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Sandra Reichrath *Editors*

Notch Signaling in Embryology and Cancer

Molecular Biology of Notch Signaling

Second Edition

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Preface

When the American scientist John S. Dexter discovered mutant fruit flies (*Drosophila melanogaster*) in his research laboratory at Olivet College (Olivet, Michigan) more than a century ago, he could not have expected the tremendous impact that the characteristic notch-wing phenotype (a nick or notch in the wingtip that gave the responsible gene the name *Notch*) would later have for many fields of biology and medicine, including embryology, genetics, and cancer.

On a first look, the Notch pathway seems delusively simple, with a direct link between an extracellular signal and transcriptional output without the requirement of an extended chain of protein intermediaries (as needed by so many other signaling pathways) representing its key feature. However, on a second, closer look, this obvious simplicity hides remarkable complexity and, consistent with its central role in many aspects of development, it has to be noted that Notch signaling has an extensive collection of mechanisms that it employs alongside of its core transcriptional machinery. During the last decades, a huge mountain of impressive scientific process has convincingly demonstrated that Notch signaling represents one of the most fascinating pathways that govern cellular core processes including cell fate decisions, embryogenesis, and adult tissue homeostasis. Therefore, it is no surprise that the first edition of *Notch Signaling in Embryology and Cancer* that was published by Landes and Springer in 2012 in the prestigious series *Advances in Experimental Medicine and Biology* was very successful, for it fulfilled the need to provide a broad audience (ranging from medical students to basic scientists, physicians, and all other health care professionals) with up-to-date information in a comprehensive, highly readable format. At this time, it was the benchmark on this topic, with individual chapters being written by highly respected experts in the field. Because of the enormous progress that has been made on this topic in recent years, we have decided that it is now the right time to publish an updated and extended version. The second edition of *Notch Signaling in Embryology and Cancer* has been expanded substantially and consequently and has been divided into three separate volumes to include many new chapters. In the different volumes of *Notch Signaling in Embryology and Cancer*, leading experts in the field present a comprehensive, highly readable overview on selected aspects of three important topics related to Notch signaling, namely the underlying molecular mechanisms that mediate its biological effects (Volume I), its role in embryogenesis (Volume II), and last but not least its relevance for pathogenesis, progression, prevention, and

therapy of cancer (Volume III). This first volume of *Notch Signaling in Embryology and Cancer* summarizes the underlying molecular mechanisms that mediate its biological effects and that was first discovered in the fruit fly, *Drosophila melanogaster*. We are convinced that it will be as successful as the previous edition and are very grateful for the willingness of all authors to contribute to this book. We would also like to express our thanks to Murugesan Tamilselvan, Anthony Dunlap, Larissa Albright, Cathrine Selvaraj, and all other members of the Springer staff for their expertise, diligence, and patience in helping us complete this book.

Enjoy the reading!

Homburg, Saarland, Germany

Jörg Reichrath
Sandra Reichrath

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A Snapshot of the Molecular Biology of Notch Signaling: Challenges and Promises

Jörg Reichrath and Sandra Reichrath

Abstract

Evolutionary conserved Notch signaling is of high importance for embryogenesis and adult tissues, representing one of the most fascinating pathways that regulate key cell fate decisions and other core processes. This chapter gives a short introduction to the first volume of the book entitled *Notch Signaling in Embryology and Cancer*, that is intended to provide both basic scientists and clinicians who seek today`s clearest understanding of the molecular mechanisms that mediate Notch signaling with an authoritative day-to-day source. On a first look, Notch signaling, that first developed in metazoans and that was first discovered in a fruit fly, seems fallaciously simple, with its key feature being a direct link between an extracellular signal and transcriptional output without the requirement of an extended chain of protein intermediaries as needed by the majority of other signaling pathways. However, on a second, closer look, this obvious simplicity hides remarkable complexity. Notch signaling, that relies on an extensive collection of mechanisms that it exerts alongside of its core transcriptional machinery, orchestrates and governs cellular development by inducing and regulating communication between adjacent cells. In general, a cell expressing the Notch receptor can be activated in trans by ligands on an adjacent cell leading to alteration of transcription and cellular fate. However, ligands also have the ability to inhibit Notch signaling and this can be accomplished when both receptor and ligands are co-expressed in cis on the same cell. The so called non-canonical Notch pathways further diversify the potential outputs of Notch, and allow it to coordinate regulation of many aspects of cell biology. Fortunately, the generation and investigation of knockout mice and other animal models have in recent years resulted in a huge volume of new scientific informations concerning Notch gene function, allowing to dissect the role of specific Notch components for human development and health, and showing promise in opening new avenues for prevention and therapy of a broad variety of independent diseases, including cancer, although this goal is still challenging.

Keywords

Notch · Notch signaling · Notch pathway · Embryonic development · Jagged · Delta like ligand

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Evolutionary conserved Notch signaling, that first developed in metazoans (Gazave et al. 2009; Richards and Degnan 2009) and that was first discovered in a fruit fly (*Drosophila melanogaster*), represents one of the most fascinating pathways

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that govern both embryonic development and adult tissue homeostasis. A huge volume of scientific evidence, that has been constantly growing during the last decades, has now convincingly shown that the Notch pathway governs, from sponges, roundworms, *Drosophila melanogaster*, and mice to humans, many key cell fate decisions and other core processes that are of high importance both for embryogenesis and in adult tissues (Andersson et al. 2011). At first glance, the Notch pathway seems fallaciously simple, with its key feature being a direct link between an extracellular signal and transcriptional output without the requirement of an extended chain of protein intermediaries as needed by so many other signaling pathways (Hunter and Giniger 2020). However, on a second, closer look, this obvious simplicity hides remarkable complexity, and consistent with its central role in many aspects of development, it has to be noted that Notch signaling has an extensive collection of mechanisms that it exerts alongside its core transcriptional machinery (Hunter and Giniger 2020). There is no doubt that the enormous scientific progress in unraveling the molecular mechanisms of Notch signaling that has been made recently has shown promise in opening new avenues for prevention and therapy of a broad variety of independent diseases, including cancer, although this goal is still challenging.

Notably, the fascinating tale that earned the gene the name *Notch* began over a century ago, when the American scientist John S. Dexter discovered at Olivet College (Olivet, Michigan, USA) the typical notched-wing phenotype (a nick or notch in the wingtip) in his stock of mutant fruit flies *Drosophila melanogaster* (Dexter 1914). The alleles responsible for this phenotype were identified 3 years later at Columbia University (New York City, New York, USA) by another American scientist, Thomas Hunt Morgan (1866–1945) (Morgan 1917). In the following years, many additional alleles were identified, that were associated with the Notch phenotype (Morgan 1928). In subsequent decades, notwithstanding the extensive research on the *Notch* locus, researchers struggled to identify the function for the *Notch* gene due to the lethality early in embryogenesis and the broad variety of phenotypic consequences of Notch mutants. Despite these challenges, the observations of John S. Dexter, Thomas Hunt Morgan and others were finally confirmed by cloning and sequencing of the mutant *Notch* locus in the research laboratories of Spyros Artavanis-Tsakonas and Michael W. Young, more than half a century later (Wharton et al. 1985; Kidd et al. 1986).

During the last decades, a broad variety of independent inherited diseases linked to defective Notch signaling has been identified, highlighting its clinical relevance. The discovery of these congenital diseases started in 1996 in patients diagnosed with CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; an autosomal dominant hereditary stroke disorder resulting in vascular dementia) (Joutel et al. 1996), with the linkage analysis-based discovery of heterozygous *NOTCH3* mutations on chromosome 19. Since these pioneer investigations, several other inherited disorders, including Adams–Oliver, Alagille, and Hajdu–Cheney syndromes, and several types of cancer, have convincingly been linked to defective Notch signaling (Li et al. 1997; Oda et al. 1997).

Interestingly, there is an emerging role of Notch as a promising therapeutic target in various malignancies, inherited diseases, and other disorders. In this context, it is of interest that in a mouse model (Notch3$^{tm1.1Ecan}$) of lateral meningocele syndrome (LMS), cancellous bone osteopenia was no longer detected after intraperitoneal administration of antibodies directed against the negative regulatory region (NRR) of Notch3 (Yu et al. 2019). In that study, anti-Notch3 NRR antibody suppressed expression of Hes1, Hey1, and Hey2 (Notch target genes), and decreased Tnfsf11 (receptor activator of NF Kappa B ligand) messenger RNA in Notch3$^{tm1.1Ecan}$ osteoblast cultures (Yu et al. 2019). This study indicates that cancellous bone osteopenia of Notch3$^{tm1.1Ecan}$ mutants can be reversed by anti-Notch3 NRR antibodies, thereby opening new avenues for treatment of bone osteopenia in LMS patients (Yu et al. 2019). Another example an emerging role of Notch as a promising therapeu-

tic target is the unilateral ureteral obstruction (UUO) mouse model, where treatment with the g-secretase inhibitor DAPT showed an amelioration of renal fibrosis including lower fibrotic levels and collagen deposition (Marquez-Exposito et al. 2020). However, the direct effect of Notch signaling pathway activation in the regulation of the ECM proteins has not been confirmed yet (Marquez-Exposito et al. 2020).

In 2012, when the first edition of "Notch Signaling in Embryology and Cancer" was published by Landes and Springer in the prestiguous series "Advances in Experimental Medicine and Biology," it was the benchmark on this topic, providing a broad audience (ranging from medical students to basic scientists, physicians and all other health care professionals) with up to date information in a comprehensive, highly readable format. Since that time, a huge mountain of new scientific findings has been build up, that, at one side underlines the many facettes and the high biological/clinical relevance of Notch signaling and at the other, further unravels the underlying molecular mechanisms. Therefore, we have decided that it is now the right time to publish an updated and extended version.

The second edition of this book has been expanded substantially to cover all aspects of this fast growing field and has been divided in three separate volumes to include additional chapters. In this new edition, leading scientists provide a comprehensive, highly readable overview on molecular mechanisms of Notch signaling (Volume I), Notch's role in embryonic development (Volume II), and last but not least, its relevance for cancer (Volume III).

This first volume gives an overview on the molecular mechanisms that mediate the biological effects of the highly conserved Notch signaling system. As outlined above, it must be emphasized that the Notch pathway seems delusorily simple, with one of its key features being a direct link between an extracellular signal and transcriptional output without the requirement for an extended chain of protein intermediaries as needed by so many other signaling pathways (Hunter and Giniger 2020). However, this apparent simplicity hides remarkable complexity, and consistent with its important role in many aspects of development, it has to be noted that Notch signaling has an extensive collection of mechanisms that it exerts alongside its core transcriptional machinery. In many biological processes, including morphological events during embryogenesis and during pathogenesis and progression of cancer, Notch-mediated coordination of the activity of gene expression with regulation of cell morphology is of high importance. Notably, Notch signaling orchestrates and governs cellular development by inducing and regulating communication between adjacent cells (Fleming 2020). In general, a cell expressing the Notch receptor can be activated in trans by ligands on an adjacent cell leading to alteration of transcription and cellular fate. However, ligands also have the ability to inhibit Notch signaling and this can be accomplished when both receptor and ligands are coexpressed in cis on the same cell. Notably, the manner in which cis-inhibition is accomplished is not entirely clear but it is known to involve several different protein domains of the ligands and the corresponding Notch receptor. While some of the protein domains involved in trans-activation are also used for cis-inhibition, others are used uniquely for each process. Other important aspects for the regulation of both canonical and noncanonical Notch signaling are phosphorylation and proteolytic cleavage of Notch (Hunter and Giniger 2020). The so-called noncanonical Notch pathways diversify the potential outputs of Notch, and allow it to coordinate regulation of many aspects of the biology of cells. Special attention should be given to the role of posttranslational modifications of Notch for noncanonical Notch signaling. Fortunately, the generation and investigation of knockout mice and other animal models have in recent years resulted in a huge mountain of new informations concerning Notch gene function, allowing to dissect the role of specific Notch components in human development and disease.

This volume is intended to provide both basic scientists and clinicians who seek the most current and clearest understanding of the molecular mechanisms that mediate Notch signaling with an authoritative day-to-day source of the same.

In Chap. 2, Brendan McIntyre and coworkers give an excellent overview of Basic Mechanisms of Notch Signaling in Development and Disease (McIntyre et al. 2020). They underline that the evolutionary conserved Notch signaling pathway is associated with the development and differentiation of all metazoans, that it is needed for proper germ layer formation and segmentation of the embryo and that it controls the timing and duration of differentiation events in a dynamic manner. As these authors further briefly summarize, perturbations of Notch signaling may result in blockades of developmental cascades, developmental anomalies, and cancers. Brendan McIntyre and coworkers conclude that an in-depth understanding of Notch signaling is thus required to comprehend the basis of development and cancer, and can be further exploited to understand and direct the outcomes of targeted cellular differentiation into desired cell types and complex tissues from pluripotent or adult stem and progenitor cells. In their chapter, Brendan McIntyre and coworkers explicitly summarize the molecular, evolutionary, and developmental basis of Notch signaling, focussing on understanding the basics of Notch signaling and its signaling control mechanisms, its developmental outcomes and perturbations leading to developmental defects, as well as have a brief look at mutations of the Notch signaling pathway causing human hereditary disorders or cancers.

In Chap. 3, Robert J. Fleming discusses explicitly the role of an extracellular region of Serrate for Ligand-induced cis-inhibition of Notch signaling (Fleming 2020). As the author points out, cellular development can be controlled by communication between adjacent cells mediated by the highly conserved Notch signaling system. He explicitly summarizes that a cell expressing the Notch receptor can be activated in trans by ligands on an adjacent cell leading to alteration of transcription and cellular fate. Robert J. Fleming further explains that ligands also have the ability to inhibit Notch signaling and that this can be accomplished when both receptor and ligands are coexpressed in cis on the same cell. Notably, the manner in which cis-inhibition is accomplished is not entirely clear but it is known to involve several different protein domains of the ligands and the corresponding Notch receptor. While some of the protein domains involved in trans-activation are also used for cis-inhibition, others are used uniquely for each process. In the chapter, the involvement of various ligand regions and the receptor are discussed in relation to their contributions to Notch signaling.

In Chap. 4, Hunter and Giniger discuss other important aspects for the regulation of canonical and noncanonical Notch signaling, namely, phosphorylation and proteolytic cleavage of Notch (Hunter and Giniger 2020). As they point out, the Notch signaling pathway seems deceptively simple, with its key feature being a direct connection between extracellular signal and transcriptional output without the need for an extended chain of protein intermediaries as required by so many other signaling paradigms. However, they discuss that this apparent simplicity hides considerable complexity and that Notch signaling, consistent with its central role in many aspects of development, has an extensive collection of mechanisms that it employs alongside its core transcriptional machinery. They convincingly summarize that these so-called noncanonical Notch pathways diversify the potential outputs of Notch, and allow it to coordinate regulation of many aspects of the biology of cells. In their chapter, Hunter and Giniger review noncanonical Notch signaling with special attention to the role of posttranslational modifications of Notch. Moreover, they also consider the importance of coordinating the activity of gene expression with regulation of cell morphology in biological processes, including axon guidance and other morphological events during embryogenesis.

In Chap. 5, Bhawana Maurya and coworkers summarize how Maheshvara a conserved RNA helicase regulates Notch signaling in *Drosophila melanogaster* (Maurya et al. 2020). They explain that gene expression is regulated at multiple steps after generation of primary RNA transcripts, including mRNA processing, stability, transport, along with co- and posttranscriptional regulation and that these processes are all controlled via involvement of multitude of RNA

binding proteins (RBPs). As they further discuss, innumerable human diseases have been associated with altered expression of these RNA binding proteins. In their chapter, the authors focus on *maheshvara* (*mahe*), which encodes a putative DEAD box RNA helicase protein in *Drosophila* and plays an important role in regulation of Notch signaling. Fine tuning of Notch signaling is required at multiple steps, since its misregulation leads to a variety of human diseases. Additionally, the authors explain that mutations in *DDX59*, a human homolog of *mahe* results in orofaciodigital syndrome associated with broad neurological phenotypes, and that drosophila *mahe* mutants show abnormal peripheral and central nervous system development that resembles neuropathology of patients harboring mutations in the *DDX59* gene. In summary, this chapter explicitly presents recent advances in our knowledge as to how *mahe* regulates Notch signaling and nervous system development.

In Chap. 6, Laura Marquez-Exposito and coworkers summarize our present understanding, how Gremlin, a member of the transforming growth factor-β (TGF-β) family, regulates Notch signaling (Marquez-Exposito et al. 2020). The authors conclude that the axis Gremlin-1/Notch plays a significative role in the embryonary development as well as some adult tissue injury, such as kidney failure. Nevertheless, more studies are needed in order to determine the intrincate functions of these signaling pathways in development and adult homeostasis.

The following chapters (7 and 8) focus on other, selected aspects of the molecular regulation of Notch signaling. In Chap. 7, Debdeep Dutta and coworkers explain the role of the Heterogeneous Nuclear Ribonucleoprotein Hrp48 and Deltex for the regulation of Notch Signaling in *Drosophila melanogaster* (Dutta et al. 2020). The authors point out that, due to its involvement in numerous developmental events, Notch signaling requires tight spatial and temporal regulation. Deltex is a cytoplasmic protein that physically binds to Notch and regulates its signaling activity in a context-dependent manner. However, as Debdeep Dutta and coworkers explain, the biology of Deltex in regulation of Notch signaling is not well explored. The authors report that Hrp48, an RNA-binding protein, was identified as an interacting partner of Deltex, and that interaction of these two proteins seemed to regulate the Notch signaling outcome in the epithelial tissue. Additionally, it was found that coexpression of Deltex and Hrp48 can lead to cell death as well as JNK activation. Debdeep Dutta and coworkers conclude that, considering the well-conserved nature of Notch, Hrp48, and Deltex, this interaction can be helpful to understand the regulation of Notch signaling both in development and disease condition.

In Chap. 8, Amanda Salviano-Silva and coworkers explicitly summarize the relevance of the interaction of long noncoding RNAs and Notch signaling for tissue homeostasis (Salviano-Silva et al. 2020). They shortly explain that Notch signaling is a crucial pathway involved in cellular development, progression, and differentiation and that deregulation of Notch signaling commonly impacts tissue homeostasis, being highly associated with proliferative disorders. As they point out, the long noncoding RNAs (lncRNAs), which are transcripts with more than 200 nucleotides that do not code for proteins, were already described as Notch signaling pathway-interacting molecules. Many of them act as important transcriptional and posttranscriptional regulators, affecting gene expression and targeting other regulatory molecules, such as miRNAs. Due to their strong impact on function and gene expression of Notch-related molecules, lncRNAs influence susceptibility to cancer and other diseases, and can be regarded as potential biomarkers and therapeutic targets. In this chapter, the authors summarize the cross talk between the Notch signaling pathway and their most important modulating lncRNAs, as well as the pathological consequences of these interactions, in different tissues.

In Chap. 9, Rajaguru Aradhya and Krzysztof Jagla report and discuss the relevance of Insulin-dependent noncanonical activation of Notch in *Drosophila*: a fascinating story of Notch-induced muscle stem cell proliferation (Aradhya and Jagla 2020). As they point out, *Notch* plays multiple roles both in development and in adult tissue

homeostasis with flagship functions being its capacity to keep precursor and stem cells in a nondifferentiated state but also its ability to activate cell proliferation that in some contexts could lead to cancer. In general, both these functions involve canonical, ligand-dependent Notch activation. However, as the authors explain a ligand-independent Notch activation has also been described in a few cellular contexts. In their chapter, Rajaguru Aradhya and Krzysztof Jagla focus on one of such contexts, *Drosophila* muscle stem cells, called AMPs, and discuss how insulin-dependent noncanonical activation of Notch pushes quiescent AMPs to proliferation.

In Chap. 10, Tsaouli and coworkers discuss the impact of NF-κB for molecular mechanisms of Notch signaling in lymphoid cell lineages development (Tsaouli et al. 2020). As they point out, Notch is a ligand–receptor interaction-triggered signaling cascade highly conserved, that influences multiple lineage decisions within the hematopoietic and the immune system, representing a recognized model of intercellular communication that plays an essential role in embryonic as well as in adult immune cell development and homeostasis. Four members belong to the family of Notch receptors (Notch1–4), and each of them plays nonredundant functions at several developmental stages. They explain that canonical and noncanonical pathways of Notch signaling are multifaceted drivers of immune cells biology and that increasing evidence highlighted Notch as an important modulator of immune responses, also in cancer microenvironment. The authors discuss, that in these contexts, multiple transduction signals, including canonical and alternative NF-κB pathways, play a relevant role. In this chapter, they first describe the critical role of Notch and NF-κB signals in lymphoid lineages developing in thymus: T natural killer cells, thymocytes, and thymic T regulatory cells. The authors also address the role played by ligand expressing cells, and given the importance of Notch/NF-κB cross talk, discuss its role in T-cell leukemia development and progression.

Last but not least, we would like to thank all authors for their excellent contributions to this book. We hope that this volume will provide a broad audience (ranging from medical students to basic scientists, physicians, and all other health care professionals) who seek the most current and clearest understanding of the molecular mechanisms of Notch signaling with up-to-date information in a comprehensive, highly readable format and with an authoritative day-to-day source of the same.

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2 Overview of Basic Mechanisms of Notch Signaling in Development and Disease

Brendan McIntyre, Takayuki Asahara, and Cantas Alev

Abstract

Notch signaling is an evolutionarily conserved pathway associated with the development and differentiation of all metazoans. It is needed for proper germ layer formation and segmentation of the embryo and controls the timing and duration of differentiation events in a dynamic manner. Perturbations of Notch signaling result in blockades of developmental cascades, developmental anomalies, and cancers. An in-depth understanding of Notch signaling is thus required to comprehend the basis of development and cancer, and can be further exploited to understand and direct the outcomes of targeted cellular differentiation into desired cell types and complex tissues from pluripotent or adult stem and progenitor cells. In this chapter, we briefly summarize the molecular, evolutionary, and developmental basis of Notch signaling. We will focus on understanding the basics of Notch signaling and its signaling control mechanisms, its developmental outcomes and perturbations leading to developmental defects, as well as have a brief look at mutations of the Notch signaling pathway causing human hereditary disorders or cancers.

Keywords

Notch pathway · Delta · Posttranslational modifications · Signaling gradients · Lateral inhibition · Notch evolution · Organogenesis · Somitogenesis · Progenitor cells · Stem cells · Differentiation

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Introduction

The evolutionarily conserved Notch signaling pathway has an essential role in metazoan development. From germ layer formation to the differentiation of specialized cell types in the embryo, Notch signaling is involved in a variety of developmental processes. Originally named over 100 years ago after the associated wing phenotype in *Drosophila* (Dexter 1914), the genomic region responsible for this mutation was identified (Morgan 1917). In the modern era of in vitro and in vivo cell-based assays; utilizing knockouts and knockin reporter constructs or CRISPR/Cas9-based genome editing studies, the intricacies of this pathway have been dissected in minute detail. In this chapter, we will summarize the basic mechanisms of Notch signaling and

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signaling control, explore the modulation and regulation of Notch activity, and look at cellular and developmental processes dependent on this pathway. We will then briefly explore the evolution of the Notch signaling pathway and focus on the role of Notch during early development and differentiation, before moving on to mutations of the Notch signaling pathway and their role in human disease.

Basic Mechanisms of the Notch Signaling Pathway

The Notch pathway is most simply illustrated in *Drosophila*, where its key components are limited to two Notch ligands, Serrate and Delta, which bind to the extracellular domain of the single-pass transmembrane Notch receptor (Fig. 2.1). The Notch receptor contains a large array of epidermal growth factor (EGF) repeats that can be modified by the addition of sugars, and a negative regulatory region, which is important for its cleavage by Furin-like convertases in the Golgi (Kidd and Lieber 2002; Siebel and Lendahl 2017). In mammals, the Notch pathway has been rendered more complex than in *Drosophila* by the addition of two Serrate orthologs (Jagged proteins), three Delta-like proteins, and four Notch receptor proteins (Kopan and Ilagan 2009). During canonical Notch signaling, when interaction of Notch with the Notch ligands Serrate or Delta (Jagged 1 and 2 and Delta-like 1, 3, or 4 in mammals) occurs, the intracellular portion of the Notch receptor is sequentially cleaved by an ADAM-family metalloproteinase in the extracellular region and the gamma-secretase complex in the transmembrane region, which frees the Notch intracellular domain (NICD). Translocation of NICD into the nucleus is then mediated by alpha-importin proteins and once in the nucleus NICD binds to the transcription

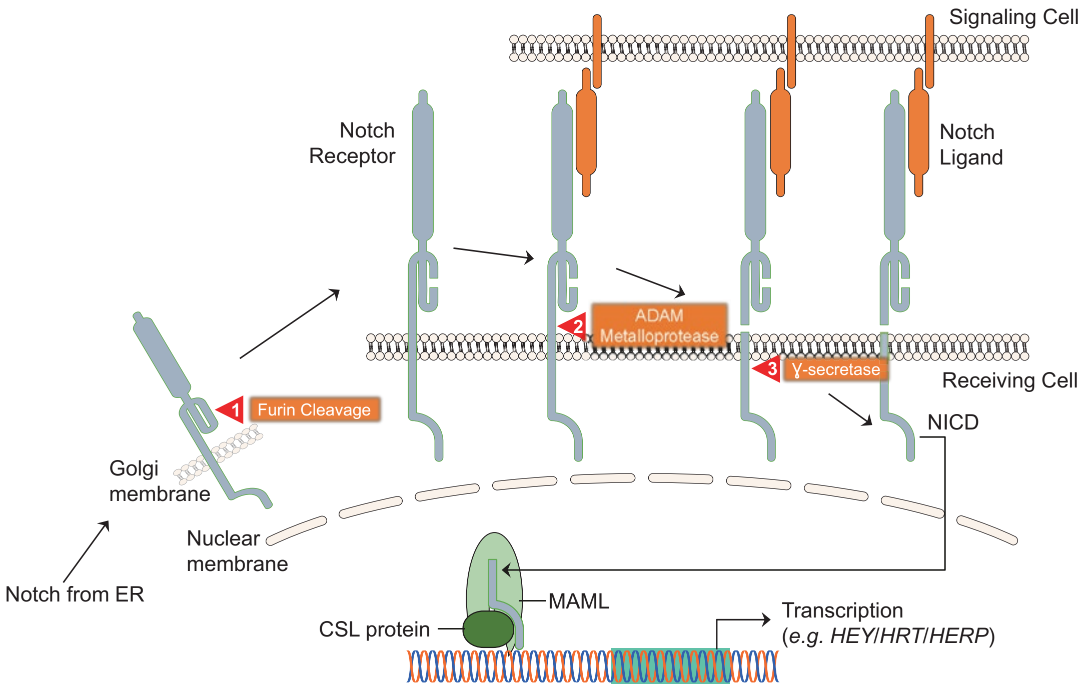

Fig. 2.1 Schematic overview of Notch signaling. Notch is processed in a series of posttranslational events mediated by Furin-like convertase and active Notch is translocated to the cell membrane. When Notch binds to ligand on the surface of an adjacent cell proteolytic cleavage by an ADAM metalloprotease and gamma-secretase results in the release of intracellular Notch (NICD) that translocates to the nucleus and together with a complex of coactivators including CSL and MAML, initiates transcription of target genes

factor Suppressor of Hairless (Su(H)), or the CSL complex in mammals (CBF1/Suppressor of Hairless/LAG-1; also known as RBPJ in mice), activating downstream targets (Fig. 2.1) (D'Souza et al. 2008; Brou et al. 2000; De Strooper et al. 1999; Okochi et al. 2002; Huenniger et al. 2010). Su(H)/CSL has a repressive role in the absence of Notch but is converted to a transcriptional activator when Notch signaling is initiated by recruitment of components of an activation complex which include Mastermind (Mastermind-like (MAML) 1–4 in mammals) and histone acetyltransferases, leading to transcriptional activation of effector genes. Among these, some of the key targets are the *HES* (Hairy and Enhancer of Split) homologs of the *Hairy* gene in *Drosophila*, and *HES* related genes, *HEY/HRT/HERP*, which all encode transcriptional repressors (Bray 2006; Masek and Andersson 2017). The action of intracellular Notch is regulated by a rapid rate of protein turnover that occurs at the PEST (rich in proline (P), glutamic acid (E), serine (S), and threonine (T)) degradation domain, which is targeted by ubiquitylation (Andersson et al. 2011). Collectively, this signal transduction cascade is known as the Canonical Notch Signaling Pathway.

The ability of Notch signaling to function is primarily dependent on cell–cell contact which brings the transmembrane Notch receptors (Notch 1–4 in mammals) and ligands, Delta-like and Jagged in mammals, together. Thus, Notch signaling provides a juxtacrine mechanism whereby neighboring cells can communicate with one another and directly influence gene activity, which has direct implications on how complex differentiation steps in multicellular organisms can be regulated in space and time (Henrique and Schweisguth 2019). Concomitantly, if a ligand and a receptor are expressed in the same cell, there is also the possibility of *cis* interactions of the Notch pathway. The current view is that interactions between cells, or *trans* interactions, lead to receptor activation, whereas ligand–receptor interactions occurring in the same cell, or *cis* interactions, are inhibitory (Siebel and Lendahl 2017; D'Souza et al. 2008). An important exception to this juxtacrine signaling mechanism has been observed in the form of extracellular microvesicles or exosomes. Exosomes loaded with high levels of Delta-like ligand-4 (DLL4) have been shown to be able to transfer their protein load and inhibit Notch signaling in vitro and in vivo (Sheldon et al. 2010). It is conceivable that a similar mechanism of DLL4 loaded exosomes could operate in a disease state such as cancer, although this has yet to be conclusively proven outside of experimental model systems.

In terms of the dynamics of Notch activity, the principle mode of action for Notch signaling occurs when nearby cells are organized in groups and are able to switch off a given trait in a neighbor, a process generally called lateral inhibition although possibly more accurately referred to as lateral specification, which is mediated through *trans* interactions (Greenwald and Rubin 1992; Greenwald 2012). A classic example of lateral inhibition can be seen in studies of bristle formation in *Drosophila* (Fig. 2.2) (Heitzler and Simpson 1991). *Trans* interactions of the Notch signaling pathway are further involved in the formation and patterning of complex embryonic structures including vertebrae, limbs, and organ buds, and will be discussed in more detail below.

Modulation and Regulation of Notch Signaling Transduction

Notch signaling can be modified at a number of different stages of the signaling cascade, acting on either the Notch receptors themselves, their ligands, ligand mediated-protein cleavage or subsequent NICD nuclear translocation and activation. The modes of activity responsible for modifying Notch signaling include posttranslational modification of the epidermal growth factor-like (EGF-like) repeats in the Notch receptor extracellular domain by oxygen-linked (O-linked) glycosylation, and downstream positive or negative feedback loops controlling receptor, ligand, or target transcription factor activity and expression.

The first step of modulation of Notch signaling occurs when newly synthesized Notch protein

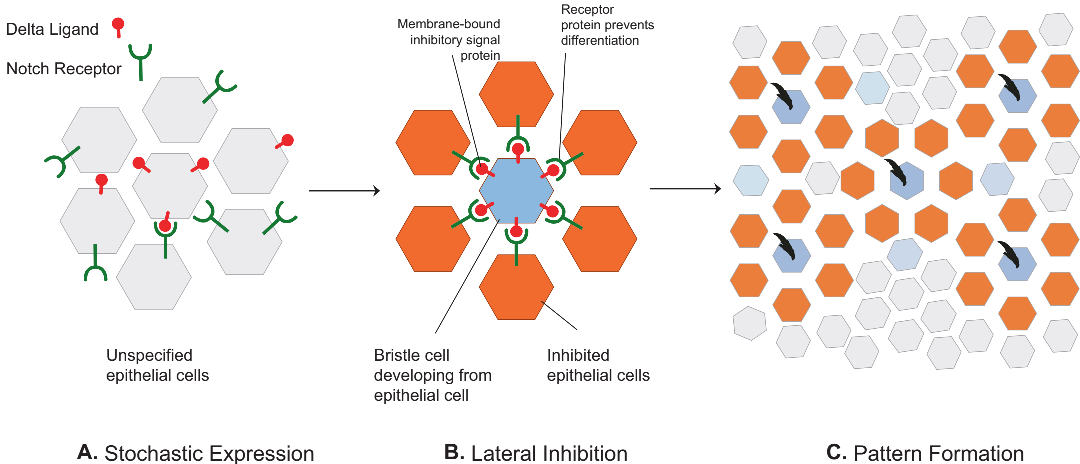

Fig. 2.2 Model of lateral inhibition mediated by Notch during bristle formation in *Drosophila*. (**a**) Stochastic expression of Notch receptor and ligand in a pool of epithelial cells. Cells with higher levels of receptor will be maintained as a progenitor pool, and cells with higher levels of ligand will downregulate receptor expression and differentiate. (**b**) Lateral inhibition where Notch expressing cells are maintained as progenitor pool and ligand expressing cells have differentiated. (**c**) Pattern formation through propagation of expression system. This type of lateral inhibitory mechanism can be observed with bristle differentiation in *Drosophila*, as pictured

is posttranslationally modified by endoplasmic reticulum-localized glycosyltransferases. The EGF-like repeats of Notch are modified by the addition of O-linked glucose or fucose, with or without the addition of a xylose added via a xylosyltransferase at their serine and threonine residues (Haines and Irvine 2003; Bakker and Gerardy-Schahn 2017). The glucosyltransferase Rumi adds O-linked glucose and the fucosyltransferase POFUT1 is responsible for adding O-linked fucose to Notch. Glycosylation and fucosylation are known to be essential for Notch signaling as both Rumi and POFUT1 loss of function result in a loss of Notch signaling activity (Acar et al. 2008; Li et al. 2017). The glycosyltransferase Fringe (Fn) is another key regulator of Notch signaling (Ju et al. 2000; Bruckner et al. 2000). Fringe elongates the O-linked fucose residues added by POFUT1 by the addition of *N*-acetylglucosamine (GlcNAc), and confers an ability to further tune the pathway's activity (LeBon et al. 2014). There is only one Fn protein in *Drosophila*, but mammals have three different related factors: Lunatic Fringe (LFNG), Maniac Fringe (MFNG), and Radical Fringe (RFNG), respectively, with the roles of LFNG and MFNG corresponding to that of Fringe in *Drosophila*, namely, in their abilities to act as inhibitors of Serrate and enhancers of Delta signaling (Panin et al. 1997). In contrast, RFNG increases the *trans* response of Notch to both ligands (Fig. 2.3) (Ladi et al. 2005). Positive and negative feedback loops are a cornerstone to Notch signaling. In two adjacent cells both expressing Delta and Notch at stochastically different levels, an outcome will occur whereby the cell with higher Notch expression remains undifferentiated and the cell with higher Delta expression will downregulate Notch signaling and differentiate. In this way, amplificatory feedback on either cell escalates Notch or Delta expression and results in divergent cell fates (Fig. 2.2).

In the mammalian system, where multiple ligands and receptors for Notch exist, feedback loops can be controlled in a more complex manner. For example, a cell expressing high levels of Notch ligand can reduce its signaling to an adjacent cell by expressing a weaker ligand that will compete with Notch receptor (Benedito et al. 2009; Petrovic et al. 2014). Different ligands also allow for different downstream responses in the nucleus. Notch 1 activation by Delta-like 1 leads to strong *Hes1* expression, whereas Notch 1 activation by Delta-like 4 results in *Hey1* expression.

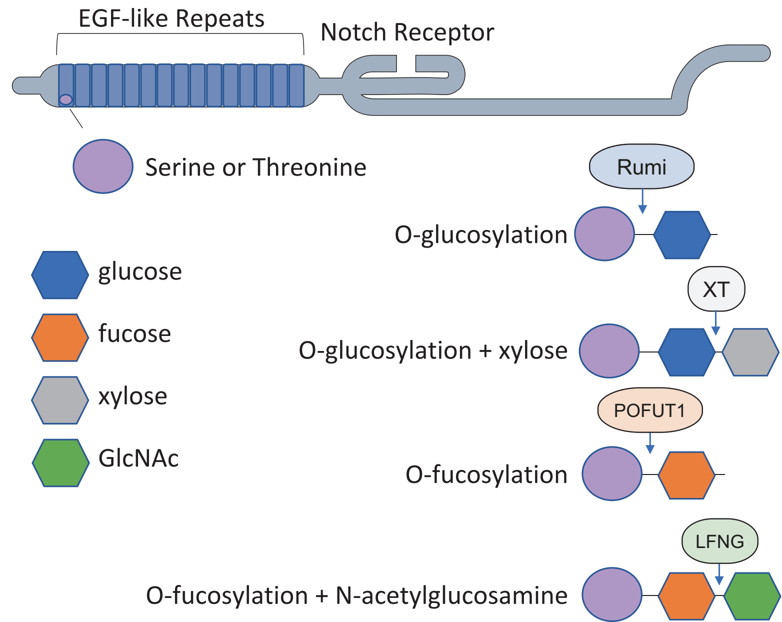

Fig. 2.3 Posttranslational modifications of the Notch receptor. Glucosyltransferase Rumi adds glucose with or without the addition of xylose molecules. Fucosyltransferase POFUT1 adds fucose to Notch receptor with or without additional *N*-acetylglucosamine (GlcNAc) addition by lunatic fringe (LFNG). Modifications occur at serine or threonine residues in the EGF-like repeats of the Notch receptor

These two transcription factors exhibit opposite effects on the process of myogenesis, despite the fact that the two inputs both effectuate NICD translocation to the nucleus (Nandagopal et al. 2018). The difference lies in how they activate Notch 1, with Delta-like 4 stimulation resulting in a sustained augmented activation of NCID and Delta-like 1 stimulation resulting in transient, pulsed activity of NICD (Nandagopal et al. 2018).

The downstream transcriptional networks activated via Notch signaling also have the role of providing either positive or negative feedback on the pathway. For instance, Notch target *HES* genes (mammalian homologs of hairy and Enhancer-of-split in *Drosophila*) are involved in preventing cellular differentiation and preserve stemness in different developmental contexts and tissues such as the forming nervous system. When activated in *trans* by Notch ligand, the receiving cell expresses *HES1* and *HES5*, which downregulate expression of Notch ligand and stimulate expression of Notch receptor, thus providing a positive feedback loop on pathway activation and preventing the cell from differentiating (Kageyama et al. 2007). A recent study using CRISPR mediated gene editing has also elucidated a similar role for Notch NICD in preserving stemness of intestinal stem cells by maintaining a positive feedback loop (Chen et al. 2017).

Evolutionary Origins of Notch Signaling

The Notch pathway is specific to metazoans, animals that undergo a three-germ layer embryonic developmental program (Gazave et al. 2009; Babonis et al. 2017). Despite this metazoan specificity, many of the enzymes associated with Notch signaling have likely evolved from co-option of existing signaling pathways, which are premetazoan in nature (Gazave et al. 2009). For instance, the evolution of the catalytic component of the gamma-secretase Presenilin-1 and its cofactors Presenilin-2, Nicastrin, and APH1 predates the evolution of metazoans as they are also found in plants (Khandelwal et al. 2007).

Single-celled Capsaspora and Choanoflagellates, also contain elements of Notch signaling such as the CSL transcription factor and proteins that are similar to Notch and Delta ligands in their domain architecture. However, these ancestral elements of Notch signaling cannot be classified as complete Notch signaling pathways, and are referred to as

"proto-Notch" in nature (King et al. 2008; Morsut et al. 2016). Indeed, the evolution of this pathway in metazoans has led to the speculation that Notch signaling was, in fact, a prerequisite to multicellular life. The mode of juxtacrine signaling mechanism utilized in Notch signal transduction would appear to give strength to this notion (Morsut et al. 2016). The basic architecture of the Notch pathway has also been elaborated upon in evolutionary time, as more complex facets of Notch signaling such as repressors (e.g., Hairless) appear to be restricted to chordates and arthropods. Further, the coactivator MAML is not found in more primitive metazoans such as aquatic cnidarians indicating that elaboration of Notch signaling occurred during evolution (Maier 2019). For further insights into the evolutionary role and emergence of the Notch signaling pathway the reader is referred to the studies of Theodosiou et al. (2009) and Shi and Stanley (2006).

Cellular and Developmental Processes Using Notch Signaling

In certain arthropods, Notch signaling has been found to be essential for the process of segmentation, notably in spiders (Stollewerk et al. 2003). Surprisingly in flies, Notch is dispensable for segmentation, indicating that a conserved program for segmentation was shared in a common ancestor and lost during subsequent divergent evolution (Shi and Stanley 2006). Germ layer formation (the formation of ectoderm, endoderm, and mesoderm) occurs prior to segmentation and Notch signaling is required for germ layer formation in *C. elegans* and sea urchins (Good et al. 2004; Peterson and McClay 2005). More specifically, Notch is involved in cellular partitioning and establishment of cellular identity (Gazave et al. 2009). During early embryonic differentiation such as epiblast formation and establishment of a head-to-tail (anterior–posterior) axis, Notch signaling does not appear to be required (Souilhol et al. 2015). Notch signaling is also dispensable for rodent and human pluripotent stem cell self-renewal (Lowell et al. 2006).

However, during gastrulation of vertebrates Notch signaling plays a regulatory role, as enforced activation of Notch signaling during this process results in a lack of mesoderm formation in both *Xenopus* (Contakos et al. 2005; Revinski et al. 2010) and mouse (Souilhol et al. 2015). Interestingly, in mouse models where Notch signaling has been abrogated by means of knocking out both *Presenilins 1* and *2*, *Csl/RBPJ*, or *Pofut1*, embryos exhibit normal development up until E8.0, well after germ layer formation, with later defects in somitogenesis, as well as neural, cardiac, and vascular defects (Donoviel et al. 1999; Oka et al. 1995; Shi and Stanley 2003). In contrast, modulating Notch signaling in anamniotes such as *Xenopus* and zebrafish has demonstrated effects on endodermal versus mesodermal specification (Contakos et al. 2005; Kikuchi et al. 2004). In aggregate, it appears that Notch signaling is required for different facets of developmental processes depending on the organism and context studied, with more direct consequences on early embryogenesis in evolutionarily ancient metazoan organisms.

Pattern formation is essential in all multicellular organisms, and Notch signaling, due to its inherent utility in coordinating direct cell-to-cell mediated control of fate and function, has a prominent role. In its most basic form, for instance, Notch mediated lateral inhibition occurs when a cell expressing Delta activates Notch signaling on all sides but creates an island where Notch signaling is turned down. This mechanism is seen at play during bristle formation of *Drosophila* (Fig. 2.2) (Cohen et al. 2010). Although this largely oversimplifies the dynamics of other factors at play, it provides an example of how Notch signaling can be used for the establishment of regularly interspersed patterns in a developing embryo. Further mathematical modeling used to examine this process has shown that Notch signaling in sensory fields transcends simple cell–cell interactions and contributes to the formation of self-organizing patterns of regular bristle rows (Corson et al. 2017).

In vertebrates, the first evidence of segmentation, the subdivision of the forming embryonic body plan into discrete segments, is observed and

established by the process of somitogenesis. Somites are the embryonic segments of vertebrates that give rise to dermis, skeletal muscle, cartilage, and bone. Somites originate in the paraxial mesoderm adjacent to the notochord on either side of the neural tube, throughout the trunk and tail of the embryo (Pourquie 2001). Somitogenesis is an important example of pattern formation in development and it too is partly controlled through rhythmically expressed genes of the Notch pathway that contribute to the formation of a developmental clock. During this process, it is known that Notch does not work independently but in combination with other signaling pathways, notably Fgf and Wnt signaling, which vary their expression in an ultradian rhythm (a recurrent period within the day rather than daily such as circadian). This ultradian rhythm, the so-called segmentation clock, cycles with approximately 2–3 h oscillation intervals in mice and shows species-specific differences in period length (Pourquie 2003; Dequeant and Pourquie 2008). FGF signaling is known to be functionally linked to Notch, and has been shown to act upstream of Notch using a conditional paraxial mesoderm FGF receptor knockout model (Wahl et al. 2007). Quantitative monitoring using mouse reporter lines has also shown that Wnt and Notch signaling are functionally linked at the level of their oscillatory activity (Sonnen et al. 2018). Strikingly, experiments using cells from randomized dissociated presomitic mesoderm from multiple transgenic-reporter mouse embryos has been shown to self-organize in vitro and mimic the synchronization and order seen in vivo in a Notch dependent manner (Tsiairis and Aulehla 2016).

Two Notch target genes, *Lfng* and *Hes7*, which exhibit oscillatory waves of periodic repression, negatively regulate the Notch pathway, with Hes7 protein autorepressing its own gene expression and that of *Lfng*, providing a molecular basis for the segmentation clock (Chen et al. 2005). Studies in the mouse have shown that this oscillatory Notch signaling is a requirement for periodicity needed for somite generation as inhibition of Notch signaling leads to a lack of somite development (Ferjentsik et al. 2009). Conversely, accelerating the segmentation clock by increasing the rate of *Hes7* expression was shown to lead to supernumerary somites in the mouse (Harima et al. 2013). The oscillatory activity of *Lfng* and *Hes7* slows down as it approaches the wave front at the boundary of new somite formation, and regions along the AP axis of the paraxial mesoderm are coordinated into different phases of cyclical expression (Fig. 2.4). The wave pattern of expression repeats for every newly formed somite and phase patterns are duplicated (Oates et al. 2012).

In mice, the basic helix–loop–helix (bHLH) transcription factor Mesp2 is known to initiate somite formation and is controlled by the Tbx6 transcription factor and cyclic Notch signaling (Takahashi et al. 2000; Morimoto et al. 2007). The Mesp2 transcription factor has been described as the master spatiotemporal regulator of somitogenesis and its expression is initiated in cells with active Notch NICD and Tbx6 in a cooperative manner (Saga et al. 1997; Wahi et al. 2016). Once Mesp2 expression is induced it rapidly acts to repress Notch signaling via destabilization of MAML, leading to a lack of CSL-NICD complex formation, and specification of a rostral vs. caudal somite identity, required for proper maturation of somites and for vertebral bone morphogenesis in mice (Sasaki et al. 2011; Saga 2012). Although somitogenesis works with different control characteristics in other model organisms such as zebrafish and chick, a network of orthologous factors, which modify and relay Notch activity are being utilized (Yabe and Takada 2016; Krol et al. 2011; Dale et al. 2003). Mutations in Notch signaling pathway members were further found to cause segmentation defects of the vertebrae such as spondylocostal dysostosis, including pathogenic mutations in *HES7* (Sparrow et al. 2008; Bessho et al. 2001), *LFNG* (Niwa et al. 2007; Sparrow et al. 2006) and *DLL3* (Bulman et al. 2000; Kusumi et al. 1998) indicating the importance of Notch signaling during not only murine but also human somitogenesis and axial skeletal development. For a more detailed look at the role of Notch and other signaling pathways on somitogenesis and the segmentation clock, the readers are referred to recent reviews

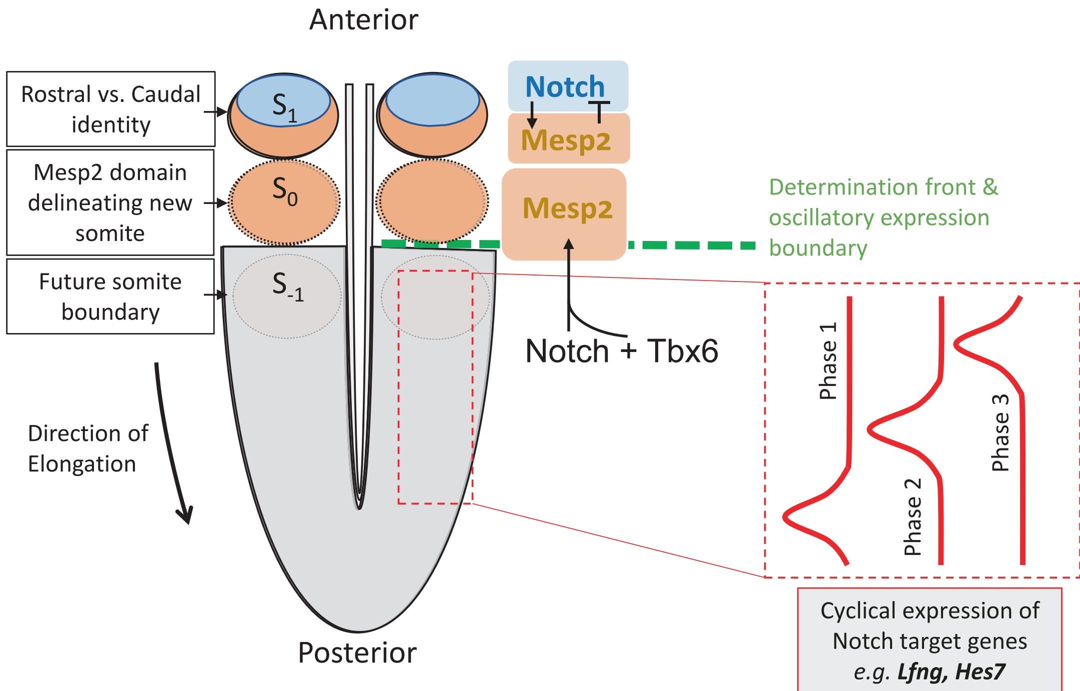

Fig. 2.4 Notch signaling in somitogenesis. Dynamic interplay between Notch and Mesp2 regulates somitogenesis and subsequent rostrocaudal identity of the somites. The molecular clock that determines somite generation is governed by the expression of Notch target genes *Lfng* and *Hes7*, which regulate Notch activity. Notch and Tbx6 cooperatively activate Mesp2 expression. A zone of Mesp2 expression delineates newly forming somites, and cyclical Notch activity occurring in waves of gene expression delineates new somite boundaries

by Pourquie et al. (Pourquie 2011; Hubaud and Pourquie 2014), Kageyama et al. (Kageyama et al. 2018; Shimojo and Kageyama 2016), and Oates et al. (Venzin and Oates 2019; Liao and Oates 2017).

Notch in Stem and Progenitor Cells

Proper control and maintenance of stem and progenitor cell pools during embryogenesis are essential for normal development and differentiation including the aforementioned processes. In the adult organism these stem and progenitor cells are also needed for tissue homeostasis and repair, and Notch signaling is used repeatedly during both differentiation/development and repair/homeostasis events (Koch et al. 2013). The role and molecular mechanism of Notch signaling cascades have been studied for neural, muscle, and intestinal stem cell maintenance in flies and vertebrates.

In zebrafish adult neural stem cells (NSC), Notch activity drives a quiescent fate, whereas blocking Notch results in NSC proliferation and subsequent differentiation (Chapouton et al. 2010). During the generation of skeletal muscle, Notch activity drives transcription of *Pax7*, which controls self-renewal of muscle progenitor/stem cells, or satellite cells. Notch signaling is also used in these cells to maintain stemness and repress myogenic gene signatures needed for terminal differentiation (Koch et al. 2013). Satellite cells of aged mice are characterized by loss of Notch activity and concomitant loss of regenerative potential. Forced Notch activation in these aged animals restores regenerative ability and results in satellite cell proliferation and differentiation to myofibers (Conboy et al. 2003). Experiments such as these give clues as to how

Notch modulation could be harnessed in the field of regenerative medicine and aging research.

Endodermal organs such as the lung, liver, and gut rely on Notch signaling in a variety of differentiation steps in their development. Lung is an organ with a noted high level of expression of Notch receptors and ligands during its development (Post et al. 2000) and Notch signaling is important for multiple steps in its formation (Rock et al. 2011; Morimoto et al. 2010, 2012). Early in development, a gradient of Notch expression arises in the proximal to distal axis in lung formation, which will give rise to ciliated epithelia and gas exchange compartments, respectively and ablation of Notch signaling results in developmental patterns skewing toward distal fates (Tsao et al. 2008, 2009). In adult lungs, Notch is involved in the homeostatic balance of secretory to ciliated cells, with both cell types derived from a common progenitor. Blocking Notch signaling through anti-Jagged mediated inhibition results in a gain of ciliated cells at the expense of a loss of secretory club cells by means of direct conversion (Lafkas et al. 2015).

In the context of the endodermal organ liver, Notch signaling plays a similar role in delineating liver cell fate decisions of progenitor hepatoblasts into hepatocytes or cholangiocytes/biliary epithelium, with its overall role being to promote formation of the latter (Siebel and Lendahl 2017; Tanimizu and Miyajima 2004; Zong et al. 2009). Furthermore, a conversion of hepatocytes to biliary cells is promoted by Notch activation during injury in the adult (Suzuki 2015).

In the final endoderm-derived organ briefly examined, the gut, proliferative Lgr5+ stem cells of the intestinal crypt are responsible for tissue self-renewal and replenishment of the cells of the rapidly cycling intestinal villi under normal homeostatic conditions. Lgr5+ cells divide and give rise to transient amplifying cells that regenerate the high turnaround of absorptive and secretory cells of the villi, and are dependent on Notch signaling in order to maintain stem cell proliferation (Gehart and Clevers 2019; van Es and Clevers 2005; van Es et al. 2005; Tian et al. 2015). The niche within which these cells reside at the base of the crypt is composed of secretory Paneth cells that escape the upward flow of cellular repopulation of the villi (Spit et al. 2018). Paneth cells express Delta-like ligand and ensure high Notch levels in Lgr5+ cells by lateral inhibition. Notch signaling in intestinal stem cells induces *Hes1* expression that suppresses the expression of *Atoh1*, the fate determination transcription factor for the secretory lineage (Spit et al. 2018; Jensen et al. 2000). Upon exiting the intestinal niche during cell proliferation, certain cells will upregulate Delta-like ligands and thus downregulate Notch, escaping progenitor programs and giving rise to Atoh1+ secretory cells (Fig. 2.5a) (Spit et al. 2018; van Es et al. 2012).

In all three of the endodermal organ systems examined here briefly, Notch is a key regulator of the balance between a stem and progenitor cell state and differentiated progeny that contribute to the formation, as well as adult homeostasis, of the organ in question, while also playing a role during the formation of cancers associated with these endoderm-derived organs (Radtke and Clevers 2005; Radtke et al. 2006; Liu et al. 2015; Xu et al. 2014; Geisler and Strazzabosco 2015; Morell et al. 2013). Further to the in vivo scenarios examined, the role of Notch signaling on the quiescence and control of the stem and progenitor state can also be exploited in the context of in vitro organoid models of human tissues and has been applied to organoid systems using Lgr5+ liver and intestinal stem cells (Sato et al. 2011; Basak et al. 2017; Leung et al. 2018; Date and Sato 2015).

Asymmetric cell division is a key aspect of aforementioned stem and progenitor cell maintenance and subsequent differentiation models, and an unequal distribution of factors within the cell contributes to either self-renewal or proliferation. One of the key factors linked to these processes is Numb. Numb is an important inhibitor of Notch, and the balance of these factors within cells is fundamentally involved in asymmetric cell division. Numb functions via promoting the degradation of Notch by targeting it for ubiquitylation. Numb is asymmetrically expressed, with higher levels contributing to differentiating cells, whereas self-renewal and maintenance of a progenitor pool results from higher Notch levels

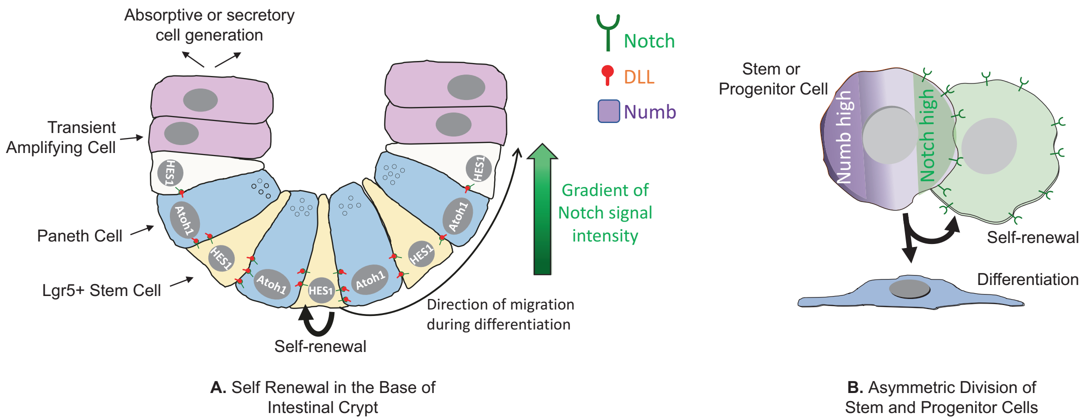

Fig. 2.5 Notch signaling in stem and progenitor cells. (**a**) In the intestinal crypt Lgr5+ intestinal stem cells express Notch receptors and Hes1. Surrounding Paneth cells express differentiation marker Atoh1 and Delta-like ligands leading to a mechanism of lateral inhibition that retains the different cellular identities in normal tissue homeostasis. Crypt cells migrate upward toward the rapidly cycling villi and lose a balance of high levels of Notch expression to differentiate into transiently amplifying cells that will generate secretory and absorptive cells of the villi. (**b**) Cell divisions in stem and progenitor pools result in asymmetry in order to replenish progenitor populations and provide differentiated cells under homeostatic conditions and in tissue repair. Asymmetric distribution of Numb, a negative regulator of Notch, provides a mechanism whereby Notch is downregulated in a part of the cell. During cycling, this part of the cell will differentiate, whereas the part of the cell retaining Notch expression self-renews

and Notch signaling activity (Fig. 2.5b) (Koch et al. 2013; Giebel and Wodarz 2012; Guo et al. 1996; Shen et al. 2002; Cheng et al. 2008; Gonczy 2008). An example of proof for this model can be found in work with transgenic mice which shows that in the adult brain, where Notch activity regulates neural stem cell identity and self-renewal, suppression of Numb expression leads to increased neural stem cell self-renewal (Aguirre et al. 2010). Taken together, the overall role of Notch signaling in stem cells homeostasis is to maintain the balance of tissue specific stem and progenitor populations in the adult organism and, in general, inhibit their differentiation. However, during embryogenesis and development, the role of Notch is not always as straightforward and is largely context dependent.

Mutations in Notch Involved in Hereditary Disorders

Due to its important role during development, knocking out the activity of key Notch pathway members in model organisms results in embryonic lethality. For instance, knockout mice for Notch receptors 1 and 4 or Notch ligands *Delta-like 1* and *Jagged 1* are all embryonic lethal with severe vascular defects (Xue et al. 1999; Krebs et al. 2000, 2004). Knockout mice targeting associated Notch pathway members or downstream Notch-target genes are also used to model developmental diseases. Examples of this are *Mesp2* KO mouse, which effectively models the vertebral segmentation defects seen in spondylocostal dysostosis (Saga et al. 1997; Makino et al. 2013), and *Notch3* KO mice, which partially recapitulate CADASIL (discussed below) (Joutel 2011). Not surprisingly, mutations in members of the Notch signaling pathway lead to a variety of human diseases, and a number of monogenic disorders are caused by mutations in key factors of the Notch signaling pathway that will be briefly discussed below (Masek and Andersson 2017).

Cerebral autosomal dominant arteriopathy or CADASIL arises from mutations in the *NOTCH3* receptor gene. It is 100% penetrant, meaning all individuals carrying these mutations acquire this disease. CADASIL was also the first monogenic disease linked to mutations in Notch (Joutel et al. 1996; Chabriat et al. 2009). Patients with this disease suffer from migraines, strokes, and dementia

later in life. CADASIL is caused by damaged blood vessels in the brain resulting from loss of vascular smooth muscle cell (vSMC) function. Mutation patterns are highly variable but always result in a NOTCH3 protein with deleted or added cysteine residues in the extracellular region of the receptor, with the mutant NOTCH3 appearing to have a role in sequestering proteins involved in vascular homeostasis, which results in granular osmiophilic material accumulation around vSMCs (Lewandowska et al. 2011; Ferrante et al. 2019). The outcome is receptor toxicity, as evidenced by the fact that homozygous carriers exhibit similar symptoms as heterozygotes. In addition to the rodent model mentioned, the disease has recently been modeled using iPSCs derived from CADASIL patients with patient lines successfully responding to Notch pathway inhibitors, giving hope to the development of future treatments (Ling et al. 2019).

A second disease arising from single mutations in a Notch pathway member is Alagille syndrome. Over 90% of patients with Alagille syndrome carry mutations in the extracellular portion of the gene encoding the Jagged 1 ligand, and the remainder with mutations in the *NOTCH2* receptor (Li et al. 1997; Oda et al. 1997). Patients with Alagille syndrome have problems associated with the function of the liver, kidneys, vertebrae and eyes as well as craniofacial abnormalities (Siebel and Lendahl 2017). Loss-of-function mutations would appear to be the cause of Alagille syndrome as *Jagged 1* heterozygous knockout mice exhibit some of the features of this disease (Huppert 2016). In contrast, Hajdu–Cheney syndrome results from activating mutations in the *NOTCH2* receptor (Simpson et al. 2011). Patients with Hajdu–Cheney syndrome experience loss of bone tissue, craniofacial defects, osteoporosis, and polycystic kidneys (Siebel and Lendahl 2017; Masek and Andersson 2017).

In addition to numerous other mutations associated with conditions such as aortic valve disease and Adams–Oliver syndrome, which are variably associated with loss-of-function mutations in *NOTCH1* and in the case of Adams–Oliver syndrome *CSL* and *DLL4*; loss of function in the Notch signaling pathway has been linked to vascular degeneration and fibrosis. Tip–stalk formation is an essential process involved in endothelial cell (EC) proliferation in angiogenesis. During this process, a VEGF expression gradient is established, with high VEGF levels in the tip cells, diminishing in the stalk cells (Blanco and Gerhardt 2013; Hellstrom et al. 2007). Under VEGF stimulation in ECs *DLL4* expression is upregulated in tip cells, which activates Notch signaling in the neighboring stalk cells, and inhibits the tip cell phenotype. Notch signaling in turn feeds back on VEGF signaling, keeping it high in the tip cells and low in stalk cells. In this way, intact Notch signaling is required for vascular homeostasis and repair. During tissue ischemia, Notch signaling is activated in the resultant tissue repair and the neovascularization that ensues. Looking at other systems, in the developing heart Notch signaling is involved in the control of valve formation, trabeculation, and myocyte proliferation (High and Epstein 2008). During heart injury, Notch signaling is required in the fibrotic heart to limit the area in which fibrosis occurs and to control maturation of cardiomyocytes (Croquelois et al. 2008). Moreover, activated Notch signaling is able to reduce myofibroblast proliferation and stimulates the expansion of cardiac precursor cell populations in animal injury models (Nemir et al. 2014).

Notch Signaling in Cancer

With an ever-growing number of studies employing deep sequencing techniques, more and more mutations involving Notch signaling have been associated with cancers. Some more recent endeavors have uncovered Notch mutations in lung and oral squamous cell carcinomas, head and neck cancers, and breast cancer (Nakagaki et al. 2017; Zhang et al. 2018; Tinhofer et al. 2016; Liang et al. 2018). Cancer mutations in Notch pathway members can be associated with either signal activation or inhibition, whereby they promote excessive self-renewal or result in

aberrant differentiation and proliferation (Siebel and Lendahl 2017).

The association of Notch with human cancers initially dates back to the mapping of translocations in patients with T-cell acute lymphoblastic leukemia (T-ALL), which result in gene truncations of *NOTCH1* (Ellisen et al. 1991). This study was also the first time that a *Drosophila* Notch homolog had been identified in mammals. In T-ALL, a mutant form of *NOTCH1* acts as an oncogene and in addition to the original translocations, there are now numerous activating mutations associated with Notch and the ubiquitin ligase *FBXW7*, which is a cofactor that targets intracellular Notch for degradation and effectively increases protein stability when it is mutated (Tosello and Ferrando 2013). Over half of all *NOTCH1* mutations are found to occur in either the C-terminal PEST domain of Notch, again resulting in prolonged Notch signal activation due to increased intracellular Notch protein stability, or the extracellular heterodimerization (HD) domain resulting in ligand independent pathway activation (Weng et al. 2004). *NOTCH1* mutations have been found in all subtypes of T-ALL, with the resultant overactivated intracellular Notch suppressing p53 mediated cellular apoptosis (Weng et al. 2004; Demarest et al. 2011).

Dampening Notch signaling with gamma-secretase inhibitors (GSIs) has shown promise in mouse models of T-ALL (Tatarek et al. 2011), but in humans GSIs have demonstrated gastrointestinal (GI) toxicity when tested clinically (DeAngelo et al. 2006), likely due to blockades of Notch signaling required for intestinal progenitor cell maintenance as mentioned above (Riccio et al. 2008). To date, the only complete report published on the treatment of T-ALL with GSIs, Pfizer's PF-03084014, encouragingly concluded from their Phase I study that further studies in solid tumors and leukemias are warranted (Papayannidis et al. 2015). At this time, there are a number of completed and ongoing clinical trials looking at GSIs for a variety of cancers such as sarcomas, gliomas, carcinomas, and pancreatic and breast cancer. However, no studies have yet made it to pivotal Phase III trials (source: ClinicalTrials.gov).

Despite having negative consequences for the GI tract, inhibiting Notch using GSIs may lead to further beneficial effects for the host when combating cancer. A study examining immune cell subtypes in head and neck cancers in an inducible mouse model found that treatment with GSIs led to a reduction in myeloid derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and immune checkpoint inhibitors, which would give an added boost to the host immune system in fighting cancer (Mao et al. 2018). Additionally, studies using standard chemotherapeutic agents such as the microtubule stabilizing agent paclitaxel (Jeong et al. 2016) or the cell cycle inhibitor doxorubicin (Li et al. 2015) have shown that Notch expressing cancer stem cells can evade their killing response, and it has been postulated that Notch sensitization such as in the case of breast cancer can be more effective in targeting tumors in combination with standard chemotherapies (Mollen et al. 2018).

Other cancers with identified Notch pathway member mutations include loss-of-function mutations in *NUMB* and gain-of-function mutation in *NOTCH1* in non-small cell lung carcinomas (Westhoff et al. 2009). Furthermore, despite having no attributable mutations identified, NOTCH1 activation has been implicated in post-chemotherapy treated colon cancers (Meng et al. 2009), and activation of NOTCH1 has been shown to drive melanoma toward a more aggressive phenotype (Liu et al. 2006). Much work has been done investigating the role of Notch signaling in breast cancer since the original publication that identified *Notch4* to be located in the insertional domain of the mammary adenocarcinoma causing mouse mammary tumor virus (MMTV) (Gallahan and Callahan 1987). Subsequent studies linked MMTV insertion to the *Notch1* gene in mice, implicating multiple roles for Notch pathway members in the initiation and progression of breast cancer (Dievart et al. 1999). Since the initial mouse studies, patient data has shown that elevated expression levels of Notch signaling factors and loss of activity of negative regulators such as NUMB correlates with a poor clinical outcome

(Reedijk et al. 2005; Dickson et al. 2007; Stylianou et al. 2006).

In HER2 positive breast cancer, standard treatment inhibits this receptor using antibody based approaches such as herceptin/trastuzumab (Mates et al. 2015). However, HER2 inhibition results in Notch signaling pathway activation, and this activation has been linked to tumor recurrence. Mouse models have shown that GSI inhibition of Notch signaling in combination with HER2 blockade results in lower tumor recurrence and indicates that the use of Notch blocking compounds as adjuvants could be more effective (Abravanel et al. 2015). Unfortunately, due to the associated GI tract toxicities, the widespread use of GSIs is keeping this promising class of therapeutics from becoming frontline treatments at this time. To this end, more specific Notch inhibitors are an attractive area of research. Looking at a parallel approach, synthetic Notch receptors, or synNotch, has been demonstrated to drive anticancer responses and could be engineered to modify Notch signaling in malignancies (Roybal et al. 2016). A synthetic biology approach combined with engineered T cell therapies provides tantalizing possibilities to treat a wide array of Notch associated and Notch independent malignancies where this cell-based mechanism could be harnessed to engineer a tailored and dosed response, specifically mediated by target cells via taking advantage of the juxtacrine signaling mechanisms needed for Notch signal activation. Studies showing proof of concept demonstrated that BiTEs, or bispecific T cell engagers, could be generated to produce a simultaneous CD3 T cell engagement and CD19 tumor cell targeting activity in response to synNotch activation (Roybal et al. 2016).

Conclusions

The Notch pathway is a multifaceted signaling mechanism implicated in and essential for development, differentiation, and stem cell maintenance. For a more detailed look at the different developmental programs involving Notch that have not been addressed in this chapter, such as neurogenesis, vasculogenesis, cardiogenesis, hematopoiesis, and the role of Notch in the development of skin, liver, intestines, and skeletal muscle, the reader is referred to the recent reviews by Siebel and Lendahl (2017) as well as Henrique and Schweisguth (2019) and subsequent chapters of this book.

Notch signaling in multicellular animals has evolved to rapidly convey cellular identity and control differentiation. It is apparent that Notch signaling is highly dynamic and studies of static gene expression by means of NGS, expression arrays or protein expression by flow cytometry and histological analyses are often not sufficient to catch and grasp the rapid turnover and timing that is at play. Notch can be quickly turned on and off as seen during wave propagation and molecular clock models during somitogenesis. Without dynamic reporter systems used to study these processes, the understanding of the role of Notch therein would be limited if not even confused. Consequently, static observations of human conditions such as cancers that mainly look at one-time assessment of Notch on or Notch off states may be limiting our understanding of the seemingly disparate roles that Notch plays in oncogenesis and tumor suppression.

In future, the power of Notch to direct differentiation and development is something that will likely be harnessed by synthetic biologists and scientists in the field of stem cell biology and regenerative medicine. Further, novel selective inhibitors of Notch signaling will be able to more precisely target the desired outcomes in Notch signaling without having off-target effects as seen with the current class of GSIs, making selective Notch inhibition a more attractive option for cancer treatment. Finally, a thorough understanding of the role of Notch signaling during fundamental developmental and homeostatic processes in animals as well as human-model systems such as stem cell-based cancer and tissue organoids will inform the contextual effects that will be used to drive and improve Notch-targeted treatment strategies.

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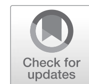

Ligand-Induced Cis-Inhibition of Notch Signaling: The Role of an Extracellular Region of Serrate

3

Robert J. Fleming

Abstract

Cellular development can be controlled by communication between adjacent cells mediated by the highly conserved Notch signaling system. A cell expressing the Notch receptor on one cell can be activated in trans by ligands on an adjacent cell leading to alteration of transcription and cellular fate. Ligands also have the ability to inhibit Notch signaling, and this can be accomplished when both receptor and ligands are coexpressed in cis on the same cell. The manner in which cis-inhibition is accomplished is not entirely clear but it is known to involve several different protein domains of the ligands and the receptor. Some of the protein domains involved in trans-activation are also used for cis-inhibition, but some are used uniquely for each process. In this work, the involvement of various ligand regions and the receptor are discussed in relation to their contributions to Notch signaling.

Keywords

Notch · Delta · Serrate · Signaling · Cis-inhibition · Regulated intramembrane proteolysis (RIP) · Bidirectional signaling · Endocytosis · Ubiquitination

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Introduction

The Notch signaling system is a highly conserved cell-to-cell communication mechanism common to most metazoans. This system is used iteratively throughout development to pattern and specify multiple cell types, primarily by mediating binary decisions in cell fate between adjacent cells (Kopan and Ilagan 2009). As the system is used to maintain stem cell populations, to define boundary structures, and to specify cellular fates, it is not surprising that mutations occurring within its component molecules are associated with developmental abnormalities and multiple types of cancers (Zhang et al. 2018; Nowell and Radtke 2017; Siebel and Lendahl 2017). Although it has many interacting regulatory features, the system's active signaling is for the most part straightforward. The Notch receptor is a type 1 transmembrane molecule that is activated by association with its membrane bound ligands (Fig. 3.1). This binding is believed to alter the conformation of a Negative Regulatory Region of Notch (NRR), allowing for access of an ADAM protease or sheddase to a membrane adjacent cleavage site. This initiates regulated intramembrane proteolysis (RIP) that releases the Notch extracellular (EC) domain in a process termed ectodomain shedding (Kopan and Ilagan 2009; Mumm et al. 2000; Lichtenthaler et al. 2018). Following release of the EC domain, a second, intramembrane cleavage event, mediated by a

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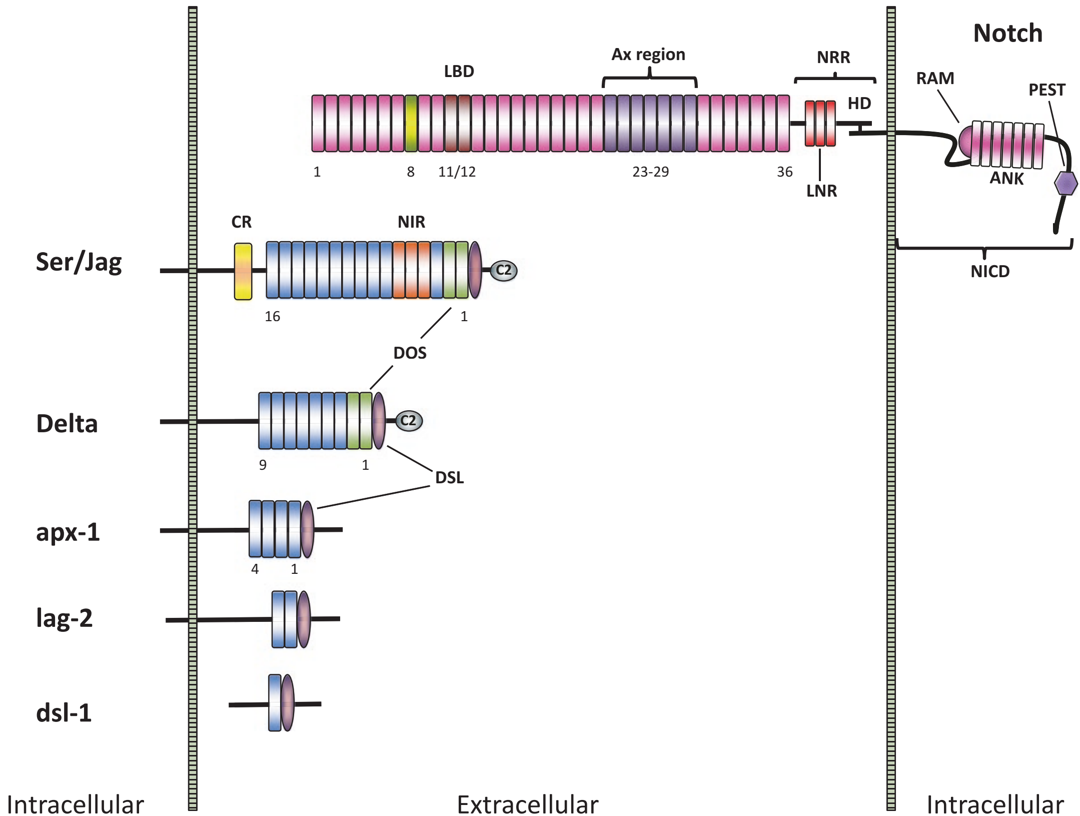

Fig. 3.1 Notch and ligand architecture. Notch (in red tones on right hand cell) and its ligands (in blue tones on left hand cell) share ELRs as a common structural feature. The extracellular ELRs of the mammalian Notch1 receptor are numbered 1–36 beginning at the N-terminal region of Notch. The minimal ligand-binding domain (LBD) requires ELRs 11 and 12. The Negative Regulatory Region (NRR) consists of the Lin-12/Notch repeats (LNR) and the heterodimerization domain (HD). This region is altered upon ligand binding to reveal an ADAM protease cleavage site. ELRs 23–29 constitute the Abruptex region in which mutations are associated with reduced cis-inhibition (de Celis and Bray 2000). ELR 8 is the site of the Notch^{jigsaw} mutation that affects Ser binding in *Drosophila* (Yamamoto et al. 2012). On the intracellular side of the membrane is the Notch intracellular domain (NICD) containing a RAM domain, Ankyrin repeats and a PEST sequence that is released to the nucleus following trans-activation. During trans-activation, the ligands would present to Notch as shown on opposing cells. ELRs (numbered from the N-terminus) in ligands range from 16 in Ser/Jag family members (Jagged1 illustrated here) to 1 in dsl-1. The Ser/Jag ligands also have a cysteine-rich segment (CR; yellow tones) that is unique to this ligand class. All ligands have a DSL segment (purple ovals) and the DL and Ser/Jag ligands have a divergent N-terminal C2 segment that may interact with the cell membrane (Suckling et al. 2017) (blue circles). The first two ELRs of the Ser and Dl ligands are known as the Delta/OSM-11 domain (DOS; green tone ELRs). Their shortened loop structures between disulfide bonded cysteines cause them to resemble the OSM proteins of *C. elegans*. The Ser/Jag family also contains the Notch inhibitory region (NIR; orange ELRs). Notch ligands in *C. elegans* (apx-1, lag-2 and dsl-1) have significant differences from the DL and SER/Jag ligands. They lack the integral DOS domain, the CR domain, have fewer ELRs and need not be membrane bound to activate Notch (Chen and Greenwald 2004; Komatsu et al. 2008) (Note dsl-1 lacks a transmembrane domain)

γ-secretase complex, results in the release of the Notch intracellular domain (NICD) (Schroeter et al. 1998). The NICD travels to the nucleus of the cell where, in conjunction with other transcriptional cofactors, it serves to regulate transcription of target genes (Nam et al. 2003; Wilson and Kovall 2006; Oswald and Kovall 2018; Bray and Gomez-Lamarca 2018).

The ligands for the system are also type 1 transmembrane molecules called DSL ligands

(named for Delta and Serrate/Jagged in flies and mammals, and lag-2 from *C. elegans*). The ligands are found in two major groupings, either the Delta or Delta-like family (termed DL in this work) or the Serrate/Jagged family (termed Ser/Jag here; named for Serrate in flies and Jagged in vertebrates). In most organisms studied, many of the main pathway components are found in multiple copies (e.g., two copies of the Notch receptor in *C. elegans* and four copies in mammals along with multiple members of each ligand family). In contrast, single gene copies for the main pathway components are present in *Drosophila*. This work will concentrate primarily on the more highly conserved *Drosophila* and vertebrate ligand families. Contrasts between these ligand types and the more diversified *C. elegans* ligand types will be made where appropriate.

Both the Notch receptor and its associated ligands have structural similarities in their extracellular domains (see Fig. 3.1). These domains are composed largely of epidermal growth factor-like repeats (ELRs) known for protein-protein interaction (Haltom and Jafar-Nejad 2015). For activation of the pathway, the ligands physically interact with Notch via the N-terminal regions of the ligands (see below), including the N-terminal C2 segment, the DSL domain, and the first two (in DLL4) or three (in Jag1) ELRs (Shimizu et al. 1999; Cordle et al. 2008a; Glittenberg et al. 2006; D'souza et al. 2008; Luca et al. 2015, 2017). Given the extensive and widespread use of the Notch signaling system during development, it is not surprising that pathway regulation and control is tightly managed. This chapter will primarily concentrate on regulatory mechanisms that affect the initiating components of the pathway, the Notch receptor and the DSL ligands. These mechanisms include protein modification by various forms of glycosylation, cellular trafficking, ligand ubiquitination, and endocytic processes.

Ligand Architecture

In addition to the overview of the Notch binding domain of the ligands previously presented, there are other key features involved with Notch activity. A domain termed the *module at the N-terminus of Notch ligands* (MNNL domain) is found in most N-terminal regions of Notch ligands. Within this domain of the DL and Ser/Jag ligands is a C2 glycosphingolipid binding motif believed to be involved with membrane recognition (see Fig. 3.1) (Hamel et al. 2010; Chillakuri et al. 2012). Although the C2 region of the ligands does not appear to be essential for ligand activity, it is capable of facilitating endocytic events on the sending cell associated with trans-activation of the receptor and can function to partially compensate for loss of Mib ubiquitination (Hamel et al. 2010) (see below). The C2 region also appears capable of directing the recycling of ligands (Heuss et al. 2013). This recycling is expected to return functional ligands to the cell surface, effectively increasing ligand concentration in the cell and potentially directing that cell toward the signal-sending fate.

The region of the canonical ligands that binds with Notch has been determined by analysis of existing human mutations and site-directed mutagenesis (Chen and Greenwald 2004; Cordle et al. 2008a; Glittenberg et al. 2006; Parks et al. 2006). These studies showed that the DSL domain is a critical region for Notch activation. This protein segment has been described as a degenerate ELR within which mutations of conserved residues lead to loss of Notch signaling (Shimizu et al. 1999; Henderson et al. 1994, 1997; Morrissette et al. 2001; Tax et al. 1994; Warthen et al. 2006). In humans, mutations of the regions N-terminal to and within the DSL domain of Jag-1 are linked to a dominant condition known as Alagille syndrome, a multisymptomatic disorder involving liver, heart, and other organ systems (Morrissette et al. 2001; Warthen et al. 2006; Gilbert and Spinner 2017). In addition, specific mutations have been made within the DSL and these mutations abolish all ligand interactions with Notch indicating that this domain is critical for Notch regulation (Glittenberg et al. 2006).

Just C-terminal to the DSL domain resides a region termed the DOS domain (named for DL and OSM-11 proteins) (Komatsu et al. 2008). This segment overlaps ELRs 1 and 2 that have unusually short loops between the disulfide

bonded cysteines in the ELR repeats giving them a unique structural configuration similar to that of OSM proteins in *C. elegans* (Komatsu et al. 2008*;* Chillakuri et al. 2012). The DOS domain is not found in mammalian Delta-like 3 and Delta-like 4 (DLL3 and DLL4), nor is it found in any *C. elegans* ligands, yet these ligands all interact with Notch (Kopan and Ilagan 2009). There is evidence that at least the *C. elegans* Notch receptors can associate with separately produced OSM-11 gene products to facilitate signaling, raising the possibility that other molecules can substitute for the DOS region of ligands lacking such domains (Komatsu et al. 2008). Adjacent to the DSL domain of the ligands are a varying number of ELRs. In the DL ligand family there are 6–9 ELRs (including the DOS ELRs) and in the Ser/Jag family there are 14–16*. C. elegans* ligands also contain ELRs, but they are significantly fewer in number (Fig. 3.1). The DOS domain and likely the third ELR of the Ser ligand facilitate binding between Notch and ligand (Shimizu et al. 1999; Cordle et al. 2008a; Glittenberg et al. 2006; Henderson et al. 1997; Fleming et al. 2013). Thus, the N-terminal sequences of the DSL ligands are essential for Notch activation. One final structural difference between the DL and Ser ligand families is the presence of a cysteine-rich region located between the final ELR and the TM region that is only found in the Ser/Jag class of ligands. This region has, as of yet, no ascribed function.

Trans-Activation of Notch

The N-terminal regions of the ligands, including the DSL and DOS domains contact the receptor using at least the Notch 11th and 12th ELRs that are believed to constitute the receptor-binding site (Rebay et al. 1991; Hambleton et al. 2004; Cordle et al. 2008b). Many of the ELRs in both the ligands and Notch are calcium binding, including ELR 12 of Notch; hence, it is not surprising that productive interactions between these molecules are calcium dependent (Chillakuri et al. 2012; Stenflo et al. 2000). Aside from ELRs 11 and 12, numerous other Notch ELRs have been shown to be critical for Notch/ligand association, presumably by altering access to the Notch binding region or by altering receptor conformation in the activation process (Yamamoto et al. 2012; Jafar-Nejad et al. 2005; Lee et al. 2013, 2017). Notch can only be activated by ligand if it is modified by O-fucosylation (Sasamura et al. 2003). Subsequent glycosylation events also modify Notch ligand interactions (Varshney and Stanley 2018). For example, modification of O-fucosylated Notch by members of the *fringe* gene family of β-1,3-*N* acetylglucosaminyl transferases generates selective activation of Notch by the Ser and DL ligands, generally inhibiting Ser-induced activation and enhancing DL-induced activation (Moloney et al. 2000; Brückner et al. 2000; Taylor et al. 2014).

The ligands can also be glycosylated although no essential functions for such modifications have yet been noted for the trans-activation of Notch. However, DLL3, a Notch ligand that does not trans-activate Notch but can inhibit Notch activity, requires O-fucosylation in vivo for murine somitogenesis. In spite of the loss of in vivo function, an *O*-fucose deficient form of the DLL3 molecule retains the ability to interact with and inhibit Notch in vitro. These conflicting outcomes suggest that either the physiological levels of expression for this ligand are critical or that DLL3 has non–Notch-related functions that depend on glycosylation (Serth et al. 2015). As a general note, many of the studies involving Notch components utilize ectopic expression, or, in cell culture, transfection of the molecules involved; hence, expression levels of these molecules are oftentimes higher than physiological levels, thereby complicating result interpretation.

Although the extracellular domains of ligand and receptor are required for physical binding between these molecules, there are also essential intracellular domains for each molecule. The NICD is the key effector of the Notch pathway. It is released from the membrane following RIP and translocates to the nucleus to affect alterations in gene transcription. The effect of expression of just the NICD is equivalent to that of a constitutively active Notch receptor (Lieber et al.

1993; Struhl et al. 1993; Jarriault et al. 1995; Struhl and Adachi 1998).

The IC domains of the ligands are essential for Notch activation. It has been demonstrated that the ligand IC domains on the signal-sending cell require modification by the *neuralized* or *mindbomb* ubiquitin ligases (Le Borgne and Schweisguth 2003; Le Borgne et al. 2005; Wang and Struhl 2005; Weinmaster and Fischer 2011). The ligand IC domains are not highly conserved in primary amino acid sequence, but they do contain lysines that have been shown to act as ubiquitination sites required for the ligand to activate Notch (Heuss et al. 2008; Daskalaki et al. 2011; Berndt et al. 2017). Following ubiquitination, endocytosis of the ligand utilizes an epsin adaptor protein and clathrin-mediated endocytosis to internalize the ligand (Wang and Struhl 2004). This constitutes a specific endocytic process that mediates some of total ligand endocytosis, but differs from bulk endocytosis of the ligand, suggesting that it has a more specialized function in ligand metabolism. In *Drosophila*, it has been demonstrated that while the DL ligand requires ubiquitination for full activity, residual function of this ligand remains when ubiquitination of the ligand is blocked either by altering the ubiquitination sites on the ligand IC domain or by eliminating the Mib1 E3 ubiquitin ligase (Berndt et al. 2017). In the same system, Ser endocytosis is nearly eliminated following the loss of the Mib1 ubiquitin ligase but Dl endocytosis continues in a near normal fashion (Wang and Struhl 2005). In the case of DL, the Neur E3 ligase can restore partial Dl activity even if ubiquitination of the ligand is prevented by altering the ubiquitination sites on the IC domain. The Dl and Neur proteins have been observed to cotraffic with one another in the cell and alterations in the levels of DL can also change the distribution of Neur (Daskalaki et al. 2011; Skwarek et al. 2007). Restoration of low-level DL function requires binding of Dl with Neur, suggesting that it is the cotrafficking of Dl and Neur that restores partial Dl activity. These functions are not shared with Ser for which signaling is not restored under similar conditions (Berndt et al. 2017). This difference between DL and Ser may help to explain why some soluble forms of DL retain the ability to activate Notch yet soluble forms of Ser do not (Shimizu et al. 2001; Qi et al. 1999; Varnum-Finney et al. 2000). Therefore, even though both ligands utilize ubiquitination for full activity, the endocytic process itself appears to be a necessary component of Notch activation.

The endocytosis of ligand in the signal-sending cell ultimately leads to Notch ectodomain shedding on the receiving cell although the exact mechanism by which this happens remains to be resolved. One suggested model for Notch trans-activation suggests that endocytosis serves to recycle the ligand through a modification pathway that generates a dynamic ligand on the cell surface capable of stimulating Notch activity (Wang and Struhl 2005). In this model, endocytosis would be expected to occur prior to the ligand making contact with the receptor in order for necessary modifications to occur. It is clear from the literature that there exist multiple paths for ligand endocytosis. For example, support for ligand recycling comes from a requirement of the Rab11 and Sec15 recycling proteins for proper activity in Notch signaling situations such as sensory organ precursor cell fate specification in *Drosophila* (Jafar-Nejad et al. 2005; Emery et al. 2005). However, other Notch dependent processes, such as germ cell and eye development, do not require these components (Banks et al. 2011; Windler and Bilder 2010). Different recycling pathway components are used during neurogenesis and pancreatic development in zebrafish. The sorting nexin family protein SNX17 regulates Jag1 protein levels by directing ligand recycling in the signaling cell. Similarly, the C2 domain of Dl and Ser in *Drosophila* and mammalian DLL1, has been shown to direct this ligand through a recycling pathway that reduces ligand degradation (Hamel et al. 2010; Heuss et al. 2013). These various endocytic routes may enhance signaling by simply returning intact ligand to the cell surface effectively increasing ligand availability, or by allowing for specific modifications to the ligands thereby increasing their activity levels with Notch. Because bona fide activated forms of the ligands that become capable of activating Notch have not been

observed, it is unclear whether these recycling pathways actually lead to ligand modification or simply recycling.

An alternate model to explain the need for epsin-/clathrin-mediated endocytosis in the signaling cell suggests that ligand endocytosis generates a pulling force on Notch in the receiving cell to alter the NRR and expose the ADAM metalloprotease cleavage site leading to RIP and Notch activation (Parks et al. 2000) (see Fig. 3.2). In this model, endocytosis is required after the ligand and receptor contact one another. Support for this model comes from studies showing that soluble Jag1 does not activate Notch in cell culture but that when the extracellular domain of Jag1 is immobilized on a plastic substrate, Notch activation can be achieved (Varnum-Finney et al. 2000). This suggests that the ligand must be anchored to activate Notch. In this experimental system, there would be no opportunity for ligand recycling. More compelling evidence for the pulling force model comes from the generation of chimeric DL and N molecules for which the extracellular interaction domain of Notch and the extracellular domain of DL had been replaced with the corresponding ligand–receptor interaction domains from the structurally unrelated mammalian follicle stimulating hormone (FSH) pair (Langridge and Struhl 2017). In these experiments, binding between ligand and receptor

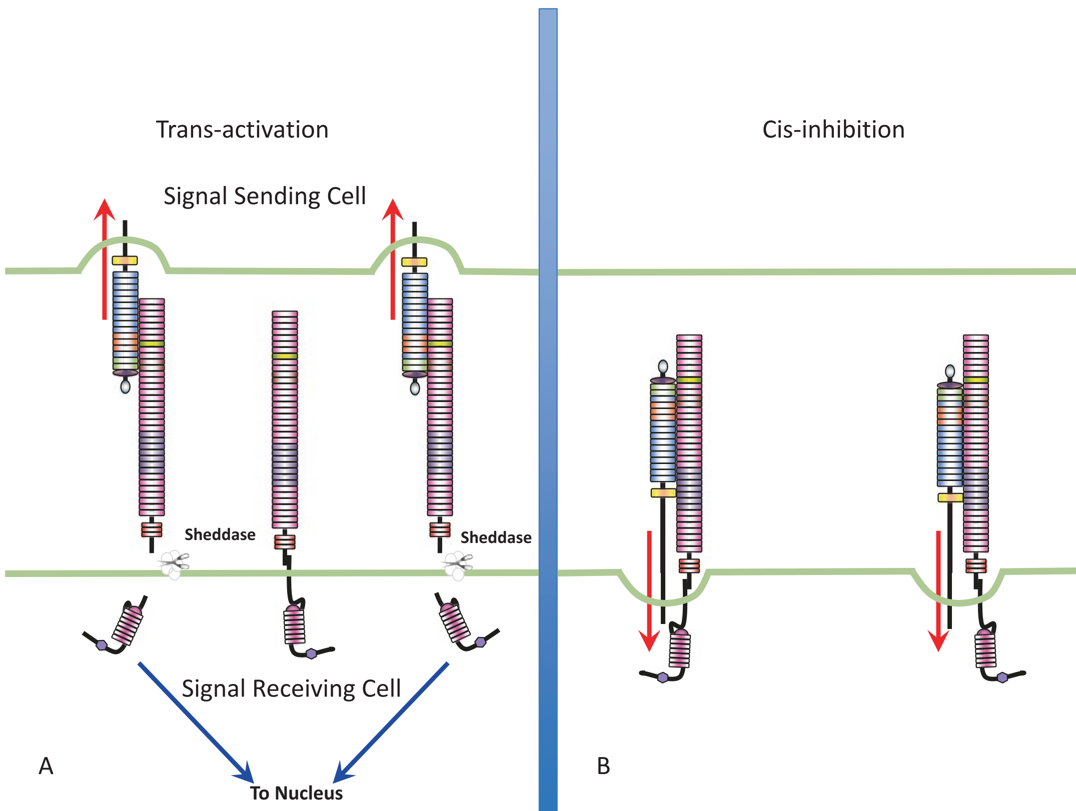

Fig. 3.2 Cis and trans Notch–ligand interactions. Interactions between Notch (red tones) and Serrate (blue tones) are illustrated. Molecules are depicted as in Fig. 3.1. (**a**) When Notch and its ligand are expressed on apposing cells, binding of ligand utilizes endocytosis in the sending cell (red arrows) to activate Notch on the receiving cell by altering the NRR region and exposing the sheddase site (scissor diagram) leading to ectodomain shedding and RIP of the receptor to release the NICD allowing it to travel to the nucleus and alter gene transcription. (**b**) When ligand and receptor are expressed on the same cell, binding of ligand does not lead to cleavage and RIP of Notch. The complex can either be removed from or inhibited from trafficking to the plasma membrane thereby inhibiting Notch activation

occurred through FSH ligand–receptor interactions. Post binding, Notch activation due to ligand endocytosis and RIP activity to release the NICD depend on Notch sequences and the DL IC domain. This study demonstrated that the interactions between the chimeric molecules effectively recapitulated normal Notch/DL interactions, including cis and trans capabilities. The trans-interactions still required epsin-mediated endocytosis of the ligand in order to activate the Notch receptor hybrid molecule. These findings argue against a recycling modification of the ligand to generate a more dynamic molecule that is capable of activating the Notch receptor since the chimeric interactions are dependent on FSH ectodomains and not Notch or DL ectodomains.

It has been suggested that epsin-/clathrin-mediated endocytosis is either stronger or more focused than bulk endocytosis and hence bulk endocytosis may be incapable of generating the necessary force to activate Notch (Langridge and Struhl 2017). In a direct measurement using single ligand molecules and magnetic tweezer manipulation, it was determined that signaling cells have the ability to generate sufficient physiological force to alter the NRR of Notch upon ligand endocytosis (Gordon et al. 2015). Although ligand modifications to extracellular regions of DL leading to Notch activation are inconsistent with the experiments described above, it remains possible that intracellular ligand modifications leading to ligand concentration or clustering could still occur in mediating interactions with the receptor.

Given the strong dependency of Notch activation on ubiquitination and endocytosis of its ligands, it can be difficult to reconcile the findings that ligands lacking transmembrane and intracellular domains exhibit varying levels of Notch activating ability in different systems. In vitro expression of *C. elegans* ligand constructs lacking TM and IC domains appear fully capable of activating Notch in vivo (Chen and Greenwald 2004; Fitzgerald and Greenwald 1995). Similarly, the dsl-I *C. elegans* ligand is a secreted protein yet is capable of receptor activation (Chen and Greenwald 2004) (see Fig. 3.1). Other Notch systems show discrepancies in Notch activation by non–membrane-bound ligand forms that often follow along the lines of in vivo vs. in vitro experimentation. When expressed in vivo, many ligand constructs lacking the TM or IC domains display dominant-negative effects that antagonize Notch activation by full-length ligand (Chitnis 1995; Sun and Artavanis-Tsakonas 1996, 1997; Hukriede and Fleming 1997; Henrique et al. 1997; Lowell et al. 2000; Lowell and Watt 2001). In contrast, in vitro studies demonstrate that the extracellular portions of some Notch ligands may retain Notch activation potential although often this potential is substantially less than for full-length ligands (Shimizu et al. 2001; Qi et al. 1999; Varnum-Finney et al. 2000). Some of these differences may be explained by differences in intracellular trafficking as described above. Other considerations include taking into account experimentally altered expression levels of the ligands relative to endogenous levels as elevated ligand concentrations may lead to difficulties in interpretation.

Cis-Inhibition

Although ligand-induced Notch activation is the expected function of the pathway, ligand-mediated cis-inhibition is another intrinsic property of the system.

It has long been recognized in *Drosophila* that the level of receptor and ligand expression on a given cell function to direct cellular fates among equivalent cells to generate signal-sending and signal-receiving cells. The Notch gene was originally characterized in *Drosophila* and the Notch locus itself is haploinsufficient. Therefore, by reducing the receptor levels by half in an animal heterozygous for the Notch gene, the fly wing margin is not formed properly giving rise to Notches (hence the gene name) in the wing. Similarly, the DL gene is also haploinsufficient such that when DL levels are reduced, the wing veins are wider or form deltas where they meet the wing margins (Lehmann et al. 1981; Vässin and Campos-Ortega 1987). If Notch is present in three doses, it produces a phenotype known as Confluens with widened wing veins and deltas

not unlike that of the DL haploinsufficiency phenotype (Welshons 1965). Taken together, these phenotypes demonstrate that the levels of receptor relative to DL ligand are critical for normal developmental processes. In addition, by simply manipulating the levels of Notch receptor relative to ligand on adjacent cells using clonal techniques, it was demonstrated that high levels of Notch activity lead cells to an epidermal fate and lower levels of Notch activity generate a neural fate in cells of the *Drosophila* notum (Heitzler and Simpson 1991, 1993). Thus, simple alteration of cellular ligand and receptor levels demonstrates dominant phenotypic properties, even in the absence of any mutation that structurally alters the proteins for these signaling components.

Cis-inhibition by the Notch system likely accounts for many of the observed phenotypes associated with alterations in ligand and receptor levels. When ligand and receptor are simultaneously expressed on a single cell, one typically observes a loss or reduction of activation for Notch even if that cell is in contact with an adjacent signal-sending cell. Interestingly, the N-terminal ligand domains involved in Notch activation also appear to be necessary for cis-inhibition because all reported cis-inhibiting ligand forms carry most if not all, of the N-terminal binding domain identified for Notch activation. At the same time, the intracellular domains of the ligands are likely dispensable for cis-inhibition as ligands lacking IC domains usually retain the ability to interact with Notch in a negative manner (Chitnis 1995; Sun and Artavanis-Tsakonas 1996, 1997; Hukriede and Fleming 1997; Henrique et al. 1997; Lowell et al. 2000; Lowell and Watt 2001; del Alamo and Schweisguth 2009).

In the developing *Drosophila* wing imaginal disk, proper developmental outcome requires alterations in cellular fate resulting from both trans-activation and cis-inhibition of Notch (Klein et al. 1997; de Celis and Bray 1997; Micchelli et al. 1997; Miller et al. 2009). Cells of the wing disk express the Notch receptor and the DL and Ser ligands on either side of the developing wing margin (Micchelli et al. 1997; Doherty et al. 1996; Panin et al. 1997). At the margin, some cells express both the Notch receptor and its ligands simultaneously. Loss of the receptor, or loss of either of the ligands at the margin generates wing margin abnormalities due to the decline in Notch signaling. However, when clones of cells simultaneously lacking both Ser and DL are produced in the wing, Notch signaling can be activated in those cells whereas they are normally unresponsive to Notch (Micchelli et al. 1997; Palmer and Deng 2015). Therefore, under normal conditions, the presence of the ligands in these cells functions to block Notch activation, even if other signal-sending cells are present and in contact with those cells. This suggests that cis-inhibition by the ligands within these cells serves an essential purpose to reduce or eliminate inappropriate Notch activation. Additionally, in the *Drosophila* eye, photoreceptor fates are controlled by DL/N signaling. Normally, Notch activity generates the R1, R6 and R7 photoreceptors from an equipotential group of precursor cells. Using clonal analysis, it was shown that levels of DL mediate cis-inhibitory interactions with Notch to specify these R1/R6 and R7 fates (Miller et al. 2009). Other experiments with similar outcomes in vertebrate angiogenesis support these findings demonstrating that cis-inhibition is a normal, essential mechanism for proper cell fate outcomes (Boareto et al. 2015). It therefore appears that cis-inhibition is an essential regulatory component for cell specification by Notch.

The actual mechanism by which cis-inhibition is mediated remains elusive and there are likely several different elements of Notch signaling in play to control it. Because ligand–receptor cis-interactions are not expected to lead to Notch ectodomain shedding and RIP due to a lack of pulling force between ligand and receptor, it is expected that cis-interactions cannot induce Notch activation (Fig. 3.2). It is believed that cis-interactions between ligand and receptor lead to endocytosis and clearing of the complex from the cell (Sprinzak et al. 2010; Bray 2016). Experimentally, reduced endocytosis appears to

enhance cis-inhibition whereas enhanced endocytosis appears to enhance trans-activation (Glittenberg et al. 2006; Overstreet et al. 2004; Baek et al. 2018). How these endocytic properties mediate cis and trans interactions in the Notch pathway are discussed below.

It is clear that during trans-activation of Notch, contact must be initiated between signaling and receiving cells and that interaction must therefore be initiated at the plasma membrane where the two cells meet and the molecules can come into contact. On the other hand, as the name implies, cis-inhibition relies on both ligand and receptor being coexpressed in the same cell. Interactions between these two molecules could therefore occur at the cell surface as it does with trans-activation or it could potentially occur within endosomes of the secretory pathway as the molecules are being transported to or from the plasma membrane. As we shall see, experiments to clarify the cellular location at which cis-inhibition occurs have yielded conflicting accounts in the literature.

The receptor and the ligands are both transmembrane in nature, so it is anticipated that they are processed through similar secretory pathways of the ER and Golgi apparatus during trafficking with the plasma membrane. It has therefore been proposed that cis-inhibition could occur by association of ligand and receptor within the secretory pathway itself, thereby targeting the complex for degradation (Sakamoto et al. 2002). Experiments supporting the intracellular association of ligand and receptor were performed by coexpressing Notch and either Dl or Ser using vertebrate Notch components in a cell culture system. Notch/Dl and Notch/Ser ligand–receptor complexes were recovered from cell lysates. In this system, even though Ser, Dl and Notch molecules were found at the cell surface by biotinylation of surface proteins, recovered Notch/Dl and Notch/Ser complexes were not biotinylated, hence were not located at the plasma membrane. This suggests that cis-interacting N/ligand complexes can be formed in the ER/Golgi secretory pathway and are not restricted to the cell surface. Further support for this model is found in the DLL3 ligand in mammals. This ligand does not appear to activate but retains the ability to cis-inhibit Notch. The DLL3 protein is found to be concentrated primarily within the Golgi apparatus of expressing cells with little DLL3 protein observed at the plasma membrane (Geffers et al. 2007). These experiments are consistent with a model where cis-inhibition is occurring during cellular trafficking of ligand and receptor hence they do not necessarily involve interactions occurring at the cell surface.

Other experiments point to cis-inhibition occurring at the plasma membrane (Glittenberg et al. 2006; Becam et al. 2010). Expression of modified Ser constructs in *Drosophila* using ligand forms with altered intracellular motifs required for ligand endocytosis demonstrated reduced or absent trans-activating potential. However, expression of these altered forms resulted in increased levels of ligand at the plasma membrane with a reduction or loss of ability to activate Notch. These altered ligand forms were abundantly located at the cell surface and were not found concentrated in intracellular vesicles, yet they retain strong cis-inhibitory properties. This suggests that their cis-inhibitory effects are a consequence of plasma membrane interaction (Glittenberg et al. 2006). In this same study, a form of Ser was modified by the addition of a strong ER retention sequence. When this Ser form was expressed in *Drosophila*, it was retained within the secretory pathway. Despite this, the protein demonstrated a dramatically reduced ability to cis-inhibit Notch. Thus, if cis-inhibition were to occur in the secretory pathway, one would have predicted that the ER-retained form should have demonstrated increased cis-inhibition of Notch, and this did not occur. Whether the discrepancies between the experiments described above indicate differences in cis-inhibition properties between vertebrate and invertebrate experimental systems or whether cis-inhibition can occur at both the cell surface and inside of the secretory pathway depending on tissue type and experimental procedures remain an area requiring additional investigation.

Ligand Protein Domains Relating to Cis-Inhibition

The Notch inhibitory effects of the ligands have been traced to several functional domains in each molecule. As stated earlier, ligand forms lacking the TM and IC domains demonstrate primarily inhibitory actions on Notch and have been shown to be secreted (Sun and Artavanis-Tsakonas 1997; Sakamoto et al. 2002; Li et al. 2007; Lobov et al. 2007). Given the proposed role for ligand endocytosis in Notch activation, the loss of activation capability for these forms can be readily understood. However, these altered ligands retain their cis-inhibitory effects upon the receptor. This means that although trans-activation of Notch relies, in part, on the ligand IC domain, the cis-inhibition property must be mediated largely though the EC domain of the ligand.

The transmembrane domains of the ligands also do not appear to hold any specific properties other than to connect the intra and extracellular portions of the ligand. Replacement of the TM domain of Ser in *Drosophila* with segments of other TM molecules does not appear to alter activating nor inhibiting properties of the ligand (Fleming, unpubl.). Therefore, at least in overexpression studies, the TM domain itself does not confer any specific attributes to the ligand. Thus, even though it has been demonstrated that ligands also undergo RIP and this would be expected to rely upon intramembrane proteolysis of the ligand by gamma secretase (see below), the TM domain itself has no demonstrable effect on ligand activity. As neither the individual TM nor IC domains of the ligands are required, cis-inhibition appears to relate primarily to the extracellular portion of the ligand.

It is possible that at least some of these ligand inhibitory properties could arise from simple competition between the nonactivating forms and normal ligands for binding with Notch. Secreted forms of TM and IC deleted ligands have been readily detected outside of the cells expressing them providing the possibility of cell surface competition (Sun and Artavanis-Tsakonas 1997; Sakamoto et al. 2002). However, simple competition between full-length and truncated ligand forms does not account for all aspects of cis-inhibition. To better understand cis-inhibition, we will examine the various regions of the ligand EC domains that are associated with cis and trans Notch interactions below.

A structural analysis of the human Jag-1 ligand revealed an intriguing and unexpected characteristic. The study examined mutations of specific amino acids conserved with Ser in *Drosophila* that alter cis and/or trans interactions between ligand and receptor. It was found by modeling of the crystal structure of the DSL and N-terminal regions of Jag-1 that the same DSL containing ligand interface has the potential to be used for both cis and trans Notch interactions. In essence, the ligand demonstrates that it could interact with Notch in two distinct orientations termed parallel and antiparallel. These alternate configurations could potentially represent ligand receptor interactions in the cis-versus-trans modes (Cordle et al. 2008b). Because cocrystals between Notch and this region of Jag-1 were not successfully generated, it has not been determined if these alternative interaction surfaces between the ligand and receptor are actually employed in vivo. A different study using crystal structures of vertebrate DLL4 did not find a similar parallel/antiparallel interface corresponding to cis-versus-trans interactions for Notch and DLL4. The study did suggest however, that there may exist sufficient interdomain flexibility regions between Notch receptors and ligands to allow the same interface to be used for both cis and trans interactions between these molecules (Luca et al. 2015; Kershaw et al. 2015). Whether the structural differences noted for the DL-versus-Ser families of ligands represent intrinsically differing mechanisms for ligand–Notch interactions, particularly as they relate to cis and trans molecular interactions, remains to be fully elucidated.

In spite of the findings that both cis and trans interactions utilize overlapping contacts between ligands and Notch, there are clearly different interaction regions between ligand and receptor used by each process. The DL and Ser family members bind to Notch using different portions of the receptor even though ELRs 11–12 of Notch are used by both ligand types (Rebay et al. 1991;

Becam et al. 2010; Harvey and Haltiwanger 2018). For example, a specific mutation of the eighth ELR of the Notch receptor, Notch^{jigsaw}, affects Ser-Notch signaling in *Drosophila* but not DL-Notch signaling demonstrating differential ligand interactions with the receptor (Yamamoto et al. 2012). Even though the Notch^{jigsaw} mutation only affects Ser signaling, it alters both trans and cis interactions with Ser implicating common interacting surfaces for both processes. As mentioned above, O-glycosylation is an essential protein modification of the Notch receptor and is required for its activity (Sasamura et al. 2003; Okajima and Irvine 2002). Modification of ELR-12 of Notch primes this ELR for additional modification of *N*-acetylglucosamine by the glycosyltransferases of the *fringe* family. Differing effects on Notch activity by different *fringe* family members in vertebrates (*Radical fringe*, *Lunatic fringe*, *Manic fringe*) have been noted (Ladi et al. 2005; Hou et al. 2012). However, in *Drosophila*, the additional modification by *fringe* allows for increased activation of Notch by the DL ligand while simultaneously reducing or eliminating activation by the SER ligand (Panin et al. 1997; Fleming et al. 1997). These experiments demonstrate that the binding of Notch with DL involves at least some differential contacts compared to the binding of Notch with SER, even though the actual Notch signal appears to be identical from each of the ligands. The mechanism of *fringe*-mediated modification of Notch on cis-versus-trans Notch interaction is somewhat controversial in the literature. Overexpression of SER in the dorsal wing disk, where Notch is modified by *fringe*, blocks Notch trans-activation but not cis-inhibition (Glittenberg et al. 2006). This suggests that contact points of Notch and SER may differ for these two types of interactions. In contrast, another study coexpressing ligand and receptor with *fringe* in cell culture experiments as well as within the *Drosophila* wing demonstrated that Notch interactions are simultaneously affected by *fringe* in both the activation and cis-inhibition roles (LeBon et al. 2014). These findings are consistent with the same Notch/ligand interaction points being utilized in cis/trans interactions. The differences in these studies may relate to the relative expression levels of ligand in the systems, strength of interactions between cis and trans effects, or the developmental timing of the experimental manipulations. Nonetheless, the evidence does support at least some differential contact being involved in the two processes.

Although the role of fringe may remain controversial for cis/trans differential interactions, it has also been demonstrated that another sugar modification of the Notch receptor, xylosylation, can specifically alter its interactions with the DL ligand. Xylosylation is mediated by the *Shams* glucoside xylosyltransferase gene during *Drosophila* wing vein morphogenesis (Lee et al. 2017). When *Shams* is nonfunctional, one observes an increase in DL/Notch trans-activation capacity. Thus, the action of *Shams* is consistent with modifying Notch to reduce trans-interaction with DL. Interestingly, *Shams* activity does not appear to significantly alter Dl/N cis-interactions. Further, these xylosylation modifications of Notch are specific to DL/Notch interaction as Ser/Notch interaction does not appear to be altered in *Shams* mutant backgrounds. Since xylosylation occurs on ELRs 16–20 of Notch (Lee et al. 2013), these findings further support differential binding interactions between receptor and ligand for cis-versus-trans conditions by DL.

A further case supporting differential contacts for cis and trans interactions between ligand and receptor is illustrated by the DLL3 ligand of mammals. This ligand appears to lack the ability to trans-activate Notch and yet, when it is coexpressed with Notch1 in cells, it retains cis-inhibition properties. The DLL3 ligand is found primarily within the Golgi apparatus of expressing cells and has a highly divergent DSL domain when aligned with other DSL ligands (Geffers et al. 2007). DLL3 also has an incomplete ELR 2 and has fewer ELRs than the activating DLL1 and DLL4 mammalian ligands (only six ELRs in DLL3 as opposed to eight ELRs found in DLL1 and DLL4). In addition to the EC domain differences, the IC domain of DLL3 is smaller than other DLL ligands and, perhaps most significantly, lacks lysine residues that represent typical ubiquitination sites for activating endocytosis

(Ladi et al. 2005). This highly divergent ligand may lack Notch activation ability for any number of reasons but since it retains cis-inhibition properties, the sequences remaining in DLL3 must be sufficient for binding with Notch during cis inhibition. Taken as a whole, the findings presented in this section imply that trans and cis ligand binding to Notch likely involves overlapping interaction domains within which there are distinct points of contact required for the different processes.

The NIR Domain of Ser/Jag

Cis-inhibition of Notch by Ser in *Drosophila* utilizes another extracellular portion of the ligand that is not required for transactivation. This portion of Ser, termed the Notch inhibitory region (NIR), is represented by ELRs 4, 5, and 6 of this molecule (Fleming et al. 2013). The experiments defining this segment were performed in the *Drosophila* wing imaginal disk allowing for definition of Notch activation and inhibition by expression of altered Ser forms. The *patched* gene promoter is active in the *Drosophila* imaginal disk along the anterior/posterior compartment borders and was used to express altered Ser forms in this pattern (Doherty et al. 1996; Hinz et al. 1994). Expression of Notch system components under the control of this promoter allows those components to be expressed at right angles to the normal endogenous control of the Ser ligand from the dorsal compartment and the DL ligand from the ventral compartment. This expression leads to Notch activation along the developing dorsal ventral boundary, leading to activation of downstream target genes such as *Cut* and *wingless* and formation of the wing margin (Micchelli et al. 1997; Doherty et al. 1996) (see Fig. 3.3). By expressing the Ser ligand along the A/P boundary, Notch activation can be observed at right angles to its normal expression pattern in the ventral wing compartment. The lack of Notch activation in the dorsal compartment is due to modification of Notch by the dorsally expressed *fringe* gene (Panin et al. 1997). When wild type Ser is expressed in this manner, Notch trans-activation is observed adjacent to the Ser expression stripe (Fig. 3.3b, c). However, in the regions where the Ser ligand is expressed at high levels, cis-inhibition of Notch is observed resulting in loss of CUT expression (Fig. 3.3b). The NIR was defined by deleting single or multiple ELRs of Ser and observing the effects of expressing these deleted forms in the wing imaginal disk. It was found that deletion of either ELR1 or ELR2 of Ser (the DOS domain) abolished both cis and trans Notch interactions, consistent with findings of other groups that these domains are required for Notch binding (Komatsu et al. 2008; Glittenberg et al. 2006). When Ser ELR3 was removed, trans-activation is absent but weak inhibition can be observed (Fleming et al. 2013). This suggests that the inhibition property is not completely dependent on ELR3, but trans-activation does require this ELR. Contrasting with these effects, single ELR deletions of ELRs 4, 5 or 6 or combined deletion of ELRs 4–6, results in the loss of cis-inhibition by Ser with no substantial alteration in the trans-activating capacity of the ligand (Fig. 3.3d–f). Removal of ELR7 did affected neither cis- nor trans-activation capabilities of the ligand. Therefore, the NIR region is defined as ELRs 4 through 6. The finding that removal of any of these ELRs leads to the same severity of phenotype suggests that the ELRs of the NIR function as a unit and do not have additive effects. That this extracellular region of Ser functions to cis-inhibit Notch provides additional evidence that cis and trans Notch interactions may share some contact sequences but also utilize at least some separable surfaces to mediate their effects. The NIR deleted forms of Ser still require an endocytic process involving epsins to activate Notch. Thus, the trans-activation interactions between cells are not altered when the NIR is removed from Ser (Fleming et al. 2013).

Interestingly, while the NIR of Ser is shared with other members of the Ser/Jag family of Notch ligands, it is not conserved with sequences within the DL ligand family (Fleming et al. 2013). Because both the Ser and DL ligands share the ability to cis-inhibit Notch, the NIR of Ser is either a separate mechanism for cis-inhibition not

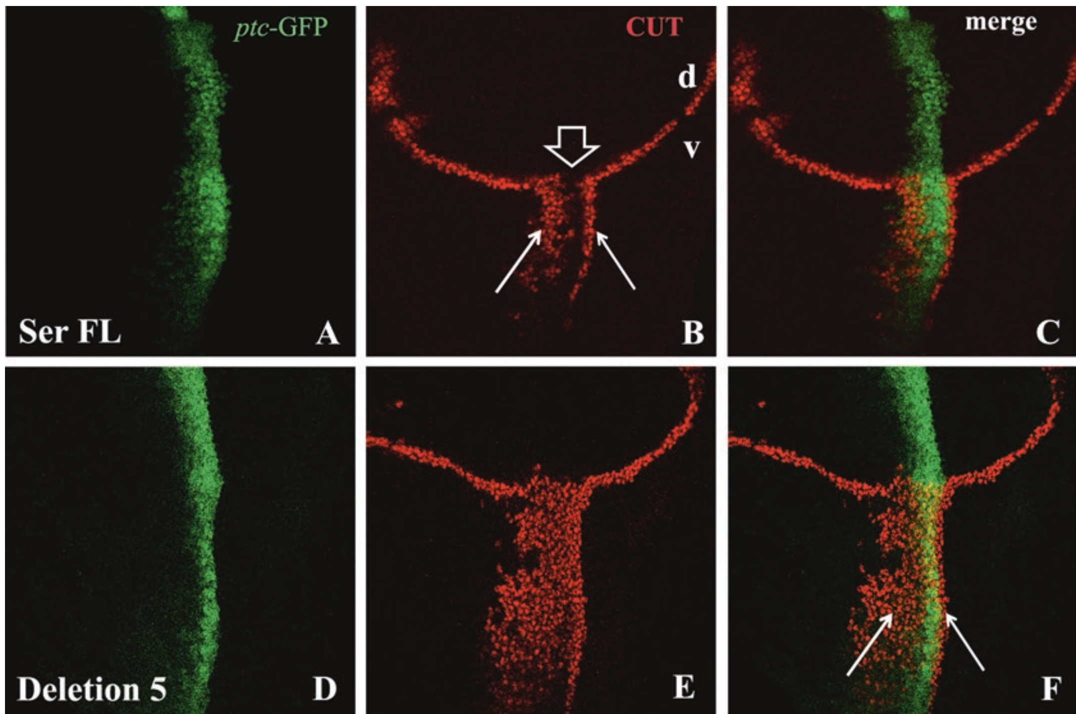

Fig. 3.3 Ser NIR-deleted forms fail to cis-inhibit Notch. Using the *Drosophila* wing imaginal disk, expression of Ser can lead to ectopic Notch activation. The *patched* (*ptc*) gene promoter was used to simultaneously express both GFP and Ser forms along the anterior–posterior wing boundary (green fluorescence in figures). The CUT transcription factor is expressed in response to high levels of Notch activation in the wing (Blochlinger et al. 1993). Antibodies directed against CUT (red fluorescence) reveal where Notch is activated in the disk. The normal margin forms as a result of endogenous Notch activation at the boundary between the dorsal (upper region, labeled d) and ventral (lower region, labeled v) of the disk. In this figure, the location of *ptc* expression within the disk is shown in the first column (*ptc*-GFP), Notch activation is shown by CUT expression in the second column, and the merge of the first two images is shown in the third column (labeled merge). Notch can only be activated by Ser in the ventral wing compartment due to the restriction of Notch activation via *fringe* modification on the dorsal side (Panin et al. 1997). (**a**) Ser is expressed at high levels throughout the *ptc* stripe. (**b**) Notch is trans-activated in cells adjacent to the *ptc* stripe (arrows) but is cis-inhibited within the *ptc* stripe due to high levels of ligand present (open arrowhead). (**c**) Merged image clearly illustrates that activation occurs adjacent to Ser expressing cells. (**d–f**) When the Ser NIR is disrupted by deleting one or more ELRs (ELR 5 deletion shown here), Notch is activated both adjacent to and within the *ptc* stripe, demonstrating the loss of Notch cis-inhibition without disruption of trans-activation. (Adapted with permission from Development (Fleming et al. 2013))

shared with DL or a comparably functioning NIR region of DL has not yet been identified. In this regard, it has been noted that in *Drosophila*, Ser appears to demonstrate stronger and distinct cis-inhibitory properties from those of DL (Klein et al. 1997; Becam et al. 2010; Li and Baker 2004). These differences between the ligand families could arise from different affinities of the ligands for Notch during cis-inhibition or they may represent different mechanistic attributes when interacting with Notch (see below).

The location of the NIR is directly adjacent to the identified Notch binding region of the ligand (Fig. 3.1). Cells expressing just the NIR ELRs of Ser are incapable of inactivating Notch suggesting that they act dependently in conjunction with the Notch binding portion of the ligand to mediate cis-inhibition. The specific mechanism by which the NIR is mediating the cis-inhibitory process on Notch remains undefined as direct interaction between the ligand NIR and Notch has not been demonstrated. It therefore remains

possible that the NIR domain functions to interact with molecules other than Notch. However, in the absence of evidence for a separate cis-inhibition pathway, it remains most likely that the NIR interacts with Notch itself.

The question arises as to whether or not ligands interact directly with Notch for cis-inhibition and if so, is that interaction restricted to Notch ELRs 11 and 12 or does it involve other Notch regions? Although such interactions remain purely speculative, it is tempting to examine the dominant *Abruptex* (*Ax*) class of Notch mutations in this regard. *Ax* alleles in *Drosophila* were initially described as ligand dependent, hyperactive Notch alleles (Heitzler and Simpson 1993; Sirén and Portin 1989; de Celis and Garcia-Bellido 1994) but are perhaps better described as mutations of Notch that exhibit a reduced ability to be inhibited by ligand (de Celis and Bray 2000). These mutations are located within ELRs 23–29 of the Notch extracellular domain (Hartley et al. 1987; Kelley et al. 1987). The *Ax* ELRs are not directly adjacent to the ligand binding ELRs 11 and 12 of Notch but reside more C-terminal to this region (Rebay et al. 1991; Lieber et al. 1993). It remains an interesting possibility that when the ligands are interacting with Notch in a cis-inhibitory manner, the Ax ELRs may reside in proximity to the NIR region of Ser ligands. Distances between ELRs in Notch and Ser are likely to be deceiving as the complete structure of these full-length molecules remains unavailable. However, low resolution structural studies of Notch that have been attempted suggest that Notch may have a more compact structure than the extended linear structure with which it is usually drawn (Chillakuri et al. 2012; Xu et al. 2005) (Fig. 3.1). It remains an intriguing, yet untested, possibility that the NIR of Ser and Ax regions of the respective molecules could be involved in a common cis-inhibition mechanism. Because the NIR region might be expected to function cooperatively with the binding domain of the ligand, mediation of cis-inhibition may only be possible when they are appropriately positioned relative to one another. This condition could help to explain why expression of only the NIR portion of Ser has no effect on Notch activity (Fleming et al. 2013). Interactions with the Ax region of Notch need not be restricted to Ser/Jag members that contain an NIR. DL members may also have the potential to interact with Ax repeats to mediate cis-inhibition.

It must be emphasized that the inferences above are purely speculative. It is entirely possible that the *Ax* region does not directly interact with the ligands at all. Alternative roles include but are not limited to the possibility that the *Ax* region forms part of the Notch architecture that serves to stabilize the NRR. If this is the case, then select mutations in this region of the receptor may serve to alter NRR stabilization thereby increasing Notch trans-activation or reducing Notch cis-inhibition potential.

Ligand Degradation and RIP

An interesting finding concerning the Notch ligands is that, like Notch itself, the ligands appear to be processed by RIP. The actual ADAM class protease that initiates this process varies from ligand to ligand and from the species of animal examined. For example, ADAM10 (aka *kuzbanian*), appears to cleave DL in flies whereas reports of either ADAM10, ADAM17 or BACE1 have been published as the likely protease cleaving Jag1 in mammals (Qi et al. 1999; Mishra-Gorur et al. 2002; Azimi and Brown 2019; LaVoie and Selkoe 2003; Coglievina et al. 2013; He et al. 2014; Hu et al. 2017; Liebler et al. 2012). Cleavage by these proteases initiates the ectodomain shedding process that precedes cleavage by the γ-secretase complex in a manner entirely analogous to RIP processing of the Notch receptor. Whereas RIP has been well documented in Notch and the released NICD has been shown to be translocated into the nucleus where it functions as a transcriptional coactivator to control Notch target genes, its function in ligand metabolism is less well defined (Kopan and Ilagan 2009). The IC domains of the majority of Notch ligands contain clusters of basic amino acids that have the potential to function as nuclear localization signals. These sequences are generally conserved in the major ligand families. Further, the IC

domain of Rat, Human and *Xenopus* Jag1, and DLL1 have been shown to localize to the nucleus when released from the membrane (LaVoie and Selkoe 2003; Liebler et al. 2012; Ascano et al. 2003; Kiyota and Kinoshita 2004). If Notch ligand IC domains can function as transcriptional regulators, then bidirectional signaling through the Notch pathway may be possible.

A growing body of evidence supports a model for ligand IC domains in signaling, even though that signaling is significantly less dramatic than that of the NICD. Expression of the Jag IC domain has been shown to selectively elevate expression of an AP-1 reporter gene (LaVoie and Selkoe 2003). Similarly, the DLL4 IC domain has demonstrated the ability to interact with the Jun transcription factor and interfere with its ability to bind with a consensus AP-1 DNA binding site (Forghany et al. 2018). Expression of human Jag1 has been shown to be capable of transforming rat kidney cells in a dose-dependent manner (Ascano et al. 2003). It has further been observed that during mammalian angiogenesis, overexpression of the DLL1 IC, Jag1 IC, or the DLL4 IC domains show an ability to reduce endothelial cell proliferation by nearly 50% indicating a possible regulatory role for these ligand IC domains (Liebler et al. 2012). Another study has shown that the DLL1 IC can bind transcription factors of the Smad family and act to enhance Smad3 transcription when located in the cell nucleus (Hiratochi et al. 2007). It is therefore possible that ligand IC domains, like the NICD, may be able to directly regulate some aspects of transcriptional control in cells.

An additional mechanism for regulating cell fate control via the IC domain of ligands that is distinct from direct transcriptional regulation has been demonstrated for DLL1 and Jag 1. In cell culture studies, the IC domains of these ligands have shown the ability to interact with and antagonize Notch transcriptional effects by destabilizing the Notch1-IC-RBP-Jk-Mastermind complex and promoting proteasomal degradation of the NICD itself (Jung et al. 2011; Metrich et al. 2015; Kim et al. 2011). Thus, direct interactions between ligand and receptor IC domains may be possible and could be responsible for mediating some aspects of cis-inhibition. However, as many of these studies use overexpression and other transfection methods to assay protein interactions and transcriptional changes, it remains unclear as to how significant a role the ligand IC domain may play in the presence of the NICD transcription complex under physiological conditions.

Models of Notch signaling that include cis-inhibition hypothesize that it may simply be the ratios of cis-versus-trans ligand that will determine signal-sending versus signal-receiving cells (Glittenberg et al. 2006; del Alamo and Schweisguth 2009; Sprinzak et al. 2010). Hence, in a signal-receiving cell, the level of Notch product would be expressed higher than that of ligand. The ligand would be titrated out, leaving Notch receptor available and in excess so that the cell would be free to receive the signal (see Fig. 3.4a). Signal-sending cells would exhibit the reciprocal outcome and have excess ligand relative to receptor to titrate the receptor away, leaving the excess ligand to allow for the production of a sending cell (see Fig. 3.4b). Support for this model has been demonstrated using a Notch activation reporter construct with varying concentrations of cis and trans DL in a cell based assay (Sprinzak et al. 2010). In this system, cis-inhibition had a sharp cut off level within cells based on the expression level of DL relative to Notch receptor in the cell. This cut off level was independent of trans-expressed DL levels. In contrast, activation of Notch within a cell generated a graded response to levels of trans-expressed DL provided that cell had expressed DL levels below the cis-inhibition cut off level. These data are consistent with Notch levels being titrated out by cis-expressed ligand, making the cell refractory to activation.

Several lines of data support this model. For example, expression of Ser alone by the *ptc* promoter results in both activation and inhibition as previously discussed (Fig. 3.3). However, if Notch is simultaneously coexpressed with Ser, thereby increasing the levels of Notch, cis-inhibition is overcome and all cells now respond to Notch activation (Klein et al. 1997; Miller et al. 2009). The titration model further suggests that by altering levels of receptor relative to

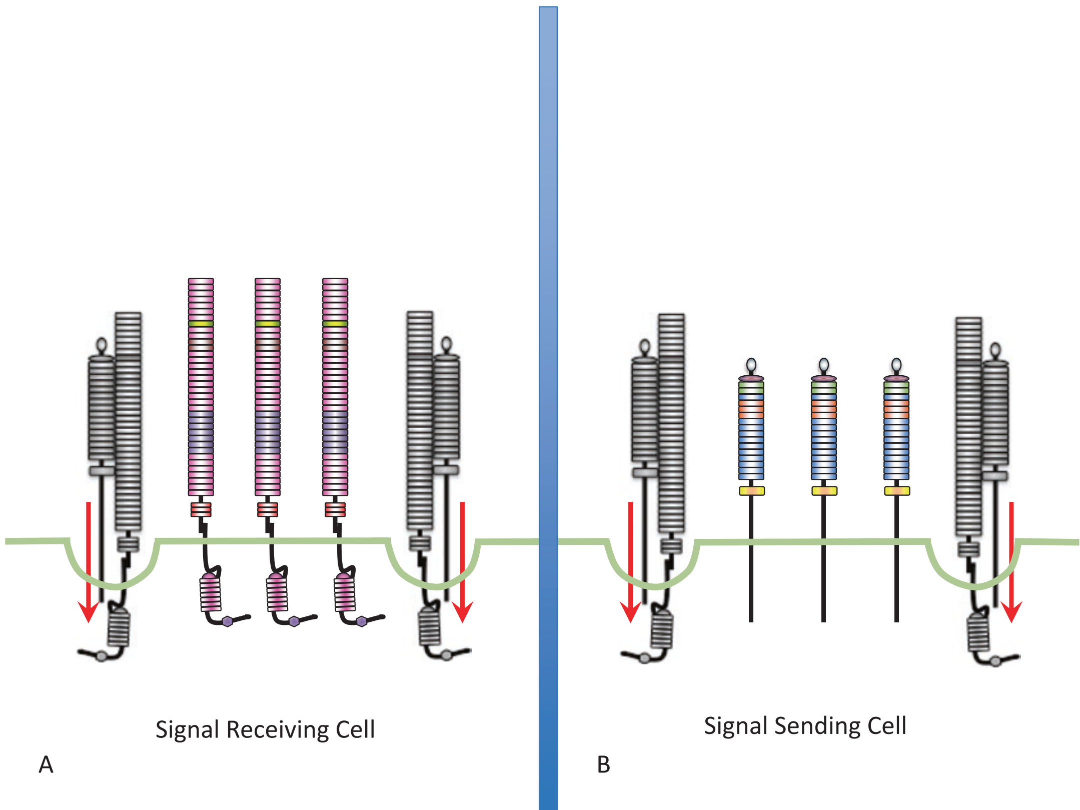

Fig. 3.4 Generation of signal-sending and signal-receiving cells through cis-inhibition. Cell fate as a cell sending versus receiving signals is highly sensitive to the levels of Notch and its ligands in a cell. When both molecules are coexpressed on a single cell, ligand–receptor complexes (shown in gray tones) can be formed and endocytosed (red arrows) into lysosomes for degradation. Thus, in cis, ligand and receptor mutually inhibit one another. When receptor is expressed at a higher level than ligand on a cell (**a**), noncomplexed Notch molecules (red tones) remain available for interaction with ligand on adjacent cells and the cell becomes capable of receiving a Notch signal. In contrast, if a cell expresses a higher level of ligand than receptor (**b**), then complexes again form, but excess ligand (blue tones) remains available to transduce a signal to an adjacent cell, thereby specifying a signal-sending cell

ligand, cellular responses can be altered (Heitzler and Simpson 1991). Experiments in the *Drosophila* wing were conducted to specifically address ligand and receptor levels (Becam et al. 2010). By manipulating the levels of endogenous Notch using clonal induction in wing imaginal disk cells, the authors showed that decreasing cellular protein levels of Notch resulted in increased levels of Ser protein presented at the cell surface. They also showed that the decrease of Ser protein in the presence of Notch was dependent on the extracellular Notch domain and, more specifically, ELRs of the receptor ligand-binding domain. Further, they demonstrated that the reduction by cis-interactions between Notch and Ser occurred at the cell surface and that the reduction in Ser levels was due, in large part, to endocytosis (Becam et al. 2010). These findings do not rule out intracellular interactions between Ser and Notch but are consistent with significant cell surface interaction. Thus, expression levels of Ser ligand correlate inversely with levels of Notch when they are being coexpressed in *Drosophila* wing cells. Interestingly, Dl protein levels were not significantly altered under these same experimental conditions suggesting that Dl may interact in cis with Notch via a mechanistically distinct process. Since it has

not been determined if the Ser/Notch cis-interactions of this study are dependent upon the presence of the NIR of Ser, it remains unclear whether or not there are alternative ways that the different ligand families interact with Notch in cis.

Concluding Remarks

It is clear that the major regions used to trans-activate and to cis-inhibit Notch are largely the same implicating the direct binding of ligand and receptor in each case. It also appears that the levels of expression for each ligand and receptor are critical for determining signal-sending versus signal-receiving cell fates. There are many properties held in common between the DL and Ser/Jag ligand families during the cis-inhibition process. The most obvious common property is that cis-inhibition requires the presence of the EC domain and can occur in the absence of the IC domain for each ligand. Other properties simultaneously show common and unique modalities for each process. For example, both Ser/Jag and DL ligands are recycled to the plasma membrane in an endocytic process termed bulk endocytosis yet different ligands utilize different recycling pathways to accomplish this (Hamel et al. 2010; Heuss et al. 2013; Jafar-Nejad et al. 2005; Emery et al. 2005; Banks et al. 2011; Windler and Bilder 2010). Ser and DL both rely upon E3 ubiquitin ligase/clathrin mediated endocytosis for full, specific Notch trans-activating ability (Wang and Struhl 2005, 2004). However, DL appears to be capable of cotrafficking with the neur E3 ligase and retaining some activating potential even when not ubiquitinated, whereas Ser does not (Berndt et al. 2017). Both ligands utilize the same primary binding site at ELRs 11 and 12 of Notch for both cis and trans activities, yet, as already described, each ligand also has independent interaction sites with the receptor and those sites can differ for cis-versus trans-interactions with Notch. Both ligand families undergo RIP and have IC domains that can generate at least limited transcriptional control of genes in expressing cells in different systems raising the possibility of bidirectional signaling in the Notch system. Glycosylation of Notch differentially affects the manner with which the receptor interacts with each class of ligand and these effects may differentially affect cis-versus-trans Notch interactions. Finally, Ser/Jag ligands have the NIR region adjacent to the trans-activation site of this ligand that demonstrates substantial cis-inhibitory properties for this ligand class. Comparable inhibitory regions on DL ligands have not been identified and may not exist. These findings suggest that each ligand class interacts with Notch to mediate cis-inhibitory roles using at least some discrete contact points.

The model that emerges from all of the above data is based on the ability of the Notch receptor to bind in both the cis and trans configurations with its ligands. Because both the receptor and ligand extracellular regions can be replaced with non–Notch-related ligand and receptor components (Langridge and Struhl 2017), it appears that the EC domains of each molecule simply mediate binding between receptor and ligand. As discussed above, each ligand family has acquired numerous, specific regulatory processes to control both the cis- and trans-binding interactions with the receptor. Trans-binding of ligand to receptor results in ubiquitination-dependent ligand endocytosis that activates the RIP of Notch on the neighboring cell and releases the NICD to mediate transcriptional regulation. Cis interactions do not activate Notch and likely target the ligand–receptor complex for degradation.

There remain several unanswered questions concerning specific processes that mediate the receptor–ligand interactions in this system. Where in the cell do cis-interactions occur? Is it at the cell surface, in the ER/Golgi system, perhaps both? Why do the ligands undergo RIP and why do their IC domains localize to the nucleus? Is there really bidirectional signaling in the Notch system? Answers to these and other questions will ultimately lead to a greater understanding of Notch-mediated cell fate determination during normal development and disease-related conditions resulting from abnormalities in the signaling components for this system.

When one follows the research conducted on Notch signaling over the past century, there is

one theme that has been consistently observed. That is, the Notch system repeatedly demonstrates unusual and unexpected mechanisms by which it mediates intercellular communication to control cellular fates. As the research continues and we further refine our understanding of the ligand–receptor interactions of this system, there are likely to be additional, unexpected cellular mechanisms remaining to be discovered.

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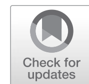

4 Phosphorylation and Proteolytic Cleavage of Notch in Canonical and Noncanonical Notch Signaling

Ginger L. Hunter and Edward Giniger

Abstract

The Notch signaling pathway seems deceptively simple, with its key feature being a direct connection between extracellular signal and transcriptional output without the need for an extended chain of protein intermediaries as required by so many other signaling paradigms. However, this apparent simplicity hides considerable complexity. Consistent with its central role in many aspects of development, Notch signaling has an extensive collection of mechanisms that it employs alongside of its core transcriptional machinery. These so-called noncanonical Notch pathways diversify the potential outputs of Notch, and allow it to coordinate regulation of many aspects of the biology of cells. Here we will review noncanonical Notch signaling with special attention to the role of posttranslational modifications of Notch. We will also consider the importance of coordinating the activity of gene expression with regulation of cell morphology in biological processes, including axon guidance and other morphological events during embryogenesis.

Keywords

Notch signaling · Posttranslational modifications · Axon guidance · Cytoskeleton

Abbreviations

CNS	Central nervous system
CSL	CBF/RBP-Jκ in mammals, Su(H) in *Drosophila*, and Lag-1 in *C. elegans*
DSL	Delta, Serrate, LAG-2
NICD	Notch intracellular domain

The Notch Pathway and Neurogenesis

Canonical Notch Signaling

Canonical Notch signaling is highly conserved, and its molecular mechanisms have been well studied. Notch (Mammalian Notch 1–4, Drosophila Notch, *C. elegans* LIN-12 and GLP-1) is a transmembrane protein that is transactivated by a DSL ligand (Figs. 4.1 and 4.2a). Extracellular EGF repeats of Notch bind to a DSL ligand on an

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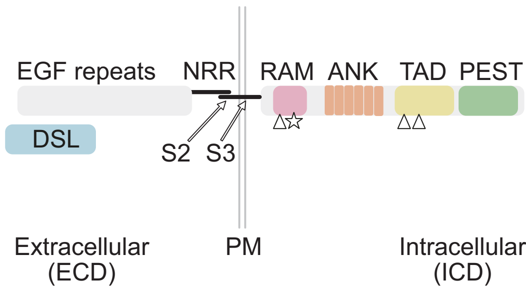

Fig. 4.1 Schematic of Drosophila Notch protein organization. *DSL* Delta/Serrate/Lag-2. The EGF repeats of the extracellular domain (ECD) are required for DSL interaction and trans-activation of Notch receptor. *NRR* negative regulatory region. In mammalian Notch, the S1 cleavage, mediated by Furin proteases also occurs in this region. S2 cleavage, mediated by Kuzbanian/ADAMs, occurs in the NRR. The S3 cleavage, mediated by the presenilin complex, occurs in the transmembrane domain spanning the plasma membrane (PM). The intracellular domain (ICD) has four major regions: Rbp-associated molecular (RAM) domain, Ankyrin (ANK) repeats, transactivation domain (TAD), and a Proline/Glutamic Acid/Serine/Threonine (PEST) degradation domain. A star indicates the Disabled binding site in the RAM domain, and the three arrowheads indicate the Su(H) (*Drosophila* CSL) binding domains

adjacent cell. Endocytosis of the ligand pulls on the extracellular domain of Notch, leading to exposure of a protease cleavage site in the negative regulatory region of the receptor and subsequent proteolytic cleavage at this region. This is followed by a second cleavage in the transmembrane domain (Gordon et al. 2015; Meloty-Kapella et al. 2012). The freed intracellular domain translocates to the nucleus to activate transcription, in a complex with its cofactors, Mastermind (Mam) and CSL. Transcriptional targets of Notch are context dependent, but often include the Enhancer of split family of transcription factors (Hes-1 and Hes-5 in mammals). While we will not explore the details of the canonical Notch signaling pathway here, other reviews have covered this excellently (Bray 2016; Kopan and Ilagan 2009).

Noncanonical Notch Signaling and Axon Guidance

In some contexts, both full-length Notch protein and its constituent domains can signal independent of interactions with CSL proteins, and sometimes without activation by ligand. Anything outside of the CSL model of Notch activation is defined broadly as noncanonical Notch signaling. The downstream consequences of noncanonical Notch signaling are therefore not necessarily associated with changes in CSL-dependent target gene expression (though they may be if canonical and noncanonical pathways are activated in parallel; see below). Here we will discuss a particularly well-characterized form of nonnuclear Notch signaling that interacts with the Abl tyrosine kinase signaling module, as well as additional mechanisms that link Notch to other signaling pathways.

Axon guidance, particularly in *Drosophila*, has become one of the best studied examples of noncanonical Notch signaling in vivo (Crowner et al. 2003; Giniger 1998; Kannan et al. 2017a; Kuzina et al. 2011; Le Gall et al. 2008). A key feature of neural circuits is the formation and maintenance of connections between developing neurons and their target cells, often in distant locations or tissues. The neurodevelopmental process by which axons grow and extend on specific paths toward their targets is called axon patterning (Dickson 2002; Stoeckli 2018). Two overlapping processes drive axon patterning: first, the ability of the axon to grow outward requires key regulators of the actin and microtubule cytoskeleton (Cammarata et al. 2016; Dent et al. 2011). Second, the axon's ability to keep to a path and ultimately find its target (guidance per se) typically requires multiple attractant or repellant cues, in the form of cell signaling molecules such as Slit, Netrins, Ephrins, and Delta, together with their receptors (Bashaw and Klein 2010). These two processes overlap as an axon's out-

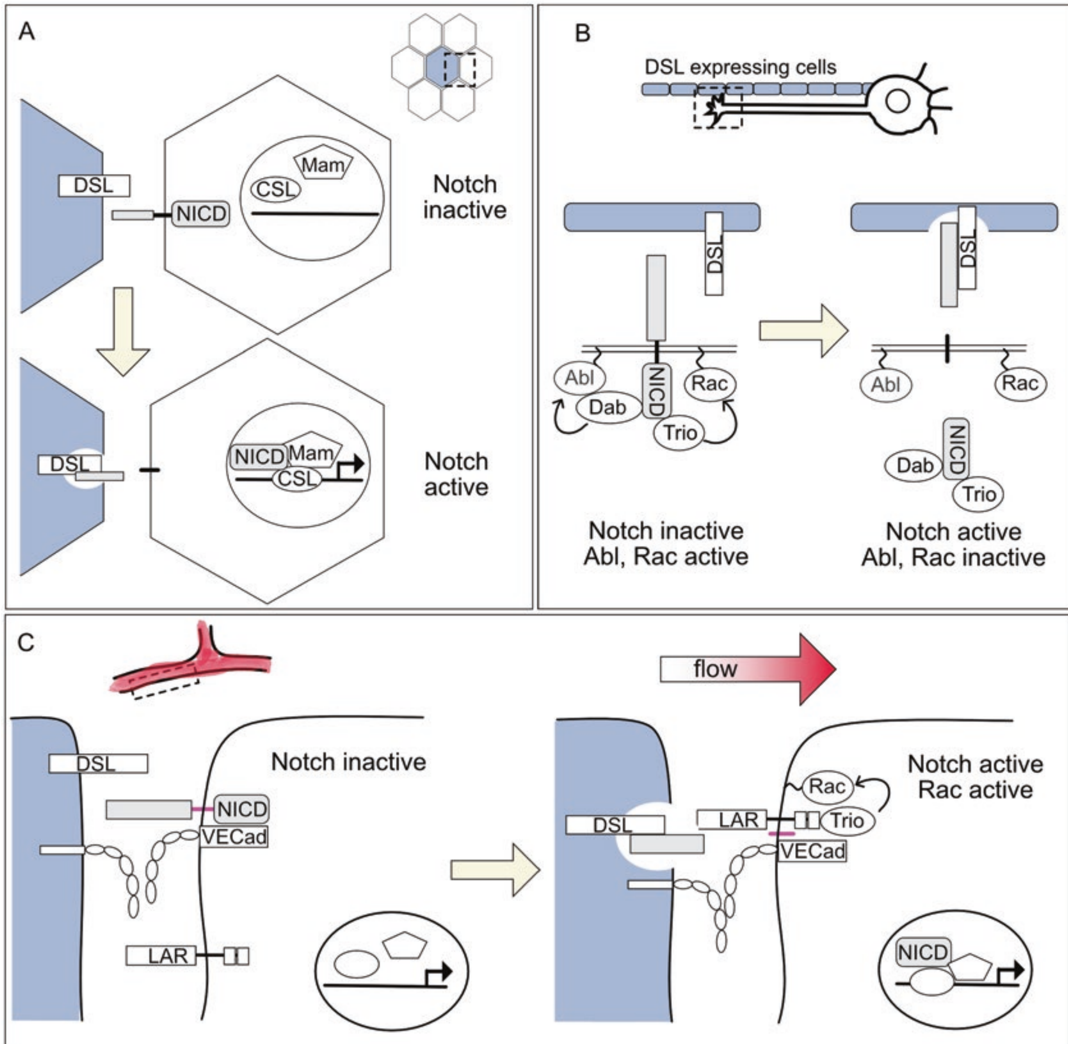

Fig. 4.2 Examples of canonical and noncanonical Notch signaling. (**a**) Cells undergoing lateral inhibition (e.g., during neurogenesis) use canonical Notch signaling to make cell fate decisions. Activation of Notch occurs upon endocytosis of bound, DSL ligand by an adjacent cell, which leads to S2 and S3 cleavage, followed by translocation of NICD to the nucleus where it interacts with CSL and Mastermind (Mam) to regulate gene expression. In this scenario, the blue DSL expressing cell is inactive for Notch and does not have NICD in the nucleus. (**b**) Neuronal cells undergoing axon pathfinding use noncanonical Notch signaling to regulate the cytoskeleton in the growth cone. Inactive Notch is complexed with Dab and Trio. The proximity of Dab and Trio to the cell cortex facilitates activation of their membrane-tethered substrates, Abl and Rac, respectively. Upon binding extracellular DSL ligand, the S2 and S3 cleavages occur. Movement of the NICD-Trio-Dab complex away from the cortex breaks up the Abl signaling complex, preventing Dab and Trio from activating the membrane-tethered Abl and Rac, respectively. (**c**) Endothelial cells lining blood vessels use noncanonical Notch signaling to regulate cell–cell junction strength in response to flow. Flow triggers the binding of Notch by DSL, followed by the S2 and S3 cleavages. The membrane bound transmembrane domain of Notch (magenta) is freed to interact with the cell–cell adhesion protein VE-cadherin (VE-cad) and the transmembrane tyrosine phosphatase LAR. LAR recruits Trio, leading to activation of Rac GTPase and the local polymerization of actin cytoskeleton to reinforce the cell–cell junction. Note that canonical signaling may still occur post-S3 cleavage, involving the nuclear association of NICD, Mam and CSL as in (**a**)

ward growth will be promoted by signaling associated with attractants and retarded or reversed by signaling associated with repellants.

The evidence for noncanonical Notch signaling during axon guidance indicates that Notch can facilitate a direct, local connection between extracellular signals and organization of the actin cytoskeleton at the cortex (i.e., near the cell membrane). In *Drosophila*, the ability of Notch to act as a guidance molecule was not immediately obvious because during *Drosophila* embryogenesis, as in other organisms, the nervous system uses Notch signaling iteratively, first to segregate neuronal precursors and establish their cell lineages (Spana and Doe 1996), and subsequently to establish its patterns of innervation. Cell identities are specified as a function of 'classical', CSL-mediated Notch signaling (Artavanis-Tsakonas and Simpson 1991; Engler et al. 2018). Multiple lines of evidence, however, have established that once the neural fates have been specified, Notch then promotes the growth and guidance of many axons, through a mechanism that is dependent on Abl tyrosine kinase (Giniger 2012).

The first evidence implicating Notch directly in axon growth and guidance exploited the ability to regulate Notch signaling with temperature sensitive (ts) mutants (Giniger 1998; Giniger et al. 1993). Shifting *Notch*^{ts} mutant embryos to the nonpermissive temperature early during neurogenesis leads to hyperplasia of the CNS, similar to other *Notch* loss-of-function mutants. Shifting them late in development, however, leads to defects in a specific set of axon patterning decisions with only limited effects on cell number and identity. More rigorously, subsequent experiments generated mutant derivatives of Notch that selectively provide either its canonical, nuclear function in neuron specification and differentiation, or its noncanonical function in axon growth and guidance (Kannan et al. 2017a; Le Gall et al. 2008).

What is the molecular basis of these two mechanisms? The Notch neurogenesis axis is controlled by interaction between the intracellular domain of Notch (NICD) and CSL, whereas the Notch axon guidance axis is governed by interaction between NICD and components of the Abl tyrosine kinase network. As a consequence, the NICD contains several protein binding motifs associated with either canonical or Abl-dependent signaling (Fig. 4.1): the former requires three conserved binding sites for its DNA-binding cofactor, Su(H) (Le Gall and Giniger 2004). One is located in the RAM domain, just downstream of the transmembrane domain, and the other two are located downstream of the ankyrin repeats (Fig. 4.1, arrowheads). In the absence of Notch, embryonic cells overselect for the neuronal cell fate resulting in hyperplasia of the CNS. Transgenic expression of a wild type Notch construct rescues the hyperplasia phenotype, but expression of a construct lacking all three NICD Su(H) binding sites does not (Le Gall et al. 2008). Abl-dependent signaling, on the other hand, is characterized by Notch activity independent of Su(H), but dependent on association with Abl pathway components, including the adaptor protein Disabled (Dab) (Crowner et al. 2003; Kannan et al. 2017a; Le Gall et al. 2008). Neither reduction of Su(H) activity nor disruption of the interaction between NICD and Su(H) impairs axon guidance; the axonal organization of the CNS in these embryos is comparable to wild type (Crowner et al. 2003; Le Gall et al. 2008). In contrast, deletion of the Dab binding site selectively ablates the axon patterning activity of Notch without reducing its cell fate function. These tests, determining the necessity of NICD/Su(H) vs. NICD/Dab interactions for a Notch-dependent signaling event, allowed rigorous discrimination between canonical and noncanonical Notch signaling. Interestingly, both mechanisms also employ sequences in the Ankyrin repeat domain of NICD as a second point of contact for their respective protein complexes. Whether those involve shared portions of the Ankyrin domain or are also separable has yet to be established.

Posttranslational Modification: Proteolytic Cleavage

Posttranslational modification of Notch receptor plays a key role in both the canonical and noncanonical signaling pathways. These changes

include proteolytic cleavages, glycosylation, fucosylation, ubiquitination, hydroxylation, and phosphorylation (Borggrefe et al. 2016; Fortini 2009; Lee et al. 2015). Below, we will first consider proteolytic cleavage of Notch and then Notch phosphorylation, as they relate to the mechanism of signaling in regulating cell morphology, especially during axon guidance.

S1 Cleavage

The first proteolytic cleavage of mammalian Notch (S1) occurs during maturation of the receptor in the Golgi of the Notch-expressing cell, prior to interactions with ligand. Notch is initially expressed as a ~300 kDa protein, which is then cleaved in the negative regulatory region by furin protease into 120 and 200 kDa pieces. The two pieces of Notch associate noncovalently prior to being expressed on the cell surface. Mutation of the S1 cleavage site in mammalian Notch1, however, does not entirely prevent cell surface presentation or block Notch activation by DSL ligand, and in fact, *Drosophila* Notch does not appear to require S1 cleavage at all for neurogenesis (Bush et al. 2001; Gordon et al. 2009; Kidd and Lieber 2002). In Notch-mediated axon guidance genetic analysis of the requirement for Furin proteases is unclear, as furin is required for the processing of several guidance molecules, including Semaphorin 3 and RGMa (Repulsive guidance molecule) (Adams et al. 1997; Monnier et al. 2002). Therefore, while a role for S1 cleavage in axon patterning has not been demonstrated to date, it cannot formally be ruled out, particularly in the mammalian nervous system.

S2 Cleavage

S2 cleavage is the first irreversible, ligand-dependent step in Notch signaling, occurring after Notch has been localized to the cell surface and engaged by DSL ligand. S2 cleavage takes place in the negative regulatory region, just C-terminal to the S1 cleavage, and is a result of the activity of an ADAM (A Disintegrin And Metalloprotease), encoded by the gene *kuzbanian* in *Drosophila* and ADAM10 in mammals (Fig. 4.1) (Fambrough et al. 1996; Lieber 2002). The outcome of this cleavage is shedding of the extracellular domain of Notch, which may remain bound to DSL ligand and transendocytosed into the ligand expressing cell (Fig. 4.2). This results in the production of a membrane bound intermediate, termed NEXT (Notch external truncation), that comprises the transmembrane domain, NICD, and a small stump of extracellular peptide, and is an obligate step in canonical signaling. In axon patterning, loss of *kuzbanian* expression in the fly embryo leads to defects consistent with known Notch functions; however, as with furins, it has been shown that Kuzbanian processes other cell surface receptors associated with axon patterning including ephrins (Hattori et al. 2000). In order to clarify the role of this cleavage in axons, use was made of Notch derivatives bearing mutations in the Notch extracellular domain that selectively block Kuzbanian-mediated S2 cleavage at elevated temperature (due to the absence of specific O-linked glycosylation events) (Kannan et al. 2017a; Leonardi et al. 2011). Indeed, these noncleavable Notch derivatives were found to be essentially inactive for providing the axon patterning function of Notch at the restrictive temperature, verifying that S2 cleavage is essential in this pathway.

ADAM10 performs S2 cleavage of Notch in mammalian systems, and mice with neural-specific loss of ADAM10 expression develop defects in brain organization consistent with defective Notch function (Jorissen et al. 2010). The Cre recombinase used to create the ADAM10 conditional knockout mouse causes ADAM10 to be absent from the definitive neurons as well as the neural precursors. Moreover, ADAM10 is known to cleave several other proteins associated with axon guidance, including Semaphorins, Ephrins and N-cadherin (Malinverno et al. 2010; O'Donnell et al. 2009; Romi et al. 2014). Therefore, it is unclear whether the observed neural phenotypes are due only to a failure during neurogenesis, or also reflect functions during axon patterning.

S3 Cleavage

The transient species NEXT rapidly undergoes S3 cleavage in the transmembrane domain, as a result of the activity of the γ-secretase complex. This results in a free NICD which can translocate to the nucleus, as well as a residual transmembrane domain. One protein of the γ-secretase complex is encoded by the gene *presenilin*, and *Drosophila* embryos lacking all *presenilin* expression resemble *Notch* null embryos (Struhl and Greenwald 1999). During axon patterning, heterozygosity for *presenilin* enhances the axon guidance defects produced by partial reduction of Notch activity, suggesting that presenilin-mediated intramembrane proteolysis is required for axonal function (Kannan et al. 2017a). The mouse genome encodes two presenilins, PS1 and PS2. They do not fully rescue each other, suggesting that they have some nonoverlapping functions; in particular, postmitotic knockout of PS1 in mouse motor neurons leads to axon guidance defects downstream of Netrin 1 signaling (Bai et al. 2011; Herreman et al. 1999; Shen et al. 1997). This may in part be because the major Netrin receptor, frazzled/DCC, also undergoes presenilin-mediated cleavage upon ligand activation (Neuhaus-Follini and Bashaw 2015; Taniguchi et al. 2003). In both *Drosophila* embryos and in cell culture, the Frazzled/DCC receptor is cleaved by γ-secretase complex to free the intracellular domain which can act as a transcription factor, in addition to Frazzled/DCC's well-known ability to locally regulate the cytoskeleton downstream of Netrin 1 binding. The parallels to Notch signaling are striking, and presenilin-mediated receptor cleavage may well represent a component of axon guidance signaling in an even broader set of guidance pathways (Seki et al. 2010; Tomita et al. 2006).

Abl Signaling and NICD

The progressive proteolysis of Notch suggests a simple model by which the receptor could be used as a switch to regulate signaling events at the cell cortex, by taking advantage of the shifting localization of NICD to change the interactions of bound proteins (Kannan et al. 2017a). The example we will discuss here is the shift of NICD from the cortex to the cytoplasm and its effects on activity of the Abl signaling pathway.

The actin cytoskeleton contributes to movement in most cells, through the formation of cellular projections called filopodia and lamellipodia, as well as contributing to cell shape through the maintenance of an actin cortex, which is associated with the intracellular face of the plasma membrane. The cytoplasmic protein tyrosine kinase, Abl, along with its cooperating factors, Enabled, Disabled, and Trio, form a signaling network that is a key regulator of actin cytoskeleton dynamics during axon guidance (Kannan et al. 2017b; Song et al. 2010). Abl itself regulates actin organization both through its kinase activity and by acting as a signaling scaffold protein. Disabled is an adaptor protein that localizes Abl and stimulates its kinase activity. Enabled is a processive actin polymerase that extends and bundles linear actin filaments, while Trio is a guanine exchange factor (GEF) for the small GTPases Rac and Rho. Together, they form the core of the Abl signaling network, which has many roles in morphology and motility. Among these roles is balancing the relative prevalence of linear vs. branched actin structures in the cell, in part to regulate the propensity of the growing axon to create filopodia or lamellipodia. Abl inhibits the activity of Enabled, which promotes extension of filopodia (Gertler et al. 1995; Grevengoed et al. 2003). At the same time, Abl promotes the activity of Trio, and in particular its Rac GEF activity that, through activation of the WAVE complex, promotes expansion of branched actin networks, including those found in lamellipodia.

The Abl pathway is known to regulate many aspects of axon patterning (Elkins et al. 1990; Hoffman 1991). The evidence that the Abl pathway specifically plays a central role in noncanonical Notch signaling stems from a series of genetic and biochemical experiments. It was first established that Abl and its accessory factors Disabled (Dab) and Trio interact genetically with Notch in axon patterning (Crowner et al. 2003; Giniger 1998; Kannan et al. 2017a; Kuzina et al. 2011; Song et al. 2010). Thus, various allelic

combinations of *Notch*, *abl*, and *dab* mutants were found to be synthetic lethal and result in specific axon patterning defects in the CNS and PNS of Drosophila embryos. In other developmental settings, Notch acts to limit the activity of the Abl/Disabled/Trio network, such that axon patterning defects of Notch mutants can be suppressed by reducing the dosage of genes in the Abl pathway. Later, physical interactions were also demonstrated between Dab, Trio, and NICD, with the phosphotyrosine binding domain of Dab binding directly to the juxtamembrane RAM domain of NICD. Consistent with this, specific deletion of the Dab-binding motif in the RAM domain ablates the ability of Notch to direct axon guidance, without affecting its ability to regulate neuron number or identity. Unexpectedly, binding of Dab and Trio to Notch was found to occur independent of signaling activation, that is, in the absence of DSL ligand. Moreover, Dab and Trio remain bound to NICD after S2 and S3 cleavage of Notch (Giniger 1998; Kannan et al. 2017a; Le Gall et al. 2008). Together, these data suggest a switch whereby release of NICD from the membrane via S3 cleavage results in the movement of the NICD/Dab/Trio complex away from the cortex (Fig. 4.2b). This separates them from their biochemical targets, Abl and Rac, both of which are tethered to the plasma membrane by fatty acylation, thus suppressing signaling downstream of Abl and Rac (Kannan et al. 2017a).

Transmembrane Domain (TMD)

After the three proteolytic cleavages have successfully led to release and nuclear translocation of the NICD, all that remains of Notch at the cell surface is the transmembrane domain, or TMD. The fate of the remnant TMD and whether it serves any purpose post-S3 cleavage is unknown for most Notch-dependent processes. However a recent study has demonstrated a role for TMD in regulating cortical F-actin organization in the vascular endothelial barrier response to shear stress (Polacheck et al. 2017). Shear stress is essentially the frictional force experienced by a cell as a consequence of fluid flow over it, in this case the flow of blood through the endothelial cell-lined lumen of blood vessels. Notch1 proteolysis is triggered by shear stress, leading to two consequences. First, canonical N1ICD signaling in the nucleus leads to changes in the expression of target genes (e.g., Hes1) in response to shear stress. Second, the remaining TMD associates with VE-cadherin, the homotypic cell–cell adhesion molecule expressed in vascular endothelial cells. Interaction between TMD and VE-cadherin recruits a transmembrane protein tyrosine phosphatase, LAR, which is bound to Trio and has the downstream effect of increasing Rac1 activity as well as strengthening endothelial cell–cell junctions, possibly through Rac1-dependent F-actin organization. In the absence of Notch1, the F-actin cytoskeleton is not recruited to the cell–cell junctions and the endothelial barrier function is weakened. Expression of TMD alone in this background is sufficient to rescue this phenotype, while continued linkage of the TMD to the Notch1 extracellular or intracellular domain can block the physical interaction of TMD with VE-cadherin and LAR. This previously unrecognized role for TMD emphasizes how noncanonical Notch signaling participates in the regulation of cytoskeleton dynamics at the cell cortex. It is also striking that the relationship of Notch and Trio is inverted in this noncanonical mechanism relative to that in the axonal signaling machinery described above, mediated through NICD. Here, acting through VE-cadherin and LAR, the Notch TMD stimulates Trio function, while Notch activation in *Drosophila* axons suppresses Trio activity. It will be interesting to see if and how TMD plays a role in organizing F-actin in other processes in vivo, including axon guidance.

Posttranslational Modification: Phosphorylation of NICD

In addition to proteolytic cleavage of Notch, phosphorylation of the NICD plays multiple roles in the activity of the Notch signaling pathway (Fig. 4.3). Phosphorylation of tyrosine, threonine, and serine residues in the NICD has been

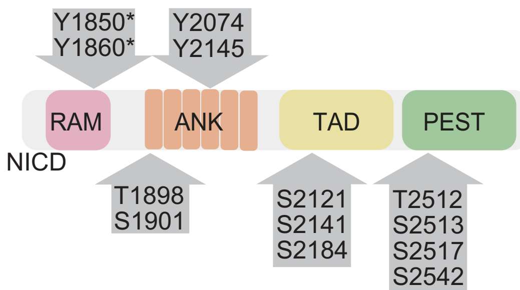

Fig. 4.3 Phosphorylation of Notch intracellular domain. Schematic of the NICD, with domains labeled as in Fig. 4.1. Y, tyrosine; S, serine; T, threonine. Amino acid numbering refers to Mammalian Notch1 except where noted with an asterisk, which refers to *Drosophila* Notch

observed in both the noncanonical and canonical Notch signaling context (Kannan et al. 2017a; Lee et al. 2015).

Tyrosine Phosphorylation of Notch, and Its Role in Axon Guidance

In *Drosophila* embryos, a small population (~5%) of Notch protein molecules is phosphorylated on tyrosine, and that tyrosine-phosphorylated population of Notch molecules is highly enriched in Dab/Trio complexes, as compared with bulk Notch (Kannan et al. 2017a). Molecular experiments identified a minimal Notch truncation derivative, Notch$^{1-2155}$, that is highly active at directing axon patterning, and that contains only three tyrosines in the ICD (Y1850, Y1860, and Y2097). Mutation of these three tyrosines to phenylalanine residues (termed 3YF mutants) largely ablates the ability of the protein to direct axon guidance in the CNS and PNS of the embryonic nervous system. In contrast, introduction of the same 3YF-mutation into a slightly longer Notch derivative that retains partial canonical signaling activity yielded a Notch protein that was as effective at suppressing neurogenic defects of a chromosomal Notch mutant as was its parent truncation allele, showing that the tyrosine phosphorylation is not necessary for CSL-mediated signaling. Perhaps surprisingly, tyrosine phosphorylation does not appear to be a requirement for association with Dab and Trio as they bind as effectively to 3YF Notch as they do to wild type Notch, placing the requirement for phosphorylation somewhere further downstream in the signaling mechanism. Furthermore, overexpression of Abl does not enhance tyrosine phosphorylation of Notch, so it is not known what kinase is responsible for the modification. Therefore, while it is clear that tyrosine phosphorylation plays an essential role in noncanonical Notch-mediated axon guidance, it is not yet known precisely which steps in the pathways require or regulate it. Mammalian Notch (1–3, but not 4) is conserved for the *Drosophila* NICD tyrosine residue Y2097 (mammalian Y2074), which has also been shown to be a target of Src kinase in human cell culture (discussed below); however, *Drosophila* Notch Y1850 and Y1860 are not conserved in mammalian Notch.

Serine, Threonine Phosphorylation of Notch, and Its Roles in Gene Regulation

Many studies have demonstrated that phosphorylation of Notch is critical for the stability and nuclear signaling strength of NICD in the context of canonical signaling. The Notch 1 intracellular domain (N1ICD) can be phosphorylated, primarily at serine and threonine residues (Fig. 4.3), and this phosphorylation seems to be substantially enhanced in N1ICD that has been released from the membrane (hyperphosphorylation). This release-dependence of phosphorylation is in contrast to the noncanonical pathway, where the full-length, uncleaved protein seems to be a target for tyrosine phosphorylation. As is also the case for tyrosine phosphorylation in the noncanonical pathway, the serine, threonine hyperphosphorylation has no effect on the ability of N1ICD to associate with its binding partner, in this case nuclear CSL proteins (Foltz and Nye 2001). Rather, phosphorylation of N1ICD plays a role in promoting the destruction of N1ICD and thus termination of the signaling event. One example of this is the role of Mastermind (MAML-1 in mammals, MAM in *Drosophila*). During signaling events, N1ICD/CSL complexes require the

activity of MAML-1, which can function both to positively and negatively regulate the signaling output: it promotes the recruitment of histone deacetylases, and an increased dwell time of NICD/CSL complexes at target loci (Fryer et al. 2002; Gomez-Lamarca et al. 2018); but it also promotes the phosphorylation of the TAD and PEST domains of N1ICD. The kinase responsible for this is CDK8 (Fryer et al. 2004). Phosphorylation of the PEST domain targets N1ICD for ubiquitination by FBW7/SEL-10 E3 ligases and subsequent degradation, terminating the Notch signal.

As might be expected given the core biological functions of Notch, both loss-of-function and gain-of-function mutations in *Notch* are directly associated with cancer states, modifying the balance of proliferation and differentiation in the cancer cells themselves (Aster et al. 2017). Some cancer states associated with increased Notch) signaling have been revealed to carry deletions that render N1ICD unable to be modified (Hoemann et al. 2000). In particular, the PEST domain is altered such that it cannot be phosphorylated, diminishing the ability of FBW7/SEL-10 ligases to degrade and terminate the signal associated with NICD. Additionally, Notch can be indirectly associated with cancer states through its effects on angiogenesis. There is substantial evidence for canonical signaling play an essential role in this process; whether noncanonical signaling also contributes has been speculated, but not investigated critically (see below).

Overlapping Action of Canonical and Noncanonical Notch Signaling

The coupling of gene expression with cell morphology is a key feature of morphogenesis. Both canonical and noncanonical Notch signaling depend on several of the same set of core proteins and posttranslational events, indeed there is evidence that these two modes of Notch signaling occur concurrently. Here we discuss two examples of Notch signaling involved in gene expression and cell morphology, and where there is potential for overlap.

Phosphorylation of Notch Dependent on Src Kinases

Research suggests that phosphoregulation of NICD stability may have overlapping implications for gene expression and cell motility mechanisms, and this could potentially be widely applicable to the interaction of migrating cells with their substrates. ILK (integrin-linked kinase) and the cytoplasmic kinase Src are both known to phosphorylate N1ICD downstream of integrin activation (LaFoya et al. 2018, 2016; Mo et al. 2007) and have also been shown to play a role in axon growth (Lilja and Ivaska 2018). ILK helps mediate interactions between focal adhesions and the actin cytoskeleton (Attwell et al. 2003), and ILK, N1ICD and FBW7 can form a trimeric complex that features phosphorylation of N1ICD at Ser2173 and leads to its ubiquitin-mediated proteasomal degradation (Mo et al. 2007).

Src family kinases appear to be involved in at least two steps of Notch signaling. First, Src has been shown to promote the association of Notch 1 and Furin protease at the golgi, prior to cell surface display (Ma et al. 2012). Src binds to the ankyrin domain of full-length Notch 1 (pre-S1 cleavage), in a manner that requires its kinase function and results in tyrosine phosphorylation of the NICD. Second, Src has also been shown to phosphorylate the ankyrin domain of N1ICD at tyrosine residue 2074 in cultured mammalian cells (LaFoya et al. 2018). Src family kinase-mediated tyrosine phosphorylation decreases the stability of N1ICD, similar to CDK8 and ILK, again leading to termination of the Notch signal. It is unclear to what extent these two functions overlap: for example, whether Src binds to, phosphorylates, and/or remains associated with the N1ICD throughout its trafficking to and from the cell surface, or whether it associates transiently during furin cleavage and post-N1ICD cleavage separately. Note also that experiments above investigating the consequences of Y2097 modification in *Drosophila* (the homolog of Y2074) might have failed to detect an effect on the half-life of the protein since such a small fraction of total Notch is tyrosine phosphorylated in the fly.

Src is also activated downstream of integrin activation by interaction with extracellular matrix, and its kinase activity is upstream of several regulators of the actin cytoskeleton, including Rho GTPases. In neurons isolated from Xenopus embryos, Src, but not Abl, activity can promote the presence of phosphorylated tyrosine residues at filopodia tips in extending axons (Robles 2005). Although the specific targets of Src at filopodia tips are not known, Src appears to function with cdc42 and PAK to regulate the actin cytoskeleton (He et al. 2015; Robles 2005). We do not yet know whether phosphorylation of the NICD could be an important element promoting cross talk between regulation of the actin cytoskeleton mediated by integrins and that mediated by noncanonical, Abl/Dab and Trio associated, Notch signaling.

Notch/Disabled Interaction in Radial Migration of Neurons and Dendrite Branching in the Mammalian Brain

Another example of potential overlap between the canonical and noncanonical Notch signaling pathways in neurodevelopment is revealed by evidence from the Reelin pathway. A critical step in the development of the mammalian brain is radial migration of neurons in the cortex (Kriegstein and Noctor 2004). *reelin* encodes a secreted glycoprotein that is required for process extension and cell body translocation by neurons resulting in cortical lamination. Reelin binds to the cell surface receptors ApoER-2 and VLDLR, leading to cytoplasmic activation of Disabled-1 (Fig. 4.4a) (Bock and Herz 2003). Interestingly, evidence suggests that Dab then binds to Notch in this pathway to regulate neuronal migration and morphology (Hashimoto-Torii et al. 2008; Sibbe et al. 2009). Reelin mutant mice exhibit lower levels of nuclear NICD as well as decreased expression of Notch target genes *Hes1* and *Hes5*. Loss of Notch1 activity mimics the phenotype of Reelin mutants, including defective neuronal morphology and reduced radial migration, while overexpression of NICD or constitutively active CSL can rescue the *reelin* phenotype. Cytoplasmic activation of Dab1 downstream of ApoER2/VLDLR involves the activity of Src family kinases that phosphorylate Dab1 at five target tyrosines (Bock and Herz 2003; Keshvara et al. 2001). Neurons that express a mutant Dab1 that is unable to be tyrosine phosphorylated (5YF) exhibit migration defects as well as lower levels of NICD in the nucleus. This suggests that Dab1 plays a role in promoting the stability of NICD, and indeed it has been shown both that wild type Dab1 can prevent Fbxw7-mediated reduction in Notch response and that overexpression of NICD can suppress the Dab1-5YF migration phenotype (Hashimoto-Torii et al. 2008). Together these data support a model where the Reelin/Notch signaling interaction relies, in part, on canonical Notch signaling.

Several lines of evidence, however, may hint at an accompanying role for the noncanonical, Notch-Abl mechanism in Reelin-dependent signaling. First, the effect of loss of Notch on neuronal morphology—especially the formation of multiple shorter and inconsistently oriented projections—and process extension during migration is reminiscent of *Drosophila* axonal phenotypes of Notch. Second, while the role of Abl in radial migration has not yet been clarified, cortical lamination depends both on the core Abl regulator, Disabled, and its core effector, Enabled. It would be surprising indeed if Abl did not participate in the process. Finally, while formal genetic nomenclature places Disabled "upstream" of Notch in radial migration, and "downstream" of Notch in *Drosophila* axon patterning, in fact, the molecular mechanism reveals that in both cases the core of the mechanism is cotrafficking of Dab and NICD away from the plasma membrane in response to receptor activation and presenilin cleavage. It therefore remains of great interest to investigate the contribution of different aspects of Notch regulation of Abl signaling in the process of cortical lamination in the mammalian brain.

Reelin/Dab and Notch are also key regulators of dendrite branching in the mammalian brain, particularly in the cortex and hippocampus. The requirement for canonical, CSL-mediated Notch signaling in regulation of dendritic branching has

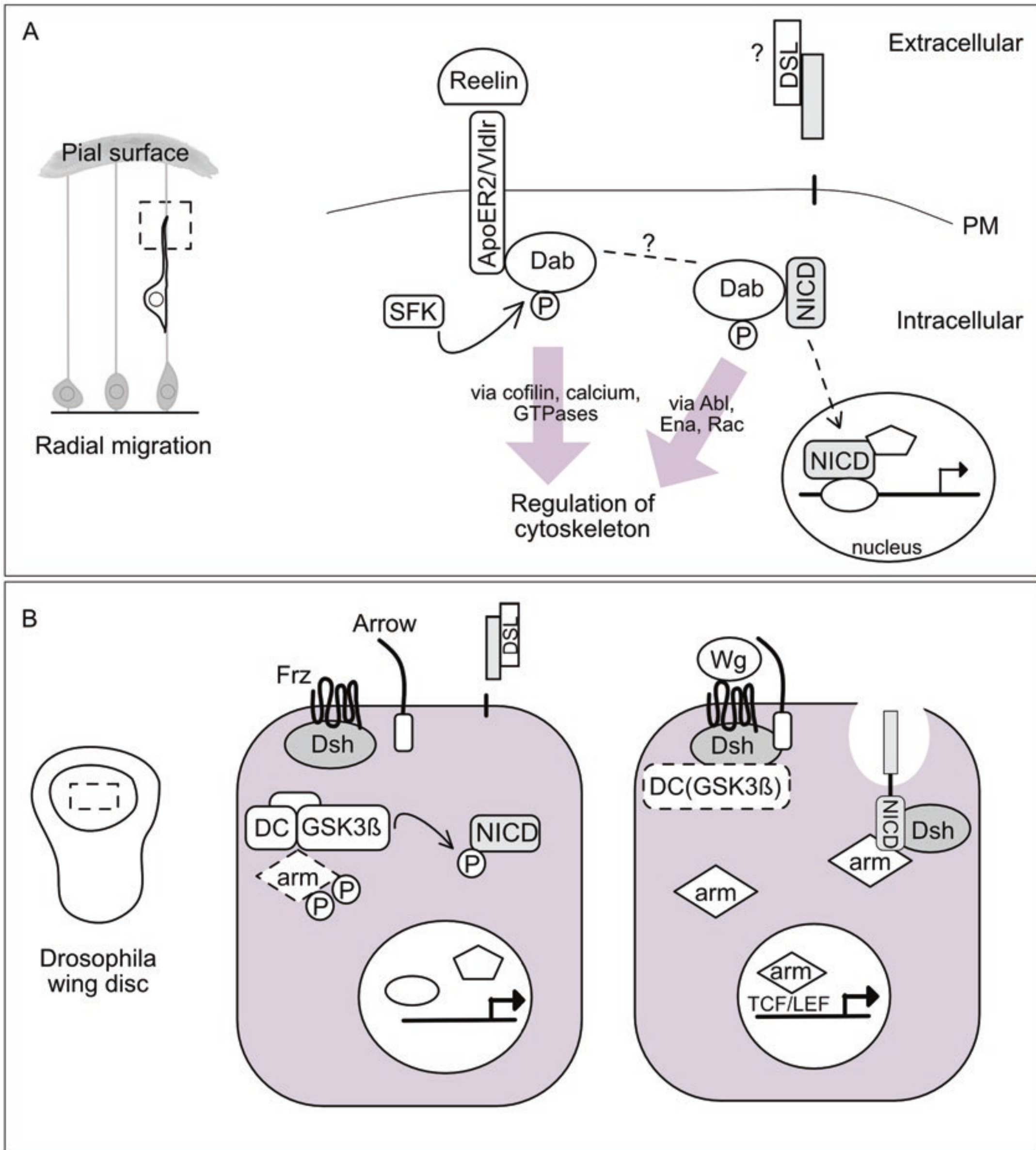

Fig. 4.4 Other noncanonical signaling mechanisms. (**a**) Radial migration of neurons in the developing mammalian brain cortex requires Reelin–Notch signaling. Reelin binds to its transmembrane receptors Vldlr and ApoER2, which leads to phosphorylation and activation of Dab by Src family kinases (SFK). Activated Dab has been shown to be upstream of several regulators of the cytoskeleton including cofilin, calcium signaling (via phosphoinositols) and GTPases. Dab is also bound to Notch, stabilizing ICD against degradation and thus stimulating expression of downstream NICD target genes. It is not known if the Dab population downstream of Reelin/SFK overlaps with the Dab population bound to NICD, and whether association with Notch modulates the interaction of Dab with Abl and its downstream signaling partners. (**b**) During pattern formation in the *Drosophila* wing disc, Notch can be inactivated by Dishevelled (Dsh) downstream of Wingless/Wnt signaling. In the absence of Wnt, beta-catenin (Arm, *Drosophila* armadillo) is hyperphosphorylated by the Destruction Complex (DC). This complex is made of many proteins, including the kinase GSK3β. GSK3β is known to phosphorylate NICD, leading to changes in protein stability. In the presence of Wnt, Dsh is activated and, along with the coactivator, Arrow (mammalian LRP), promotes inactivation of the DC/GSK3β. This allows stabilization of cytoplasmic beta-catenin/Arm, as well as nuclear translocation of beta-catenin/Arm leading to changes in gene expression through interactions with cofactors, TCF/LEF. Dsh has also been shown to bind the NICD and promote Notch endocytosis, leading to downregulation of Notch signaling activity. Unphosphorylated beta-catenin/arm is also known to bind to NICD, which promotes beta-catenin/arm stabilization; it is not known what effect this has on Notch signaling

been well established (Franklin et al. 1999; Redmond et al. 2000; Šestan et al. 1999), as has the role of Reelin/Dab signaling (Bosch et al. 2016; Howell et al. 1999; Niu et al. 2008). It is not known whether Disabled-mediated noncanonical Notch signaling also contributes to the final patterning of the dendritic tree. Indeed, preliminary experiments in *Drosophila* suggest that Notch modulates dendrite branching of sensory neurons, and hint that it may do so with contributions from both nuclear and cytoplasmic Notch signaling mechanisms (M. Shivalkar and EG, unpublished observations).

Other Forms of Noncanonical Notch Signaling

Noncanonical Notch signaling, both in the sense of signaling not mediated by CSL-activated gene expression and signaling that locally controls cell morphogenesis and motility, does not exclusively involve interactions with Abl, Trio, and Dab. Here we briefly discuss three additional noncanonical mechanisms that have been proposed and their implications for neuronal development.

Wnt

Wnt is one of the key signaling pathways that regulate embryonic development. Canonical Wnt signaling hinges on the phosphorylation status and nuclear translocation of beta-catenin (*Drosophila* armadillo, Arm) (Peifer et al. 1994; Salic et al. 2000; Yost et al. 1996). Briefly, in the absence of a Wnt signal (*Drosophila* wingless, Wg; Fig. 4.4b), a destruction complex comprising several proteins, including the kinase GSK3β, phosphorylates beta-catenin/Arm, targeting the protein for ubiquitin-mediated proteasomal destruction. In the presence of Wnt/Wg, the Frizzled/Arrow/Dishevelled complex recruits and inactivates the destruction complex, which allows stabilization and accumulation of beta-catenin/Arm in the cytoplasm, followed by nuclear translocation of beta-catenin/Arm and its interaction with cofactors that are transcriptional regulators.

Several lines of evidence suggest that Wnt signaling intersects with the regulation of Notch signaling. The Martinez-Arias group has shown that many components of the Wnt signaling pathway bind to Notch or interact genetically with the Notch signaling pathway (Hayward et al. 2005; Muñoz-Descalzo et al. 2011, 2010; Sanders et al. 2009). In the developing wing disc, for example, the cytoplasmic Wnt effector protein Dishevelled binds to NICD and this interaction contributes to the endocytosis of full length Notch independent of ligand interactions. Axin and Apc, two additional proteins found in the destruction complex, also contribute to Notch trafficking: loss-of-function mutant clones for either Axin or Apc lead to delayed endocytosis of Notch from the cell surface. Together this suggests that Wnt signaling can act to downregulate Notch signaling. Conversely, endosomal recycling of Notch can downregulate Wnt signaling. Full length Notch binds to beta-catenin/arm, which leads to the downregulation of Wnt signaling via coendocytosis with Notch. GSK3β (*Drosophila shaggy*, *sgg*) is not required for Notch trafficking, but has been shown to phosphorylate and affect the stability of NICD in CSL-dependent transcriptional activation (Espinosa et al. 2003; Foltz et al. 2002).

The classic system for studying these interactions in the fly is the developing wing disc which has stereotypical tissue axes established by signaling networks including Wnt and Notch. However, Wnt signaling is also an important axon guidance mechanism, especially via alternate beta-catenin/arm-independent pathways: planar cell polarity (PCP) and Derailed (sometimes termed noncanonical Wnt signaling). Extracellular Wnts, especially Wnt4, promote directional growth of some axons (Lyuksyutova et al. 2003; Shafer et al. 2011; Zhang et al. 2007). Mutations in genes of the Wnt-mediated PCP pathway, which overlap in part with components of the canonical Wnt pathway, including Dishevelled and Frizzled, can lead to neuron migration phenotypes. PCP pathway output can

mediate changes in gene expression, but notably also affect the activity of several cytoskeletal regulators, including the Rho GTPases. Wnt5 can also mediate axon guidance though its interactions with the receptor tyrosine kinase Derailed (Keeble et al. 2006; Yoshikawa et al. 2003). In this context, Wnt5 binds to Derailed on growing axons to act as a repellent during development of the *Drosophila* CNS. This activity does not require beta-catenin/Arm. In summary, the Wnt signaling pathway, like Notch, independently regulates cell fate and axon guidance, and it displays bidirectional interactions with Notch during patterning of the *Drosophila* wing disc. It remains to be investigated whether these Wnt/Notch interactions also play a role in axon guidance.

Tor

Tor complex 1 (TORC1) is associated with cell growth and protein synthesis, while TORC2 is associated with regulation of the cytoskeleton via the activity of Rac GTPase (Grider et al. 2009; Jacinto et al. 2004). Independently, both TORCs have been shown to interact with noncanonical Notch signaling pathways toward the regulation of cell growth and apoptosis, as well as during neuronal stem cell differentiation (Lee et al. 2013; Perumalsamy et al. 2009). Both of these studies describe CSL-independent mechanisms by which NICD, after S3 cleavage, remains in the cytoplasm and interacts with TORCs and Akt in order to regulate the differentiation of neural stem cells or to inhibit apoptosis in cultured cells, respectively. Cell morphology, and in particular axon guidance, is also regulated by Tor signaling, downstream of PI3K/AKT and Ras signaling cascades (Laplante and Sabatini 2009). Both TORCs contribute to neuronal morphology in both developmental and regenerative contexts (Abe et al. 2010; Knox et al. 2007). However, it is unknown to what extent these aspects of TORC signaling rely on their noncanonical interactions with Notch.

STAT3

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway functions in a variety of developmental processes, especially those downstream of growth factor and cytokine signaling (Rawlings et al. 2004). Following receptor activation, a variety of STATs can be phosphorylated downstream of JAK, which promotes their translocation to the nucleus and changes in target gene expression. STAT3 in particular has been associated with a role in neurogenesis and axonal regeneration (Androutsellis-Theotokis et al. 2006; Cao et al. 2010; Hong and Song 2015; Leibinger et al. 2013). The cytokine interleukin 6 (IL-6) promotes the elongation of axons after injury in cell culture and in vivo, via the activation of STAT3. Interestingly, the IL-6/JAK/STAT3 signaling module has been shown to be regulated by a noncanonical Notch process in breast cancer cells (Jin et al. 2013). Jin et al. show specifically that cytoplasmic NICD is required for an increase in IL-6 production. Stimulation of IL-6 does not require the CSL binding domain of NICD, and is unaffected by mutations in CSL that abolish its ability to bind to DNA. This evidence suggests a role for noncanonical Notch signaling upstream of the IL-6/JAK/STAT3 signaling module, although it remains to be seen how widely applicable this relationship is, for example, whether it plays a role in promoting axon regeneration. Similarly, tyrosine phosphorylation of STAT3 can by induced by Notch, in response to DSL ligand, at a time scale that is much too fast to be accounted for by a transcription-mediated mechanism, again suggesting the action of a non-CSL process, possibly mediated through mTOR (Androutsellis-Theotokis et al. 2006)

Open Questions

Throughout this review, we have focused on examples of canonical and noncanonical Notch signaling, especially with respect to the role of posttranslational Notch regulation in neurogenesis

and axon guidance. Although there are numerous processes in morphogenesis that require canonical Notch signaling, generally it is unknown whether noncanonical signaling also participates in these processes. One example is that of branching morphogenesis, for example, the development of the vasculature and trachea. Both of these processes rely upon axon guidance molecules and receptors to coordinate the movement of cells as they establish branching patterns (Adams and Eichmann 2010). There is a clear role for canonical Notch signaling in promoting the growth of new branches, using a lateral inhibition-like mechanism to maintain tip vs. stalk cell identity (Guarani et al. 2011; Hellström et al. 2007; Llimargas 1999). This process takes input from VEGF and FGF signaling pathways, leading to changes in the behavior and morphology of the DSL-expressing tip cell, including the formation of actin-based structures like filopodia. The morphological changes that are observed bear striking resemblance to the changes that occur in axonal growth cones in the local, Abl-mediated response to Notch activation. However, although the similarities are suggestive, we do not yet know what role, if any, noncanonical Notch signaling plays in morphogenetic processes that require canonical Notch signaling. Part of the reason for this is because of the profound effects of Notch on fate and differentiation, which may mask local, noncanonical effects. When studying the role of Notch signaling in new development contexts, identifying canonical and noncanonical contributions requires separating the transcriptional and non-transcriptional effects. For example, it becomes necessary to test rigorously whether CSL is involved in a Notch-dependent process, in parallel with assaying mutations in NICD that selectively abolish relevant noncanonical interactions (e.g., Dab binding).

The coordination of gene expression with acute signaling events at the cell cortex is a feature of several of the core developmental signaling pathways; however, we often think of these in terms of a chain reaction, rather than a network of discrete events that can bifurcate at any point. Notch signaling is a prime example of how a single mechanism can bifurcate to achieve multiple consequences: in the context of developing neural circuits, nuclear NICD participates in the regulation of gene expression to establish neuronal identity, in parallel with which cytoplasmic NICD or full length Notch can participate in the regulation of the cytoskeleton, among other targets, to achieve coordinated effects on cell identity and morphology. Are these two mechanisms simply parallel features of the same process, generally speaking the cleavage of NICD and its movement away from the cell cortex? In that case, it may be reasonable to speculate that biological processes that have already shown a role for canonical Notch could be explored for additional, local, noncanonical effects. Further studies will be critical to determine how widely noncanonical mechanisms are applied in development, both by Notch and by other signaling receptors.

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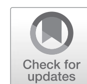

5 Maheshvara, a Conserved RNA Helicase, Regulates Notch Signaling in *Drosophila melanogaster*

Bhawana Maurya, Satya Surabhi,
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Abstract

Gene expression is regulated at multiple steps after generation of primary RNA transcripts, including mRNA processing, stability, and transport, along with co- and post-transcriptional regulation. These processes are controlled via the involvement of a multitude of RNA binding proteins (RBPs). Innumerable human diseases have been associated with altered expression of RNA binding proteins. In this chapter we have focused on *Maheshvara* (*mahe*) which encodes a putative DEAD box RNA helicase protein in *Drosophila*. We have recently reported that *mahe* plays an important role in regulation of Notch signaling. Fine tuning of Notch signaling is required at multiple steps and it's misregulation leads to a variety of human diseases. Additionally, mutation in *DDX59*, a human homolog of *mahe* results in broad neurological phenotypes associated with orofaciodigital syndrome. *Drosophila mahe* mutants show abnormal peripheral and central nervous system development that resemble neuropathology of patients having mutation in *DDX59* gene. This chapter will help in advancing the knowledge as to how *mahe* regulates Notch signaling and nervous system development.

Keywords

Maheshvara · Notch signaling · RNA binding protein · DDX59 · Deltex · Cut · Wingless

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Introduction

RNA binding proteins (RBPs) are involved in all aspects of mRNA biology. RBPs regulate numerous physiological processes like splicing, post-transcriptional editing, ribosome biogenesis, RNA transport, decay, and translation. RBPs along with their target RNAs form dynamic ribonucleoprotein complexes (RNPs). Thus, they influence the structure, biogenesis, transport, decay, and translation of RNAs. Many RBPs have well-defined RNA binding domains (RBDs) and bind to their target RNA by recognising specific RNA sequence and structure. RBPs are classified into different classes, based on the type of

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RNA binding domains, namely, RNA recognition motifs (RRMs), zinc-fingers, KH domains, DEAD-Box, Pumilio, and double-stranded RNA binding motifs (dsRBMs) (Cléry and Allain 2012). A single RBP may have multiple copies of the same RBD or a combination of different RBDs. These RBDs enhance RNA binding affinity as well as its specificity through the recognition of nucleotides within the RNA sequences. In addition to RBD, auxiliary domains and flanking regions also confer the ability of binding and assembly of RBP with RNA (Rudolph and Klostermeier 2015). Increasing evidence supports the fact that many of these RBPs can bind to DNA, along with assembling into extensive protein–RNA and protein–protein interactions. These interactions play important role in a variety of cellular processes.

RBPs play an important role during development through post-transcriptional regulation of their target RNA, that may start anytime between birth to death of an RNA. Using a wide variety of animal models, many RBPs have been identified as key factors needed during embryonic, germ line, and somatic cell development. RBPs are crucial during embryonic development as early embryonic development rely on the correct localization, translation, and polyadenylation of target RNA. *Drosophila* RBPs such as Oskar, Nanos, Tudor, Staufen, Piwi, and Vasa have been identified during early oogenesis; however, the precise role of only a few RBPs have been characterized. Vasa is essential for translation and localization of germ line specific mRNA, pole plasm assembly, and for proper oogenesis. Twister (Tst) regulates RNA localization and protein biosynthesis. Additionally, RBPs like Staufen and Dbp5 helps in RNA transport from nucleus to cytoplasm as well associates with the transcriptional machinery (Hodge et al. 2011; Noble et al. 2011). Belle (Bel), the *Drosophila* homolog of yeast Ded1 and human DDX3, plays an important role during oogenesis. Some of the RBPs like Maleless act as a coactivator, and Gemin3 acts as a corepressor during transcription. Ded1 in yeast has been reported to be a translation initiation factor and helps 40S ribosome to scan mRNA, it is also a part of RNP cofactor capping complex (Senissar et al. 2014; Gupta et al. 2018). Kurz DEAD box protein which is similar to DHX37 regulates nuclear pre-mRNA splicing. Pasilla (Ps), which is similar to the RNA binding protein NOVA2, regulates nuclear mRNA splicing. Bicaudal, a translational regulator, plays a vital role during oogenesis (Gamberi et al. 2006).

During development, a number of signaling pathways are required for a wide variety of processes like cell-type specification, cell division, pattern formation, and survival. Further, RBPs regulate gene expression during different signaling pathways. Some of the well-known signaling mechanisms in *Drosophila* are the bone morphogenetic protein/transforming growth factor (BMP/TGF) pathway, Wnt pathway, c-Jun N-terminal kinase (JNK) pathway, Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) pathway, activating receptor tyrosine kinases (RTKs), and the Notch signaling pathway. Among the various signaling pathways, Notch signaling is an evolutionary conserved cell communication system which has been studied for nearly a century. The *Notch* mutant in *Drosophila* was identified in 1913 by Thomas Hunt Morgan's group based on its wing notching phenotype (Morgan 1916). Additionally, *Notch* null mutants show embryonic lethality and "neurogenic" phenotype due to defect in lateral inhibition during neurogenesis, along with wing vein and mechanosensory bristles phenotype (Lindsley and Zimm 1992; Lehmann et al. 1981). Studies of Notch gene revealed that it encodes a large transmembrane receptor-like protein (Wharton et al. 1985, 1985; Kidd et al. 1986; Artavanis-Tsakonas et al. 1983; Artavanis-Tsakonas and Muskavitch 2010). *Drosophila* model provides a very good framework for studying various signaling pathways including Notch signaling. The information thus obtained was then validated in various organisms including Human. Fly genome contains a single gene encoding Notch receptor, while worm and humans have two (*lin-12* and *glp-1*) and four (*NOTCH notch1–4*) loci respectively. Notch signaling controls various processes such as lateral inhibition, lineage decision, differentiation, proliferation, and apoptosis in multiple tissues needed at almost all developmental stages.

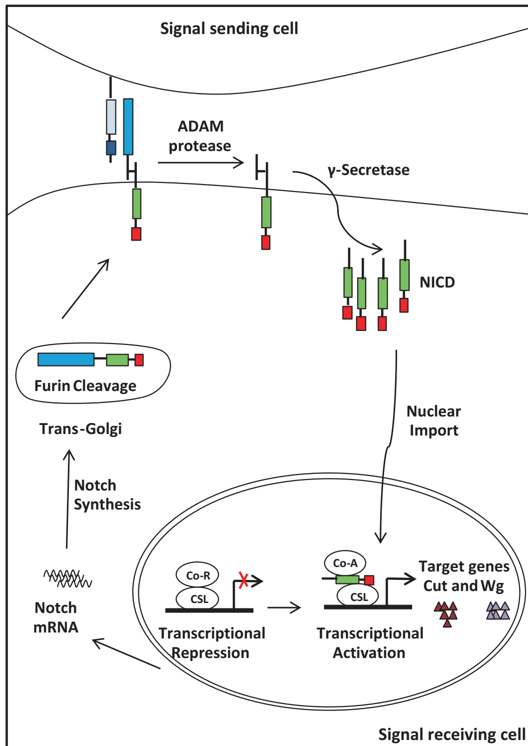
Mutation in various members of Notch signaling pathway leads to developmental defects and diseases like T-ALL (T-cell acute lymphoblastic leukemia), aortic valve disease, Alagille syndrome, CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), mucoepidermoid carcinoma, secondary acute myeloid leukemia, and spondylocostal dysostosis (Conkright et al. 2003; Enlund et al. 2004; Weng et al. 2004; McDaniell et al. 2006; Simpson et al. 2011; Joutel et al. 2004).

The core Notch pathway consists of Notch, a cell surface receptor which is expressed as a processed heterodimer. Notch protein is synthesized in ER and is trafficked to the Golgi body. During trafficking through Golgi complex, full-length Notch receptor, which is synthesized in the signal-receiving cell, undergoes Furin-dependent cleavage (S1 cleavage) in Notch extracellular domain (NECD) (Logeat et al. 1998). During Notch synthesis and secretion, the receptor undergoes posttranslational modification like O-linked glycosylation and O-linked fucosylation. These modifications are needed for proper folding and interaction of canonical Notch receptor with its ligands Delta and Serrate (Rana and Haltiwanger 2011). Signal-sending cells express ligands like Delta and Serrate which interact with the Notch receptor located on apposing cells. Following the ligand and receptor binding a second Notch Extracellular Domain (NECD) cleavage (S2 cleavage) is initiated by a metalloproteinase Kuzbanian in *Drosophila*. These result in release of majority of the extracellular domain leaving a membrane bound portion of Notch receptor. This is further followed by S3 cleavage comprising γ-secretase complex that contains Presenilin and leads to subsequent release of the Notch intracellular domain (NICD) from the membrane (Struhl and Greenwald 1999; Brou et al. 2000). The released NICD then translocates to the nucleus and forms transcriptional complex with a CBF1/Suppressor of Hairless/LAG-1 (CSL), Mastermind (Mam) family of DNA binding protein and initiates transcription of Notch target genes. In the absence of NICD, the corepressors remain bound to the CSL complex and suppress transcription of Notch target genes (Bray 2006; Kopan and Ilagan 2009).

Over the past several years multiple studies have reported noncanonical pathways for Notch signaling. Noncanonical Notch signaling may or may not require involvement of CSL and can be Notch ligand independent. In vivo studies in *Drosophila* have shown that even in the absence of ligand or CSL complex, Notch inhibits the selection of muscle progenitors from mesoderm in Notch loss-of-function mutants (Rusconi and Corbin 1998). Another study shows γ-secretase-independent noncanonical Notch signaling in postmitotic neurons which is involved in synaptic protein expression. As Notch signaling plays important role in nearly all the tissues it has to be regulated at multiple levels such as recycling, trafficking and degradation by different proteins. In the next section we shall focus on the role of RNA binding protein in Notch signaling (Fig. 5.1).

RNA Binding Proteins in Notch Signaling

A number of genetic screens have unraveled RNA binding proteins which play significant role during Notch signaling. Although the precise mechanism through which they interact or regulate Notch signaling is yet to be explored. Control of gene expression is a highly regulated program which is determined by combination of regulatory factors and post-translational modification of the RNA transcripts. Post-transcriptional control includes splicing, polyadenylation, localization, stability, and degradation. RNA binding proteins in a variety of combinations regulate the post-transcriptional gene regulatory network. For instance, *hiiragi* (*hrg*), a gene encoding poly(A) polymerase (PAP), regulates Notch at the level of mRNA processing at the 3′ end. Mutations in *Drosophila hiiragi* shows notched wing phenotype similar to that of Notch mutant phenotype (Murata et al. 2001; Juge et al. 2002). Pumilio RNA binding proteins in human, PUM1 and PUM2, regulate mRNAs of a number of protein-coding genes as well as noncoding RNAs. PUM also regulates Notch signaling at different levels by repressing ligands JAG1 and JAG2 (Serrate in *Drosophila*). Adam10 metalloproteinase which



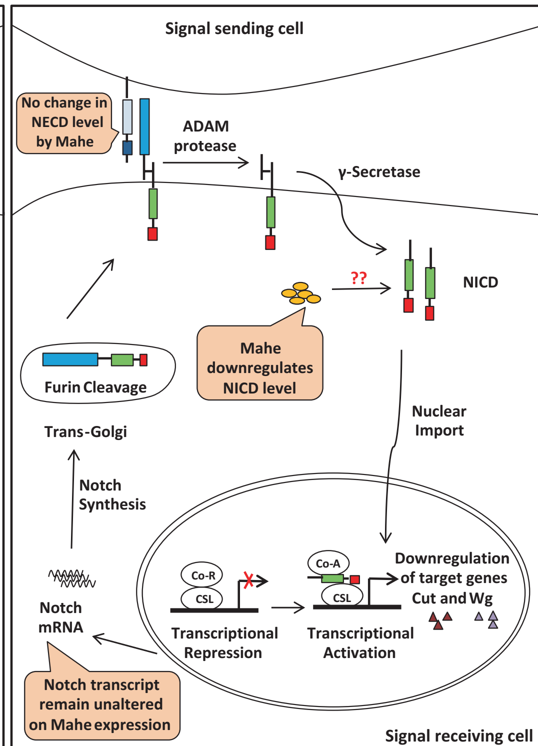


Fig. 5.1 (**a**) Notch is synthesized in endoplasmic reticulum followed by its processing in Golgi complex and is then transported to the membrane. Notch receptor interacts with the ligand of apposing cell and undergoes series of cleavages. ADAM protease removes the NECD by cleaving at site 2 (S2), γ-secretase then cleaves Notch within transmembrane domain at site 3 (S3) to release NICD. NICD translocates to the nucleus and binds to CSL and the activating complex leads to transcriptional activation of downstream target genes like Cut, Wingless, and Vestigial. (**b**) Overexpression of Mahe results in alteration of Notch signaling. However, Mahe does not change the levels of Notch transcripts or NECD upon its ectopic expression. Interestingly, Mahe overexpression reduces the level of NICD which results in downregulation of target genes Cut and Wg

cleaves Notch receptor, during angiogenesis represses THEM100, a Notch transmembrane component (Penton et al. 2012; Saftig and Lichtenthaler 2015). Factors which are activated by PUMs include Mastermind-like protein 1 (MAML1), a transcriptional coactivator protein, and a DNA-binding transcription factor Dp-2 (TFDP2) (Ribeiro and Wallberg 2009). Overall, PUM broadly affects Notch signaling pathway and Notch mediated processes. Split End RNA binding protein (SPEN) family members, SPEN, RBM15, and RBM15B, play important role in regulating Notch signaling during hematopoiesis spanning across *Drosophila* to mammals (Su et al. 2015). IGF2 mRNA binding proteins (IMPs) harboring four conserved RNA binding domain bind to *oskar* and *gurken* mRNAs in oocyte and localize with them. Imp controls the timing of somatic follicle cell development in *Drosophila* ovary. It has been reported that *imp* mutant delays follicle cell differentiation and formation of columnar epithelium at the time of oocyte development. These *imp*mutants are epistatic to *delta* mutants. Also, *imp* mutant phenotype has been shown to be rescued by overexpression of Kuzbanian, that carries out a metalloprotease-mediated S2 cleavage of Notch receptor. It has been shown that *imp* regulates timing of egg chamber development by regulating Notch signaling temporally and spatially (Fic et al.

2019). Another RNA binding protein Musashi (MSI), an evolutionary conserved family of RBPs first identified as a regulator of asymmetric cell division in *Drosophila* (Okano et al. 2002, 2005; Nakamura et al. 1994) too has been implicated in regulation of Notch. Mammals have two members MSI1 and MSI2 which play role in several processes like apoptosis, differentiation, cell-cycle regulation and stem cell maintenance during nervous system development and other developmental processes. Mammalian homolog of MSI1 suppress translation of Numb protein which antagonizes Notch signaling by binding to it's mRNA, thus promote Notch signaling (Spana and Doe 1996; Shen et al. 2002). Interestingly, proteasome degradation plays a role in inhibiting Notch signaling by degrading Notch-ICD; thus, downregulation of 26S proteasome is expected to stabilize NICD. In case of cancer stem cells (CSCs), Musashi binds to mRNA of NF-YA which encodes a transcription factor required for proteasome subunit expression and thus helps in downregulation of 26S proteasome expression, leading to persistent Notch signaling (Lagadec et al. 2014). Okabe and group have shown that Musashi plays a role in asymmetric cell division by regulating *tramtrack69* (*ttk*), a repressor for zinc-finger transcription which alters Notch signaling in *Drosophila* (Okabe et al. 2001). *Drosophila hephaestus* gene encodes a putative RNA binding protein that regulates wing margin and wing vein pattern formation during wing tissue development. It attenuates Notch pathway by altering Notch activity following Delta dependent ligand activation (Dansereau et al. 2002). In this chapter we will discuss in details about a novel RNA binding protein Maheshvara, that has been recently identified as a regulator of Notch signaling in *Drosophila melanogaster* (Surabhi et al. 2015).

Maheshvara a Putative DEAD Box Helicase

maheshvara (*mahe*) has been mapped to X chromosome at cytogenetic position 7C3 to 7C4. It comprises of five exons, which codes for two putative annotated transcripts*mahe-RB* and *mahe-RC* with a predicted size of 8.365 kb and 6.384 kb respectively (www.flybase.org). *mahe* codes for a 945-amino acid-long protein with a predicted molecular weight of 110 kDa. Mahe has orthologs in *Homo sapiens*, *Mus musculus*, *Caenorhabditis elegans*, *Danio rerio*, and *Saccharomyces cerevisiae* which have been identified based on their conserved domains. It is evolutionarily conserved in human *DDX5/DDX59*, worm *DDX17*, yeast *DBP2*, and fly *Rm62*, thereby indicating that it is highly conserved across taxa (Surabhi et al. 2015; Salpietro et al. 2018). Mahe protein encodes a highly conserved ATP-dependent RNA helicase with DEAD-box domain, Helicase superfamily 1/2 ATP-binding domain, Helicase, C-terminal, RNA helicase, P-loop containing nucleoside triphosphate hydrolase, and Q-motif (www.flybase.org). Based on crystal structure of PRP5 Swiss model 2.0 a variety of different motifs have been predicted, like Domain D1 and D2 which comprises of the protein structure of Mahe. The D1 and D2 domains consists of Q, I, Ia, Ib, Ic, II (DEAD) and III, IV, IVa, V, Va, and VI motifs, respectively. The name has been annotated as DEAD box RNA helicase since it includes Asp, Glu, Ala, Asp, or DEAD amino acid sequences in the motif II (DEAD). These motifs are vital for RNA binding, ATP binding as well as for intramolecular interactions with other RNA or proteins. Analysis of Belle, Vasa and Mss116p DEAD box RNA helicases have unraveled the various functions of motifs and the unwinding mechanism by theses RNA helicases (Sengoku et al. 2006). Vasa was the first RNA helicase to be identified, further its X-ray structural analysis had revealed that Vasa binds to a single stranded RNA resulting in a sharp bend in the RNA duplex. This bend in the duplex and ATP dependent activity of the D1 domain unwinds the RNA (Linder et al. 1989; Linder and Lasko 2006). Moreover, studies on yeast Mss1p DEAD box RNA helicase has shown that it can unwind the RNA duplex by local strand separation. Biochemical and structural analyses have unraveled that out of the two domains, D1 is responsible for ATP binding and D2 helps in RNA duplex recognition. Usually, the helicase

domain D1 and D2 are widely spread or remains in an "open" state form in the absence of any substrate, while the binding of substrate forms a closed state followed by RNA unwinding in an ATP-dependent manner. This mode of duplex unwinding by local strand separation is different from translocation based on duplex unwinding mechanism without affecting the RNA duplex globally. Mahe contains all the domains similar to that of other DEAD box RNA helicases indicating that Mahe might be acting through similar unwinding mechanism (Surabhi et al. 2015).

Expression of *maheshvara* During Development of *Drosophila*

RT-PCR analysis revealed tissue specific temporal expression pattern of *mahe* transcripts during different stages of development. Interestingly, *mahe* shows a dynamic pattern of expression in salivary gland, wing disc, eye-antennal disc, leg disc, and brain of larval tissues. Fluorescence in situ hybridization (FISH) depicted the spatial expression pattern of *mahe* transcript at different embryonic stages of development along with maternal transcript deposition during embryogenesis from stage 1 to 3. Additionally, it manifests strong neuronal expression during neuroblast development, specifically at stage 11 of embryonic development. Furthermore, *mahe* is expressed in the central nervous system (CNS) of *Drosophila* at stage 16 embryo. Neuroblasts are the neural stem cells of developing *Drosophila* brain which proliferate and differentiate to form diverse neurons and glia cells.

Immunohistochemical analysis revealed dynamic expression of Mahe protein in different tissues during development like in larval wing disc, eye disc, salivary gland, and brain. Interestingly strong expression can be seen in specific neurons of the ventral nerve cord and optic lobe of third instar larval brain. Similarly, *mahe* is expressed in the photoreceptor cells of eye-antennal disc, while in larval salivary gland Mahe shows prominent nuclear localization along with weak cytoplasmic expression. Mahe shows strong expression in the developing central nervous system as well as in peripheral nervous system. The dynamic expression of Mahe during *Drosophila* development and it's enrichment in the nervous system indicated it's significant role during development and neurogenesis (Surabhi et al. 2015).

maheshvara Overexpression Resembles Notch Loss-of-Function Phenotype

In order to understand the function of *mahe* on fly development, *mahe* was ectopically expressed in different tissues with the help of UAS/GAL4 system in fly. UAS-GAL4 is a transcription activation system derived from yeast. The GAL4 protein serves as the transcriptional activator, and it's expression can be regulated by any upstream enhancer/promoter. Fly lines expressing GAL4 results in tissue specific expression. The GAL4 transcription factor expressed under the desired promoter binds to (UAS) upstream activating enhancer sequence that harbors DNA elements recognized by GAL4 protein. Our gene of interest is tagged downstream of UAS sequence and for ectopic or overexpression it is crossed to the GAL4 driver line under tissue specific promoter. Upon crossing of the two fly lines, GAL4 protein binds to the UAS sequence and recruits the transcriptional machinery to the upstream site and induces downstream gene expression of the desired gene in targeted tissues. Using this approach *UAS-HA-mahe* flies, were generated and crossed with a variety of tissue-specific GAL4 driver lines. Overexpression of *mahe* driven by *engrailed-GAL4* and *Actin-GAL4* led to embryonic lethality. Additionally, ectopic expression driven by eye specific *eyeless-GAL4* resulted in significant reduction of eye-antennal disc along with massive reduction in eye size in both male and female adult flies. Similarly, there was massive reduction in overall size of salivary gland along with its nucleus upon *mahe* overexpression with *patched-GAL4*. Interestingly, *mahe* overexpression with *patched-GAL4* along the anterior–posterior (A/P) boundary of the wing disc results in wing notching phenotype together with

reduced distance between the veins L3 and L4, which resembled Notch loss-of-function phenotype. Furthermore, balding phenotype or loss of sensory bristle was also seen. All these phenotypes phenocopies Notch-loss-of-function phenotype, suggesting the role of *mahe* in regulation of Notch signaling in *Drosophila* (Surabhi et al. 2015).

Genetic Interaction of *mahe* with Notch and *deltex* Alleles

Genetic interaction of *mahe* with mutant alleles of genes involved in the Notch signaling pathway further supported the prediction of the influence of *mahe* on Notch signaling. Trans-heterozygous combination of N^{54l9} a null allele of Notch and *mahe* overexpression resulted in 100% reduced eye phenotype. Coexpression of Mahe along with NICD (Notch intercellular-domain) rescued the proliferation caused by Notch (NICD) overexpression in eye-antennal disc, suggesting the antagonistic role of *mahe* in Notch signaling. Additionally, *mahe* interacts with *deltex* (*dx*) a cytoplasmic protein which is required for fine tuning of Notch pathway outcomes. Deltex regulates Notch signaling both positively and negatively and helps in transport of Notch receptor from early endosome to lysosome (Hori et al. 2011; Wilkin et al. 2008; Yamada et al. 2011; Matsuno et al. 1995; Mukherjee et al. 2005). Further, dx^{152} a loss-of-function allele of *deltex* showed suppression of *mahe* overexpression phenotype in 32% flies. Ectopic *deltex* expression by *C96-GAL4* along the wing margin results in mild bristle loss. Coexpression of both *mahe* and *deltex* with *C96-GAL4* results in wing nicking phenotype, indicating that *mahe* and *deltex* genetically interact to downregulate of Notch signaling. Moreover, Mastermind (*mam*) a transcriptional coactivator in the Notch pathway when coexpressed with *mahe* enhanced wing notching and serration phenotype, when compared to that of overexpression of Mam dominant negative form alone along the wing margin. Similar to *mahe* overexpression phenotype, hypomorphic alleles of *mahe* also shows wing notching phenotype in combination with N*54l9* allele of Notch. Thus the genetic interaction studies clearly signifies the role of *mahe* in regulation of Notch signaling (Surabhi et al. 2015).

Ectopic Expression of *mahe* Leads to Downregulation of Notch Signaling

Notch signaling operates in many cell types and at various developmental stages. The classical Notch signaling plays a role in lateral cell inhibition during mechanosensory bristle development from sensory organ precursor (SOPs) where it restricts cell fate. Notch signaling has important role in formation of wing margin at the time of *Drosophila* wing development. It has been reported that active Notch signaling is needed for proper expression of Wingless at the wing margin (Matsuno et al. 1995; Mukherjee et al. 2005). Similarly, at D/V boundary of wing disc, Notch signaling is required for expression of Cut and Vestigial the downstream targets of Notch. Synergistic action of these targets, Wingless, Cut and Vestigial ultimately regulates the wing cell proliferation and margin formation at the D/V boundary in wing. Ectopic expression of *mahe* along the D/V boundary leads to wing notching phenotype. Additionally, loss of Cut and Wingless upon *mahe* overexpression in wing disc, shows downregulation of Notch signaling.

Notch protein expression was also examined along the D/V boundary, as its downstream targets Cut and Wg were downregulated. Further, Notch protein expression was checked using anti-Notch antibody raised against NICD in wing imaginal disk. Significant reduction in the amount of endogenous Notch levels was exhibited by ectopic *mahe* at the D/V boundary of wing imaginal disc as compared to neighboring wild type cells. In contrast, ectopic *mahe* did not bring about any change in the levels of full length Notch protein as detected by antibody raised against NECD. Thus, we concluded that full-length Notch receptor remains unaltered upon *mahe* overexpression.

Notch levels were also checked upon coexpression of both *mahe* and *dx*. Since ectopic expression of both the genes results in wing nicking phenotype, levels of Notch downstream target Cut was examined. Cut expression was massively lost along the D/V boundary of wing disc in comparison to that of *deltex* alone, which exhibits ectopic expression of Cut. This clearly depicts that *mahe* together with *deltex* negatively regulates Notch signaling outcome (Surabhi et al. 2015).

Maheshvara Is Associated with Nervous System Development

Mahe is evolutionary conserved across various species ranging from yeast to human. It has been recently reported that *DDX59* is the human ortholog of *Drosophila* Mahe. Whole-exome sequencing (WES) was performed on an Italian family reported of having Oliver syndrome (OS; MIM# 258200) (Salpietro et al. 2005) along with orofaciodigital syndrome and postaxial polydactyly (PAP). Additionally, the affected children showed neurological abnormalities which included seizure and delay in developmental milestones along with microcephaly and cognitive impairment. WES revealed homozygous frameshift deletion in *DDX59* (c.185del: p.Phe62fs*13) as a causal variant for the disease pathogenesis. A separate study showed involvement of *DDX59* gene in orofaciodigital syndrome type V with PAP. This study further revealed the role of *DDX59* gene in midline development of nervous system. Interestingly, bioinformatics prediction revealed that single base deletion can result in nonsense mRNA decay (NMD) or early truncation of protein. Real-time PCR from affected and unaffected controls did not led to complete NMD, instead the levels of mutant cDNA transcripts were lowered in cell lines in comparison to that of the wild type control. Moreover, the c.185del of a single T nucleotide exhibits a frameshift at position 62 amino-acid residue. This frameshift results into a truncated protein due to generation of premature stop codon leading to deletion of evolutionary conserved motifs such as PTRELA, TPGR, and DEAD and thus alters the protein structure. Interestingly, *DDX59* expression was found in human central nervous system indicating it's role in nervous system development. In order to better understand the underlying disease pathogenesis, *Drosophila* model was used to examine whether *mahe*, the *Drosophila* ortholog of human *DDX59* is crucial for proper central nervous system development and maintenance of normal life span.

As observed earlier, *mahe* is expressed in the developing nervous system suggesting it's role in neurogenesis. To further test this hypothesis and to examine its connection with disease pathology, *mahe* loss-of-function mutants were examined for neuronal defects. Immunostaining was carried out with anti-Elav antibody since it marks differentiated neurons and 22C10 or Futsch which stains neurons and axonal projections. Paralleling the symptoms exhibited by patients, *mahe* mutants too showed significant embryonic defects in both peripheral nervous system (PNS) and developing central nervous system (CNS). In *mahe* loss-of-function mutants, the commissures were underdeveloped or fused, resulting in widening of distance in CNS between the longitudinal connectives in comparison to that of the wild-type CNS. In addition to this embryonic CNS appeared disconnected. Moreover, mutant embryos also exhibited midline longitudinal axon disorganization, along with incomplete ventral cord, all of which resembled the phenotype associated with ciliopathy-associated syndromes. Moreover, survival assay was carried out using homozygous hypomorphic allele $mahe^{EP1347}$ and viable mutant flies exhibited shortened life span in comparison to that of the wild-type flies. These studies using mutant *mahe* clearly depicts the vital role of this novel RNA helicase in proper neuronal development as well as for maintenance of normal life span in *Drosophila*, which parallels patient pathogenesis. Thus, functional analysis using *Drosophila* model clearly signifies the role of genetic variants identified in *DDX59* as the pathogenic disease causing mutation affecting the children in the Italian family (Salpietro et al. 2018).

Summary

Notch signaling is an evolutionary conserved pathway that plays fundamental role during development of multicellular organism. Notch pathway mediates juxtacrine signaling and regulates cell fate, cell differentiation and cell proliferation (Artavanis-Tsakonas et al. 1999; Kopan 2002). Genome wide screening studies have identified multiple modifiers and regulators of Notch signaling. Among these RNA binding proteins have been shown to regulate Notch pathway at multiple levels. It has been reported that a number of different RNA binding proteins like Numb, Musashi, and Pumilio modulates Notch signaling. In this chapter we have discussed in detail about a novel RNA binding protein Mahe in *Drosophila*, in addition to the already well-studied RBPs. We have seen that *mahe* regulates Notch signaling by downregulating the pathway. However, the exact mechanism through which *mahe* regulates Notch pathway is yet to be unraveled.

Studies indicate enrichment of *mahe* in developing nervous system of flies. Interestingly, loss-of-function *mahe* mutants exhibits shortened life span of flies with abnormalities in nervous system development. This suggests that DEAD box RNA helicase *mahe* is needed for development of nervous system and is vital for maintaining normal life span. However, the abnormalities shown by the patients resemble CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) syndrome, which is known to be caused by alteration in Notch signaling pathway. Studies have shown that mutation in *Notch3* gene cause CADASIL which usually affects highly conserved cysteine residues within the epidermal growth factor-like repeat domain, which is an extracellular part of the Notch receptor (Joutel et al. 2000). Similarly, *mahe* human ortholog of *DDX59* has been identified as a novel regulator of Notch signaling in *Drosophila*. This putative DEAD box RNA helicase is highly conserved across species further indicating that its function might be conserved. Thus we reinstate that Notch being a very vital molecule needs fine-tuned regulation at multiple levels and *maheshvara*/*mahe* is an additional component added to the ever growing list of Notch regulators.

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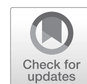

6 Molecular Regulation of Notch Signaling by Gremlin

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Abstract

Gremlin is a member of the TGF-β superfamily that can act as a BMP antagonist, and recently, has been described as a ligand of the vascular endothelial growth factor receptor 2 (VEGFR2). Gremlin shares properties with the Notch signaling pathway. Both participate in embryonic development and are reactivated in pathological conditions. Gremlin is emerging as a potential therapeutic target and biomarker of renal diseases. Here we review the role of the Gremlin–VEGFR2 axis in renal damage and downstream signaling mechanisms, such as Notch pathway.

Keywords

Gremlin · Notch · TGF-β · BMP · VEGFR2 · Renal damage · Fibrosis · Inflammation

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Gremlin-1

Description

GREM-1 gene was first identified and isolated under the name of *drm* (down-regulated in *mos*-transformed cells) in rat cells (Topol et al. 1997). GREM-1 sequence is highly conserved along evolution and the human gene GREM-1 has been mapped to chromosome 15q13–q15. The Gremlin-1 protein is formed by 184 amino acids (accession numbers: nucleotide NM_013372, protein NP_037504.1) and, similar to other TGF-β family members, presents a cysteine-rich region and a cysteine-knot motif. The predicted amino acid sequence analysis showed several potential residues for phosphorylation and glycosylation linked to the N-terminal, and signals for the nuclear localization at the C-terminal region. Although almost all these sites have been identified, the nuclear localization signals have not been confirmed yet (Mezzano et al. 2018) (Fig. 6.1).

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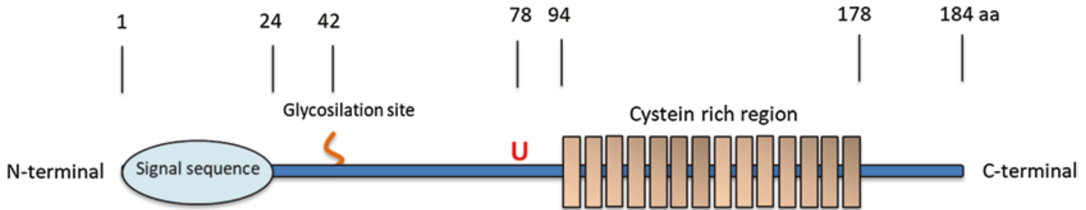

Fig. 6.1 Amino acid sequence of human Gremlin-1. Gremlin-1 is a 184 aa protein, which its principal characteristic is the cysteine region from 94 to 178 aa which is highly conserved along evolution. From the Nt to the Ct there are a signal sequence, a glycosylation site, ubiquitination site (U) and some phosphorylation sites between those (Mezzano et al. 2018)

TGF-β Superfamily

The superfamily of TGF-β comprises more than 40 evolutionary conserved members, including three TGF-β cytokines, four activins, four neurotropic factors, and 21 bone morphogenetic proteins (BMPs) and the BMP antagonists (Rider and Mulloy 2017). All these proteins share common features: (1) they exhibit the presence of a cysteine-knot module in their structure, (2) they are secreted as soluble proteins, (3) they are tightly regulated, and (4) several proteins bind to heparin and heparan sulfate polysaccharides, which retain them in the extracellular matrix where they act as paracrine cytokines in their soluble form (Rider and Mulloy 2017). Some authors guessed that the divergence in the differences in the number of cysteine knot motifs and their binding to heparin/heparan sulfate could be the explanation of the different functions they have within the family (Rider and Mulloy 2017). In this regard, the Cerberus or DAN (CAN) family act as natural antagonists of the BMP family, with 8 cystine knot motifs in this case. This family consists on Cerberus, Coco, Dan, Gremlin-1, Gremlin-2/PRDC, Sclerostin, and USAG-1. Among these antagonists, only Gremlin-1, Gremlin-2/PRDC, and Sclerostin bind to the polysaccharides heparin/heparan sulfate. This binding is not only significant for blocking the diffusion of the proteins, but also for being negative regulators of the BMP signaling pathway (Rider and Mulloy 2017).

Gremlin-1 as a BMP Antagonist

Gremlin-1 was described as an antagonist of the BMPs by David R. Hsu and colleagues in 1998 in a *Xenopus* model (Hsu et al. 1998). The BMP signaling pathway, their ligands and their antagonists, are conserved along the evolution, and they are involved in processes as important as limb generation and organ development, such as bone or kidney (Brazil et al. 2015; Walsh et al. 2010). The canonical BMP signaling pathway is activated when a dimer of the BMP ligands binds to the BMP receptor II, which binds and phosphorylates the BMP receptor I. Then, Smad 1, 5, and 8 are activated by phosphorylation and form a complex with the Co-Smad4. Both proteins are translocated to the nucleus acting as a transcription factor regulating the gene expression of some targets of the pathway, such as Smad6 (Fig. 6.2). The BMP antagonist proteins, such as gremlin-1, are extracellularly combined with the ligands thus impeding them to activate the BMP receptor (Brazil et al. 2015). Apart from the main role of this pathway during the embryo development, defects in the expression or function of these proteins cause disruptions in the adult tissue homeostasis (Walsh et al. 2010). In an elegant study by Khokha et al. (2003) they demonstrated that direct *grem-1* gene mutation in some mouse embryos caused aberrant limb patterning and digitization (Khokha et al. 2003). Moreover, the homozygous Gremlin-1 knockout mice are lethal after birth (Walsh et al. 2010). All these data highlight the fact that the expression

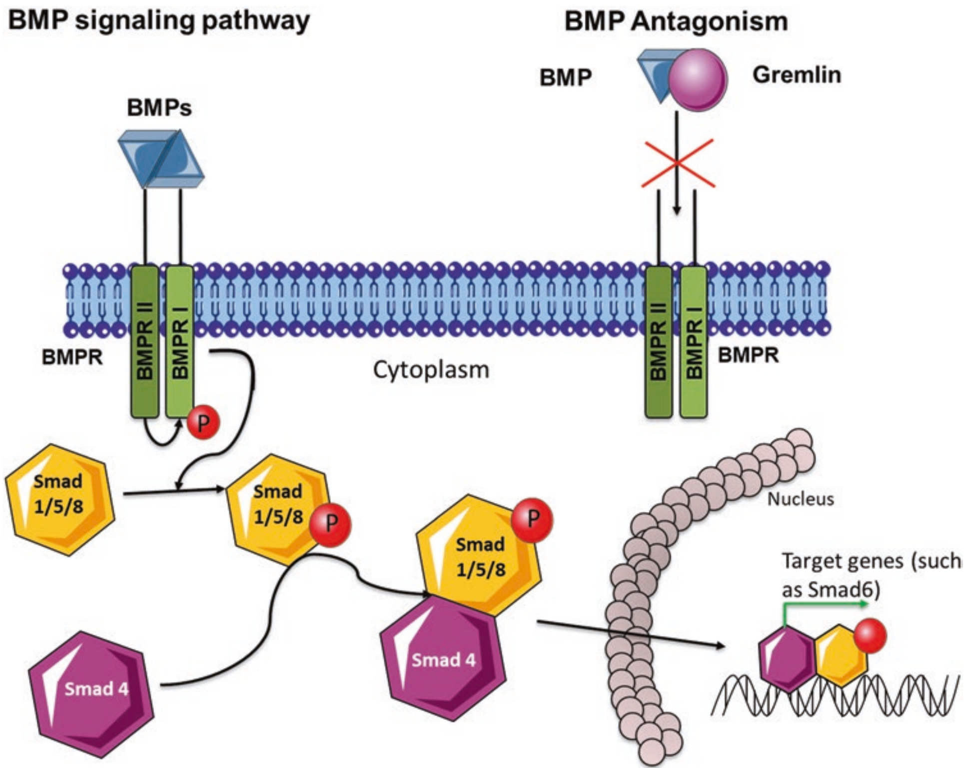

Fig. 6.2 Gremlin-1 is a BMP signaling antagonist. Briefly, a dimer of BMPs ligand binds to its receptor (BMPR) and triggers some phosphorylations leading to Smad 1/5/8 pathway activation. Gremlin-1 directly binds to BMP ligands, blocking the activation of this pathway (Brazil et al. 2015)

and normal function of Gremlin-1 is completely necessary in the embryonic development.

Gremlin-1 Induction Under Pathological Conditions

Under pathological conditions, the reexpression of development genes and key signaling pathways has been described. An early work discovered that Gremlin-1 expression was induced in rat mesangial cells incubated with high glucose or TGF-β and in the kidney of rats with streptozotocin-induced renal diabetic nephropathy (McMahon et al. 2002). Later, this group described Gremlin induction in human diabetic nephropathy (Dolan et al. 2005). Overexpression of Gremlin-1 has also been found in other pathological conditions (Erdmann et al. 2015). In human osteoarthritis, Gremlin-1 was found in chondrocytes from cartilage, but not in healthy chondrocytes, suggesting this protein as a possible therapeutic target for this disease (Tardif et al. 2004).

Some experimental studies assessed the aggravation of the renal damage due to Gremlin-1 overexpression. In an experimental mouse model of folic acid-induced nephropathy, the specific overexpression of Gremlin-1 in the tubules increased the renal damage, mainly mediated by upregulation of local proinflammatory and profibrotic factors (Droguett et al. 2014). Similar findings were showed in a Streptozotocin-induced diabetic nephropathy mouse model in which the specific tubular overexpression of Gremlin-1 associated to a further decrease of renal function, and podocyte damage (Marchant et al. 2015). In human diabetic nephropathy, there was a positive correlation between Gremlin-1 expression levels and tubulointerstitial fibrosis score, suggesting a deleterious role of Gremlin-1 in human renal diseases

(Dolan et al. 2005). Related to this, the heterozygous deletion of Gremlin-1 in a diabetic kidney disease model decreased some renal diabetic markers, as well as fibrotic and renal damage indicators when compared to the wild-type group (Roxburgh et al. 2009).

Role of Gremlin-1 in Fibrosis and EMT

Fibrosis is characterized by an excessive accumulation of extracellular matrix proteins (ECM), such as collagens and fibronectin, which are overexpressed by some cells, mainly activated myofibroblasts. This fibrotic response is naturally produced by the organism in physiological processes. However, when the fibrotic response is exacerbated, it could induce tissue damage and function failure (Lee and Kalluri 2010). Our group discovered that Gremlin-1 increased the expression of stimulated profibrotic factors and the production of ECM components including CTGF, fibronectin, collagen and PAI-1 in murine renal fibroblasts in vitro (Rodrigues-Diez et al. 2013) (Fig. 6.3). Other in vitro studies have found that Gremlin-1 can regulate ECM production in different cell types (Mezzano et al. 2018). However, the direct effect of Gremlin-1 in fibrotic process has not been completely demonstrated.

The origin of myofibroblasts in the fibrotic process varies among tissues and pathological conditions and includes resident fibroblasts, pericytes, bone marrow derived cells, fibrocytes and cellular transformations from epithelial or endothelial cells, mechanisms known as epithelial-to-mesenchymal transition (EMT) or endothelial-to-mesenchymal transition (EndoMT), respectively (Zeisberg and Kalluri 2013; Leaf and Duffield 2017).

In the kidney, partial EMT of tubular epithelial cells is now accepted as a process involved in renal damage progression. Partial EMT consists on polarized epithelial cells losing their phenotype and function, including permeability and polarity, associated to an aberrant senescence-related secretome, that includes pro-inflammatory and profibrotic proteins, all these changes contribute to the pathogenic of renal damage (Leaf and Duffield 2017). Studies in knockout mice for key EMT genes have demonstrated the role of this process in renal damage (Grande et al. 2015). The contribution of EMT to renal fibrosis is a matter of intense debate;

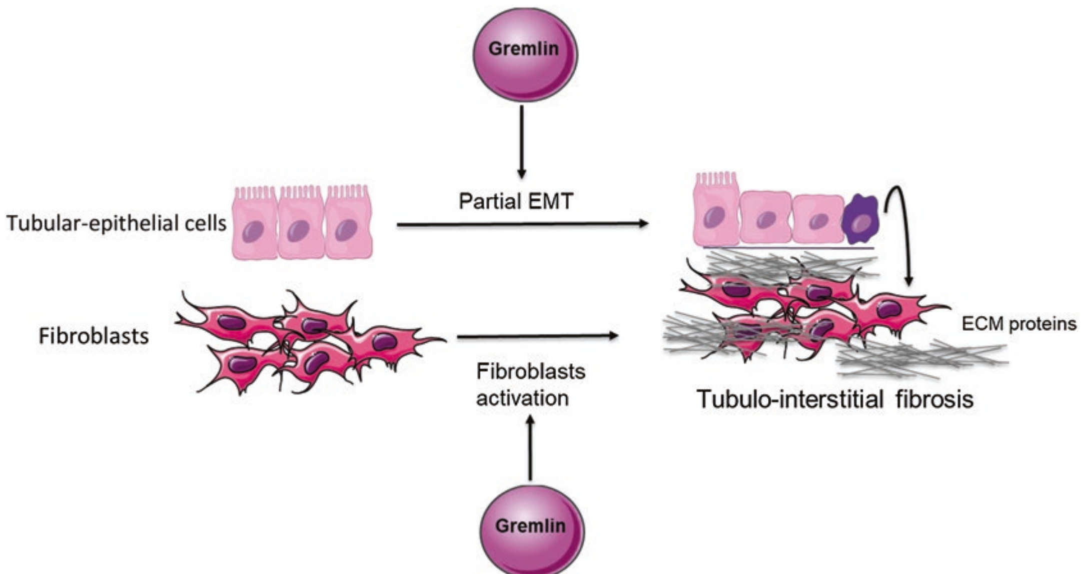

Fig. 6.3 Gremlin-mediated partial EMT and fibroblasts activation in the kidney. Gremlin-1 is known to produce partial EMT in epithelial cells, as well as activating local fibroblasts, thus producing an increase in extracellular matrix components (ECM) and subsequent tubulointerstitial fibrosis

however, EMT-related changes are an initial step in renal damage and an important potential therapeutic target.

Gremlin-1 induced the transformation of cultured human tubular epithelial cells from an epithelial to a mesenchymal phenotype, characterized by the induction of myofibroblast phenotype (like vimentin and α-SMA) and the loss of cadherins and cytokeratins (Rodrigues-Diez et al. 2013). In progressive human renal diseases Gremlin-1 overexpression was associated to loss of E-cadherin and increased α-SMA and correlates to tubulointerstitial fibrosis (Mezzano et al. 2018). These data suggest that Gremlin-1 induced EMT in tubular epithelial cells can contribute to renal damage progression (Fig. 6.3).

Importantly, in progressive human renal diseases we described Gremlin-1 mRNA and protein up-regulation at the glomerular crescents (Mezzano et al. 2007), and, more recently, we suggested that Gremlin-1 could be a novel urinary biomarker of ANCA-associated renal vasculitis (Droguett et al. 2019). The formation of cellular glomerular crescent occurs by the disruption of glomerular capillaries and subsequent immune infiltration of the Bowman's space. This inflammatory response can active the parietal epithelial cells to proliferate and change its phenotype to myofibroblast-like cells, inducing EMT, as well as the activation of other cells including monocytes/macrophages and fibroblasts, and therefore contribute to the crescent formation (Bariety et al. 2003). Our data showing Gremlin-1 expression in proliferating parietal epithelial cells and monocytes within the crescents support the hypothesis of a pathogenic role of Gremlin-1 in crescents formation (Droguett et al. 2019). However, future studies are necessary to confirm these results.

TGF-β1 is known as the main profibrotic factor, since regulates ECM production in fibroblasts and induces EMT phenotype changes in many cells (Meng et al. 2016; Derynck and Budi 2019), being the Smad3 signaling pathway the most important fibrotic pathway in many fibrotic-related disorders (Meng et al. 2016). In renal cells we have described a positive loop between Gremlin-1 and TGF-β1 leading to fibrosis (Fig. 6.4). In the short-term,

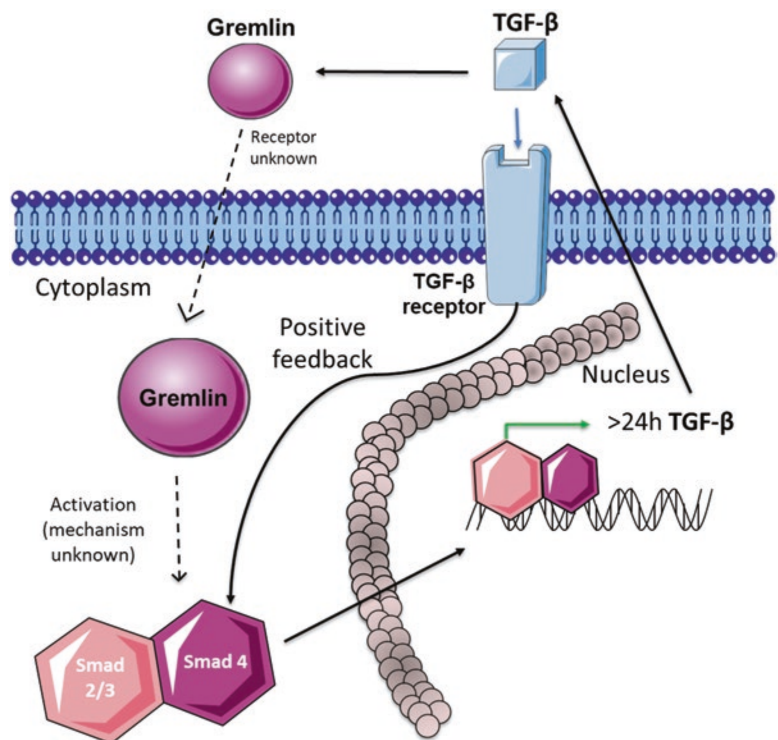

Fig. 6.4 Gremlin activates the TGF-β/Smad pathway. In the short-term**,** Gremlin is activating the Smad proteins in a TGF-β independent manner, leading to the transcription of several genes including TGF-β. In the long-term, TGF-β activation is maintaining this loop by increasing Gremlin-1 expression (Rodrigues-Diez et al. 2014)

Gremlin-1 induced the activation of the Smad signaling pathway in a TGF-β independent manner, whereas in the long term, TGF-β helped to maintain the Smad pathway activated and produced an overexpression of Gremlin-1 (Rodrigues-Diez et al. 2013, 2014) contributing to a profibrotic positive loop. Moreover, in vitro studies showed that Gremlin-1 gene silencing inhibited TGF-β1 responses and that treatment with a neutralizing TGF-β1 antibody inhibited Gremlin-1 fibrotic responses. A scheme of these mechanisms is shown in Fig. 6.4.

The role of Gremlin-1 as a downstream mediator of fibrogenic responses was not only assessed in renal cells and tissue, but also in an experimental mouse model of chronic pancreatitis (Staloch et al. 2015). In this model, authors demonstrated Gremlin-1 overexpression, as well as in human biopsies from chronic pancreatitis. Accordingly, the use of a heterozygous knockout of Gremlin-1 decreased the fibrogenic components in the chronic pancreatitis model. Moreover, the expression of Gremlin-1 induced by TGF-β was demonstrated in vitro as well (Staloch et al. 2015).

In idiopathic pulmonary fibrosis, Gremlin-1 overexpression promoted an exacerbated inhibition of BMP-4, which is known that produces a consequent epithelial degeneration leading to an increase in the subset of myofibroblast population (Koli et al. 2006).

Gremlin-1 Binds to the Vascular Endothelial Growth Factor Receptor 2

After the discovering of direct functions of Gremlin-1 in cultured cells, such as cell growth regulation, EMT, and angiogenesis, some groups were searching for a potential Gremlin-1 receptor. The group of Mitola described a new proangiogenic role of Gremlin-1, BMP-independent and mediated by direct Gremlin-1 binding to Heparan sulfate motifs (Stabile et al. 2007). Moreover, they assessed in vivo that stimulating chick embryos with Gremlin-1 caused more vascularization when compared to control groups. This effect was involving MAP kinases activation, such as ERK (Stabile et al. 2007). Later studies of this group discovered that Gremlin-1 directly binds to the Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) using a Surface Plasmon Resonance assay and described a functional binding of Gremlin-1 to VEGFR2 in cultured endothelial cells involved in angiogenesis (Mitola et al. 2010). The VEGFR2 activation elicited by Gremlin-1 was similar to that produced by its canonical ligand, VEGFA. These authors used the kinase VEGFR2 inhibitor called SU5416 to confirm this receptor activation by Gremlin-1. This finding remarked that Gremlin-1, apart from being a BMP antagonist cytokine, could directly activate cells via VEGFR2 signaling pathway. In addition, the VEGFR2-mediated Gremlin-1 proangiogenic effect was due to Gremlin-1 capacity to bind to Heparan sulfate motifs (Chiodelli et al. 2011). The downstream mechanisms involved in Gremlin-1/VEGFR2 effects in endothelial cells include the activation of the MAP kinases family as ERK1/2 and finally, the activation of the CREB protein, which translocates to the nucleus and provokes migration, tube formation and proinflammatory cytokine expression, which leads to an increase of the infiltrating inflammatory cells (Corsini et al. 2014).

After these findings, we demonstrated that VEGFR2 was a functional receptor of Gremlin-1 in the kidney (Lavoz et al. 2015). First, we described in cultured human renal epithelial cells that Gremlin-1 binds to VEGFR2 and activates its signaling pathway. Both VEGFR2 pharmacological blockade, by SU5416, and VEGFR2 gene silencing blocked Gremlin-induced cellular responses. Moreover, in vivo studies using a mouse model of direct Cy5-labeled Gremlin-1 to the renal parenchyma showed the maximal intensity of the fluorescent marker after 15 min of fluorescent protein injection located in proximal tubular epithelial cells and associated to VEGFR2 activation. Moreover, colocalization of Gremlin-1 binding and phosphorylated-VEGFR2 expression was

found in the same proximal tubular epithelial cells. Interestingly, the evaluation of renal biopsies of human progressive kidney diseases showed induction of Gremlin-1 expression associated to phosphorylated-VEGFR2 in the tubular epithelial cells (Lavoz et al. 2015), suggesting that activation of the Gremlin-1/VEGFR2 pathway participates in renal damage.

The Gremlin-1/VEGFR2 activation in the kidney was linked to the regulation of inflammation. Gremlin-1 in vivo and in vitro activated the NF-κB signaling pathway and upregulated proinflammatory genes leading to the recruitment of immune infiltrating cells, such as CD3+ cells and macrophages, in the kidney. The NF-κB pathway is a key regulator of inflammation (Sanz et al. 2010). In human and experimental kidney diseases, elevated renal NF-κB activity correlates with upregulation of proinflammatory parameters (Mezzano et al. 2004; Sanz et al. 2010). In the model of Unilateral Ureteral Obstruction (UUO), Gremlin-1 was overexpressed in the proximal tubular epithelial cells, and mice treatment with the VEGFR2 kinase inhibitor SU5416 blocked NF-κB activation and diminished renal inflammation (Lavoz et al. 2015). These mechanisms of Gremlin-1 in endothelial and epithelial cells are summarized in Fig. 6.5.

Gremlin-1 and Cancer

Related to EMT, some researchers have found that Gremlin-1 is involved in some types of cancer (Namkoong et al. 2006). In a mesothelioma tumor, Gremlin-1 overexpression was first assessed (Tamminen et al. 2013), and these authors observed a colocalization of this BMP

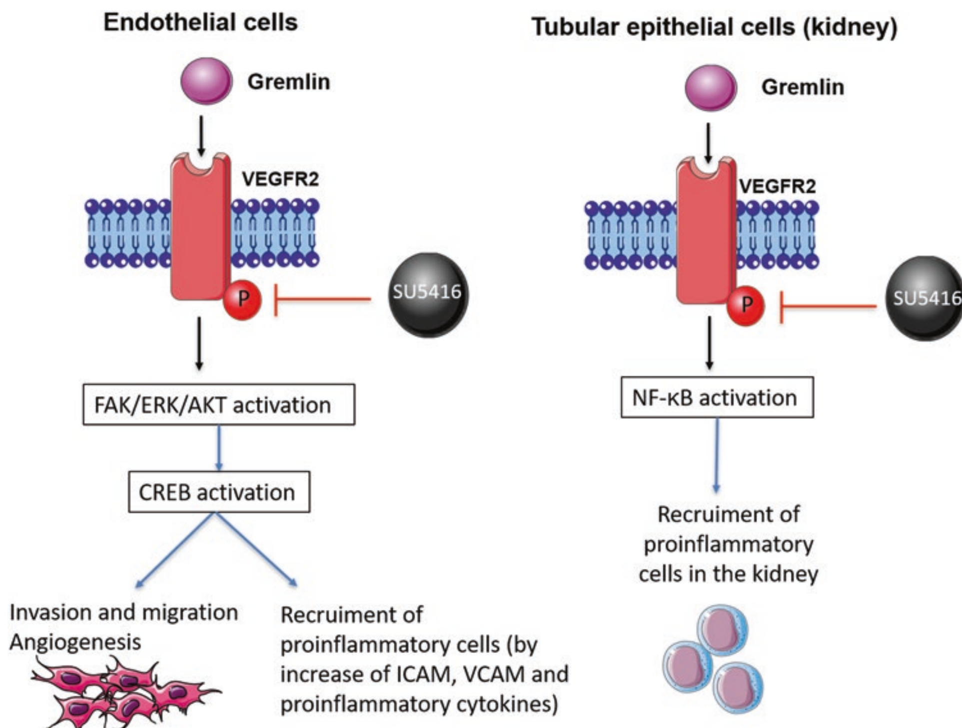

Fig. 6.5 Gremlin binds and activate VEGFR2 in endothelial and tubular epithelial cells. Due to its ability to bind and activate VEGFR2, Gremlin-a activates some intracellular kinases which provokes the translocation to the nucleus of CREB protein and the subsequent expression of migration, invasion and tube formation proteins, as well as proinflammatory cytokines such as ICAM and VCAM, in endothelial cells (Corsini et al. 2014). In tubular epithelial cells from kidney, Gremlin via VEGFR2 can induce the activation of NF-κB and this triggers the recruitment of proinflammatory cells in the kidney (Lavoz et al. 2015)

inhibitor with fibrillin microfibrils. After this, a direct relation of Gremlin-1 with migration and invasion in this type of mesothelioma cancer was found (Yin et al. 2017). They discovered a TGF-β dependent and independent action of Gremlin-1 in EMT, as well as more vascularization of the tumors. This remarks that Gremlin-1 in EMT is playing a role in fibrotic responses and in the invasion and migration of some tumors.

As commented before, Gremlin-1 binds and neutralize directly BMP-2, not only forming dimers but also oligomers, in order to inhibit the BMP signaling pathways (Kišonaitė et al. 2016). However, Gremlin-1, when acting as a covalent dimer, has been found to bind and activate VEGFR2, whereas acting as a monomer is an antagonist of this receptor (Grillo et al. 2016). This inhibition was tested with mutated and endogenous monomeric Gremlin-1 in vitro in tumor formation (Grillo et al. 2016). BMP and VEGFR2 independent actions of has been assessed in some types of tumors (Kim et al. 2012), thus guessing that other different receptors for this cytokine should exist, but they have not been established yet (Mezzano et al. 2018).

Notch Signaling Pathway

The Notch signaling pathway involves several receptors (Notch 1, 2, 3, and 4) and canonical (Jagged-1, 2 and Delta-like 1-3-4) and noncanonical (DLK1, 2) ligands. These are highly conserved during evolution, and their expression are needed for the correct formation of some organs and tissues, since this pathway regulates different cellular fates, such as differentiation, proliferation or apoptosis (Marquez-Exposito et al. 2018a). Mutations on these components are known to cause several syndromes and diseases, such as some arteriopathies and Alagille syndrome, among others (Marquez-Exposito et al. 2018a).

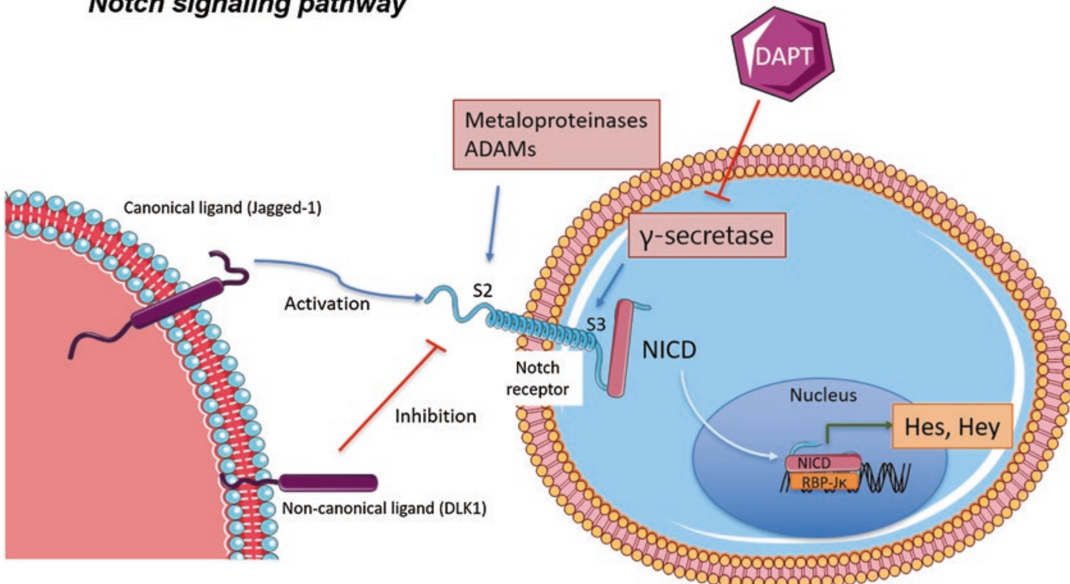

Fig. 6.6 Notch signaling pathway activation and inhibition. Some canonical ligands (such as Jagged-1) activate the route, whereas the noncanonical ligands (such as DLK1) inhibit it. Two proteolytic cleavages are needed in order to release the Notch Intracellular Domain (NICD) and translocate it to the nucleus, one mediated extracellularly by ADAMs metalloproteinases and one intracellularly by the γ-secretase. In the nucleus, NICD binds to the RBP-Jκ forming a complex and expressing the effector genes, Hes and Hey. The γ-secretase inhibitors, such as DAPT, blocks the activation of the pathway by inhibiting the second cleavage (Marquez-Exposito et al. 2018a)

The Notch signaling pathway is activated when a canonical ligand, as Jagged-1, binds to the receptor (Fig. 6.6). This triggers a cleavage by the ADAMs metalloproteinases in the external site 2 (S2) of the Notch receptor. After this, a second cleavage by the γ-secretase is needed in order to release the Notch Intracellular Domain (NICD) and be translocated to the nucleus. Here, the NICD forms a transcriptional complex with the coactivator RBP-jκ in the DNA and the effector genes of the pathway (HES, HEY) are expressed (Marquez-Exposito et al. 2018a).

Notch Signaling in Renal Development and Disease

The activation of this signaling pathway is necessary for the correct nephrogenesis during embryonary development and it is inhibited in the adult kidney. However, Notch pathway is reactivated when a renal damage occurs (Marquez-Exposito et al. 2018a). In some human renal diseases such as glomerulonephritis or diabetic nephropathy, among others, some components of the Notch pathway were overexpressed in renal progenitors, podocytes, and tubular cells (Murea et al. 2010). Interestingly, our group elucidated that in hypertensive nephropathy the Jagged-1/Notch-1 pathway was not being overexpressed nor activated (Lavoz et al. 2014), remarking that angiotensin II is not activating the Notch pathway in the kidney (Lavoz et al. 2012). Furthermore, the Notch-1 upregulation was related to albuminuria in glomerulosclerosis and with tubulointerstitial fibrosis when NICD was found active in tubules. Jagged-1 was observed to be the main activator of these responses, due to its overexpression (Murea et al. 2010). Therefore, some authors tried to use different approaches to inhibit Notch activation to ameliorate experimental kidney damage, including UUO and folic acid nephropathy, mainly by inhibiting fibroblast proliferation and, therefore, decreasing fibrosis (Bielesz et al. 2010; Han et al. 2017; Marquez-Exposito et al. 2018a). However, the inhibition of Notch pathway was not improving the renal injury in the acute phase of folic acid nephropathy (Wyss et al. 2017), thus suggesting that the blockade of this pathway is not effective in all types of renal failure.

Recently, the beneficial effect of Notch inhibition in models of experimental inflammation has also been described, including renal diseases (Cheng et al. 2015; Niewczas et al. 2019; Lavoz et al. 2018). In an experimental model of rheumatoid arthritis, the inhibition of the Notch pathway activation ameliorated the inflammatory response and the arthritis progression (Park et al. 2015). In an allergic asthma disease linked to an activation of the Th17 immune response, the treatment with the γ-secretase inhibitor blocked Notch activation and the subsequent Th17 inflammatory response (Zhang et al. 2015). The γ-secretase inhibitors are being evaluated in some clinical trials of different pathologies, including leukemia, melanoma, and Alzheimer's disease (NCT00594568, NCT00762411, NCT01193868, NCT01196416, NCT01981551). If positive results are obtained, Notch inhibition could represent a therapeutic option for the treatment of several diseases.

Relation Between Gremlin-1/VEGFR2 Signaling and Notch Pathway

Walsh et al. described in 2008 that Gremlin-1, Jagged-1 and Hes-1 possessed more similarities than expected. They share a common promoter region, conserved along evolution. Furthermore, they were all reactivated by TGF-β1 in tubular epithelial cells and overexpressed in the human diabetic nephropathy disease (Walsh et al. 2008).

Our research group was the first to demonstrate that Gremlin-1 is reexpressing Jagged-1 and thus, activating the Notch signaling pathway in human tubular epithelial cells and in mouse kidney experimental models (Lavoz et al. 2018). First, the expression of Jagged-1 by Gremlin-1 was studied in human tubular epithelial cells. Gene level expression of Jagged-1 started 3 h after the stimulation with Gremlin-1 and reached the peak at 24 h. Besides, Gremlin-1 also upregulated Notch-1 mRNA levels, and after 48 h a

nuclear NICD staining was observed by confocal microscopy, suggesting that Gremlin-1 was activating the Notch signaling pathway by upregulating Jagged-1 expression. Furthermore, this expression was BMP-independent but VEGFR2 dependent, since the addition of the BMP ligands did not modify the Jagged-1 expression whereas the addition of the VEGFR2 kinase inhibitor SU5416 diminished Jagged-1 production and NICD nuclear localization. The role of VEGFR2 in the regulation of the Notch pathway was confirmed by transfecting cells with a VEGFR2 siRNA (Lavoz et al. 2018).

The activation of the Notch signaling pathway was further demonstrated in vivo in a murine model of Gremlin-1-induced kidney injury. In Gremlin-1-treated mice, Jagged-1 overexpression was markedly found in the cytoplasm of tubular epithelial cells associated to a nuclear localization of NICD. Moreover, these cells also presented activation of VEGFR2 signaling. Importantly, the blockade of VEGFR2 by treatment of mice with SU5416 blocked the expression of Jagged-1 and the Notch signaling pathway activation, in several kidney damage models including UUO, suggesting that the Gremlin-1–VEGFR2 axis is involved in the Notch pathway activation in renal injury (Lavoz et al. 2018) (Fig. 6.7).

Functional Consequences: Inflammation

The functional consequences of Gremlin-1/VEGFR2/Notch activation in the kidney have been evaluated using a model of direct administration of recombinant Gremlin-1 in the mouse kidney. Gremlin-injected mice increased the Notch pathway activation linked to an increase in the inflammatory infiltration in the kidney. Treatment with the γ-secretase inhibitor DAPT did decrease the proinflammatory response observed in the Gremlin-injected mice as well as in the UUO mice. Moreover, tubular epithelial cells treated with DAPT and stimulated with Gremlin-1 diminished the mRNA expression of the proinflammatory cytokines. Interestingly, in human glomerulonephritis Gremlin-1 was expressed by infiltrating immune cells, including activated macrophages (Droguett et al. 2019). The activation of the NF-κB pathway by Gremlin-1 was reverted when mice where pretreated with DAPT. Moreover, the direct effect of DAPT in inflammatory events and NF-κB pathway was confirmed in vitro (Lavoz et al. 2018). Some evidences suggest a cross talk between Notch and NF-κB pathways. Studies in cancer cells showed that Notch regulates gene transcription of NF-κB components (Osipo et al. 2008; Schwarzer and Jundt 2011), and in endothelial cells, Jagged-1 induced the transactivation of adhesion molecules by physical interaction of NICD, and p65 NF-κB (Nus et al. 2016). Whether or not Gremlin-1 activation of the Notch signaling pathway could be involved in the regulation of inflammation in other pathological conditions should be evaluated.

Functional Consequences: EMT and Fibrosis

Our group suggested that Gremlin-1 was mediating EMT via VEGFR2/Notch activation in tubular epithelial cells (Marquez-Exposito et al. 2018b). The blockade of Notch with DAPT restored Gremlin-induced EMT. In a wound healing assay performed to evaluate the capacity of the cells to migrate and proliferate, Gremlin-1 showed increased migration and/or proliferation prevented by DAPT pretreatment (Marquez-Exposito et al. 2018b). Furthermore, we have assessed Gremlin-1 effects in proliferation through the study of the proliferation marker PCNA. In the Gremlin-injected mice, PCNA increased significantly and the treatment with SU5416 or DAPT reverted this upregulation (Lavoz et al. 2018), suggesting that Notch pathway through the activation of Gremlin-1–VEGFR2 axis is mediating proliferation (Fig. 6.7).

In the UUO model, treatment with the γ-secretase inhibitor DAPT or the VEGFR2 kinase inhibitor SU5416 showed an amelioration of renal fibrosis including lower fibrotic levels and collagen deposition (Marquez-Exposito et al.

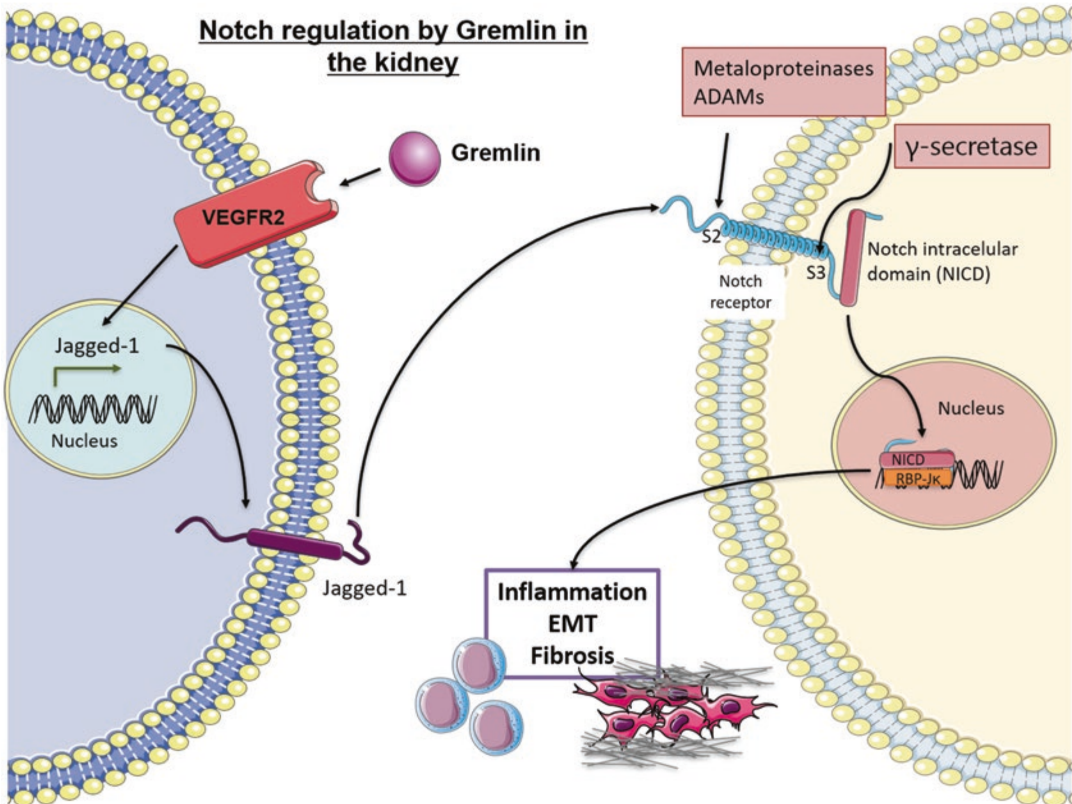

Fig. 6.7 Gremlin via VEGFR2 activates Notch pathway in the kidney associated to renal inflammation, EMT and fibrosis. Gremlin-1 through VEGFR2 activation produces the jagged-1 expression which is capable of activate Notch pathway in the kidney and disbalance the proinflammatory signals as well as the epithelial to mesenchymal transition and an increase in ECM deposition and subsequent fibrosis (Lavoz et al. 2018; Marquez-Exposito et al. 2018b)

2018b). However, the direct effect of Notch signaling pathway activation in the regulation of the ECM proteins has not been confirmed yet.

As a conclusion of this chapter, the Gremlin-1–Notch axis plays a significant role in the embryonary development as well as some adult tissue injury, such as in kidney failure. Nevertheless, more studies are needed in order to determine the intricate functions of these signaling pathways in the development and the adult homeostasis.

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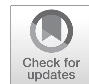

7 Regulation of Notch Signaling in *Drosophila melanogaster*: The Role of the Heterogeneous Nuclear Ribonucleoprotein Hrp48 and Deltex

Debdeep Dutta, Mousumi Mutsuddi, and Ashim Mukherjee

Abstract

Notch signaling is an evolutionarily conserved pathway that plays a central role in a number of cellular events during metazoan development. Due to its involvement in numerous developmental events, Notch signaling requires tight spatial and temporal regulation. Deltex is a cytoplasmic protein that physically binds to the Notch and regulates its signaling activity in a context-dependent manner. However, the biology of Deltex in regulation of Notch signaling is not well explored. For a better understanding of Deltex activity in the regulatory circuit of Notch pathway, a co-IP-based screening was performed. Hrp48, an RNA-binding protein, was identified as an interacting partner of Deltex in that screening. Interaction of these two proteins seemed to regulate the Notch signaling outcome in the epithelial tissue. Additionally, it was found that coexpression of Deltex and Hrp48 can lead to cell death as well as JNK activation. Considering the fact of well conserved nature of Notch as well as both of these two proteins, namely, Hrp48 and Deltex, this interaction can be helpful to understand the regulation of Notch signaling both in development and disease condition.

Keywords

Deltex · Hrp48 · Notch signaling · *Drosophila*

Overview of Notch Signaling

Notch signaling is essentially required for the regulation of a spectrum of cellular events like cell fate determination, cellular differentiation, stem cell maintenance, proliferation, and apoptosis during development of multicellular organisms (Artavanis-Tsakonas et al. 1995, 1999). This juxtacrine signaling circuit gets switched on by the binding of the ligand from one cell to the receptor of its neighboring cell. The outcome of Notch pathway can vary greatly depending on the signal strength and the cellular as well as developmental context. Any mutation affecting the Notch receptor or any of the Notch pathway component leads to alteration in the signaling outcome, and it results in a number of human diseases like Allagile syndrome, CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), neurodegenerative disorders, cancer, familial

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cardiomyopathy, and stroke (Penton et al. 2012; Mašek and Andersson 2017).

Notch is a single pass transmembrane protein (300 kDa) consists of two domains, namely, Extracellular domain (NECD) and Intracellular Domain (NICD). The NECD is responsible for ligand–receptor interaction, and the NICD acts as the signal transducer by functioning as an activator of the downstream genes. Mammals have four Notch isoforms (Notch1–4), while *Drosophila* has single Notch receptor (Andersson et al. 2011; Kopan and Ilagan 2009). However, all of the mammalian Notch proteins share a strong structural homology with that of the *Drosophila* Notch protein (Portin 2002; Bray 2006; Fiuza and Martinez-Arias 2007). The extracellular domain of Notch consists of 29–36 EGF (Epidermal Growth Factor)-like repeats and a LNR (Notch-related region in *Caenorhabditis elegans* LIN-1). These structural modules in the NECD help in proper activation of Notch receptor (Bray 2006; Fiuza and Martinez-Arias 2007). On the other hand, Notch intracellular domain is made up of a RAM (RBPjk association module) domain, an NLS (Nuclear Localization Signal), seven ankyrin repeats, and a C-terminal PEST (proline/glutamic acid/serine/threonine-rich) sequence (Artavanis-Tsakonas et al. 1995; Bray 2006; Fiuza and Martinez-Arias 2007).

In canonical Notch signaling pathway, the DSL (Delta/Serrate/LAG-2)-family ligands, which are expressed from the neighboring cell, bind to the ECD of Notch receptor to initiate the signaling cascade. There are five Notch ligands (Delta-like 1, 3, 4 and Jagged 1, 2) found in mammals, whereas in *Drosophila* only two ligands (Delta and Serrate) have been reported (Andersson et al. 2011; Kopan and Ilagan 2009). Similar to the receptor, mammalian DSL ligands share structural homology with their *Drosophila* counterparts. These ligands are type one transmembrane protein containing an N-terminal DSL motif, a DOS domain (Delta and OSM-11-like proteins), a tandem array of extracellular EGF-like repeats, and C-terminal PDZ ligand binding motif (Kopan and Ilagan 2009; Moretti and Brou 2013).

Primarily, Notch is synthesized as a single-pass precursor protein, and a series of post-translational modifications (PTM) makes it a functional dimeric receptor. The first PTM is glycosylation, more specifically fucosylation, which starts within the trans-Golgi network of the cell. Subsequently, a S1 cleavage by a furin convertase makes Notch a heterodimeric protein that remains attached together by non-covalent interactions (Tien et al. 2009; Logeat et al. 1998; Nichols et al. 2007). Finally, the receptor reaches to the cell membrane as a heterodimer to act as the active receptor. Once the ligand binds to the extracellular domain of Notch receptor, the receptor further undergoes an extracellular S2 cleavage by ADAM/TACE family proteases [Kuzbanian in *Drosophila* and ADAM10 and ADAM17 in mammals] (Lieber et al. 2002; Pan and Rubin 1997). This cleavage generates an intermediate membrane-tethered Notch receptor known as NEXT (Notch extracellular truncation) fragment. NEXT acts as a substrate for Presenilin group of proteases, which are responsible for the S3 intramembrane cleavage of the receptor (Strooper et al. 1999; Struhl and Greenwald 1999; Struhl and Greenwald 2001; Ye et al. 1999). As a result of S3 cleavage, membrane-tethered NICD is released from the membrane into the cytoplasm. Subsequently, NICD translocates into the nucleus of cell with the help of Importin α3 (Sachan et al. 2013). Within the nucleus, NICD forms a multiprotein complex via interaction with DNA-binding protein CSL [CBF1/Su(H)/LAG-1] and transcriptional coactivators like Mastermind (Mam) in *Drosophila*/Mastermind-Like (MAML) in mammals to initiate transcriptional activation of Notch targets genes, which transcribe for basic helix loop helix family of transcription factors (Lai et al. 2000). Recently, we have reported that a chromatin-modeling protein Hat-trick (Htk) is a component of Notch-Su(H) activation complex, and it positively regulates Notch signaling (Singh et al. 2019).

Additionally, non-canonical activation of Notch signaling by a molecule other than the DSL ligand(s) and/or a ligand that lacks a DSL motif has been reported. For example, CSL ligand-independent activation of Notch signaling was found to hamper differentiation of muscle progenitor cells from the mesodermal cells in

Drosophila (Rusconi and Corbin 1998). Another study conducted in *Drosophila* reported involvement of Hif-α in activating non-canonical Notch pathway during blood cell development and differentiation (Mukherjee et al. 2011). Recent views suggest a possible role of Deltex (Dx) in context-specific activation of non-canonical Notch signaling in *D. melanogaster* (Hori et al. 2011, 2012). Different signaling pathways, such as Wg and JNK signaling, profoundly influence CSL and/or ligand-independent Notch signaling in a non-canonical manner, thereby influencing a wide variety of developmental events in both *Drosophila* and mammals (Andersen et al. 2012; Arias et al. 2002; Zecchini et al. 1999).

Due to its strong influence in a variety of developmental events, Notch signaling is intricately regulated at multiple levels. For example, post-translational modifications of both ligand and receptor play an important role in regulation, activation, and maintenance of appropriate signal activity. A number of biochemical events, namely, proteolysis, glycosylation, phosphorylation, hydroxylation, and ubiquitination, take place at various steps from synthesis to maturation of the components as well as during successful signaling in order to maintain the proper signaling outcome (Fortini 2009).

Deltex, A Cytoplasmic Modulator of Notch Signaling

Similar to the *Notch*, *deltex* is another evolutionarily conserved gene. While human genome contains five *deltex* genes, namely, *DTX1*, *DTX2*, *DTX3*, *DTX3L*, and *DTX4*, *Drosophila* has only one *deltex* (*dx*) gene (Kishi et al. 2001; Matsuno et al. 1998; Mitsiadis et al. 2001; Thang et al. 2015). Due to this reason, it becomes easier to study the function of this gene in *Drosophila*. In *Drosophila*, the gene is present on the X-chromosome, and the flies carrying recessive viable alleles of *deltex* have scorable phenotypes in wing, eye, bristle, and ocelli in the adult flies (Gorman and Girton 1992; Xu and Artavanis-Tsakonas 1990).

In fact, the first *dx* mutation was identified as a suppressor of *Notch* mutations in *Drosophila*. Additionally, it was found that *deltex* interacts with *Delta* and *mastermind*, two other neurogenic genes that are involved in regulation of Notch signaling pathway (Xu and Artavanis-Tsakonas 1990). Thus, in early 1990s, genetic interaction studies suggested Deltex as a modifier of Notch-dependent cell fate specification event during development (Gorman and Girton 1992). Subsequent set of studies using null mutant allele of this gene established its role in cell and tissue-specific regulation as well as developmental regulation of Notch pathway (Fuwa et al. 2006).

Dx is an 82-kDa protein that consists of 737 amino acids. A significant presence of glutamine (11.26%), histidine (5.97%), and serine (11.94%) residues makes this protein basic in nature with a pI of ~9.8 (Busseau et al. 1994). In *Drosophila* embryonic and imaginal tissues, Dx is ubiquitously expressed in the cytoplasm (Busseau et al. 1994), where it colocalizes with cytoplasmic Notch protein (Diederich et al. 1994).

There are two Gly-rich OPA repeats present in Dx, and these repeats helps in a structural demarcation of broadly three domains in the protein (Fig. 7.1). Interestingly, the structural conservation of these domains of Dx is preserved in its mammalian homologs (Matsuno et al. 1998). First 1–303 amino acids of Dx make Domain I, which is responsible for its interaction with the N-terminal ANK-repeats of NICD. Domain II and III (306–737 amino acids) consist of a Proline-rich motif and a RING (Really Interesting New Gene)-H2 zinc finger motif (Matsuno et al. 1995). As Proline-rich motif helps in interaction with SH3 domain-containing proteins, it was predicted that Dx might also have interacting partner(s) with a SH3 domain. Subsequent experiment using yeast two-hybrid assay revealed that both human and *Drosophila* Dx can bind to a RTK (receptor tyrosine kinase) family protein named Grb2, which is a SH3 domain-containing protein (Matsuno et al. 1998). The RING-finger motif in domain III is a well conserved region among the DTX-family proteins. Because of the presence of this C-terminal RING domain, Dx is

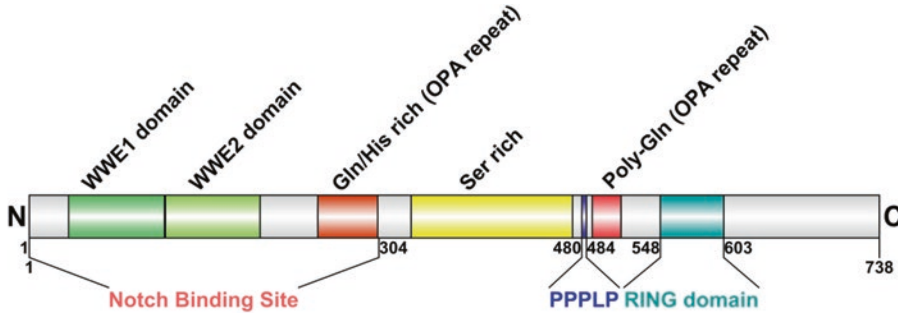

Fig. 7.1 Structure of Deltex protein. The N-terminal of Deltex is required for its binding with Notch-ICD. The C-terminal contains a RING domain that suggests a putative E3 ligase activity of this protein. Additionally, it contains a proline-rich motif (PPPLP) that might facilitate the interaction of Deltex with a SH3 domain containing protein

considered as a putative E3 ubiquitin ligase. It is suggested that alone Dx can promote a monoubiquitinated state of NICD (Baron 2012; Hori et al. 2011). However, other studies in *Drosophila* discovered that the RING-finger motif is necessary for the self-association of Dx proteins, and it plays an important role for proper functioning of the Dx protein (Matsuno et al. 2002). Additionally, it has also been reported that Dx with either deleted Proline-rich motif or deleted Notch-binding domain can act as a dominant-negative form in different developmental contexts (Capilla et al. 2012; Matsuno et al. 2002). Altogether, these studies shed a light on the importance of all three domains of the protein for its complete activity. In fact, all three domains of Dx have been shown to be necessary for the stabilization of Notch protein during its late endosomal trafficking (Hori et al. 2004).

Dx is probably the only protein that can regulate Notch signaling both in a positive as well as negative manner depending on the cellular context. However, the exact functioning of Dx is still not very clear even after three decade-long research. After the initial genetic interaction studies between the alleles of *dx* and *Notch* (Xu and Artavanis-Tsakonas 1990; Gorman and Girton 1992; Busseau et al. 1994), Diederich and coworkers reported that Dx can bind to Notch to positively regulate its signaling activity (Diederich et al. 1994). Additionally, they put forward the hypothesis that Dx can interact with Notch in a ligand-independent manner to mediate vesicular trafficking of Notch protein (Diederich et al. 1994). Subsequent experiments establish the fact that alone Dx can act as a positive regulator of Notch pathway. Using fruit fly model, it was shown that overexpression of the domain I of Dx as well as full length Dx protein led to eye, wing, and bristle morphology phenotypes that were comparable to that of activated Notch (NICD) overexpression phenotypes. Additionally, it was observed that overexpression of either the domain I of Dx or full length Dx or activated Notch (NICD) was able to rescue *dx* mutant phenotype. Their study also suggested that by binding to ANK repeats of NICD, Dx might help in nuclear translocation of Su(H) to activate Notch signaling (Matsuno et al. 1995). Altogether these data suggest a role of Dx in positive regulation of Notch signaling (Matsuno et al. 1995). In subsequent time, studies have put a question on the effect of interaction between Dx and Su(H) on Notch signaling (Fuwa et al. 2006; Hori et al. 2004; Koelzer and Klein 2006; Matsuno et al. 1995; Ramain et al. 2001). However, it is important to note that the interaction between Dx and Su(H) has a profound effect over Notch signaling outcome.

Continuous effort to understand the mechanistic detail of interaction between Dx and Notch and its effect on Notch signaling has helped to elucidate the role of Dx in vesicular trafficking of Notch receptor. Overexpression studies demonstrated that Dx mediates trafficking of membrane-bound Notch into the cytoplasmic late endosomal vesicles and helps to increase the half-life of vesicular Notch protein (Hori et al. 2004). Blockage in

transport of Notch into the late endosomal vesicle affects Dx-mediated Notch signal activation (Hori et al. 2004). Subsequently, Dx was shown to interact with the components of other endocytic trafficking machinery, namely, HOPS and AP-3 complexes, to influence Notch pathway (Wilkin et al. 2008). Dx interacts with the HOPS and AP-3 complexes and this interaction helps full-length Notch receptor to be endocytosed in the limiting membrane of late endosome and/or lysosome (Wilkin et al. 2008). It was proposed to prevent the degradation of Notch in the lumen of lysosome. In addition to that, presence of full-length Notch on the limiting membrane of multivesicular bodies facilitates Presenilin-mediated proteolytic cleavage of Notch. Thus, it turns on a ligand-independent Notch signal activation (Wilkin et al. 2008). The role of endogenous Dx in trafficking of Notch receptor was first explored by Yamada et al. in 2011. It was demonstrated that endogenous Dx is required not only for the membrane to endocytic trafficking of Notch, but also it is required for the trafficking of Notch from endosome to the lysosome at a later stage (Yamada et al. 2011). Using *dx* null tissue, it was demonstrated that in the absence of Dx, transport of Notch to the lysosome for degradation was affected. Alternatively, Notch was misrouted to a vesicular compartment of unknown identity (Yamada et al. 2011). Their study also helped to identify two routes, namely, canonical and Dx-mediated activation of Notch pathway. It was revealed that canonical activation of Notch pathway occurs before incorporation of Notch into multivesicular body. On the other hand, activation of Dx-mediated Notch signaling happens at the time Notch is transported from the multivesicular body (Yamada et al. 2011). Altogether these studies were instrumental to unravel the function of Dx as a regulator of Notch pathway by assisting the vesicular trafficking of the receptor.

The first demonstration of Dx activity as a negative regulator of Notch signaling was carried out by Mukherjee et al. in 2005. In that study, Dx was reported to interact genetically as well as physically with Kurtz, a nonvisual beta-arrestin protein (Mukherjee et al. 2005). It was found that Dx forms a trimeric complex with Kurtz and Notch. This interaction leads to poly-ubiquitination mediated proteasomal degradation of Notch (Mukherjee et al. 2005). This study revealed a new function of Dx, thereby conferring it a status of a context-dependent regulator of Notch signaling. Further study identified a protein called Shrub as an important modulator of Dx-Kurtz synergy (Hori et al. 2011). Shrub is a component of ESCRT-III endosomal trafficking complex. Depending on the Dx-mediated ubiquitination state of Notch, Shurb modulates the rate-limiting step in ligand-independent late endosomal activation of the receptor (Hori et al. 2011).

Dx is quite well-studied for its association with Notch. However, less is known about the other genetic and physical interacting partners of Dx. Earlier, Dx has been reported to interact with Supressor of deltex [Su(dx)], a HECT E3 ubiquitin ligase that function as a negative regulator of Notch signaling. As suggested by its name, Su(dx) suppresses the function of Dx. Initially it was postulated that by blocking the activity of Dx, a positive regulator of Notch, Su(dx) might downregulate the Notch signaling pathway (Fostier et al. 1998). In fact, this was the first study to identify the involvement of Su(dx) as a regulator of Notch pathway and demonstrated a triaxial interaction among Dx, Su(dx), and Notch. Later studies revealed that Su(dx) ubiquitinates Notch to promote the endosomal degradation of Notch receptor. This actually helps in negative regulation of Notch pathway (Wilkin and Baron 2005; Wilkin et al. 2004). Some other interactors of Dx have been identified in the past. As mentioned before, Kurtz is another protein that shows both genetic and physical interaction with Dx to negatively influence the outcome of Notch pathway (Mukherjee et al. 2005). In 2011, Ramain et al. reported Dishevelled (Dsh), a component of Wingless signaling pathway, as a genetic interactor of Dx. Dsh was reported to negatively regulate the activity of Dx. This, in turn, represses the Dx-dependent function of Notch (Ramain et al. 2001). This study was instrumental to link Notch and Wingless signaling, two fundamental pathways important for metazoan development. Additionally, it shed a light on the fact that Dsh and Dx might control a common target, JNK (Ramain et al. 2001). In addition to these, a few

candidates were identified in the Dx interactome. For example, dTARF2, a component of Eiger-JNK signaling, was found to interact with Dx genetically as well as physically. Also, when expressed together, it leads to downregulation of Notch signaling (Mishra et al. 2014). Recently, Dx was reported to interact with Eiger, the sole TNF homolog in *Drosophila*. The interaction between Dx and Eiger not only affected Notch pathway, but also influenced Eiger-induced cell death (Dutta et al. 2018a). Additional example includes the newly identified DEAD-box helicase family gene, *Maheshvara* (*Mahe*), which codes for an RNA-binding protein. Mahe was demonstrated to interact genetically with *dx*, and this interaction might have an effect on Notch signaling outcome (Surabhi et al. 2015). In recent past, we identified another RNA binding protein Hrp48 as an interactor of Dx (Dutta et al. 2017).

Heterogeneous Nuclear Ribonucleoprotein Hrp48

Hrp48 is a 48 kDa protein that belongs to hnRNP A/B family. The gene encoding Hrp48 is known as *Hrb27C*. Hrp48 plays a vital role in regulation of different facets of RNA metabolism including its stability, transport, splicing, and translational regulation (Matunis et al. 1992; Dreyfuss et al. 2002; Kalifa et al. 2009). In *Drosophila*, Hrp48 protein is essentially required for the survival of the organism (Matunis et al. 1992; Hammond et al. 1997). In *Drosophila*, 10 hnRNP proteins have been so far reported, and Hrp48 is one of the most abundant hnRNPs found in fruit-fly (Matunis et al. 1992; Hammond et al. 1997). Structurally, the Hrp48 protein consists of two N-terminal RBD (RNA-Binding Domain) domains/RNA recognition motif and a C-terminal Glycine-rich motif (Fig. 7.2). RBD domain is important for the interaction of this protein with RNAs, whereas the glycine-rich motif helps in both RNA–protein as well as protein–protein interaction (Matunis et al. 1992; Hammond et al. 1997).

Previously, Hrp48 has been studied for its regulatory involvement in post-transcriptional as well as translational modulation of mRNAs during oogenesis in *Drosophila* (Nelson et al. 2007; Sibel et al. 1994). Depending on the cellular context and the interacting partner protein, Hrp48 can act either as a repressor or as a activator of mRNA translation during the oogenesis (Nelson et al. 2007). During *Drosophila* oogenesis, Hrp48 plays an instrumental role in localization and translational control of *oskar* and *gurken* mRNAs (Geng and Macdonald 2006; Giorgi and Moore 2007; Goodrich et al. 2004; Huynh et al. 2004; Kalifa et al. 2009; Yano et al. 2004). Also, Hrp48 was reported to influence somatic inhibition by negatively regulating the splicing mechanism (Siebel et al. 1994).

In recent past, a series of studies has shed light into the involvement of Hrp48 in biological events other than oogenesis in *Drosophila*. For example, Bruckert and coworkers have found that Hrp48 plays a role in central nervous system of *Drosophila*. It was found that Hrp48 is involved in growth and development of Mushroom body neurons (Bruckert et al. 2015). Other reports suggest a protective role of Hrp48 in degenerative neuronal pathophysiology (Appocher et al. 2017; Ritson et al. 2010). More recently, Hrp48 has been reported to bind in the 3'UTR of msl-2 mRNA and

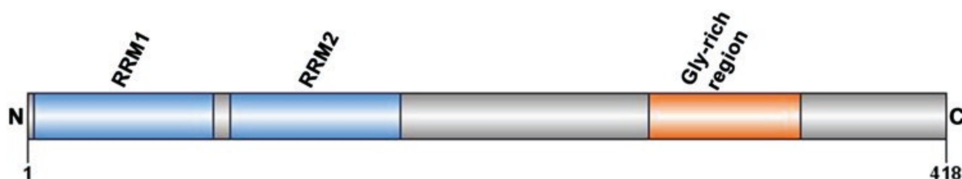

Fig. 7.2 Structure of Hrp48 protein. The N-terminal region of Hrp48 contains two RNA recognition motifs (RRM), which are also known as RNA-binding domain (RBD). These RRMs facilitate RNA–protein interaction. On the other hand, the C-terminal region contains a Gly-rich region, which helps in protein–protein interaction

to act as a cofactor for Sex-lethal (Sxl)-mediated translational repression of msl-2 (Szostak et al. 2018). Also, Hrp48 has been found to regulate a number of signaling pathways including Notch, JNK and Hippo pathway (Dutta et al. 2017, 2018b; Mach et al. 2018; Ren et al. 2018).

Suissa et al. (2010) first identified a connection between Hrp48 and Notch signaling. In fact, their study identified Hrp48 as a positive regulator of Notch signaling. Previously, it was reported that Sex-lethal (Sxl), an RNA-binding protein that specifies the female-specific development in *Drosophila*, can downregulate the activity of Notch pathway by putatively binding to the 3’-UTR of Notch mRNA during development (Penn and Schedl 2007).Using third instar larval wing disk of *Drosophila* as a model, it was demonstrated that Hrp48 represses the negative effect of Sxl on Notch in a sex-specific manner (Suissa et al. 2010). Both Notch protein as well as its signaling activity was found to be increased and decreased as per the enhanced and reduced dose of Hrp48, respectively. However, Sxl is expressed in females, not in males. Thus, the impact of Hrp48-mediated positive regulation over the Notch signaling was observed mostly in a female-specific manner.

Impact of the Interaction Between Hrp48 and Deltex on Notch Signaling

Earlier, a mass spectrometry-based analysis was carried out to identify novel interacting partners of Dx protein, and in this screen Hrp48 was identified as an interacting partner of Dx (Dutta et al. 2017). As Hrp48 is an RNA-binding protein, it was further checked whether the interaction of Dx and Hrp48 is an RNA-dependent or not. For that, the protein samples were treated with RNase, and it did not affect the interaction indicating the association between Dx and Hrp48 is an RNA-independent protein-protein interaction. Additionally, using in-vitro pull-down assay, the N-terminal Notch binding domain of Deltex was identified as the probable interacting site with Hrp48 protein. In wild-type tissue as well as the tissue in which both of these proteins were overexpressed, Dx and Hrp48 were colocalized in the cytoplasm. To understand their functional interaction in vivo, a genetic assay was employed. Hemizygous *deltex*^{null} male flies show a wing vein thickening phenotype with delta formation at the distal tip of the wing vein in adult wings. However, heterozygous *Hrb27C* females with reduced expression of Hrp48 protein do not show any obvious wing phenotype. However, when the dose of Hrp48 was reduced in *deltex*^{null} hemizygous background, the wing phenotype was enhanced markedly (Dutta et al. 2017). In some cases, notching at the tip of the adult wing was also observed, which indicates that the interaction of Dx and Hrp48 is functionally active during wing development. And, as Notch signaling plays an important role during wing margin formation, it was hypothesized that the interaction between Dx and Hrp48 might be influencing the Notch signaling pathway. Therefore, the expression pattern of Cut protein, a reliable marker of Notch signaling was checked in the dorsoventral (D/V) boundary of the larval wing disk (Neumann and Cohen 1996). D/V boundary of the larval wing disk forms the margin of a wing in adult flies. A decrease in Cut expression was noted in the D/V boundary of the wing disks, where the dose of Hrp48 was reduced in *deltex* null background. This ensures the possibility that Dx and Hrp48 might act in a controlling circuit during wing development by regulating Notch signaling outcome.

Subsequently, a gain-of-function assay was employed, where both of these proteins were coexpressed using *UAS-GAL4* system (Brand and Perrimon 1993). Overexpression of only Hrp48 in the D/V boundary of the wing disk did not affect the adult wings. These look similar to that of wild-type wings. On the other hand, overexpression of Dx results in minor irregularities in the wing marginal bristle pattern and occasional shortening of L4 vein of the adult wing. Interestingly, when both of these proteins were coexpressed in the wing, a wing-serration phenotype was observed (Dutta et al. 2017). However, any sex-specific alteration of the wing serration phenotype was not noticed when these proteins

were expressed together. This sex-neutral phenotype indicates that Hrp48, along with Dx, can regulate Notch signaling in a Sxl-independent manner. In Dx-Hrp48 coexpression background, a comparable level of Sxl protein was observed justifying the observed sex-neutral phenotype.

Wing serration phenotype is a classical Notch loss-of-function phenotype. Therefore, in order to confirm the involvement of Notch in this case, the dose of Notch was increased and decreased in Dx-Hrp48 coexpressed background. When the dose of Notch was reduced using RNAi technology, the wing serration phenotype got severed. Also, when the dose of Notch was increased, Dx-Hrp48 mediated wing serration phenotype was rescued. This genetic assay was compelling to the notion that the coexpression of Dx and Hrp48 can downregulate Notch signaling activity (Dutta et al. 2017). Subsequently, to check the level of Notch signaling activity, expression of Cut and Wingless, two different markers of Notch signaling was checked at the D/V boundary of the wing disk (Micchelli et al. 1997; Neumann and Cohen 1996; Rulifson and Blair 1995). Expression of both of these two markers was affected in Dx and Hrp48 coexpression background. Interestingly, expression of either Dx or Hrp48 does not affect the expression of these proteins in the D/V boundary. These results confirm that coexpression of Dx and Hrp48 can lead to a downregulation of Notch signaling.

Further, to understand how this downregulation of Notch signaling activity was achieved, we hypothesized two probable contributing factors. First, coexpression of Dx and Hrp48 might affect Notch at the transcript level. However, we could not detect any significant change in the transcript level of Notch in Dx and Hrp48 coexpressed condition. Secondly, the downregulation was probably achieved at the protein level of Notch. To check the level of Notch protein, first, we performed immunostaining to check the level of Notch in the wing disk. We observed that the cytoplasmic Notch was reduced when both Dx and Hrp48 was coexpressed. This observation was in line with the previous data showing a downregulation of Notch signaling activity in the same background. There were two possible reasons for the observation that the cytoplasmic Notch protein was reduced. Either Notch was degraded, or its trafficking to the cytoplasm was affected. In the western blots, no obvious change at the level of Notch protein was observed. It discarded the possibility of degradation of Notch due to coexpression of Dx and Hrp48. Subsequently, the level of membrane-bound Notch was checked using a detergent-free staining. An increase in the Notch protein level was noticed in the Dx and Hrp48 coexpression background. Together these observations suggested that interaction of Dx and Hrp48 might affect the transport of Notch from the membrane to the cytoplasm, thus affecting the signaling outcome.

Perspectives

Notch signaling is involved in a number of developmental as well as physiological events, and misregulated Notch signaling leads to a diseased condition. Sensitivity of Notch signaling outcome depends on the multiple factors including stability and maintenance of receptor and ligand, interaction with other proteins, as well as crosstalk with other signaling pathways. For that reason, knowledge about various spatiotemporal regulatory mechanisms of Notch signaling can be helpful for a better comprehension and management of Notch-signaling-related human pathologies.

Deltex, being a critical regulator of Notch signaling, has been reported to be involved in a number of pathologies including cancer of different tissues such as diffuse large B-cell lymphoma (DLBCL), Glioblastoma, and osteosarcoma (Gupta-Rossi et al. 2003; Huber et al. 2013; Meriranta et al. 2017; Zhang et al. 2010). Recurrent driver mutations in *DTX1*, the closest mammalian ortholog of *Drosophila dx*, has been identified as a cause of DLBCL pathogenesis (Meriranta et al. 2017). In case of osteosarcoma, *DTX-1* modulates Notch signaling activity, thereby increasing the cancer cell invasiveness (Zhang et al. 2010). Another example includes Glioblastoma, where the glial cells of the CNS (central nervous system) transforms into a malignant tumor. In these cells, oncogenic *DTX1*

activates non-canonical Notch signaling along with other mitogenic pathways like MAPK/ERK as well as RTK/PI3K/PKB signaling. Altogether, hyperactivity of DTX-1 leads to increased invasiveness of the cancer cells resulting into an aggravated form of Glioblastoma (Huber et al. 2013).

Therefore, considering the involvement of Dx in cancer pathogenesis, critical influence of Dx over Notch signaling, and its context-dependent regulatory activities, it is important to understand the biology of this protein. Identification of Hrp48 as a new interacting candidate of Dx might be helpful to understand biology of Dx, and the role of this interaction in regulation of Notch signaling. Effect of interaction of these two proteins on the Notch signaling and its crosstalk with other pathways like JNK signaling could led us better understanding of the involvement of Notch in a pathogenic condition.

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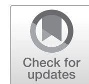

8 Interaction of Long Noncoding RNAs and Notch Signaling: Implications for Tissue Homeostasis Loss

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Abstract

The Notch signaling is a crucial pathway involved in cellular development, progression, and differentiation. Deregulation of Notch signaling pathway commonly impacts tissue homeostasis, being highly associated with proliferative disorders. The long noncoding RNAs (lncRNAs), which are transcripts with more than 200 nucleotides that do not code for proteins, were already described as Notch signaling pathway-interacting molecules. Many of them act as important transcriptional and posttranscriptional regulators, affecting gene expression and targeting other regulatory molecules, such as miRNAs. Due to their strong impact on function and gene expression of Notch-related molecules, lncRNAs influence susceptibility to cancer and other diseases, and can be regarded as potential biomarkers and therapeutic targets. Along this chapter, we summarize the cross talk between the Notch signaling pathway and their most important modulating lncRNAs, as well as the pathological consequences of these interactions, in different tissues.

Keywords

LncRNAs · Notch signaling · Tissue expression · Homeostasis loss

Introduction

The Notch signaling pathway has a key role in normal cellular growth and development, regulating cell differentiation, proliferation, migration, angiogenesis, and other physiological processes (Artavanis-Tsakonas et al. 1999). This highly conserved cell signaling pathway is composed by a type I transmembrane Notch receptors (NOTCH1 to 4), Notch ligands (Jagged-1 (JAG1), JAG2, Delta-Like1 (DLL1), DLL3, and DLL4), and other regulatory and downstream effector molecules, such as HES (*hairy and enhancer of split*) and HEY (*hairy/enhancer-of-split related with YRPW motif*) subfamilies. The canonical

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Notch signaling activation occurs when a Notch ligand binds to its receptor on a neighbor cell, triggering a two-step proteolytic cleavage of the Notch receptor. The first cleavage is mediated by enzymes of the ADAM (*a disintegrin and metalloproteinase*) family, and the second by γ-secretase, cleaving the Notch receptor and releasing the Notch intracellular domain (NICD). NICD is then translocated to the cell nucleus, where it interacts with CSL (an acronym for *CBF1/RBPJ* in mammals, *suppressor of hairless* in *D. melanogaster*, and *Lag-1* in *C. elegans*) transcription factors to activate the transcription of effector genes (Mizutani et al. 2001; Miele 2006; Kopan and Ilagan 2009).

Due to the role of Notch pathway in the balance between cell proliferation, differentiation and apoptosis, it is not surprising that some alterations in Notch signaling are associated with tumorigenesis (Wilson and Radtke 2006; Wang et al. 2010). An aberrant Notch signaling pathway has been associated with cancer recurrence, metastasis, resistance to treatment and other oncogenic-related processes. It may act alone or in cross talk with other oncogenic pathways (Wilson and Radtke 2006; Reicher et al. 2018). Moreover, the Notch signaling pathway is an important regulator of Epithelial-Mesenchymal Transition (EMT), a process by which epithelial cells undergo remarkable morphologic changes to become mesenchymal stem cells (MSCs), inducing their tumor migration and invasion properties (Klymkowsky and Savagner 2009; Wang et al. 2010; De Craene and Berx 2013). After EMT, cells lose cohesiveness, and acquire resistance to apoptosis and enhanced ability to migrate. The main molecular features of EMT are the loss of expression and function of epithelial markers such as E-cadherin, Claudin and Occludin, as well as the overexpression of mesenchymal cell markers, such as N-cadherin and Vimentin (Turley et al. 2008; Scanlon et al. 2013) and important transcription factors, such as SNAI1, SNAI2, TWIST1, TWIST2, ZEB1 and ZEB2 (Yang and Weinberg 2008; De Craene and Berx 2013).

Certain Notch-related molecules are currently being investigated for clinical application, such as γ-secretase inhibitors (GSI), which inhibit the final step of proteolytic activation of Notch receptors. Another clinical approach being developed is the blockage of a Notch ligand (Dll4) with monoclonal antibodies, resulting in disorganized angiogenesis, without formation of functional capillaries (Takebe et al. 2015).

Among the Notch-related molecules and targets, there are different types of nucleic acids categories, besides the protein-coding molecules (Wang et al. 2010; Reicher et al. 2018). Since the popularization of high-throughput sequencings, it is becoming increasingly clear that the genome noncoding regions are far from being "junk DNA," and the human transcriptome is much more complex than previously thought. Several genes have been discovered to transcribe RNA molecules with no protein-coding potential, but with distinct expression patterns and important roles in gene regulation: they are the noncoding RNAs (ncRNAs) (Harrow et al. 2012; Iyer et al. 2015). The ncRNAs represent the major part of human transcriptome, and currently receive increasing attention in the pursuit to comprehend cell and tissue physiology, as well as pathological processes and diseases (Cipolla et al. 2018; Salviano-Silva et al. 2018; Oliveira et al. 2018). Among the diverse classifications of ncRNAs, many studies highlight the microRNAs (miRNAs) and the long noncoding RNAs (lncRNAs). In general, while the miRNAs mainly act as posttranscriptional negative regulators by inhibiting translation or degrading mRNA transcripts, the lncRNAs present an extensive range of pre- and posttranscriptional regulatory mechanisms, including miRNA targeting (as "sponges" of miRNAs) (Salviano-Silva et al. 2018). Besides, both categories of ncRNAs have been identified as crucial molecules in Notch signaling, the role of lncRNAs relative to this pathway is still poorly explored. Throughout this chapter, we will discuss the main findings about lncRNAs and Notch signaling in different tissues, as well as the relevance of this interaction in diseases.

Long Noncoding RNAs

The lncRNAs are defined as noncoding transcripts with more than 200 nucleotides (Derrien et al. 2012; Harrow et al. 2012) and participate in important cellular physiological processes interacting with DNA, RNA and proteins. Several lncRNAs are transcribed by RNA polymerase II, present alternative splicing and may or not be polyadenylated (Derrien et al. 2012; Harrow et al. 2012; Salviano-Silva et al. 2018). The lncRNA secondary structure is formed by molecular folds, as a result of base pairing of complementary sequences, dynamically exposing specific functional motifs that allow its interaction with other molecules (Pegueroles and Gabaldón 2016; Smith and Mattick 2017).

According to their localization in relation to the nearest coding gene, the lncRNAs are categorized as intergenic (lincRNAs), sense (overlapped or intronic), antisense (intronic or Natural Antisense Transcripts—NAT), or bidirectional (divergent) (Fig. 8.1a). In general, lncRNAs are involved in several regulatory processes (Fig. 8.1b), acting by different functional mechanisms, modulating gene transcription, splicing, translation, nuclear and cytoplasmic traffic, and imprinting, among other processes. The lncRNAs can even be processed to short RNAs, such as miRNAs. Furthermore, lncRNAs are also related to epigenetic mechanisms, inducing chromatin remodeling and altering the recruitment of RNA polymerase II, transcription factors and other crucial proteins to gene expression (Wapinski and Chang 2011; Shi et al. 2013; Salviano-Silva et al. 2018).

The lncRNAs are strictly regulated and often present cell-specific expression patterns, orchestrating distinct biological processes, of which many are restricted to developmental stages (Dinger et al. 2008; Mercer et al. 2008; Salviano-Silva et al. 2018). Deregulation in lncRNAs expression may result in homeostasis loss, and has been associated with several disorders, including cancer. Concerning to cancer hallmarks (Hanahan and Weinberg 2011), lncRNA deregulation was already described altering the proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion/metastasis, reprogramming of energy metabolism, evading immune destruction, and inducing inflammation and genome instability (Oliveira et al. 2018).

Several lncRNAs were associated to different proliferative signaling pathways, such as PI3K-Akt, NF-kB, WNT, and the Notch signaling (Sun et al. 2018). In addition, lncRNAs may affect EMT through the Notch signaling pathway (directly or indirectly), also contributing to tumor development (Gao et al. 2017; Yang et al. 2017). Further, similarly to the lncRNAs, the Notch targets present tissue-specific expression patterns. Thus, Notch regulates different genes in distinct tissues (Borggrefe and Oswald 2009), as discussed along this chapter.

Cross Talk Between Long Noncoding RNAs and Notch Signaling in Different Tissues and Respective Disease Conditions

Activation of Notch signaling strongly contributes to cell proliferation, differentiation, and survival, physiologically for stem cells or pathologically for malignant cells (Fox et al. 2008). Thus, fine-tuning regulation of Notch signaling molecules is important to maintain tissue homeostasis, but can be disturbed by direct or indirect interactions with aberrantly expressed genes (Fig. 8.2). Among them, various lncRNAs were described to be positively or negatively correlated with Notch signaling and other related molecules (such as miRNAs). This tissue-specific cross talk between lncRNAs and the Notch-signaling pathway is highly associated with Notch-related disorders, especially carcinogenesis. Finally, we summarize lncRNAs that modulate or are regulated by Notch signaling pathway in the homeostasis loss (Table 8.1).

LncRNAs and Notch Signaling in Nervous Tissue

In neural stem cells, Notch signaling pathway has an important role in neuronal differentiation inhibition

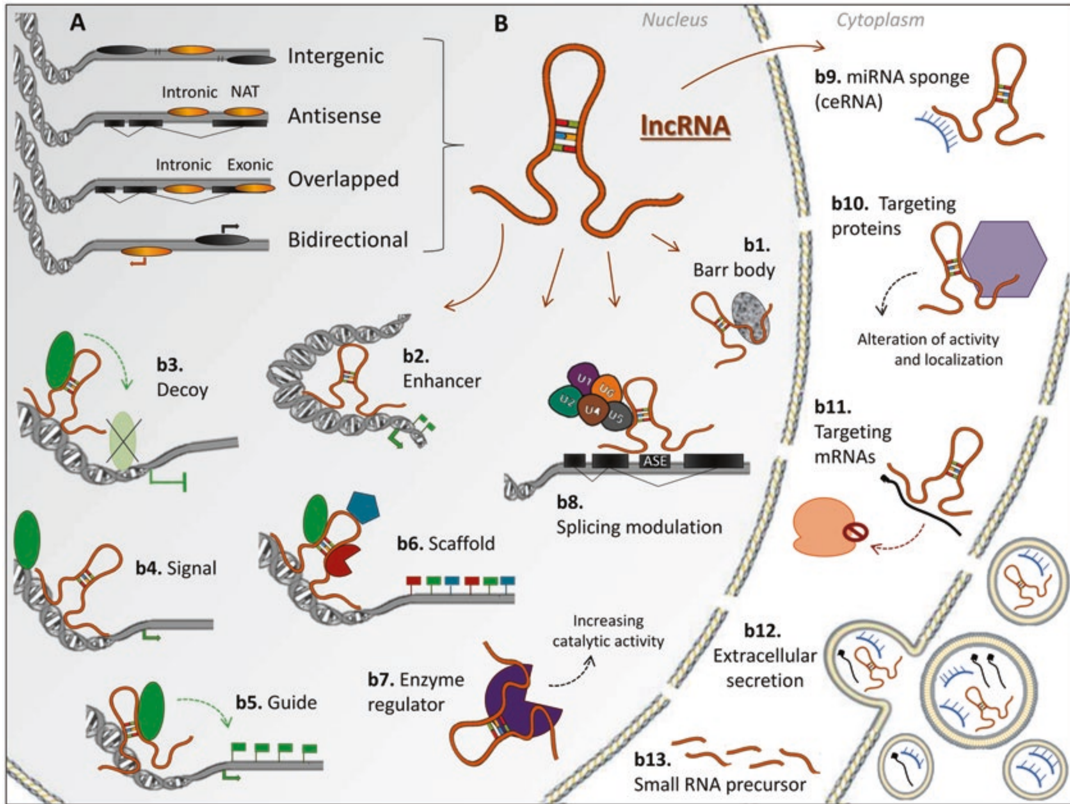

Fig. 8.1 Long noncoding RNAs: genomic location and regulatory mechanisms. (**a**) Nomenclature of lncRNA genes (gold ellipses), according to their genomic location relative to the nearest coding gene (black ellipses) and/or to exons of coding genes (black rectangles). (**b**) lncRNAs functions: (**b1**) lncRNAs involved in inactivation of X chromosome in females, such as the Barr body lncRNA component XIST; (**b2**) acting as enhancers, inducing transcription in cis or in trans; (**b3**) a decoy to regulatory proteins, such as transcription factors and chromatin modifiers, blocking their binding to DNA; (**b4**) as molecular signals, activating or silencing gene expression through signaling to regulatory pathways; (**b5**) Guiding proteins (in general, chromatin modifiers) to specific target sites; (**b6**) as scaffolds, binding different proteins and forming ribonucleoprotein (RNP) complexes, which also affect gene expression; (**b7**) interacting with enzymes, such as kinases, regulating/enhancing their catalytic activity and altering their signalization; (**b8**) modulating alternative splicing of primary transcripts; (**b9**) as competing endogenous RNA (ceRNA), serving as a sponge for microRNAs (miRNAs), blocking their effect; (**b10**) targeting proteins, forming molecular complexes which can block or induce functional effects, or even alter their location in the cell; (**b11**) targeting messenger RNAs (mRNAs), inhibiting their translation in ribosomes. In addition, lncRNAs can be (**b12**) transferred to other cells by extracellular vesicles (EVs); (**b13**) precursors of miRNAs and other regulatory small RNA. An lncRNA can act by multiple regulatory mechanisms, in both the nucleus and/or in the cytoplasm. The **b12** itself is not exactly a regulatory feature; however, the release of these functional lncRNAs through EVs is a way of regulating genes, RNAs, or proteins in other tissues. *ASE*—alternatively spliced exon. This image contains illustrations obtained in Mind the Graph Infographic Platform, and is original from the article of Salviano-Silva et al. (published in Non-Coding RNA, by MDPI in 2018) (Salviano-Silva et al. 2018), being reproduced with authors' permissions

(Louvi and Artavanis-Tsakonas 2006). It promotes neuronal self-renewal and represses neurogenic and differentiation programs in brain tumors, acting through similar (but still not well understood) mechanisms to those regulating stem cells during neural development (Pierfelice et al. 2008). LncRNAs regulate the expression of molecules of the Notch signaling pathway, through both direct and indirect interactions, which may disturb neural homeostasis and contribute to carcinomas of the central nervous system (CNS) and ischemic stroke. Some of them are summarized below.

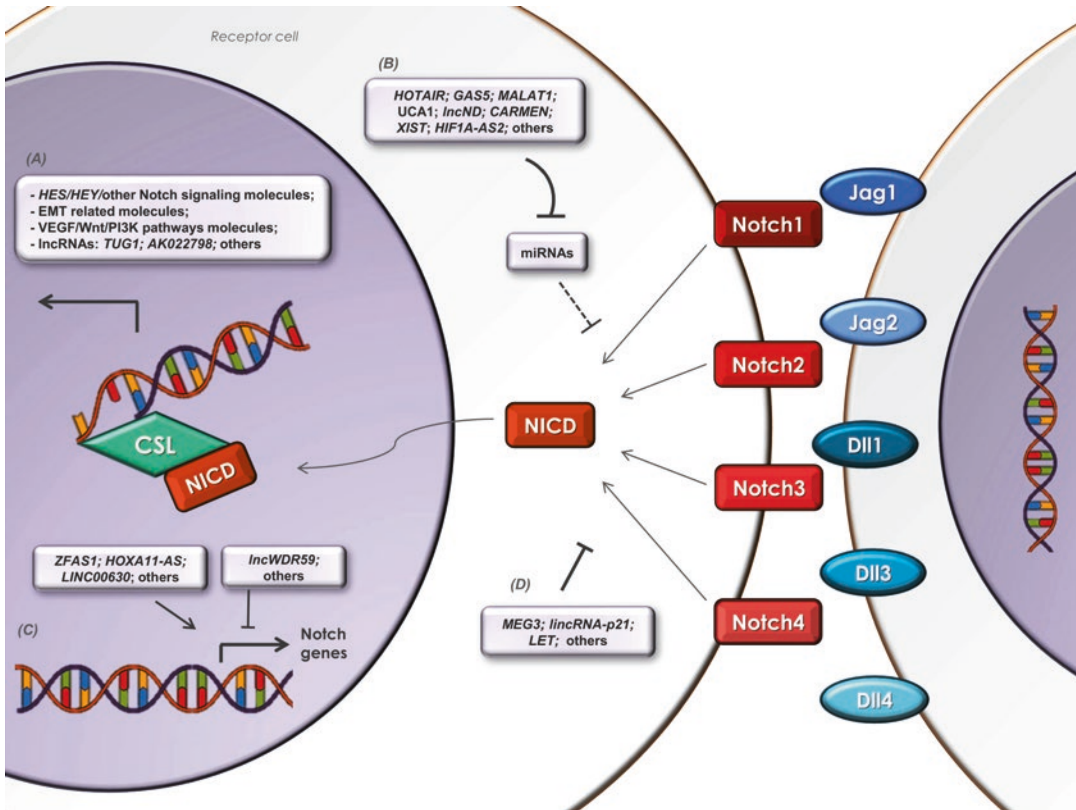

Fig. 8.2 Long noncoding RNAs in Notch signaling pathway. Notch transmembrane receptors (in red: NOTCH1-4) and ligands (in blue: JAG1/2 and DLL1/3/4) randomly bind to release the NICD, which in turn, enters the cell nucleus and interacts with CSL to control gene expression, mainly upregulating coding and noncoding genes of proliferative pathways (**a**). This process is supported by lncRNAs, which inhibit miRNAs involved in repression of Notch signaling at distinct stages (**b**), or directly bind the Notch pathway genes, enhancing their expression, or directly bind Notch-repressor genes, inhibiting their expression (**c**). Moreover, some lncRNAs can inhibit Notch signaling molecules, attenuating the proliferative effects (**d**). Further, some lncRNAs can act in one or more of these processes and can be or not associated with different diseases. This image contains illustrations obtained in Mind the Graph Infographic Platform

The lncND (*lncRNA for Neurodevelopment*) acts in the early regulation of neuronal differentiation, during the development of the human brain. In neuroblastoma cells, lncND was shown to act as sponge for miR-143-3p, a miRNA which binds mRNA and negatively regulate *NOTCH1* and *NOTCH2* genes. The downregulation of *lncND* leads to increased levels of miR-143-3p, inhibiting the mRNA and protein expressions of Notch1 and Notch2, and the activation of their downstream targets, which reduces cell proliferation and induces cell differentiation to neurons. In contrast, *lncND* overexpression in vivo results in Notch signaling increasing and expansion of radial glia (Rani et al. 2016).

Gliomas are the most prevalent type of CNS malignancies, being categorized in four histological grades (I–IV), according to the WHO (World Health Organization) classification (Ostrom et al. 2015). In glioma stem cells (GSCs), NOTCH1 activation induces the expression of *TUG1* (*Taurine-Upregulated Gene 1*), a lncRNA associated with many types of cancer. In higher histological grades, Notch-regulated TUG1 sponges miR-145, maintaining the expression of stemness-associated genes, such as the transcription factor *SOX2* (*Sex determining region Y-box 2*) and the oncogene *MYC* (*v-myc avian myelocytomatosis viral oncogene homolog*). In addition, TUG1 interacts with the Polycomb Repressive Complex 2 (PRC2) to

Table 8.1 Resume of lncRNAs interacting with molecules of Notch signaling pathway in the homeostasis loss

lncRNA	Molecular mechanisms/ direct and indirect targets	Consequence/function	Disease association	References
AK022798	Induced by NOTCH1; increases MRP1 and P-glycoprotein; decreases Caspase-3	Drug resistance; cell viability of resistant gastric cancer cells; reduction of apoptosis	Upregulated in gastric cancer	Hang et al. (2015)
ANRIL	Decreases miR-99a, thus increasing BMI1, NOTCH1, mTOR, and p70S6K	Inhibition of apoptosis and promotion of tumorigenesis	Upregulated in gastric cancer	Liu et al. (2018a)
CBR3-AS1 (PlncRNA-1)	Increases NOTCH1, HES1, and JAG1	Induction of cell proliferation, colony formation, and apoptosis	Upregulated in glioma/higher grades	Wang et al. (2018a)
FOXD2-AS1	Increases HES1, NICD, N-cadherin, and SNAI1; decreases E-cadherin	Induction of cell proliferation, invasion, and migration	Upregulated in colorectal cancer	Yang et al. (2017)
FAM83H-AS1	Positively correlates with NOTCH1 and HES1	Induction of cell proliferation and migration; reduction of apoptosis	Upregulated in colorectal cancer	Lu et al. (2017)
GAS5	Sponges miR-137, increasing NOTCH1 and NICD; inhibition of Caspase-3	Induction of cell viability and reduction of apoptosis	Upregulated in ischemic stroke	Chen et al. (2018)
	Negatively regulated by NOTCH1, which is increased in breast cancer	Induction of cell proliferation	Downregulated in breast cancer	Pei and Wang (2015)
GHET1	Negatively regulates KLF2, increasing NOTCH1 and HIF-1α	Induction of cell proliferation; inhibition of cell cycle and apoptosis	Upregulated in prostate cancer	Zhu et al. (2019)
HIF1A-AS2	Sponges miR-153-3p, increasing HIF-1α, NOTCH1, and VEGF	Induction of cell viability, migration, and angiogenesis	Upregulated in ischemic stroke	Li et al. (2017)
HOTAIR	Sponges miR-613, increasing NOTCH3	Induction of tumor growth	Upregulated in pancreatic cancer	Cai et al. (2017)
	Increases NOTCH1, HES1, P300, β-catenin, N-cadherin, Vimentin, SNAIL, and TWIST	Induction of tumor growth, invasion, and metastasis	Upregulated in cervical cancer	Lee et al. (2016)
	Increases NOTCH1	Reduced HOTAIR (and NOTCH1) levels increases apoptosis	Downregulated in premature ovarian failure	Zhao and Dong (2018)
	Increases NOTCH1 and JAG1	Induction of proliferation and invasion	Upregulated in retinoblastoma	Dong et al. (2016)
LET	Decreases NICD1 levels	Reduced LET levels induces cell proliferation, migration and invasion, and inhibits cell cycle arrest and apoptosis	Downregulated in non-small cell lung cancer	Li et al. (2018a)

(continued)

Table 8.1 (continued)

lncRNA	Molecular mechanisms/ direct and indirect targets	Consequence/function	Disease association	References
LINC00630	Interacts with HDAC1 and DDX23, activating Notch signaling (predicted)	Induction of cell proliferation, invasion, and metastasis	Upregulated in non-small cell lung cancer	Mao et al. (2017)
LINC00974	Sponges miR-642, increasing KRT19, and thus NOTCH1, JAG1, DTX1, TGFBR1, SMAD2, and SMAD3	Increased tumor size and metastasis and decreased tumor differentiation grade	Upregulated in hepatocellular cancer; plasma fraction suggested as biomarker	Tang et al. (2014)
linc-OIP5	Positively correlated with YAP, which increases JAG1, NOTCH1, and HES1	Induction of proliferation, migration, and tumor growth	Upregulated in glioma/higher grades	Hu et al. (2017)
lincRNA-p21	Decreases HES1, NICD, N-cadherin and SNAI1L; Increases E-cadherin and Claudin-1	Inhibition of invasion and lung metastasis of hepatocellular cancer	Downregulated in hepatocellular cancer	Jia et al. (2016)
Lnc34a	Sponges miR-34a, inducing Notch and Wnt signaling pathways	Induction of cell proliferation, self-renewal, and tumor progression	Upregulated in colorectal cancer and colon cancer stem cells	Bu et al. (2013)/Wang et al. (2016)
lnc-AC004696.1–1, lnc-BACH1–1 and lnc-IRF2–3	Associated with NOTCH1 mutations	B cell tumorigenesis and adverse prognostic factors	Upregulated in chronic lymphocytic leukemia	Ronchetti et al. (2016)
lnc-C1orf132–1	Associated with NOTCH1 mutations	B cell tumorigenesis and adverse prognostic factors	Downregulated in chronic lymphocytic leukemia	Ronchetti et al. (2016)
lnc-LFAR1	Bind with SMAD2/3 to increase NOTCH2, NOTCH3, HES1, TGFB, SMAD2, and SMAD3 transcriptions	Proliferation of hepatic stellate cells; hepatocytes apoptosis induction; liver injury and fibrosis	Upregulated in hepatic stellate cells during fibrogenesis	Zhang et al. (2017a)
LncND	Sponges miR-143-3p, increasing NOTCH1 and NOTCH2	Induction of cell proliferation; reduced differentiation; expansion of radial glia	Upregulated in neuroblastoma	Rani et al. (2016)
LNCRNA00673	Increases NOTCH1 and NOTCH3	Induction of cell proliferation and metastasis	Upregulated in hepatocellular cancer	Chen et al. (2017a)
lncWDR59	Targeted by miR-103, thus increasing NUMB and decreasing NOTCH1; miR-103 also increases oxidized low-density lipoprotein (oxLDL) levels	Inactive lncWDR59 results in inhibition of endothelial proliferation, hyperlipidemia, and oxLDL-induced mitotic aberrations	Downregulated in atherosclerosis	Natarelli et al. (2018)
LUNAR	Positively regulated by NOTCH1; cis-regulates IGF1R expression	Enhance the IGF1R mRNA expression and sustain IGF1 signaling.	Upregulated in T cell acute lymphoblastic leukemia	Trimarchi et al. (2014)

(continued)

Table 8.1 (continued)

lncRNA	Molecular mechanisms/ direct and indirect targets	Consequence/function	Disease association	References
MALAT1	Sponges miR-217, increasing Sirt1, which increases NOTCH1, NOTCH2, and NOTCH3	Alleviate in hypoxia-induced cell injury	Upregulated after hypoxia-induced cardiomyocyte injury	Yao et al. (2019)
	Sponges miR-124, increasing JAG1	Induction of tumor growth and metastasis	Upregulated in tongue cancer	Zhang et al. (2017b)
MEG3	Decreases HES1, DLL4, and VEGF	Reduced MEG3 levels induces angiogenesis	Downregulated in CNS tumors	Gordon et al. (2010)/He et al. (2017)
	Decreases NICD, HES1, and HEY1	Reduced MEG3 levels increases endothelial cell proliferation and migration, stimulating angiogenesis	Downregulated in ischemic stroke	Liu et al. (2017)
	Decreases NOTCH1 and HES1	Reduced MEG3 levels increases cell proliferation	Downregulated in endometrial cancer	Guo et al. (2016)
	Decreases NOTCH1, VEGFA, m-TOR, P70S6K, BCL-XL and PI3K	Reduced MEG3 levels increase EC cell viability, migration invasion, and tumor growth	Downregulated in endometrial cancer (mainly poorly/ moderately differentiated)	Sun et al. (2017)
	Decreases HES1, HEY1, TP73 and SOX2; increases SNAI2 and YAP1	Induction of basal cell differentiation; tissue remodeling	Upregulated in idiopathic pulmonary fibrosis	Gokey et al. (2018)
NALT (RP11-611D20.2)	Increases NOTCH1, through cis-regulation	Induction of cell proliferation	Upregulated in pediatric T cell acute lymphoblastic leukemia	Wang et al. (2015)
PAUPAR	Inhibits the expression of HES1 gene	Reduced PAUPAR levels induces tumor progression, colony formation, and migration	Downregulated in uveal melanoma	Ding et al. (2016)
PVT1	Sponges miR-146a, increasing NOTCH1	Induction of tumor growth and migration	Upregulated in prostate cancer	Liu et al. (2016a)
RAMP2-AS1	Decreases NOTCH3	Suppression of cell cycle progress and proliferation	Downregulated in glioblastoma	Liu et al. (2016b)
RP11-567G11.1	Increases JAG1, HES1, HES5, and MATH1	Induction of stemness, cell proliferation, and cell cycle progression; inhibition of apoptosis	Upregulated in poorly differentiated pancreatic cancer; suppression associated with gemcitabine treatment efficiency	Huang et al. (2019)
SNHG1	Increases NOTCH1, HES1, N-cadherin, and Vimentin; reduces E-cadherin	Induction of cell proliferation and migration	Upregulated in pancreatic cancer	Cui et al. (2019)

(continued)

Table 8.1 (continued)

lncRNA	Molecular mechanisms/ direct and indirect targets	Consequence/function	Disease association	References
SNHG12	Increases NOTCH1, HES1, P21, Vimentin, and N-cadherin, while decreases E-cadherin	Induction of cell proliferation and inhibition of apoptosis	Upregulated in nasopharyngeal cancer	Liu et al. (2018b)
SRA	Increases MMP-9, MMP-2, VEGFA, NOTCH1, HES1, P300, β-catenin, Vimentin, SNAI1, and TWIST; decreases E-cadherin	Induction of cell proliferation, migration, and invasion	Upregulated in cervical cancer	Eoh et al. (2017)
TUG1	Induced by NOTCH1 activation; sponge for miR-145; interacts with PRC2	Stemness maintenance; uncontrolled self-renewal	Upregulated in glioma/higher grades	Katsushima et al. (2016)
UCA1	Sponges miR-124, increasing JAG1, NOTCH1, and Vimentin, while decreases E-cadherin	Invasion induction	Upregulated in tongue cancer	Zhang et al. (2019)
XIST	Sponges miR-137, increasing NOTCH1	Induction of cell proliferation	Upregulated in non-small cell lung cancer	Wang et al. (2018b)
ZFAS1	Increases HES1, NICD, N-cadherin, and SNAI1; reduces E-cadherin	Apoptosis suppression and induction of cell proliferation, migration, and invasion	Upregulated in glioma/higher grades	Gao et al. (2017)

promote locus-specific epigenetic regulation, repressing differentiation genes. Thus, uncontrolled self-renewal of GSCs is the ultimate consequence of NOTCH1 activation and *TUG1* overexpression (Katsushima et al. 2016).

The lncRNA *ZFAS1* (*ZNFX1 or Zinc Finger 1 NFX1-type antisense RNA 1*) is implicated in glioma progression, being upregulated in glioma cells and in higher WHO grades (III and IV). In vitro silencing of *ZFAS1* induces apoptosis and decreases the proliferation, migration, and invasion of glioma cells. Moreover, silenced *ZFAS1* decreases HES1 (homolog 1 of HES, critical for cellular self-renewal) and NICD levels, as well as the levels of the EMT-related proteins N-cadherin and SNAI1, while increasing E-cadherin. The deregulated expression of these pivotal proteins underpins the importance of the lncRNA ZFAS1 in the development, progression, and poor prognosis of glioma patients, by activating the Notch signaling pathway and stimulating the EMT process (Gao et al. 2017).

The expression of *CBR3-AS1* (also known as *PlncRNA-1—prostate cancer-upregulated long noncoding RNA 1*) increases in glioma cell lines, especially in higher glioma grades. The knockdown of *CBR3-AS1* resulted in downregulation of *NOTCH1*, *JAG1*, and *HES1*, while inhibition of the Notch signaling pathway reverses the proliferation, colony formation, and apoptosis in cells overexpressing *CBR3-AS1* (Wang et al. 2018a).

Similarly, the lncRNA gene *linc-OIP5* (*Opa-interacting protein 5 lincRNA*) is also upregulated in glioma cells lines, especially in the higher advanced grades III/IV. Knockdown of *linc-OIP5* suppresses proliferation and migration of glioma cell lines in vitro and inhibits tumor growth in vivo. Furthermore, in comparison with adjacent normal tissues, the expression of *linc-OIP5* in human glioma tissue positively correlates with expression of *YAP* (*Yes-Associated Protein 1*), a

well-known upregulated gene in gliomas. Interestingly, YAP expression and protein levels are reduced in glioma cells lines after *linc-OIP5* knockdown, as well as of JAG1, NOTCH1, and HES1 (Hu et al. 2017).

Among the 225 differentially expressed lncRNAs in a microarray analysis of glioblastoma (a grade IV glioma type) specimens, *RAMP2-AS1* was found downregulated. RAMP2-AS1 negatively regulates *NOTCH3*, whose mRNA and protein expressions are enhanced in glioblastoma. Reduced levels of RAMP2-AS1 in glioblastoma are also correlated with poor prognosis. Moreover, overexpression of *RAMP2-AS1* reduces the expression of *NOTCH3* in vitro and in vivo, blocking cell cycle progress and cell proliferation in glioblastoma (Liu et al. 2016b).

The lncRNA MEG3 (*maternally expressed gene 3*) is expressed in many normal tissues. Considered as a tumor suppressor gene, its expression decreases in primary tumors, including those of the CNS (He et al. 2017). Brains of *Meg3*-null murine embryos present aberrant expression of angiogenic genes, important for blood vessel development (as *vascular endothelial growth factor—Vegf*) and for Notch signaling (as *Hes1* and *Dll4*), increasing formation and density of microvessels. This suggests that MEG3 inhibits VEGF-mediated angiogenesis and Notch signaling pathway in the brain (Gordon et al. 2010). This lncRNA is also downregulated after ischemic stroke (IS) in mice. In contrast to cancer, angiogenesis is beneficial after IS, inducing endogenous recovery mechanisms that contribute to neurogenesis and neuronal plasticity. *Meg3* knockdown increases endothelial cell proliferation and migration. It also increases the protein levels of *Nicd*, *Hes1*, and *Hey1*, both in endothelial cells and in rats with IS (through middle cerebral artery occlusion—MCAO), increasing the formation of functional microvessels in the peri-infarct area in vivo. Moreover, inhibition of Notch signaling abolished angiogenic activity. Thus, *MEG3* silencing seems to reduce brain lesions and to improve functional recovery after IS, inducing angiogenesis via Notch signaling pathway (Liu et al. 2017).

The lncRNA HIF1A-AS2 (*hypoxia inducible factor 1 subunit alpha antisense 2*), previously associated with hypoxia processes in cancer, activates Notch signaling by increasing the expression of its antisense coding gene *HIF1A*, through negative regulation of miR-153-3p. *HIF1A-AS2* is upregulated in human umbilical vein endothelial cells (HUVECs), under hypoxia conditions. This lncRNA acts as sponge for miR-153-3p, which in turn, negatively regulates *HIF1A-AS2* expression, indicating a reciprocal regulation of both RNAs. MiR-153-3p also targets HIF-1α mRNA, inhibiting its translation. In HUVECs under hypoxia, the knockdown of *HIF1A-AS2* increased miR-153-3p and inhibited HIF-1α and NOTCH1 protein levels, resulting in decreased cell viability, migration, and angiogenesis. Angiogenesis was also inhibited by knockdown of *HIF1A*, as well as by overexpression of *miR-153-3p*. In addition, permanent MCAO rats presented enhanced angiogenesis, as well as increased Hif-1α, Vegfa, and Notch1 levels, and decreased miR-153-3p levels in infarcted areas. Taken together, the negative regulation of miR-153-3p by HIF1A-AS2 promotes the expression of *HIF1A*, as well as of *VEGFA* and *NOTCH1*, enhancing cell viability, migration, and angiogenesis in IS (Li et al. 2017).

Controversially, Notch signaling also contributes to IS-induced neuronal injury through the lncRNA GAS5 (*growth arrest-specific transcript 5*) (Chen et al. 2018). Neurons exposed to oxygen and glucose deprivation (OGD) or to MCAO surgery upregulate *Gas5* in mice. GAS5 acts as sponge for miR-137 (Chen et al. 2018), a miRNA that inhibits Notch1 translation (Shi et al. 2017). *Gas5* knockdown resulted in *miR-137* upregulation, lower Notch1 and NICD levels, higher cell viability and reduction of caspase-3 activity and apoptosis in neurons submitted to OGD. *Notch1* downregulation also reversed Gas5 effects on reduced cell viability. These positive effects were lost after miR-137 inhibition, reinforcing the crit-

ical role of GAS5 and miR-137 in Notch signaling regulation in IS (Chen et al. 2018).

LncRNAs and Notch Signaling in Hepatic and Pancreatic Tissues

Liver and pancreas are both endocrine and exocrine glands responsible for metabolism and secretion of lipids (liver), digestive enzymes and hormones (pancreas), with crucial roles in the digestive system. Their tissues develop from common progenitors of the distal foregut, where the Notch signaling has a role in lineage differentiation of stem cells (McCracken and Wells 2012).

The well-known lncRNA *HOTAIR* (*Hox transcript antisense intergenic RNA*) is upregulated in pancreatic cancer (PC) tissue and cell lines. HOTAIR sponges miR-613, an oncosuppressor miRNA which prevents the translation of the Notch3 mRNA. Knockdown of *Hotair*, as well as the overexpression of *miR-613*, decreased Notch3 levels and tumor growth in vivo. Thus, HOTAIR seems to act as a competing endogenous lncRNA for miR-613, increasing NOTCH3 expression and contributing to pancreatic carcinogenesis (Cai et al. 2017).

SNHG1 (*Small Nucleolar RNA Host Gene 1*) is another lncRNA overexpressed in PC cell lines, suggested to promote pancreas tumorigenesis via Notch signaling pathway. *SNHG1* knockdown decreased mRNA and protein levels of Notch1, Hes1, N-cadherin, and Vimentin, while induced E-cadherin. These alterations inhibited cell proliferation and migration in vitro and were reversed by *NOTCH1* overexpression (Cui et al. 2019).

Poorly differentiated PC tissues do also overexpress the lncRNA *RP11-567G11.1*. In vitro depletion of *RP11-567G11.1* induced apoptosis and decreased PC stemness, cell proliferation and cell cycle progression, accompanied by decreased protein levels of JAG1, HES1, HES5, and MATH1 (*Meprin-associated Traf homology domain containing 1*). Interestingly, *RP11-567G11.1* suppression in PC cells increased the effects of gemcitabine, a first-line chemotherapy treatment for PC patients (Huang et al. 2019).

Regarding the hepatic tissue, there are at least 150 ncRNAs regulated by insulin in murine liver. Among them, Gm15441 regulates fatty acid oxidation and lipid accumulation in hepatocytes. Gm15441 is downregulated in euglycemic clamp conditions, as well as the Notch signaling pathway, but 100-fold upregulated after fasting and refeeding experiments (Batista et al. 2019).

The expression of *LINC00974* is upregulated in hepatocellular carcinoma (HCC). This lncRNA acts as sponge for miR-642, a microRNA which inhibits *KRT19* (keratin 19) mRNA translation. Upregulation of *LINC00974*, and consequently of *KRT19*, is associated with decreased tumor differentiation grade, increased tumor size, and metastasis. Overexpression of *KRT19* increased the Notch signaling proteins NOTCH1, JAG1, and DTX1, as well as the TGF-β signaling related-molecules TGFBR1, SMAD2, and SMAD3. In contrast, *LINC00974* knockdown reduced HCC cell proliferation and migration in vitro (even in the presence of KRT19), as well as tumor growth and metastasis in vivo. Furthermore, the plasma fraction of LINC00974 was suggested to act as biomarker in prediction of HCC growth and metastasis (Tang et al. 2014).

The *LNCRNA00673* is also upregulated in HCC tissues and cell lines. In vitro overexpression of *LNCRNA00673* promoted cell proliferation and invasion, as well as metastasis in vivo. *LNCRNA00673* silencing, in turn, decreased NOTCH1 and NOTCH3 protein levels, induced apoptosis and inhibited proliferation and invasion of HCC cells, while repressing tumor formation in vivo. These results reinforce the proliferative and metastatic effects of LNCRNA00673 in HCC, via Notch signaling pathway (Chen et al. 2017a).

The tumor suppressor lncRNA *lincRNA-p21* is a direct transcriptional target of the tumor suppressor protein p53, and it also feeds back to upregulate p53 gene expression. In vitro overexpression of *lincRNA-p21* decreased the levels of the Notch signaling proteins HES1 and NICD, as well as the EMT-related proteins N-cadherin and SNAI1, while increasing E-cadherin and Claudin-1 levels. Quite the opposite was observed with *lincRNA-p21* silencing, as occurs in HCC tissues and cell lines. As expected, *lin-*

cRNA-p21 overexpression inhibited HCC invasion in vitro, and decreased lung metastasis of HCC in vivo. These results indicate that decreased levels of lncRNA-p21 in HCC are associated with activation of the Notch signaling pathway, inducing EMT and contributing to HCC metastasis (Jia et al. 2016).

Furthermore, among the lncRNAs differentially expressed in liver fibrosis, the *lnc-LFAR1* (*liver fibrosis-associated lncRNA1*) is specifically upregulated in hepatic stellate cells (HepSCs) during fibrogenesis. Lnc-LFAR1 contributes to phosphorylation of the transcription factors SMAD2/3 in cytoplasm, and directly binds them. The association between lnc-LFAR and SMAD2/3 promotes their binding to the promoters of target genes, such as *NOTCH2*, *NOTCH3*, *HES1*, *TGFB*, *SMAD2*, and *SMAD3*, thus activating Notch and TGFβ pathways. This results in a positive feedback loop, which reactivates SMAD2/3, TGFβ, and Notch pathways, inducing proliferation of HepSCs and hepatocyte apoptosis, leading to liver injury and fibrosis (Zhang et al. 2017a).

LncRNAs and Notch Signaling in Gastrointestinal Tissue

The Notch signaling pathway presents specific expression and distribution patterns in gastrointestinal tissues, being a key determinant of gastrointestinal epithelial self-renewal differentiation and cancer (Guilmeau 2012; Yao et al. 2017). It has been suggested as one of the most commonly activated pathway in malignancies of the gastrointestinal tract, including stomach and colorectal cancer (Pan et al. 2017).

The expression of *ANRIL* (*antisense noncoding RNA in the INK4 locus*) is increased in gastric cancer (GC) cell lines. This lncRNA negatively regulates the expression of miR-99a, which represses *BMI1* (*B-lymphoma Mo-MLV insertion region 1*, also known as *polycomb ring finger 1*) translation. Increased ANRIL levels, and consequently of BMI1 protein, inhibit apoptosis and increase the mRNA and protein levels of Notch1, mTOR, and p70S6K. Moreover, *ANRIL* knockdown decreases BMI1 protein levels, which induces apoptosis and suppresses cell viability, migration, and invasion. These results suggest that ANRIL promotes gastric tumorigenesis through activation of BMI1, and consequently of Notch and mTOR signaling pathways, via miR-99a suppression (Liu et al. 2018a).

In GC cell lines resistant to cisplatin treatment, mRNA and protein levels of Notch1 are increased. NOTCH1 overexpression, in turn, upregulates the lncRNA *AK022798*, as well as the protein levels of MRP1 (Multidrug Resistance associated Protein I) and P-glycoprotein, both related with drug resistance. As expected, the overexpressed NOTCH1 promotes drug resistance and decreases apoptosis in these cell lines. Moreover, silencing of *AK022798* decreased MRP1 and P-glycoprotein, and induced caspase-3 and apoptosis, reducing cell viability of resistant GC cells (Hang et al. 2015).

In colorectal cancer (CRC), the lncRNA FOXD2-AS1 acts as a tumor promoter, being enhanced in CRC tissues and cells lines. *FOXD2-AS1* knockdown decreased the levels of HES1, NICD, N-cadherin and SNAI1, while increased E-cadherin. Moreover, the downregulation of *FOXD2-AS1* suppressed CRC cell proliferation, invasion, and migration. These results reveal that FOXD2-AS1 appears to be a critical player in CRC, and might promote CRC development via EMT and Notch signaling pathway (Yang et al. 2017).

FAM83H-AS1 is another lncRNA overexpressed in CRC tissues and cell lines. The higher levels of FAM83H-AS1 were positively correlated with *NOTCH1* and *HES1* expression levels, and associated with clinical features and poor prognosis of CRC. *FAM83H-AS1* knockdown inhibited cell proliferation and migration, while inducing apoptosis. Interestingly, the antiproliferative effects of this silenced *FAM83H-AS1* were reversed by the NOTCH1 activator JAG1. Thus, FAM83H-AS1 seems to present a proliferative role in CRC, through activation of Notch signaling pathway (Lu et al. 2017).

Furthermore, the lncRNA Lnc34a is upregulated in CRC and in colon cancer stem cells (CCSC), where it epigenetically represses the

expression of miR-34a (Wang et al. 2016), a miRNA which inhibits CCSC self-renewal by targeting molecules of Notch and Wnt signaling pathways (Bu et al. 2013). Lnc34a increases self-renewal and tumorigenesis of CCSCs, being correlated with CRC proliferation and progression (Wang et al. 2016). However, understanding of the direct cross talk between Lnc34a and the Notch signaling still requires investigation.

LncRNAs and Notch Signaling in Female Reproductive System Tissues

The female reproductive system is a complex system designed to carry out several functions, comprising internal and external organs, such as the uterus (including the endometrium and cervix), ovaries, the genitals, and a crucial transient organ—the placenta. Due to its complexity, maintaining the global homeostasis of female reproductive tissues also represents an overwhelming task. Disturbance of such homeostasis may result in some common associated outcomes, as premature ovarian failure and gynecological malignancies (including endometrial, cervical and ovarian cancers) (Bates and Bowling 2013; Zavesky et al. 2015).

Different lncRNAs seem to interact with the Notch signaling pathway molecules, affecting the female reproductive homeostasis. One example is MEG3, already mentioned in this chapter as a tumor suppressor lncRNA, whose expression is decreased in endometrial cancer (EC) tissues and cell lines. *MEG3* overexpression inhibits cell proliferation and decreases the mRNA and protein levels of Notch1 and Hes1, *both* in vitro and in vivo. Interestingly, the presence of the NOTCH1 activator JAG1 reversed the inhibition properties of MEG3 on cell proliferation. On the other hand, *MEG3* silencing promoted cell proliferation, which could be repressed by the NOTCH1 inhibitor GSI. Taken together, the lncRNA *MEG3* is downregulated in EC, which increases cell proliferation through the Notch signaling pathway (Guo et al. 2016). In concordance with its suppressive effects, MEG3 also presented lower levels in women with poor/moderate-differentiated EC, than in those with well-differentiated EC. *MEG3* overexpression inhibited EC cell viability, migration, and invasion, and repressed the expression of molecules involved in distinct signaling pathways, such as NOTCH1, VEGFA, m-TOR, P70S6K, BCL-XL, and PI3K. Likewise, MEG3 suppressed tumor growth in vivo. Therefore, it is clear that MEG3 stands as a tumor-suppressive agent, reducing EC tumorigenesis and progression through different pathways, as both Notch and PI3K signaling pathways (Sun et al. 2017).

Besides the involvement of the lncRNAs in EC, others have been related to cervical cancer (CC) development. The lncRNA SRA (*Steroid Receptor RNA Activator*), whose expression is enhanced in CC, was suggested to induce cell proliferation and tumor invasion through upregulation of MMP-9 (Matrix Metalloproteinase-9), MMP-2 (Matrix Metalloproteinase-2) and VEGFA expressions, as well as the Notch signaling pathway in CC cell lines. Knockdown of SRA decreased cell proliferation, migration and invasion, as also repressed MMP-9, MMP-2, VEGFA, NOTCH1, HES1, P300, β-catenin, Vimentin, SNAI1, and TWIST expressions, while overexpressed E-cadherin. Thus, the lncRNA SRA seems to function as a key regulator of Notch and other signaling mechanisms involved in CC, contributing to cervical carcinogenesis (Eoh et al. 2017).

Another important lncRNA involved in CC is the aforementioned HOTAIR. First, it was found upregulated in CC tissues and associated with lymph node metastasis and reduction of overall survival. *HOTAIR* knockdown in CC cell lines reduced cell proliferation, migration, and invasion, and also decreased the expressions of *VEGF*, *MMP-9* and EMT-related genes (Kim et al. 2015). Subsequently, HOTAIR was also shown to increase CC growth and invasion by targeting the Notch pathway. HOTAIR levels are higher in the serum of CC patients, being associated with tumor size and lymph node metastasis. *HOTAIR* overexpression in CC cell lines also enhances cell proliferation and invasion, while its knockdown inhibits these malignant properties and increases apopto-

sis. In vivo overexpression of *Hotair* revealed this lncRNA as a strong inducer of tumor growth, modulating the expression of EMT and of Notch/Wnt signaling pathway-related genes. Moreover, it resulted in increased mRNA and protein levels of Notch1, Hes1, p300, β-catenin, N-cadherin, Vimentin, SNAIL, and TWIST. These results reinforce HOTAIR's role as a tumor promoter in CC through EMT and the Notch signaling pathway, affecting cell growth, invasion and cancer metastasis (Lee et al. 2016).

Besides its impact on cervical tissue, HOTAIR also affects the ovarian tissue homeostasis. *HOTAIR* is downregulated in ovarian tissues and serum samples of patients with premature ovarian failure, accompanied by a downregulated *NOTCH1*. In ovarian cell lines, *HOTAIR* overexpression increased NOTCH1 protein levels and reduced apoptosis, whereas the use of GSI inhibited NOTCH1 and increased apoptosis. Hence, *HOTAIR* overexpression seems to be an interesting strategy to prevent premature ovarian failure by inducing NOTCH1 (Zhao and Dong 2018).

LncRNAs and Notch Signaling in Breast Tissue

During normal development, the Notch signaling pathway controls cellular growth and differentiation of breast epithelial cells. Contrarily, deregulation of Notch signaling components (especially those related to angiogenesis) impacts breast tissue homeostasis, and as consequence, breast cancer (BC) may arise (Lamy et al. 2017; Kontomanolis et al. 2018). Such deregulation seems to occur at early noninvasive stages of BC (Mittal et al. 2009; Zardawi et al. 2010), being crucial for tumor progression as well (Yuan et al. 2015). Although a valuable amount of studies support the Notch signaling involvement in breast tissue homeostasis, as well as on BC development, few studies report the cross talk between lncRNAs and Notch signaling in such contexts.

One example is the aforementioned lncRNA GAS5 and its regulator NOTCH1. In contrast to certain tumors, NOTCH1 appears to promote breast cells proliferation by negatively regulating *GAS5* expression, which was demonstrated both in vivo and in vitro. Increased Notch1 mRNA and protein levels were observed in BC, in contrast to decreased *GAS5* expression. Additionally, *NOTCH1* silencing resulted in increased GAS5 levels, which in turn, inhibited cell proliferation. Thus, an important interplay between NOTCH1 and GAS5 is suggested to occur in BC development (Pei and Wang 2015).

Moreover, the well-known lncRNA H19 (*imprinted maternally expressed transcript*) may also cross talk with components of the Notch signaling pathway, affecting breast cells phenotype. Further, H19 is an estrogen-regulated breast oncogene, involved in estrogen-induced proliferation of both normal and malignant estrogen receptor positive (ER^+) breast epithelial cells, acting as a modulator required for endocrine therapy resistance in ER^+ BC cells (Basak et al. 2015). Increased *H19* expression occurred in endocrine therapy resistant (ETR) cells after chemotherapy, while decreased expression of *H19* overcame resistance to those agents. Interestingly, the Notch signaling pathway regulates *H19* expression in ETR cells. The use of a pan Notch inhibitor reversed resistance to Tamoxifen and Fulvestrant in an H19-dependent manner in these cells. Thus, it demonstrates the interplay between H19 and Notch elements, and its crucial impact on breast cells fate (Basak et al. 2018).

LncRNAs and Notch Signaling in Lung Tissue

The pulmonary homeostasis is maintained by tightly regulated processes, during lung development, during childhood lung growth, and at the "aging lung" (Bush 2016; Lloyd and Marsland 2017). Deregulation of lncRNAs interacting with the Notch signaling pathway may lead to lung diseases, as idiopathic pulmonary fibrosis (IPF) and lung malignancies.

IPF is a chronic interstitial lung disease causing fibrotic remodeling of the peripheral lung, leading to respiratory failure. The lncRNA *MEG3* is overexpressed in IPF epithelial cells, along with basal cell–related genes (such as *TP63*, *SOX2*, *STAT3*,

and *KRT14*), being associated with enhanced cell migration and regulation of basal cell identity. Moreover, MEG3 suppressed the *HES1* and *HEY1*, as well as the transcription factors TP73 and SOX2, supporting a role for MEG3 in a gene regulatory network of basal cell differentiation. In addition, MEG3 induced EMT/cell migration–related genes (such as *SNAI2* and *YAP1*), all of which are predicted to interact in a network activated in IPF epithelial cells. Together, these data demonstrate that MEG3 induced basal cell genes and suppressed genes associated with terminal differentiation of airway cells, supporting its role in basal cell differentiation, which may contribute to IPF tissue remodeling (Gokey et al. 2018).

Decreased levels of the lncRNA LET (*low expression in tumor*) were observed in non-small cell lung cancer (NSCLC) samples and cell lines. Decreased *LET* expression was associated with advanced tumor stages and poorer overall survival of NSCLC patients. *LET* overexpression suppressed cell proliferation, migration and invasion, and promoted cell cycle arrest and apoptosis in NSCLC cells. As expected, *LET* knockdown caused an opposite effect, suggesting LET's tumor-suppressive role in NSCLC. Its overexpression in NSCLC cells reduced the levels of NICD1, while *LET* knockdown increased it. Accordingly, NSCLC lung tissues with high levels of LET presented lower levels of NICD1 (Li et al. 2018a).

The *X-inactive specific transcript* (*XIST*) was one of the first lncRNAs described and has a well-known role in X inactivation in females. *XIST* is upregulated in NSCLC tissues and cell lines. XIST sponges miR-137, a miRNA which in turn, targets Notch1 mRNA. Moreover, the XIST-miR-137 axis was observed to regulate cell proliferation and TGF-β1-induced EMT, suggesting that the XIST-miR-137-Notch-1 axis may play an important role in NSCLC progression (Wang et al. 2018b).

The lncRNA LINC00630 activates the Notch signaling pathway and promote metastasis in NSCLC cells, interacting with HDAC1 (Histone Deacetylases 1) and DDX23 (DEAD-box helicase 23). *LINC00630* is upregulated in NSCLC, especially in metastatic tumor tissues, and it is associated with tumor size and stage. *LINC00630* overexpression increased cell proliferation and metastasis in vitro and in vivo, while the opposite effects occurred with *LINC00630* knockdown in vitro. Moreover, LINC00630 levels are positively correlated with HDAC1 and DDX23 in NSCLC tissues, both identified with LINC00630-binding elements. In addition, the Notch signaling pathway is the mainly involved in the affected functional processes in silenced-*LINC00630* cells. These data suggest that LINC00630 could bind with HDAC1 and DDX23, and regulates NSCLC cells invasion and proliferation, possibly through the Notch signaling pathway (Mao et al. 2017).

The potential cross talk between LINC00630 and the Notch signaling pathway needs further functional investigation. Similarly, other lncRNAs that have been described in NSCLC, suppressing tumor metastasis by reversing EMT, as well as promoting tumor metastasis by inducing EMT (Chen et al. 2017b), may also regulate cell invasion and proliferation through the Notch signaling pathway. Since this pathway promotes EMT through interaction with several transcriptional factors, lncRNAs that were found to impact EMT may exert their effects through Notch elements. Examples of lncRNAs that suppress tumor metastasis by reversing EMT or promote tumor metastasis by inducing EMT are BANCR (*BRAF-activated noncoding RNA*) (Sun et al. 2014) and MALAT1 (*Metastasis Associated Lung Adenocarcinoma Transcript 1*, also known as *NEAT2*) (Ji et al. 2003; Shen et al. 2015), respectively. The potential involvement between Notch elements and some of the above-mentioned lncRNAs on NSCLC remains to be explored.

LncRNAs and Notch Signaling in T and B Lymphocytes

Notch signaling pathway plays important roles within the development of embryonic hematopoietic stem cells and influences multiple lineage decisions of developing lymphoid and myeloid cells. The role of Notch signaling has been particularly well documented in the T cell-compartment, where NOTCH1/DLL4 interactions are crucial to induce T-lineage dif-

ferentiation at the expense of other hematopoietic lineages (De Obaldia et al. 2013).

The aforementioned lncRNA SRA has been reported as a positive regulator of Notch signaling by associating with the DDX5 protein in T cells. SRA is required for the recruitment of histone acetyltransferase P300 to Notch target genes, acting as a transcriptional activator of these genes (Jung et al. 2013).

The lncRNA RP11-611D20.2 can modulate the expression of *NOTCH1* through *cis*-regulation. RP11-611D20.2 overexpression has been described in pediatric T cell acute lymphoblastic leukemia (T-ALL), reason why it was called "*Notch1 associated lncRNA in T-ALL*" (NALT). In T-ALL, higher NALT levels were correlated with increased *NOTCH1* expression. Moreover, functional studies demonstrated that NALT is a transcriptional activator of *NOTCH1* (Wang et al. 2015).

An interesting study established a lncRNA compendium that is under control of Notch signaling during normal and malignant thymocyte development. The authors investigated lncRNA expression following pharmacological Notch signaling inhibition in the T-ALL cell line CUTLL1, as well as in normal human thymocytes. They identified a total of 40 Notch-driven lncRNAs, thereby revealing a novel layer in the molecular machinery that mediates Notch signaling (Durinck et al. 2014).

A large number of T-ALL specific lncRNAs targeting the Notch pathway were identified by a large scale RNA-sequencing study. The lncRNA *LUNAR* (*Leukemia-induced Noncoding Activator RNA*) is overexpressed in T-ALL and its expression was downregulated after Notch signaling inhibition. This lncRNA stood out as a *cis*-regulator of IGF1R (insulin-like growth factor receptor 1), already described as involved in T-ALL (Trimarchi et al. 2014).

Considering B cells, lncRNA expression can be associated with *NOTCH1* mutations and influence chronic lymphocytic leukemia (CLL). A lncRNA profile was obtained for CLL and compared with subpopulations of normal B cells. Among the differentially expressed lncRNAs in *NOTCH1* mutated CLLs, *lnc-IRF2-3*, *lnc-AC004696.1-1*, and *lnc-BACH1-1* were upregulated, while *lnc-C1orf132-1* was downregulated. All of them were deregulated in CLL subgroups with adverse prognosis (Ronchetti et al. 2016).

Overall, in T cells and probably other hematopoietic cells, lncRNAs regulate the Notch signaling pathway, playing a role in hematopoiesis, lymphocyte development and function, and consequently in disorders related with aberrant T cell growth.

LncRNAs and Notch Signaling in Cardiac Tissue

Different lncRNAs emerged as key players in cardiac differentiation, development, and regeneration, being essential for specific maturation of pluripotent stem cells and/or resident cardiac precursor cells (CPCs) into cardiomyocytes (Ounzain et al. 2015b). The expression of the lncRNA *CARMEN* (*Cardiac Mesoderm Enhancer-associated ncRNA*) was increased in CPCs from human fetal heart, regulating cardiac specification and differentiation (Ounzain et al. 2015a). CARMEN is also a Notch-responsive enhancer, capable of modulating miR-143 and miR-145 expressions in various developmental contexts (Boucher et al. 2011). Evaluating the cardiogenic potential of CPCs isolated from adult human hearts, CARMEN was demonstrated to control the expression of miR-143/145 and target these miRNAs through the Notch signaling pathway. Thus, CARMEN/miR-145/143 axis seems to have an important role in cardiomyocytes lineage specification (Plaisance et al. 2016).

Furthermore, the lncRNA DIGIT (*Divergent to GSC Induced by TGF-b family signaling*) has an important role in endothelial cell growth, migration and tube formation. After *DIGIT* silencing in human microvascular endothelial cells (HMEC-1), a reduction in cell viability, migration, tube-like structure formation, and apoptosis induction was observed. Additionally, DIGIT acts as sponge for miR-134, and the antigrowth, antimigratory, and anti–tube formation functions of *DIGIT* silencing were abolished by *miR-134* suppression. Further, miR-134 targets and inhibits Bm1 mRNA translation, involved in activation of PI3K/AKT

and Notch signaling pathways. In *BMI1* suppressed-cells, protein levels of p-PI3K, p-AKT, NOTCH1, NOTCH2, and NOTCH3 are decreased, while the opposite effects are observed in cells overexpressing *BMI1*. Thus, DIGIT accelerates tube formation of vascular endothelial cells (EC) through indirect activation of BMI1 and of Notch signaling pathway, via miR-134 inhibition (Miao et al. 2018).

An interplay between lncRNAs, miRNAs, and Notch signaling was also shown for atherosclerosis. MiR-103 targets the lncWDR59 (*lncRNA WD Repeated Domain 59*), impeding its interaction and blockage of the Notch1-inhibitor NUMB. This results in decreased NOTCH1 levels, and as consequence, in inhibition of endothelial proliferation. Moreover, miR-103 increases oxidized low-density lipoprotein (oxLDL) levels and hyperlipidemia. It enhances the susceptibility of proliferating ECs to oxLDL-induced mitotic aberrations, characterized by an increased micronuclei formation and DNA damage accumulation, by affecting Notch1-related β-catenin coactivation. Thus, miR-103 programs ECs toward a maladapted phenotype, which may promote atherosclerosis (Natarelli et al. 2018).

Additionally, the aforementioned lncRNA MALAT1 seems to prevent cardiomyocyte injury. Cardiomyocyte cell lines submitted to hypoxia present a decrease in cell viability and upregulated *MALAT1* expression. MALAT1 negatively regulates miR-217, a miRNA which inhibits Sirt1 (Sirtuin 1) translation. On its turn, *SIRT1* overexpression alleviates hypoxia-induced cell injury by activating PI3K/AKT and Notch pathways. *MALAT1* knockdown aggravated the hypoxia-induced cell injury by upregulating miR-217, influencing cell viability, migration, invasion and apoptosis. Moreover, *NOTCH1*, *NOTCH2*, and *NOTCH3* expression levels were decreased after hypoxia treatment and further downregulated after *SIRT1* silencing. Therefore, the MALAT1/miR-217/SIRT1 axis seems to exert an important role in modulating hypoxia-induced cardiomyocyte injury, through the Notch signaling pathway (Yao et al. 2019).

LncRNAs and Notch Signaling in Other Tissues

The importance of lncRNAs has been described in virtually all body tissues, and in several of them, the Notch signaling pathway is also implicated. In addition to those previously mentioned, one-off studies have reported Notch signaling and lncRNAs interaction in diverse other tissues.

Notch signaling has been shown to maintain bone homeostasis by controlling the commitment, differentiation, and function of cells in both the osteoblast and osteoclast lineages (Yamada et al. 2003; Sekine et al. 2012), processes regulated by lncRNAs. The lncRNA LINC00311 is an osteoclast inducing factor by inhibiting *DLL3* expression. Osteoclasts overexpressing *LINC00311* exhibited increased expression and protein levels of *NOTCH2* and *TRPA*, while decreased *DLL3*, *NOTCH1*, *JAG1*, and *HES1*. Moreover, overexpression of *LINC00311* is associated with osteoclast proliferation and inhibition of apoptosis (Wang et al. 2018c). Furthermore, the aforementioned lncRNA H19 is induced in MSCs by BMP9 (*Bone Morphogenetic Protein 9*, an important signaling for osteogenic activity) in early stages of BMP9 stimulation. H19 is coexpressed with osteogenic markers and influences osteogenic differentiation from MSCs, by modulating the expression of a group of miRNAs (such as *miR-107*, *miR-27b*, *miR-106b*, *miR125a*, and *miR17*) that can target Notch receptors and/or ligands (Liao et al. 2017).

The lncRNA HULC (*High Upregulated in Liver Cancer*) increases the cell viability of adipose-derived stem cells (ADSCs), and induces the differentiation of these mesenchymal ADSCs into epithelial and smooth-muscle-like cells. This physiological process is regulated by BMP9, with participation of the Wnt/β-catenin pathway and deactivation of the Notch signaling pathway. In *HULC* overexpressed ADSCs, both transcriptional and protein levels of BMP9 were increased, resulting in upregulation of *WNT3A*, *WNT5A*, and *B-Catenin* and downregulation of *NOTCH1*,

NOTCH2, and *NOTCH3*, which in turn, induced the expression of epithelial and smooth-muscle protein markers. After *BMP9* silencing, the upregulation effects on Wnt/β-catenin molecules and downregulation effects in Notch signaling molecules were reversed, as well as the epithelial/smooth expression patterns. The results suggest that HULC promotes differentiation of ADSCs into epithelial and smooth muscle-like cells, via the BMP9/Wnt/β-catenin/Notch signaling network, representing a useful approach for epithelial reconstruction (Li et al. 2018b).

In the ocular tissue, the aforementioned lncRNA gene HOTAIR is upregulated in retinoblastoma tissues and Y79 retinoblastoma cell line. Concomitant with HOTAIR, the protein levels of NOTCH1 and JAG1 are increased in Y79 cells. In vitro knockdown of *HOTAIR* reduced proliferation and invasion, rescued the expression patterns of key cell cycle-related proteins and decreased NOTCH1, JAG1, HES1, and HEY1 levels. Moreover, *Hotair* knockdown also inhibited Notch signaling and tumor sizes in vivo. These results suggest HOTAIR as a tumor-promoting gene in retinoblastoma, due to Notch signaling activation (Dong et al. 2016). Still regarding intraocular cancer, the lncRNA gene *PAUPAR* (*PAX6 Upstream Antisense RNA*) is downregulated in uveal melanoma (UM) tissues and cell lines. PAUPAR negatively regulates the *HES1* gene (via histone H3K4 methylation), which in turn, is induced in UM. The overexpression of *PAUPAR* suppressed *HES1*, reducing tumor colony formation and migration in vitro, and tumor progression in vivo. Thus, PAUPAR seems to be an oncosuppressor lncRNA in UM, through interaction with Notch signaling pathway (Ding et al. 2016).

The lncRNAs *MALAT1* and *UCA1* (*urothelial cancer associated 1*) are upregulated in tongue cancer (TC), especially in tongue metastatic tissues. Both lncRNAs target miR-124, which inhibits Jag1 mRNA translation, getting suppressed in TC (Zhang et al. 2017b, 2019). By miR-124 inhibition, MALAT1 increases *JAG1* expression, promoting TC growth and metastasis (Zhang et al. 2017b). UCA1 shows similar mechanisms in TC, with the addition of EMT and TGFβ1 stimulation. Overexpression of TGFβ in tongue cell lines also induces *UCA1* expression, increasing the protein levels of Vimentin, while decreasing E-cadherin. *UCA1* knockdown increased E-cadherin and reduced Vimentin and cell invasion capacity. Moreover, inhibition of miR-124 induced TGFβ1 stimulation, and consequently, the protein levels of Vimentin, JAG1, and NOTCH1. These results indicate that the UCA1/miR-124 axis regulate TGFβ1-induced EMT and TC invasion, via Notch signaling activation (Zhang et al. 2019).

Another example of Notch regulation by a miRNA-lncRNA effect can be observed in prostate tissue and prostate cancer (PrC). The lncRNA PVT1 (*plasmacytoma variant translocation 1*) regulates PrC cell viability and apoptosis depending on miR-146a. The miR-146a was downregulated and negatively correlated with PVT1 levels in PrC. MiR-146a can be regarded as an inhibitor of tumor growth and migration, reducing *NOTCH1* translation and downregulating the Notch pathway (Liu et al. 2016a). Furthermore, the expression of the lncRNA *GHET1* (*gastric carcinoma high expressed transcript 1*) is upregulated in PrC tissues and negatively correlated with *KLF2*, a tumor suppressor gene. *GHET1* knockdown in PrC cell lines inhibited proliferation and induced cell cycle arrest, while promoted apoptosis and increased KLF2 expression. Moreover, suppression of *GHET1* decreased the protein levels of NOTCH1 and HIF-1α, which was attenuated by KLF2 silencing. These results demonstrate the tumor effects of GHET1 on prostate, through suppression of KLF2 and Notch signaling activation (Zhu et al. 2019).

Last but not least, expression of the lncRNA gene *SNHG12* (*Small Nucleolar RNA Host Gene 12*) is upregulated in nasopharyngeal cancer (NPC) tissues and cell lines, being associated with poor prognosis. Silencing of *SNHG12* inhibited proliferation and induced apoptosis of NPC cells, while upregulated *E-cadherin* and downregulated *Vimentin* and *N-cadherin* expressions. Moreover, *SNHG12* knockdown decreased the NOTCH1, HES1 and P21 protein levels. These results suggest that SNHG12 pro-

motes nasopharyngeal carcinogenesis, through activation of EMT and Notch signaling pathway (Liu et al. 2018b).

Taken all together, these examples highlight Notch signaling pathway complexity and add lncRNAs as important players in the modulation of this pathway.

Conclusions

lncRNAs are noncoding transcripts with specific expression patterns in tissues and with the ability to interact with different molecules, regulating pre- and posttranscriptional expressions, through various molecular mechanisms. In this chapter, we revise the lncRNAs described to interact with distinct Notch signaling molecules, whose deregulation affect the expression patterns of different genes, contributing to the loss of tissue homeostasis (Table 8.1).

The lncRNAs affect (and are affected by) Notch signaling components and modify downstream pathways, influencing cell processes as differentiation, proliferation, cell cycle, apoptosis, migration, invasion, and others. Aberrant expressions of lncRNAs and alterations in their interactions with Notch signaling molecules were described in various tissues. In several of them, such deregulations are highly associated with tumorigenesis. Thus, lncRNAs are increasingly being proposed as therapeutic targets and biomarkers. However, despite the increasing number of studies investigating the cross talk between lncRNAs and proliferative signaling pathways, there is still a lot to explore. Therefore, lncRNAs can be better investigated and would contribute to the diagnosis, treatment and prognosis of cancer and other diseases.

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Insulin-dependent Non-canonical Activation of Notch in Drosophila: A Story of Notch-Induced Muscle Stem Cell Proliferation

Rajaguru Aradhya and Krzysztof Jagla

Abstract

Notch plays multiple roles both in development and in adult tissue homeostasis. *Notch* was first identified in *Drosophila* in which it has then been extensively studied. Among the flag-ship Notch functions we could mention its capacity to keep precursor and stem cells in a nondifferentiated state but also its ability to activate cell proliferation that in some contexts could led to cancer. In general, both these functions involve, canonical, ligand-dependent Notch activation. However, a ligand-independent Notch activation has also been described in a few cellular contexts. Here, we focus on one of such contexts, *Drosophila* muscle stem cells, called AMPs, and discuss how insulin-dependent noncanonical activation of Notch pushes quiescent AMPs to proliferation.

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Muscle stem cells · Noncanonical Notch signaling · Insulin · Proliferation · *Drosophila*

Introduction

It is now more than hundred years when Thomas H. Morgan identified in *Drosophila* alleles causing the notched-wing phenotypes, which later turned out to be mutants of a highly important gene, called then *Notch*. Over the last decades, hundreds of studies allowed to establish that Notch pathway regulates key cell decisions both in the development and in the adult stage of most multicellular organisms including humans. In a large portion of cellular contexts studied so far the Notch expression was associated to the undifferentiated cell populations and its function dedicated either to maintain cell stemness or to promote cell proliferation. It is now well known that depending on cell contexts Notch could interact with other signaling pathways that further diversify its involvements. In this chapter we discuss interplay between insulin/TOR and Notch pathways in *Drosophila* muscle stem cells that push them from the quiescent state to proliferation. In this particular context insulin provides cues to activate Notch in the ligand-independent way involving ubiquitin ligase Deltex and a component of ESCRT-III complex, Shrub. This noncanonical Notch acti-

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vation appears to be a way to make muscle stem cells competent to enter cell cycle and to leave quiescent state for which canonical Notch also plays a role. But before presenting this particular Notch in work scenario, we will first travel one century back.

Drosophila and Notch: A Century-Old Tale

Although Thomas H. Morgan and his graduate students popularized the use of *Drosophila* as a model organism for understanding genetics, and discovered many mutant phenotypes, it was John S. Dexter who observed the notched-wing phenotype in his stock of flies during 1914 (Dexter 1914). While working in Olivet College, Michigan, he observed a nick or notch in the wingtip of a few *Drosophila* lines in his lab, which came to be known as the notched-wing phenotype, or Notch. Three years later, Morgan identified the alleles responsible for this phenotype (Morgan 1917), and in the following years, with the help of his students, found additional alleles associated with the Notch phenotype (Morgan 1928). Most of the alleles were lethal, but they would give rise to the classical nick phenotype in the wingtip and bristle phenotype specifically in female flies, indicating that this locus might be associated with the X chromosome (Morgan 1928). This observation was finally confirmed by Spyros Artavanis-Tsakonas and Michael W. Young more than 60 years later (in 1980) by cloning and sequencing of the mutant *Notch* locus (Wharton et al. 1985; Kidd et al. 1986). Several other loci were later discovered that gave similar notched-wing phenotypes, but also variations in bristle number, neurogenic phenotypes and embryonic developmental defects (Lieber et al. 1993; Zhong et al. 1997; Go and Artavanis-Tsakonas 1998). This broad spectrum of associated phenotypes indicated that there were several downstream effectors of the Notch pathway involved in a plethora of biological processes (Artavanis-Tsakonas et al. 1995).

Highly Conserved Molecules of the Notch Pathway: From Worms to Humans

The discovery of orthologous *Notch* genes in *Caenorhabditis elegans*, termed *lin-12* and *glp-1*, led to the first insights that the pathway had a major role in the development of multicellular organisms (Greenwald et al. 1983; Austin and Kimble 1987; Priess et al. 1987). Several mutant alleles of both loci in *C. elegans* showed severe cell fate transformations in different tissues. *Lin-12* was shown to regulate cell fate decisions dose-dependently in deciding the specification of one cell type over the other (Greenwald et al. 1983), and *glp-1* in regulating the mitotic versus meiotic cell divisions in the germ line precursors (Austin and Kimble 1987; Priess et al. 1987). It is well known that Notch signaling plays an important role across taxa, controlling a wealth of biological processes, such as cell proliferation, cell fate decisions, stem cell self-renewal, and differentiation (Artavanis-Tsakonas et al. 1999; Bray 2006; Fortini 2009; Andersson et al. 2011; Liu et al. 2010; Hori et al. 2013; Penton et al. 2012), and these functions have been extensively reviewed in Anderson et al. (2011). The Notch pathway has also been shown to be involved in several pathological conditions in mammals, including cancer (Penton et al. 2012).

Although it is now well established that the Notch pathway regulates several important molecular mechanisms both in the development and in the adult stage of most multicellular organisms, the core molecules involved in the pathway are surprisingly few in number compared for example to the Wnt, RTK, or BMP pathways (Andersson et al. 2011). Right from the initial discovery of Notch molecules in worms and flies it was clear that the upstream signaling cascade began with a transmembrane receptor, named Notch receptor in flies (*Drosophila*) and Lin-12 in worms (*C. elegans*) (Greenwald 1998). A single Notch receptor performs the entire function in the lower organisms, but in mammals there are four different Notch receptors involved in the core pathway (Sato et al. 2012). In all organisms, the Notch receptor is made up of a

type I transmembrane protein (Sato et al. 2012). Each receptor is made up of a large extracellular domain, which often consists of 29–36 EGF repeats, three Lin-12 Notch repeats and a heterodimerization domain. This is followed by a single transmembrane domain, which in turn continues as a large intracellular domain consisting of an RBPJk association module (RAM) domain, a nuclear localization signal (NLS) domain, seven ankyrin repeats (ANK), and the proline/glutamic acid/serine/threonine-rich (PEST) domain (Gordon et al. 2008, 2009; Sanchez-Irizarry et al. 2004; Steinbuck and Susan 2018; Kopan and Ilagan 2009).

The activation of the Notch pathway is initiated when appropriate ligands from adjacent cells bind to the receptor. Similar to Notch receptors, the Notch ligands also have a typical Type I transmembrane domain (Sato et al. 2012; Gordon et al. 2008, 2009; Sanchez-Irizarry et al. 2004; Steinbuck and Susan 2018; Kopan and Ilagan 2009). Notch ligands belong to a small family of proteins that depends on the organism (Delta/Serrate for *Drosophila*, Lag-2/Apx-1 for *C. elegans* and Delta 1–4/Jagged 1–2 for mammals) (Bray 2006; Borggrefe and Oswald 2009; D'Souza et al. 2008). Binding of the appropriate ligand leads to a structural modification, and finally to a series of proteolytic cleavages on the Notch receptors. Of these, the main ones are S2 cleavage near the extracellular domain by ADAM (a disintegrin and metalloprotease) secretase and an intramembrane S3 cleavage by γ-secretase releasing the NICD (Notch intracellular domain) fragment which migrates toward the nucleus (Bozkulak and Weinmaster 2009; Van Tetering et al. 2009; Mumm et al. 2000; Li et al. 2009; Bergmans and De Strooper 2010; Okochi et al. 2002). In the nucleus, the active NICD binds, with high affinity, with a class of transcription factors, termed CSL, (CBF1 in mammals, Su(H) in *Drosophila*, and Lag1 in *C. elegans*) and with some coactivators to switch on the expression of Notch downstream genes such as E(spl) "Enhancer of split complex" (in flies) and HES family (in mammals). These downstream effectors carry out the diverse functions in different tissues initiated by the Notch signaling (Bray 2006; Fortini 2009). This mode of the Notch pathway is part of the canonical mode of activation that contributes to most of the cellular functions of the Notch. However, there is ample evidence for the presence of noncanonical Notch signaling in which the activation of the Notch pathway occurs by binding of the receptor with noncanonical Notch ligands or without the cleavage of intracellular domain or interaction of the downstream effectors, which are not part of CSL. These modes of noncanonical activation of the Notch pathway are reviewed in D'Souza et al. (Steinbuck and Susan 2018; D'Souza et al. 2010) and discussed here in the following subsections.

Notch Signaling and Cell Proliferation

Among the multitude of molecular functions regulated by Notch signaling, its effect on the rate of cell proliferation is of great importance for studying development and cancer (Bolós et al. 2007). During the development of multicellular organisms, three key steps dominate the major processes: specification of the cell precursors, proliferation of the progenitor cells committed toward a particular lineage, and finally the differentiation process that brings the cells an armory of proteins to perform the specified biological functions in that lineage. Notch signaling was found to have a crucial role in both specification and proliferation mechanisms almost two decades ago (Artavanis-Tsakonas et al. 1995; Go et al. 1998), and it is now well known that Notch and its target genes control aspects of specification, proliferation and in a few cases cell fate decisions (e.g., sensory organ formation in *Drosophila*) (Huang et al. 1991; Blochlinger et al. 1990). However, in all these scenarios the Notch expression is confined to the undifferentiated cell populations. The presence of Notch pathway components tends to inhibit the differentiation mechanism. The interconnection between the cell lineage progression and Notch signaling is discussed in Koch et al. (2013), indicating that the level of Notch is instructive in the fine tuning of the above processes. Most of the

differentiated cells do not express Notch, except for some cells arising from the neural stem cell division. Here, Notch tends to accumulate in the differentiated accessory cells like glia, and is absent from its sibling neuronal cells (Furman and Bukharina 2008; Huang et al. 1991; Blochlinger et al. 1990; Koch et al. 2013). This mode of differential expression depends on the asymmetric segregation of Numb and Hairless, strong antagonists of the Notch signaling pathway (Roegiers and Jan 2004; Wirtz-Peitz et al. 2008; Lu et al. 1998).

In this chapter we focus mainly on the role of Notch signaling in initiating the proliferation of previously quiescent cells. The multifaceted role of Notch signaling during development has been thoroughly investigated in the past two decades. The large number of individual functions of Notch in the development of several mammalian tissues (summarized in Andersson et al. 2011) suggests that the Notch pathway might regulate several unrelated processes, but deeper observation indicates that all these processes are the outcome of three important cellular mechanisms; specification, proliferation and differentiation. Proliferation is the process through which the developing embryo will grow in size by increasing cell numbers. It is one of the highly regulated developmental mechanisms in which the slightest anomaly will result in variation in expected cell numbers, and lead to abnormal development (Thomas 2005). The orchestrated coordination between proliferation and programmed cell death defines the total cell number and thereby the final size of the tissues, organs and the organism as a whole at the end of development (Thomas 2005). Proliferation can also occur in the adult stage, resulting in tissue regeneration or homeostasis (Krafts 2010). Like in embryonic stages, any abnormalities in the proliferation events in the adult stage can also lead to pathological conditions and in particular to cancer (Farber 1995; Feitelson et al. 2015). As discussed below, other signaling pathways, and specifically the insulin/PI3K/TOR pathway, may influence tissue growth and cell proliferation, suggesting interconnection with Notch (Baker 2009; Gancz and Gilboa 2013).

Insulin/PI3K/TOR Pathway in Regulating Tissue Growth and Metabolism

In humans, the insulin peptide controls glucose and lipid metabolism, thereby regulating systemic energy homeostasis (Sharma et al. 2008). Mammalian insulin secreted by pancreatic beta cells binds to insulin receptors (IR) and insulin-like growth factor receptors (IGF-1) activating a complex signal transduction mechanism involving several key players, such as PI3K, FOXO, mTOR, Akt, aPKC, Ras, 4EBP, and S6K, and ultimately leading to tissue growth and metabolic responses (Boucher et al. 2014; De Meyts 2016; Bevan 2001). Most of the components, mechanisms and functions of the insulin/TOR pathway are well conserved in *Drosophila*. Like in mammals, it plays a crucial role in energy metabolism and in maintaining tissue/cell growth and proliferation (Grewal 2009). In *Drosophila* ovarian tissue it has been found that the insulin/TOR pathway is not only required for the maintenance of germ-line stem cell populations (Hsu and Drummond-Barbosa 2009; Baker 2009; Gancz and Gilboa 2013; LaFever et al. 2010; Chou and Hsu 2017; Eliazer and Buszczak 2011), but is also essential for controlling the growth rate of the developing egg relative to the surrounding somatic cell sheet (Mendes and Mirth 2016; Cavaliere et al. 2005). However, it was only recently that the source of insulin production and the nature of the insulin-like peptides in *Drosophila* were discovered (Kannan and Fridell 2013). The counterparts of insulin in *Drosophila*, termed insulin-like peptides (DILPs) are produced mainly by insulin-secreting cells (ISCs) located in the brain (Kannan and Fridell 2013). In *Drosophila*, eight DILPs have so far been discovered, and the ISCs are known to secrete three of them, DILP 2, 3, and 5, that are responsible for most of the canonical insulin signaling in *Drosophila* as summarized in Nässel et al. (2015). However, a few recent studies indicate that some of the key DILPs, especially those involved in cell proliferation, are secreted by nonneuronal tissues such as glial cells and larval body wall muscles, illustrating the additional endocrine

activity of these tissues (Chell and Brand 2010; Sousa-Nunes et al. 2011; Aradhya et al. 2015). Similarly, the novel role of the insulin/TOR pathway in initiating the cell proliferation from the quiescent state (Chell and Brand 2010; Sousa-Nunes et al. 2011; Aradhya et al. 2015) is also a recent discovery, and is discussed in detail further on in the chapter.

Drosophila Adult Muscle Precursors (AMPs); A Model to Study Muscle Stem Cells

Adult stem cells are the main source of postnatal tissue homeostasis and repair. During growth of all multicellular organisms, a pool of undifferentiated and totipotent embryonic stem cells gives rise to all types of cells required for functioning of the adult organism (https://stemcells.nih.gov/info/2001report/chapter4.ht). On the other hand, adult stem cells, though formed during embryonic development, reside in the differentiated tissues as dormant stem cells that can be reactivated (Rumman et al. 2015). In the past few decades, evidence for undifferentiated cells present throughout fully differentiated tissues in postnatal organisms has progressed with the discovery, analysis and use of adult stem cells in several degenerative diseases (Conrad and Huss 2005; Boyette and Tuan 2014; Prentice 2019). Adult stem cells have now been found in almost every type of tissue in mammals, from skin to gut epithelium, and from nervous to muscle systems (Montagnani et al. 2016). Briefly, once specified, adult stem cells remain quiescent for a long period of time, and when activated by signals from the tissue damage response, they proliferate and migrate to the site of injury to repair the damaged cells (Körbling et al. 2003).

Satellite cells are the adult muscle stem cells situated under the basal lamina of the differentiated skeletal muscle fibers in mammalian and nonmammalian vertebrate species (Wang and Rudnicki 2012). They are solely responsible for the repair of damaged muscle tissue that has undergone recent trauma or injury (Wang and Rudnicki 2012). Unlike the typical skeletal muscle fibers, which are known for their syncytial nature, the satellite cells are mononucleated small cells that do not express any muscle-specific protein such as actin and myosin. Instead they express the paired box transcription factor Pax7, a muscle stem cell marker that is essential for satellite cell specification (Seale et al. 2000). Among other muscle stem cells that we know of, satellite cells were the first to be identified nearly 60 years ago. In 1961, with the help of electron microscopy, two studies (Katz 1961; Mauro 1961) showed the presence of small quiescent cells at the top of the muscle fiber. These were termed satellite cells because of their satellite position at the top of the long muscle fiber. Several non-satellite muscle stem cells had also been discovered through the expression of particular markers, such as Side population cells (SP cells) expressing CD31 antigen (Gussoni et al. 1999; Asakura et al. 2002; Majka et al. 2003), PDGFRα$^+$ cells (Uezumi et al. 2010), and pericytes with the combinatorial expression of NG2, CD146, PDGFRβ (Crisan et al. 2008). Although several studies have indicated that the non-satellite muscle stem cells could contribute to regeneration of the muscle fiber (Boppart et al. 2013), the satellite cells are the main actors in muscle regeneration, and the "workhorses" of skeletal muscle repair. Genetic ablation of Pax7$^+$ cells effectively removed the entire satellite cell population. With this information, four independent studies have shown that the complete loss of satellite cells leads to a drastic reduction in the regeneration of the damaged muscle fiber even though intact non-satellite muscle stem cells were identified in the same animal (Lepper et al. 2011; McCarthy et al. 2011; Murphy et al. 2011; Sambasivan et al. 2011). The identification, heterogeneity, development, and regenerative capacity of the satellite cells are well summarized in Relaix and Zammit (2012).

Adult muscle precursors (AMPs) are the only type of muscle stem cells in *Drosophila* that share several features with mammalian satellite cells. AMPs were first described by Bate et al. (1991), as persistent Twist-expressing myoblasts. AMPs originate from the embryonic muscle progenitors and are the

source of all adult muscles in *Drosophila*. Similar to satellite cells, AMPs remain undifferentiated and mitotically quiescent and are able to proliferate and generate all the adult muscle tissue through either regeneration or de novo formation (Aradhya et al. 2015; Bate et al. 1991, 1993; Broadie and Bate 1991; Figeac et al. 2010). Thus AMPs serves as an attractive model to study muscle stem cells in *Drosophila*, an invertebrate model widely used for its biological closeness to humans. Until recently, studies on AMPs were confined to the pupal stages, in which several groups have studied in detail how the proliferated myoblasts migrate to the muscle formation site, fuse and give rise to the differentiated muscle fibers (Gunage et al. 2017). Except for the initial work described by Bate and his group (Bate et al. 1991, 1993; Broadie and Bate 1991), very few studies have been done on the nature of embryonic AMPs and the mechanisms that maintain their quiescent properties. This might in part be due to lack of suitable genetic tools to study the embryonic AMPs or the molecular markers that could be used to specifically label the AMPs. In the last 5 years, findings by Aradhya et al. have shed more light on the embryonic AMPs (Aradhya et al. 2015). These were made possible by novel AMP-specific genetic tools that were generated as a part of the study. They include an AMP-specific driver line and a GFP sensor line (Aradhya et al. 2015; Figeac et al. 2010). In the initial study, they compiled several molecular markers that could be used to label the AMPs specifically during embryonic stages. Also, through an extensive genetic screen, they found that AMPs were specified via a rhomboid-involving EGF signaling pathway. An interesting outcome of this study using AMP-specific driver lines and live imaging was that AMPs have highly irregular morphologies and are interconnected through long cytoplasmic filopodia (Figeac et al. 2010). This was a surprising result because AMPs in the embryonic stage were thought to be situated far from each other and never to contact until proliferation began, as visualized by staining with the transcription factor Twist (Bate et al. 1991). A few years later the group published another equally far-reaching finding that showed that the differentiated muscle fiber with which AMPs have been closely associated acts as a suitable niche for them to maintain quiescence, and helps survival (Aradhya et al. 2015). Importantly, this study showed for the first time the nature of the molecular mechanisms that drive the AMPs toward proliferation at the end of the larval period (Aradhya et al. 2015), which will be discussed in detail further on in this chapter.

AMPs have always been considered as the source of adult muscles and required only during pupal metamorphosis (Broadie and Bate 1991). However, the satellite-like cells that could be associated with the adult muscles in a quiescent form and have the capacity to regenerate the damaged muscle fibers were not identified until recently, mainly because the markers used to identify undifferentiated AMPs did not show any signal in the mature adult muscles. But very recently VijayRaghavan's group have discovered, with the help of a novel AMP-specific driver line (Zfh1-GAL4) (Puretskaia et al. 2017), that in *Drosophila* there are indeed quiescent and undifferentiated muscle stem cells residing alongside the fully differentiated adult muscle fibers (Chaturvedi et al. 2017). This was aided by another experiment using clonal analysis methods, which proved that these novel adult muscle stem cells were in fact the descendants of the embryonic AMPs reactivated during the larval stages (Chaturvedi et al. 2017). The study indicated that some of the myoblasts escaped from the differentiation during pupal metamorphosis and retained their undifferentiated nature to become adult muscle stem cells in postpupal stages. This study brings the *Drosophila* AMPs even closer to mammalian satellite cells, indicating that they could represent an attractive model system to address unexplored aspects of muscle stem cell functions. The current views on the *Drosophila* muscle stem cells and the genetic tools that are available for further studies have been extensively discussed in Gunage et al. (2017).

Drosophila AMPs and Notch; Multiple Roles in Quiescence, Proliferation, and Differentiation

As discussed earlier in this chapter, Notch expression is a hallmark of the undifferentiated state (Bigas and Espinosa 2018). Except in very rare cases, all stem-like precursors maintain the Notch expression in their undifferentiated state, and the expression of Notch signaling components is reduced as soon as the differentiation has begun (Liu et al. 2010). However, the role of Notch signaling in deciding on two reciprocal biological events for a given cell; quiescence and proliferation, has been found highly variable in different biological contexts and in different tissues/taxa (Liu et al. 2010; Bigas and Espinosa 2018).

Drosophila AMPs arise from muscle progenitors, which divide asymmetrically, giving rise to a muscle founder cell and an AMP cell. Before this division, the muscle progenitors segregate through lateral inhibition from a group of equivalent cells called a promuscular cluster (Carmena et al. 1995). Hence the muscle progenitors retain a higher Notch activity than surrounding cells. The asymmetric cell division leads to unequal distribution of Numb protein, which accumulates in muscle founder cells, thus inhibiting Notch and leading to founder cell differentiation. However, AMPs, founder cell siblings, lack the Numb protein and hence continue to express Notch and its downstream targets (Carmena et al. 1995; Rushton et al. 1995) (Fig. 9.1a).

The targets of the Notch pathway expressed in the AMPs include Hole in muscles (Him) (Liotta et al. 2007) and Zinc finger homeodomain 1 (Zfh1) (Figeac et al. 2010), the *Drosophila* homolog of ZEB, both of which are able to suppress the Mef2-driven myogenic differentiation. Moreover, another Notch target and readout of Notch activity, E(spl)M6 (Rebeiz et al. 2002), was shown to label the embryonic AMPs. Thus Notch signaling, which also regulates quiescence and proliferation of vertebrate satellite cells (Conboy and Rando 2002) could play an evolutionarily conserved role in the maintenance of muscle stem cells.

Along with these markers, the AMPs in the third instar larvae are also known to express Cut, which is associated with Notch signaling in several contexts (Blochlinger et al. 1993; Sudarsan et al. 2001). Once the myoblasts begin to

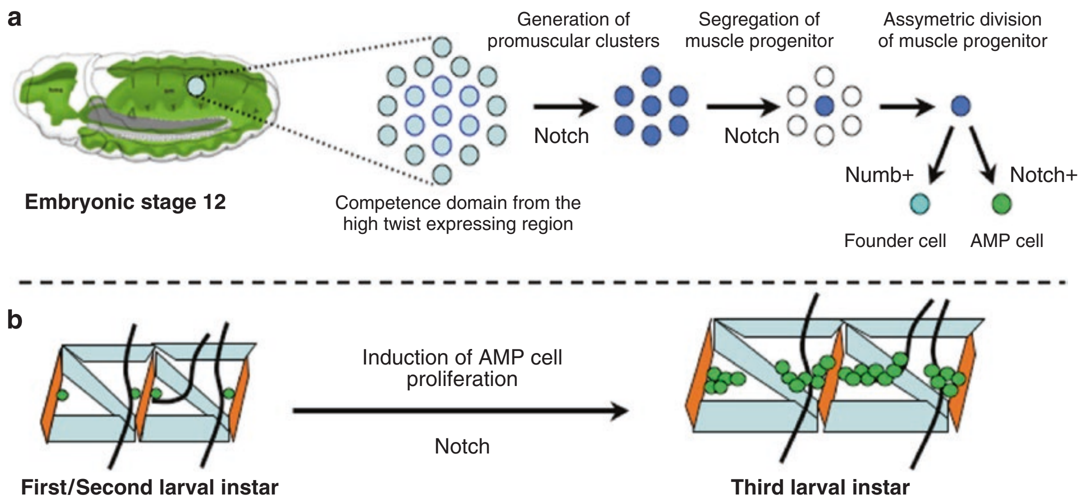

Fig. 9.1 Panel **a**—Stochastic expression of Notch signaling in the lineage of adult muscle precursors (AMPs) during its specification in the embryonic stages. OR The selective expression of Notch signaling only in the cell lineages that gives rise to AMPs during the embryonic development, and its sibling cells lacks Notch expression, hence differentiates. Panel **b**—Diagram illustrating the onset of proliferation of AMPs during the transition between second and third larval instar which is an indicative of different functions of Notch signaling in the same cells, but in different time points

differentiate into mature muscle fibers, the expression of Notch pathway components collapses, while the muscle differentiation markers such as dMef2, start to appear (Roy and VijayRaghavan 1999). The *Zfh1* gene, expressed in both embryonic/larval AMPs (Figeac et al. 2010) and the satellite-like cells associated with the differentiated muscle fibers in the adult fly (Chaturvedi et al. 2017), is also known to be regulated by Notch signaling (Lee and Lundell 2007; Bernard et al. 2010). The enhancer region of the *Zfh1* carries Su(H) binding sites, and a recent study has shown that removal of these sites affects the expression of Zfh1 and influences the behavior of the satellite-like muscle stem cells of the adult *Drosophila* (Boukhatmi and Bray 2018).

Hence we see that Notch signaling is active in AMPs both during quiescence and the proliferative stage, but not during differentiation (Sudarsan et al. 2001; Roy and VijayRaghavan 1999) (Fig. 9.1b). It is hard to imagine how the same signaling pathway can regulate two contrasting processes in the same cell. In vertebrates most of the studies show that Notch acts as a proproliferative agent leading to the accumulation of progenitor cells from the precursors (Grotek et al. 2013). However, it is now clear that Notch can act on both proliferative and quiescent conditions in different stages of development, undifferentiated precursors and cancer cells according to the different circumstances. Although it is not well understood how Notch signaling regulates such diverse processes in the same lineage, it is believed that the varying level of Notch activity itself may be responsible for different biological activities (Perdigoto et al. 2011; Ninov et al. 2012). Furthermore, the influence of other genes and signaling pathways that potentially interact with Notch, in the same cell at a given time point, could also modify Notch signaling and its effect on proliferation. Depending on the gene circuitry, the behavior of Notch signaling can range from cell-autonomous effects to non–cell-autonomous effects (Ho and Artavanis-Tsakonas 2016) and from ligand-dependent activation to ligand-independent activation (Steinbuck and Susan 2018; D'Souza et al. 2010; Hori et al. 2012; Palmer and Deng 2015). In the next sections we describe in detail the effect of noncanonical Notch signaling on AMP proliferation.

Cross Talk Between Insulin/TOR, Myc, and Notch During Initiation of AMP Proliferation

As stated earlier, the molecular mechanisms that govern the quiescent properties of embryonic AMPs and the initiation of their proliferation were largely unknown until recently. The studies of Aradhya et al. (2015) resulted from a large-scale genetic screen in which the individual components of several signaling pathways were manipulated specifically in AMPs using appropriate genetic tools. From the initial analysis it was observed that overexpression of the constitutively activated form of Notch intracellular domain (NICD), specifically in AMPs, gave a dramatic increase in AMP proliferation in the third instar *Drosophila* larvae. This indicated that it is the low level of Notch signaling in embryonic AMPs that keeps them in the quiescent state, and during the third instar larval stage the activity of the Notch pathway increases, leading to the initiation of the proliferation (Aradhya et al. 2015). However, it was not clear how the upregulation of the Notch activity in AMPs took place during the larval period. As discussed previously, during the canonical mode of Notch signaling, the activation of the pathway takes place when appropriate ligands from the neighboring cells bind to the Notch receptor of the effector cell (Artavanis-Tsakonas et al. 1999; Bray 2006; Fortini 2009; Andersson et al. 2011). Aradhya et al. (2015) used the UAS-GAL4 system available for *Drosophila* (Brand and Perrimon 1993) and downregulated the two known Notch ligands, Delta and Serrate, using double-stranded RNA interference (RNAi) in the surrounding tissues that might be physically contacting the AMPs during the larval period. They used specific GAL4 drivers for the somatic body wall muscles, neurons and glial cells to overexpress short interference RNA (siRNA) against Delta and Serrate. Surprisingly, no change in AMP proliferation

was detected in any of the above contexts. Also, overexpression of a dominant-negative form of Notch receptor (ECN) in AMPs, which is lacking the extracellular domain and has been shown to repress the ligand-dependent mode of Notch signaling (Rebay et al. 1993), did not change the rate of AMP reactivation (Aradhya et al. 2015). This suggested that the Notch activity in the larval AMPs might be under the regulation of a non-canonical mode that includes a ligand-independent mode of activation. In parallel, the role of Myc (dMyc in *Drosophila*) in regulating the proliferation of AMPs has also been analyzed. As previously described (Bernard et al. 2010), dMyc carries Su(H) binding sites and is an important downstream effector of the Notch signaling in *Drosophila* myoblast cell lines. The role of Myc/dMyc in metabolism, growth and proliferation (Bernard and Eilers 2006; Bretones et al. 2015; Gallant 2013) is thus well established, making it a good candidate for a role in AMP proliferation acting downstream of Notch. As predicted, the RNAi-driven attenuation of dMyc, specifically in AMPs, gave a very marked reduction of AMP proliferation (Aradhya et al. 2015). The late third instar larvae in which dMyc was knocked down had a similar number of AMPs to that of embryonic stages, indicating that AMPs failed to leave quiescence, although in the control larvae they would have undergone several rounds of proliferation, giving rise to pool of myoblasts. Similarly, overexpression of dMyc resulted in a significant increase in the overall rate of AMP proliferation. This indicates that dMyc plays a critical role in initiating and maintaining AMP proliferation and is most downstream in the signaling cascade (Aradhya et al. 2015).

The low-level expression of Notch in AMPs prior to the onset of proliferation (Figeac et al. 2010), and lack of phenotypic change in AMP proliferation when the Notch ligands are altered in the surrounding tissues (Aradhya et al. 2015), indicate that there must be another crucial signaling pathway driving the AMPs out of quiescence. Also, it seems that this pathway, through an unknown mechanism, increases the level of Notch activity in AMPs in a ligand-independent context (Aradhya et al. 2015). *Drosophila* neural stem cells, also termed neuroblasts, display features similar to those of AMPs. They are specified in the early embryonic stages, remain quiescent throughout the embryonic and early larval instars, and proliferate to give rise to several progenies in the third instar stage (Homem and Knoblich 2012). Two independent studies by the groups of Gould and Brand indicate that the entry of neuroblasts into the cell cycle is determined by the systemic metabolic/nutritional state, sensed by insulin/TOR signaling pathways, thus triggering the responses required for proliferation (Chell and Brand 2010; Sousa-Nunes et al. 2011). Through series of experimental data, these findings showed that fat cells sensed the nutritional status of the organism before the pupal metamorphosis and relayed signals to the glial cells in the central nervous system, which in turn secreted a specific insulin-like peptide (dILP6) to activate insulin/TOR signaling in the dormant neuroblasts and drive them toward proliferation. Inspired by these studies, Aradhya et al. (2015) manipulated several key components of the insulin/TOR signaling pathway specifically in AMPs and it has been observed that similar to neuroblasts, these signaling pathways also positively regulate the initiation of proliferation in AMPs. With these results they went on to search for the source of insulin ligands, *Drosophila* insulin-like peptides (dILPs) to activate the insulin/TOR signaling in AMPs. By genetically manipulating individual dILPs known to be expressed in the larval period, in the neighboring tissues like glia, somatic muscle and neurons, it was found that dILP6 specifically expressed from somatic body wall muscles was required for activation on AMP proliferation (Aradhya et al. 2015). This proved the dual supporting nature of differentiated body wall muscles displaying a suitable niche environment during both the quiescent state of embryonic AMPs and in their proliferative state during the third larval instar.

The similar roles of insulin/TOR and Notch signaling pathways in driving the quiescent AMPs toward the cell cycle, invite the speculation that these pathways might be interacting with each other in regulating the above process. By performing genetic complementation

experiments, Aradhya et al. (2015) showed that the overproliferation phenotype displayed by AMPs due to the overexpression of insulin/TOR pathway components was significantly rescued by the coexpression of RNAi against Notch receptor. Similarly, the Notch overexpression phenotype was rescued by coexpression of RNAi against dMyc, indicating the presence of an insulin-Notch-dMyc signaling cascade, which acts together in activating the cell cycle of quiescent AMPs. These results were supplemented by measuring the amount of protein expression for both NICD and dMyc in the context of insulin and Notch overexpression, respectively. There are few findings in either mammals or *Drosophila* that show an interaction between insulin and Notch signaling pathways in different contexts (Hsu and Drummond-Barbosa 2009; Valenti et al. 2013; Billiard et al. 2018; Foronda et al. 2014). However, there was no previous evidence for the direct activation of Notch receptor by insulin signaling through a ligand-independent mechanism. Puzzled by these novel findings, the authors analyzed the involvement of previously described components of noncanonical ligand-independent activation of Notch signaling (Steinbuck and Susan 2018; D'Souza et al. 2010; Hori et al. 2012; Palmer and Deng 2015) in the reactivation of AMPs.

Insulin Signaling Activates Notch Pathway in Deltex- and Shrub-mediated Ligand-independent Mode to Reactivate AMPs

The ubiquitin ligase Deltex (Dlx), has been shown to play a key role in ligand-independent activation of Notch signaling in *Drosophila* wing disc epithelial cells (Hori et al. 2012). It exerts this effect by increasing its monoubiquitinated state (Hori et al. 2012). When Aradhya et al. (2015) performed immunostaining of proliferating AMPs using anti-Deltex antibody, they found an increase in the amount of punctate expression of Deltex protein compared to the control. Similarly, overexpression of Deltex in AMPs or downregulation of Suppressor of Deltex (Su(Dx)), an inhibitor of Deltex, gave an increased number of AMPs. However, genetically manipulating Kurtz (Krz), a nonvisual β-arrestin homolog, in AMPs led to overproliferation of AMPs in both overexpression and downregulation contexts. Kurtz binds with Deltex to the Notch receptor, leading subsequently to its polyubiquitination and degradation (Hori et al. 2012). The action of Kurtz on AMP proliferation in both directions suggests that fine-tuning of Krz and Dlx is required for the activation of the Notch receptor in a ligand-independent manner in AMPs. This possibility was supported by data showing that both reduction of Deltex by RNAi and overexpression of S(u)Dx in AMPs lead to an increase in AMP proliferation. Similarly, overexpression of both Deltex and Kurtz simultaneously did not show any change in the number of AMPs. In parallel, Aradhya et al. analyzed another important player in the ligand-independent mode of Notch activation, Shrub, a component of ESCRT-III complex, helps Notch receptor degradation in multivesicular bodies (MVBs) (Hori et al. 2012). Hence it is a negative regulator of ligand-independent activation, and downregulation of Shrub in AMPs gave rise to a substantial increase in AMP proliferation. To support these results, authors performed genetic complementation experiments in which overexpression of Dlx or Krz in insulin gain-of-function contexts elevated the rate of AMP proliferation compared to the overexpression of insulin activity alone. Also, overexpression of Dlx in AMPs in which the insulin signaling had been attenuated by the expression of an inhibitor, PTEN, resulted in a rate of proliferation similar to the wild-type scenario. In conclusion, the studies by Aradhya et al. (2015) have yielded several novel findings. They demonstrate the role of insulin and Notch signaling in regulating the quiescent vs. proliferative status of *Drosophila* AMPs. Importantly, activation of Notch by the insulin pathway involves a Deltex and Shrub-mediated noncanonical ligand-independent mode of signaling cascade. The outcomes of this study are illustrated in the Fig. 9.2.

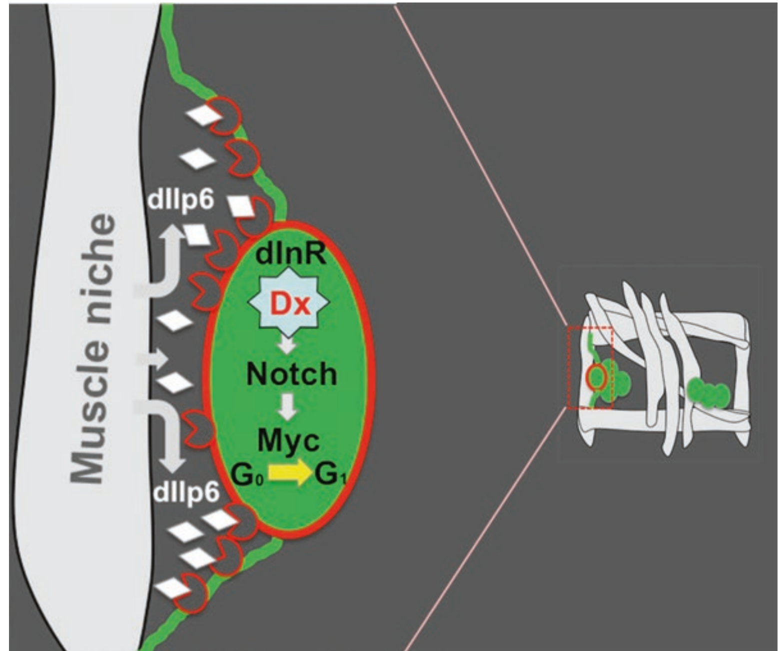

Fig. 9.2 Scheme illustrating the muscle niche-induced Insulin/Notch/dMyc cascade governing the AMP proliferation. These AMPs are reactivated at mid-second larval instar. The reception of the inductive dIlp6 signal emitted by the muscle niche is facilitated by the projected by the AMPs filopodia. In reactivated AMP (depicted in red), activation of the Insulin pathway leads to a Deltex-involving activation of Notch and induces AMP proliferation through the Notch target Myc

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Molecular Mechanisms of Notch Signaling in Lymphoid Cell Lineages Development: NF-κB and Beyond

10

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Abstract

Notch is a ligand–receptor interaction-triggered signaling cascade highly conserved, that influences multiple lineage decisions within the hematopoietic and the immune system. It is a recognized model of intercellular communication that plays an essential role in embryonic as well as in adult immune cell development and homeostasis. Four members belong to the family of Notch receptors (Notch1–4), and each of them plays nonredundant functions at several developmental stages. Canonical and noncanonical pathways of Notch signaling are multifaceted drivers of immune cells biology. In fact, increasing evidence highlighted Notch as an important modulator of immune responses, also in cancer microenvironment. In these contexts, multiple transduction signals, including canonical and alternative NF-κB pathways, play a relevant role. In this chapter, we will first describe the critical role of Notch and NF-κB signals in lymphoid lineages developing in thymus: natural killer T cells, thymocytes, and thymic T regulatory cells. We will address also the role played by ligand expressing cells. Given the importance of Notch/NF-κB cross talk, its role in T-cell leukemia development and progression will be discussed.

Keywords

Notch · NF-κB · Innate immune cells · T-cells · T-cell leukemogenesis

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Abbreviations

ANK	Ankyrin domain
DSL	Delta-Serrate
IKK	Inhibitor of KAPPA-B kinase complex
Jagged	Jag
N1ICD	Notch1 intracellular domain
N2ICD	Notch2 intracellular domain
N3ICD	Notch3 intracellular domain
NF-κB	Nuclear Factor-κB
NKT	Natural Killer T cells
PEST	Proline (P), glutamic acid (E), serine (S), and threonine (T)-rich protein

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PTCRa	Invariant pre-Talpha chain of the pre-TCR receptor
T-ALL	T-cell acute lymphoblastic leukemia
Tregs	T regulatory cells

Introduction

Notch is an evolutionary conserved protein and controls cell fate specification by regulating cell proliferation, differentiation, apoptosis, and survival in many invertebrates and vertebrates.

To date in mammals, are known four different Notch receptors (Notch1, 2, 3, or 4) able to interact with five different ligands, Delta-like1 (DLL1), Delta-like3 (DLL3), Delta-like4 (DLL4), Jagged1 (Jag1), and Jagged2 (Jag2). The different Notch ligands, as well as the four Notch receptors, are transmembrane proteins characterized by common structural features, but they differ in the number of EGF-like repeats and domain composition. All ligands present an amino-terminal domain called DSL (Delta, Serrate, and Lag-2) involved in receptor binding, which is followed by EGF-like repeats. A cysteine-rich domain close to the plasma membrane is located only in Jag1 and Jag2 ligands downstream of the EGF-like repeats. Notch receptors present in their extracellular portion several EGF-like repeats (36 in Notch1 and Notch2, 34 in Notch3, and 29 in Notch4), followed by three cysteine-rich LIN domains that prevent ligand-independent activation and the heterodimerization domain (HD).

The cytoplasmic portion of the receptors contains a RAM domain followed by six ankyrin repeats (ANK) that bind to the CSL transcription factor, two nuclear localization signals (NLS), a transactivation domain (TAD; present only in Notch1 and Notch2), and a PEST domain rich in the amino acids proline(P), glutamic acid (E), serine (S), and threonine (T) with a role in regulating protein stability (see also The molecular basis of Notch signaling: an overview by Asahara (Kwon et al. 2012)). Notch receptors are type I transmembrane heterodimeric receptors. After translation the Notch protein before being localized in the membrane in order to function as a receptor, undergoes Furin-dependent cleavage (S1 cleavage) and fringe-dependent glycosylation, only then as a heterodimer it is transported to the cell surface and is ready to act as a receptor able to trigger the Notch signaling. This pathway can be activated either upon ligand interaction (canonical Notch pathway) or independently (noncanonical Notch pathway). The canonical Notch signaling initiates when the extracellular domain of Notch (NECD) interacts with a ligand, that interaction triggers sequential proteolytic cleavages in the Notch receptor by ADAM 10 protease (S2 cleavage) and by γ-secretase complex (S3 cleavage). At the end of these two cleavages the release of Notch extracellular and intracellular regions occurs. While the extracellular region is endocytosed by the ligand-expressing cells (via Mindbomb-dependent and neuralized-dependent mechanism), the intracellular domain (ICD) of Notch receptor translocates into the nucleus of the receptor-expressing cell. In the nucleus NICD interacts with CSL (also known as CBF1 in humans, suppressor of hairless in Drosophila, RBPJk in the mouse, and Lag-1 in *Caenorhabditis elegans*) helping in the release of transcriptional corepressors bound to it and in the recruitment of coactivators such as Mastermind-like (MAML), p300, and PCAF (Bray 2016). The canonical complex is now able to activate the transcription of Notch target genes such as Hes1, Deltex1, pre-TCRα, and c-Myc. Based on their structural differences, Notch1 and Notch3 receptors have been reported to have a different ability to transactivate depending on the binding site (CSL) orientation and distribution on the promoter (reviewed in Bellavia et al. 2018).

Alternatively, a RBPJ-independent Notch signaling can be activated, that in turn can lead to the transcription of Notch target genes independently of ligand binding. In this case, Notch interacts with a plethora of intracellular proteins, such as AKT, mTOR, NF-κB, mitofusin, and CARMA (Perumalsamy et al. 2009, 2010; Shin et al. 2014) that can influence Notch function (Ayaz and Osborne 2014).

Moreover, the function of NICD as a transcription factor (TF) can be inhibited by MINT

(Msx2-interacting nuclear target protein) and LRF (Leukemia/lymphoma-related factor) or can be turned off by modulators as Numb and F-box ubiquitin ligase (Fbxw7), which interacts with nuclear NICD and prepares it for proteasomal degradation. The Notch target gene-NRARP is another negative regulator of Notch signaling, by binding to NICD activated complex mediates loss of NICD (Lamar et al. 2001).

Finally, noncanonical and nonnuclear Notch signaling occurs in T-cells and involve the mammalian target of rapamycin (mTOR) complex 2 (mTORC2), a key protein kinase which controls cellular metabolism and growth. In T cell-context, NICD interacts with mTORC2 in the cytosol and activates Akt signal to prevent loss of mitochondrial function and then nuclear damage, thus promoting cell survival (Perumalsamy et al. 2010).

Notch in Lymphoid Cell Lineages

Notch signaling, through complex transcriptional programs, regulates homing, proliferation, survival and differentiation of immune cells (Radtke et al. 2010). All cells of the immune system are derived from multipotent hematopoietic stem cells (HSC), which differentiate into a common lymphoid progenitor (CLP) or into a common myeloid progenitor. The CLPs in turn will further differentiate to generate either T or B or natural killer (NK) cells according to the microenvironmental stimuli. Although Notch is present in these immune cells, its role in early T-cell development and during mature T cell function is most prominent and better studied (reviewed in Amsen et al. 2015); Shah and Zuniga-Pflucker (2014) and in Rothemberg et al. (Yui et al. 2010)). Given the important role of Notch as a mediator of cell fate choices, in this chapter it will be discussed its role mainly in intrathymic T-cell differentiation. Developmental progression of lymphocytes is regulated also by NF-κB signals and together with Notch contributes to the maturation of the cells.

The NF-κB Pathway

Recently excellent reviews highlighted the key role of NF-κB in differentiation, proliferation and survival programs of immune cells (Zhang et al. 2017a; Taniguchi and Karin 2018). Deregulated NF-κB signaling can affect these physiologic programs; certainly, its constitutive activation is detected in many types of cancer cells. Constitutively activated NF-κB transcription factors (TFs) have been associated with several aspects of tumorigenesis, such as promotion of cancer-cell proliferation, prevention of apoptosis, and increase of tumor's angiogenic and metastatic potential (Karin et al. 2002).

It is now accepted that inflammation is a critical component of tumor progression. Many cancers arise from sites of infection, chronic irritation and inflammation. It is now becoming clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration (Pasparakis et al. 2002). In particular, NF-κB provides a critical link between inflammation and cancer through its ability to upregulate the expression of tumor promoting cytokines, such as IL-6 or TNF-α, and survival genes, such as Bcl-XL (Karin et al. 2002).

The NF-κB family comprises five members (RelA, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52) that are combined as homo- or heterodimers to bind DNA and regulate gene transcription. All family members contain the characteristic Rel homology domain (RHD), responsible for DNA binding, dimerization, and nuclear localization. The RelA (p65), RelB, and c-Rel subunits contain transactivating domains (TADs) that interact with transcriptional coactivators to control gene expression. The p50 and p52 proteins, which derive from proteolytic processing of the p105 and p100 precursor proteins, respectively, do not contain TADs, and can only control gene transcription through dimerization with other NF-κB subunits or interaction with other transcriptional regulators (e.g., Bcl3). In the steady-state, the

NF-κB dimers are localized in the cytoplasm bound to inhibitory IkB proteins. The IkB proteins (IκBα, IκBβ, IκBε, p100, p105, and Bcl3) are characterized by the presence of an ankyrin repeat domain, which interacts with and inhibits the RHD domain of NF-κB proteins. Thus, only when IκB proteins are degraded or proteolytically processed, upon cell stimulation and IκB kinase (IKK) activation, then NF-κB factors translocate to the nucleus and become activated. Two different NF-κB pathways (Fig. 10.1) have been described that are activated by different stimuli and participate in different biological functions (Jost and Ruland 2007).

The canonical pathway is characterized by differential requirement for IKK subunits. The IKK complex consists of two kinase subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO. Particularly IKKβ can regulate activation of the canonical pathway through phosphorylation of inhibitors of IKK (IKBs). This phosphorylation allows the translocation of p65/p50 heterodimers from the cytoplasm to the nucleus. The "canonical" pathway is triggered by microbial products and proinflammatory cytokines such as TNF (Tumor Necrosis Factor) α and IL-1. In particular TNF stimulation results in a complex cascade of signaling events that can trigger either cell death or survival (Zhang et al. 2017a) (Fig. 10.1). Ligation of TNF receptor 1 (TNFR1) results in TRAF2/TRAF5 (ubiquitin ligases) and RIP1 (Receptor Interacting Protein domain1) recruitment, which is TRADD (TNFR1-associated death domain) dependent. TRAF2 causes ubiquitination of RIP1 and also recruits IKK to the receptor complex, where binding of IKKγ/NEMO to ubiquitinated RIP1 stabilizes IKK interaction with the receptor complex. This promotes TAB interaction with TRAF2 and TAK1, leading to TAK1 activation that may

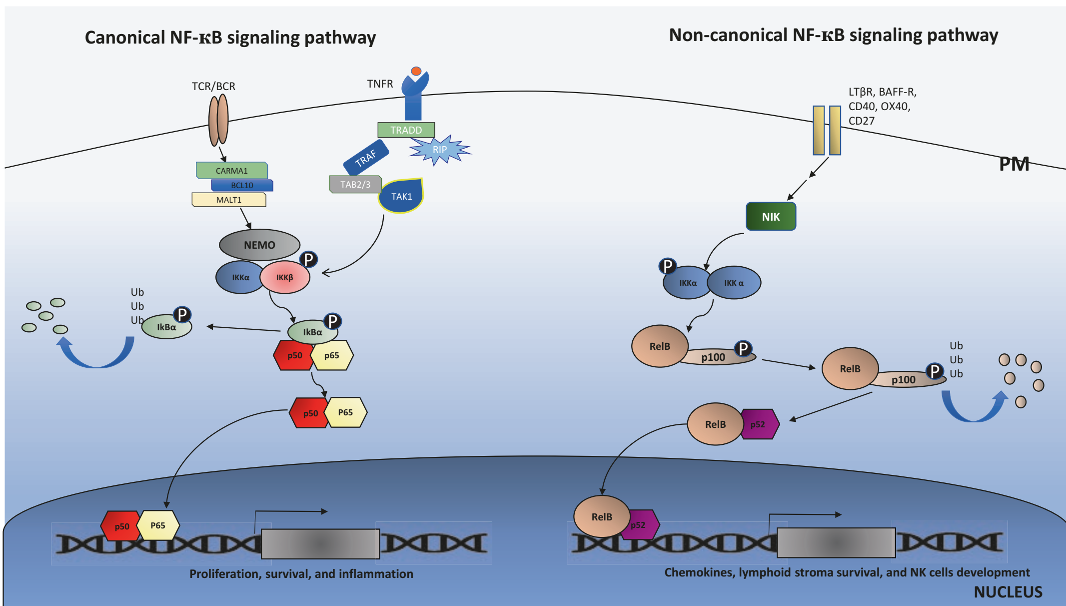

Fig. 10.1 The canonical and noncanonical NF-κB signal transduction pathway. Signals by T-cell receptor (TCR), B-cell recptor (BCR), tumor necrosis factor rceptor (TNFR) as well as IL1-R and Toll-like Receptors (TLRs) activate tha canonical pathway. They use a wide variety of signaling adaptors to engage and activate the IKKβ that in turn phosphorylates IκBα on serine residues. This event will release the p65/p50 complex. Free p65/p50 NF-κB heterodimers can translocate to the nucleus and regulate target gene transcription. Activation of the noncanonical pathway is triggered by receptors such as lymphotoxin-β-R(LTBR), B cell-activating factor receptor (BAFFR), CD40, OX40, and CD27, and triggers IKKα phosphorylation and activation by NIK. Activated IKKα then phosphorylates p100 on serine residues, leading to p100 polyubiquitination and partial processing to p52. Free p52/RelB heterodimers translocate to the nucleus to regulate target genes

phosphorylate IKKβ, finally forming the TNFR complex I. Triggering IKK activity in this complex results in activation of downstream NF-κB and promotion of cell survival (Webb et al. 2019). A failure to maintain the stability of complex I results in the formation of one of the cell death inducing complexes that can be RIPK1 kinase independent or RIPK1 kinase dependent (Webb et al. 2019).

Conversely, IKKα homodimer is required for the activation of the alternative pathway of NFκB through the phosphorylation and processing of p100, the precursor for p52 (Fig. 10.1). In this case the most common noncanonical heterodimer is RelB/p52 (Taniguchi and Karin 2018). In particular the upstream kinase that activates IKKα in this pathway has been identified as NIK (NF-κB inducing kinase) and the pathway is completely independent by IKKβ and IKKγ.

Most of the information about the relative role of Notch and NF-κB cross talk in lymphoid lineages development derived from the study of murine models, bearing specific mutation in members of the two pathways.

Notch and NF-κB Coupling in Bone Marrow Differentiation Programs

Notch signaling may represent an ideal candidate for instructing communication between HSC and their niche. It requires cell-to-cell contact for its activation. However, the critical role of Notch signaling in regulating HSC self-renewal is still unclear. Conditional deletion of Jagged1 or Notch1 from BM cells has no effect on HSC maintenance (Kushwah et al. 2014). Accordingly, inhibition of canonical Notch signaling independently of Notch ligand receptor usage, via dominant-negative MAML or by inactivating RBPjK, did not reveal any HSC defects (Radtke et al. 2010; Maillard et al. 2008). Overall, it is supported the notion that canonical Notch signaling is dispensable in maintaining adult HSCs, but contrasting data has been reported (Duncan et al. 2005). Although dispensable for HSC maintenance, there are evidences that in BM-residing CLPs Notch signaling is repressed for the normal BM homeostasis, in order to avoid ectopic T cell differentiation (Maeda et al. 2007; Radtke et al. 2013; Shang et al. 2016). Notch signaling is a “gatekeeper” between self-renewal and commitment of HSCs. Indeed, Notch is downregulated as HSCs differentiate and its integration with Wnt signaling is essential to maintain the stem cell state (Duncan et al. 2005). In mice, Notch1 is required, but Notch2 is dispensable for HSC specification (Yuan et al. 2010).

Notwithstanding, knowledge of the mechanisms by which stromal cells support normal hematopoiesis is lesser known and still more complex in neoplasia. In fact, in vitro experiments revealed that human CD34+CD38+ cells differently respond to Jag1 or DLL1 stromal stimuli, by increasing Notch1 or Notch3, respectively, thus reflecting the requirement of a specific Notch ligand–receptor combination for sequential maturational stages of CD34+ (Neves et al. 2006). Moreover, embryonic HSC development requires Notch signaling in the endothelial compartment. In support, endothelial expression of Notch ligands (Jag1) is essential for the self-renewal and repopulation of Notch-dependent HSC (Butler et al. 2010). Therefore, Notch ligand–receptor signaling has a central role in stroma-mediated effects, that once subverted may sustain leukemia development.

Recent studies indicated that the activation of both canonical and noncanonical NF-κB pathway regulates adult HSC homeostasis and function intrinsically. By suspecting compensatory effects of different NF-κB members, combined Rel and RelA mutations lead to several hematopoietic defects during embryogenesis. Noncanonical NF-κB signaling is critically implicated also in HSC self-renewal in adult mammalian hematopoiesis (Espin-Palazon and Traver 2016). On the other hand, deregulated canonical NF-κB signals in HSCs cause a complete depletion of HSC pool, pancytopenia, bone marrow failure, and premature death (Nakagawa and Rathinam 2018).

Given the importance of Notch signals in BM progenitors biology, the NF-κB pathway was demonstrated to be a very good partner of Notch in HSC differentiation programs. A common feature of this Notch/NF-κB interplay is that the two

partners can induce a direct and often reciprocal effect. Initially, an interesting paper by Cheng P et al. demonstrated that Notch1 could regulate the DNA binding activity of NF-κB as well as its ability to activate transcription in Hematopoietic Progenitor Cells (HPC), thus contributing to differentiation and function of hematopoietic cells (Cheng et al. 2001). In HPC, Notch1 exerts its effect via transcriptional regulation of canonical and noncanonical members of the NF-κB family (p65, p50, RelB, cRel). On the reverse, a functional cross talk between the NF-κB pathway and Notch signaling in HSC has been implicated in the control of balance between HSC renewal and lineage commitment in a Fanconi anemia model. In this case, NF-κB regulates the Notch pathway, probably by influencing the expression of Notch target genes.

Additionally, the lymphostromal cross talk requires the activity of both pathways to commit HSC cells. Notch1 and Jagged1 in collaboration with canonical NF-κB activation and via TNFα establish HSC fate, indicating the requirement for inflammatory signals released by primitive neutrophils in HSC generation (Espin-Palazon et al. 2014). Interferon-gamma (IFNγ) is involved in this process, as a downstream target of Notch. Conversely, Notch signaling is repressed to permit HSC emergence (Butko et al. 2016).

T Cell Development: An Overview

T cells are generated from pluripotent HSCs and derive their name from their maturation in the thymus. The thymus is a bilobed organ located just above the heart and provides a highly specialized microenvironment that guarantees the development of T cells (Ardavin et al. 1993). The structure of the thymus and the presence of different cell types, like epithelial and dendritic cells, make the thymus indispensable for T cell development (Jenkinson et al. 1992).

As thymocytes mature, they move from the cortex to the medulla while undergoing a series of phenotypic, genetic and functional changes. During T cell development (Fig. 10.2) the thymocytes can be divided in four main subsets based on the expression of CD4 and CD8 coreceptor (Germain 2002). The earliest progenitor cells entering the thymus from the BM are defined as a CD4−CD8− Double Negative (DN) subpopulation of thymocytes. In mice the DN stage can be divided in four different stages by the expression of CD44 and CD25 (IL2-R α chain) (Godfrey et al. 1993).

Accordingly, the earliest subtype of DN cells in mice is classified as CD44+CD25−(DN1). The differentiation then proceeds via the CD44+CD25+(DN2) stage, afterward CD44 is downregulated and the CD44−CD25+(DN3) cells start to rearrange their TCRβ genes (Germain 2002). The TCRβ chain is subsequently expressed together with the surrogate TCRα chain of pre-Tα and CD3 components on the cell surface, forming the pre-TCR complex.

The successful rearrangement of TCRβ chain is a prerequisite for survival and subsequent proliferation of αβ thymocytes. This is considered the first checkpoint of T cell maturation (Robey and Fowlkes 1994). Finally, pre-TCR/CD3 signaling promotes cell proliferation but inhibits apoptosis to allow for transition to the DN4 stage.

Mainstream development then further proceeds predominantly through an $\alpha\beta TCR^{low}$ immature single-positive stage (iCD8/iCD4) to the major CD4+CD8+ double-positive (DP) subset of thymocytes (Robey and Fowlkes 1994). Positive selection of DP to CD4+ or CD8+ single-positive (SP) thymocytes is driven by weak recognition of self-antigens (self-AGs) presented on cortical thymic epithelial cells in the context of MHC class II or class I, respectively (Hare et al. 1999, 2000). Failure to recognize self-AGs leads to elimination of thymocytes (death by neglect); strong recognition of self-AGs also leads to elimination (negative selection). Negative selection begins in the cortex but may occur predominantly in the medulla, where self-AGs are presented on dendritic cells (DCs) and on medullary thymic epithelial cells (mTECs) (Bommhardt et al. 2004).

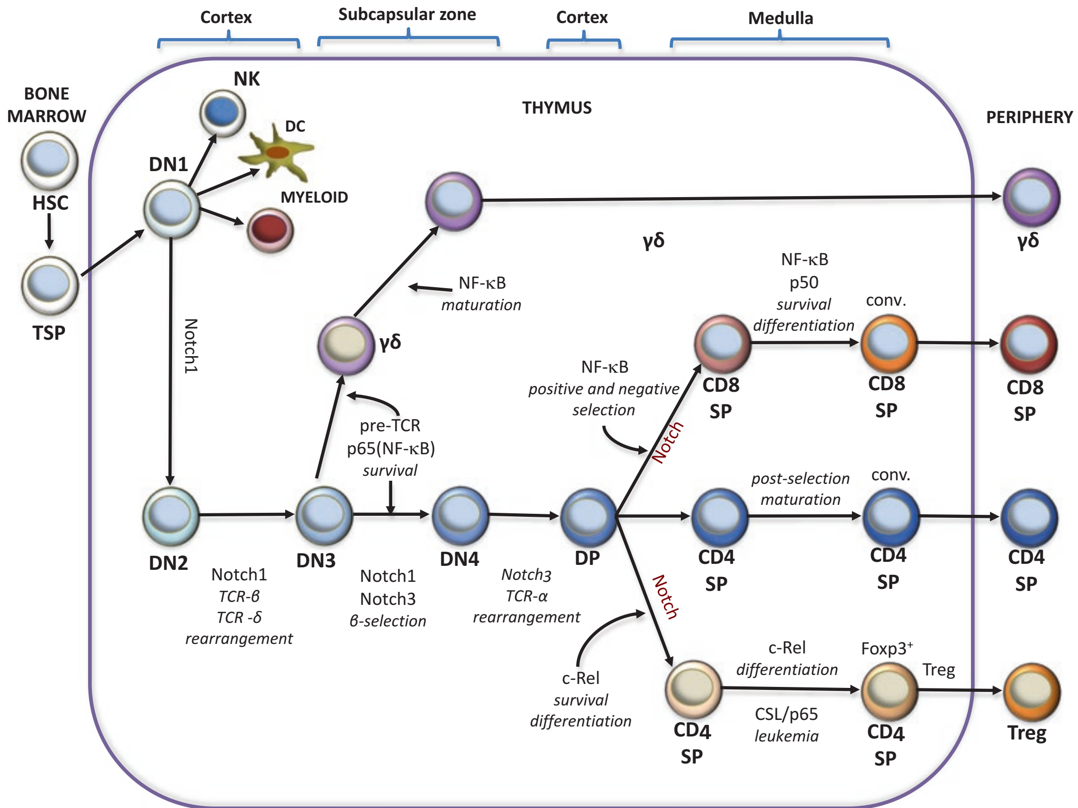

Fig. 10.2 Overview of thymic T-cell development. The individual stages of intrathymic T cell maturation from Hematopietic stem cells (HSC) to thymocyte-seeding progenitor (TSP) up to the fully mature CD4 and CD8 single positive (SP) cells. Dendritic cells (DC), natural killer (NK)

Notch and NF-κB in Natural Killer T Cell Development

Natural Killer T (NKT) cells are a lymphocyte subset with important immunoregulatory properties. They are implicated in different immune responses that can vary from suppression of autoimmunity to tumor rejection by exhibiting a potent NK-like cytotoxic activity in the thymus. NKT cells develop in thymus from BM precursors (DN1) in a Notch- and IL7-dependent manner, and their homeostasis was found to be controlled by Notch (Yamamoto et al. 2019). Critical for NKT cell differentiation is also the NF-κB signaling. Distinct members of the Rel/NF-κB family of transcription factors are required for normal development of thymic NKT cells. Different studies demonstrated that RelA regulates the NK1.1− to NK1.1+ transition during NKT cell development. Indeed, studies by Elewaut et al. (2003) demonstrated that the activation of NF-κB via the classical IKBa-regulated pathway was required in a cell autonomous manner for the transition of NK1.1- negative precursors that express the TCR Vα 14-Jα 18 chain to mature NK1.1+ NKT cells (Elewaut et al. 2003). In addition, NF-κB1, c-Rel, and RelA were found not essential in early NKT cell development (before NK1.1 expression), but each of them was found to play a non redundant role in later stages of NKT cell maturation and function (Stankovic et al. 2011). RelA is also required for both IL15 and IL7-induced proliferation of CD44hiNK1.1- NKT cell precursors (Vallabhapurapu et al. 2008).

A proposed mechanism by which NF-κB regulates NKT cell development is through activa-

tion of the invariant NKT cell receptor, that after its activation induces IL15 receptor α and γ chains' expression in an NF-κB-dependent manner (Vallabhapurapu et al. 2008). Invariant NKT cells exert Janus-like functions (Wilson and Delovitch 2003) and studies of CD4-specific ablation of Notch1 and Notch2 (in N1N2$^{lox/lox}$ CD4-Cre mice) showed an increase of invariant NKT cells in thymus (Oh et al. 2015). These evidences would argue in favor of Notch and NF-κB as converging signals in invariant NKT cell development.

NF-κB also regulates TCR-induced expression of lymphotoxin (LT)-α and LT-β within the NKT cells. LT-α and LT-β are involved in lymphoid organogenesis and are indispensable for the differentiation of Vα14i NKT cells. Those cytokines through interaction with the LT-β receptor induce a unique signaling cascade which leads to the activation of the transcription factor RelB through activation of NF-κB inducing kinase (NIK) (Franki et al. 2005). Thus, another important player for the development of NKT cells is the thymic stroma, that is not required for the positive selection of these cells but plays a prominent role in their terminal differentiation (Franki et al. 2005). Indeed, lack of intrinsic RelB in thymic epithelial cells resulted in a reduced population of mTECs and in an impaired development of thymic invariant NKT cells, thus non canonical NF-κB signals can shape for a suitable microenvironment (Elewaut et al. 2003; Jin and Zhu 2018). Also other studies support the role of noncanonical NF-κB signaling in thymic stromal cells as an extrinsic mechanism involved in regulating NKT cell generation. Finally, data from different studies demonstrate that the NIK-mediated activation of RelB in thymic stroma is also important for the development of these immune cells.

Notch Signaling Dictates Early Events in Thymocyte Development

Thymocyte progressive maturation is a complex dynamic process, driven by ordered stromal signals delivered to thymus-seeding progenitors (TSPs) that migrate throughout different thymus compartments. Thymic epithelial cells (TEC) have a double role to sustain immature and mature T-cell development and to modulate their maturation and function, which is also influenced by thymocyte progenitors. This bidirectional cell-to-cell interaction is known as "thymus cross talk." Along the four documented DN stages, Notch signals progressively drive maturation of T-cells and inhibit multiple cell fate potentials (Fig. 10.2). A CLP derived from the BM enters the thymus through the corticomedullary junction, where corticoepithelial cells express DLL4, that will drive DN1 cell toward the formation of mature T cells. In fact, DLL4-deleted thymic epithelial cells promote B-cell fate (reviewed in Shah and Zuniga-Pflucker 2014), thus suggesting Notch1 as a B-cell fate suppressor. Nevertheless, inactivation of DLL4 but not DLL1 in TECs results in a complete block in T-cell development. In response to increased Notch signal, DN1 precursors proliferate and differentiate to DN2 cells and express CD25 (Yui et al. 2010). At this stage, DLL1-Notch2 signaling promotes the early DN differentiation (DN1 and DN2) up to the DN3 stage (Besseyrias et al. 2007). During the transition from DN2 to DN3 subsets a marked decrease in proliferation is inversely correlated to an increased expression of Notch target genes (Hes-1 and Deltex) (Yui et al. 2010). Therefore, a strong Notch signaling is required. Two T-cell subsets are generated at DN3 stage, by acquiring either the αβ or the γδ T-cell antigen receptor (TCR) (Garcia-Leon et al. 2018). Both TCRs imply rearrangement of the gene segments to produce a functional TCR. Most Notch1-induced T-cell progenitors develop along αβ lineage, which involves a complex journey in the thymus cortex and medulla, to finally generate mature CD4+ and CD8+ SP T-cells. In contrast to murine T-cell development, constitutive expression of Notch in human thymocyte progenitors efficiently generates γδT-cells (Garcia-Peydro et al. 2003). Unlike the role of DLL1–4 in mouse, human cortical TECs express Jag2 and mediate Notch3 signaling in γδT-cell commitment and development (Garcia-Leon et al. 2018). In murine thymocytes, Notch3 is highly expressed at the

beginning of the DN3 stage (Visan et al. 2006; Felli et al. 1999). In comparison to the other Notch receptors, Notch4-deficient mice do not exhibit any defects in the hematopoietic system (Krebs et al. 2000).

DN-to-DP Thymocyte Transition: Not a Question of Notch Redundancy

The persistence of Notch1 signals is required through the DN2 stage to suppress other non-T cell potentials (NK, macrophage-granulocyte, and dendritic cell fate) (Yuan et al. 2010). Upon rearrangement of the T-cell receptor beta locus (Tcr-β), DN3 thymocytes express a functional TCRβ that when combined with the pTα chain forms the pre-TCR. This receptor, in combination with CXCR4, has a critical role during DN3 to DN4 differentiation thus contributing to continued T cell development beyond β-selection (Janas et al. 2010). DN3/DN4 transition is critically driven by Notch1 and Notch3 function. The redundancy is normally ensured by two safety systems with overlapping functions. This is not the case of Notch1 and Notch3 receptors. Interesting experiments in thymocytes, demonstrated that during differentiation Notch1 cannot compensate for the absence of Notch3, and conversely, Notch3 cannot compensate for the lack of Notch1 (Shi et al. 2011). Additionally, this study by Shi et al. reported that, despite the absence of Notch1, the other receptor, Notch3, was still expressed. In contrast to the lethality of Notch1 gene deletion, mice with inactivated Notch3, by insertion of a gene trap, exhibited reduced thymic size and cellularity (~15%), in agreement with 10-week old Notch3-deficient mice previously described (Kitamoto et al. 2005). Both reports showed no impairment in T cell differentiation. Overall, Notch1 is believed to be absolutely required early in lymphopoietic process. Nevertheless, these two receptors have non-overlapping function.

In addition, to further complicate the Notch network within the thymus, there is a different spatial distribution of ligands (and receptors), not only partitioned between thymocyte and epithelial cells, but also in different compartment of the thymus: cortex (DLL1, Jag2) and medulla (Jag1) (Garcia-Leon et al. 2018; Felli et al. 1999). All Notch ligands except DLL3 are expressed in the thymus. DLL4 is the essential nonredundant ligand promoting Notch1 dependent T cell fate specification and maturation in mice. This data is suggestive of a Notch receptor with different roles depending on the thymus microenvironment stimuli.

The generation of DP cells from DN4 progenitors are hindered in the absence of Notch signal (Huang et al. 2003). DP thymocytes express low levels of Notch1, but Notch2 and Notch3 are expressed, indicating a stage-specific expression and function of the members of this receptor family. Maturation of thymocytes to the DP stage induced downregulation of DLL4 on cortical TECs (cTECs), suggesting a negative feed-back loop between developing thymocytes and cTECs.

The development of thymocytes depends on glucose for energy metabolism as well as proliferation. Notch signaling and IL-7 are essential for maintaining thymocyte glucose metabolism and cell viability during thymic selection. This is a specific requirement of CD4+CD8+ thymocytes, but not of DN or SP thymic subtypes. The glucose-mediated survival of DP thymocytes occurs through upregulation of NF-κB dependent antiapoptotic survival factors (Ramakrishnan et al. 2011). The binding of CD8 coreceptor and TCR to MHC class I ligands induce DP to CD8+ SP transition, while binding between CD4 coreceptor and TCR to MHC class II induces progressive maturation to CD4+ SP cells. Two excellent papers by Deftos and colleagues demonstrated the critical role of Notch1 in driving the final step in the maturation of CD4+ SP and CD8+ SP αβ T cells in the thymus (Deftos et al. 1998, 2000).

NF-κB, a Tale of Two Pathways in DN-to-DP Transition

Signal transduction pathways, including those driven by the Notch family of transmembrane receptors, the intracellular glucocorticoid receptor,

the mitogen-activated protein (MAP)-kinase cascade and NF-κB, determine the fate of the developing thymocytes in response to external stimuli such as cell surface molecules, like TCR, cytokines, and hormones (Bommhardt et al. 2004).

The presence of pre-TCRs on DN3 stage of thymocytes provides important signaling for the progression into the DN4/DP stage (Taghon and Rothenberg 2008). These cells contain significant level of nuclear NF-κB activity, presumably due to the activation by the preantigen receptor (Voll et al. 2000). Failure to assemble a pre-TCR receptor causes the elimination of these cells. In Addition, IκB superrepressor-mediated interference with NF-κB activation in pre-TCR expressing thymocytes leads to their apoptosis due to interference with NF-κB-mediated induction of antiapoptotic Bcl-2 protein (Voll et al. 2000). In a mouse model with constitutively active IKKβ, DN thymocytes progress to DP stage even in the absence of pre-TCR in RAG-deficient mice (Voll et al. 2000). As we discussed above, after the rearrangement of α and β chain of TCR the DN T cells will progress to be DP (Bommhardt et al. 2004). At this time the thymocytes are subjected firstly to positive and then to negative selection, while they physically move from the thymic cortex to medulla (Boehm 2008). Surviving thymocytes CD4 or CD8 SP undergo further maturation (Fig. 10.2), exit from the thymus and enter into the peripheral circulation as mature näive T cells (Bommhardt et al. 2004). The role of NF-κB in selection remains controversial; NF-κB appears to have both positive and negative effects due to the TCR signal strength. In the last years studies on a mouse model lacking protein of the NF-κB pathway allowed to study better the role of this TF in T cell development. Studies in mice expressing IkB super-repressor transgene have suggested a role of NF-κB in positive selection of CD8 and, to a lesser degree, of CD4 SP thymocytes (Mora et al. 2001). On the other hand, NF-κB is also involved in promoting apoptosis of DP thymocytes mimicked by α-CD3 treatment in vivo (Hettmann et al. 1999), and in mouse model with TCR that strongly recognize MHC class I and II (Mora et al. 2001). To further support the role of the canonical NF-κB pathway, the study by Schmidt-Supprian and colleagues shows that mice with conditional ablation of IKKγ/NEMO or of IKKβ in thymocyte generated significantly fewer CD8+ SP thymocytes, and had no peripheral T cells, including CD4+ SP (Schmidt-Supprian et al. 2004). A very recent paper showed how the survival of SP thymocytes depends on the developmental control of RIPK1 signaling but is IKK-independent (Webb et al. 2019). Webb and colleagues demonstrated that IKK is required to protect RelA/cRel/p50 deficient thymocytes from RIPK1-dependent cell death, underscoring the NF-κB-independent function of IKK in thymic development. Thus, the role of NF-κB during the development of thymocytes is really controversial. Additionally, the circumstances under which NF-κB is activated in thymocytes during positive and negative selection are not fully understood. We hypothesize that if TCR signaling is responsible of this selection, then it must also activate NF-κB by an "unconventional" pathway to generate mature SP thymocytes (Felli et al. 2005; Jost et al. 2007). Essential components of the conventional pathway include PKCθ and Carma1, Bcl-10, and Malt1 (CBM complex). Alternatively, NF-κB can be activated by other signals, which are independent of TCR like Tak1. In response to many NF-κB activating signals, Tak1 functions upstream of the IKK complex (Fig. 10.1). Loss of Tak1 reduced the number of SP thymocytes due to increased apoptosis (Lin and Wang 2004).

Notch and NF-κB Converging Signals in Early Thymic Development of T-Cells

The first evidence of Notch/NF-κB connection in T cells came with a study reporting the ability of TAN-1, a translocation-associated Notch homolog, to engage NF-κB transcription factors (Guan et al. 1996). These authors also identified the cytoplasmic portion of Notch as the domain that prevents p50 homodimer suppressive effect and induces target genes transcription in T-cell leukemia. Interesting studies by the Osborne's group demonstrated that active Notch1 can increase the

activity of NF-κB by a direct interaction with p50/c-Rel. This interaction retains the active NF-κB heterodimer in the nucleus, thus leading to sustained NF-κB activity (Shin et al. 2006).

In the following years, reports demonstrated that Notch and NF-κB are good partners, which influence each other reciprocally. Both c-Rel and RelA can trigger Notch signaling by inducing Jagged1 ligand expression (Bash et al. 1999). On the reverse, hyperactive Notch3 induces a constitutive activation of canonical NF-κB in transgenic mice (Bellavia et al. 2000). This event results in increased number of thymocytes in young mice, particularly late DN cells with a failure to down-modulate CD25 expression at DP stage. These DN cells also display decreased apoptosis and increased proliferation programs. In T cells, Notch signaling importantly mediates G1/S entry in cell cycle progression via cyclin D3, which is transcriptionally regulated by both NF-κB and CSL nuclear factors (Joshi et al. 2009). Therefore, the cooperativity of the two signaling pathways induces T cell proliferation. Hypothetically, this mechanism could regulate T lymphocyte development. In fact, mice lacking cyclinD3 show defective thymocyte development, with a marked deficit of DP T cells, and lack of the proliferative burst during DN3 to DN4 transition (Sicinska et al. 2003). Notably, developing thymocytes exhibit constitutive NF-κB activity. Pre-TCR can induce NF-κB in a ligand independent manner to deliver selective survival signals during thymocyte development. In immature thymocytes (Fig. 10.3), survival programs are indeed activated by Notch3/pre-TCR signals that upregulating the canonical NF-κB pathway trigger transcription of cyclinD1, Bcl2-A1, IL-7 receptor α (Vacca et al. 2006). In contrast, pre-TCR deletion allows for only alternative NF-κB activation by Notch3 resulting in transcriptional activation of Bcl2-A1, IL7 receptor, both playing a crucial role in the early stages of thymocyte differentiation. Therefore, pre-TCR discriminates the ability of Notch to activate the canonical and alternative NF-κB pathways in immature thymocytes.

During DN to DP transition, pre-T cells require different signals in order to promote their proliferative expansion and differentiation. In the thymus, progressive maturation of T-cells is regulated by the expression levels of different TFs. Most of them regulate early events in T cell development and can be activated by Notch. One of these is calcineurin, which dephosphorylates a number of substrates, prominently NFAT family members, allowing for their nuclear translocation and transcriptional activity (Li et al. 2011). In normal T-cells, the cooperation between NFAT

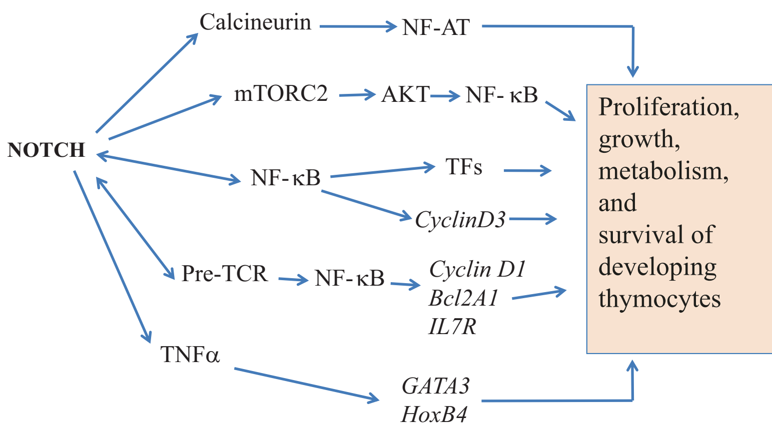

Fig. 10.3 Common pathways physiologically regulated by Notch signaling and deregulated by aberrant Notch signaling in T-cell leukemia. Double arrowed lines define the cross talk between the partners. *TFs* transcription factors, *mTORC2* mammalian target of rapamycin complex 2, *NF-AT* nuclear factor of activated T cells, *pre-TCR* pre T cell receptor composed by the invariant preTα chain, *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B cells, *IL-7Ra* interleukin 7 receptor a, *TNFα* tumor necrosis factor α, *GATA3* GATA-binding protein 3

factors (e.g., NFATc1) with different transcriptional partners leads to the expression of distinct set of genes, including inflammatory cytokines, thus eliciting multiple effects (Macian 2005). NFATc1 activity suppresses the B-lineage potential of immature thymocytes. Any alteration, such as above or below threshold levels of NFATc1 TF activity is critical for T-cell development and may not consolidate T-lineage commitment (Klein-Hessling et al. 2016). NFATc1 activity critically influences Notch-pre-TCR signaling required for normal DN3 differentiation to T cells. The nuclear levels of this TF increase from DN1 to DN3 stage. Notch3 hyperexpression can downregulate NF-ATc1 and hence thymocyte differentiation in T-ALL.

Another critical TF is GATA-3 whose specific role is to restrict the ability of CLP to differentiate into B cell, early after they enter the thymus (Rothenberg 2013). More recently, coculture experiments of human HSCs with OP-9DL1 cells (expressing DLL1) demonstrated that TNFα positively regulate the transcription of two nuclear factors, GATA3 and HoxB4 (Fig. 10.3), in a Notch-dependent manner (Dos Santos Schiavinato et al. 2016). Consequently, in early T-cell development, a potential interconnected transcriptional network can be suggested by the presence of NF-κB and CSL sites within the promoters of all these genes (Notch1, TNFalpha, GATA3, and HoxB4). Additionally, other critical T-cell genes, such as Tcf7, Runx1, Ets, and Lef1 are also maximally upregulated at the DN3 stage (Yui et al. 2010). All the products of these genes are related to NF-κB, mostly by acting as transcriptional partners or as regulators of the pathway.

In early thymocyte development, Notch in collaboration with other signals regulates pre-T cell proliferation and differentiation efficiency. Rictor is an essential component of TORC2 and it has been demonstrated that T lineage cells need an intact mTORC2 to fully perform Notch-driven biological effects at the DN to DP transition. In thymocytes, mTORC2 relays a Notch signal to regulate canonical NF-κB nuclear translocation. In this context, Akt mediates mTORC2 signaling downstream from Notch. Consequently, Rictor-deficient pre-T cells, with decreased NF-κB and Akt activity, showed an impaired proliferation and differentiation efficiency. Further the study by Keunwook et al. (Lee et al. 2012) suggested that Rictor is required in cell-autonomous processes of normal thymocyte development, both in establishing normal numbers of cells and in promoting the DN to DP transition.

Tregs Another Story of Notch and NF-κB Interconnection in T-Cell Differentiation

Regulatory T (Treg) cells are a subset of CD4+ T-cells. They are crucial for the maintenance of immunological tolerance. Consequently, the alterations in Treg differentiation programs and/or functions are implicated in autoimmunity. Moreover, Tregs are also implied in tissue repair, the control of proinflammatory immune responses, as well as the prevention of the immune response to tumors.

Treg cells (Tregs) are defined by the expression of their master transcriptional regulator, the Forkhead box P3 (Foxp3) factor. There are two major classes of CD4+ Tregs. The Tregs derived from the thymus are termed naturally occurring Tregs (nTregs), while when induced from naïve CD4+ T-cells in the presence of TGFβ and IL-2, in vitro are called induced Tregs (iTregs), or in vivo pTregs. nTregs also express α chain of IL-2R or CD25 (CD4+CD25+Foxp3+). Another class of Tregs, less explored, are CD8+ suppressor T-cells that express Foxp3 (Tang et al. 2005; Dinesh et al. 2010).

The activation of TCR signals that finally induce NF-κB, are critical for the development and cell inhibitory action of Tregs. Defects of the TCR signaling complex (including CARMA1, Bcl10, MALT1, Protein KinaseCθ (PKCθ), and IKKβ) can impair development of nTregs, whereas conventional T-cell development seems to be less affected. The study by Gupta et al. (2008) demonstrated that PKC-θ- mediated TCR signals are required for the activation of peripheral naïve T cells, but they are dispensable for their thymic development. Following TCR

engagement, activated NF-κB transcription factors, particularly nuclear c-Rel, play an important role in thymic Treg development (Fig. 10.2), including the transition from CD4+CD8+(DP) to nTreg cell progenitors (CD4+CD25+Foxp3−) before Foxp3 expression (Fulford et al. 2015). Recently, it has been suggested that negative regulators of NF-κB (A20 and CYLD) hold the thymic development of nTregs in check. In mice deficient for A20 in T lineage cells, but with enhanced NF-κB activation, there is a quantitative enlargement of nTreg (increased in term of proportions and absolute numbers) and peripheral Treg compartments (Fischer et al. 2017). In T cells, PKCθ and CYLD are antagonists in the activation of NF-κB. Activation of the classical NF-κB transcription factor can also be triggered by the cytosolic interaction between Notch1-ICD and the components (PKCθ and CARMA1) of T cell signalosome (Shin et al. 2014). Another Notch, Notch3 can enhance also PKCθ-mediated IKKβ-dependent NF-κB activation.

Hypothetically, it could be suggested that Notch1 and Notch3 can regulate the balance between positive (PKCθ) and/or negative (A20 and CYLD) regulator of NF-κB-dependent Tregs generation and function. Similarly, mice lacking IKKβ or NF-κB factors (p50 or cRel) show impaired Treg cell development (Deenick et al. 2010). Conversely, transgenic mice with constitutively active IKKβ display an increased number of thymic Foxp3+ Tregs (Long et al. 2009). Overall, these evidences suggest how important are the canonical NF-κB-mediated cell-intrinsic mechanisms that regulate Tregs development. Despite this, the noncanonical NF-κB pathway does not have any evident role in Treg cell-intrinsic mechanisms. Conversely, in murine model germ line deletion of RelB in thymic medulla cells induces autoimmunity and an expansion of Tregs (Cowan et al. 2013; Li et al. 2018). These evidences further stress the critical role mTEC in nTreg differentiation.

Many reports from several groups strengthened the role of Notch in Treg differentiation and function. The study of Vigouroux et al. (2003) demonstrated with coculture experiments that Epstein-Barr virus lymphoblastoid B cells overexpressing Jag1 with T-cells could induce the generation of Tregs. Notch1 can control nTreg survival and suppressor activity. This Notch effect is performed through a non nuclear mechanism implying the direct interaction between NICD and members of the autophagy system. These authors also suggest that Notch1 integration with autophagy holds implications for Notch regulated cell-fate decisions governing differentiation. The cell-autonomous role for Notch signaling in nTregs biology was initially proposed by our group. Indeed, observations in a transgenic murine model with a constitutive activation of the N3ICD in immature thymocytes showed a reproducible increase of CD4+CD25+Foxp3+ T-cells and Treg specific cytokine (IL-10) with respect to the wild-type, in thymus and spleen. Consequently, transgenic mice were protected from streptozotocin-induced autoimmune diabetes (Anastasi et al. 2003). In this model, the cooperation of hyperactive Notch3, pre-TCR, and constitutively active canonical NF-κB enhances Foxp3 gene transcription, thereby regulating Tregs generation (Barbarulo et al. 2011; Ferrandino et al. 2018a).

Multiple signaling pathways converge on Foxp3 promoter. Three different groups demonstrated the critical role of canonical c-Rel transcription factor in regulating Foxp3 gene expression (Long et al. 2009; Isomura et al. 2009; Ruan et al. 2009). cRel in cooperation with NF-AT binds to the Foxp3 to form a specific complex composed by c-Rel, p65, SMAD3 (mother against DPP3), NF-ATc2, and CREB (cAMP response element-binding protein) in order to transactivate gene transcription. The study of Ou-Yang and colleagues (Ou-Yang et al. 2009) revealed that Notch signaling regulates the Foxp3 promoter through RBP-jK and Hes-1–dependent mechanisms. These authors demonstrated, in freshly isolated Tregs, that the N1ICD/RBPjK complex is bound to Foxp3 promoter. Overall, Foxp3 promoter is an integration site for Notch and NF-κB signaling in determining Treg identity and function. Nevertheless, Foxp3 is required for suppressive activity and transcriptional repression. To this end, overexpressed Foxp3 may indirectly impair translocation of NF-κB into the nucleus.

Two important studies demonstrated that Notch and NF-κB signaling cross talk is also extremely important in regulating iTregs biology. Samon and colleagues revealed the cooperation between Notch1 and TGFβ in regulating Foxp3 expression and the maintenance of peripheral iTregs (Samon et al. 2008). Interesting studies by Osborne's group demonstrated Th1 and iTreg differentiation is driven by a noncanonical Notch signaling RBPjK-independent which likely occurs through NF-κB (Ferrandino et al. 2018a). Compendiously, all the evidences reported so far sustain that Notch and NF-κB alternatively behave as driver or passenger in T lymphocyte development.

Notch and NF-κB Pathways Underlie Molecular Mechanism in T-Cell Leukemia

Given its function in cell fate decision, Notch has been implicated in many solid and hematological malignancies. Its prominent role in the differentiation and function of T-cells, also implicate Notch in the immune system diseases. During T-cell development, deregulated Notch signaling and enhanced NF-κB activity may result in malignant transformation. Paradigmatic is the development of T-cell acute lymphoblastic leukemia (T-ALL) associated to immature T-cell deregulation. T-ALL is an aggressive leukemia, which represents 15% and 25% of ALLs seen in children and adults (Aster 2005).

Misregulation of Notch signaling, in particular Notch1 and Notch3, represents a prominent oncogenic pathway in T-ALL. In fact 50% of human T-ALL patients show activating Notch1 mutations (Mansour et al. 2006), whereas overexpression of Notch3 is a common finding in human T-ALL (Bellavia et al. 2002). Moreover, rare Notch3 mutations have been detected in T-ALL (Bernasconi-Elias et al. 2016). Overall, these studies have increased the interest in the pathogenesis of T-ALL and expanded the role of Notch in the molecular mechanisms involved in tumorigenesis.

In particular Ordentlich and colleagues demonstrated that Notch and its downstream target Deltex act on E2A-encoded E47 by inhibiting signaling through Ras (Bain et al. 1997). Most T-ALLs induced by other oncoproteins such as TAL1, LMO1, or LMO2 are characterized by inhibition of the transcriptional activity of the E2A proteins, suggesting that E2A could be an essential pathway in the leukemogenesis of T-ALL (Chervinsky et al. 1999).

Additional studies revealed that the c-Myc protoncogene involved in cellular growth is a critical direct downstream target gene of Notch1 in leukemogenesis (Sharma et al. 2006).

Notch signaling has been shown to be a potent regulator of cell cycle. In particular Sicinska and colleagues have studied the function of cyclin D3 in T-cell leukemogenesis and the correlation with Notch signaling (Sicinska et al. 2003). Cyclin D3 deficiency inhibits T-ALL induced by Notch1, suggesting the requirement of cyclin D3 for the growth of T-ALL cells.

Other studies from Beverly group proposed that Notch suppresses p53 in lymphomagenesis through repression of the ARF-Mdm2-p53 pathway that is involved in the regulation of apoptosis (Beverly et al. 2005).

However, increasing evidence revealed the key role of the cross-talk between Notch and NF-κB pathways in T-ALL development, suggesting NF-κB signaling as one of the major mediators of Notch-induced oncogenic transformation (Vilimas et al. 2007).

In particular Espinosa and colleagues (Espinosa et al. 2010) demonstrated that Hes1, a target of oncogenic Notch1, is able to induce the activation of the NF-κB pathway in human T-ALL lines and animal models of disease in two different ways. First Hes1 is able to modulate the IκBα protein stability facilitating IKBα degradation that is an important inhibitor of canonical pathway of NF-κB. Hes1 is also a key mediator of Notch-induced transformation by targeting the deubiquitinase CYLD that is a negative regulator of IKK activity and is normally suppressed in human T-ALL (Espinosa et al. 2010).

Notch can cooperate or counteract other signaling pathways in T-ALL. Calcineurin (Cn) is a calcium-dependent serine/threonine phosphatase implicated in a variety of physiological and developmental processes, including the immune system. Recently, this signaling pathway has been implicated in the induction and progression of hematological malignancies. Indeed, nuclear NFAT2 was found in cases of Burkitt's lymphoma, diffuse large B cell lymphoma, and aggressive T-cell lymphoma (Gachet and Ghysdael 2009).

In T-ALL, Cn was reported to contribute to leukemogenesis in N1ICD and ETV6-JAK2 mouse models of T-ALL, in which sustained activation of the pathway by microenvironmental cues leads to constitutive dephosphorylation of NFAT (Medyouf et al. 2007).

The molecular mechanisms that account for sustained activation of Cn in leukemic cells remain to be identified, but may require signaling from the in vivo tumor microenvironment and have been shown to be independent of TCR and pre-TCR expression (the main Cn activators in normal T progenitors), at least in the ETV6-JAK2 mouse model (Medyouf et al. 2007). Although pre-TCR signaling is vital for proliferation and differentiation of thymocytes, at the same time it is crucial that the preTCR signals are switched off along differentiation. Failure to silence Ptcra will lead to uncontrolled proliferation resulting in T-ALL development. NF-ATc1 has been demonstrated to have a tumor suppressor activity by switching off the Ptcra expression and thus preventing T-ALL development in an experimental setting of hyperactive Notch3 (Klein-Hessling et al. 2016).

In the past years, also the miRNAs have been involved in the regulation of physiological and pathological processes including human leukemias (Schotte et al. 2012).

Our group demonstrated that Notch signaling and NF-κB are able to increase miR-223 gene expression, which in turn downregulates the expression of the oncosuppressor FBXW7, known to regulate negatively Notch signaling, thus suggesting that the Notch/miR-223/FBXW7 axis may reinforce Notch signaling effect in T-ALL (Kumar et al. 2014).

Notch can also cooperate with NF-κB indirectly, by modulating the mechanisms that induce the release of TFs sequestered in the cytoplasm. In particular Asb2 is able to target IKba for destruction and thus is able to free NF-κB from an inhibitory status. Notch can also upregulate Asb2 transcription and NF-κB activation in T-ALL cells (Yu et al. 2018). Inhibition of Asb2 expression can significantly decrease Notch-induced NF-κB activation, suggesting that Notch signaling mediates NF-κB activation through Asb2.

Notch family and NF-κB are not involved only in the molecular mechanism of T-ALL but in 2015 Xu and colleagues (2015) found mutation of Notch1 in chronic lymphocytic leukemia and accordingly constitutive activation of NF-κB. Patients carrying Notch1 mutations had a poor prognosis and high level of NF-κB.

Although Notch and NF-κB are involved in leukemogenesis, the mechanism of propagation and disseminations of leukemia cells are still unclear. CXCR4 and other transmembrane receptor seem to have an important role in this aspect of leukemia. In particular CXCR4 is also involved in survival, proliferation and dissemination of cancer, including acute lymphoblastic and myeloid leukemia (ALL, AML). The chemokine receptor CXCR4 mediates cell anchorage into BM microenvironment and is overexpressed in 25–30% of patients with acute myeloid leukemia (AML) (Zhang et al. 2017b).

Recent studies in our laboratory have shown that deregulated Notch3 signaling enhances CXCR4 cell-surface expression and migratory ability of CD4+CD8+ thymocytes, contributing to "pre-leukemic" cell propagation, early in disease progression (Ferrandino et al. 2018b).

Moreover, hyperactive Notch3 is able to sustain CXCR4 surface expression by modulating the phosphorylation of β-arrestin1 that is involved in the internalization of CXCR4. Activated Notch3 can constitutively phosphorylate β-arrestin, thus enhancing CXCR4 cell surface expression (Ferrandino et al. 2018b).

All of these studies show how Notch, NF-κB, CXCR4 are involved in the leukemia contest and how a combined therapy can be an important and future approach against it.

Conclusion and Perspectives

Notch and NF-κB are two main signal transduction pathways with multiple and diverse functions, from differentiation to proliferation and/or survival of different cell types. They have an essential role in regulating lymphocyte differentiation and represent prominent signals in T cell development and maturation at several stages. There also is increasing evidence that their deregulation in early T cell differentiation steps can be strongly associated to leukemia development. An emerging factor is represented by the multiple level of interaction between these two pathways modulating cell-intrinsic as well as cell-extrinsic cues in lymphocyte functions. Both Notch and NF-κB pathways are composed by several members with a highly versatile and cell context–dependent activity, and these elements may complicate extrapolation of experimental results. Members of both signaling systems are nonredundant by performing nonoverlapping functions. Controversies in these signals require studies in-depth to clarify the importance and the functions of Notch and NF-κB as fundamental partners in immune cell biology. Experimental design should also reach a consensus regarding how they regulate progressive maturation and immune response.

To make the scenario more complex both Notch and NF-κB pathways, besides being interconnected with each other, interfere with other important developmental signaling, thus requiring a critical balance among them in order to prevent disease development.

In future, all this information could be taken into consideration for further studies and when designing a combined targeted therapy, including Notch inhibition.

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