detail in Sect. 13.3, Tyr35 autoinhibits Rheb GTP hydrolysis, and the slow intrinsic hydrolysis of GTP can be increased tenfold by a Y35A Rheb mutation (Mazhab-Jafari et al. 2012). However Y35A is insensitive to the GAP activity of TSC2; thus, Rheb Y35A impaired the regulation of mTOR by growth factors (Mazhab-Jafari et al. 2012). The accelerated catalysis of Rheb Y35A occurs through a noncanonical catalytic mechanism involving D65 rather than Q64 (Mazhab-Jafari et al. 2012). The D65A mutation increases GTP loading of Rheb, but failed to stimulate mTOR, possibly because mutation of this region of switch II disrupts mTOR interaction (Mazhab-Jafari et al. 2012).

Both constitutively active and inactive Rheb mutants have been engineered by mutation of Gly63, which coordinates the hydrolytic water molecule (H_2O^{cat}) and the γ-phosphate. The G63A mutation displaces H_2O^{cat} thus impairing GTP hydrolysis whereas G63V blocks the γ-phosphate and can only accommodate GDP (Mazhab-Jafari et al. 2014). Thus Rheb G63A and G63V are constitutively activated and inactive variants, respectively.

Rheb localization can be disrupted by mutation of the CaaX box. The Rheb C181S mutation blocks Rheb farnesylation, thus localization and signaling (Buerger et al. 2006), whereas a M184L mutation leads to geranylgeranylation instead of farnesylation of the CaaX box (Mavrakis et al. 2008).

Finally, a number of Rheb mutations have been identified in various tumour types, and await characterization to determine how they may affect Rheb function (discussed in more detail in Sect. 13.7).

13.6 Rheb Protein–Protein Interactions

It is quite common for small GTPase proteins to interact with several other proteins, including multiple GAPs and GEFs, as well as several effector proteins that can stimulate more than one signaling pathway. Indeed, a series of recent reports suggest that this trend extends to Rheb as well. In addition to TSC2 and mTOR (discussed in Sect. 13.2), Rheb has been reported to interact with a number of other proteins, including putative GEFs, effector proteins, guanine nucleotide dissociation inhibitor-like solubilizing proteins, as well as proteins that inhibit Rheb by sequestration or phosphorylation. These interactions implicate Rheb in diverse cellular functions that extend beyond mTOR signaling.

13.6.1 Identity of a Rheb GEF

The identity and existence of a GEF for Rheb remain unresolved. The low intrinsic GTPase activity and rapid intrinsic nucleotide exchange exhibited by Rheb initially led to the suggestion that a GEF may not be required to maintain Rheb in its highly activated state. Since then, three proteins have been proposed as putative GEFs for Rheb; however, some of these findings have been controversial.

13.6.1.1 Translationally Controlled Tumor Protein (TCTP)

Based on studies in *Drosophila*, Hsu et al. proposed that the small, conserved protein translationally controlled tumor protein (dTCTP), which is highly expressed in various tumours, plays an essential role in growth and proliferation by activating Rheb (Hsu et al. 2007). RNAi-mediated knockdown of dTCTP reduced cell size, cell number, and organ size, similar to mutation of *Drosophila* Rheb. Genetically, TCTP was epistatic to Tsc1 and Rheb, but upstream of S6K, suggesting that it functions in the TSC pathway. Overexpressed TCTP and Rheb were coimmunoprecipitated in 293T cells and a direct interaction was demonstrated in vitro by pull-down experiments. TCTP preferentially interacted with nucleotide-free Rheb and also stimulated release of GDP, suggestive of a GEF function, although relatively high concentrations of TCTP (equimolar to Rheb) were required for modest enhancement of nucleotide exchange. Further, Rheb activation was decreased modestly by knockdown of TCTP in *Drosophila* S2 cells (Hsu et al. 2007). Structurally, TCTP is similar to the Mss4/Dss4 family of proteins, which are guanine nucleotide-free chaperones (GFCs) that bind nucleotide-free Rab GTPases, and also exhibit weak GEF activity (Thaw et al. 2001). Molecular dynamics simulations were used to model the interaction between Rheb and TCTP, and predict residues involved in the interaction, which were subsequently verified using mutagenesis experiments (Dong et al. 2009).

The identification of TCTP as a putative Rheb GEF was of great interest to the field; however, some laboratories subsequently reported that these findings could not be reproduced. Rehmann et al. reported that they could not detect exchange activity of human TCTP towards Rheb in vitro using a mant-tagged nucleotide fluorescence-based exchange assay, nor could an interaction between TCTP and Rheb be detected by NMR spectroscopy (Rehmann et al. 2008), consistent with unpublished results from our laboratory. Rehmann et al. also reported that depletion of TCTP had no effect on phosphorylation of the mTOR substrate S6K in vivo (Rehmann et al. 2008). Further they point out that TCTP has two insertions relative to Mss4 that would be expected to occlude Rheb binding in the manner by which Rab interacts with Mss4 (Rehmann et al. 2008). Wang et al. studied the role of TCTP in mammalian TORC1 signaling, and concluded that neither depletion nor overexpression of TCTP affected mTORC1 signaling, and no stable interaction between TCTP and Rheb, was observed (Wang et al. 2008a).

Alternatively, Dong et al. reported results confirming that human TCTP (hTCTP) interacts with human Rheb (hRheb) and accelerates GDP release, although once again high concentrations of TCTP (in excess over Rheb) were required to achieve a modest increase in nucleotide exchange (Dong et al. 2009). By contrast, Ras nucleotide exchange is enhanced by the catalytic domain of the RasGEF Sos at a ratio of only 1:10,000 (Smith et al. 2013).

13.6.1.2 Protein Associated with Myc

The E3 ubiquitin ligase protein associated with Myc (PAM) was also proposed as a regulator of the mTOR pathway that may directly activate Rheb by facilitating nucleotide exchange (Maeurer et al. 2009). Activation of mTOR by sphingosine-1-phosphate treatment was found to be dependent on PAM, and PAM purified to near homogeneity from HeLa cells promoted Rheb nucleotide exchange in vitro, although once again a high PAM concentration was required to see this effect (Maeurer et al. 2009). PAM is a large 510 kDa protein and it remains to be determined whether an isolated recombinant domain possesses GEF activity for Rheb. Intriguingly, PAM contains two domains with homology to regulator of chromosome condensation 1 (RCC1), which is a well-studied Ran GEF (Renault et al. 1998).

13.6.1.3 αβ-Tubulin

Another group proposed that soluble αβ-tubulin acts as a Rheb activator, reporting that soluble αβ-tubulin directly binds and activates Rheb in vivo (Lee et al. 2013). The deacetylated form of αβ-tubulin was found to have higher affinity for Rheb than the acetylated form and it was proposed that the balance of tubulin acetylation throughout the cell cycle may influence Rheb activation in a temporal manner. It has not been demonstrated in vitro that tubulin possesses GEF activity for Rheb, as

the GTP-binding and intrinsic GTPase activities of tubulin confound the conventional GEF assays (Lee et al. 2013), thus it remains unknown whether the reported effects of tubulin on Rheb activation are direct or indirect. Interestingly, tubulin is known to form complexes with heterotrimeric G proteins that result in the activation of Gα (Rasenick et al. 2004).

13.6.2 Noncanonical Rheb Effector Proteins

While Rheb's functional role in stimulating the phosphorylation of mTOR kinase substrates has been well demonstrated, some questions remain regarding whether this is mediated through a direct interaction or indirect mechanisms. For example, activation of the related PIKK kinase phosphoinositide 3-kinase (PI3K) by Ras occurs through a direct interaction with the Ras-binding domain (RBD) (Pacold et al. 2000), a mode that is common amongst effectors of Ras subfamily GTPases. However, the primary sequence of mTOR lacks an identifiable RBD. Rheb has been reported to bind the N-terminal lobe of the mTOR kinase domain (amino acids 1967–2191) using truncations and pulldowns, but this interaction is not GTP dependent (Long et al. 2005), and a direct interaction has not been confirmed in vitro with purified recombinant constructs. Alternate mechanisms proposed for mTOR activation by Rheb are discussed below, along with interactions with other putative effector proteins that implicate Rheb in additional cellular pathways.

13.6.2.1 Raf Kinase

There have been several reports of inhibitory interactions of Rheb with Raf kinases. Rheb was originally reported to antagonize Ras signaling and transformation (Clark et al. 1997). An interaction between Rheb and Raf-1 kinase was reported and it was proposed that Rheb and H-Ras function together to integrate cAMP and growth factor signaling, as PKA phosphorylation of Raf-1 inhibited binding of H-Ras but enhanced Rheb binding (Yee and Worley 1997). Expression of Rheb was also reported to inhibit B-Raf kinase (Im et al. 2002) through an mTOR-independent interaction that is enhanced by serum and did not require farnesylation (Karbowniczek et al. 2004). Rheb was found to inhibit the association of B-Raf with H-Ras and decrease the phosphorylation and heterodimerization of B-Raf and C-Raf, as well as their kinase activity (Karbowniczek et al. 2006). In vitro pulldowns have confirmed the direct interaction of Rheb with the RBD of Raf (Uhlenbrock et al. 2009); however the affinity of activated Rheb for the isolated Ras-binding domain (RBD) of c-Raf was shown by NMR to be 1,000-fold lower than that of Ras; thus it is not clear whether Raf kinases are physiological effectors of Rheb in the cell (Karassek et al. 2010). However the interaction between Ras and full length Raf-1 is promoted by the Raf cysteine-rich domain (Brtva et al. 1995),

thus the relative affinities of Rheb and Ras for full length Raf kinases in vivo remain unknown.

13.6.2.2 FK506-Binding Protein 38

An alternate mechanism by which Rheb might regulate mTOR through FK506-binding protein 38 (FKBP38) was proposed by Jiang and coworkers (Bai et al. 2007). FKBP38 belongs to the peptidyl prolyl cis/trans isomerase family of FKBPs, and contains a region of homology to FKBP12. In the presence of rapamycin, FKBP12 forms a tripartite complex with the FKBP12-rapamycin-binding domain (FRB) of mTOR and inhibits mTOR kinase function (Choi et al. 1996; Vilella-Bach et al. 1999). Bai et al reported that endogenous Rheb coimmunoprecipitates with FKBP38 from HEK293 cell lysates, and that recombinant Rheb and FKBP38 interact in vitro, with the activated form of Rheb exhibiting higher affinity than Rheb-GDP. FKBP38 was found to inhibit mTORC1 kinase activity, and this was antagonized by overexpression of Rheb. Thus a model was proposed in which Rheb-GTP binds FKBP38 in an amino acid- and serum-sensitive manner, displacing FKBP38 from mTOR thus reactivating the kinase (Bai et al. 2007).

Subsequently, this model has been challenged by other groups. Wang et al. confirmed an interaction between Rheb and FKBP38 in vivo, but reported that FKBP38 did not inhibit mTORC1 signaling, and that FKBP38 binding to mTOR was not regulated by amino acids or insulin (Wang et al. 2008a). Interestingly the farnesylation-deficient Rheb mutant C181S failed to interact with FKBP38, which contains a trans-membrane domain at its C terminus, suggesting any interaction may require colocalization on a membrane (Wang et al. 2008a). Uhlenbrock et al. reported that they did not detect an interaction between FKBP38 and Rheb using in vitro pull-down experiments or solution-binding assays, regardless of the nucleotide bound (Uhlenbrock et al. 2009). These authors noted that the in vitro Rheb-FKBP38 interactions reported by Jiang et al. were performed in the absence of magnesium, where small GTPase proteins fail to bind guanine nucleotides and are thus unstable. Finally, immunoprecipitated mTORC1 that does not contain FKBP38 is still activated by Rheb, indicating that repression of FKBP38 inhibition is not involved in mTORC1 activation by Rheb in vitro (Sato et al. 2009).

FKBP38 interacts with the anti-apoptotic proteins Bcl-2 and Bcl-XL, and Ma et al. proposed that Rheb binding to FKBP38 plays a role in modulating apoptosis by regulating these interactions (Ma et al. 2010). FKBP38 recruits Bcl-2 and Bcl-XL to the mitochondria. According to their proposal, Rheb-GTP promotes release of Bcl-XL from FKBP38, thus allowing this anti-apoptotic protein to inhibit the pro-apoptotic activities of Bak (Ma et al. 2010). Thus the activation of Rheb leads to suppression of apoptosis in this model.

13.6.2.3 Nix and LC3-II

Interactions between Rheb and mitochondrial proteins were also reported to regulate mitophagy (Melser et al. 2013), a process that removes damaged mitochondria. Stimulation of mitochondrial oxidative phosphorylation increases the degradation and renewal of mitochondria. High oxidative phosphorylation was also found to recruit Rheb to the mitochondrial outer membrane in a farnesylation-dependent manner, where it interacts with Nix and LC3-II, thus promoting mitophagy (Melser et al. 2013). Nix is known as the mitochondrial autophagic receptor and LC3-II is an autophagosomal protein. These interactions have not yet been demonstrated to be direct and the binding sites have not been demonstrated; however, it was proposed that mitochondrial Rheb promotes interaction between Nix and LC3, leading to autophagosomal engulfment (Melser et al. 2013).

13.6.2.4 Bcl-2/Adenovirus E1B 19-kDa Interacting Protein 3

mTOR signaling is inhibited in response to hypoxia, and it has been proposed that this is mediated by an inhibitory interaction between Rheb and Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (Bnip3) (Li et al. 2007), a Bcl-2 superfamily pro-death protein that is induced by hypoxia. The Bnip3 protein contains a C-terminal transmembrane domain and a central Bcl-2 homology 3 (BH3) domain. Bnip3 was reported to interact with Rheb in coimmunoprecipitation and FRET assays in a manner that requires Rheb farnesylation and membrane localization of Bnip3. Bnip3 overexpression modestly decreased Rheb GTP levels and inhibited mTOR signaling, dependent on the N-terminal region of Bnip3. Knockdown and overexpression of Bnip3 demonstrated that Bnip3 mediates the inhibition of the mTOR pathway in response to hypoxia (Li et al. 2007).

13.6.2.5 Inhibition of Aggresome Formation

Activated Rheb was reported to sensitize cell death in response to misfolded proteins by inhibiting aggresome formation in a manner that is independent of TOR complex 1 (Zhou et al. 2009). Formation of aggresomes facilitates degradation of misfolded proteins and this process is defective in cells bearing TSC mutations, which undergo apoptosis when misfolded proteins accumulate. Activated Rheb was reported to block aggresome formation by disrupting the binding of misfolded ubiquitinylated proteins to dynein, thus inhibiting their transportation for degradation (Zhou et al. 2009). The specific Rheb-binding targets that mediate this effect remain to be identified. Rheb activation thus coordinately promotes protein synthesis through mTORC1, and independently inhibits protein degradation by inhibiting formation of aggresomes.

13.6.2.6 Phospholipase D (PLD)

Phosphatidic acid (PA) was identified as a lipid messenger that mediates mitogenic mTORC1 activation through an interaction with the FRB (Fang et al. 2001). It was later proposed that the PA-generating enzyme phospholipase D (PLD) may be activated by Rheb as an effector protein (Sun et al. 2008). Rheb was found to activate PLD1 in vivo and activation of mTOR by overexpressed Rheb requires PLD1. It was demonstrated in vitro that Rheb interacts directly with immunoprecipitated mammalian PLD1 and activates its enzymatic activity in a GTP-dependent manner. However purified recombinant PLD1 was not activated by Rheb suggesting a requirement for another factor that remains unidentified (Sun et al. 2008).

13.6.3 Inhibitors of Rheb Signaling

A number of proteins have been proposed to interact with Rheb and inhibit signaling by sequestering Rheb. In addition, phosphorylation of Rheb was found to inhibit Rheb by disruption of nucleotide binding.

13.6.3.1 Phosphodiesterase 4D

The second messenger cyclic AMP (cAMP) was shown to be involved in regulation of mTOR. Kim et al. proposed that the cAMP hydrolyzing enzyme phosphodiesterase 4D (PDE4D) acts as a cAMP sensor that binds and sequesters Rheb, thus providing a putative mechanism linking cAMP levels to mTOR signaling (Kim et al. 2010). They reported that endogenous PDE4D5 and Rheb coimmunoprecipitate in several cell types through an interaction that requires the PDE catalytic domain, is independent of the nucleotide bound to Rheb, and does not alter Rheb's GDP/GTP ratio. Increased levels of the PDE substrate cAMP disrupt this interaction allowing Rheb to bind and activate mTOR (Kim et al. 2010). This noncanonical function of PDE4D5 appears to be independent of cAMP regulation of PKA. However elevated cAMP can also inhibit mTORC1 activation through a PKA-dependent mechanism that is independent of Rheb (Xie et al. 2011).

13.6.3.2 Glyceraldehyde-3-Phosphate Dehydrogenase

The same group proposed a conceptually analogous mechanism by which Rheb could be regulated in response to glucose. They reported that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) directly interacts with Rheb to regulate mTORC1 activity in response to glycolytic flux (Lee et al. 2009). GAPDH was

identified in Rheb immmunoprecipitates and the two proteins were shown to interact in vivo in a manner that does not depend on the nucleotide bound to Rheb but is destabilized by the glycolytic intermediate Gly-3-P (Lee et al. 2009). It was proposed that GAPDH binding to Rheb prevents its activation of mTOR under low-glucose conditions whereas high glycolytic flux promotes release of Rheb to restore mTOR signaling (Lee et al. 2009). This represents a mode of mTOR regulation in response to glucose availability that is independent of AMPK and TSC2 signaling. Consistent with this, increased expression of the glucose transporter GLUT1 enhances mTORC1 activity, even in the absence of functional TSC2. Enhanced GLUT1 expression increased binding of mTOR to Rheb while decreasing the association of GAPDH with Rheb (Buller et al. 2011). The interaction between GAPDH and Rheb was characterized by atomic force microscopy (AFM), which confirmed that the interaction was inhibited by the presence of Gly-3-P (Kim et al. 2011a)

13.6.3.3 Rabin8

Rabin8, which is known to function as a GEF for a small GTPase of another subfamily, Rab8, was immunoprecipitated with Rheb. Overexpression of Rabin8 was reported to decrease mTOR signaling, whereas Rabin8 knockdown stimulated mTOR, suggesting that rather than acting as a GEF for Rheb, Rabin 8 may sequester Rheb in a stable complex (Parkhitko et al. 2011).

13.6.3.4 NMDA Receptor

An interaction between Rheb and the NMDA Receptor subunit NR3A was identified in a yeast two-hybrid system, and confirmed for overexpressed proteins in mammalian cells, as well as by co-immunoprecipitation of endogenous proteins from membrane fractions of rat brain. It was suggested that NR3A sequesters synaptic Rheb to inhibit mTOR signaling (Sucher et al. 2010), although this has not been demonstrated. The reported interaction has not been shown to be direct and it is not known whether it depends on farnesylation or nucleotide binding of Rheb.

13.6.3.5 p38-Regulated/Activated Kinase

Rheb signaling can also be inhibited by phosphorylation. Zheng et al. showed that p38β mitogen-activated protein kinase (MAPK) and its downstream kinase p38-regulated/activated kinase (PRAK) are activated by energy starvation and suppress mTOR signaling through a mechanism that does not involve phosphorylation of TSC1/2. PRAK was found to directly phosphorylate Rheb at Ser130, which has the effect of impairing nucleotide binding, and thus inhibiting mTORC1 activation (Zheng et al. 2011). Ser130 does not form part of the

nucleotide-binding site, although phosphorylation may exert an allosteric effect on the proximal G4 box.

13.6.4 Guanine Nucleotide Dissociation Inhibitors

13.6.4.1 Rod Rhodopsin-Sensitive cGMP 3′,5′-Cyclic Phosphodiesterase 6D Delta Subunit (PDEδ)

Guanine nucleotide dissociation inhibitors (GDIs) act as solubilizing factors to transport lipidated small GTPase proteins through the cytoplasm between membranes (Goody et al. 2005). GDIs are best characterized for GTPases of the Rho and Rab subfamilies, but more recently PDEδ was proposed to function as a solubilizing factor for farnesylated Ras-subfamily proteins, including Rheb (Hanzal-Bayer et al. 2002; Ismail et al. 2011). PDEδ is a noncatalytic subunit of retinal rod rhodopsin-sensitive cGMP 3′,5′-cyclic phosphodiesterase required for solubilization of the holoenzyme (Florio et al. 1996), but also known to interact with interact with and solubilize prenylated small GTPase proteins (Nancy et al. 2002). Ismail et al. solved a structure of farnesylated, fully C-terminally modified Rheb-GDP in complex with PDEδ. The interaction with PDEδ occurs predominantly through the Rheb farnesyl moiety and is thus nucleotide-independent. This interaction is regulated by activated Arl2-GTP or Arl3-GTP, which interact with PDEδ to induce release of the farnesylated protein through an allosteric mechanism (Ismail et al. 2011). A similar interaction between PDEδ and KRAS was targeted as a therapeutic approach for cancer. Disruption of the interaction between PDEδ and KRAS by small molecules that selectively bind with high affinity to the prenyl-binding pocket of PDEδ alters the subcellular localization of KRas and suppresses its oncogenic signaling (Zimmermann et al. 2013).

13.7 Physiological Functions of Rheb in Health and Disease

13.7.1 Insights from Yeast, C. elegans*, and Drosophila*

Studies in yeasts have implicated Rheb in regulating arginine uptake. In the budding yeast *S. cerevisiae*, Rheb inhibits the uptake of the basic amino acids arginine and lysine (Urano et al. 2000). Budding yeast lacks both TSC genes and TOR is not activated by Rheb in this species. In contrast, the fission yeast *S. pombe* produces both TSC1 and TSC2, which form a complex that inactivates Rheb, and Rheb is coupled to activation of TOR2 in *S. pombe*. Thus fission yeast provides a genetically manipulable model system for studying the TSC–Rheb–TOR axis (Aspuria et al. 2007). Disruption of TSC1/2 in *S. pombe* reduces uptake of arginine,

which can be reversed by the expression of the dominant negative Rheb mutant D60K (Matsumoto et al. 2002; van Slegtenhorst et al. 2004). Deletion of Rheb arrests cell growth of fission yeast in a manner resembling nitrogen starvation (Mach et al. 2000), which suggested a role for Rheb in signaling the availability of nutrients.

In *Drosophila* S2 cells, dRheb regulates production of ribosomes, protein synthesis, and cell size (Hall et al. 2007), and in the developing organism dRheb is required for both cell growth and cell cycle progression (Patel et al. 2003). dRheb overexpression promotes tissue growth in fly development, resulting in increased cell size and accumulation of S-phase cells. dRheb is required for viability of the organism and for cell growth, while its inhibition results in G1 arrest (Patel et al. 2003). Overexpression of dRheb in central brain neurons (mushroom bodies or insulin producing cells) of Drosophila stimulated growth resulting in enlarged axon projections and cell bodies (Brown et al. 2012).

In many species, dietary restriction (calorie restriction or intermittent fasting) is known to extend lifespan, and inhibition of (m)TOR mimics this effect in many species, including mice (Harrison et al. 2009). In *Caenorhabditis elegans*, Rheb appears to have two roles in lifespan regulation. RNAi-mediated RHEB-1 knockdown extends lifespan, whereas intermittent fasting requires RHEB-1 and mTOR to extend lifespan. Most of the genes upregulated by fasting in *C. elegans* required RHEB-1 (Honjoh et al. 2009). These results underscore a significant role of Rheb and the (m)TOR pathway in longevity in mammals and lower organisms.

13.7.2 Rheb in Mammalian Development and Metabolism

The mTOR signaling pathway regulates growth and homeostasis in response to a wide variety of environmental conditions, and its deregulation is associated with numerous pathologies including cancer, tumor syndromes, type II diabetes, aging, and neurological diseases (Dazert and Hall 2011; Laplante and Sabatini 2013). As an activator of mTORC1, Rheb has a putative role in many of the functions of this mTOR complex. Here, we discuss physiological roles of Rheb in mammals inferred from phenotypes associated with its knockout or overexpression.

Rheb1 is essential for murine development, as Rheb1-deficient embryos die in gestation (embryonic day 12) with impaired development of the cardiovascular system (Goorden et al. 2011). The proliferation of Rheb(−/−) embryonic fibroblasts was impaired, associated with decreased TORC1 activity (Goorden et al. 2011). Consistently overexpression of Rheb1 via an adenoviral vector in adult rat ventricular cardiomyocytes activated mTORC1 signaling and stimulated cell growth in an mTORC1-dependent manner (Wang et al. 2008b).

Cardiac-specific Rheb-deficient mice died at postnatal day 8–10 with reduced heart-to-body weight ratios and impaired sarcomere maturation (Tamai et al. 2013). Rheb(−/−) hearts exhibited reduced phosphorylation of S6 and 4E-BP1 beyond

postnatal day 5, indicating that Rheb and mTOR become critical for cardiac hypertrophic growth in this period of development (Tamai et al. 2013).

By contrast to the lethal homozygous deletion of Rheb-1, deletion of a single Rheb1 allele from cardiomyocytes did not impair heart function or survival, but was cryoprotective against some conditions. Hemizygous deletion of Rheb reduced pathologic heart remodeling following myocardial infarction and also reduced pressure-induced cardiac hypertrophy (Wu et al. 2013). Remarkably, an mTOR inhibitor isolated from Chinese herbs (As-IV) was similarly cryoprotective in these models, suggesting a therapeutic strategy for patients with pathological cardiac remodeling or hypertrophy (Wu et al. 2013).

mTOR is known to be required for myogenesis; however, Rheb was reported to suppress skeletal muscle differentiation (Ge et al. 2011). Overexpression of Rheb inhibits differentiation of C2C12 mouse myoblasts and Rheb knockdown enhances differentiation, whereas mTOR knockdown impairs differentiation. The negative regulation of myogenesis by Rheb was mediated by suppression of insulin receptor substrate 1 (Ge et al. 2011).

In the central nervous system, conditional deletion of Rheb1 in neuronal progenitor cells reduced the size of the brain, and demonstrated that Rheb1 (but not Rheb2) is required for mTORC1 activation and myelination in postnatal brain development (Zou et al. 2011). Rheb signaling is neurotrophic in the adult mammalian central nervous system and promotes the preservation and restoration of axons. Viral transduction of substantia nigra dopaminergic neurons with constitutively active Rheb(S16H) induced mTOR activation and axon regrowth following neurotoxin-induced damage (Kim et al. 2011b, 2012). Rheb expression is induced in the rat brain cortex by injection of lipopolysaccharide (LPS) in a neuroinflammation model, and Rheb knockdown or mTOR inhibition suggested that Rheb may promote astrocytic proliferation and neuronal apoptosis in this model (Cao et al. 2013).

Rheb and mTOR also play a role in the endocrine regulation of energy metabolism at the whole-organism level. Pancreatic beta-cells are critical for maintenance of glucose homeostasis through production of insulin. Transgenic mice overexpressing Rheb in pancreatic beta-cells exhibited upregulation of mTORC1 signaling and increased beta-cell mass and cell size. These mice showed higher insulin secretion, improved glucose tolerance, and resistance to hyperglycemia, suggesting a therapeutic strategy for stimulating insulin production in diabetes (Hamada et al. 2009). mTORC1 signaling plays an important role in adipogenesis (Laplante and Sabatini 2009). In adipose cells, stimulation of mTOR signaling by constitutively activated Rheb induces production of leptin, a hormone that regulates food intake and body weight in response to feeding (Chakrabarti et al. 2008). Expression of constitutively active Rheb in cultured adipocytes activates mTORC1 signaling and inhibits expression of adipose triglyceride lipase and hormone-sensitive lipase, associated with reduced lipolysis, increased de novo lipogenesis, and intracellular accumulation of triglycerides, thus promoting fat storage (Chakrabarti et al. 2010).

Rheb also plays an essential role in hematopoiesis and development of the immune system. Conditional knockout of Rheb in T-cells caused specific defects in T-cell differentiation although T-cell proliferation was not blocked (Delgoffe et al. 2011). The Rheb2 isoform has been shown to play a role in maintaining hematopoietic progenitor cell (HPC) growth (Campbell et al. 2009). Rheb2 overexpression in mouse HPC cell lines enhanced mTOR signaling and stimulated proliferation, colony formation and survival of primary HPCs. Rheb2 expression promoted expansion of hematopoietic stem cells (HSCs), but also impaired overall repopulation of HSCs in transplantation recipients, consistent with observations that sustained proliferation can lead to exhaustion and loss of ability to repopulate (Campbell et al. 2009).

Clearly the Rheb proteins both play critical roles in development and energy metabolism, controlling a variety of mammalian physiologies ranging from development of muscle cells, neurons, and immune system cells to regulating glucose homeostasis. Determination of the potential roles of Rheb in other organ systems will require development of additional conditional knockout models. The extent to which Rheb1 and Rheb2 have overlapping versus distinct physiological roles remains to be determined.

13.7.3 Rheb in Apoptosis and Autophagy

As discussed in Sect. 13.6, Rheb has been reported to interact with proteins involved in apoptosis pathways. It has been proposed that Rheb-mTOR signaling may switch from a proliferative function to a cell death function, depending on the cellular context (Ehrkamp et al. 2013). Karassek et al. showed that overexpression of Rheb in HeLa cells enhanced the apoptotic effects induced by toxic stresses in an mTORC1-dependent manner, and that this effect was reversed by Rheb knockdown (Karassek et al. 2010). The mechanism by which Rheb promotes apoptosis was not defined but may involve apoptosis signal-regulating kinase 1 (ASK-1), as knockdown of this kinase inhibited Rheb's enhancement of apoptosis. Ma et al. proposed that Rheb may regulate apoptosis by modulating the sequestration of Bcl-2 and Bcl-XL by FKBP38 (Ma et al. 2010) (discussed in more detail in Sect. 13.6). Rheb has also been observed to sensitize induction of apoptosis by misfolded proteins by inhibiting aggresome formation in a TOR-independent manner (Zhou et al. 2009). Loss of TSC1 or TSC2 and activation of Rheb sensitizes cells to ER stress and promotes apoptosis, implicating TSC1/TSC2 and Rheb in the unfolded protein response and cell survival (Kang et al. 2011).

Autophagy is a protective process by which digestion of cellular contents releases nutrients under starvation conditions to maintain critical cellular functions and avoid cell death. mTOR signaling is known to inhibit autophagy, and this process is frequently suppressed in tumors. Rheb, which is frequently activated in tumours, may contribute to impairing this protective autophagic function in cancer (Ehrkamp et al. 2013). During energy deprivation in cardiomyocytes, Rheb is

inactivated, which suppresses mTOR signaling, increases autophagy, and improves cardiomyocyte survival. In mouse models of obesity, cardiac activation of Rheb and mTORC1 become deregulated and inhibit cardiac autophagy resulting in increased ischemic injury. Inhibition of mTORC1 restored autophagy and reduced cardiac damage (Sciarretta et al. 2012).

Treatment of a macrophage cell line with H_2O_2 was found to induce autophagic cell death by promoting ubiquitination and degradation of Rheb, thus suppressing mTOR signaling (Seo et al. 2011). Interestingly, regulation of autophagy by Rheb may be exploited to destroy the intracellular pathogen *Mycobacterium tuberculosis* (Wang et al. 2013). *Mycobacterium* is difficult to eradicate but may be controlled by enhancing autophagy. MicroRNA-155 expression was enhanced by mycobacterial infection, and overexpression of MicroRNA-155 accelerated autophagy, promoting maturation of mycobacterial phagosomes in macrophages and decreasing mycobacterial survival. MicroRNA-155 was shown to promote autophagy by targeting Rheb mRNA and decreasing Rheb levels (Wang et al. 2013). In addition to autophagy, Rheb was also found to promote mitophagy induced by mitochondrial energy status (Melser et al. 2013) (details in Sect. 13.6).

13.7.4 Rheb in Pathogenesis

13.7.4.1 Tuberous Sclerosis

Tuberous sclerosis (TSC) is an autosomal dominant tumor syndrome that affects ~1/6,000 births, and is characterized by the development of benign tumors (hamartomas) affecting multiple organs. The vast majority (~80 %) of TSC cases are associated with mutations of the tumor suppressor genes TSC1 or TSC2 that encode the proteins hamartin and tuberin, respectively (Crino et al. 2006; Huang and Manning 2008). Tuberin, a 1807-a.a. protein, and hamartin, a 1164-a.a. protein, function as a heterodimer (van Slegtenhorst et al. 1998) and hamartin stabilizes tuberin (Hodges et al. 2001; Nellist et al. 2001). Tuberin contains a GAP domain near its C terminus (residues 1525–1742) (Benvenuto et al. 2000; Chong-Kopera et al. 2006) that acts on Rheb (Castro et al. 2003; Garami et al. 2003; Inoki et al. 2003a). mTOR is hyperactivated in TSC hamartomas, strongly implicating deregulated Rheb activation as the major element in the pathogenesis of tuberous sclerosis, although Rheb-independent TSC1 or TSC2 functions may also contribute (Neuman and Henske 2011).

The GAP domain of TSC2 is homologous to Rap1GAP and inactivates Rheb by providing an asparagine thumb residue that catalyzes hydrolysis of GTP (Daumke et al. 2004; Scrima et al. 2008) as discussed in Sect. 13.3. Loss of TSC1/2 GAP function is thus thought to contribute to TSC phenotypes and it has been demonstrated that disease-associated mutations of the putative asparagine thumb catalytic residue (N1643) of TSC2 eliminate GAP activity for Rheb in vivo (Garami et al. 2003) and in vitro (Marshall et al. 2009). Currently thousands of unique

DNA variations and mutations have been identified in the TSC1 and TSC2 genes of TSC patients, including missense and nonsense, frame shift, and truncation mutations [Leiden Open Variation Database (LOVD)]. Most nonsense mutations in TSC2 would be expected to abolish GAP activity since the GAP domain resides near the C terminus. Characterization of TSC-associated point mutations has shown that some but not all mutations within the GAP domain impair catalytic activity in vitro (Marshall et al. 2009). Other mutations disrupt association of TSC1 with TSC2, reducing levels of TSC2, and/or causing mislocalization of TSC2 from endomembranes where it encounters Rheb (Hoogeveen-Westerveld et al. 2011, 2013).

Larcher et al. identified a feedback mechanism in TSC-null cells in which Rheb activates AMPK, thus suggesting an mTOR-independent role for Rheb in proliferation. Adenosine 5′-monophosphate-activated protein kinase (AMPK) is activated under low glucose conditions, and acts to suppress energy-expensive anabolic processes. Consistent with this role, AMPK phosphorylates and activates TSC1/2 thus inhibiting mTOR signaling and protein production. In an mTORC1-independent feedback loop, Rheb was found to activate AMPK, and reduce levels of cyclin-dependent kinase inhibitor p27(KIP1) (p27), thus activating Cdk2 and proliferation in TSC2-null cells (Lacher et al. 2010, 2011).

Tuberous sclerosis exhibits tremendous diversity in its clinical manifestations and severity of disease. Despite extensive research on the TSC1/2 genes and proteins, there is a poor understanding of how associated phenotypes relate to different mutation types. Treatment is limited to rapamycin and its analogs, which are only approved for brain subependymal giant cell astrocytomas (SEGAs) that cannot be treated surgically. Future work on structural and functional analysis of the TSC1/2 proteins may provide new insights into how the TSC1/2 proteins may be stabilized or activated to maintain GAP function towards Rheb.

13.7.4.2 Rheb and mTOR in Cancer

The vital role of the mTOR signaling pathway in cell growth and tumorigenesis is highlighted by the finding that multiple signaling components upstream and downstream of mTOR are frequently altered in a wide variety of human cancers, resulting in mTOR hyperactivation (Bjornsti and Houghton 2004; Rosner et al. 2008; Dazert and Hall 2011; Laplante and Sabatini 2013). Thus mTOR has emerged as a target of anticancer therapies (Petroulakis et al. 2006; Sabatini 2006). Several lines of evidence implicate the mTOR activator Rheb in tumorigenesis. Knockdown of Rheb results in cell-cycle arrest in the G0/G1 phase, while its overexpression leads to an increase in S-phase cells (Mach et al. 2000; Patel et al. 2003). Constitutively activated Rheb mutants can induce oncogenic transformation in cell culture (Jiang and Vogt 2008), and Rheb overexpression is sufficient for carcinogenesis of skin epithelial cells (Lu et al. 2010) and can induce rapid development of aggressive lymphomas (Mavrakis et al. 2008). Both the expression of Rheb as well as its activation state are elevated in some cancer cell lines (Gromov

et al. 1995; Im et al. 2002; Eom et al. 2008; Mavrakis et al. 2008; Nardella et al. 2008). Elevated levels of Rheb protein are associated with an increase in its activation state, presumably due to limiting TSC2 GAP activity (Im et al. 2002).

Prostate: Rheb is overexpressed in many human prostate cancer cell lines (Nardella et al. 2008). Transgenic mice overexpressing Rheb specifically in the prostate exhibited hyperplasia and low-grade neoplasia, but also senescence and limited Akt activation through a negative feedback loop (Nardella et al. 2008). In combination with PTEN haploinsufficiency, Rheb overexpression promotes tumorigenesis (Nardella et al. 2008). Rheb was found to be highly expressed in aggressive, androgen-independent prostate cancer cell lines and cancer tissues (Kobayashi et al. 2010). Rheb promotes proliferation of prostate cancer cells, and proliferation of prostate cancer cell lines can be suppressed by Rheb knockdown or inhibition or mTOR by rapamycin (Kobayashi et al. 2010).

Breast Rheb is a key determinant of 17-beta estradiol (E(2))-dependent proliferation of the MCF-7 breast cell line (Yu and Henske 2006). E(2) induces AKT phosphorylation and inactivation of TSC2, thus increasing Rheb activation, and Rheb knockdown blocked E(2)-stimulation of MCF-7 cell proliferation (Yu and Henske 2006). Rheb upregulation correlates with poor prognosis in breast, as well as head and neck cancers (Lu et al. 2010).

Skin Most malignant melanomas exhibit overactivation of mTOR signaling, and it was found that proliferation of three of six melanoma cell lines could be partially blocked by rapamycin or a farnesyl transferase inhibitor (FTI-277), thus implicating Rheb in the pathogenesis (Karbowniczek et al. 2008). Rheb is overexpressed in human squamous cancer cell lines, and expression of Rheb at similar levels in murine basal keratinocytes led to development of skin tumors in transgenic mice (Lu et al. 2010). In this model, Rheb promoted carcinogenesis through multiple oncogenic mechanisms and tumor persistence was mTORC1 dependent.

Cancer-Associated Mutations of Rheb While it is appreciated that deregulation of Rheb occurs in many cancers and contributes to oncogenic processes, until recently there were no reports of Rheb mutations in cancer. Recently high-throughput cancer genome sequencing projects have identified ~20 mutations in Rheb (S21L, T23M/K, I24V, G29S, Y35N, T42N/I, V49E, E53D, D60H, G63W, Q72E, N79S, L137F, E139D/K/G, A173T, and G177C) occurring in various tumor types including lung, breast, endometrium, urinary tract, colon, stomach, and kidney (Catalogue of somatic mutations in cancer, COSMIC). Rheb mutations occur somewhat rarely in tumors, with the highest frequency (1 %) in patients with kidney clear cell carcinoma (Lawrence et al. 2014). To date, no characterization of these Rheb mutations has been reported, thus it is currently not known whether individual mutations may be oncogenic "driver" mutations, or how they may impact Rheb function. The mutations are widely distributed throughout the Rheb sequence with weak clustering around the G-box motifs and switch regions. In contrast, cancer-associated mutations of Ras isoforms occur primarily at codons 12, 13, and 61 (Prior et al. 2012). Interestingly, mutation of Rheb Tyr35 has been

observed recurrently in kidney and endometrial cancers (Lawrence et al. 2014). Tyr35 is in switch I and covers the nucleotide-binding pocket (Yu et al. 2005). Mutation of Tyr35 (Y35A/F) was previously shown to significantly enhance Rheb's intrinsic hydrolysis of GTP, but rendered it insensitive to TSC2 GAP (Mazhab-Jafari et al. 2012).

13.7.4.3 Rheb and mTOR in Cancer Treatment

Based on the established role of mTOR signaling in cancer, there has been tremendous interest in therapeutic targeting of this pathway. Rapamycin and its synthetic analogs (Rapalogs) inhibit mTORC1 phosphorylation of a subset of its substrates (Kang et al. 2013). Rapalogs have shown some clinical promise and are approved for treatment of advanced-stage renal cell carcinoma, but have proven less effective than expected against many cancer types (Wander et al. 2011). This may be because mTOR inhibition reduces cell growth but may not cause cell death, and can lead to activation of AKT through multiple negative feedback loops in mTOR signaling. More recently, a second generation of mTOR kinase inhibitors were developed that directly compete with ATP binding to more completely inhibit the kinase activity of mTORC1 as well as mTORC2 (Zaytseva et al. 2012). By inhibiting phosphorylation of rapamycin-resistant mTOR substrates (particularly 4E-BP), these kinase inhibitors may more effectively block proliferation, and are currently under clinical evaluation (Zaytseva et al. 2012)

Alternatively, mTORC1 signaling could be controlled by preventing its activation by Rheb. Farnesyltransferase inhibitors (FTIs) were developed to block the transforming activity of oncogenic Ras mutants, which requires posttranslational modification by farnesylation and membrane localization (Berndt et al. 2011). FTIs exhibited limited clinical success. Whereas they have demonstrated antitumour activity in a subset of cancer patients, their efficacy was not dependent on Ras mutation status (Berndt et al. 2011). Thus there has been interest in understanding why some tumors are sensitive to FTIs and identifying farnesylated proteins that mediate the antitumour effects of FTIs in these cells. Inhibition of Rheb appears to be responsible for some of the antitumor effects of FTIs (Mavrakis et al. 2008). Lymphomas with high expression of Rheb have increased mTOR activity and exhibited enhanced sensitivity to FTIs, whereas these lymphomas can be rendered resistant to FTIs by expressing a farnesylation-independent, geranylgeranylated Rheb mutant (M184L) (Mavrakis et al. 2008).

Ding et al. showed that inhibition of Rheb by FTIs impairs mTOR signaling and induces apoptosis in acute myelogenous leukemia (AML) cells. The apoptotic mechanism was proposed to involve stabilization and increased levels of the pro-apoptotic proteins Puma and Bax (Ding et al. 2013). Expression of farnesylation-independent Rheb M184L reduced induction of apoptosis by FTIs, as did knockdown of Bax or Puma, confirming the importance of Rheb as a FTI target. The relative importance of farnesylation targets may be dependent on cell

type, as inhibition of Ras and Bim upregulation was critical for the effects of FTIs in malignant lymphoid cells.

Together these observations suggest that Rheb farnesylation is an important target for the anticancer activity of FTIs in some tumours. However FTIs have off-target effects on other GTPases as well as other diverse proteins (Basso et al. 2006), and inhibition of farnesyltransferase can be partially overcome by the compensating activity of geranylgeranyltransferase (Whyte et al. 1997). Novel Rheb inhibitors could prove useful in tumors displaying aberrant Rheb activation and mTOR activity. Although small GTPase proteins have traditionally been viewed as intractable drug targets, recent progress with the development of Ras inhibitors (Maurer et al. 2012; Sun et al. 2012; Shima et al. 2013) has renewed interest in this endeavor.

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Part III
Rho Subfamily

Chapter 14
Classical Rho Proteins: Biochemistry of Molecular Switch Function and Regulation

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Abstract Rho family proteins are involved in an array of cellular processes by modulating cytoskeletal organization, transcription, and cell cycle progression. The signaling functions of Rho family proteins are based on the formation of distinctive protein–protein complexes with their regulators and effectors. A necessary precondition for such differential interactions is an intact molecular switch function, which is a hallmark of most members of the Rho family. Such classical Rho proteins cycle between an inactive GDP-bound state and an active GTP-bound state. They specifically interact via a consensus-binding sites called switch I and II with three structurally and functionally unrelated classes of regulatory proteins, such as guanine nucleotide dissociation inhibitors (GDIs), guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs). Extensive studies in the last 25 years have provided invaluable insights into the molecular mechanisms underlying regulation and signal transduction of the Rho family proteins. In this chapter, we will review common features of Rho protein regulations and highlight specific aspects of their structure–function relationships.

Keywords Effector • GAP • GDI • GEF • Rho GTPase • Switch region

Abbreviations

A	Aliphatic amino acid
Bcr	Breakpoint cluster region protein
C	Cysteine
CZH	CDM-zizimin homology

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Db1	Diffuse B-cell lymphoma
DH	Dbl homology domain
DHR1&2	DOCK-homology regions 1 and 2
ERM	Ezrin/radixin/moesin
GAPs	GTPase-activating proteins
GDIs	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
Gln	Glutamine
Gly	Glycine
GTP	Guanosine triphosphate
$p75^{NTR}$	Neurotrophin receptor p75
PAK1	p21-activated kinase 1
PH	Pleckstrin homology domain
PKA	Protein kinase A
PKC	Protein kinase C
P-loop	Phosphate-binding loop
X	Any amino acid

14.1 General Introduction

The role of the Rho family proteins as signaling molecules in controlling a large number of fundamental cellular processes is largely dependent on a functional molecular switch between a GDP-bound, inactive state and a GTP-bound, active state (Dvorsky and Ahmadian 2004). This function underlies a so-called GTPase cycle consisting of two different, slow biochemical reactions, the GDP/GTP exchange and the GTP hydrolysis. The cellular regulation of this cycle involves guanine nucleotide exchange factors (GEFs), which accelerate the intrinsic nucleotide exchange, and GTPase-activating proteins (GAPs), which stimulate the intrinsic GTP hydrolysis activity (Cherfils and Zeghouf 2013). Rho protein function requires both posttranslational modification by isoprenyl groups and membrane association. Therefore, Rho proteins underlie a third control mechanism that directs their membrane targeting to specific subcellular sites. This mechanism is achieved by the function of guanine nucleotide dissociation inhibitors (GDIs), which bind selectively to prenylated Rho proteins and control their cycle between cytosol and membrane. Activation of Rho proteins results in their association with effector molecules that subsequently activate a wide variety of downstream signaling cascades (Bishop and Hall 2000; Burridge and Wennerberg 2004), thereby regulating many important physiological and pathophysiological processes in eukaryotic cells (Etienne-Manneville and Hall 2002; Heasman and Ridley 2008) (see Chap. 16). In the following, the biochemical properties of the Rho proteins and their regulatory cycles will be described in detail. Figure 14.1 schematically summarizes the regulatory mechanism of the Rho proteins.

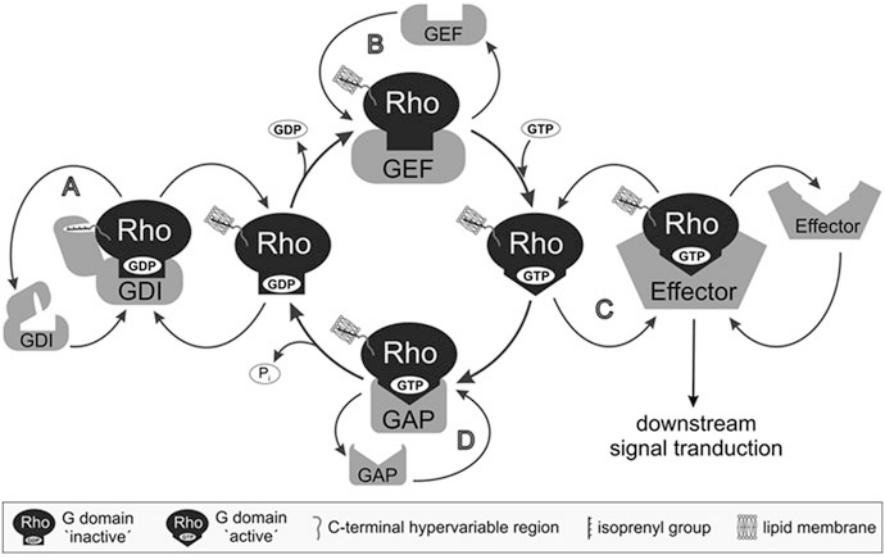

Fig. 14.1 Molecular principles of regulation and signaling of Rho Proteins. Most members of the Rho family act as molecular switches by cycling between an inactive, GDP-bound state and an active GTP-bound state. They interact specifically with four structurally and functionally unrelated classes of proteins: (**a**) In resting cells, guanine nucleotide dissociation inhibitors (GDIs) sequestrate the Rho proteins from the membrane by binding to the lipid anchor and create an inactivated cytosolic pool. (**b**) In stimulated cells, different classes of membrane receptors activate guanine nucleotide exchange factors (GEFs), which in turn activate their substrate Rho proteins by accelerating the slow intrinsic exchange of GDP for GTP and turn on the signal transduction. (**c**) The active GTP-bound Rho proteins interact with and activate their targets (the downstream effectors) to evoke a variety of intracellular responses. (**d**) GTPase-activating proteins (GAPs) negatively regulate the switch by stimulating the slow intrinsic GTP hydrolysis activity of the Rho proteins and turn off the signal transduction

14.2 Rho Family and the Molecular Switch Mechanism

Members of the GTP-binding proteins of the Rho family have emerged as key regulatory molecules that couple changes in the extracellular environment to intracellular signal transduction pathways. So far, 20 human members of the Rho family have been identified, which can be divided into six distinct subfamilies based on their sequence homology: (1) Rho (RhoA, RhoB, RhoC); (2) Rac (Rac1, Rac1b, Rac2, Rac3, RhoG); (3) Cdc42 (Cdc42, G25K, TC10, TCL, RhoU/Wrch1, RhoV/Chp); (4) RhoD (RhoD, Rif); (5) Rnd (Rnd1, Rnd2, Rnd3); (6) RhoH/TTF (Boureux et al. 2007; Jaiswal et al. 2013a, b; Wennerberg and Der 2004).

Rho family proteins are approximately 21–25 kDa in size typically containing a conserved GDP/GTP-binding domain (called G domain) and a C-terminal hypervariable region ending with a consensus sequence known as CAAX (C is cysteine, A is any aliphatic amino acid, and X is any amino acid). The G domain consists of five conserved sequence motifs (G1-G5) that are involved in nucleotide binding and

hydrolysis (Wittinghofer and Vetter 2011). In the cycle between the inactive and active states at least two regions of the protein, switch I (G2) and Switch II (G3), undergo structural rearrangements and transmit the "OFF" to "ON" signal to downstream effectors (Fig. 14.1) (Dvorsky and Ahmadian 2004). Subcellular localization of Rho proteins at different cellular membranes, that is known to be critical for their biological activity, is achieved by a series of posttranslational modifications at a cysteine residue in the CAAX motif, including isoprenylation (geranylgeranyl or farnesyl), endoproteolysis, and carboxyl methylation (Roberts et al. 2008).

A characteristic region of Rho family GTPases is the insert helix (amino acids 124–136, RhoA numbering) that may play a role in effector activation and downstream process (Thapar et al. 2002). Although the function of the insert helix has not been elucidated yet, it has been reported to be involved in the Rho-dependent activation of ROCK (Zong et al. 2001), phospholipase D (Walker and Brown 2002) and mDia (Lammers et al. 2008; Rose et al. 2005), and in the Rac-dependent activation of p67phox (Joneson and Bar-Sagi 1997; Karnoub et al. 2001; Nisimoto et al. 1997) and Plexin B1 (Bouguet-Bonnet and Buck 2008).

Although the majority of the Rho family proteins are remarkably inefficient GTP hydrolyzing enzymes, in quiescent cells they rest in an inactive state because the GTP hydrolysis is in average two orders of magnitude faster than the GDP/GTP exchange (Jaiswal et al. 2013a, b). Such different intrinsic activities provide the basis for a two-state molecular switch mechanism, which highly depends on the regulatory functions of GEFs and GAPs that directly control ON and OFF states of classical type of Rho proteins (Fig. 14.1). Eleven out of twenty members of the Rho family belong to these classical molecular switches, namely RhoA, RhoB, RhoC, Rac1, Rac2, Rac3, RhoG, Cdc42, G25K, TC10, and TCL (Jaiswal et al. 2013a, b).

The atypical Rho family members, including Rnd1, Rnd2, Rnd3, Rac1b, RhoH/TTF, Wrch1, RhoD, and Rif, have been proposed to accumulate in the GTP-bound form in cells due to various biochemical properties (Jaiswal et al. 2013a, b). Rnd1, Rnd2, Rnd3, and RhoH/TTF represent a completely distinct group of proteins within the Rho family (Riou et al. 2010; Troeger et al. 2013), as they do not share several conserved and essential amino acids, including Gly-12 (Rac1 numbering) in the G1 motif (also called phosphate-binding loop or P-loop) and Gln-61 (Rac1 numbering) in the G3 motif or switch II region. The role of these residues in GTP hydrolysis is well described for Ras oncogene in human cancers (Chaps. 6 and 7). Thus, they can be considered as GTPase-deficient Rho-related GTP-binding proteins (Fiegen et al. 2002; Garavini et al. 2002; Gu et al. 2005; Li et al. 2002) (see also Chap. 15). Another example is Rac1b, which is an alternative splice variant of Rac1 and contains a 19-amino acid insertion next to the switch II region (Jordan et al. 1999). Rac1b exhibits different biochemical properties as compared to the other Rac isoforms (Fiegen et al. 2004; Haeusler et al. 2006), including an accelerated GEF-independent GDP/GTP exchange and an impaired GTP hydrolysis (Fiegen et al. 2004). RhoD and Rif are involved in the regulation of actin dynamics (Fan and Mellor 2012; Gad and Aspenstrom 2010) and exhibit a strikingly faster nucleotide exchange than GTP hydrolysis similarly to Rac1b and thus persist

mainly in the active state under resting conditions (Jaiswal et al. 2013a, b). Wrch1, a Cdc42-like protein that has been reported to be a fast cycling protein (Shutes et al. 2006), resembles in this context Rac1b, RhoD, and Rif (Jaiswal et al. 2013a, b). These atypical members of the Rho family with their distinctive biochemical features do not follow the classical switch mechanism and may thus require additional forms of regulation.

14.3 Guanine Nucleotide Dissociation Inhibitors

Multiple functions have been originally described for the Rho-specific GDIs, including the inhibition of the GDP/GTP exchange (Hiraoka et al. 1992; Ohga et al. 1989), the intrinsic and GAP-stimulated GTP hydrolysis (Chuang et al. 1993; Hancock and Hall 1993; Hart et al. 1992), and the interaction with the downstream effectors (Pick et al. 1993). However, it is generally accepted that in resting cells, RhoGDIs target the isoprenyl anchor and sequester Rho proteins from their site of action at the membrane in the cytosol (Boulter and Garcia-Mata 2010; Garcia-Mata et al. 2011).

RhoGDIs undergo a high affinity interaction with the Rho proteins using an N-terminal regulatory arm contacting the switch regions and a C-terminal domain binding the isoprenyl group (Tnimov et al. 2012). In contrast to the large number of RhoGEFs and RhoGAPs, there are only three known RhoGDIs in human (DerMardirossian and Bokoch 2005). RhoGDI-1 (also called RhoGDIα) is ubiquitously expressed (Fukumoto et al. 1990), whereas RhoGDI-2 (also called RhoGDIβ, LyGDI, or D4GDI) is predominantly found in hematopoietic tissues and lymphocytes (Leonard et al. 1992; Scherle et al. 1993) and RhoGDI-3 (also called RhoGDIγ) in lung, brain, and testis (Adra et al. 1997; Zalcman et al. 1996).

Despite intensive research over the last two decades, the molecular basis by which GDI proteins associate and extract the Rho GTPases from the membrane remains to be investigated. The neurotrophin receptor p75 ($p75^{NTR}$) and ezrin/radixin/moesin (ERM) proteins have been proposed to displace the Rho proteins from the RhoGDI complex resulting in reassociation with the cell membrane (Takahashi et al. 1997; Yamashita and Tohyama 2003). Another regulatory mechanism is RhoGDI phosphorylation. RhoGDI has been shown to be phosphorylated by serine/threonine p21-activated kinase 1 (PAK1), protein kinase A (PKA), protein kinase C (PKC), and the tyrosine kinase Src, thereby decreasing the ability of RhoGDI to form a complex with the Rho proteins, including RhoA, Rac1, and Cdc42 (DerMardirossian et al. 2004, 2006).

14.4 Guanine Nucleotide Exchange Factors

GEFs are able to selectively bind to their respective Rho proteins and accelerate the exchange of tightly bound GDP for GTP. A common mechanism utilized by GEFs is to strongly reduce the affinity of the bound GDP, leading to its displacement and the subsequent association with GTP (Cherfils and Chardin 1999; Guo et al. 2005). This reaction involves several stages, including an intermediate state of the GEF in the complex with the nucleotide-free Rho protein. This intermediate does not accumulate in the cell and rapidly dissociates because of the high intracellular GTP concentration leading to the formation of the active Rho·GTP complex. The main reason therefore is that the binding affinity of nucleotide-free Rho protein is significantly higher for GTP than for the GEF proteins (Cherfils and Chardin 1999; Hutchinson and Eccleston 2000). Cellular activation of the Rho proteins and their cellular signaling can be selectively uncoupled from the GEFs by overexpressing dominant negative mutants of the Rho proteins (e.g., threonine 17 in Rac1 and Cdc42 or threonine 19 in RhoA to asparagine) (Heasman and Ridley 2008). Such mutations decrease the affinity of the Rho protein to nucleotide resulting in a so-called dominant negative behavior (Rossman et al. 2002). As a consequence, dominant negative mutants form a tight complex with their cognate GEFs and thus prevent them from activating the endogenous Rho proteins.

RhoGEFs of the diffuse B-cell lymphoma (Dbl) family directly activate the proteins of the Rho family (Cook et al. 2013; Jaiswal et al. 2013a, b). The prototype of this GEF family is the Dbl protein, which was isolated as an oncogenic product from diffuse B-cell lymphoma cells in an oncogene screen (Eva et al. 1988; Srivastava et al. 1986), and has been later reported to act on Cdc42 (Hart et al. 1991). The Dbl family consists of 74 members in human (Jaiswal et al. 2013a, b) with evolutionary conserved orthologs in fly (23 members), yeast (6 members), worm (18 members) (Schmidt and Hall 2002; Venter et al. 2001), and slime mold (45 members) (Vlahou and Rivero 2006). Human Dbl family proteins have recently been grouped into functionally distinct categories based on both their catalytic efficiencies and their sequence–structure relationship (Jaiswal et al. 2013a, b). The members of the Dbl family are characterized by a unique Dbl homology (DH) domain (Aittaleb et al. 2010; Erickson and Cerione 2004; Hoffman and Cerione 2002; Jaiswal et al. 2011; Viaud et al. 2012). The DH domain is a highly efficient catalytic machine (Rossman et al. 2005) that is able to accelerate the nucleotide exchange of Rho proteins up to 10^7-fold (Jaiswal et al. 2011, 2013a, b), as efficiently as the RanGEF RCC1 (Klebe et al. 1995) and *Salmonella typhimurium* effector SopE (see below) (Bulgin et al. 2010; Rudolph et al. 1999). The DH domain is often preceded by a pleckstrin homology (PH) domain indicating an essential and conserved function. A model for PH domain-assisted nucleotide exchange has been proposed for some GEFs, such as Dbl, Dbs, and Trio (Rossman et al. 2005). Herein the PH domain serves multiple roles in signaling events anchoring GEFs to the membrane (via phosphoinositides) and directing them

towards their interacting GTPases which are already localized to the membrane (Rossman et al. 2005).

In addition to the DH-PH tandem, Dbl family proteins are highly diverse and contain additional domains with different functions, including SH2, SH3, CH, RGS, PDZ, and IQ domains for interaction with other proteins; BAR, PH FYVE, C1, and C2 domains for interaction with membrane lipids; and other functional domains like Ser/Thr kinase, RasGEF, RhoGAP, and RanGEF (Cook et al. 2013). These additional domains have been implicated in autoregulation, subcellular localization, and connection to upstream signals (Dubash et al. 2007; Rossman et al. 2005). Spatiotemporal regulation of the Dbl proteins has been implicated to specifically initiate activation of substrate Rho proteins (Jaiswal et al. 2013a, b) and to control a broad spectrum of normal and pathological cellular functions (Dubash et al. 2007; Hall and Lalli 2010; Mulinari and Hacker 2010; Mulloy et al. 2010; Schmidt and Hall 2002). Thus, it is evident that members of the Dbl protein family are attractive therapeutic targets for a variety of diseases (Bos et al. 2007; Loirand et al. 2008; Vigil et al. 2010).

Apart from conventional Dbl family RhoGEFs there are two additional proteins families, which do not share any sequence and structural similarity with each other. The dedicator of cytokinesis (DOCK) or CDM-zizimin homology (CZH) family RhoGEFs are characterized by two conserved regions, known as the DOCK-homology regions 1 and 2 (DHR1 and DHR2) domains (Meller et al. 2005; Rittinger 2009). This type of GEFs employs their DHR2 domain to activate specially Rac and Cdc42 proteins (Meller et al. 2005). Another Rho protein-specific GEF family, represented by the SopE/WxxxE-type exchange factors, is classified as type III effector proteins of bacterial pathogens (Bulgin et al. 2010). They mimic functionally, but not structurally, eukaryotic GEFs by efficiently activating Rac1 and Cdc42 and thus induce "the trigger mechanism of cell entry" (see Chap. 4) (Bulgin et al. 2010; Rudolph et al. 1999).

14.5 GTPase-Activating Proteins

Hydrolysis of the bound GTP is the timing mechanism that terminates signal transduction of the Rho family proteins and returns them to their GDP-bound inactive state (Jaiswal et al. 2012). The intrinsic GTP hydrolysis (GTPase) reaction is usually slow, but can be stimulated by several orders of magnitude through interaction with Rho-specific GAPs (Eberth et al. 2005; Fidyk and Cerione 2002; Zhang and Zheng 1998). The RhoGAP family is defined by the presence of a conserved catalytic GAP domain which is sufficient for the interaction with Rho proteins and mediating accelerated catalysis (Scheffzek and Ahmadian 2005). The GAP domain supplies a conserved arginine residue, termed "arginine finger", into the GTP-binding site of the cognate Rho protein, in order to stabilize the transition state and catalyze the GTP hydrolysis reaction (Nassar et al. 1998; Rittinger et al. 1997). A similar mechanism is utilized by other small GTP-binding proteins

(Scheffzek and Ahmadian 2005), including Ras, Rab, and Arf, although the sequence and folding of the respective GAP families are different (Ismail et al. 2010; Pan et al. 2006; Scheffzek et al. 1997). Masking the catalytic arginine finger is an elegant mechanism for the inhibition of the GAP activity. This has been recently shown for the tumor suppressor protein DLC1, a RhoGAP, which is competitively and selectively inhibited by the SH3 domain of p120RasGAP (Jaiswal et al. 2014).

RhoGAP insensitivity can be achieved by the substitution of either the catalytic arginine of the GAP domain (Fidyk and Cerione 2002; Graham et al. 1999) or amino acids critical for the GTP hydrolysis in Rho proteins, e.g., Glycine 12 and Glutamine 61 in Rac1 and Cdc42 or Glycine 14 and Glutamine 63 in RhoA, which are known as the constitutive active mutants (Ahmadian et al. 1997; Graham et al. 1999). Most remarkably, a similar mechanistic strategy has been mimicked by bacterial GAPs (see Chap. 4), such as the *Salmonella typhimurium* virulence factor SptP, the *Pseudomonas aeruginosa* cytotoxin ExoS, and *Yersinia pestis* YopE, even though they do not share any sequence or structural similarity to eukaryotic RhoGAP domains (Evdokimov et al. 2002; Stebbins and Galan 2000; Wurtele et al. 2001).

The first RhoGAP, p50RhoGAP, was identified by biochemical analysis of human spleen cell extracts in the presence of recombinant RhoA (Garrett et al. 1989). Since then more than 80 RhoGAP containing proteins have been identified in eukaryotes, ranging from yeast to human (Lancaster et al. 1994; Moon and Zheng 2003). The RhoGAP domain (also known as Bcr-homology, BH domain) containing proteins are present throughout the genome and rarely cluster in specific chromosomal regions (Peck et al. 2002). The majority of the RhoGAP family members are frequently accompanied by several other functional domains and motifs implicated in tight regulation and membrane targeting (Eberth et al. 2009; Moon and Zheng 2003; Tcherkezian and Lamarche-Vane 2007). Numerous mechanisms have been shown to affect the specificity and the catalytic activity of the RhoGAPs, e.g., intramolecular autoinhibition (Eberth et al. 2009), posttranslational modification (Minoshima et al. 2003), and regulation by interaction with lipid membrane (Ligeti et al. 2004) and proteins (Yang et al. 2009).

14.6 Conclusions

Abnormal activation of Rho proteins has been shown to play a crucial role in cancer, infectious and cognitive disorders, and cardiovascular diseases. However, several tasks have to be yet accomplished in order to understand the complexity of Rho proteins signaling: (1) The Rho family comprises of 20 signaling proteins, of which only RhoA, Rac1, and Cdc42 have been comprehensively studied so far. The functions of the other less-characterized members of this protein family await detailed investigation. (2) Despite intensive research over the last two decades, the mechanisms by which RhoGDIs associate and extract the Rho proteins from the

membrane and the factors displacing the Rho protein from the complex with RhoGDI remain to be elucidated. (3) For the regulation of the 22 Rho proteins, a tremendous number of their regulatory proteins (>74 GEFs and >80 GAPs) exist in the human genome. How these regulators selectively recognize their Rho protein targets is not well understood and majority of GEFs and GAPs in humans so far remain uncharacterized. (4) Most of the GEFs and GAPs themselves need to be regulated and require activation through the relief of autoinhibitory elements (Chow et al. 2013; Eberth et al. 2009; Jaiswal et al. 2011; Mitin et al. 2007; Moskwa et al. 2005; Rojas et al. 2007; Yohe et al. 2008). With a few exceptions (Cherfils and Zeghouf 2013; Mayer et al. 2013), it is conceptually still unclear how such autoregulatory mechanisms are operated. A better understanding of the specificity and the mode of action of these regulatory proteins is not only fundamentally important for many aspects of biology but is also a master key for the development of drugs against a variety of diseases caused by aberrant functions of Rho proteins.

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Chapter 15
Atypical Rho Family Members

Barbara Borda-d'Agua, Elvira Infante, Philippe Riou, Virginia Tajadura, and Anne J. Ridley

Abstract Of the 20 Rho GTP-binding proteins in humans, 8 have atypical properties, which are also unusual within the Ras superfamily. These atypical proteins fall into four subfamilies: RhoU/RhoV, Rnd1/Rnd2/Rnd3, RhoH and RhoBTB1/RhoBTB2. These proteins are known or predicted to be predominantly GTP-bound in cells, because of changes in their ability to exchange GDP for GTP or to hydrolyse GTP. Apart from RhoH, they also have N-terminal and C-terminal extensions that give them unique interacting partners and functions. For example, RhoU can bind SH3 domain-containing proteins, Rnd proteins can bind to 14-3-3 proteins, and RhoBTB proteins can interact via their BTB domains with cullin-3, which is involved in proteasomal degradation. The proteins have been implicated in diverse functions, including cell adhesion and migration, vesicle trafficking and cell proliferation.

Keywords RhoU/RhoV • RhoH • Rnd proteins • RhoBTB proteins • Cytoskeleton • Cell cycle

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15.1 Introduction

Twelve of the 20 Rho family GTP-binding proteins in humans cycle between a GTP-bound active form and a GDP-bound inactive form and are regulated by GEFs, GAPs and in some cases RhoGDIs. The other 8 family members are considered non-classical because they do not appear to be regulated in the same way, and also have unique N-terminal and C-terminal extensions to the standard G-domain. These atypical Rho members fall into two categories. First, RhoU and RhoV have a high intrinsic nucleotide exchange rate in vitro, which means they are mostly GTP-bound in cells and unlikely to be regulated by GEFs. Second, the Rnd (Rnd1, Rnd2 and Rnd3/RhoE), RhoH and RhoBTB proteins (RhoBTB1 and RhoBTB2) have amino acid substitutions that prevent them from hydrolysing GTP. These amino acid substitutions are at the equivalent amino acids that are mutated in Ras oncogenes, and known to prevent/reduce GTP hydrolysis, so that the proteins are constitutively GTP-bound. This means that they are regulated in different ways to the GTP/GDP switch.

Of the non-classical Rho proteins, Rnd3 was the first to be discovered, when it was cloned as a p190RhoGAP-interacting protein in 1996 (Foster et al. 1996). Here we describe the structure and function of the eight non-classical Rho proteins.

15.2 RhoU and RhoV

15.2.1 Evolution and Structure

RhoV (also known as Chp) was first discovered as a p21-activated kinase-2 (PAK2)-interacting protein in 1998 (Aronheim et al. 1998), whereas RhoU (also known as Wrch1) was identified as a Wnt-inducible gene in 2001 (Tao et al. 2001). RhoU and RhoV form a subfamily within the Rho family and are most closely related to Cdc42: RhoU and RhoV share 55.4 % of total amino acid identity and 43.5 % with Cdc42. A *RhoU*/*RhoV* gene first appeared in evolution in Coelomates, including *Drosophila melanogaster* and *C. elegans*, and both genes are highly conserved across vertebrates (Boureux et al. 2007).

RhoU and RhoV proteins have a high intrinsic guanine nucleotide exchange rate in vitro and are therefore likely to be constitutively GTP-bound in cells (Chenette et al. 2005; Saras et al. 2004). Both RhoU and RhoV have an N-terminal and C-terminal extension compared to Rac and Cdc42. In particular the N-terminal extension of RhoU is proline-rich and has three potential SH3-binding PxxP motifs, one of which can bind the SH3-containing adaptor proteins Grb2 and Nck (Risse et al. 2013).

15.2.2 Regulation

RhoU was first isolated and cloned as a Wnt-inducible gene. The role of Wnt1 signalling in development and tumorigenesis is mediated by its target genes, and Wnt1 acts through RhoU to induce transformation of mouse mammary epithelial cells (Tao et al. 2001). Subsequent analysis in vivo showed that upregulation of canonical Wnt signalling increased expression of *RhoU* in mouse embryos (Loebel et al. 2011). Conversely, the transcription factor Sox17 reduced *RhoU* expression (Loebel et al. 2011). Expression of both *RhoU* and *RhoV* is increased in T-acute lymphoblastic leukaemia (T-ALL) compared to normal T cells, and *RhoU* was shown to be induced by Notch1, which is frequently mutated in T-ALL (Bhavsar et al. 2013).

Unlike other Rho family members, RhoU and RhoV do not undergo isoprenylation but are modified by palmitoylation, which is the post-translational covalent addition of the 16-carbon fatty acid palmitate (Berzat et al. 2005; Chenette et al. 2005). Palmitoylation is a reversible process, which allows proteins to associate transiently with membranes, regulating their localisation and trafficking (Baekkeskov and Kanaani 2009). RhoU and RhoV localisation are not affected by inhibitors of protein prenylation but by inhibitors of protein palmitoylation (Berzat et al. 2005; Chenette et al. 2005). RhoU localisation to the plasma membrane and biological activity is reduced by Src-induced tyrosine phosphorylation at Y254 near the C-terminus (Fig. 15.1) (Alan et al. 2010).

15.2.3 Known Binding Partners

Several binding partners for RhoU have been identified (Fig. 15.1), but so far RhoV has only been reported to bind to PAKs. RhoU and RhoV are both able to bind to and activate several of the six PAK family members, which are well known as effectors of Rac and Cdc42 (Aronheim et al. 1998; Tao et al. 2001; Weisz Hubsman et al. 2007). PAKs regulate cell migration and invasion and their overexpression and/or hyperactivation is observed in several human tumours (Dummler et al. 2009). Overexpression of RhoV induces lamellipodia possibly through interaction with PAK2 (Aronheim et al. 1998). N-terminal deletion of RhoU enhances its ability to bind PAK1 (Shutes et al. 2004). RhoV overexpression induces downregulation of PAK1 (Weisz Hubsman et al. 2007).

Although RhoU and RhoV do not appear to be regulated by GEFs, ARHGAP30 and the closely related CdGAP interact with RhoU (Naji et al. 2011). RhoU was found to bind ARHGAP30 and CdGAP in co-immunoprecipitation assays in fibroblasts. Overexpression of ARHGAP30 phenocopied RhoU in inducing filopodium formation and stress fibre disassembly, suggesting a role downstream of RhoU (Naji et al. 2011).

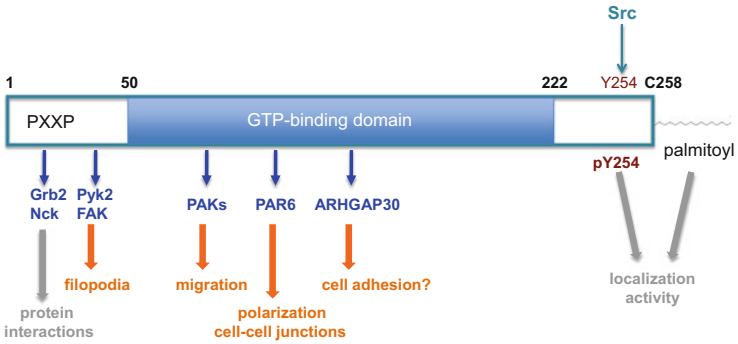

Fig. 15.1 Domain organisation and interacting partners of RhoU. RhoU has an N-terminal and C-terminal extension on each side of the GTP-binding domain. The C-terminus is palmitoylated. The tyrosine phosphorylation site near the C-terminus of RhoU is indicated. Known or possible functions of interacting partners of RhoU are shown

The proline-rich region at the N-terminus of RhoU binds to SH3 domains in several proteins, including the second and third SH3 domains of Nck2 (Saras et al. 2004) and the adaptor protein Grb2, which increases RhoU activity (Shutes et al. 2004). Interestingly, after epidermal growth factor (EGF) stimulation RhoU colocalises with the EGF receptor on endosomes in a Grb2-dependent manner in pancreatic cancer cells (Zhang et al. 2011).

RhoU also binds the non-receptor tyrosine kinase Pyk2. The interaction requires RhoU to be in a GTP-bound form and also involves the N-terminal proline-rich extension (Ruusala and Aspenstrom 2008). The interaction depends on the presence and activity of Src and has a role in filopodium formation in fibroblasts. In H1299 non-small cell lung cancer cells RhoU relocates to the plasma membrane upon serum stimulation. Serum stimulation induces Src-mediated tyrosine phosphorylation of RhoU on Tyr254. This phosphorylation decreases GTPase activity and the ability of RhoU to interact with downstream effectors (Alan et al. 2010).

Finally, RhoU was shown to bind to the scaffolding protein Par6 in epithelial cells (Brady et al. 2009), which is best known for its role in regulating polarity downstream of Cdc42 (Chen and Zhang 2013). This interaction was shown to affect epithelial cell TJ assembly and actin organisation.

15.2.4 Functions

RhoU and RhoV have been implicated in adhesion and migration, as well as cell proliferation and transformation. For example, overexpression of RhoU or RhoV has been shown to stimulate lamellipodial or filopodial extensions and/or integrin-based focal adhesions (Aronheim et al. 1998; Aspenstrom et al. 2004; Chuang et al. 2007). In osteoclasts, RhoU localises to podosomes and influences integrin

signalling (Brazier et al. 2009). In HeLa cells RhoU localises to focal adhesions, and RhoU depletion increased focal adhesion formation and inhibited cell migration (Chuang et al. 2007). On the other hand, RhoU depletion in T-ALL cells reduced adhesion to fibronectin and also migration. Notch1 depletion also decreased the adhesion and migration of T-ALL cell lines suggesting that Notch1 could affect these processes through RhoU (Bhavsar et al. 2013). Similarly, cranial neural crest cells were shown to adhere poorly to fibronectin when depleted of RhoU and had a rounded morphology (Fort et al. 2011). RhoU therefore plays an important role in adhesion of cells to extracellular matrix, which impacts on cell migration.

Both RhoU and RhoV have been shown to be important for neural crest development in *Xenopus laevis*. RhoV is required for the differentiation of neural crest cells, while RhoU is necessary for neural crest cell migration in vivo (Fort et al. 2011). *RhoU* is highly expressed in the developing foregut endoderm in embryonic mice (Loebel et al. 2011). In embryonic stem (ES) cells in vitro, RhoU knockdown reduced endoderm differentiation but enhanced mesodermal differentiation. Embryos derived from these ES cells showed multiple defects, including disrupted epithelial morphology of the gut endoderm associated with altered F-actin distribution.

RhoU or RhoV overexpression induces cell proliferation and transformation in fibroblasts (Shutes et al. 2004; Chenette et al. 2005; Tao et al. 2001). Deletion of the N-terminal extension of RhoU or RhoV enhanced their transforming activity suggesting a negative regulatory role of this domain. Moreover an intact C-terminus is required for transformation by RhoV (Chenette et al. 2005).

In contrast to the ability of RhoV to induce fibroblast transformation, in PC12 neuronal cells RhoV overexpression induced apoptosis and activation of JNK signalling via both death receptor-mediated and mitochondrial apoptotic pathways as determined by caspase-8 and caspase-9 activation (Shepelev et al. 2011). This suggests a possible role of RhoV in regulating apoptosis in a JNK-dependent manner.

15.3 Rnd Proteins

15.3.1 Evolution and Structure

There are three *Rnd* genes in humans: *Rnd1*, *Rnd2* and *Rnd3* (also known as *RhoE*). Rnd3 was the first member of the three to be identified, as a binding partner for the RhoA GAP, p190RhoGAP (Foster et al. 1996). *Rnd1* and *Rnd2* cDNAs were subsequently cloned in a screen for genes containing sequences with homology to the effector domain of RhoA (Nobes et al. 1998).

The Rnd proteins form a distinct branch of the Rho family, only slightly more closely related to RhoA, RhoB and RhoC than any other Rho family member

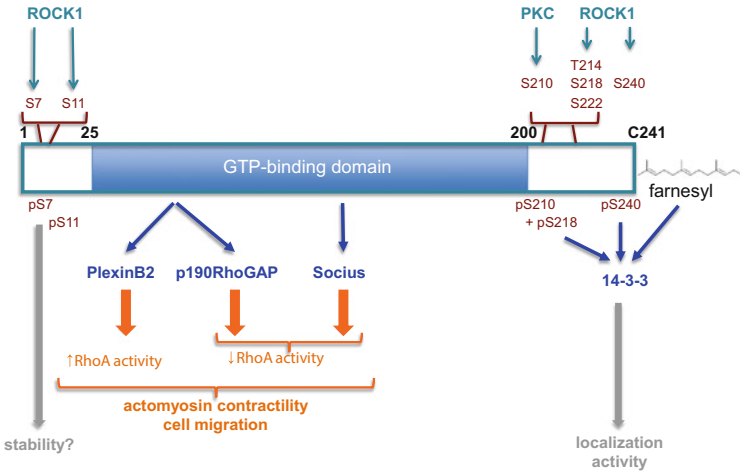

Fig. 15.2 Domain organisation and interacting partners of Rnd3. Rnd3 has an N-terminal and C-terminal extension on each side of the GTP-binding domain, which contain seven Ser/Thr phosphorylation sites. Kinases phosphorylating these sites are indicated at the *top*. The C-terminus is farnesylated. Known or possible functions of interacting partners of Rnd3 are shown

(Boureux et al. 2007). The three human Rnd proteins share 54–63 % amino acid sequence identity pairwise (Nobes et al. 1998). Rnd proteins have characteristic C-terminal extensions of 30–32 amino acids, specific to each protein, and Rnd1 and Rnd3 also have N-terminal extensions of 8 and 18 amino acids, respectively, compared to other Rho family members (Fig. 15.2). The Rnd subfamily appeared relatively late in evolution and are only present in vertebrates (Boureux et al. 2007).

Rnd proteins do not hydrolyse GTP, because they have substitutions in specific amino acids that are critical for GTPase activity: they naturally have Ser or Val at the position that corresponds to Ras Gly12 and Ser at the positions that correspond to Ras Ala59 and Ras Gln61 (Nobes et al. 1998). Any one of these substitutions in Ras decreases its intrinsic GTPase rate and prevents GAP-mediated GTPase stimulation, leading to it being constitutively GTP-bound (Fernandez-Medarde and Santos 2011). Indeed, Rnd3 is constitutively GTP-bound within cells (Foster et al. 1996). Furthermore, Rnd1 and Rnd3 have very low affinity for GDP (Guasch et al. 1998; Nobes et al. 1998). The crystal structure of the core domain of Rnd3 is highly similar to that of RhoA-GTP, but has critical differences that explain why it does not hydrolyse GTP and suggest why its affinity for GDP is very low (Garavini et al. 2002; Fiegen et al. 2002). The co-crystal structure of Rnd3 with the N-terminal kinase domain of ROCK1 shows how its N- and C-terminal extensions can be substrates for ROCK1 (see below) (Komander et al. 2008). The crystal structure of Rnd1 is available (PDB 2CLS), and it has also been crystallised together with Rho-binding regions of two plexin receptors, Plexin B1 and Plexin A2 (Wang et al. 2011a), which are potential targets for Rnd function (see below).

15.3.2 Regulation

The expression of the three *Rnd* genes is highly regulated, most particularly *Rnd3* expression, for which changes have been reported in response to diverse stimuli and conditions (Riou et al. 2010). *Rnd* genes show highly specific gene expression patterns during development, in particular in the brain. For example, *Rnd2* expression is induced in the developing cerebral cortex by the transcription factor neurogenin2 (Heng et al. 2008), but is suppressed by the transcription factors COUP-TFI and RP58 in different regions of the cortex (Alfano et al. 2011; Heng et al. 2013). Together, these transcription factors probably act to fine-tune Rnd2 levels to regulate cortical neuron migration. Another proneural factor, Ascl1, was also found to control neuronal migration by inducing the expression of *Rnd3* (Pacary et al. 2011). *Rnd3* is also downregulated by miRNAs in some cancer cell lines and colorectal cancer (Xia et al. 2010; Luo et al. 2012), which appears to be linked to Rnd3 function in inhibiting proliferation rather than migration (see below).

Rnd proteins are post-translationally modified by addition of a 15-carbon farnesyl group at the C-terminus (Foster et al. 1996; Roberts et al. 2008). Since Rnd proteins do not cycle between active GTP-bound and inactive GDP-bound forms, they are not controlled by GEFs and GAPs. Instead, the main mechanism for regulating their function is via phosphorylation. Rnd3 is phosphorylated on its N- and C-terminal extensions by ROCK1 and PKC, inducing its translocation from membranes to the cytosol (Riento et al. 2005; Madigan et al. 2009). Phosphorylated Rnd3 as well as Rnd1 and Rnd2 interact with the dimeric phosphoSer/Thr-binding protein 14-3-3; this interaction requires Rnd farnesylation as well as phosphorylation of a Ser adjacent to the farnesyl group (Riou et al. 2013). This phospho-dependent interaction with 14-3-3 inhibits Rnd3 interaction with effectors and hence its function, by inducing its translocation from the plasma membrane to the cytosol.

As well as phosphorylation, Rnd3 protein levels are regulated by proteasomal degradation. During cell cycle progression, Rnd3 accumulates during G1 and then levels rapidly decrease at the G1/S phase transition. Rnd3 interacts with and is targeted for degradation by the F-box protein Skp2, a substrate receptor that links its substrates to the SCF Cullin-1 E3 ligase complex (Lonjedo et al. 2013).

15.3.3 Binding Partners

Rnd3 was initially identified as an interacting partner for p190RhoGAP (Foster et al. 1996) Fig. 15.2), which subsequently was reported to bind to all three Rnd proteins (Wennerberg et al. 2003). Rnd3 but not Rnd1 or Rnd2 binds to the serine/threonine kinase ROCK1, and is regulated by ROCK1 phosphorylation (Riento et al. 2005; Komander et al. 2008). Indeed, phosphorylation of Rnd proteins leads to

their interaction with 14-3-3 proteins, which inhibit Rnd signalling (Riou et al. 2013) (Fig. 15.2). All three Rnd proteins have been shown to interact with plexins, which are transmembrane receptors for Semaphorin ligands (Hota and Buck 2012). Plexins have a Rho-binding domain in their intracellular region, inserted within a R-Ras/Rap GAP domain.

A number of other interacting partners of Rnd proteins have been identified (Riou et al. 2010), several of which were identified in yeast two hybrid screens. So far relatively little is known about their functions in Rnd signalling.

15.3.4 Functions

The best-known function of Rnd proteins is to regulate actomyosin contractility and hence cell migration (Fig. 15.2); Rnd3 also inhibits cell cycle progression and proliferation (Riou et al. 2010). Rnd1 and Rnd3 induce loss of stress fibres and cell rounding (hence the name Rnd) in a variety of cell types. Rnd3 also stimulates neurite extension in PC12 neuronal cells and axonal outgrowth of hippocampal neurons (Talens-Visconti et al. 2010; Peris et al. 2012). One way in which Rnd proteins regulate cell morphology is by inhibiting the Rho/ROCK signalling pathway by binding to and stimulating the GAP activity of p190RhoGAP. This reduces the level of active GTP-bound RhoA, hence decreasing actomyosin contractility (Wennerberg et al. 2003). Surprisingly, in endothelial cells Rnd3 induces stress fibres through RhoB (Gottesbuhren et al. 2013).

Rnd3 affects cell migration in a variety of cell types (Riou et al. 2010). For example, in osteoclasts Rnd3 is required for both migration and podosome turnover, through its ability to activate the actin regulator cofilin (Georgess et al. 2014). Rnd3 depletion reduces bone resorption by osteoclasts through its effects on podosomes. In the developing mouse cortex, Rnd3 contributes to neuronal migration (Pacary et al. 2011). *Rnd3*-depleted mice have strongly impaired innervation of muscles and hence profound motor impairment (Mocholi et al. 2011), consistent with defective neuronal migration and axon outgrowth.

Unlike Rnd1 and Rnd3, Rnd2 appears to induce rather than inhibit stress fibres and actomyosin contractility in cultured cells. In HeLa and PC12 neuronal cells, Rnd2 acts through its target pragmin and RhoA to induce contraction (Tanaka et al. 2006), whereas in endothelial cells it increases stress fibres by inducing RhoB expression (Gottesbuhren et al. 2013). By contrast, Rnd2 has been found to inhibit RhoA activity in neurons of the mouse cortex in vivo and to be important for neuronal migration (Pacary et al. 2011). The cytoskeletal effects of Rnd proteins are therefore dependent on cell type and context.

As well as affecting the actin cytoskeleton and cell migration, Rnd3 inhibits proliferation in fibroblasts in vitro (Villalonga et al. 2004) and neuronal progenitors in the embryonic mouse cortex in vivo (Pacary et al. 2013). Rnd3 inhibits cell cycle progression by inhibiting translation of CyclinD1 and the transcription factor Myc, most probably by mediating phosphorylation of the translational repressor 4E-BP1

(Villalonga et al. 2009; Villalonga et al. 2004). Whether other Rnd proteins affect cell cycle progression is not yet known.

15.4 RhoH

15.4.1 Evolution and Structure

RhoH, originally known as *TTF* (translocation three to four), was discovered as a fusion transcript with the oncogene *BCL6*/*LAZ3* in B-cell non-Hodgkin lymphoma (NHL). The translocation results in the exchange of the 5′ regulatory sequences between *BCL6* and *RhoH*, that in some cases affect the expression of both transcripts (Preudhomme et al. 2000).

RhoH is only found in vertebrates, and its phylogenetic origin is uncertain. It is possible that *RhoH* was gained by horizontal gene transfer from a distant species (Boureux et al. 2007). This hypothesis is supported by the absence of introns in the *RhoH* coding sequence. RhoH shows low identity to other members of the Rho family: less than 45 % identity to the closest member, Cdc42. RhoH also has a poorly conserved Rho family-specific insert, which is normally a 13 amino acid motif involved in binding to regulators and effectors. Like the Rnd proteins, RhoH has amino acid substitutions in the residues Gly12 and Gln61 (Ras numbering), involved in GTP binding and hydrolysis, and thus it is GTPase-deficient and permanently GTP-bound (Li et al. 2002).

15.4.2 Regulation

The expression of *RhoH* was reported to be restricted to cell lines of the lymphoid lineage, and in tissues, *RhoH* is most highly expressed in the thymus, spleen and bone marrow (BM) (Li et al. 2002). *RhoH* is also expressed in haematopoietic progenitor cells (HPCs) and to a lesser extent in myeloid and erythroid human lineages (Gu et al. 2005). Different transcription start sites along with alternative splicing of the 5′ exons of *RhoH* produce transcripts with different 5′UTRs (Lahousse et al. 2004). Specific expression of some of these transcripts is observed in B, T and other haematopoietic cell lines, which might lead to differences in protein expression. *RhoH* expression is reduced by T cell receptor (TCR) activation or by treatment of T cells with PMA, a PKC activator (Li et al. 2002). The 5′ end of *RhoH* is targeted by aberrant somatic hypermutation in lymphomas, but whether this alters the expression levels of RhoH is not clear (Lahousse et al. 2004; Preudhomme et al. 2000; Fueller and Kubatzky 2008).

The half-life of RhoH protein is less than 3 h in Jurkat T cells (Troeger et al. 2013). A motif (LFSINE) at the C-terminus of RhoH is involved in targeting

RhoH for lysosomal degradation. Deletion of the LFSINE motif results in RhoH protein accumulation but does not affect RhoH localisation or function (Troeger et al. 2013).

RhoH can be prenylated at the C-terminus by the addition of either a farnesyl or a geranylgeranyl lipid moiety in vitro (Fueller and Kubatzky 2008), and only treatment with both farnesyl and geranylgeranyl transferase inhibitors causes a complete translocation of RhoH from the plasma membrane to the cytosol (Roberts et al. 2008).

RhoH function is also regulated by tyrosine phosphorylation of an ITAM (immunoreceptor tyrosine-based activation motif)-like motif, which overlaps with the Switch II region (Gu et al. 2006). RhoH is tyrosine phosphorylated upon activation of the TCR, promoting interaction with the tyrosine kinase ZAP70 (see below).

15.4.3 Binding Partners

Among the known regulators and effectors of Rho GTPases, RhoH binds in vitro to the three different RhoGDIs (α, β and γ) (Li et al. 2002) and the p21-activated kinases, PAK1, 2, 5 and 6 (Wu and Frost 2006; Wang et al. 2010). RhoH activates PAK1 in Jurkat cells stimulated with high levels of SDF1α, a chemoattractant for T cells (Wang et al. 2010). RhoH has Val42 and Val44, which are necessary in Cdc42 for PAK1 binding (Morreale et al. 2000; Shin et al. 2013), but if these residues are important for RhoH-PAK1 interaction is not known.

RhoH also interacts with the T cell tyrosine kinase Zap70 following TCR engagement (Fig. 15.3). The binding appears to involve tyrosine phosphorylation of the RhoH ITAM-like motif, which then interacts with Zap70 SH2 domains (Gu et al. 2006). In resting T cells, RhoH binds the tyrosine kinase Lck and helps maintaining the kinase in an inactive state. TCR engagement induces release of RhoH from Lck and Lck activation (Wang et al. 2011b).

15.4.4 Functions

RhoH function has predominantly been studied in T cells, in which it affects both chemotaxis and TCR signalling (Fig. 15.3), at least in part by affecting the activity of other GTPases.

RhoH overexpression inhibits the Rac1/RhoA- and Rac1/Cdc42-mediated activation of NFκB and p38 respectively (Li et al. 2002). In HPCs, RhoH overexpression inhibits localisation of Rac1 at the plasma membrane and the consequent actin polymerisation, whereas *RhoH* $^{-/-}$ HPCs have higher Rac1-GTP levels than control cells (Chae et al. 2008). The increase in Rac1 levels in *RhoH*$^{-/-}$ HPCs or RhoH-depleted cells promotes proliferation and chemotaxis in

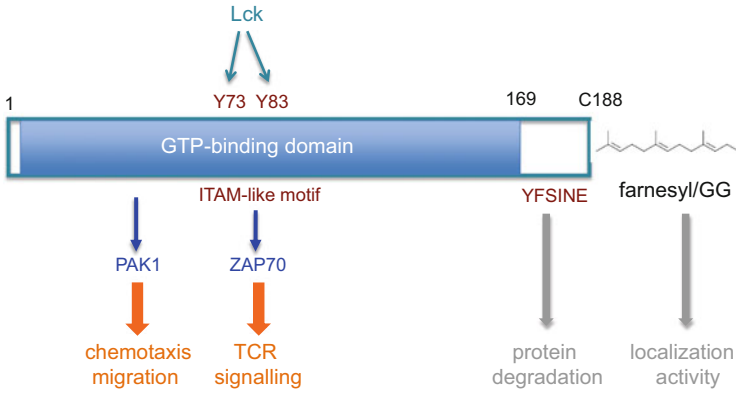

Fig. 15.3 Domain organisation and interacting partners of RhoH. RhoH has an ITAM-like motif within the GTP-binding domain, which can be tyrosine phosphorylated, and a C-terminal sequence involved in targeting RhoH for degradation (YFSINE). The C-terminus can be farnesylated or geranylgeranylated (GG). Known or possible functions of interacting partners of RhoH are shown

the presence of the chemokine SDF1α, a process that involves PAK1 and RhoH (Wang et al. 2010). Whether RhoH regulates Rac1 levels through PAK1 or other effectors remains unclear. RhoH overexpression also inhibits chemokine-induced Rap1 activation (Baker et al. 2012), which reduces T-cell adhesion by the lymphocyte-specific integrin LFA-1 (αLβ2), thereby impairing chemokine-induced chemotaxis (Cherry et al. 2004; Baker et al. 2012). In contrast, mouse *RhoH*$^{-/-}$ thymocytes had reduced adhesion to ICAM-1, the major ligand for LFA-1 (Dorn et al. 2007), and RhoH increases TCR-induced Rap1 and LFA-1 activation (Baker et al. 2012). The role of RhoH in adhesion therefore depends on the signalling context.

RhoH$^{-/-}$ mice have impaired TCR signalling and TCR-mediated thymocyte selection and maturation, resulting in fewer mature T cells in the thymus as well as peripheral lymphoid tissues compared to wild-type mice (Dorn et al. 2007; Gu et al. 2006). RhoH was reported to induce the recruitment of Zap70 to the activated TCR and thereby mediate downstream signalling required for the positive selection and maturation of thymocytes (Gu et al. 2006) (Fig. 15.1). On the other hand, in resting T cells RhoH appears to maintain Lck in an inactive state. Reduction of RhoH levels results in Lck autoactivation and constitutive TCR signalling (Wang et al. 2011b).

Interestingly, a loss-of-function mutation in RhoH, *Y38X,* has been found in two patients with *Epidermodysplasia verruciformis* (EV), a primary immunodeficiency disease in which patients have increased susceptibility to papilloma viruses and have abnormalities in several T cell populations (Crequer et al. 2012; Troeger and Williams 2013). The molecular basis for the contribution of RhoH deficiency to the disease remains to be established.

In contrast to T cells, B cell development is not severely affected in *RhoH*$^{-/-}$ mice. However, *RhoH* depletion delays development of B-cell chronic lymphocytic

leukaemia (CLL) in a mouse model (Troeger et al. 2012; Sanchez-Aguilera et al. 2010). Human CLL is characterised by accumulation of mature B lymphocytes in peripheral blood, bone marrow and secondary lymphoid tissues. RhoH mRNA levels are higher in primary human CLL samples and its expression positively correlates with the protein levels of Zap70, a poor prognosis factor in CLL that contributes to B cell receptor (BCR) signalling. Indeed, BCR signalling was reduced in *RhoH*$^{-/-}$ splenocytes (Sanchez-Aguilera et al. 2010).

RhoH affects the interaction between CLL cells and stromal cells that contributes to accumulation in the bone marrow and CLL progression (Troeger et al. 2012). *RhoH*$^{-/-}$ CLL cells failed to interact with monocytic nursing cells and had reduced chemotaxis towards chemokines in vitro, which might be due to higher Rac1 and RhoA activation compared to wild-type cells. This in turn could explain the delayed CLL progression.

15.5 RhoBTB Proteins

15.5.1 Evolution and Structure

RhoBTB proteins were initially identified as Rho-related proteins in Dictyostelium (Rivero et al. 2001). *RhoBTB* genes are present in vertebrates (*RhoBTB1-3*), Drosophila (*RhoBTB*) and Dictyostelium (*RacA*), but have been lost in *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, plants and fungi (Berthold et al. 2008b; Chang et al. 2006). RacA in *Dictyostelium discoideum* has considerable sequence differences when compared to the human RhoBTBs (Chang et al. 2006). Both human and mouse RhoBTB1 and RhoBTB2 are closely related, whereas RhoBTB3 is divergent (Ramos et al. 2002). RhoBTB1 and RhoBTB2 were shown in phylogeny studies to belong to the Rho family, but RhoBTB3 was excluded as a Rho family member based on its GTP-binding domain (Boureux et al. 2007). RhoBTB3 will therefore not be discussed here.

RhoBTB proteins have a modular organisation that is quite different from typical Ras superfamily small GTP-binding proteins (Fig. 15.4). They have a GTP-binding domain at the N-terminus, followed by a proline-rich domain, two tandem BTB (*B*ric-a-Brac, *T*ramtrack, and *B*road Complex) domains, and a C–terminal region (Berthold et al. 2008b). The GTP-binding domain of RhoBTB1 and RhoBTB2 contains the hypervariable insert characteristic of Rho proteins; however, this insert is rich in charged residues and longer than in other Rho family members (Berthold et al. 2008b). Additionally, this atypical domain contains two insertions and one deletion together with a few other differences compared to the consensus GTP-binding domain of most GTPases (Rivero et al. 2001). Moreover, in RhoBTB1 and RhoBTB2 the glycine residue (equivalent of Gly12 in Ras) is substituted by an asparagine. Because these changes affect amino acids that are essential for GTP hydrolysis, the GTP-binding domain in RhoBTBs is predicted to

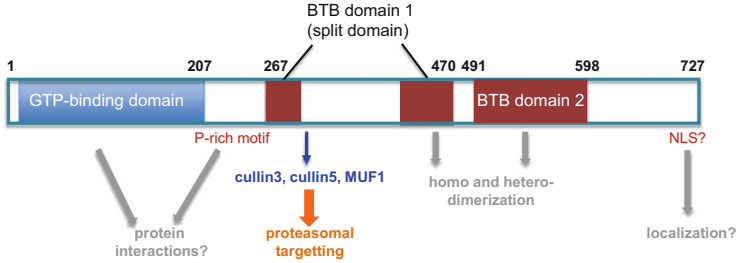

Fig. 15.4 Domain organisation and interacting partners of RhoBTB2. RhoBTB2 consists of an N-terminal GTP-binding-like domain, proline (P)-rich motif, two tandem BTB domains (the first of which is split), and a putative NLS sequence at the C-terminus. Interacting partners are shown, as well as known or potential functions of different domains

be GTPase-deficient. It has been reported that RhoBTB2 does not even bind GTP in vitro (Chang et al. 2006), but so far RhoBTB1 has not been tested.

The first BTB domain in RhoBTB1 and RhoBTB2 contains an insertion of about 115 amino acids which is predicted to generate a loop (Berthold et al. 2008b). BTB domains can form homo- and heterodimers (Stogios et al. 2005; Aravind and Koonin 1999). Unlike other Rho family members, RhoBTB1 and RhoBTB2 do not have any prenylation or palmitoylation sites (Ramos et al. 2002). The C-terminal domain has a potential NLS sequence, and RhoBTB2 has been reported to localise in part to the nucleus (Aspenstrom et al. 2007; Chang et al. 2006).

15.5.2 Regulation

RhoBTB1 is ubiquitously expressed in all tissues examined, whereas *RhoBTB2* is more abundant in neural tissues than other tissues (Ramos et al. 2002). *RhoBTB1* is a target gene for peroxisome proliferator-activated receptor-γ (PPARγ), which is a nuclear hormone receptor involved in vascular wall remodelling (Pelham et al. 2012). *RhoBTB2* is a direct target of the E2F1 transcription factor, which regulates cell cycle progression (Freeman et al. 2008). *RhoBTB1* and *RhoBTB2* expression is altered in some cancers. For example, *RhoBTB1* was found to be a target for the microRNA miR-31 in human colon cancer (Xu et al. 2013). The *RhoBTB2* gene promoter is reported to be methylated and *RhoBTB2* mRNA levels downregulated in breast cancers compared to normal breast tissue (Tang et al. 2013).

RhoBTB2 has been hypothesised to be a tumour suppressor gene because it is deleted in some human breast cancers (hence its original name, Deleted in breast cancer 2; DBC2) (Hamaguchi et al. 2002). *RhoBTB2* expression is also reduced in lung and bladder cancers compared to normal tissues, either through allelic loss or gene silencing (Berthold et al. 2008b). *RhoBTB1* is deleted in a small percentage of head and neck squamous cell carcinomas (Beder et al. 2006); however, it has also

been shown to be upregulated in some cancer cell lines (Vega and Ridley 2008; Ramos et al. 2002).

15.5.3 Binding Partners and Functions

RhoBTBs do not interact with the known Rho family targets WASP, PAK1, or Rhotekin and do not affect cell shape or cytoskeletal organisation (Aspenstrom et al. 2004). The only characterised interaction partner for RhoBTB1 and RhoBTB2 is cullin-3 (Berthold et al. 2008a; Wilkins et al. 2004) (Fig. 15.4).

Cullin-3 (Cul3) is a scaffolding protein that links RING E3 ligases to their substrates: BTB-containing proteins act as substrate adaptors in the Cul3 complexes, bringing substrates close to E3 ligases (Lydeard et al. 2013). The first BTB domain of RhoBTB proteins interacts with the N-terminal region of Cul3 (Berthold et al. 2008a). It is therefore possible that RhoBTBs bring substrates to the Cul3 E3 ligase complex, either through their second BTB domain or the GTP-binding domain (Fig. 15.4). The protein MUF1 has recently been identified as a substrate for RhoBTB-Cul3 ubiquitin ligase complexes (Schenkova et al. 2012). Interestingly, RhoBTB2 is itself a target for Cul3-dependent ubiquitination, and the interaction between RhoBTB2 and Cul3 is disrupted by a RhoBTB2 mutation found in lung cancer (Wilkins et al. 2004).

RhoBTB1 has recently been linked to RhoA ubiquitination and degradation (Pelham et al. 2012). RhoA is targeted for proteasomal degradation by the Cul3 complex through binding to BACURD, a BTB-containing substrate adaptor (Chen et al. 2009). Expression of a dominant negative form of the nuclear hormone PPARγ in vascular smooth muscle leads to hypertension and vascular dysfunction. This correlates with reduced *RhoBTB1* mRNA levels (see above) and increased RhoA protein levels, suggesting that RhoBTB1 might regulate RhoA degradation. Cul3 depletion or inhibition similarly increased RhoA levels and increased blood pressure in vivo, although whether RhoBTB1 contributes to these effects is not clear (Pelham et al. 2012).

Several diverse functions for RhoBTB proteins have been described, including vesicle trafficking, gene expression and cell proliferation, but whether these are linked to their interactions with Cul3 are not clear.

RhoBTB proteins have been suggested to affect vesicle trafficking, due to their perinuclear and vesicular localisation (Aspenstrom et al. 2004). Indeed, RhoBTB2 depletion impaired protein transport from the endoplasmic reticulum to the Golgi apparatus (Chang et al. 2006). In Drosophila larvae, the phenotype of neuromuscular junction overgrowth induced by a dominant negative form of NSF (N-ethylmaleimide sensitive factor) can be suppressed by *RhoBTB* expression. NSF is an ATPase that participates in SNARE-dependent vesicle trafficking (Laviolette et al. 2005; Zhao et al. 2007).

Gene expression analysis of HeLa cells depleted of RhoBTB2 with siRNA revealed that 247 genes were upregulated and 433 downregulated. These genes

were related to cell-cycle control, cytoskeletal regulation, apoptosis and intracellular trafficking (Siripurapu et al. 2005). Another screen performed in normal primary human bronchial epithelial cells revealed only two genes that were affected by RhoBTB2 depletion. *CXCL14*/*BRAK* was one of these downmodulated genes (McKinnon et al. 2008). CXCL14 is a chemokine that is normally secreted by epithelial cells, but its expression is altered in a wide range of carcinomas and epithelial cancers. Further studies performed using head and neck squamous cell carcinoma cell line have revealed that RhoBTB2 loss of expression correlated with CXCL14 diminished secretion. CXCL14 works as a chemoattractant for dendritic cells (Shellenberger et al. 2004), monocytes (Kurth et al. 2001) and natural killer cells (Starnes et al. 2006) which suggest that CXCL14 loss of expression might induce a reduced level of immune protection, conferring therefore survival capacity to tumour cells.

RhoBTB2 inhibits cell proliferation in a breast cancer cell line lacking endogenous *RhoBTB2* expression (Hamaguchi et al. 2002), at least in part by inducing a decrease in Cyclin D1 levels (Yoshihara et al. 2007). On the other hand, *RhoBTB2* is a target of the E2F1 transcription factor, which contributes to cell cycle progression (Freeman et al. 2008). Acute RhoBTB2 overexpression (48 h) induced transient S-phase entry, but at later timepoints S-phase entry and proliferation were reduced (Freeman et al. 2008). This implies that the effects of RhoBTB2 on cell cycle progression are context-dependent, which might reflect targeting of different substrates for Cul3 complex-mediated degradation.

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Chapter 16
Molecular Structures, Cellular Functions, and Physiological Roles of Rho Effectors

Toshimasa Ishizaki and Shuh Narumiya

Abstract Rho GTPase is a regulator controlling the cytoskeleton in multiple contexts such as cell migration, adhesion, and cytokinesis. Upon binding to GTP, Rho exerts its functions through downstream Rho effectors such as ROCK/Rho-kinase/ROK, mDia, Citron, PKN, Rhophilin, and Rhotekin. Our knowledge about the functions of Rho effectors has accumulated since their discoveries in the mid-1990s through in vitro studies using heterologous expression in cultured cells and in vivo studies using gene targeting strategy as well as pharmaceutical intervention. In this chapter, we summarize findings obtained by these studies and discuss their implications.

Keywords Actin cytoskeleton • Rho • Rock (Rho-kinase) • mDia • PKN • Rhophilin • Rhotekin • In vitro functions

16.1 ROCK

16.1.1 Molecular Structure, Isoforms, Activity, and Activation Mechanism

ROCK/Rho-kinase/ROK is the best characterized of the Rho effectors. It belongs to the AGC family of serine/threonine kinases (Ishizaki et al. 1996; Leung et al. 1995; Matsui et al. 1996; Nakagawa et al. 1996) and contains two members, ROCK1 (also

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referred to as Rho-kinaseβ/ROKβ) and ROCK2 (also referred to as Rho-kinaseα/ROKα). ROCK1 and ROCK2 transcripts are ubiquitously but differentially expressed in tissues. ROCK1 is preferentially expressed in the lung, liver, spleen, kidney, and testis, whereas ROCK2 is most highly expressed in the brain and heart (Nakagawa et al. 1996). Both kinases are composed of the N-terminal kinase domain followed by the central coiled-coil domain containing a Rho-binding domain (RBD) and the C-terminal pleckstrin-homology (PH) domain with an internal cysteine-rich domain (Fig. 16.1). The two isoforms share 65 % overall homology and 92 % identity in the kinase domain (Ishizaki et al. 1996; Leung et al. 1995; Matsui et al. 1996; Nakagawa et al. 1996). The active form of Rho directly binds to the C-terminal region of the coiled-coil domain of ROCK, leading to activation of the catalytic activity of ROCK. Studies of structural analysis revealed that eliciting the kinase activity of ROCK requires both N- and C-terminal extension segments in addition to its core catalytic domain (Fig. 16.1). These segments contribute to dimer formation of ROCK, keeping its catalytic domain in an active conformation (Jacobs et al. 2006; Yamaguchi et al. 2006). Besides binding to the active form of Rho, ROCKs are activated by cleavage at the C-terminus by caspase-3 and granzyme B, which results in production of constitutively active N-terminal fragments that facilitate plasma membrane blebbing during apoptosis (Coleman et al. 2001; Sebbagh et al. 2001, 2005).

16.1.2 ROCK Inhibitors

Functions of ROCK have been extensively clarified compared with those of other Rho effectors partly because of the discovery of a ROCK specific inhibitor, Y-27632 (Uehata et al. 1997). This compound inhibits agonist-induced contraction of vascular and bronchial smooth muscles through the inhibition of the calcium sensitization mechanism of smooth muscle contraction. Photoaffinity labeling using a ^{125}I-labeled analog of Y-27632 identified the target protein of this compound as ROCK1. Currently, three chemically synthetic compounds, Y-27632, fasudil (also called as HA1077), and H-1152, are widely utilized as ROCK inhibitors in many studies (Narumiya et al. 2000; Olson 2008). They inhibit kinase activity in a manner competitive with ATP, but are nonselective compounds for the two isoforms. These ROCK inhibitors have been utilized not only as tools for studies on ROCK functions but also have been evaluated for their potential as therapeutic drugs (see below).

16.1.3 Functions in the Cell

ROCK1 and ROCK2 have been reported to phosphorylate multiple target proteins such as myosin-binding subunit of myosin light chain phosphatase 1 (MYPT1)

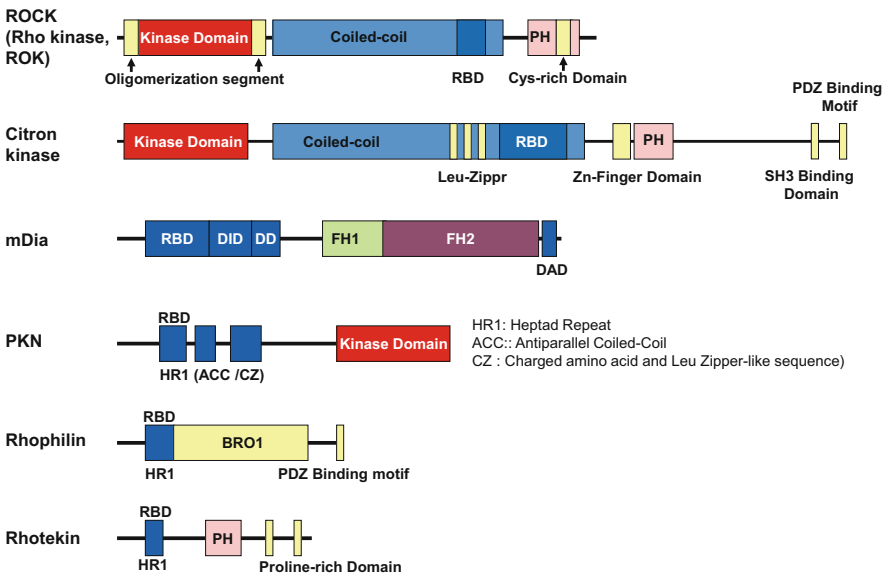

Fig. 16.1 Schematic representation of domain structure of Rho effector molecules

(Kimura et al. 1996), myosin light chain (MLC) (Amano et al. 1996a), Lin-11 Isl-1 Mec-3 kinase (LIMK) (Maekawa et al 1999), ezrin/radixin/moesin (ERM) (Matsui et al. 1998), Na^+-H^+ exchanger 1 (NHE1) (Tominaga et al. 1998), adducin (Kimura et al. 1998), calponin (Kaneko et al. 2000), myristoylated alanine-rich C-kinase substrate (MARCKS) (Nagumo et al. 2001), collapsin response mediator protein-2 (CRMP-2) (Arimura et al. 2000), and Par3 (Nakayama et al. 2008). Due to the high degree of homology between their catalytic domains, ROCK1 and ROCK2 are believed to share more than 10 substrates including MYPT1, MLC, and LIMK and elicit redundant biological functions. Among these, functions of ROCK-mediated phosphorylation of MYPT1, MLC, and LIMK have been well characterized (Fig. 16.2). ROCK can increase the amount of phosphorylated MLC either by directly phosphorylating MLC or indirectly by inactivating MLC phosphatase, both resulting in stimulation of actomyosin-based contractility in the cell (Amano et al. 1996a; Kimura et al. 1996). LIMK is activated upon phosphorylation by ROCK and phosphorylates the downstream target molecule, colifin. Cofilin, which is a member of actin depolymerization factor (ADF), is involved in severing actin filaments. Phosphorylation of colifin by the ROCK-LIMK pathway inhibits its actin severing activity, leading to stabilization of actin filaments in the cell (Maekawa et al. 1999). Both the ROCK/MYPT1/MLC and ROCK/LIMK/Cofilin pathways are involved in Rho-mediated reorganization of the actin cytoskeleton in various fundamental cell processes such as cell to substrate adhesion and migration (Itoh et al. 1999; Tsuji et al. 2002), cell to cell adhesion (Sahai and Marshall 2002), transcription (Chihara et al. 1997), apoptosis (Coleman et al. 2001; Sebbagh et al. 2001), axonogenesis in neurons (Bito et al. 2000; Hirose et al. 1998), and

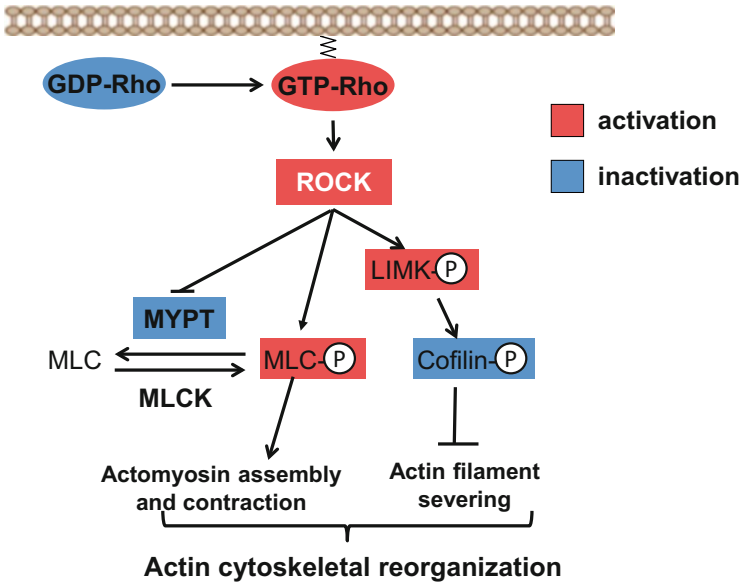

Fig. 16.2 Molecular mechanisms of actin cytoskeleton reorganization through ROCK. Upon binding to the active form of Rho, ROCK is activated and then phosphorylates MLC and MYPT. Thereby, the amount of phosphorylated MLC in the cell is increased, resulting in the activation of actomyosin assembly and the generation of cell contractility. On the other hand, ROCK also phosphorylates and activates LIMK, who then phosphorylates and inactivates cofilin, leading to the stabilization of actin filament through the inhibition of actin severing activity of cofilin

polarization and transmigration of immune cells including T cells (Heasman et al. 2010). Recently, it was also reported that ROCK is involved in apoptosis of dissociated human embryonic stem (hES) cells (Ohgushi and Sasai 2011). One characteristic feature of human pluripotent stem cells (hPSCs) such as human iPS and embryonic stem (ES) cells is their susceptibility to dissociation-induced apoptosis. Watanabe et al. (2007) reported that treatment of hES cells with a ROCK inhibitor, Y-27632, rescues them from dissociation-induced apoptosis. They also showed that the addition of Y-27632 to cell culture medium increases colony formation of dissociated hES cells and facilitates the selective subcloning of hES cells post-gene transfer. Based on these findings, ROCK inhibitor has recently been used in stem cell research for a variety of applications associated with cell dissociation such as passaging, expansion, cryopreservation, gene transfer, differentiation induction, and cell sorting.

ROCK 1 and ROCK2 are thought to play redundant roles in cells because of the high degree of sequence homology of their kinase domains, and currently used ROCK inhibitors are not selective to either isoform. Therefore, little has been done to distinguish the isoform-specific functions of ROCK. Yoneda et al. (2005; 2007) used siRNAs specific for each of the ROCK isoforms and showed that ROCK1 is

essential for stress fiber formation, whereas ROCK2 appears to be necessary for phagocytosis and cell contraction. Hopefully, future studies will show which isoform is functionally dominant in each of the ROCK-mediated cellular processes described above.

16.1.4 Functions In Vivo in the Body

16.1.4.1 Roles of ROCK During Embryogenesis

ROCK inhibitors have been used in various studies to dissect the roles of ROCK in vivo, one being those examining the role of ROCK in embryonic development. Wei et al. (2001) reported that pharmacological inhibition of ROCK in chicken embryos in culture results in defects in both cardiac tube formation and neural tube closure. During neural tube closure, F-actin in the neural epithelium is predominantly localized as a dense band encircling each cell at the apical tip of the adherens junction (AJ). Many genes involved in the closure of the neural plate during embryonic development have been identified (Copp et al. 2003). One of them is Shroom3, which is an actin-binding protein localized around AJs in neuroepithelial cells (Haigo et al. 2003; Hildebrand and Soriano 1999; Hildebrand 2005). Nishimura and Takeichi (2008) showed that ROCK directly binds to Shroom3, and this association recruits ROCK to AJs of neuroepithelial cells, where ROCK enhances MLC phosphorylation and generates myosin-based contractility of the encircling apical actin filaments, which is indispensable for the closure of the neural plate. The same group further reported an activation mechanism of ROCK at AJs in neuroepithelial cells (Nishimura et al. 2012). They found that Celsr1, the planer cell polarity (PCP) regulator that localizes to AJs in the neural plate, recruits Dishevelled and Frizzled, which in turn activate PDZ-RhoGEF to activate Rho-ROCK signaling. Activation of ROCK then results in anisotropic contraction of the AJs to make a tubular structure (Fig. 16.3). Similar polarized actomyosin contraction is used in making tissue architecture. Studies using Y-27632 (Eiraku et al. 2011) have shown that ROCK activity is important for eye-cup formation during development. By ex vivo differentiation of ES cell culture and two-photon microscopy, it was demonstrated that invagination of the retinal epithelium to form a cup shape requires actomyosin contraction induced by ROCK activity.

In parallel to the use of ROCK inhibitors, gene-targeting technology is also used to dissect the function of ROCK in vivo. Thumkeo et al. (2003) first generated KO mice deficient in ROCK and examined the in vivo roles of ROCK. Deletion of ROCK2 in mice results in intrauterine growth retardation due to increased thrombotic tendency in the labyrinth layer of the placenta. On the other hand, ROCK1 deficiency impairs closure of eyelid and body wall through epithelial sheet contraction during embryogenesis (Shimizu et al. 2005). A subsequent study in C57BL/6 genetic background further demonstrated that ROCK2 also plays a similar role in eyelid and body wall closure during development (Thumkeo et al. 2005). The same

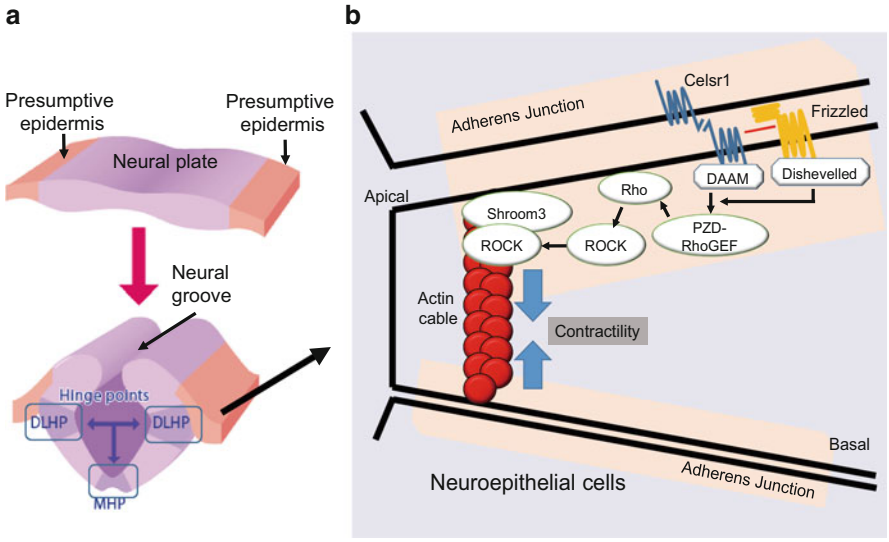

Fig. 16.3 Mechanisms of neural plate bending through ROCK signaling during embryogenesis. (**a**) Folding begins as the medial neural hinge point (MHP) cells change their shape, while the presumptive epidermal cells move toward the center. The neural folds are elevated as presumptive epidermis continues to move toward the dorsal midline. Convergence of the neural folds occurs as the dorsolateral hinge point (DLHP) cells become wedge-shaped and epidermal cells push toward the center. (**b**) In the bending neural plates, Celsr1 is concentrated in the adherens junctions (AJs). At these AJs, Celsr1 cooperates with Dishevelled, DAAM1, and the PDZ-RhoGEF to activate ROCK. ROCK is recruited to AJs through the binding to Shroom3. Thereby, actomyosin-dependent contractility is generated at the apical area in neuroepithelial cells, leading to the bending of the neural plate at hinge points

phenotype was observed in ROCK1/2 double heterozygous mice, suggesting a functional redundancy between ROCK1 and ROCK2 (Shimizu et al. 2005; Thumkeo et al. 2005). On the other hand, Kamijo et al.(2011) found that ROCK1/2 double homozygous knockout mouse embryos survived until the blastocyst stage, but no embryos were observed after E8, indicating that ROCK activity is required between the blastocyst stage and E8 (Kamijo et al. 2011; Thumkeo et al. 2005). They also found that $ROCK1^{-/-}ROCK2^{+/-}$ and $ROCK1^{+/-}ROCK2^{-/-}$ mice exhibit embryonic lethality around E9.5 due to impaired vasculature development in the yolk sac (Kamijo et al. 2011). In contrast to $ROCK2^{-/-}$ mice with a C57B/6 background, ROCK2 knockout mice with a CD1 background survive at a high rate without any defects during embryogenesis (Duffy et al. 2009; Zhou et al. 2009). Using these mice, Zhou et al. (2009) reported that ROCK2 knockout mice are impaired in both basal synaptic transmission and hippocampal long-term potentiation, and Duffy et al. (2009) reported enhanced axonal growth after spinal cord injury in these mice.

16.1.4.2 ROCK and Cancer

Rho proteins are frequently overexpressed in human cancers including colon, breast, lung, and testicular germ cell tumors (Sahai and Marshall 2003b). Aberrant expression and activation of RhoA and RhoC are thought to promote the tumorigenic, invasive, and metastatic potential of certain clinical cancers (Karlsson et al. 2009; Sahai and Marshall 2003b; Wheeler and Ridley 2004). The clinical significance of Rho signaling in cancer is further suggested by the discovery that deleted liver cancer 1 (DLC-1), a RhoA GAP, acts as a tumor suppressor in humans (Lahoz and Hall 2008; Xue et al. 2008). Depletion of DLC-1 causes hyperactivation of Rho signaling, which presumably results in tumorigenesis in collaboration with other oncogenes such as Myc and Ras (Xue et al. 2008). Consistently, Rho activity is required for malignant cell transformation in vitro. For example, expression of a dominant-negative RhoA mutant suppresses oncogenic Ras-induced focus formation in NIH 3T3 cells, and conversely, co-expression of Raf and a dominant active RhoA mutant facilitates focus formation (Qiu et al. 1995). In addition, Rho GEFs such as Dbl and Ect2 have potent transforming activities in cultured cells in vitro (Rossman et al. 2005). Involvement of ROCK in tumors has been extensively examined by the use of its inhibitors, such as Y-27632 (Narumiya et al. 2000; Uehata et al. 1997), and the Rho-ROCK pathway has been strongly implicated in tumor cell migration, metastasis, and invasion. For example, Itoh et al. (1999) reported that transfection of dominant active mutants of ROCK conferred hepatoma MM1 cells invasive activity independent of Rho and serum, whereas expression of a dominant negative ROCK mutant or treatment with Y-27632 substantially attenuated their invasion in vitro. Furthermore, continuous infusion of Y-27632 in situ markedly reduced dissemination and tumor nodule formation of MM1 cells injected into the peritoneal cavity of syngeneic rats. Similarly, expression of dominant-negative ROCK1 in Li7, a highly motile and metastatic hepatocellular cancer (HCC) cell line, resulted in reduced cell motility in vitro and suppressed metastatic dissemination of these cells in vivo in an orthotopic implantation model (Genda et al. 1999). Then, how does ROCK function in migration of tumor cells? Recent advances in real-time in situ imaging and the use of in vitro 3-dimensional invasion models have brought a wealth of deep insights into the invasion process. Sahai and Marshall (2003a) have shown that cancer cells can adopt two different types of motility, amoeboid or elongated, dependent on the conditions in 3-D environment, and Rho-ROCK signaling is critical in the former and promotes proteolysis-independent amoeboid types of movement of cancer cells. Such action of ROCK is not limited to tumor cells themselves. Tumor cells can move either as individual cells as shown above or in a collective fashion. In the latter mode of movement, carcinoma-associated fibroblasts (CAFs), including a large number of myofibroblasts, interact with carcinoma cells and other stroma cells, and this interaction is crucial for the development of aggressive tumors. CAFs are also known to facilitate the conversion of incipient tumor cells into highly malignant cells which can spread to and infiltrate distant organs. By analyzing the action of

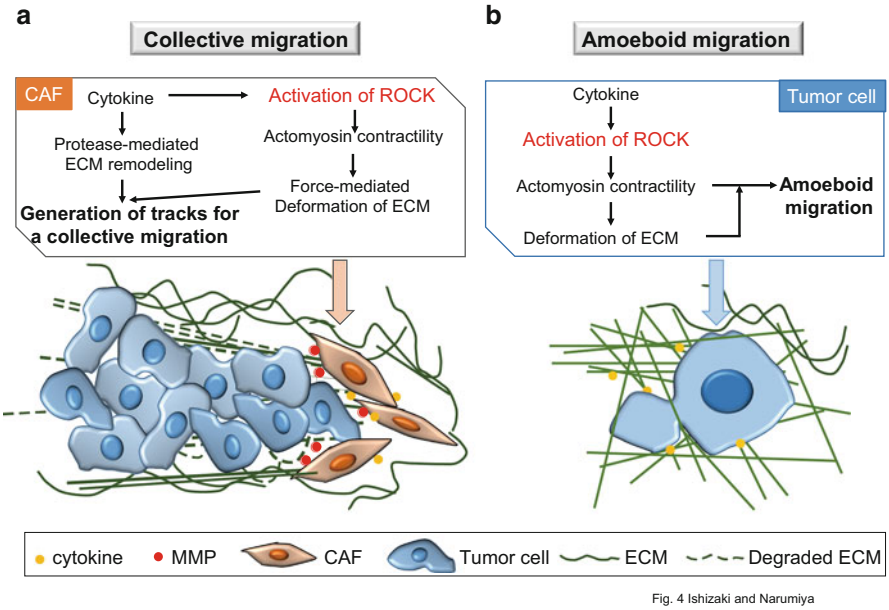

Fig. 16.4 Roles of actomyosin contractility through activation of ROCK in a collective migration (**a**) and an amoeboid migration (**b**)

CAFs in the 3-D culture, Gaggioli et al. (2007) found that CAFs utilize the Rho-ROCK pathway and remodel ECM through contractile forces and proteolytic activity to generate tracks for migration of cancer cells. Sanz-Moreno et al. (2011) further analyzed the activating mechanism of CAFs and showed that the IL-6 family cytokines through glycoprotein 130 (gp130)-Janus kinase 1(JAK1)-signal transducer and activator of transcription 3 (STAT3) signaling pathway regulate actomyosin contractility in a ROCK-dependent manner in CAFs, for generation of tracks for the collective invasion of human squamous-cell carcinoma cells (Fig. 16.4). The same author moreover reported that this cytokine signaling is also used by individual human melanoma cells to facilitate their amoeboid type motility (Fig. 16.4).

Cytokine signaling described above is one of the environmental factors affecting tumor growth. Another environmental factor is stiffness of tissues that is thought to be an active participant in tumor growth and progression (DuFort et al. 2011). There is a report that ROCK regulates the stiffness of tissues surrounding tumors. Samuel et al generated transgenic mice conditionally expressing constitutively active ROCK2 in the skin by driving it under the keratin-14 promotor (Samuel et al. 2009) and examined how ROCK activation affects tissue homeostasis and tumor development (Samuel et al. 2011). They showed that contractile forces through ROCK activation cause increased tissue stiffness and promote β-catenin-mediated hyperproliferation and skin thickening. Using the DMBA-TPA-induced, two-stage chemical skin carcinogenesis model, they further found that the ROCK

activation increases the number of papilloma, growth, and malignant cell progression, and this phenotype is inhibited by pharmacological inhibition of ROCK with Y-27632. These results indicate that ROCK is involved in tumor cell invasion and demonstrate its potential as a therapeutic target.

Because Rho functions in Ras-induced cell transformation as described above, it is likely that ROCK is involved not only in tumor cell motility and invasion but also directly in malignant cell transformation and tumorigenesis. Previously, Sahai et al. (1999) reported that Y-27632 not only blocked focus formation by RhoA, Dbl, or mNET1 but significantly inhibited focus formation by Ras, and that active ROCK mutant exhibited, albeit weakly, the ability to cooperate with activated Raf in focus formation. These findings suggest that ROCK functions downstream of Ras and is directly involved in Ras-induced cell transformation. Recently, Kumar et al. (2012) performed RNAi targeting on 7,000 human genes in non-small-cell lung cancer (NSCLC) expressing K-ras mutation and screened essential genes functioning downstream of Ras for cell transformation and tumorigenesis. They found that GATA2, a transcriptional factor, is essential for growth and survival of Ras-transformed but not wild-type cells. The authors then performed microarray and ChIP sequencing to identify the target genes of GATA2, and found three major pathways essential to support of Ras-induced transformation, one being Rho-ROCK pathway. Depletion of ROCK1 by RNAi or pharmacological inhibition of ROCK by fausdil combined with inhibition of another critical pathway, proteasome, induces substantial suppression of oncogenic *Kras*-driven tumorigenesis in vivo.

16.1.4.3 ROCK and Cardiovascular Diseases

Hypertension is a risk factor for a variety of cardiovascular diseases and is characterized by high arterial pressure resulting from increased peripheral vascular resistance, which can be attributed to both enhanced contractility of vascular smooth muscle cells (VSMCs) and arterial wall remodeling. VSMC contraction is initiated by both Ca^{2+}- dependent and Ca^{2+}-independent mechanisms. An increase in $[Ca^{2+}]_i$ leads to activation of myosin light chain kinase, myosin phosphorylation, and ultimately to an increase in contraction. The major mechanism of Ca^{2+}-independent contraction, which is known as Ca sensitization, is mediated by inhibition of myosin light chain phosphatase (MLCP) through the activation of Rho, leading to increased MLC phosphorylation and VSMC contraction (Somlyo et al. 1994). A ROCK inhibitor, Y-27632, suppresses arterial and tracheal contraction induced by various agonists such as phenylephrine, histamine, acetylcholine, serotonin, endothelin, and a thromboxane agonist, U-46619. Further analysis revealed that Y-27632 inhibits GTPγS-induced contraction, but has no effect on Ca^{2+}-induced contraction, suggesting that this compound specifically inhibits Ca-independent contraction (Uehata et al. 1997). Utilizing this compound in various animal models of experimental hypertension, blocking ROCK activity with Y27632 was found to lower blood pressure in spontaneously hypertensive rats (SHR),

deoxycorticosterone-acetate (DOCA)/salt-treated rats, and rats with renal hypertension. Thus, this initial study demonstrated that Rho-ROCK signaling plays an important role in blood pressure control in the body by regulating Ca-sensitization pathway in VSMCs.

In addition to VSMC contraction, the blood pressure is regulated by nitric oxide (NO) derived from endothelial cells (ECs) that induces relaxation of VSMCs. Reduced bioavailability of NO could therefore be a cause of hypertension and is caused by reduced expression of eNOS in ECs. Several studies show that ROCK is also involved in regulation of eNOS expression. For example, hypoxia and thrombin downregulate eNOS activity through destabilization of eNOS mRNA, and this effect is reversed by treatment with C3 transferase and ROCK inhibitors, Y-27632 and fasdil (Eto et al. 2001; Rikitake et al. 2005; Satoh et al. 2011; Takemoto et al. 2002). In vivo in the cerebral stroke model of middle cerebral artery ligation, inhibition of ROCK by fasudil and Y27632 increases eNOS mRNA and activity, which is correlated with increased cerebral blood flow to both ischemic and non-ischemic brain areas (Rikitake et al. 2005). Furthermore, treatment of ECs with fasudil was also reported to lead to rapid phosphorylation and activation of eNOS through the phosphatidylinositol (PI)-3 kinase-protein kinase Akt pathway, and this action exerts protective effects on myocardial infarction induced by transient coronary artery occlusion (Wolfrum et al. 2004). Thus, ROCK signaling negatively modulates eNOS at both mRNA and protein levels. In addition to these functions in VSMCs and ECs, ROCK has been implicated in reactive oxygen spices (ROS) generation and VSMC proliferation in relation to cardiovascular diseases (Satoh et al. 2011). Among ROCK inhibitors, fasudil has been in clinical use since 1995 in Japan for the prevention and treatment of cerebral vasospasm after surgery for subarachnoid hemorrhages in a well-tolerated and safe manner (Suzuki et al. 2007), and this drug is also evaluated for indication in other cardiovascular disorders such as acute ischemic stroke, stable angina pectoris, coronary artery spasm, heart failure-associated vascular resistance and constriction, pulmonary arterial hypertension, essential hypertension, atherosclerosis, and aortic stiffness (Olson 2008).

16.1.4.4 ROCK and Other Diseases

In addition, ROCK has also attracted interest as a potential target for treatment of axon degeneration, glaucoma, osteoporosis, erectile dysfunction, and insulin resistance (Olson 2008).

Axon degeneration occurs frequently in many types of chronic neurodegenerative diseases and in injuries to axons caused by toxic, ischemic, or traumatic insults (Coleman and Perry 2002; Raff et al. 2002). Axonal regeneration in the injured CNS is hampered by multiple inhibitory molecules, many of which are known to activate Rho signaling pathway, either directly or indirectly (Schmandke et al. 2007). In vitro, activation of Rho and ROCK induces neurite retraction or growth cone collapse (Bito et al. 2000; Hirose et al. 1998; Wahl et al. 2000).

Conversely, inhibition of Rho/ROCK signaling by either expression of a dominant negative Rho mutant or by treatment with C3 exoenzyme or Y-27632 inhibits neurite retraction on inhibitory molecules including MAG, Nogo, and myelin substrates and promotes outgrowth (Dergham et al. 2002; Lehmann et al. 1999; Niederöst et al. 2002; Yamashita et al. 2002). Indeed, several studies have shown that ROCK inhibitor treatment promotes axonal regeneration/sprouting in spinal cord injury models (Chan et al. 2005; Fournier et al. 2003; Hara et al. 2000; Tanaka et al. 2004). These results suggest that inhibition of ROCK might be beneficial for axonal regeneration of the injured CNS.

Glaucoma is an eye disease in which elevated intraocular pressure ultimately damages the optic nerve, leading to progressive, irreversible vision loss. Accumulating evidence indicates that inhibition of ROCK activity in the eye facilitates aqueous humor outflow through the trabecular meshwork and lowers intraocular pressure and achieves significant benefits in patients with glaucoma (Honjo et al. 2001a, b; Tian and Kaufman 2005; Rao et al. 2001; Tokushige et al. 2007; Waki et al. 2001; Whitlock et al. 2009). ROCK inhibitors may also exert beneficial effects by enhancing ocular blood flow, retinal ganglion cell survival, and axon regeneration. This use of a ROCK inhibitor, K-115, is now in phase III clinical trial. Bone continuously undergoes remodeling. The bone remodeling consists of two distinct processes, one being bone resorption by osteoclasts and the other bone formation by osteoblasts. An imbalance in bone resorption and bone formation results in bone loss that eventually leads to osteoporosis. Chellaiah et al. (2003) showed that Rho/ROCK signaling increases the surface expression of a hyaluronan receptor CD44 in osteoclast cells. They also demonstrated that neutralized CD44 antibody inhibits migration and bone resorption of osteoclasts in vitro. These results suggest that activation of Rho/ROCK signaling enhances bone resorption by osteoclasts. On the other hand, other studies have indicated that Rho signaling inhibits proliferation and differentiation of osteoblasts. *Pasteurella multocida* toxin (PMT), a bacterial toxin that activates Rho, inhibits osteoblast differentiation through the downregulation of BMP-2 and BMP-4 expression (Harmey et al. 2004). Moreover, Y-27632 stimulates the expression of BMP-4 and osteoblast markers, ALP and OC. Taken together, this evidence suggests that the Rho/ROCK pathway regulates the bone remodeling cycle, and activation of this pathway may bias the balance to bone resorption.

Penile erection requires the relaxation of cavernosal smooth muscle, leading to an increase in blood flow and distension of corpus cavernosum, an erection. Erectile dysfunction (ED) may be due to an inability of penile smooth muscles to relax. It has been shown that pharmacological inhibition of ROCK improves erectile function in several rat models including those of aging and castration (Chitaley et al. 2001; Rajasekaran et al. 2005; Teixeira et al. 2005; Wingard et al. 2003).

Activation of RhoA/ROCK also increases the association between ROCK and IRS-1, leading to inhibition of insulin signaling (Begum et al. 2002). Kanda et al. (2006) demonstrated that treatment with fasudil for 4 weeks corrected glucose and lipid metabolism in obese Zucker rats by improving insulin signaling in skeletal muscles. On the other hand, Kim's group showed that dominant, negative ROCK

decreases insulin-stimulated glucose transport in L6 muscle cells, isolated soleus muscles ex vivo, and 3T3-L1 adipocytes by impairing PI3K activity (Furukawa et al. 2005). Further analysis revealed that global ROCK1 deficienct mice in the FVB genetic background show insulin resistance (Lee et al. 2009). Overall, the role of ROCK in glucose homeostasis and diabetes is still obscure, and further analysis is needed.

16.2 mDia

16.2.1 Molecular Structure, Isoforms, Activity, and Activation Mechanism

mDia, the mammalian homolog of *Drosophila* Diaphanous, was originally identified by yeast two-hybrid screening as a molecule binding to the GTP-bound Rho (Watanabe et al. 1997). Three mDia isoforms, mDia1, mDia2, and mDia3, are expressed in mammalian cells. mDia is composed of, from the N-terminus to the C-terminus, a Rho-binding domain (RBD), Dia-inhibitory domain (DID), dimerization domain (DD), coiled-coil domain (CC), formin homology 1(FH1) and formin homology 2 (FH2) domains, and Dia auto-regulatory domain (DAD) (Fig. 16.1). mDia belongs to formin homology (FH) family of proteins, defined by the presence of FH1 and FH2 domains. The FH1 domain contains multiple discrete stretches of contiguous proline residues capable of binding profilin, an abundant actin monomer-binding protein. The FH2 domain from two mDia molecules forms a head–tail dimer like a doughnut. This FH2 dimer encircles the barbed end of an actin filament. By this FH2 dimer, mDia, as well as other formin family proteins, catalyzes actin nucleation and polymerization while they remain on the elongating barbed end, known as processive filament elongation. The FH1 domain is located immediately N-terminal to the FH2 domain and presents actin monomers rapidly to the FH2 dimer to add them onto the barbed end of the filament (Paul and Pollard 2009).

The activation mechanism of mDia has been well studied (Sakamoto et al. 2012b). In resting state, mDia is thought to be auto-inhibited via intramolecular interaction between DID and DAD, which inhibits the ability of FH1–FH2 to nucleate and elongate actin filaments. In response to stimuli, the active GTP-bound form of Rho accumulates and binds to mDia RBD. This binding disrupts the DID–DAD interaction and leads to activation of mDia. Activated mDia then induces actin polymerization through the FH1–FH2 domain as described above. Endogenous mDia1 is mainly distributed diffusely in the cytoplasm. Notably, release of auto-inhibition by activated Rho is suggested to induce not only actin polymerizing activity but also membrane localization of mDia in the cell, and this membrane targeting is prerequisite for initiating actin nucleation and polymerization in the cell. Molecular mechanism of membrane targeting of mDia has been studied using

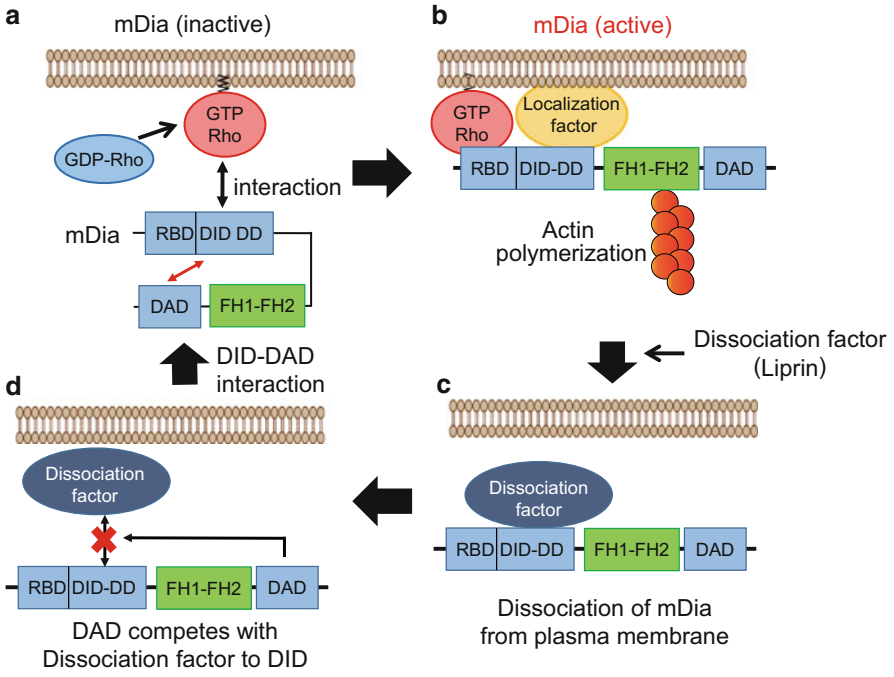

Fig. 16.5 Proposed mechanisms of regulation of mDia. (**a**) Upon activation of Rho, mDia binds to the active form of Rho through its Rho-binding domain (RBD). (**b**) mDia binding to Rho is localized on the plasma membrane and stabilized by the binding to localization factor(s) through its DID-DD, leading to actin polymerization. (**c**) When mDia binds to dissociation factor(s) such as liprin-α through its DID-DD, mDia dissociates from the plasma membrane. (**d**) DAD of mDia competes with dissociation factor(s) to DID, and DID–DAD interaction may form, resulting in inactivation of mDia

truncation fragments. Such studies have revealed that membrane localization of mDia is mediated through its DID-DD-CC regions in addition to its binding to Rho. These results suggest that, in addition to regulation by Rho, the activity and localization of mDia are regulated by its interaction with DID-DD-CC-binding proteins. Sakamoto et al. (2012a) isolated liprin-α as a mDia DID-DD-CC-binding protein. Liprin-α is a cytosolic protein and can sequester mDia from the membrane. Because depletion of liprin-α by RNAi induces massive stress fibers in an mDia-dependent manner, the authors proposed that membrane localization of mDia, consequently its activity, is regulated by competitive binding by liprin-α in the cytosol and unknown target protein(s) in the membrane (Fig. 16.5).

16.2.2 Functions in the Cell

Watanabe et al. (1999) isolated mDia as a Rho effector and first reported that expression of mDia1 induces production of actin filaments. They further found the activation mechanism of mDia as described above and that mDia works in collaboration with ROCK to form actin stress fibers in cultured cells. Since these reports, evidence on cellular functions of mDia through actin polymerization activity mainly studied on mDia1 has accumulated. mDia is involved in various cellular processes such as filopodia formation (Goh and Ahmed 2012), mechanotransduction (Higashida et al. 2013; Jégou et al. 2013; Riveline et al. 2001), cell morphogenesis, polarization and migration (Brandt et al. 2007; Tsuji et al. 2002; Yamana et al. 2006), axonogenesis in primary culture of cerebellar granule neurons (Arakawa et al. 2003), and exocrine vesicle secretion in the apical membrane (Geron et al. 2013). Furthermore, mDia-mediated assembly of actin filaments in the cytoplasm changes the ratio of monomeric to filamentous actin in cells, and this change is crucial for elicitation of transcriptional activity of serum response factor (SRF). In this process, actin monomers sequester a transcriptional co-activator megakaryocytic acute leukemia (MAL) through its binding in the cytoplasm (Juliano 2009). When the amount of monomeric actin is decreased by actin assembly, MAL traffics into the nucleus and promotes transcriptional activity through association with SRF. Recently, Baarlink et al. (2013) reported that the same mechanism operates in the nucleus. They found that serum addition stimulates rapid and transient mDia-dependent actin polymerization within the nucleus, which further accelerates MAL association with SRF and promotes transcriptional activity. Previously, Miki et al. (2009) reported that mDia2 shuttles between the cytoplasm and nucleus through importin- and CRM1-mediated nuclear transport mechanism. Thus, actin dynamics modulated by mDia are crucial not only for regulation of cell morphology and motility but also for transcriptional control in the nucleus. Another function of mDia-induced actin assembly is its action in cell division. Diaphanous, a *Drosophila melanogaster* homolog of mDia, is essential for cytokinesis. Looking for a candidate among three mammalian mDia isoforms, Watanabe et al. (2008) found that mDia2 is apparently the main mDia isoform functioning in cytokinesis. It is clearly localized at the cleavage furrow during cytokinesis and its depletion by RNAi produces multinucleate cells. In addition to binding to RhoA, mDia2 also binds to anillin through its DID, and this binding specifies the mDia2 localization to the cleavage furrow, which restricts the actomyosin-dependent contractility in the cleavage furrow and therefore is critical for successful cytokinesis (Watanabe et al. 2010). mDia2 is also involved in filopodia formation (Beli et al. 2008; Pellegrin and Mellor 2005; Yang et al. 2007), red blood cell development (Ji et al. 2008), and endosome trafficking in cultured fibroblasts (Wallar et al. 2006). As for specific action of mDia3, Yasuda et al. (2004) examined its cellular function in HeLa cells and revealed that mDia3 is indispensable for normal chromosome alignment in HeLa cells. A recent independent work confirmed the findings by Yasuda et al. and further suggests that AuroraB

kinase phosphorylates and regulates mDia3 in this process (Cheng et al. 2010). mDia3 is also involved in endocytosis downstream of RhoD (Gasman et al. 2003).

In addition to the action of mDia on the actin cytoskeleton, mDia regulates microtubule stabilization and orientation (Ishizaki et al. 2001). Several works suggest that mDia can stabilize microtubules independently of its actin nucleation activity (Bartolini and Gundersen 2010). Studies revealed that mDia1 and mDia2 appear to stabilize MTs both through direct binding (Bartolini et al. 2008; Gaillard et al. 2011) and/or by altering the posttranslational modification of tubulin (Bartolini et al. 2008; Thurston et al. 2012; Wen et al. 2004). Direct binding is supported by the interaction of mDia with MT plus-end tracking proteins such as EB1 and APC (Wen et al. 2004). Recently, Gaillard et al. (2011) showed that of the FH1–FH2 and C-terminus of mDia2 binds microtubules with 1:1 ratio at submicromolar affinity and microtubules inhibit mDia-induced actin assembly, suggesting that mDia interaction with the two cytoskeletal elements modulate its activities to these cytoskeletons.

16.2.3 Functions In Vivo in the Body

Compared to extensive studies on its functions in the cell and compared to studies on ROCK, little has been performed previously on in vivo functions of mDia, which is perhaps due to the lack of convenient tool. However, recent generation of knockout mice deficient in each mDia isoform has enabled studies on their roles in vivo. These studies have revealed that mDia isoforms contribute to tissue homeostasis and architecture through their action on actin cytoskeleton in vivo. The first KO mice generated was mDia1$^{-/-}$ mice. Intriguingly, homozygous mDia1$^{-/-}$ mice are viable and develop apparently normally. However, detailed analysis revealed that they have homeostatic defects particularly in lymphoid and myeloid organs. Sakata et al. (2007) found that the number of T cells in secondary lymphoid organs is significantly decreased in mDia1$^{-/-}$ mice. They further found that mDia1$^{-/-}$ T cells have defects in homing to lymph nodes due to impaired T cell chemotaxis and migration. These T cells also showed impaired responses to TCR stimulation. This phenotype of mDia1$^{-/-}$ mice was confirmed by Eisenmann et al. (2007). As for other types of cells involved in immune response, the loss of mDia1 also impairs migration and antigen presentation of dendritic cells, and consequently, the DC-dependent delayed hypersensitivity response was attenuated (Tanizaki et al. 2010). Furthermore, mDia1$^{-/-}$ mice aged more than 400 days spontaneously develop myelodysplastic syndrome (MDS) characterized by hyperplastic bone marrow, extramedullary hematopoiesis, splenomegaly, and abnormally shaped erythrocytes and immature myeloid progenitor cells in peripheral blood (Peng et al. 2007). This phenotype is enhanced and the onset is accelerated by combined loss of RhoB (DeWard et al. 2009). These results together with the finding that DIAPH1 (human mDia1 gene) is located at 5q31.3 and lies between two commonly deleted regions in MDS patients led the authors suggest that mDia1

acts as a node in a tumor-suppressor network involving multiple 5q gene products (Eisenmann et al. 2009). However, in humans it is known that a mutation of DIAPH1 is associated with progressive deafness (Lynch et al. 1997). The DIAPH1 mRNA containing this mutation is translated to produce a truncated protein with 32 amino acid deletion from the C-terminal end. Although the underlying molecular mechanism is still unknown, this mutation may induce a gain-of-function phenotype by disrupting the auto-inhibition mechanism of mDia1 and a cause of the deafness may be aberrant actin assembly in hair cells.

As mentioned above, the major function of mDia2 in the cell is to control cytokinesis during the cell cycle. Deletion of the mDia2 gene was therefore expected to lead to embryonic lethality at the early stage of embryogenesis due to impaired cell division. However, mDia2 knockout embryos develop until E12.5 and then die in utero. Watanabe et al. (2013b) analyzed the cause of this embryonic lethality of mDia2 knockout mice and found that mDia2-deficient mice that survive at E11.5 exhibit severe anemia. When mDia2-deficient erythroid progenitor cells are cultured, they differentiate until pro-erythroblasts, which then exhibit cytokinesis failure in late differentiation stages with decreased accumulation of F-actin in the cleavage furrow, and become multinucleated. Multinucleate mDia2$^{-/-}$ erythroblasts could extrude their nuclei with F-actin accumulation, albeit at a lower frequency, to give rise to big enucleated cells, which is contrary to the results previously reported in mDia2–depleted erythroblasts (Ji et al. 2008). It has also been reported that mDia2 is involved in human disease named auditory neuropathy (AUNA1), a rare form of deafness characterized by an absent or abnormal auditory brainstem response with preservation of outer hair cell function. Schoen et al found a point mutation in the 5′UTR of the human DIAPH3 gene (mDia2) that leads to overexpression of DIAPH3 and causes a progressive non-syndromic auditory neuropathy (Schoen et al. 2010). The authors confirmed the progressive hearing loss phenotype observed in human patients in two independent transgenic mouse lines over-expressing mDia2 and found abnormalities of the inner hair cell (IHC) stereocilia and a loss of IHC ribbons (Schoen et al. 2013).

As for the in vivo role of mDia3, its role in ovarian function was suggested by human study on premature ovarian failure (POF). In some patients with X-linked POF, a region of the chromosome, located at Xq21, is interrupted by a break point (Bione et al. 1998). Mapping of the gene responsible for POF identified DIAPH2, which is a human homolog of mDia3, and has a break point in the last intron. These results suggest a possible role of mDia3 in the development of the ovary and fertility. However, mDia3 knockout mice are fertile and develop apparently normally (Shinohara et al. 2012; Thumkeo et al. 2011). Mild phenotype of mDia1$^{-/-}$ and mDia3$^{-/-}$ mice indicates possible functional redundancy between the two mDia proteins. To address this issue, mDia1/3 double knockout mice were generated by crossing mice deficient in mDia1 with mDia3 KO mice. Thumkeo et al. (2011) found that mDia3 is co-localized with an actin belt connecting the apical surface of neuroepithelial cells, and that loss of mDia1/3 abolishes this actin belt and disrupts the apical integrity of the neuroepithelial surface (Fig. 16.6). Impaired neuroepithelial integrity then causes dysplastic mass formation, which

occasionally leads to occlusion of outflow of cerebrospinal fluid and causes hydrocephalus. Such dysplastic masses can also form in the central canal of the developing spinal cord and disrupt the midline chemoattractant gradient, which impairs axon guidance of the corticospinal neurons and the spinal cord interneurons, resulting in an abnormal rabbit-hopping gait (Toyoda et al. 2013). Consistently, these phenotypes have also been observed in RhoA knockout mice (Katayama et al. 2011, 2012). Shinohara et al. (2012) further showed that in the developing brain of mDia1/3 double KO mice, tangential migration of cortical and olfactory inhibitory interneuron progenitors, but not radial migration of excitatory neuron progenitors, is specifically impaired. They further showed that these defects reflect impairment of mDia-mediated actin cytoskeleton reorganization in migrating subventricular zone neuroblasts, precursors of interneuron. Beside of these phenotypes, mDia1/3 double knockout mice display abnormalities in coat color (T.I and S. N., unpublished). Thus, mDia isoforms contribute to formation of proper tissue architecture and maintenance of tissue homeostasis through their regulation of the actin cytoskeleton in a variety of cells.

16.3 Citron

16.3.1 Molecular Structure, Isoforms, Activity, Activation Mechanism, and Functions in the Cell

Citron was first identified by yeast two-hybrid system as a Rho- and Rac-binding protein in 1995 (Madaule et al. 1995). There are two isoforms of Citron, the longer form, Citron-K, containing a N-terminal kinase domain and the shorter form, Citron-N without a kinase domain (Di Cunto et al. 1998; Madaule et al. 1998). mRNAs coding for the two proteins are transcribed from the identical gene, but it remains unknown whether they are produced by alternative transcriptional initiation or alternative splicing. Citron-K and Citron-N share a central coiled-coil domain, followed by a zinc-finger domain, PH domain, a proline-rich domain (SH3-binding domain), and a PDZ-binding domain at the C-terminus (Fig. 16.1). Citron-K is highly expressed in proliferating cells (Di Cunto et al. 2000). Earlier studies indicated involvement of Citron-K in constriction of the contractile ring during cytokinesis (Madaule et al. 1998; Yamashiro et al. 2003). Later studies, however, revealed that Citron functions primarily in abscission to complete cytokinesis (Echard et al. 2004; Naim et al. 2004). Recent studies further demonstrated that the coiled-coil domain and not the kinase domain or C-terminal region of Citron is indispensable for its function of completion of cytokinesis (Bassi et al. 2011, 2013; Gai et al. 2011; Serres et al. 2012; Watanabe et al. 2013a). In the coiled-coil domain, the C-terminal portion is responsible for the localization in the cleavage furrow through Rho-binding, and a cluster-forming region concentrates Citron-K as a ring in the mid-body. On the other hand, the N-terminal half of

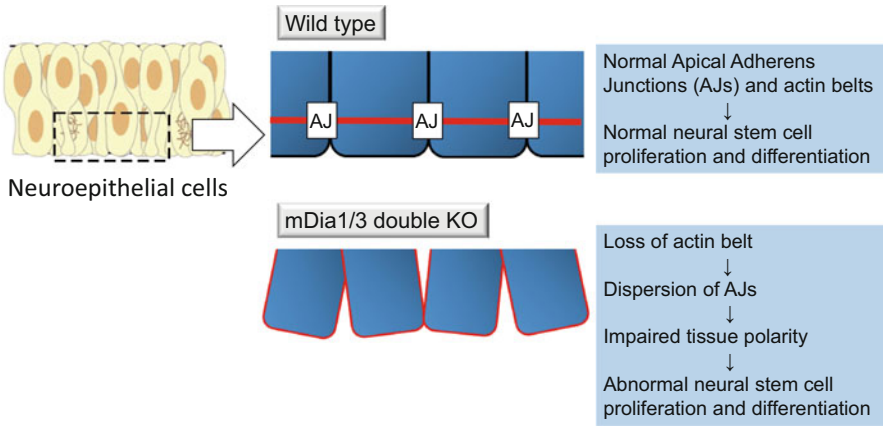

Fig. 16.6 mDia1/3 is indispensable for the proliferation and differentiation of neural stem cells through maintenance of actin belt at adherens junctions

the coiled-coil domain interacts with KIF-14, and this interaction is required for the timely transfer of Citron-K to the midbody after furrow ingression (Fig. 16.7). It is thought that, once the midbody is properly formed by the action of Citron-K, ESCORT molecules are recruited there and cleavage of the bridge occurs. In addition to abscission, it was also reported that Citron-K is involved in HIV virion production through modulating exocytosis. Loomis et al. (2006) showed that both the C-terminal half of the coiled-coil domain containing RBD and the zinc-finger domain are necessary for this process. Contrary to Citron-K, Citron-N is abundantly expressed in post-mitotic mature neurons (Di Cunto et al. 2000). Citron-N interacts with PSD-95 through its PDZ-binding domain, and this association contributes to the localization of Citron-N at the postsynaptic density (Furuyashiki et al. 1999; Zhang et al. 1999). However, the physiological importance of this interaction remains unknown. As for neuronal function, it was reported that Citron-N is involved in organization of Golgi apparatus in cultured hippocampal neurons through local regulation of the actin cytoskeleton assembly in collaboration with Rho, ROCK-II, and profilin-IIa (Camera et al. 2003). The same authors also reported that Citron-N recruits actin filaments and Golgi membrane at dendritic spines in primary neurons and is essential for maturation of dendritic spine in cultured cells and in vivo in the brain (Camera et al. 2008).

16.3.2 Functions In Vivo in the Body

Di Cunto et al. (2000) generated KO mice selectively deficient in Citron-K (Citron-$K^{null/null}$) by devising a gene targeting approach that produces a null allele for this particular isoform and spares Citron-N expression. Mice lacking Citron-K grow at a

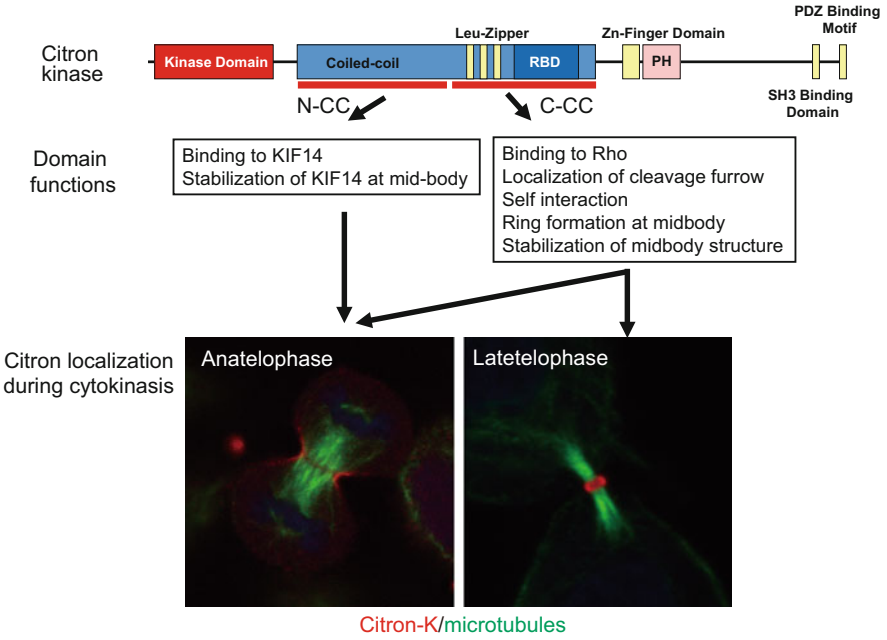

Fig. 16.7 Distinct roles of coiled-coil (CC) of Citron-K during cytokinesis. Domain structure (*upper*) and roles of CC during cytokinesis (*middle*) are shown. N-terminal part of CC and C-terminal part of CC are referred as N-CC and C-CC, respectively (*Bottom*). HeLa cells during cytokinesis are stained with Citon-K antibody (*red*) and tubulin (*green*)

slower rate around P10 and display ataxia. The authors found that Citron-K knockout mice have a defect in neurogenesis, with depletion of specific neuronal populations such as olfactory, hippocampal, and cerebellar granule neurons. In these, cytokinesis failure is followed by massive apoptosis in neuronal progenitor cells during brain development, which might be a causative reason of the phenotypic abnormality. Consistently, a similar phenotype is seen in flathead mutant rats, which Sarkisian et al. (2002) found have a single base deletion in exon 1 of the kinase domain of the Citron-K gene and contain no Citron-K protein. In addition to such phenotype in the brain, the loss of Citron-K results in abnormal cytokinesis and polyploidity of spermatogonia, which is again followed by induction of programmed cell death (Di Cunto et al. 2002). These results indicate that Citron-K plays a critical role in cytokinesis of several cell types in vivo. On the other hand, a Citron-N-specific knockout mouse has not been generated, yet. However, a mouse line lacking both Citron-K and Citron-N isoforms was generated. To address the in vivo function of Citron-N, Camera et al generated such a mouse line ($Citron^{null/null}$) lacking both Citron-K and Citron-N and investigated the function of Citron-N by comparing the two lines, $Ciron\text{-}K^{null/null}$ and $Citron^{null/null}$ mice (Camera et al. 2008). Although there was no difference on the cytokinesis defect in neuroblasts between the two lines, $Citron^{null/null}$ showed the immature morphology

of dendritic spines, spines of stubby and filopodia appearance, in primary cultured hippocampal neurons, and in cortical pyramidal neurons in vivo. Although the authors found that Citron-N is associated with actin filaments and ROCK in the spine, the detailed underlying mechanism by which Citron-N regulates the morphology of dendritic spines remains unclear.

16.4 Protein Kinase N (PKN)

16.4.1 Molecular Structure, Isoforms, Activity, and Functions in the Cell

The protein kinase N (PKN) (also called PRK) family of serine/threonine kinases comprises three isoforms, termed PKN1, PKN2, and PKN3 (Mukai 2003). These isoforms are closely related and exhibit variation within their regulatory domains located at the N-terminus (Fig. 16.1). PKN not only binds to Rho (Amano et al. 1996b; Lu and Settleman 1999) but also responds to phosphoinositides (Palmer et al. 1995) and fatty acids such as arachidonic, linoleic, and oleic acid (Kitagawa et al. 1995; Lim et al. 2005; Mukai et al. 1994). By apoptotic stimuli, PKN2 is proteolytically cleaved, and the C-terminal fragment directly binds to and inhibits Akt (Koh et al. 2000; Takahashi et al. 1998). In addition, PDK1, an Akt activator, is also inhibited by PKN1 and PKN2 (Balendran et al. 2000; Biondi et al. 2000; Dettori et al. 2009; Wick et al. 2000). This indicates that PKN may negatively regulate AKT signaling. It was also reported that PKN is involved in several cellular functions including cell migration (Lachmann et al. 2011), cell adhesion (Calautti et al. 2002), vesicle transport (Torbett et al. 2003), apoptosis (Takahashi et al. 1998), glucose transport (Standaert et al. 1998), and cell cycle regulation (Misaki et al. 2001). Furthermore, the individual isoform can be activated upon specific receptor signaling such as the androgen receptor for PKN1 (Metzger et al. 2003), CD44 for PKN2 (Bourguignon et al. 2007), and insulin for PKN3 (Leenders et al. 2004) suggesting that each isoform might have specific roles. However, how PKN acts in these various cellular events are still largely unknown.

16.4.2 Functions In Vivo in the Body

In vivo roles of PKN were first reported in Drosophila. Lu and Settleman (1999) showed that the loss of PKN in Drosophila as well as Rho1 mutation impairs the dorsal closure, suggesting that PKN works downstream of Rho and is required for the cell shape change during embryogenesis. Recently, Yasui et al. (2012) generated PKN1 knockout mice and analyzed their phenotype. PKN1 knockout mice are born in a Mendelian ratio and exhibit normal appearance. However, after more than

30 weeks, they spontaneously form germinal centers in the spleen in the absence of immunization or infection and develop autoimmune-like disease. PKN1$^{-/-}$ B-cells were hyperresponsive and had increased phosphorylated Akt1 levels upon BCR stimulation. These results led the authors to suggest that PKN1 negatively regulates AKT in vivo and is indispensable for physiologically appropriate germinal center B-cell selection through regulation of AKT activity upon BCR stimulation.

16.5 Rhophilin

Rhophilin was identified as a GTP-Rho-binding protein by yeast two-hybrid screening using the active form RhoA as a bait (Watanabe et al. 1996). To date, two Rhophilin isoforms, Rhophilin-1 and Rhophilin-2, are known in mammals. Both isoforms of Rhophilin are composed of N-terminal RBD and C-terminal proline-rich and PDZ domains. Rhophilin-1 is highly expressed in testis, kidney, and, at a lower level, in brain and stomach (Nakamura et al. 1999). Rhophilin-1 is specifically localized in sperm flagella (Nakamura et al. 1999). Although the function of Rhophilin in sperm is unknown, the presence of a PDZ domain at the C-terminus of Rhophilin suggests that Rhophilin acts as an adaptor molecule. The PDZ domain of Rhophilin contributes to its binding to another sperm protein, Ropporin (Fujita et al. 2000). The amino acid sequence of Ropporin showed high homology to that of the regulatory subunit of type II cAMP-dependent protein kinase, which is involved in dimerization and binding to A-kinase anchoring proteins. Electron microscopy revealed that Ropporin is mostly localized to the inner surface of the fibrous sheath of the sperm while Rhophilin is present in the outer surface of the outer dense fiber, suggesting that Rhophilin and Ropporin may form a complex in sperm flagella. Despite limited tissue distribution of Rhophilin, the function of Rhophilin-1 remains unknown. On the other hand, Rhophilin-2 is ubiquitously expressed (Peck et al. 2002). Rhophilin-2 was initially isolated by differential screening of a chronically thyrotropin (TSH)-stimulated dog thyroid cDNA library (Behrends et al. 2005). Steuve et al. (2006) showed that Rhophilin-2 is localized to late endosomes in a RhoB-dependent fashion. However, the physiological significance of Rhophilin-2 localization to late endosomes remains unknown. A Rhophilin-2 knockout mouse line was already generated, but no apparent abnormality has been observed (Behrends et al. 2005).

16.6 Rhotekin

Rhotekin was identified as a Rho-binding protein by yeast two-hybrid screening (Reid et al. 1996). There are two isoforms of Rhotekin (Rhotekin1 and Rhotekin2) expressed in mammalian cells. While Rhotekin1 is ubiquitously expressed, expression of Rhotekin 2 is only found in a particular lineage of lymphocytes such as in

CD4 positive T-cells and bone marrow-derived B-cells (Collier et al. 2004). Rhotekin is highly expressed in human gastric cancer and many cancer-derived cell lines, while its expression is low in normal cells. Liu et al. (2004) found that overexpression of Rhotekin in cells with low Rhotekin expression conferred resistance to apoptosis induced by serum starvation and sodium butyrate, while depletion of Rhotekin by RNAi sensitized cells to apoptosis. They further showed that overexpressed Rhotekin activated NFκB in a Rho-dependent manner, and this activation is required for anti-apoptotic activity. Biochemically, the Rho-binding domain (RBD) of Rhotekin binds selectively to GTP-Rho with high affinity and has been widely used to detect the amount of the active GTP-bound form of Rho proteins in cell and tissue lysates.

16.7 Future Perspective

Research on Rho effectors and their signaling has attracted much attention since the discovery of the first Rho effector, Citron, in 1995. Since then, knowledge of the functions of individual Rho effector proteins in vitro and in vivo has been accumulating. In the future, analysis of tissue-specific Rho effector knockout mice is expected to give us much more knowledge of their physiological functions. Notably, while a number of Rho effector proteins are occasionally expressed in the same cells simultaneously, their functional relationship and spatiotemporal activation mechanism remain largely unknown. In parallel with dissection of functions of each effector molecule, comprehensive dissection of Rho effector crosstalk is indispensable for understanding the physiological significance of Rho signaling.

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Chapter 17
Principles Driving the Spatial Organization of Rho GTPase Signaling at Synapses

Scott H. Soderling and Linda Van Aelst

Abstract The Rho proteins play critical roles in numerous aspects of neuronal development, and mutations in their regulators (GEFs and GAPs) and effectors underlie multiple neurodevelopmental and neurological disorders. How Rho GTPase-mediated signaling can have a hand in regulating so many different neurobiological processes remains a challenging question. An emerging theme is that GAPs and GEFs, through their spatial/temporal regulation and/or through additional protein–protein interactions, cooperate in making connections between upstream signals and the downstream signaling output, engaging distinct effector proteins. This chapter focuses on recent evidence illustrating distinct modes of regulation and specialized roles of Rho regulators particularly in the context of synaptic structure, function, and plasticity, and how their dysregulation affects behavioral processes and contributes to disease.

Keywords Rho regulators • Rho effectors • Neuronal development • Synaptic structure and function • Brain disorders

17.1 Introduction

Rho-family GTPases (typified by Rho, Rac, and Cdc42) are a branch of the Ras superfamily of small G-proteins, consisting of 22 different Rho GTPases. They function as intracellular molecular switches that, among other functions, rapidly activate actin polymerization and reorganization in vivo (Van Aelst and D'Souza-

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Schorey 1997; Hall 2012). Rho-family GTPases are active when bound to GTP, which induces a conformational alteration that realigns two surface regions, known as switch one and two regions (Abdul-Manan et al. 1999). Upon realignment, the switch regions bind to and modulate the activity of a wide variety of downstream effectors, including kinases and regulators of actin polymerization (Bishop and Hall 2000). Activation of Rho GTPases is mediated by Guanine Nucleotide Exchange Factors (GEFs), while inactivation is accelerated by GTPase Activating Proteins (GAPs) (Cook et al. 2013; Bos et al. 2007; Cherfils and Zeghouf 2013).

The birth of the Rho-family GTPase field was the discovery of Rho (*R*as *ho*molog) (Madaule and Axel 1985), the founding member of the Rho family. Interestingly, Rho was first identified from the sea slug Aplasia, using a low stringency screen for homologs of the alpha subunit of human chorionic gonadotropin (hCG). The resulting cDNA clone however showed no significant similarity to hCG but to Ras and was evolutionally conserved in humans, distinguishing it as a new branch of the Ras superfamily of GTPases. It was subsequently shown in the 1990s by Alan Hall's laboratory to potently regulate actin formation in cells (Paterson et al. 1990). These seminal observations drove the formation of a new field of study, which rapidly expanded, and has shown that the breadth of cellular functions regulated by Rho-family GTPase activity is truly remarkable.

Almost 1 % of human proteins are either regulators or effectors for the 22 different Rho GTPases (Jaffe and Hall 2005). Consistent with the wide variety of interaction partners, the regulation of Rho-family GTPase signaling pathways drive many key functions of developing and mature neural networks, including polarization, axonal guidance, dendritic arborization, intracellular trafficking, migration, and synapse formation and plasticity (Govek et al. 2005; Luo 2000; Tolias et al. 2011; Tahirovic and Bradke 2009; Guan and Rao 2003; Hall and Lalli 2010; Saneyoshi et al. 2008; Lai and Ip 2013; Colgan and Yasuda 2013). The dysregulation of Rho-family GTPase pathways are also associated with some of the most enigmatic neuropsychiatric disorders, including intellectual disability, schizophrenia, and autism (Newey et al. 2005; Tolias et al. 2011; van Galen and Ramakers 2005; van Bokhoven 2011; Boda et al. 2010; DeGeer and Lamarche-Vane 2013; Nadif Kasri and Van Aelst 2008).

Yet the discoveries that the Rho GTPases occupy a central role in so many different neurobiological functions have also led to several conundrums. For example, how can specificity be achieved downstream of the activation of Rho, Rac, or Cdc42 when they seemingly have a role in so many cell functions? This problem is exemplified by one of the remarkable features of the Rho-family GTPases, which is their ability to interact with many different regulators and effectors. For example, Rac activity is regulated by several different GEFs or GAPs, many of which are co-expressed in the same cell. Furthermore, once activated, Rac can bind to and modulate the activity of an even larger number of different downstream effectors.

The large excess of regulators and effectors when compared to Rho GTPases means that individual GTPases do not function as simple binary switches. Rather, they behave as signaling multiplexers that can pair a given upstream cue with a

specific cellular effector. The most important unanswered question in the field is how a GTPase achieves specificity when faced with such a large diversity of potential interactions? Assembling the correct complement of regulators and effectors to fulfill specific neuronal functions is a major challenge in the field. Here we review literature to propose that specificity of neuronal Rho GTPase functions is achieved by three general mechanisms: (1) *Input Targeting*—interactions between GAPs and GEFs with receptors; (2) *Signaling Clustering* by scaffolding and linker proteins; and (3) *Effector Clustering*—linking GAPs and GEFs to downstream Rho GTPase targets. For space considerations we primarily focus on examples of these mechanisms that operate to regulate the functions of synapses. However these mechanisms are also used to drive many other important tasks, including neural migration and axonal outgrowth and guidance.

17.2 Input Targeting

Initial responses to external stimuli are transduced by the initial engagement of neuronal transmembrane receptors, resulting in the rapid organization of adhesion, trans-synaptic morphogenesis, and electrical responses to neurotransmitters. Thus, an efficient mechanism to integrate specific responses to ligand binding is to physically couple signaling molecules to receptors, ensuring the spatial and temporal specificity between the initiation and propagation of synaptic signals. Recent studies have uncovered several mechanisms by which GEFs and GAPs for Rho GTPases are specifically tethered to receptors, and in many cases regulated by neuronal receptor activity (Fig. 17.1a). These studies reveal that receptor binding is an important mechanism to specify the timing of synapse development as well as distinguish between excitatory versus inhibitory synapses.

17.2.1 Bidirectional Regulation of Excitatory Synapse Formation by Eph Receptor and GEF Complexes

Eph receptors represent a large class of receptor tyrosine kinases that are classified as either EphA or EphB receptors by their preference for a certain type of ephrin ligands (Lai and Ip 2009). EphR-ephrin interactions are critical for excitatory synaptogenesis, a process that must be regulated to allow for coordinated pre- and postsynaptic specialization at the correct time and place during development (Sheffler-Collins and Dalva 2012; Hruska and Dalva 2012). The molecular mechanisms by which EphB receptors regulate postsynaptic development have been elucidated by several studies, which highlight a central role for receptor tethering of Rho-family GEFs. Surprisingly, differential GEF anchoring to these receptors is important for both the inhibition and promotion of dendritic spine formation. Thus,

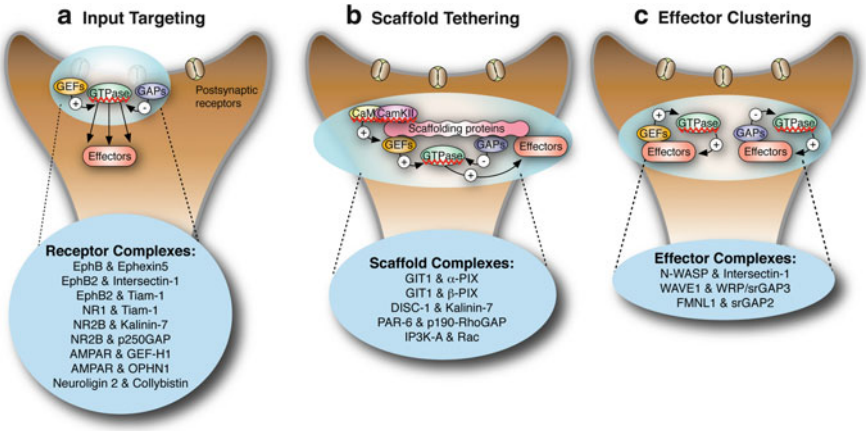

Fig. 17.1 Schematic of the organizational principles directing Rho-family GTPase signaling at synapses. Three different mechanisms to organize Rho-family GTPase-based signaling complexes are shown. In each panel the postsynaptic dendritic spine is shown with different schematics of signaling complexes. Note that some complexes exist within inhibitory synapses (i.e., Neuroligin 2 and Collybisitin) rather than excitatory spines. (**a**) Input targeting representing protein–protein complexes of Rho-family GTPase regulators (GEFs and GAPs) with different receptors present at synapses. (**b**) Scaffold tethering representing the formation of complexes of GEFs or GAPs along with downstream Rho-family effector proteins within a single protein complex. Note scaffolding proteins such as GIT1 also incorporate regulators of GEFs and GAPs such as kinases to these protein complexes. (**c**) Effector clustering occurs when GAPs or GEFs are in physical complex with Rho-family GTPase effectors. This can allow for specific pairing of GTPase regulators to individual downstream targets. Specific examples of protein interactions representing each mode of signaling regulation are shown below

it appears the sequential recruitment of different GEFs to EphB receptors coordinate the developmental timing of synapse development.

Early in dendritic development, before the initiation of spinogenesis, EphB receptors are present but are likely to remain unbound to ephrins until a presynaptic bouton makes contact. In this pre-synaptogenesis state, EphB receptors are in complex with the GEF ephexin-5 (E5) (Margolis et al. 2010). E5 functions to specifically activate RhoA. Active RhoA inhibits dendritic spine formation (Luo 2000; Nakayama et al. 2000; Tashiro et al. 2000). Mice lacking E5 have significantly reduced levels of activated RhoA, indicating it is a major regulator of neuronal RhoA in vivo. E5 knockout mice exhibit elevated numbers of excitatory synapses, indicating that the GEF activity of E5 limits synaptogenesis. Importantly, EphB receptor activation by ephrin binding (which promotes spine formation) initiates the tyrosine phosphorylation of E5, triggering its recognition as a substrate by the ubiquitin ligase Ube3a. Ube3a-mediated ubiquination leads to proteasomal degradation and loss of E5, alleviating the E5-activated RhoA brake on synaptogenesis. Loss of Ube3a is the primary cause of Angelman's Syndrome (Kishino et al. 1997; Matsuura et al. 1997), suggesting that altered regulation of E5 levels may contribute to the synaptic abnormalities in these syndromes.

Consistent with this possibility, Ube3a mutant mice, a model for Angelman's syndrome, have elevated levels of E5 (Margolis et al. 2010).

GEF-EphB interactions, however, orchestrate not only the RhoA inhibition of synaptogenesis but also the subsequent promotion of synaptogenesis (Irie and Yamaguchi 2002). EphB2 ligand binding potently stimulates Cdc42 activation in neurons in a time course that corresponds with EphB2 auto-phosphorylation, suggesting that activation of EphB2 is closely linked to Cdc42 activation. Co-immunoprecipitation analysis demonstrated that EphB2 activates Cdc42 by binding to the N-terminal region of Intersectin-1, which is a brain enriched Cdc42 GEF (Irie and Yamaguchi 2002; Thomas et al. 2009). Intersectin-1 has very low basal activity, which is stimulated upon its binding to EphB2. Importantly, Cdc42 activity is critical for the maturation of spines, in part by activating the Cdc42 effector protein N-WASP. N-WASP is a member of larger family of proteins (including WAVE1) whose activation stimulates Arp2/3-dependent polymerization of branched actin filaments that are required for spine head development during the transition from dendritic filopodia to spines (Wegner et al. 2008; Hotulainen et al. 2009). As discussed in the effector clustering section, Intersectin-1 interacts with N-WASP as well (Hussain et al. 2001), suggesting a model of tight spatial and temporal regulation of Cdc42 activation and effector binding within an EphB2 complex. Interestingly, activation of EphB2 also triggers the recruitment of the Rac GEF Tiam-1 to sites of new synaptic contacts, resulting in the phosphorylation of Tiam-1 and subsequent activation of Rac, the latter being important for spine formation (Tolias et al. 2011). Thus Rac and Cdc42 activities downstream of EphB2 are likely to cooperate to facilitate spine formation.

17.2.2 Focal Regulation of Rac by NMDA Receptor Tethering of GEFs and GAPs

Upon maturation, excitatory postsynaptic spines contain a protein-rich postsynaptic density (PSD) containing arrays of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)- and *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. These receptors organize signaling complexes of Rho-family GTPase regulatory proteins and are anchored within the PSD by several associated actin cytoskeletal proteins (Tada and Sheng 2006; Newpher and Ehlers 2009).

NMDA receptors (NMDARs) are tetramers predominately composed of NR1 and NR2 subunits. Their synaptic activity-dependent activation produces modification of synaptic AMPARs and forms the basis of the well-established LTP (long-term potentiation) and LTD (long-term depression) forms of synaptic plasticity (Sudhof and Malenka 2008; Kessels and Malinow 2009; Huganir and Nicoll 2013). NMDARs mediate calcium influx into the spine and are required for Rho-family GTPase activation and actin polymerization during spine structural plasticity associated with LTP (Govek et al. 2005; Cingolani and Goda 2008; Tolias et al. 2011;

Kiraly et al. 2010a; Carlisle and Kennedy 2005). One mechanism driving this is the specific association between the aforementioned Rac GEF Tiam-1 and the NR1 NMDAR subunit (Tolias et al. 2005). Activation of NR1 induces the rapid phosphorylation and activation of Tiam-1, presumably by calcium activation of CaMKII. Tiam-1 activation of Rac mediates the induction of dendritic arborization and spine development downstream of NR1 activity. The importance of Tiam-1 tethering to NR1 could be to facilitate its phosphorylation by CaMKII, which binds to the NR2B subunit and is thereby locked in an active conformation (Bayer et al. 2001; Sanhueza et al. 2011). Localization of Tiam-1 to NR1 may also bias its activation by placing it within NMDAR local calcium micro-domains in the spine. In this way, Tiam-1 can be positioned for the rapid phosphorylation by NMDA-mediated CaMKII activation. As Tiam-1 forms also a stable interaction with EphB2, it is noteworthy that EphB2 is important for NMDAR clustering and function (Dalva et al. 2000; Takasu et al. 2002), thus placing Tiam-1 as a bridge between these receptors.

NMDARs are also anchored within the postsynaptic density to actin by the actin binding and crosslinking protein, α-actinin-2 (Wyszynski et al. 1997). Activity-dependent rundown of NMDAR current is enhanced by destabilizing actin and is blocked by stabilizing actin filaments, suggesting that tethering of NMDAR to the actin cytoskeleton reduces its synaptic turnover (Rosenmund and Westbrook 1993). Kalirin-7 is a PSD enriched Rac-specific GEF. It plays a role in spine development and structural and functional plasticity (Penzes and Jones 2008) and stabilizes the NMDAR through a specific interaction with the juxtamembrane region of the NR2B subunit (Kiraly et al. 2011). It is thought that this binding may be competitive with an AP2-binding site on NR2B that is important for stimulating NMDAR endocytosis. Indeed, Kalirin-7 knockout mice have reduced levels of synaptic NR2B, supporting the notion that it normally stabilizes the receptor at the synapse. Importantly, Kalirin-7- and NR2B-deficient mice exhibit overlapping behavioral phenotypes, including a deficit in conditioned place preference for cocaine (Lemtiri-Chlieh et al. 2011; Kiraly et al. 2010b; Kiraly et al. 2011). This suggests that the loss of Kalirin-7 interaction with NR2B in the Kalirin-7 knockout mice may be important for the learning involved in this aspect of addiction.

The intracellular tail of NR2B additionally tethers the Rho-family GAP p250GAP (also known as RICS) (Nakazawa et al. 2003; Okabe et al. 2003). p250GAP knockdown increases Rac activity and increases spine number and miniature excitatory postsynaptic potential (mEPSP) frequency, suggesting that this GAP is important for regulating synaptogenesis via inhibition of Rac activity (Impey et al. 2010). Importantly, the effect of p250GAP knockdown requires the activity of Kalirin-7. Thus, the intriguing possibility exists that p250GAP and Kalirin-7 coordinate both the activation and inactivation of Rac by local clustering via NR2B. It is unclear if such a complex would be important for regulating synaptogenesis, synaptic plasticity, or both. The regulation of p250GAP activity is unknown, but it is possible that it could be clustered with NR2B to specifically regulate pools of Rac activated by NMDAR-associated Kalirin-7. Finally, it should be noted that p250GAP has recently been identified as a risk loci for schizophrenia-

associated disorders (Ohi et al. 2012). This is interesting given the evidence linking NMDAR hypofunction to schizophrenia (Coyle et al. 2003; Snyder and Gao 2013), yet additional work with larger cohorts will be required to determine its possible importance.

17.2.3 Interplay Between AMPA Receptors and RhoA Activity via GEFs and GAPs

While Rac regulators are linked to NMDARs, it is intriguing that RhoA regulators have been found in a complex with AMPARs. In a screen for AMPAR-binding proteins, Kang et al identified the RhoA-specific GEF GEF-H1/Lfc as a component of the AMPAR complex in the brain (Kang et al. 2009). This interaction was found to be important for AMPAR activity-dependent regulation of spine development (Kang et al. 2009). Previous studies had implicated AMPAR activity in the stabilization of dendritic spines, with inhibition of AMPAR activity significantly reducing spine density (McKinney et al. 1999; Fischer et al. 2000). The underlying signaling mechanisms remained however elusive. Kang et al. demonstrated that the decrease in spine density caused by blocking AMPAR activity was associated with an increase in RhoA activity. Even more importantly Kang el al. showed that knockdown of GEF-H1 expression was able to eliminate both the decrease in spine density and increase in RhoA activity. Thus, an increase in AMPAR activity is likely to trigger the inactivation of GEF-H1 and consequently RhoA activity, thereby stabilizing spine structure. How AMPAR activity influences GEF-H1/Lfc function remains currently unknown. Notably, in spines GEF-H1/Lfc also forms a complex with Spinophilin and Neurabin, two actin interacting proteins. Association between GEF-H1/Lfc and these proteins was reported to modulate the actin cytoskeleton in a Rho-dependent manner, thereby contributing to spine development (Ryan et al. 2005).

The Rho-GAP OPHN1 was also found to form a complex with AMPARs (Nadif Kasri et al. 2009). OPHN1, however, does not seem to be regulated by AMPAR activity, but instead OPHN1 regulates the stabilization of synaptic AMPARs. In particular, NMDAR activation was shown to drive OPHN1 into dendritic spines, where it then forms a complex with AMPAR. In turn, OPHN1 signaling regulates activity-dependent AMPAR stabilization, as well as maintenance of spine structure, thereby permitting synaptic maturation and plasticity. The mechanism by which OPHN1 controls these events involves its Rho-GAP activity and a RhoA/Rho-kinase signaling pathway (Nadif Kasri et al. 2009; Govek et al. 2004). A likely scenario is that spine enriched OPHN1 contributes to the stabilization of AMPARs by locally inactivating RhoA/Rho-kinase activities and modulating actin dynamics (i.e., in the proximity of AMPARs). Consequently, decreased or defective OPHN1 signaling results in destabilization of synaptic AMPARs, leading to impairment in synapse maturation and plasticity and eventually loss of spines. This is of particular

relevance, as *OPHN1* mutations have been identified in families with mental retardation associated with cerebellar hypoplasia and lateral ventricle enlargement (Billuart et al. 1998; Bergmann et al. 2003; Philip et al. 2003; des Portes et al. 2004; Zanni et al. 2005). Thus, glutamatergic dysfunction and defects in early circuitry development caused by OPHN1 mutations could be an important contributory factor to the cognitive deficits observed in *OPHN1* patients. Noteworthy, OPHN1 has also been implicated in another form of plasticity, namely mGluR-LTD that relies on the activation of group I metabotropic glutamate receptors (mGluRs) and occurs at later developmental stages (Nadif Kasri et al. 2011). In this case, however, OPHN1, whose expression is rapidly induced by mGluR activation, exerts its effects via interaction with members of the endophilin-A family, endophilin A2 and A3 (Endo 2/3), which mediate the downregulation of surface AMPARs during mGluR-LTD (Nadif Kasri et al. 2011). Thus, OPHN1 likely operates during adulthood to weaken synapses in response to behaviorally relevant stimuli. In light of the previously reported role for LTD in behavioral flexibility and novelty detection (Kemp and Manahan-Vaughan 2007; Luscher and Huber 2010), the requirement of OPHN1 in mGluR-LTD could offer an intriguing potential explanation for some of the behavioral deficits exhibited by *OPHN1* patients.

17.2.4 Specification of Inhibitory Synapses by a Neuroligin 2 and Collybistin Interaction

Although excitatory synapses receive a disproportionate amount of attention, it should be emphasized that Rho-family GTPase signaling is likely to be important for inhibitory synapse formation as well. Collybistin, a Cdc42 GEF, is mutated in human disorders of epilepsy/hyperekplexia and intellectual disability (Shimojima et al. 2011; Lesca et al. 2011; Kalscheuer et al. 2009; Marco et al. 2008; Harvey et al. 2004). One such mutation that is associated with epilepsy is a missense mutation of a critical residue within the SH3 domain of collybistin (Harvey et al. 2004). Recently it was discovered that this SH3 domain is selective for binding to neuroligin 2, an organizer of nascent inhibitory synapses that mediates trans-synaptic neuroligin/neurexin interactions (Poulopoulos et al. 2009). It was previously shown that collybistin facilitates the membrane targeting of gephryin, the primary scaffolding protein of inhibitory synapses, but that the SH3 domain of collybistin inhibits this function (Harvey et al. 2004; Kins et al. 2000; Papadopoulos et al. 2007). Gephyrin interacts with a conserved 15 amino acid region that is present in all neuroligins (Poulopoulos et al. 2009). This suggested that there must be a molecular mechanism to specify gephryin clustering at developing neuroligin 2 inhibitory synapses, but not at developing excitatory synapses organized by neuroligin 1. Thus, the specific interaction between collybistin and neuroligin 2 appears to explain how specific clustering of gephyrin can occur at inhibitory synapses. Importantly, collybistin knockout mice exhibit a striking loss of

inhibitory synapses, which is accompanied by significant changes in hippocampal plasticity. These effects are associated with increased levels of anxiety and impaired spatial learning (Papadopoulos et al. 2007). As yet, it is still not clear what the exact role of the GEF domain is for collybistin during inhibitory synapse development or maintenance (Papadopoulos and Soykan 2011). Collybistin is a Cdc42 GEF, but it remains to be seen whether Cdc42 regulates inhibitory synapse formation or maintenance (Tyagarajan et al. 2011; Reddy-Alla et al. 2010). It is possible that future work will identify additional Rho-family GTPases that are activated by collybistin that regulate inhibitory synapses. It should be noted that the Rac GAPs, srGAP2 and WRP/srGAP3, also interact directly with gephryin and appear to facilitate inhibitory synapse formation (Okada et al. 2011). Indeed, loss of WRP in mice results in reduced densities of gephyrin and GABA-A receptor clusters in the hippocampal formation. Thus, inhibitory synapse function is likely to be fine-tuned by coordinated action of GEFs and GAPs organized by a neuroligin 2/gephryin complex, yet the relevant GTPases remain to be clarified.

17.3 Scaffolding and Linker Proteins Focus Rho GTPase Signaling

Transfer of information from one enzyme to the next in cell signaling cascades is often organized around protein scaffolds. These platforms for signaling allow for increased signal efficiency, signaling precision, and can also facilitate the diversity of cellular functions a given enzyme can regulate (Pawson and Scott 2010). Given that a single Rho-family GTPase can regulate multiple different cellular processes, this has emerged as an important mechanism for specifying the whens and wheres of a Rho-family GTPase action (Fig. 17.1b).

17.3.1 A GIT1 and Rac Signaling Scaffold Involved in Synaptogenesis and Attention Deficit Hyperactivity Disorder

GIT1 (G-protein-coupled receptor kinase-interacting protein 1) is a multifunctional scaffolding and adaptor protein composed of multiple domains (Hoefen and Berk 2006). This includes a GAP domain for Arf GTPases as well as a Spa Homology Domain that binds to the Rac and Cdc42 GEFs α-PIX and β-PIX. The interaction between GIT1 and β-PIX is of high affinity, in the nanomolar range, and may be organized in a heteropentameric structure containing a GIT1 dimer and β-PIX trimer (Schlenker and Rittinger 2009). The functional implications of this higher order structure are unknown, but may facilitate the coordinated binding of multiple signaling molecules with β-PIX. β-PIX GEF activity is enhanced within the context

of GIT1 by the ability of GIT1 to also scaffold the CaMKKβ and CaMKI kinases (Saneyoshi et al. 2008). Kinases often interact with scaffolding proteins in order to preferentially target their activity towards substrates. Indeed, CaMKKβ phosphorylation of CaMKI is induced by NMDA-mediated calcium influx, activating the CaMKI-mediated phosphorylation of β-PIX at serine 516. This potently stimulates its GEF activity towards Rac and is required for excitatory synapse development. Interestingly, β-PIX also binds to PAK (p21 activated kinase) via an N-terminal SH3 domain (Mott et al. 2005), suggesting that the GIT1/β-PIX/PAK complex can organize and regulate β-PIX GEF activity and the Rac-mediated activation of PAK. Loss of GIT1 in mice results in reduced dendritic arborization and spine density and in multiple Attention Deficit Hyperactivity Disorder (ADHD)-like behaviors (Won et al. 2011; Menon et al. 2010; Schmalzigaug et al. 2009). Importantly, GIT1 SNPs (single nucleotide polymorphisms) that reduce GIT1 expression are associated with human ADHD (Won et al. 2011). In mice, GIT1 loss specifically reduces Rac1 activation, but does not alter the active levels of the Arf GTPase Arf6, supporting a key role of GIT1 in Rac signaling (Won et al. 2011). Surprisingly GIT1 knockout mice also have specific impairments in pre-synaptic inhibitory input, indicating that GIT1 additionally regulates presynaptic organization and actin dynamics. This is supported by other studies reporting that β-PIX regulates actin polymerization required for synaptic vesicle recruitment during initial axonal bouton formation (Sun and Bamji 2011).

17.3.2 Keeping Rac Activation in Check by a Disc-1/PSD95/Kalirin-7 Complex

While the GIT1 signalosome serves to cluster β-PIX and downstream effectors of Rac, it is also important to insure that Rac is held inactive until the appropriate moment. Recent work identified the Rac GEF Kalirin-7 in a complex with Disc-1 and PSD-95 that limits Rac activation (Hayashi-Takagi et al. 2010). Disc-1 is a schizophrenia susceptibility gene originally identified as a causal mutation in a Scottish family with significant psychosis (Millar et al. 2000; St Clair et al. 1990). PSD-95 is the major structural protein of the excitatory post-synaptic density that links multiple signaling proteins to receptors at the postsynaptic membrane (Kim and Sheng 2004). Disc-1 binding to Kalirin-7 inhibits its ability to bind to and promote Rac activation (Hayashi-Takagi et al. 2010). Importantly, the complex between Disc-1, Kalirin-7, and PSD95 is rapidly disassembled in response to synaptic activity and NMDA activation. The release of Kalirin-7 from the inhibitory complex corresponds with a rapid activation of synaptic Rac. Although the effect of PSD95 on Kalirin-7 may be to enhance its localization to the PSD, its dissociation in response to synaptic activity may also facilitate Rac activation. For example, PSD95 interacts with the Rac GAPs BCR as well as with ABR that could further oppose Rac activation (Oh et al. 2010). The long-term consequences of

overactive Rac signaling or the loss of Disc-1 were also assessed, as glutamatergic synapse dysfunction is implicated in schizophrenia (Hayashi-Takagi et al. 2010). Interestingly, it was found that over time active Rac, or the loss of Disc-1, significantly decreased spine size, which might be related to spine abnormalities observed in postmortem schizophrenia samples. These results, in combination with the GIT1 studies, reveal how scaffolding proteins can bidirectionally modulate Rac signaling in space and time and indicate that the loss of this regulation may be associated with neuropsychiatric disorders.

17.3.3 Tuning p190-RhoGAP Function by PAR-6 and Arg to Control RhoA Activity in Spine and Dendrite Stabilization

As mentioned above, in addition to Rac, the spatial-temporal control of RhoA levels/activity is also critical for the proper formation and stabilization of dendritic spines. While it is known that RhoA levels during synaptogenesis are regulated by Ube3a-mediated ubiquitination and degradation of the Rho GEF ephexin-5, the regulation of RhoA levels/activity in maturing spines to govern their maintenance/stabilization is an enduring question. One key emerging player in the stabilization of spines as well as dendritic branches is the p190A-RhoGAP (p190), which is expressed at high levels in the adolescent/mature brain (Lamprecht et al. 2002; Settleman 2003). Somewhat unexpectedly, the polarity protein PAR-6 in a complex with atypical PKC (aPKC), but independent of its interaction with PAR-3, was found to contribute to spine maintenance, by reducing the activity of RhoA in spines in a p190-dependent manner (Zhang and Macara 2008). Indeed, evidence was presented that PAR-6, via its N-terminal PB1 domain, binds to and recruits aPKC to spines. Spine localized aPKC in turn either directly or indirectly triggers the phosphorylation of p190, thereby locally inactivating RhoA activity and contributing to the stabilization of spines (Zhang and Macara 2008). The upstream input(s) that regulate PAR-6 in neurons still remain(s) unknown. While in other systems, this involves the binding of PAR-6 via its CRIB domain to Cdc42-GTP; this does not seem to be the case in neurons (Zhang and Macara 2008). A possible scenario could be that the PAR-6/aPKC complex is coupled to AMPARs, as described above for the Rho GEF GEF-H1/Lfc.

Additionally, p190 was found to be phosphorylated in neurons by Arg (Abl-related gene), a member of the Abl non-receptor tyrosine kinase family (Hernandez et al. 2004; Sfakianos et al. 2007). Neurons in mice that lack Arg develop normally through postnatal day P21 (P21); however by P42 these mice lose dendritic spines and synapses and display reductions in dendritic arbor size complexity. Notably, these deficits are coincident with the impairment in memory tasks by the loss of Arg in mice (Sfakianos et al. 2007; Kerrisk and Koleske 2013). Arg promotes phosphorylation of p190, which then can bind to two SH2 domains in

p120RasGAP (p120). p190 is recruited to the plasma membrane by the PH and CalB domains of p120 GAP, where it then diminishes RhoA activity (Bradley et al. 2006). Intriguingly, the Arg/p190-mediated reduction in RhoA activity, while critical for dendrite arbor stability, does not appear to be involved in spine stabilization (Lin et al. 2013). Indeed, reducing RhoA activity in Arg knockdown neurons blocked dendrite loss, but did not rescue the spine/synapse loss observed in these neurons. Instead, spine destabilization in Arg knockdown neurons was prevented by blocking NMDAR-dependent relocalization of cortactin from spines or by forcing cortactin into spines via fusion to an actin-binding region of Arg (Lin et al. 2013). Thus, the Arg-p190 axis preserves dendrite structure in early adulthood by attenuating Rho activity, while Arg interacts with the NMDAR and cortactin to control spine stabilization. Together with the PAR6 studies, these results indicate that dependent on what protein complex p190 is in, it dampens RhoA activity to control either spine or dendrite stabilization.

17.3.4 Anchoring Rac by IP3K-A to Actin During Synaptic Plasticity

Although the primary mechanism to regulate Rho-family GTPase signaling is by the focal targeting of their regulators, the targeting of Rho-family GTPases themselves by scaffolding proteins can also occur. Perhaps the best example of this is IP3K-A, an F-actin-binding lipid kinase (Schell et al. 2001). Prior work had established that IP3K-A is highly expressed in neurons and phosphorylates inositol 1,4,5-trisphosphate (IP3) to generate inositol(1,3,4,5)tetra-kisphosphate (IP4). Thus it modulates intracellular calcium release mediated by IP3 (Choi et al. 1990; Irvine et al. 1986). Surprisingly, it was found that IP3K-A directly modulates actin polymerization in cells, independent of its kinase activity (Windhorst et al. 2008; Kim et al. 2009). IP3K-A is recruited to spines by synaptic activity via its F-actin-binding domain and was found to bind selectively to activated Rac (Kim et al. 2009). Importantly, the binding of active Rac to IP3K-A did not occlude the ability of downstream effectors such as PAK to simultaneously bind Rac, suggesting that IP3K-A could scaffold activated Rac to F-actin in a way that potentiated its ability to further stimulate actin remodeling. The role of IP3K-A in regulating Rac activity and targeting is likely to be important, as IP3K-A knockout mice exhibit profound deficits in synaptic plasticity as well as learning and memory paradigms (Kim et al. 2009).

17.4 Effector Clustering: Linking GAPs and GEFs to Downstream Rho GTPase Targets

In order to influence cellular physiology, Rho-family GTPases must bind to and regulate protein effectors. Rho effector proteins have evolved several domains, which can function as specific docking sites for GTP-bound Rho GTPases. Canonical activity-dependent GTPase-binding domains include the Cdc42/Rac Interactive Binding (CRIB) domain from the PAK kinases; the Protein kinase C-related homology region 1 (HR1) domain typified by Rho-associated kinase, PKN, and Rhotekin kinase; and the GTPase Binding Domains (GBD) of formins (Burbelo et al. 1995; Shibata et al. 1996; Flynn et al. 1998; Rose et al. 2005). One of the most efficient mechanisms to regulate how Rho-family GTPase signaling can shape cellular responses is to physically couple the regulators of their activation to downstream effectors (Fig. 17.1C). In this way, effectors can be selectivity tuned to the action of specific Rho-family GTPases. Additionally, this type of interaction allows for bidirectional coordination of signaling events, with effectors sometimes influencing the activity of GEFs and GAPs upon binding. Alternatively, GAP or GEF binding may directly influence effector activity in addition to modulating their activation by Rho-family GTPases. Although this is a relatively newer concept for Rho-family signal integration, several important examples have emerged which are discussed below.

17.4.1 Enhancing Cdc42 Signaling by an Intersectin-l and N-WASP Complex

One of the earliest examples of this type of signaling cascade organization came from the observation that the long splice variant of the endocytic protein, Intersectin-l, contains an additional DH-PH domain specific for Cdc42 activation that is not found in the short splice variant (Intersectin-s) (Hussain et al. 2001; Thomas et al. 2009; Pucharcos et al. 1999). While Intersectin-s is widely expressed in many cell types, Intersectin-l is almost exclusively neuronal. Surprisingly, it was found that although the DH domain of Intersectin-l could specifically bind to and activate Cdc42, full-length Intersectin-l does not, suggesting that Intersectin-l exists in an autoinhibited state (Hussain et al. 2001). Furthermore, the ability of Intersectin-l to stimulate actin dynamics in cells is blocked by inhibitory N-WASP activity (a Cdc42 effector that activates Arp2/3-dependent actin polymerization), suggesting a link between Intersectin-l, Cdc42, and N-WASP (Hussain et al. 2001). Activation of Intersectin-l is mediated by binding to N-WASP via the SH3 domain(s) of Intersectin-l with the proline-rich domain of N-WASP. The release of Intersectin-l inhibition is likely mediated by an N-WASP interaction with the fifth SH3 domain (SH3E) of Intersectin-l (Zamanian and Kelly 2003). Surprisingly, the mechanism of Intersectin-l inhibition is probably distinct from

other SH3-inhibited GEFs, since mutation of the proline-binding groove of SH3E does not alter its inhibition, suggesting that inhibition and its release are not via a direct competition for SH3 domain ligand binding. Consistent with this, recent crystal structures have suggested that the Intersectin-l SH3E domain uses an interface distinct from proline ligand binding to interact with the DH domain, which may occlude GTPase binding (Ahmad and Lim 2010). As discussed above, the Intersectin-l and N-WASP interaction is important for EphB regulation of synaptogenesis and spine maturation. Recent work also supports a role for Intersectin-l and N-WASP in facilitating somato-dendritic endocytosis, which may involve actin-mediated pushing of clathrin-coated vesicles into cells during scission (Thomas et al. 2009; Merrifield et al. 2004; Benesch et al. 2005).

17.4.2 GAP-Mediated Control of Rac1 Signaling to WAVE1

Like N-WASP, WAVE1 is a Rho-family GTPase effector protein, expressed throughout the CNS and whose function is to activate Arp2/3 complex-mediated branched actin filament polymerization (Padrick and Rosen 2010; Pollitt and Insall 2009). Instead of functioning downstream of Cdc42, WAVE1 senses Rac activation to regulate spine morphogenesis and activity-dependent synaptic plasticity such as LTP and LTD (Soderling et al. 2007). Analysis of WAVE1-deficient mice indicates it is critical for many behaviors, including anxiety, sensorimotor function, and learning and memory (Soderling et al. 2003). Mass spectrometry analysis of WAVE1-associated proteins led to the identification of the mechanism by which WAVE1 senses Rac activation and how this activation is tuned by negative feedback (Eden et al. 2002). Active Rac binds to the Rac effector CYFIP1 (also known as SRA-1) and induces the dissociation of CYFIP1 and several associated inhibitory proteins (Abi-1/2 and Nap1), allowing WAVE1 to interact with and stimulate Arp2/3-dependent actin polymerization. Interestingly, analysis of the WAVE1 complex of proteins also identified a neuronal Rac GAP protein, WRP (also known as srGAP3) (Soderling et al. 2002). WRP contains a carboxyl-terminal SH3 domain that binds directly to WAVE1 within the poly-proline-rich region, analogous to the Intersectin-l and N-WASP complex. Notably, WAVE1 mice mutants for the WRP-binding site display abnormal dendritic spines, altered plasticity, and subtle deficits in memory, indicating that the regulation of Rac activity within the WAVE1 complex is a crucial feature of the signaling pathway (Soderling et al. 2007). Moreover, WRP is also likely to regulate WAVE1-mediated actin dynamics in specific spatial contexts, as it contains a unique N-terminal inverse F-BAR domain that senses and induces dendritic filopodial formation during the earliest stages of spine formation (Carlson et al. 2011). WRP has been implicated in several human neuropsychiatric and developmental disorders, including intellectual disability associated with 3p-syndrome and schizophrenia (Endris et al. 2002; Addington and Rapoport 2009; Wilson et al. 2011). Consistent with a role of

WRP in contributing to these syndromes, multiple aspects of these disorders are modeled in mice lacking WRP (Carlson et al. 2011; Waltereit et al. 2012).

17.4.3 Regulation of Formin-Mediated Actin Remodeling by SrGAP2

In addition to the regulation of N-WASP and WAVE1, Rho GTPases also potently stimulate actin remodeling through the regulation of the Diaphanous-related formins. Formins form a large family of proteins (15 in mammalians) whose actin regulatory properties are kept in check by an autoinhibition mechanism (Chesarone et al. 2010). Rho GTPases physically disrupt this autoinhibition by binding within a GTPase-binding domain (Rose et al. 2005; Otomo et al. 2005). Relieving this autoinhibiton through GTP-dependent binding unmasks formin activity, resulting in either actin polymerization of linear filaments or actin filament severing, depending on the type of formin. Recently it was discovered that the Rac GAP srGAP2, a close homolog of WRP, binds the formin FMNL1 through its SH3 domain, analogous to the association of WRP with WAVE1 (Mason et al. 2011). srGAP2 is implicated in neocortical development by facilitating the formation of leading edge processes of migrating newborn neurons that are necessary to effectively migrate to the correct laminar position within the cortical plate as well as dendritic spine maturation (Guerrier et al. 2009; Charrier et al. 2012). Of note, srGAP2 has two main duplicates in humans (SRGAP2B and SRGAP2C), which encode a truncated F-BAR domain that interacts with ancestral SRGAP2 to inhibit its function. Interestingly, expression of the SRGAP2C paralog in mouse cortical neurons in vivo phenocopies srGAP2 deficiency, leading to the emergence of human-specific features, including neoteny during spine maturation and increased density of longer spines (Charrier et al. 2012; Dennis et al. 2012). The interaction of srGAP2 with FMNL1 does not occur until after Rac has activated the formin, meaning the formation of the complex is temporally regulated by an activity-dependent conformational change (Mason et al. 2011). srGAP2 binds to a critical region of the formin that appears to be required for FMNL1 activity, which is to sever actin filaments in response to active Rac. The in vivo role of actin severing is still unclear, but is likely to result in remodeling of existing actin networks into newly polymerized filaments by exposing barbed ends of actin that are competent for additional actin subunit assembly. Remarkably, reconstitution of the complex using purified components showed that the srGAP2 SH3 domain potently inhibits the FMNL1 actin severing activity (Mason et al. 2011). Together these data indicate that upon activation, srGAP2 binds FMNL1 and shuts off both the upstream activation signal Rac and the functional output of severing actin filaments. In this way the srGAP2 and FMNL1 complex may function as a timing mechanism to limit the extent of actin severing in vivo.

17.5 Future Directions

The sophistication and nuances of Rho-family GTPase signaling are only matched by the diversity of the neurophysiologic processes they regulate. The above studies have begun to illuminate the molecular mechanisms through which they achieve these important functions and how the dysfunction of their regulation ultimately leads to neurodevelopmental and neuropsychiatric disorders. Proteomic analysis of GTPase regulators suggests that their incorporation into molecular complexes is likely to be a common and important theme (Okada et al. 2011). Beyond identifying and characterizing the complexes, however, new technologies and concepts will be required to decode the importance of the spatial and temporal regulation of Rho-family signaling. Recent advances in super-resolution imaging, including PALM, SIM, STORM, and array tomography promise to help reveal where these complexes exist in synaptic space, leading to new insights into their possible functions (Schermelleh et al. 2010; Ahmed 2011; Triller and Choquet 2008). Additionally the ability to monitor the dynamics of sub-synaptic pools of actin using these techniques will likely be paired with the genetic disruption of specific complexes, allowing the field to delve more deeply into not only cataloging the nanometer scale location of these complexes but also their functional relevance at high resolution. 2-photon FLIM imaging of the spatial and temporal activity of Rho GTPases promises to reveal new insights into how these pathways are orchestrated at submicron and millisecond timescales. For example, recent work has demonstrated that the induction of spine-specific LTP leads to activation of Rho and Cdc42 activation, but with differing spatial profiles (Murakoshi et al. 2011). These approaches may also be combined with disruptions of specific GEF and GAP complexes, using high resolution imaging of activity reporters to reveal their importance in space and in time. New advances in light-gated regulation of Rho-GTPase activity using genetically encoded photo-switches, such as the LOV (light, oxygen, voltage) domain, also promises new avenues to investigate how GTPase activity modulates specific neuronal functions with the spatial resolution of light diffraction (Wu et al. 2009). As recently demonstrated for the role of Rac in addiction, when combined with technology for optogenetics, it promises to reveal the importance of GTPase signaling in specific brain regions under behavioral paradigms (Dietz et al. 2012). Most of these new imaging advances, however, will need to be paired with a deeper understanding of the biochemical nature of how GTPase signaling complexes are physically put together in order to manipulate their activity in a spatial manner. Caution must also be exerted when overexpressing proteins to understand their functions, particularly the GTPases. For example it has recently been shown that the Rho-family GDIs are limiting, and that the overexpression of one GTPase may alter the activity and localization of other endogenous GTPases by outcompeting the limiting pool of GDI (Boulter et al. 2010). Thus, a combinatorial approach, using biochemical, genetic, and new imaging approaches to dissect and understand how GTPase signaling is organized in space and time, will likely be required. Yet the rewards for such approaches will

be great as it is already clear that Rho GTPases govern the whens and wheres of neuronal development and synaptic responses.

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Chapter 18
Rho GTPases in Cancer

Jon W. Erickson, Marc A. Antonyak, Reina Fuji, and Richard A. Cerione

Abstract Rho GTPases play well-established roles in regulating cell growth and migration. Here we review how alterations in the functioning of their upstream activators [guanine nucleotide exchange factors (GEFs)] and negative regulators (GTPase-activating proteins) can contribute to the development of the transformed state. We also describe a more recent discovery of a novel signaling connection between these GTPases and elevated glutamine metabolism in cancer cells. This was based on the identification of a small molecule inhibitor that blocks the transformation of fibroblasts by oncogenic Dbl (for Diffuse B cell lymphoma), a Rho GEF, as well as inhibits the growth of human breast cancer and B lymphoma cells, and shrinks tumors induced by these cancer cells in mice. The effects of the small molecule inhibitor were specific for transformed/cancer cells, as it did not inhibit the growth of normal cells. The target of this inhibitor was shown to be an isoform of the metabolic enzyme glutaminase (GLS1), which catalyzes the hydrolysis of glutamine to glutamate. Transformed/cancer cells show markedly elevated levels of GLS1 activity, which are dependent on the activation of Rho GTPases. We further discuss that an important outcome of the metabolic changes exhibited by cancer cells is the generation of vesicular structures (microvesicles) that contain signaling proteins, metabolic enzymes, RNA transcripts, and microRNA. Microvesicles, by transferring these components to recipient cells, are capable of

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conferring transformed properties on to their acceptor cells and thus have been suggested to play important roles in cancer progression. Collectively, these results shed new light on how Rho GTPases contribute to the development of human malignancies.

Keywords Rho GTPases • Cancer • DLC1 • Glutamine metabolism • Microvesicles

18.1 Regulators of the Rho GTPase Nucleotide Bound State and Their Roles in Tumorigenesis and Metastatic Cancer

Since the discovery and cloning of the prototypical mammalian Rho GTPases in the late 1980s, much interest has been focused on identifying their regulatory proteins, their downstream effectors, and how their signaling partners coordinate the pathways that underlie observed pathologies, including cancer. As with all low molecular weight (small) GTPases of the Ras family, Rho proteins act as tightly regulated bimodal molecular switches that exchange and hydrolyze GTP according to their regulation by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs; see Fig. 18.1). The active or GTP-bound form of the GTPase targets a host of effector proteins that regulate a similarly large number of signaling pathways that orchestrate changes in cell motility and morphology, proliferation, transcription, and cellular trafficking. How then do aberrations in the regulation of the nucleotide-bound state of Rho GTPases manifest themselves in cell transformation and what are the relevant signal transduction pathways that contribute to abnormal cell growth?

18.1.1 Turning on the Switch: The Discovery of Rho GTPase Guanine Nucleotide Exchange Factors and Their Role in Cellular Transformation

Following the discovery and molecular cloning of Rac, Rho, and Cdc42, attention turned to the likelihood that all were tightly regulated by auxiliary regulatory proteins that would presumably control their guanine nucleotide-bound state and the amplitude of their downstream signaling activities. Indeed, the recognition that the Ras GTPase is commonly mutated in cancer (Malumbres and Barbacid 2003) and the subsequent discovery of the guanine nucleotide exchange factor (GEF) Son of Sevenless (SOS; Rogge et al. 1991), as well as the Ras GTPase-activating protein p120GAP (Trahey and McCormick 1987), revealed the components of a complete GTPase cycle that in all likelihood was conserved (in some form) and present for all GTPases. The clues to what might promote guanine nucleotide exchange on Rho

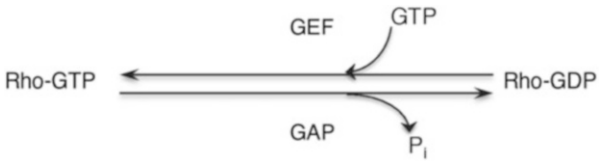

Fig. 18.1 The Rho GTPase bimodal switch is regulated by *G*uanine Nucleotide *E*xchange *F*actor (GEFs) and *G*TPase-*A*ctivating *P*roteins (GAPs)

GTPases came from yeast genetics, where analysis of cell division cycle (cdc) mutants in *Saccharomyces cerevisiae* and identified sequence similarities between the *cdc24* gene and a potently transforming mammalian gene derived from a diffuse B-cell lymphoma, led to the identification of the first Rho GTPase GEF, Dbl (Eva and Aaronson 1985; Hart et al. 1991). Sequence analysis of Dbl revealed conserved tandem domains in the yeast and mammalian genes that were designated DH (Dbl homology) and PH (pleckstrin homology) (Ron et al. 1989, 1991).

Remarkably, two-dimensional cell culture transformation assays revealed Dbl to be as potently transforming as oncogenic Ras, implicating Rho GTPases and their regulatory proteins in the pathways leading to cellular transformation. Since the initial discovery of Dbl and the identification of the DH–PH domains as the Rho GTPase binding site harboring the nucleotide exchange motif, upwards of 70 additional Dbl-like GEFs have been identified (Bernards 2006). Many of these similarly display potent transformation activity when assayed in two-dimensional tissue culture upon their transfection into NIH 3T3 cells, although in almost all cases, only the truncated version of the protein with intact DH–PH domains promotes oncogenesis (Whitehead et al. 1997). This finding is nicely illustrated in the case of Dbl, where successive N-terminal deletion mutants show increasingly potent transformation activity (Fig. 18.2). The increase in transformation activity appears to be related to a change in the intracellular location of oncogenic Dbl, relative to proto-Dbl, because although oncogenic Dbl lacks the N-terminal half of the proto-Dbl protein, it shows essentially the same in vitro nucleotide exchange activity as proto-Dbl (Fig. 18.3). Moreover, tethering the oncogenic Dbl protein with either a Golgi cytoplasmic leaflet localization tail (i.e., the C terminus of Golgin-84) or confining it to the inner leaflet of the plasma membrane abolished its transformation capability (McBrayer 2008). The potent transforming activity of the N-terminally truncated DH–PH containing proteins is therefore most likely a consequence of their spatially unrestricted nucleotide exchange activity that leaves all the cellular Rho GTPases, including those in the cytosol normally sequestered there by the RhoGDI, part of the exchangeable target pool of Rho GTPases (Johnson 2011).

These demonstrated effects of N-terminal truncation on the unregulated nucleotide exchange activity of Dbl family members raised the possibility that Rho GTPase GEFs might be causative agents in human cancers. This has been found to be largely not the case, as very few mutations (including N-terminal truncations) in Dbl family GEFs have been identified in the cancer genome and, of these, most occur outside of the conserved domains responsible for GEF activity (Rossman

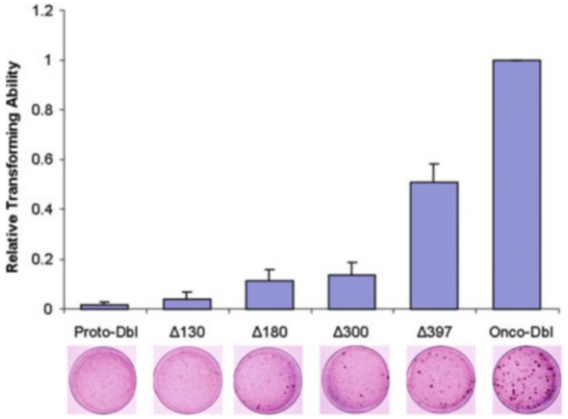

Fig. 18.2 Transformation of NIH-3T3 cells by proto-Dbl N-terminal truncation constructs. The number of amino acids for each truncation mutant is indicated (Onco-Dbl lacks the first 497 amino acids). Dbl constructs were transiently transfected into NIH-3T3 cells and allowed to grow for 14 days in DMEM/5 % serum. Plates were fixed and stained with 0.4 % crystal violet. Focus formation is plotted as a percentage of Onco-Dbl-induced foci

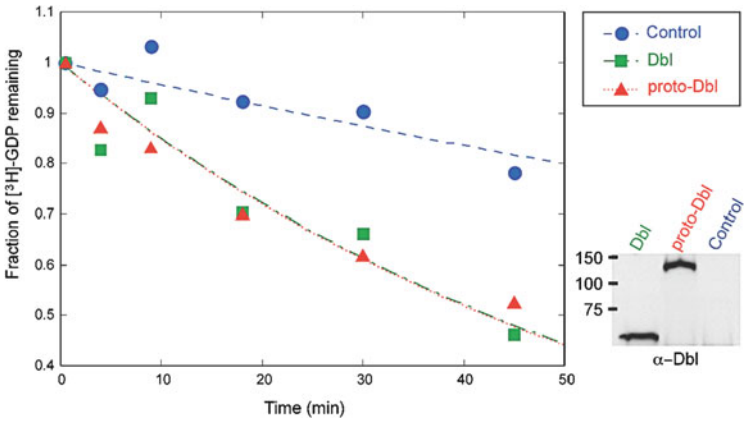

Fig. 18.3 In vitro GEF-mediated nucleotide exchange assays of proto-Dbl versus oncogenic Dbl. GEF activity was measured as the rate of dissociation of pre-loaded [^{3}H]-GDP from Cdc42 catalyzed by the Dbl proteins. Equivalent amounts of proto-Dbl and oncogenic Dbl were immunoprecipitated with Dbl antibody from transiently transfected COS-7 cell lysates and used in the reaction. Lysates from untransfected cells were used as the negative control

et al. 2005). Similarly, the occurrence of mutations in Rho GTPases themselves that would confer a constitutively active state (i.e., analogous to the Ras(G12V) oncoprotein) has not been observed in the cancer genome to date (Alan and Lundquist 2013), although "fast cycling" mutations in Rac1, that have been shown to promote transformation in two-dimensional culture in a

GEF-independent manner, do occur in a subpopulation of melanomas (Davis et al. 2013).

Despite the dearth of identifiable "driver" mutations in Rho GTPases or their activating GEFs, there have been numerous reports that collectively argue for a critical role of activated Rho pathways in human cancer cell biology (Clark et al. 2000; Ma et al. 2007; Shibue et al. 2013; Mardilovich et al. 2012). Mechanistically, hyper-activated Rho GTPase-signaling appears to be very often caused by overexpression of a single GTPase or a combination of Rho, Rac and Cdc42 (Fritz et al. 1999). As a consequence, even in the absence of any mutation that would serve to shift the proportion of active GTPase in the cell, the mere abundance of a given Rho protein ensures, by mass action, a higher level of active GTPase and, consequently, hyperstimulation of downstream signaling pathways.

This critical role for Rho protein overexpression in metastatic cancer was brought to the fore by the microarray study of Clark et al., where RhoC was found to be highly overexpressed in resected and recultured human and murine pulmonary lung tumor cells following their reintroduction into nude mice (Clark et al. 2000). Subsequently, a number of studies have linked metastatic progression with RhoA and RhoC overexpression in clinical models, supporting a major role for Rho signaling pathways in malignant neoplastic homing and colonization (Fritz et al. 2002; van Golen et al. 2000; Imamura et al. 1999).

18.2 Turning Off the Switch: GAPs in the Cancer Genome

Following the discovery of GTPase-activating proteins (GAPs) for Ras (Trahey and McCormick 1987; Xu et al. 1990), interest turned towards the possibility that similar cellular activities might exist for the purpose of down-regulating Rho protein signaling. Along with the initial identification of the p120 Ras GAP protein, there was the exciting discovery that a well-studied inheritable neoplasm, neurofibromatosis, was attributable to a point mutation in a gene encoding a second Ras GAP protein, neurofibromin 1, a negative regulator of Ras activity (Xu et al. 1990). The identification of a tumor suppressor protein activity for Ras soon after prompted a search for a similar factor in Rho GTPase signaling pathways, leading to the cloning and characterization of p50Cdc42GAP (also known as p50RhoGAP) reported in 1993 (Barfod et al. 1993; Lancaster et al. 1994). Interestingly, despite the conserved structures of Ras and Rho and, to a lesser degree, their primary sequence similarities, Ras and Rho subfamily-specific GAPs share neither structural nor sequence homology. Since the initial identification of the first Rho GAP, the sequencing of the human genome revealed a surprisingly large number of proteins (~70) containing the limit Rho GAP (Kandpal 2006).

Although p50Cdc42GAP itself can serve to suppress increased fibroblast proliferation in cell culture when induced by "fast cycling" Cdc42 or by oncogenic Dbl (Fidyk et al. 2006), only a minority of the Rho GAP family members characterized to date have been implicated in clinically observed malignancies. By far the most

prominent example is the Rho GAP domain-containing protein Deleted in Liver Cancer 1 or DLC1 (Lahoz and Hall 2008). DLC1 is encoded by a gene located on human chromosome region 8p22 that was initially found to be absent or suppressed in a host of cancer cell lines and primary tumor tissues (Yuan et al. 2003). The loss of DLC1 expression is thought to be due, in many cases, to the hyper-methylation of DLC1 promoter regions. This observation may provide therapeutic opportunities in the future, as DNA methyl-transferase inhibitors have been demonstrated to reverse DLC1 suppression and restore normal expression levels and normal growth in cancer cells (Kim et al. 2003; Wong et al. 2003; Yuan et al. 2004). The frequency of DLC1 loss in human cancers is one of the striking outcomes of subsequent DLC1 gene analysis, as deletion or loss of expression of DLC1 occurs in ~50 % of liver, breast, and lung cancers, as well as ~70 % of colon cancers, making DLC1 a tumor suppressor rivaling p53 in its absence in cancer.

Interestingly, DLC1, in vivo, is predicted to be a fairly specific Rho GAP, pointing to RhoA or RhoC signaling pathways as critical contributors to transformation phenotypes in cancer cell lines as well as primary tumors. The implied importance of Rho signaling pathways in cancer progression, inferred from the frequency of the DLC1 deletion cited above, raises the possibility that inhibition of the downstream effectors of Rho GTPases may offer opportunities for therapeutic intervention. There are currently several Rho kinase (i.e., p160Rock for Rho-associated, coiled-coil-containing protein kinase) inhibitors under investigation for possible application in treating malignancies as part of a multi-platform approach to inhibiting cancer cell growth and metastasis (Rath and Olson 2012).

In order to illustrate the wide-ranging effects that aberrant Rho signaling might achieve in cancer progression, we describe below two relatively new examples of the downstream consequences of Rho signaling. First, Rho GTPase signaling has been linked to the changes in metabolism, specifically glutamine metabolism, that often accompany neoplastic transformation (Erickson and Cerione 2010; Wang et al. 2010). In fact, active RhoA,C and Cdc42 pools are elevated in many cancers, probably through a combination of Rho GTPase overexpression, as well as DLC1 deletion or epigenetic suppression. Many of these cancer cell lines exhibit altered glutamine metabolism as well. In addition to their influence on cancer cell metabolism, Rho GTPases trigger a signaling pathway that leads to the biogenesis of shedded plasma membrane-derived microvesicles that are produced from cancer cells. Remarkably, the formation of these microvesicles can be reversed by treatment of cells with the Rho kinase inhibitor, Y27632 (see below). These Rho-dependent microvesicles represent a novel form of cancer cell–cell communication and are currently an area of great interest, as they represent an unexpected but potentially critical mechanism whereby cancer cells communicate with the tumor microenvironment (D'Souza-Schorey and Clancy 2012; Antonyak et al. 2011).

18.3 Rho GTPases Regulate Glutamine Metabolism in Cancer Cells

18.3.1 The Early Clues from a Small Molecule Inhibitor of Rho GTPase-Dependent Transformation

The importance of cellular metabolism in the development of cancer goes back to the early observations of Warburg that tumor cells exhibit enhanced glycolytic activity (i.e., the "Warburg effect") (DeBerardinis et al. 2007). This phenomenon has been receiving a great deal of renewed attention (DeBerardinis et al. 2007, 2008; Pederson 1978). Two major events characterize the metabolic changes that cancer cells undergo (Fig. 18.4). The first is an acceleration of many of the steps in the glycolytic pathway, with the penultimate step being attenuated as a result of the tyrosine phosphorylation of the M2 isoform of pyruvate kinase, which catalyzes the conversion of phosphoenolpyruvate to pyruvate (DeBerardinis et al. 2008; Pederson 1978). This results in most of the pyruvate that is generated through the glycolytic pathway being converted to lactic acid (by lactate dehydrogenase) instead of acetyl coA which normally would help to initiate the TCA cycle. These events then prompt the second major change in cancer cell metabolism, namely the elevations in glutamine metabolism (often referred to as "glutamine addiction") that occur through the accelerated hydrolysis of glutamine to glutamate by members of the glutaminase family, and the subsequent conversion of glutamate to α-ketoglutarate (by glutamate dehydrogenase). The enhanced production of α-ketoglutarate is used to feed the TCA cycle (Fig. 18.4). Metabolic flux experiments using ^{13}C-NMR have demonstrated that while proliferating cancer cells exhibit a pronounced Warburg effect, their TCA cycle remains intact, and that it is necessary to replenish metabolic intermediates for the production of NADPH for fatty acid synthesis, to provide the carbon necessary for nucleotide synthesis as well as for the production of asparagine and arginine, and to serve as a major anaplerotic source of oxaloacetate (DeBerardinis et al. 2007). Moreover, cancer cells use elevated glutamine metabolism to carry out what is referred to as reductive carboxylation, with α-ketoglutarate dehydrogenase driving the pathway in reverse such that α-ketoglutarate ultimately leads to the production of citrate (Mullen et al. 2011). The end result of these changes in cancer cell metabolism is that the utilization of glucose metabolites is shifted from energy production to anabolic processes.

We recently discovered a new role for Rho GTPases in cancer progression through a previously unappreciated connection between these signaling proteins and cellular metabolism. Specifically, we found that the hyper-activation of Cdc42 as well as related Rho GTPases (i.e., Rac1, RhoA, and RhoC) that occurs in transformed cells and different cancer cell lines signals the up-regulation of a mitochondrial enzyme, glutaminase (Wang et al. 2010). The discovery that glutaminase expression and activity are significantly increased in response to the hyper-

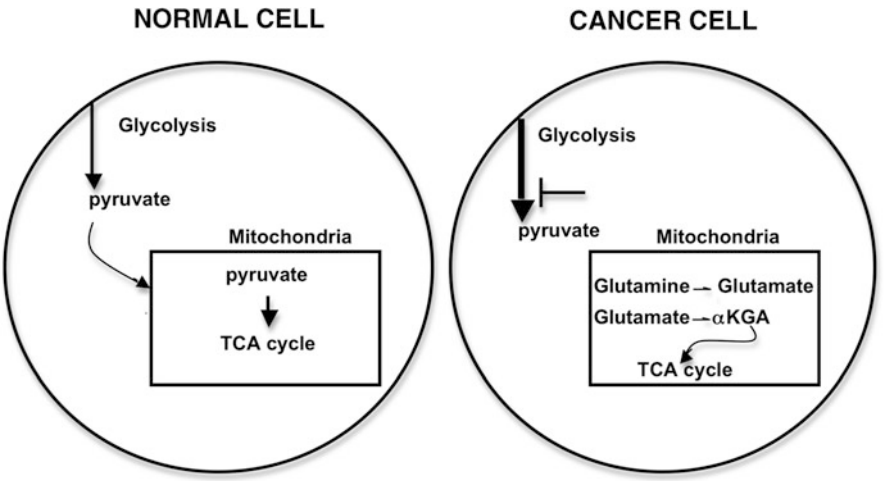

Fig. 18.4 Cancer cells undergo metabolic reprogramming. The glycolytic pathway in normal cells (*left*) is used to generate pyruvate that enters the citric acid cycle in the mitochondria. In cancer cells (*right*), many of the enzymes of the glycolytic pathway are up-regulated and/or activated, although M2 pyruvate kinase activity, which catalyzes the penultimate step in the pathway, is inhibited. This appears to preferentially direct the conversion of pyruvate to lactate acid (as catalyzed by lactate dehydrogenase). Elevated glutamine metabolism, through the up-regulation and/or activation of glutaminase (which converts glutamine to glutamate) and glutamate dehydrogenase (converting glutamate to α-ketoglutarate), is then essential for "feeding" the TCA cycle in cancer cells

activation of Rho GTPases offers some interesting new insights into how the demands of proliferating cells for accelerated glutamine metabolism are met.

The discovery of a signaling connection between Rho GTPases and glutamine metabolism stemmed from efforts to identify small molecules that inhibit the aberrant signaling activity of Rho GTPases in transformed/cancer cells and thus act to block oncogenic transformation. By screening NIH 3T3 cells expressing oncogenic Dbl for molecules that inhibited their ability to become transformed, an 8-bromo-derivatized benzo(a)phenanthridinone, designated from here on as 968, was identified as being an effective inhibitor (Fig. 18.5), working over a concentration range of 1–10 μM. Subtle changes in the structure of 968, such as simply removing the bromine or the dimethyl amine from the phenyl ring, dramatically affected its inhibitory activity (Wang et al. 2010). 968 was also found to be capable of inhibiting transformation induced by the constitutively active, fast-cycling Cdc42(F28L), Rac(F28L), and RhoC(F30L) mutants, which are able to spontaneously exchange GDP for GTP in the absence of a GEF (Wang et al. 2010). Importantly, 968 did not inhibit the growth nor alter the morphology of normal cells (Wang et al. 2010), indicating that this small molecule targets events downstream from Rho GTPases that are specific for transformed cells.

Rho GTPases have been suggested to be overexpressed and/or hyper-activated in human breast cancer cells (Kleer et al. 2002; Clark et al. 2000; Burbelo et al. 2004).

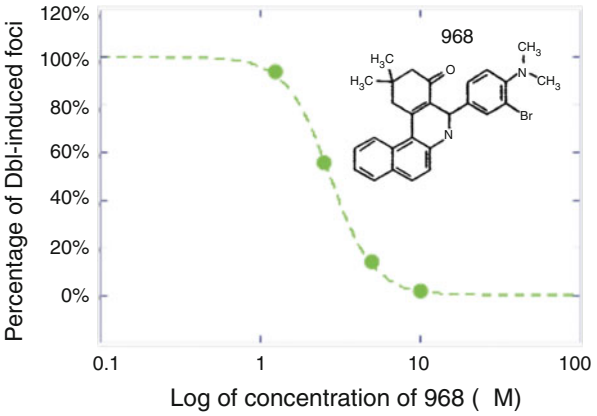

Fig. 18.5 968 inhibits Dbl-induced transformation. Cells were transiently transfected with oncogenic Dbl and cultured for 14 days, while treated with different amounts of 968 added to the growth media. Tissue culture plates were fixed with 3.7 % formaldehyde in PBS and stained with crystal violet for counting foci. *Inset*: Chemical structure of 968

For example, RhoA and RhoC are hyper-activated in the highly invasive MDAMB231 and SKBR3 breast cancer cell lines, compared to normal human mammary epithelial cells (HMECs), and the growth of these cancer cells in low serum or in soft agar was severely compromised when RhoA and RhoC levels were knocked down using siRNAs (Wang et al. 2010). 968 inhibited the ability of these breast cancer cells to form colonies in soft agar, as effectively as it blocked Dbl-induced colony formation in NIH 3T3 cells (Wang et al. 2010). Similarly, 968 inhibited their ability to grow to high density, whereas it had little if any effect on the growth or morphology of HMECs (Wang et al. 2010).

18.3.2 The Identification of a Link Between Rho GTPases and Glutamine Metabolism in Transformed/Cancer Cells

Surprisingly, the protein target for 968 turned out not to be a Rho GTPase or one of its immediate binding partners, but instead a specific isoform of the mitochondrial enzyme, glutaminase. The identification was made through biochemical experiments using biotin-labeled 968. When immobilized to streptavidin beads, the biotin-labeled 968 precipitated a protein from Cdc42(F28L)-transformed NIH 3T3 cells that was shown by micro-sequencing analysis to be the mouse homolog of human glutaminase C (GAC). Mammals contain two distinct but structurally related genes encoding proteins collectively referred to as glutaminase, with one form being highly expressed in liver (thus referred to as liver-type glutaminase or GLS2), and another form that is found in kidney and a number of other tissues including many transformed cells and is referred to as the kidney-type enzyme or GLS1 (Curthoys 1995; Kenny et al. 2003; Robinson et al. 2007). GAC is a splice-variant of GLS1.

Mitochondrial preparations from cells expressing oncogenic Dbl showed markedly higher levels of glutaminase activity compared to control, non-transformed fibroblasts (Wang et al. 2010). While recombinant glutaminase preparations are absolutely dependent on inorganic phosphate (an allosteric regulator) for activity, mitochondrial preparations from transformed cells exhibit significant levels of enzyme activity even in the absence of added phosphate. Mitochondrial preparations from SKBR3 cells, as well as MDAMB231 cells, also exhibited significantly higher basal glutaminase activity, compared to normal HMECs. Knockdowns of RhoA and RhoC in breast cancer cells reduced their basal glutaminase activity, without significantly affecting phosphate stimulation of the enzyme, indicating that the increased basal activity was Rho GTPase-dependent (Wang et al. 2010).

The importance of glutaminase activity for Rho GTPase-dependent transformation was demonstrated in knockdown experiments where siRNAs targeting both isoforms of GLS1 strongly inhibited the ability of cells expressing constitutively active Cdc42, as well as cells expressing oncogenic Dbl, Rac, and RhoC (Wang et al. 2010), to exhibit anchorage-independent growth and form colonies in soft agar. Knocking down both isoforms of GLS1 also strongly inhibited MDAMB231 and SKBR3 cells from growing in soft agar (Wang et al. 2010).

18.4 Rho GTPases and Their Role in How Cancer Cells Communicate with Their Environment

The high rates of glutamine metabolism exhibited by tumor cells, together with their elevated levels of glycolysis, are needed for the synthesis of growth regulatory proteins, and for the stimulation of fatty acid synthesis to provide new lipid membranes, as well as to satisfy the hefty energy requirements needed for cells to reach the transformed state. However, a new and potentially important outcome of the metabolic reprogramming of cancer cells is the biogenesis of vesicular structures that are specifically formed and shed from the surfaces of cancer cells.

18.4.1 Microvesicles in Cancer Progression

An exciting development in cancer cell biology has come from the discovery that cancer cells generate and shed small vesicles from their surfaces with sizes on the order of 1-2 μm in diameter. These vesicular structures are referred to by various names including extracellular shed vesicles, microvesicles, and oncosomes. Their relatively large size distinguishes them from the smaller shed vesicles called exosomes, which are released as an outcome of the exocytosis of multivesiclular bodies (Al-Nedawi et al. 2009; Cocucci et al. 2009; Ratajczak et al. 2006).

What makes microvesicles particularly interesting is that they contain a wide range of components from their parent cancer cells, including cell surface receptors and a number of other signaling proteins, RNA transcripts, microRNAs, and even in some cases DNA, as well as metabolic enzymes (Skog et al. 2008; Al-Nedawi et al. 2008; Graner et al. 2009; Di Vizio et al. 2009; Mathivanan and Simpson 2009; Ginestra et al. 1998; Graves et al. 2004; Muralidharan-Chari et al. 2010). Thus, microvesicles have the potential to serve as "satellites" of intercellular communication during cancer progression. An important role for microvesicles in cancer progression was first suggested from studies involving the EGFRvIII, a truncated version of the EGFR lacking the EGF-binding site (i.e., residues 6–273) that has been implicated in aggressive forms of brain cancer (Sugawa et al. 1990; Ekstrand et al. 1991; Kuan et al. 2001; Luwor et al. 2004; Heimberger et al. 2005). The microvesicle-mediated transfer of the EGFRvIII between glioblastoma cells significantly enhanced their signaling and transformed characteristics (Al-Nedawi et al. 2008). Microvesicles have been shown to be capable of transferring receptor proteins and matrix-degrading enzymes between cancer cells, thus further enhancing the transformed phenotypes and invasive capability of the recipient cells. Especially intriguing is the possibility that microvesicles might have broader consequences by altering the microenvironment at the primary tumor site, as well as by contributing to metastasis, and in particular, to the development of the pre-metastatic niche. Indeed, an unexpected and exciting discovery was that microvesicles also provide a mechanism of communication between cancer cells and normal (non-transformed) cells. Specifically, microvesicles were shown to contain polymeric fibronectin that plays an essential role in their ability, when shed from cancer cells (e.g., MDAMB231 breast cancer cells or U87 glioblastoma cells) to dock onto fibroblasts and induce their transformation, i.e., their ability to undergo anchorage-independent growth (Antonyak et al. 2011).

Efforts to determine how microvesicles form on the surfaces of cancer cells showed that the RhoA GTPase plays a critical role (Li et al. 2012). A signaling pathway was delineated that starts with the activation of RhoA, then binds and activates the serine/threonine kinase p160Rock. Activated p160Rock then signals to LIM kinase, leading to the phosphorylation of cofilin, which negatively affects its actin-severing activity. The RhoA-signaling pathway, by regulating actin cytoskeletal architecture, enables actin filaments to form the base of the maturing microvesicles, such that blocking any step in this pathway (e.g., by inhibiting p160Rock with the inhibitor Y27632) eliminates microvesicle formation in different cancer cells. The elucidation of this signaling pathway also sheds light on the question of why certain aggressive cancer cell lines such as MDAMB231 breast cancer cells are constitutively generating microvesicles, i.e., as compared to other cancer cell lines such as HeLa, which form microvesicles in a strictly EGF-dependent fashion (Li et al. 2012). The reason is likely that the RhoA-signaling pathway is constitutively activated in MDAMB231 cells due to the loss of the negative regulatory protein, DLC1. When the cDNA encoding DLC1 was introduced into MDAMB231 cells, the ability of the breast cancer cells to generate microvesicles was markedly reduced (Fig. 18.6).

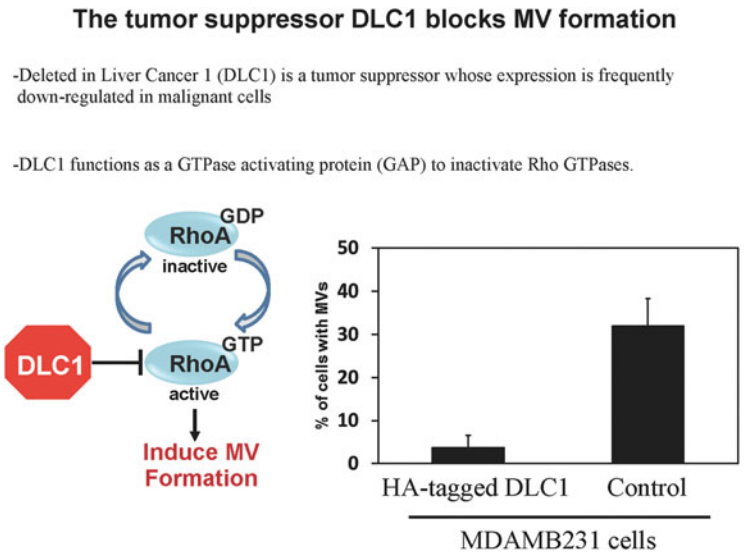

Fig. 18.6 DLC1 encodes a RhoGAP. The absence of DLC1 in MDAMB231 breast cancer cells leads to the constitutive activation of RhoA and the constitutive generation of microvesicles. The transfection of a plasmid encoding HA (hemaglutinin)-tagged DLC1 into MDAMB231 cells eliminated MV formation

18.4.2 A Role for Rho GTPases and Their Impact on Cellular Glutamine Metabolism in Microvesicle Formation

The finding that RhoA signaling is essential for the maturation of microvesicles on the surfaces of cancer cells, coupled with the link between the hyper-activation of Rho GTPases and the activation of glutamine metabolism, makes it attractive to consider that there might be a connection between these metabolic changes and microvesicle formation. Indeed it is not much of a stretch to consider that the bioenergetic requirements to generate microvesicles, and the need to replace the plasma membrane upon the shedding of these vesicles, would give rise to some connection between the metabolic program of cancer cells and their propensity to generate microvesicles. Interestingly, the first clue for such a connection came from early studies of the effects of the GAC inhibitor 968 on the formation of fibronectin clusters along the surfaces of cancer cells. These clusters were eliminated when the cells were treated with 968 (Fig. 18.7). Initially, the molecular basis by which these fibronectin clusters were able to form was not well understood, although it was possible to correlate the ability of 968 analogs to inhibit the formation of the clusters, with the effectiveness of the different compounds in blocking the transformed features of cancer cells. Ultimately, it was determined that the fibronectin clusters were the outcome of the crosslinking of fibronectin along the outer surface of microvesicles, as catalyzed by tissue transglutaminase, an acyl

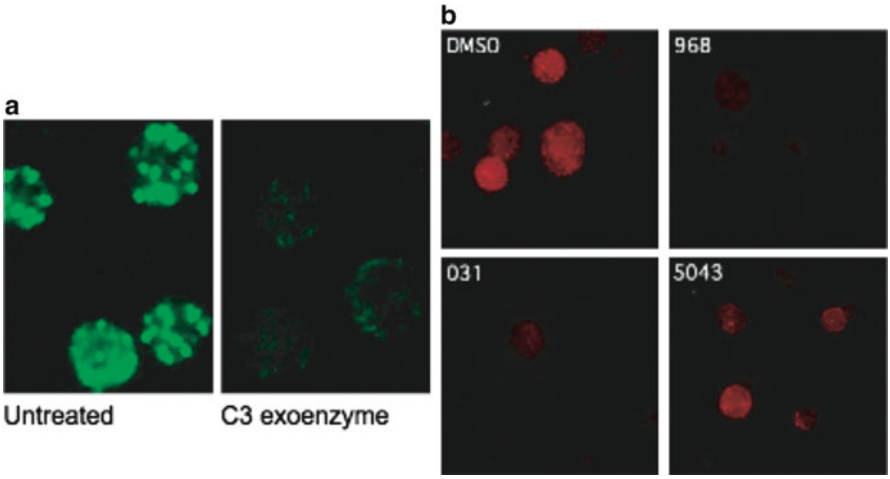

Fig. 18.7 The detection of polymeric fibronectin due to microvesicle formation on the surfaces of MTF7 breast cancer cells is inhibited by blocking Rho GTPase signaling and glutaminase activity. (**a**) (*Left*) Immunofluorescence (*green*) of globular cell-surface polymeric fibronectin assembly on MTF7 cells after 2 h in suspension in DMEM supplemented with 20 % FBS. (*Right*) Identically cultured MTF7 cells pretreated with the *C. botulinum* toxin, exoenzyme C3 transferase which blocks RhoA-signaling. (**b**) Treatment of cells with 10 μM 968 and 031 (an analog that also blocks Dbl-transformation) inhibits polymeric fibronectin assembly (*red*) as compared to DMSO and the less active analog, 5043

transferase that is a primary cargo of these vesicles. The cross-linked fibronectin was then shown to play an important role in the ability of microvesicles to dock onto fibroblasts and markedly alter their inherent signaling capabilities such that they adopted many characteristics of transformed cells (Antonyak et al. 2011). Based on the original clue obtained from the effects of 968 on the formation of fibronectin clusters, it was then possible to show that this small molecule inhibitor blocks microvesicle formation, as read-out in immunofluorescence experiments where microvesicle formation was monitored by staining for tissue transglutaminase (Antonyak et al. 2012; Wilson et al. 2013).

18.5 Concluding Remarks

When taken together, these latest findings now raise some intriguing possibilities regarding the relative importance of the activation of glutamine metabolism and the generation of microvesicles in the roles played by Rho GTPases in malignant transformation and how these actions might give rise to new therapeutic targets. However, they also highlight a number of key questions that need to be addressed in the future. Among these is the question of how Rho GTPase signaling results in the "activation" of glutamine metabolism in cancer cells? Do Rho GTPases send

signals that up-regulate the expression of GAC? Indeed, recent work from our laboratory suggests that Rho GTPases, by signaling to the N-terminal c-Jun kinase (JNK) and the transcription factor c-Jun, up-regulate GAC expression in cancer cells (M. Lukey, in preparation). The transcription factor NFκB, which has been shown to be a downstream target of Rho GTPases (Van Aelst and D'Souza-Schorey 1997; Biswas et al. 2000), has also been implicated in Rho GTPase-signaling to GAC, although not by directly mediating the transcriptional regulation of GAC expression (Wang et al. 2010). Studies performed in B-lymphoma cells and in prostate cancer cells demonstrated a connection between c-Myc and GLS1/GAC expression (Gao et al. 2009). This was shown to be the outcome of the c-Myc-dependent regulation of microRNA expression, which resulted in a reversal of the microRNA-mediated repression of the translation of RNA transcripts encoding GLS1/GAC. Since c-Myc is activated downstream of NFκB in some cellular contexts (La Rosa et al. 1994), this might explain the role played by NFκB in the Rho GTPase-dependent activation of glutamine metabolism.

Rho GTPases are likely to be involved in additional forms of regulation of GAC, i.e., aside from the up-regulation of its expression. Whereas the transient expression of GAC, alone, in NIH 3T3 cells was insufficient to induce foci, the expression of GAC in cells stably expressing the Cdc42(F28L) mutant, which exhibits only weak focus-forming activity, caused a dramatic increase in foci matching the strong response induced by oncogenic Dbl (Wang et al. 2010). This increase in focus-forming capability was blocked by 968 and did not occur when the catalytically dead GAC(S291A) mutant was co-expressed with Cdc42(F28L) (Wang et al. 2010). These findings would seem to indicate that activated Cdc42, as well as other Rho GTPases, send signals that directly increase the enzymatic activity of GAC. Thus, an important future goal will be to define the nature of these signals, and in particular determine whether they are the outcome of specific posttranslational modifications of GAC and/or represent the promotion of an interaction (s) between GAC and other cellular binding partners that influence its activation status, as this may well yield additional targets of therapeutic value.

Similarly, a number of questions will need to be answered regarding the role of Rho GTPases in microvesicle formation. These include identifying the signaling pathways necessary for loading microvesicles with protein cargo, as well as those responsible for the loading of RNA transcripts and microRNAs. It seems likely that specific sets of signals will be involved for loading these different classes of cargo, given that various types of membrane-associated proteins, including RhoA and other Rho GTPases, rarely appear in microvesicles, whereas surprisingly, some nuclear proteins like the Ran GTPase are among the microvesicle cargo. Another important question concerns what signals ensure that microvesicles are actually shed from the surfaces of cancer cells. The RhoA-dependent signaling pathway that leads to microvesicle maturation on the surfaces of cancer cells shares similarities with the signals that result in the formation of cell surface structures called "membrane blebs." However, unlike microvesicles, membrane blebs are not shed but rather are retracted back into the cell. This suggests that a specific signal (s) enables mature microvesicles to be discharged from the cancer cell surface.

Preliminary results from our laboratory have suggested that other members of the Rho GTPase family may play important roles in microvesicle formation and function, with Rac directing the loading of protein cargo into microvesicles and Cdc42 signaling their shedding from the cell surface (M. Antonyak and L. Desrochers, unpublished data). Future studies will need to be directed at verifying these roles as well as identifying other potential signals that result in the loading of cargo into microvesicles and the shedding of microvesicles from the cell surface.

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