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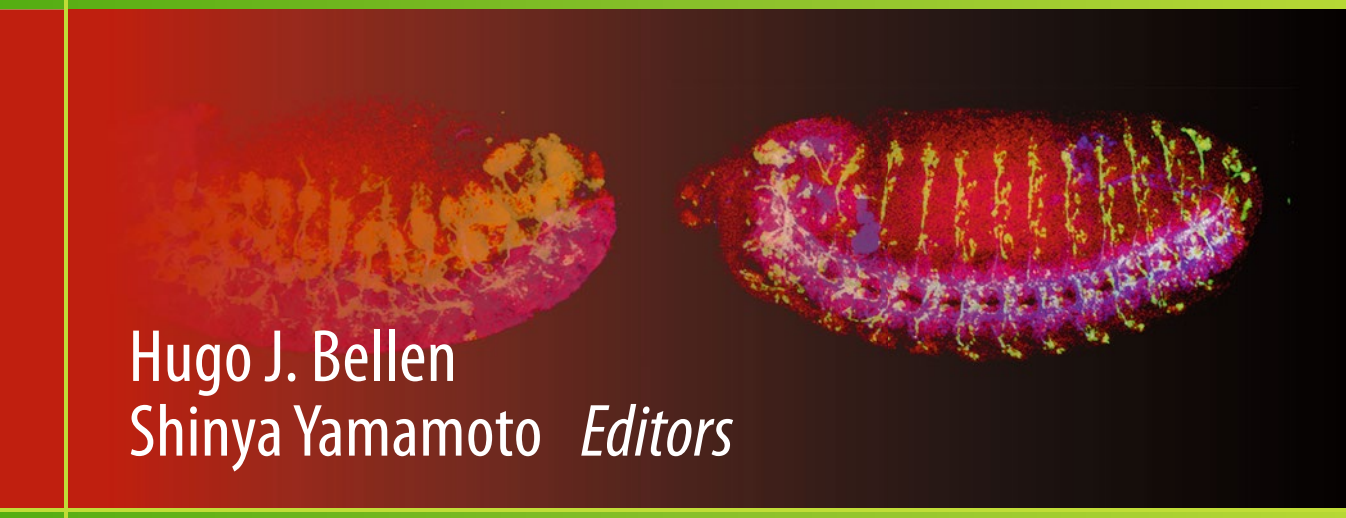

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Notch Signaling

Methods and Protocols

Humana Press

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Notch Signaling

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Preface

Notch signaling has a long history that originated 100 ago. However, 98 % or more of the knowledge related to Notch signaling has been gathered in the past 30 years, and the period between 1990 and 2013 has been exciting, both because of the extent of basic knowledge accumulated and potential implications for therapy in diseases associated with Notch. Most of the methods discussed in this book have been developed in the past 10–15 years and they cover a wide array of approaches related or based on mouse and human cell lines, flies, and mice. The first set of chapters focus on genetic methods in flies and mice, methods to image Notch signaling in live organisms or cells, techniques to monitor Notch activity in cells, and procedures to visualize oscillation associated with Notch signaling in cells and tissues. The next set of chapters focus on molecular, biochemical, and bioinformatics aspects of Notch signaling and include analyzing the Notch interactome, posttranslational modifications of Notch, ligand binding assays, and methods to assess proteolytic cleavage and transcriptional targets. Finally, strategies to diminish Notch signaling using small molecules, anti-Notch antibodies, and anti-ligand antibodies are discussed.

It is impossible to cover all methods using all organisms related to Notch signaling, but we believe that these 25 chapters will be a valuable contribution to hundreds of labs and thousands of scientists who pursue this research area.

We are especially grateful to Karen L. Schulze who provided advice and performed skillful editing. We thank all authors for their expert contributions and their diligence.

Houston, TX, USA

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Chapter 1

Introduction to Notch Signaling

Shinya Yamamoto, Karen L. Schulze, and Hugo J. Bellen

Abstract

Notch signaling is probably the most widely used intercellular communication pathway. The *Notch* mutant in the fruit fly *Drosophila melanogaster* was isolated about 100 years ago at the dawn of genetics. Since then, research on *Notch* and its related genes in flies, worms, mice, and human has led to the establishment of an evolutionarily conserved signaling pathway, the Notch signaling pathway. In the past few decades, molecular cloning of the Notch signaling components as well as genetic, cell biological, biochemical, structural, and bioinformatic approaches have uncovered the basic molecular logic of the pathway. In addition, genetic screens and systems approaches have led to the expansion of the list of genes that interact and fine-tune the pathway in a context specific manner. Furthermore, recent human genetic and genomic studies have led to the discovery that Notch plays a role in numerous diseases such as congenital disorders, stroke, and especially cancer. Pharmacological studies are actively pursuing key components of the pathway as drug targets for potential therapy. In this chapter, we will provide a brief historical overview of Notch signaling research and discuss the basic principles of Notch signaling, focusing on the unique features of this pathway when compared to other signaling pathways. Further studies to understand and manipulate Notch signaling in vivo in model organisms and in clinical settings will require a combination of a number of different approaches that are discussed throughout this book.

Key words Review, Notch signaling, History, Development and disease, Experimental approaches

1 Historical Overview

Notch was "discovered" in the laboratory of Thomas Hunt Morgan in March of 1913 [1, 2]. The oldest publication record of description of a "notch" defect in *Drosophila*, a loss of wing margin tissue from the distal tip of the wing, goes back to 1914 by John S. Dexter [3]. The first allele of *Notch* was established in 1917 [4]. In addition to notched wings, heterozygous *Notch* mutant flies were reported to exhibit additional wing vein and bristle abnormalities [5], providing a glimpse of its pleiotropic nature. The first link between Notch and development was established by the pioneering work on hemizygous *Notch* mutant embryos by Donald F. Poulson in the 1930s [6, 7]. *Notch* mutant embryos lacked mesodermal and endodermal tissue while most of the remaining

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ectodermal tissue produced nervous system at the expense of hypodermal cells. This unique phenotype, later called the "neurogenic" phenotype [8], was one of the first indications that *Notch* functions during cell–cell signaling. Furthermore, genetic screens looking for mutants with similar neurogenic phenotypes lead to the identification of core Notch signaling components such as *Delta*, *mastermind*, and *Enhancer of Split* (*E(spl)*) [8, 9]. In addition, analysis and genetic screens focusing on wing notching and bristle defects lead to the identification of genes such as *Suppressor of Hairless* (*Su(H)*, also known as CSL), *Serrate*, and other factors of the pathway [10, 11].

An important breakthrough was the cloning [12, 13] and sequencing of the *Notch* gene [14, 15]. Notch was shown to be a very large transmembrane domain protein with large extracellular and intracellular domains. Molecular characterization of the *Notch* gene in *Drosophila* lead to the identification of direct homologs in other species including *C. elegans* (*lin-12* and *glp-1*) [16–18] and vertebrates (*Xotch* and *TAN-1*) [19, 20], expanding the Notch field from *Drosophila* to other model organisms and human biology. Evidence that Notch functions as a receptor of a novel intercellular signaling pathway accumulated during the late 1980s and the early 1990s [21–23]. Pioneering work in *C. elegans* made the link between Notch activation and γ-secretase activity [24–27], which later led to the model that the intracellular portion of the Notch receptor translocates into the nucleus and directly regulates transcription [28–31]. Identification of Presenilin, a key protein in Alzheimer's disease pathogenesis, as the core catalytic subunit of the γ-secretase complex responsible for the proteolytic cleavage of Notch broadened the significance of Notch studies in biomedical research [32]. Biochemical and structural approaches have also greatly contributed to the understanding of chemical, physical, and mechanistic properties of Notch signal regulations [33].

In parallel to the efforts to reveal the genes and mechanisms that coordinate the Notch signaling pathway using model organisms and cultured cell lines, research also uncovered a strong link between Notch and a diverse set of human diseases [34]. Mutations in the receptors and the ligands have been shown to be causative for the pathogenesis of multiple developmental disorders, and misregulation of the pathway has been linked to an array of tumors in various tissues [35]. The core components of the Notch signaling pathway have emerged as major drug targets for anticancer therapy, although less important or context specific components of the pathway may be much better targets as the core components are almost certainly extremely pleiotropic and are required almost continuously in gut, hematopoiesis, skin, bone, etc. [36]. More recently, an active debate is ongoing whether Notch signaling pathway dysregulation may participate in neurological and psychiatric disorders as well [37, 38].

In summary, Notch research that started out by noticing a mild phenotype at the tip of the *Drosophila* wing has grown into an interdisciplinary field involving hundreds if not thousands of geneticists, developmental, cell, molecular, structural biologists, bioinformaticians, chemists, and clinicians.

1.1 Unique Properties of Notch Signaling

Compared to other intercellular signaling pathways such as Wnt, Hedgehog, and TGF-β/BMP, Notch signaling pathway is unique in multiple aspects. First, canonical Notch signaling occurs in a "juxtacrine" manner meaning that the signaling takes place between juxtaposed neighboring cells and requires direct cell–cell contact, while most other signaling pathways rely on paracrine signaling mediated by ligands that are secreted and reach distant cells through diffusion and/or active transport mechanisms. This is because both ligands and receptors of the canonical Notch pathway are transmembrane proteins that are embedded into the membrane of the cells [39]. However, some noncanonical Notch signaling events can be mediated by a secreted ligand in a paracrine manner, which has been documented in *C. elegans* [40].

Second, Notch signaling is extremely dose sensitive due to the lack of a signal amplification step or utilization of secondary messengers to transmit the signal from the cell surface to the nucleus. Notch signaling is mediated through the release and translocation of the intracellular domain of Notch (NICD) into the nucleus and NICD directly functions as a transcriptional coactivator. *Notch* in *Drosophila* is one of the very few examples where the locus is haploinsufficient, and a duplication also causes visible phenotypes. Furthermore, *Delta*, which encodes one of the two Notch signaling ligands in *Drosophila*, also exhibits haploinsufficient phenotypes in flies [41]. Strict dosage dependence of Notch signaling during development is also observed in mammals, including human [42, 43]. In addition, both hyper- and hypo-activation of the pathway are associated with different types of cancer in mice [44–46] and in humans [47–51]. These studies point out that a tight regulation of signal activity in both the signal sending and receiving cells is crucial for optimal signal output in physiological settings.

Third, Notch is a highly pleiotropic signaling pathway whose output depends on developmental and cellular contexts. For example, Notch signaling can be used for lateral inhibition, cell fate decisions, and in an inductive or a permissive manner to select out certain cell types, induce specific cell fates, and define boundaries during morphogenesis in *Drosophila* [23, 52]. In addition, activation or inhibition of the pathway can lead to a wide range of cellular responses such as proliferation, differentiation, or cell death depending on the context and cell type. Finally, activation of the pathway is now being associated with synaptic plasticity and learning

and memory in both fly [53–57] and mammalian nervous systems [58–60], suggesting a post-developmental role of Notch in differentiated cells.

In sum, given these characteristic properties, Notch signaling can be thought of as a "double-edged sword" that needs to be carefully controlled and constantly monitored for proper activity.

2 Core Components of Notch Signaling

The core components of the Notch pathway are depicted in Fig. 1. The ligand binds to the receptor at the interface between the two signaling cells. This leads to the release of the intracellular portion of the receptor that translocates into the nucleus, and interacts with a transcription factor and coactivators to activate transcription. However, due to the unique properties of Notch signaling and the extreme dosage sensitivity, numerous factors seem to have evolved to fine-tune the activity of the Notch signaling pathway in different tissues, cell types, and contexts [61, 62]. Some of these factors are general regulators and affect the core components of the canonical Notch signaling pathway in all contexts, whereas others are context and tissue specific regulators. In addition, although many of the phenotypes caused by dysregulation of the pathway can be explained by the canonical signaling pathway, several phenomena exist where we need to consider a noncanonical branch of Notch signaling that is mediated without the involvement of certain core canonical pathway members [63]. Here, we mainly focus on the receptors, ligands, and nuclear factors (NICD, CSL, and Mastermind) that comprise the core of the Notch signaling pathway.

2.1 Receptors and Ligands

Notch receptors are large multidomain type I transmembrane proteins (Fig. 2) [33, 39]. Four Notch paralogs (Notch1-4) are present in mammals including human, while only one Notch homolog exists in *Drosophila*. Notch receptors are translated in the endoplasmic reticulum (ER) and travel to the plasma membrane through the exocytic pathway while undergoing numerous post-translational modification events. In the ER, the signal sequence at the N-terminus is cleaved off and the extracellular domain of Notch, most of which consists of EGF repeats (EGFrs), undergoes sugar modifications mediated by protein glycosyltransferases [64]. For example, O-fucosylation by O-fut1 (Pofut1 in mammals) and subsequent elongation of this sugar chain with O-GlcNAc by Fringe (Lunatic, Manic, and Radical Fringe in mammals) in the Golgi complex modulates the ligand selectivity of the Notch receptor [65–67]. In addition, O-glucosylation by Rumi (Poglut in mammals) in the ER is necessary for receptor activation [68], while further elongation of this chain with O-xylose negatively regulates

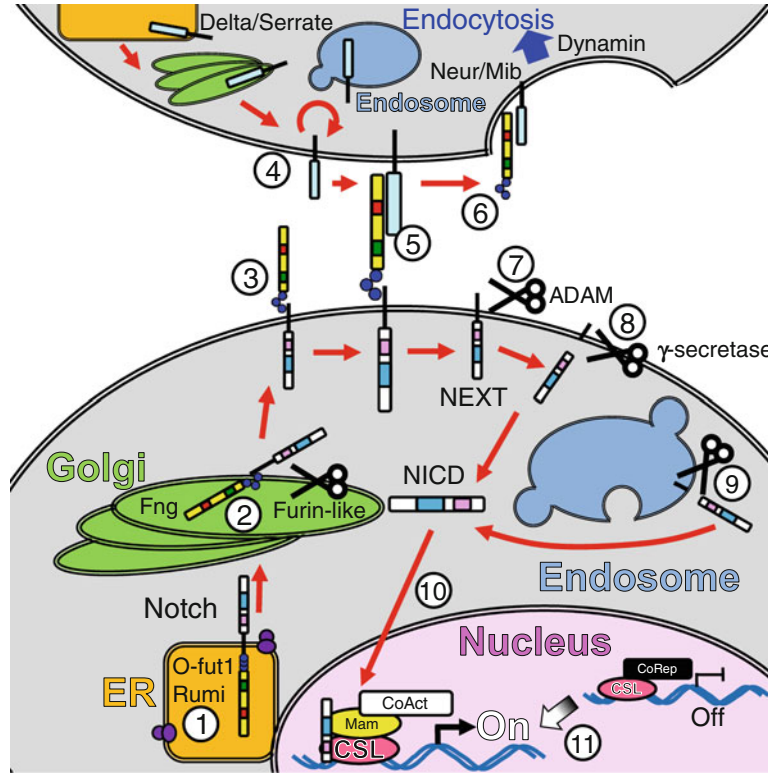

Fig. 1 A simplified diagram of the canonical Notch signaling pathway. For simplicity, the nomenclature of proteins depicted is based on *Drosophila*. Notch signaling occurs between juxtaposed signal sending cell (*top*) and signaling receiving cell (*bottom*). (1) Notch is translated in the rough ER and becomes glycosylated by glycosyltransferases such as O-fut1 and Rumi. (2) Notch traffics to the Golgi complex and undergoes S1 cleavage by Furin-like proteases. In addition, elongation of O-fucose chains on EGFrs occurs in cells that express Fringe. (3) Notch traffics to the cell surface. (4) Notch ligands, Delta and Serrate, are translated in the ER and traffic to the cell surface through the Golgi complex. Endocytosis and recycling of the ligands towards the site of ligand–receptor interaction is critical in certain contexts. (5) Ligands and Notch receptor physically interact. (6) The ligand–receptor complex is endocytosed into the signal sending cell. E3 ubiquitin ligases Neur or Mib and endocytic proteins such as Dynamin are essential for this *trans*-endocytosis. This physically "pulls" the Notch receptor so that conformational changes to reveal the S2 cleavage site can occur. (7) Notch undergoes S2 cleavage by ADAM proteases to generate NEXT. (8) NEXT undergoes S3 cleavage by the γ-secretase complex to release NICD. (9) γ-secretase complex cleavage of Notch/NEXT can also occur on the endosomal membrane. (10) NICD translocates into the nucleus. (11) NICD interacts with CSL and Mam on the target DNA. In the absence of NICD, CSL recruits corepressors to turn off gene expression. When NICD binds CSL and Mam, corepressors become replaced by coactivators to turn on gene expression. See main text for abbreviations

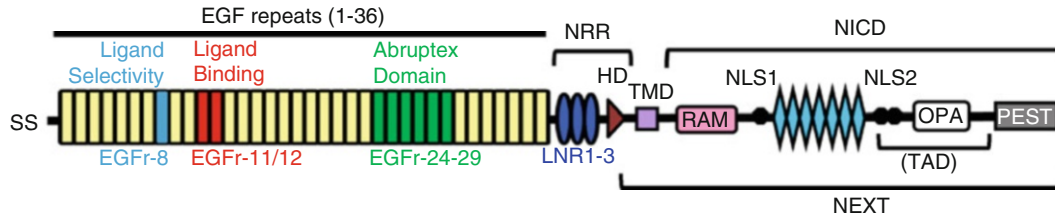

Fig. 2 Schematic diagram of the structure of the Notch receptor. For simplicity, *Drosophila* Notch receptor is depicted. Notch receptors are type I transmembrane proteins that have a signal sequence (SS) at the N terminal. The SS becomes cleaved off after translation. The majority of the extracellular domain consists of EGFrs. 36 EGFrs are present in *Drosophila* Notch. EGFrs-11 and -12 are essential for ligand binding, and EGFr-8 is involved in ligand selectivity. EGFrs-24 ~ 29 (Abruptex domain) are involved in negative regulation of the signaling. The NRR consists of three LNR domains and a HD motif. S1 and S2 cleavage sites are present here. The S3 cleavage occurs in the transmembrane domain (TMD). The NICD consists of a RAM domain, seven ANK repeats, several NLS, the TAD, and the PEST domain. The RAM domain and ANK repeats interact with CSL and Mam. NLS are required for nuclear translocation of NICD. TAD is involved in the recruitment of additional coactivators. PEST domain is necessary for proteasome mediated degradation of NICD for signal termination. See text for abbreviations

Notch signaling [69]. The Notch extracellular domain undergoes its first proteolytic cleavage (S1) in the Golgi complex by furin-like proteases [70]. While the S1 cleavage is not absolutely required for signal activation in flies as well as in mammals, this processing is thought to contribute to the net signaling activity by facilitating exocytosis of Notch [71, 72]. The cleaved fragments are held together through non-covalent interactions at the heterodimerization (HD) domain, and the receptor subsequently traffics to the cell surface to interact with its ligands.

Both ligands, Delta and Serrate (grouped together as DSL family ligands), are type I transmembrane proteins with a large extracellular domain and a relatively short intracellular domain [39, 40]. In mammals, three Delta-family ligands (Dll1, Dll3, and Dll4) and two Serrate-family ligands (Jagged1 and Jagged2) are present. Dll2 was initially identified in *Xenopus* [73], but its ortholog was later found to be absent in mammalian species, hence Dll2 is not present in mammalian nomenclature. The ligands are also synthesized in the ER, trafficked through the Golgi complex, and exocytosed. Since the ligands need to meet their receptors at the interface of the two signaling cells, vesicular trafficking to the membrane and endocytosis and recycling of proteins play important roles in fine-tuning the signaling strength [74–76]. Furthermore, upon ligand–receptor interactions, the ligand–receptor complex becomes endocytosed into the signal sending cell (trans-endocytosis) creating a "pulling force" that leads to a conformational change that promotes receptor activation. Mono-ubiquitination of the intracellular domains of ligands by the E3 ligase Neuralized (Neur) or Mindbomb (Mib) is critical for this

ligand endocytosis. In the absence of ligand ubiquitination, the ligand–receptors can interact but fail to activate the signal. Interestingly, the intracellular domain of Dll3 lacks ubiquitination sites and is thought to act as a decoy ligand in vivo.

Upon ligand binding and endocytosis of the ligand–receptor complex, the Notch receptor undergoes a conformational change that reveals a proteolytic cleavage site that permits cleavage by ADAM (a disintegrin and metalloproteinase) proteases. This leads to the second (S2) cleavage of the Notch receptor and the release of the extracellular domain [77]. This S2 cleaved Notch is still embedded in the membrane and is often referred to as the Notch extracellular truncated form (NEXT). NEXT is the substrate of the γ-secretase complex, an intramembrane protease [78]. Hence, Notch, via NEXT, undergoes a third (S3) cleavage that releases the NICD from the membrane. Where the cleavage is occurring within the cell is still a matter of debate [76]. The NICD that is freed from the membrane can translocate into the nucleus by the nuclear import machinery to engage in transcription regulation [79].

2.2 Nuclear Complex

NICD consists of a single RAM (RBP-jκ Associated Molecule) domain, seven ankyrin repeats (ANK), a transactivation domain (TAD), and a PEST (proline (P)/glutamic acid (E)/serine (S)/threonine (T)-rich motif) sequence at the carboxy-terminus (Fig. 2). In the fly NICD, a poly-glutamine (Q)-rich domain is present within the TAD, which is referred to as the OPA domain [80]. In addition, NICD carries multiple nuclear localization sequences (NLS) [29] as well as target sites for multiple posttranslational modifications such as phosphorylation [81] and ubiquitination [82].

In the nucleus, NICD interacts with a DNA binding transcription factor CSL (also known as *Su(H)* in *Drosophila*, *RBP-jκ* in mammals) and a coactivator Mastermind (*Mam* in Drosophila, *MAML1, MAML2, MAML3* in mammals) through its RAM and ANK domains [83, 84]. In the absence of NICD, CSL binds to its consensus sequence on the DNA and recruits transcription corepressors such as Hairless, CtBP (C-terminal Binding Protein), and Groucho to further recruit histone deacetylases (HDACs) and other repressive cofactors to negatively regulate the expression of Notch target genes [85–90]. Upon Notch signaling activation and NICD binding to CSL and Mam, the corepressor complex is disassembled, leading to derepression of the gene targets [91–93] and recruitment of the transcription activation complex including histone acetyltransferases (HATs) and chromatin remodeling complexes [94–97]. To prevent continuous signal activation, Notch signaling is shut off through phosphorylation of NICD by kinases such as cyclin-dependent kinase-8 (CDK8) [82] followed by poly-ubiquitination via E3 ubiquitin ligases such as SEL10/FBXW7 [98]. Poly-ubiquitination of NICD leads to proteasome mediated degradation and termination of the signal [99–101].

3 Fine-Tuning Notch Signaling in Development and in Disease

As a molecule of Notch is consumed upon a single round of signal activation through proteolytic processing at the plasma membrane and eventual degradation in the nucleus, proteins and factors that regulate any of the above mentioned events during signal activation and termination have the potential to regulate and fine-tune the output of the canonical pathway [52, 61, 62]. For example, the requirement of *trans*-endocytosis of the ligand–receptor complex for signal activation allows an additional layer of fine-tuning the signal strength at the cell surface. In addition to the ability of the ligands and receptors to interact (*in trans*) with one another at the interface of the signaling cells, the two can interact (*in cis*) within the same cell when co-expressed. Since the *cis*-interaction does not permit the conformational change of the Notch receptor required for S2 cleavage, and the ligands expressed in *cis* and *trans* are thought to compete for the ligand–binding domain of the Notch receptor, *cis*-interactions lead to negative regulation of signal activation (*cis-inhibition*) [102]. Furthermore, Notch receptors that bind to the ligand in *cis* reduce the concentration of the ligand that can *trans-activate* the neighboring cells [103, 104]. Thus, *cis*-inhibition not only inhibits the signal receptive ability of the signal receiving cell, but also the signal transmission ability of the signal sending cell (mutual inactivation). By altering the amount of ligands and receptors expressed in a cell, and by modulating the affinity of the ligands and receptors through posttranslational modifications such as glycosylation, numerous scenarios of Notch activation patterns can be generated in a tissue of interest [105].

While ligand-mediated activation of Notch signaling is thought to be responsible for most physiologic Notch signaling events, ligand-independent activation of Notch receptors can occur in vitro and in vivo and has been linked to pathogenic events such as cancer. In cultured cells, Notch can be activated in a ligand-independent fashion by chelating extracellular Ca^{2+} [106, 107]. This has been used as a convenient experimental manipulation to monitor and follow the time course of Notch activation. Ligand-independent activation of Notch can also be seen in vivo in a number of mutants in which endocytic trafficking towards the lysosome is disrupted [108, 109]. If Notch is not degraded properly in the lysosome, a conformational change in the extracellular domain of Notch and/or dissociation of the heterodimer is thought to happen in the endolysosomal pathway [76]. This leads to ectopic production of NEXT, which in turn undergoes S3 cleavage at the endosome/lysosome membrane to generate a functional NICD. Furthermore, ligand-independent activation of Notch can occur when a specific domain of the Notch extracellular domain is mutated.