Chapter 2
Noncanonical Notch Signaling

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Abstract Discovered nearly a century ago, today, Notch is known to mediate several biological processes through the canonical pathway that involves ligands, RBPJ, proteases, and coactivators. However, recent studies in vertebrates and invertebrates reveal that Notch can also exert its effect independent of RBPJ, in a noncanonical fashion. These studies demonstrate that Notch can exert its noncanonical role not only through nuclear partners but also via nonnuclear or cytosolic interactions. Additionally, there now is increasing evidence that Notch signaling can be initiated in a "noncanonical" fashion, independent of ligands. In this review, we detail the different cytosolic and nuclear, noncanonical interactions of Notch and discuss how they affect signaling processes in various contexts. We also discuss the evidence for ligand-independent, noncanonical initiation of Notch signaling.

Keywords Notch signaling · Noncanonical · RBPJ-independent signaling · Noncanonical Notch · Deltex · Akt · Abl · Cell survival · β-catenin · NF-κB

2.1 Introduction

The Notch signaling pathway is ancient and conserved throughout evolution. As detailed in previous chapters, Notch signaling was first described in *Drosophila* in 1916 as a mutation that gave rise to aberrant or "notched" wings in flies [55]. With the advent of molecular biology, the genetics of Notch signaling in *Drosophila* was uncovered at the molecular level revealing a single gene encoding one Notch receptor and two genes encoding the ligands, Delta and Serrate [6]. One of the first reports of a mammalian homologue of Notch is the elegant description of an oncogenic form of Notch by Sklar and colleagues in human T lymphoblastic leukemia (T-ALL) [24]. This landmark study provides the first observation of Notch

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expression in the immune system and importantly demonstrates that Notch expression can be oncogenic. Over the ensuing 25 years, it has become apparent that the Notch family of proteins are critical regulators of development in numerous vertebrate cell lineages, and, in many instances, deregulated Notch expression can be oncogenic [41].

The canonical Notch signaling pathway, observed in a wide variety of organisms, involves activation of Notch, initiated by ligand binding, followed by proteolysis through an ADAM protease and subsequent cleavage by gamma secretase. This two-step proteolysis leads to release of the intracellular domain of Notch (NICD) which rapidly translocates to the nucleus where NICD binds the DNA-binding protein, CSL/RBPJ, displacing corepressors and recruiting coactivators such as p300 and Mastermind, leading to the initiation of CSL-regulated transcription [11]. This canonical Notch signaling pathway is known to regulate many functions ascribed to Notch, and, in these instances, deletion of CSL/RBPJ usually phenocopies deletion of Notch. However, in recent years, it has become obvious that canonical Notch signaling does not account for all Notch function. We now recognize that noncanonical Notch signaling plays an important role in many Notch-driven processes. Indeed, recent evidence from *Nematostella*, the cnidarian sea anemone, suggests that the canonical Notch signaling pathway emerged after the divergence of the cnidarian-bilaterian lineages [45]. These data imply that noncanonical Notch signaling may be the ancestral signaling pathway and canonical Notch signaling evolved specifically in the bilaterian lineage.

The term noncanonical Notch signaling was originally coined to describe signaling events that are Notch dependent but do not rely on CSL/RBPJ. It was first observed in *Drosophila* using a genetic approach that examined the precise requirement of various components of the Notch signaling pathway. More recently, as our understanding of noncanonical Notch signaling has been refined, it is apparent that, at least in some instances, noncanonical Notch signaling may occur in the cytosol. We refer to this as noncanonical, cytosolic Notch signaling. Noncanonical Notch signaling can also occur in the nucleus, and we refer to this as noncanonical, nuclear Notch signaling. In the following sections, we will highlight several examples of noncanonical Notch signaling that have been observed in organisms as diverse as flies, mice, and humans and discuss the therapeutic implications of noncanonical Notch signaling.

2.2 Noncanonical, Cytosolic Notch Signaling

The notion that Notch plays a role in the cytosol is strengthened by the early observation that endogenous Notch is rarely observed in the nucleus and is mostly detected in the cytoplasm and/or cell membrane [7]. This implies that Notch may interact with various molecular partners in nonnuclear environments, affecting their function posttranslationally. Studies as early as in the 1990s have described

cytoplasmic interactions of Notch that are different from the usual, previously explored, nuclear roles of Notch. However, lack of follow-up studies exploring these interactions and their biological implications prevents us from strongly categorizing these molecular factors as noncanonical partners of Notch. In this section, we illustrate the abovementioned cytosolic interactions of Notch in addition to several other studies that provide solid evidence for the nonnuclear, noncanonical pathway of Notch signaling.

2.2.1 Notch and Deltex

In the early 1990s, in an attempt to identify interacting partners of Notch, Artavanis-Tsakonas and colleagues uncovered a small number of genes called the "Notch group" [5, 91]. This group comprised the following genes – *Delta*, *Serrate*, *Enhancer of split*, *Mastermind*, *Strawberry*, *Notch*, and *Deltex* [20]. Following this, pioneering work by the Artavanis-Tsakonas lab in 1994 revealed a cytosolic interaction between Deltex and the ankyrin repeats of Notch, a first of its kind interaction for Notch [20]. The ankyrin repeats form part of the intracellular domain of the Notch receptor and constitute the most conserved region between Notch and its vertebrate counterparts [81]. These repeats have been reported to be vital for Notch-mediated signaling events by several groups [68, 69]. Deltex is a cytoplasmic protein of unknown biochemical function that is ubiquitously expressed throughout development [12]. Using three techniques – in vivo co-localization, *Drosophila* cultured cell expression assay, and yeast interaction trap assay – Diederich et al. identified Deltex as the first cytoplasmic protein known to interact with Notch ankyrin repeats, implicating Deltex in the Notch signaling pathway. Further in 1995, the same group elucidated the role of Deltex in Notch signaling using *Drosophila* as their system [52]. In this study, they described a model for the action of Deltex, wherein the Deltex-Notch interaction antagonizes the interaction between Su(H) and Notch, thus preventing the cytoplasmic retention of Su(H). [Note: Su(H) is *Drosophila* CSL.] Therefore, Artavanis-Tsakonas and colleagues were the first to uncover a cytoplasmic interaction of Notch, providing the first clue of an alternate, nonnuclear role of Notch.

Shedding more light on the Deltex-Notch interaction, a study in 1998 described E47, a protein that is essential for B lymphocyte development, as a novel target of Notch [60]. Ordentlich et al. provide convincing evidence of inhibition of full-length E47 by cytoplasmic Notch1 and Notch2. Additionally, they showed that this inhibition did not correlate with the ability of Notch to activate RBPJ/CBF1. Furthermore, E47 was also inhibited by the cytoplasmic, Notch-interacting protein, Deltex, independent of CBF1/RBPJ. Therefore, in addition to identifying E47 as a novel target of Notch, this study also showed that the pathway that connects Notch and E47 is independent of RBPJ and also involves Deltex. These data not only add to the Deltex-Notch story but also present the first indication of a cytosolic and noncanonical mode of Notch signaling.

2.2.2 An Early Description of RBPJ-Independent Notch Signaling

In the canonical pathway, Notch controls cell fate by activating expression of the transcriptional regulator RBPJ which upregulates the expression of the Hes-1 gene, a well-characterized Notch target gene [35]. One of the earliest studies providing compelling evidence of an RBPJ-independent role of Notch came almost two decades ago from Shawber and colleagues [75]. They demonstrated that constitutively active forms of Notch inhibit muscle differentiation in mouse myoblasts but do not interact with RBPJ (referred to as CBF1 in this paper) or upregulate Hes1 gene expression. The authors generated truncated, cytoplasmic forms of Notch1 that lack the Notch/RBPJ interaction sequences, and showed that, although these cytoplasmic forms cannot interact with RBPJ or upregulate Hes1, they can, nonetheless, prevent muscle cell differentiation when stably expressed in mouse myoblasts. While the mechanism by which this form of Notch prevents muscle cell differentiation was not explored in this study, it is suggestive of cytoplasmic molecular partners of Notch that propagate its signal independent of RBPJ. In addition to being one of the first reports of an RBPJ-independent role of Notch in mammals, this study also provided further proof of cytosolic functions of Notch even though these cytosolic functions were not explored in this study.

2.2.3 Notch and Abl

Another study describing a cytosolic, noncanonical function of Notch was conducted by Giniger in 1998 [30]. He showed that modest reduction in Notch levels, in the context of an Abl mutation, results in synthetic lethality and defects in *Drosophila* axon extension. Abl is a cytosolic, tyrosine kinase that is widely expressed and is involved in the development of various tissues [32, 79, 82]. It is one of the first cellular genes implicated in a common human cancer [23, 26]. More recently, a pivotal role has been established for Abl in axon patterning, particularly in *Drosophila*, where it contributes to the growth and guidance of many developing axons [29, 89]. Giniger found that Notch is present in extending axons and in growth cones of *Drosophila* and that the Abl accessory protein, Disabled, binds directly to NICD in vitro, providing the first link between the Notch and Abl pathways. These results led to speculations that Abl and its associated accessory factors might be involved in an alternate, noncanonical, Su(H)-independent signaling pathway of Notch in *Drosophila*.

Several years later in 2003, Giniger and colleagues provided additional evidence for the Notch-Abl interaction while examining the path taken by the intersegmental nerve b (ISNb) axons to approach its muscle targets [17]. The ISNb axons, which innervate body wall muscles, exit the central nervous system and reach a turning point to innervate specific target muscles in *Drosophila* [44, 84]. Crowner et al.

showed that the turning of the ISNb axons requires interaction of Notch with components of the Abl pathway and its accessory proteins. However, genetic interaction experiments failed to provide evidence for a role of the canonical, Su(H)-dependent pathway in this process. Further in 2008, Giniger and colleagues reevaluated axon guidance in *Drosophila* and provided genetic and biochemical evidence for a Su(H)-dependent Notch pathway for cell fate specification, whereas axon guidance required cytosolic interaction of Notch with Abl [27]. In this way, the Giniger group presents convincing evidence for both the canonical, Su(H)-dependent Notch pathway and the noncanonical, nonnuclear Notch pathway in the *Drosophila* nervous system.

2.2.4 Notch in Apoptosis and Cell Survival

Notch has been linked with apoptosis/cell survival for several years [18, 36, 58, 67]. Studies in the recent past attribute this anti-apoptotic role of Notch to the noncanonical, membrane-tethered or cytoplasm-localized form of Notch. Insightful studies from the Sarin laboratory have revealed the mechanisms by which Notch regulates apoptosis. In an attempt to determine if Notch mediates apoptosis in model T cell lines, Sade et al. found that ectopic expression of the intracellular domain of Notch1 confers protection against diverse apoptotic stimuli [72]. This anti-apoptotic activity results from NICD-induced increased expression of Bcl-x_L, FLIP, and IAP-2, components of three major families of anti-apoptotic proteins. Using pharmacological inhibitors and dominant-negative experiments, they showed that NICD-mediated anti-apoptotic function requires phosphatidylinositol 3-kinase (PI3K)-dependent activation of the serine/threonine kinase Akt/PKB, through the tyrosine kinase, $p56^{lck}$. They further demonstrated that endogenous Notch1 associates with PI3K and $p56^{lck}$, both of which are membrane-localized signaling complexes, lending further support to a noncanonical, cytosolic function of Notch.

In 2009, the Sarin laboratory identified a novel Notch-mediated signaling pathway that favors cell survival [64]. Here, Notch inhibits apoptosis triggered by neglect or nutrient withdrawal in mammalian cells by integrating its signal with the mTOR-Rictor signaling complex, ultimately resulting in activation of the kinase Akt/PKB. Their data reveal that, although Notch processing is required for the activation of the cascade, NICD activity did not require CSL-mediated transcription, suggesting a role for the noncanonical Notch signaling pathway. Moreover, spatial constraint experiments showed that enforced nuclear retention of NICD abrogates the anti-apoptotic activity, whereas the membrane-tethered form of NICD blocks apoptosis through mTOR-Rictor and Akt-dependent signaling. This suggests that cytoplasmic localization of NICD is required for its anti-apoptotic function. Further in 2010, Perumalsamy et al. investigated the mechanisms underlying the anti-apoptotic activity of Notch with regard to intersections with mitochondrial events [63]. They showed that Notch activity inhibits apoptosis induced by Bax, a proapoptotic protein from the Bcl2 family of proteins that determines mitochondrial

involvement in apoptotic cascades. This activity of Notch required ligand-dependent processing to generate NICD but was independent of the canonical, nuclear interactions of Notch. Indeed, similar to results from previous studies from the Sarin laboratory, this anti-apoptotic activity was compromised by forced nuclear retention of NICD and recapitulated by NICD recombinants localized outside the nucleus. Experiments using siRNA and dominant-negative constructs revealed that the kinase Akt is an intermediate in the Notch-mediated anti-apoptotic pathway and that this process requires Mitofusin 1 and Mitofusin 2 (Mfn 1 and Mfn 2). Mitofusins are mitochondrial remodeling proteins that coordinately regulate mitochondrial fusion [13]. Therefore, Sarin and colleagues have identified a nonnuclear, Notch-Akt-Mfn-mediated anti-apoptotic signaling pathway, thus laying the foundation for further analysis of a noncanonical Notch pathway that regulates cell survival.

Further in 2012, Sarin and colleagues went on to identify the cellular and molecular patterns of Notch activity that govern survival outcomes of mature T cells following their activation [65]. They describe a ligand-dependent, noncanonical Notch activation pathway coupled with a spatial pattern of Notch that protects T_{regs} from apoptosis caused by cytokine withdrawal. The survival of T_{regs} was mediated by the interaction of Notch signaling with PI3K signaling and mammalian target of rapamycin complex 2 (mTORC2), wherein biochemical studies revealed a membrane-proximal complex of NICD and the mTORC2 component, Rictor. Interestingly, they found that induced Tregs (iT_{regs}) and effector T cells, where nuclear NICD is predominant, were susceptible to cytokine withdrawal-induced apoptosis. Reconstitution with the nuclear excluded forms of Notch1 protected iT_{regs} and Notch$^{-/-}$ T_{regs} from apoptosis, whereas the nuclear-localized forms failed to do so, again showing that NICD activity outside the nucleus accounts for its anti-apoptotic activity.

Liu et al. revealed another mechanism that contributes to the survival effect of Notch [49]. The X-linked inhibitor of apoptosis protein (XIAP), one of the best characterized members of caspase inhibitors, is often overexpressed in malignant cells and elevated XIAP levels increasing resistance to apoptosis [19, 74]. Liu and colleagues have shown that NICD inhibits the degradation of XIAP during apoptosis by binding directly to XIAP, thereby blocking the binding of E2 ubiquitin-conjugating enzymes and preventing the in vitro and in vivo ubiquitination of XIAP. The authors also examined whether the interaction of Notch with the cytosolic protein XIAP is possible and found that NICD is able to bind XIAP in the cytoplasm, describing another cytosolic, noncanonical interaction of Notch.

2.2.5 Notch and Akt

A number of vital biological processes such as DNA synthesis, gene expression, neurotransmission, and hormonal storage and release are regulated by discrete subcellular pools of zinc [16, 34, 48]. Previous studies have demonstrated that zinc activates both PI3K and Akt [25, 40], thereby indirectly implicating a role for zinc

in Notch signaling. Based on these observations, a group in Korea investigated the crosstalk between zinc and Notch1 signaling [9]. Here, they showed that zinc acts as a negative regulator of Notch signaling by causing the cytoplasmic retention of not only NICD but also RBPJ, and this prevents their interaction both in vitro and in vivo. Their data further reveal that this cytoplasmic retention of NICD is a consequence of the activation of the PI3K-Akt signaling pathway. However, the mechanism of the zinc-mediated suppression of Notch signaling via PI3K-Akt and the biological implications of this downregulation are not understood. Nevertheless, the cytoplasmic retention of Notch and inhibition of Notch/RBPJ binding due to zinc give rise to speculation that this may be yet another instance of nonnuclear, RBPJ-independent signaling through Notch.

Another study in 2008 describing the cytoplasmic localization of Notch was conducted by the Shin group [80]. They explored the effect of Akt on NICD-mediated transcription in 293 T and Cos7 cells. Using luciferase reporter constructs, they demonstrated that constitutively active Akt downregulates NICD-dependent transcription. The CSL family protein, RBPJ/CBF1, recruits a corepressor complex involving SMRT and HDAC1 to exert its inhibitory effect on transcription after binding to DNA. Therefore, the authors further determined if this inhibition of Notch-dependent transcription by Akt is due to the effect of corepressors or HDAC activity and found that this downregulation is independent of both factors. In fact, the Akt-induced inhibition of Notch-mediated transcription was because Akt inhibited proper nuclear localization of NICD. Co-expression of the Akt isoforms resulted in cytoplasmic mislocalization of NICD. This, in turn, can lead to reduced expression of canonical Notch targets as less NICD is available in the nucleus to bind RBPJ allowing for more cytoplasmic, potential noncanonical interactions of Notch.

A hallmark of all stem cells is the maintenance of a delicate balance between differentiation and self-renewal, impairments which can lead to tumorigenesis or lineage depletion [21, 56]. In mammalian neural stem cells (NSCs) and *Drosophila* neuroblasts, the self-renewal versus differentiation decision requires Notch signaling [4, 86]; however, the mechanism by which Notch regulates these processes is not well defined. Shedding light on this aspect, a 2013 study described a new mechanism where canonical Notch signaling cooperates with a noncanonical Notch pathway to mediate Notch-directed NSC regulation [46]. In the noncanonical pathway, Notch activates the mTORC2/Akt pathway by interacting with PTEN-induced kinase 1 (PINK1) to influence mitochondrial function and enhance *Drosophila* neuroblast growth and proliferation. PINK1 is a mitochondrial serine/threonine kinase that is critical in regulating mTORC2 activity and influences mitochondrial function and dynamics [90]. Experiments exploring the mechanism by which Notch and PINK1 interact to influence mitochondrial function showed enrichment of full-length Notch at the mitochondria and the presence of increased mitochondrial Notch on PINK1 overexpression. These results demonstrate that Notch can exert effects directly at the mitochondrial membrane. Further, through co-immunoprecipitation studies, the authors showed that PINK1 and Notch physically associate in the mitochondria of human NSCs and in glioblastoma multiforme cells. These results identify a novel

noncanonical role for Notch, where it regulates mTORC2/Akt activity by directly interacting with a mitochondrial kinase PINK1, thereby influencing mitochondrial function.

2.2.6 Notch and β-Catenin

In 2009, the Srivastava lab showed that Notch1 antagonizes the Wnt/β-catenin signaling pathway, which promotes the expansion of cardiac progenitor cells by reducing the levels of active β-catenin in these cells [42]. More recently, they expanded these studies to determine the mechanism by which Notch negatively regulates β-catenin and explore this interaction in other stem cell types [43]. Their data in embryonic stem cells provide evidence for the negative regulation of active β-catenin by Notch and reveal that this regulation is independent of RBPJ-mediated transcription. To further determine if this regulation involves a physical interaction between Notch and β-catenin, the authors performed co-immunoprecipitation studies and found that Notch does, indeed, physically associate with active β-catenin and that this is the membrane-tethered form of Notch. This study, therefore, highlights a different role for Notch than the known, nuclear, canonical role, where a membrane-bound form of Notch physically associates with active β-catenin and negatively regulates it through the adaptor protein, Numb.

Lending further credence to the Notch-β-catenin interaction, Acosta et al. showed that Notch interacts with β-catenin in a nonnuclear fashion in *Xenopus* blastula cells and regulates early *Xenopus* development in a CSL-independent manner [1]. Wnt signaling has been shown to be important during early development in vertebrates; however, the role of Notch at these stages is still not well understood. Therefore, the authors set out to determine if Wnt signaling and Notch interact during early stages of *Xenopus* development. Overexpression of NICD alone resulted in accumulation of Notch in both the cytoplasm and nuclei of *Xenopus* blastula cells. However, upon co-expressing NICD with β-catenin, NICD was located on cell-cell junctions and not in the nuclei, whereas β-catenin was degraded. This suggests that Notch interacts with β-catenin in a nonnuclear fashion and regulates its degradation, perhaps through endosomal trafficking. Therefore, this study contributes to another nonnuclear, CSL-independent mode of Notch signaling.

2.3 Noncanonical, Nuclear Notch Signaling

In addition to the numerous accounts of noncanonical, cytosolic Notch signaling highlighted above, there are a few examples of noncanonical, nuclear Notch signaling. A recent paper from Chiang and colleagues questions the conventional canonical Notch signaling pathway in the nucleus where a lone NICD/Mastermind/RBPJ complex regulates all Notch-responsive genes [66]. In this report, the authors

demonstrate a direct physical interaction between Notch1 and Zmiz1, a member of the *p*rotein *i*nhibitor of *a*ctivated *S*TAT (PIAS) family of coactivators. The authors show that Zmiz1 and Notch interaction is important for both T cell development and leukemogenesis, but this interaction plays no role in other Notch-mediated events such as myeloid suppression or intestinal homeostasis. The data in this report suggest a previously unrecognized intricacy in the proteins that comprise the Notch/RBPJ complex. Whereas some genes are clearly regulated by canonical Notch/RBPJ complexes, others require coactivator Zmiz1. Although not directly addressed in this report, it is interesting to speculate that Zmiz1/Notch1 may regulate a unique subset of genes in the absence of RBPJ.

Additionally, a recent report from Kastner and Chan and colleagues examines the role of Ikaros in shaping the repertoire of Notch target genes in T cells [28]. In this study, DNA-binding complexes of NICD/Ikaros were identified in the absence of RBPJ. These complexes are likely repressive since the genes associated with these complexes were only expressed when cells are treated with a gamma-secretase inhibitor. Thus, another form of noncanonical Notch signaling may be a physical interaction of NICD with Ikaros that acts to repress associated genes.

2.4 Notch and NF-κB: A Player in Both Cytosolic and Nuclear Noncanonical Notch Signaling

Many investigations point to connections between NF-κB and Notch signaling pathways [61]. In some situations, NF-κB signaling can initiate Notch signaling either by direct or indirect interaction between the two pathways suggesting that NF-κB acts upstream of Notch [14]. As early as 1996, Guan and coworkers demonstrated a physical interaction between Notch1 and the p50 subunit of the NF-κB complex suggesting another level of interaction between these two pathways [88]. Following up on this observation, our group and others have observed physical interactions between Notch1 and several cytosolic proteins involved in NF-κB signaling [61, 77], and these are discussed below.

2.4.1 Notch/NF-κB Interactions in the Cytosol

A recent study investigating the effects of hyperactivated Notch signaling in breast cancer identified IL-6 as a novel target of Notch in basal breast tumor cells, where Notch upregulates IL-6 expression [37]. This Notch-induced increase in IL-6 levels further activates the JAK-STAT signaling pathway in both an autocrine and paracrine fashion and is controlled by the cellular p53 status and two proteins from the NF-κB signaling pathway – IKKα and IKKβ. Transfection of a NICD construct that lacks the CSL-binding RAM domain upregulated IL-6 expression but not the expression of the canonical target gene Hes1, suggesting a noncanonical mode of Notch action.

Furthermore, transfection of a NICD-ER fusion protein that is retained in the cytoplasm in the absence of tamoxifen upregulated IL-6 expression irrespective of the presence of tamoxifen, whereas the canonical target gene Nrarp was upregulated only in the presence of tamoxifen. This indicated that cytoplasmic localization of NICD is sufficient to upregulate IL-6, while nuclear translocation is necessary for the activation of the canonical target gene, Nrarp.

Early studies from our lab and others demonstrated that signaling through the T cell receptor (TCR) results in rapid activation of Notch1 signaling [2, 62], and these reports provided the first link of TCR signaling to Notch activation. Our data also linked TCR activation of Notch1 to triggering NF-κB activity, suggesting that Notch1 activation may drive NF-κB activity [62]. Following on this observation, we asked whether Notch1 physically interacts with NF-κB family members in lymphocytes and determined that Notch1 is found in a complex with either p50 or c-rel, two NF-κB family members, and is responsible for shuttling p50 and c-rel into the nucleus [78]. Using biochemical approaches, as well as confocal microscopy, we showed that NICD directly interacts with NF-κB and competes with IκBα, leading to retention of NF-κB in the nucleus. These data show that Notch1 plays a key role in the cytosol in escorting NF-κB into the nucleus and, in the nucleus, in promoting retention of the NF-κB complex, suggesting that Notch1/NF-κB interactions may occur in both the cytosol and the nucleus [78].

TCR-mediated signaling also induces the formation of the CBM complex (comprising CARMA1, BCL10, and MALT1) that is essential for TCR-induced NF-κB activation [70, 71, 87]. Signaling through TCR activates Notch proteins, which have also been implicated in NF-κB activation [78]. However, the molecular interactions that link Notch signaling through TCR to early events in NF-κB activation remain largely unknown. In collaboration with our colleagues, we revealed a novel cytosolic function of Notch1 where it acts as a scaffold protein and influences the formation of the CBM complex [77]. Via two distinct approaches, lipid rafts and a novel approach called biomolecular fluorescence complementation (BiFC), we demonstrated that cytosolic Notch1 physically interacts with the components of the CBM complex following stimulation through the TCR. Additionally, experiments using a luciferase reporter assay and Notch1 constructs with localization restricted to the cytoplasm or to the plasma membrane showed that nonnuclear Notch1 can enhance NF-κB transcriptional activity in stimulated T cells. Thus, this study presents a novel model where Notch1 has the ability to function in the cytoplasm to facilitate early events during T cell activation, by physically interacting with the CBM complex and initiating NF-κB signaling.

2.4.2 Notch/NF-κB Interactions in the Nucleus

A close examination of NF-κB- and CSL-consensus binding sites reveals an interesting observation. The DNA sequence recognized by CSL/RBPJ and NF-κB is quite similar and potentially overlapping. While not all CSL-binding motifs are

subsets of a larger NF-κB response element, NF-κB consensus sites incorporate a nested CSL-binding site [47, 51, 85]. This observation suggests that CSL/RBPJ and NF-κB may compete for the same DNA-binding site [31].

As described above, we and others have demonstrated that TCR signaling results in rapid activation of Notch1 and blockade of Notch using various strategies, including inhibition of γ-secretase (GSI) as well as deletion of Notch1 [2, 62], result in diminished T cell activation and proliferation and reduced cytokine secretion [50, 54, 59]. To better understand how Notch1 regulates expression of various cytokine genes, we conducted ChIP analysis of promoters from a variety of cytokine genes expressed in CD4 T cells. In such studies, we identified complexes of Notch and NF-κB1 (p50), as well as Notch and c-Rel bound to DNA in the promoter region of the *IFNγ* gene [78] indicating that there may be other nuclear partners, in addition to CSL, that cooperate with Notch to regulate target genes. We followed up these observations with an examination of the promoters of several other T cell-specific genes including IL-2, granzyme B, perforin, and cyclin D3 and the transcription factors T-bet and EOMES [15, 38, 54, 78]. In all cases, we found that Notch-1 could be found in a complex with either p50 or c-rel suggesting that Notch/NF-κB complexes may regulate transcription independent of CSL/RBPJ. In some instances (EOMES, granzyme B, and perforin), we reported evidence suggesting that these complexes may form independent of CSL [38, 54, 78].

The observation that several T cell-specific genes may be regulated by Notch/NF-κB complexes led us to question whether CSL/RBPJ is required for Notch-dependent T cell function. When T cells are activated by engagement of antigen with the T cell receptor, cell division and rapid proliferation are induced. This phase of proliferation is accompanied by the production and subsequent secretion of T cell-specific cytokines such as IL-2 and interferon-γ. Additionally, if provided with appropriate signals, $CD4^+$ T cells can differentiate into one of several different effector cell subsets including T helper-1 (Th1), T helper-2 (Th2), T helper-17 (Th17), or T regulatory (Treg) cells. Notch signaling has been implicated in the activation and proliferation of T cells as well as in the production of cytokines and the differentiation of naïve T cells into various effector cell subsets [3, 8, 39, 59, 73]. We asked whether canonical Notch signaling was required for these functions [22]. In these experiments, $CD4^+$ T cells were isolated from either wild-type animals or animals in which $Notch1^{-/-}$ or $RBPJ^{-/-}$ was conditionally deleted in peripheral T cells and activated in vitro through cross-linking the T cell receptor. Surprisingly, we observed that while conditional deletion of Notch1 abrogated the ability of $CD4^+$ T cells to proliferate in response to TCR stimulation, T cells from conditional deletion of RBPJ proliferated as well and perhaps even better than wild-type T cells indicating T cell activation was not dependent upon RBPJ. We then asked if the ability to produce IL-2 and interferon-γ was dependent upon RBPJ, and again, we found that $CD4^+$ T cells from T cells lacking RBPJ expression produced as much or more IL-2 and interferon-γ than wild-type T cells. Lastly, we asked whether naïve $CD4^+$ T cells could differentiate into Th1 effector cells in the absence of RBPJ expression. Once again, we observed that $CD4^+$T cells lacking RBPJ readily differentiated into Th1 effectors. Taken together these data indicate that activation,

proliferation, and differentiation of CD4+ T cells can occur in the absence of RBPJ expression and imply that these functions occur through a noncanonical Notch signaling pathway.

Based on our prior observation that localized Notch-1/NF-κB complexes on both the IL-2 and interferon-γ promoters, we asked whether Notch signaling regulates $CD4^+$ T cell activation, proliferation, and differentiation in concert with NF-κB. We used $CD4^+$ T cells from wild type mice or animals with conditional deletion of either Notch-1 or RBPJ and treated these cells with DHMEQ, an NF-κB inhibitor [83]. The results from these experiments showed that, as expected, Notch1-deleted $CD4^+$ T cells did not proliferate or produce IL-2 or interferon-γ when treated with either DMSO control or DHMEQ. However, in contrast, RBPJ-deficient $CD4^+$ T cells both proliferated and produced cytokines when treated with DMSO, but the ability to proliferate and secrete IL-2 and interferon-γ was greatly reduced by treatment with DHMEQ. These data suggest that noncanonical Notch1 signaling in $CD4^+$ T cells occurs, at least in part, through NF-κB. Whether this interaction takes place in the nucleus or cytosol remains to be determined.

2.5 Other Forms of Noncanonical Notch Signaling

Just as we recognize that Notch signaling can occur in the absence of RBPJ, it is also becoming clear that Notch signaling may be initiated in the absence of ligand binding. While this is not necessarily noncanonical as defined above, these reports indicate that Notch signaling can also be initiated in a "noncanonical" fashion. Mukherjee and colleagues found that in *Drosophila* crystal cells, a myeloid-like cell that circulates in the hemolymph, HIF-α (the *Drosophila* homologue of HIF-1α) stabilizes Notch in the endosome and enables cleavage of Notch by γ-secretase, thus releasing the active form of Notch into the cytosol [57]. Interestingly, this activation of Notch is totally independent of interaction with Notch ligands (Fig. 2.1a).

Another example of ligand-independent interaction of Notch in *Drosophila* comes from the report by Hori et al. [33] where Notch interaction in the endosomal compartment with the ESCRT protein Shrub diverts Notch from lysosomal degradation into a signaling pathway and initiates ligand-independent Notch signaling. This process is context dependent and only occurs in selected cell types during *Drosophila* development. It remains to be determined whether the ESCRT pathway or the HIF-α-dependent pathway of ligand-independent activation of Notch signaling occurs in vertebrates. However, it is tempting to speculate that because activation through the T cell receptor leads to rapid activation of HIF-1α in $CD4^+$T cells, HIF-1α may play a role in ligand-independent Notch activation in mammalian T cells as well [53].

Lastly, another example of "noncanonical" Notch signaling comes from the recent reports that Notch ligands have been found in exosomes. In particular, Sheldon et al. demonstrated that endothelial cells produce exosomes containing the Delta ligand Dll4 [76]. Exosomes are small extracellular membrane vesicles that

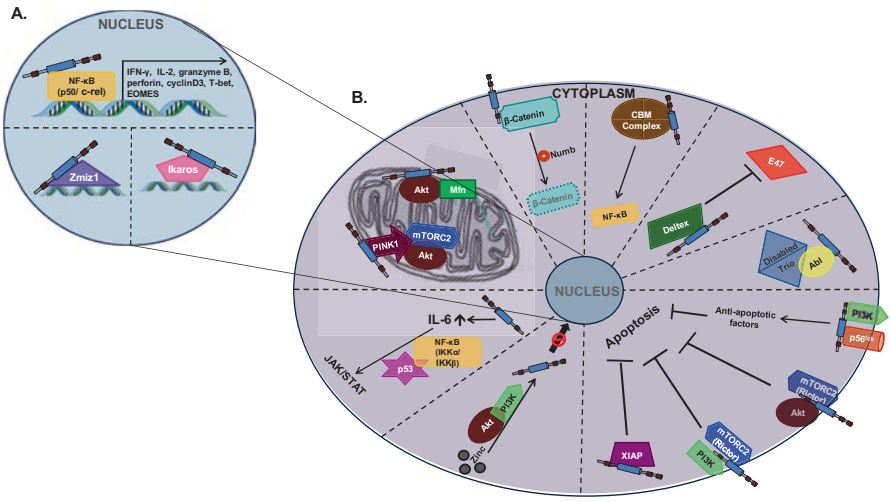

Fig. 2.1 (**a**) Noncanonical, nuclear Notch signaling. (**b**) Noncanonical, cytosolic Notch signaling

originate from endosomes. These vesicles are released from a wide variety of cell types and are postulated to influence cell signaling by interactions with cells either in the near vicinity or at more distant sites. Interestingly, DLL ligands are thought to require endocytosis to become functional [10]; therefore the incorporation of these ligands into exosomes is not surprising. One can readily see how exosomes containing Notch ligands might influence Notch signaling in a cell contact-independent fashion. In the report by Sheldon et al. [76], Dll4-containing exosomes produced by endothelial cells were shown to interact with Notch expressed in cells at a distant site. Surprisingly, rather than activating the Notch signaling pathway, these Dll4 exosomes instead inhibit Notch signaling and cause a developmental switch in the recipient cell resulting in a change in phenotype from endothelial cell to tip cell (Fig. 2.1b). During angiogenesis, a tip cell promotes the growth of new blood vessels; hence, the transfer of Dll4 exosomes to endothelial cells could promote vascularization. However, a more recent report employing Dll4 containing exosomes in a 3D microfluidic device reported a different effect and suggested that the exosomes initiate Notch signaling rather than suppressing Notch signaling. In any case, it is likely that exosomes can influence Notch signaling at distant sites.

2.6 Concluding Remarks

As highlighted above, signaling through the Notch receptor is not only complicated but also versatile, with important biological implications in several systems. The phenotype of mutations in Notch was discovered almost a century ago and the molecular components of the canonical Notch signaling pathway determined within

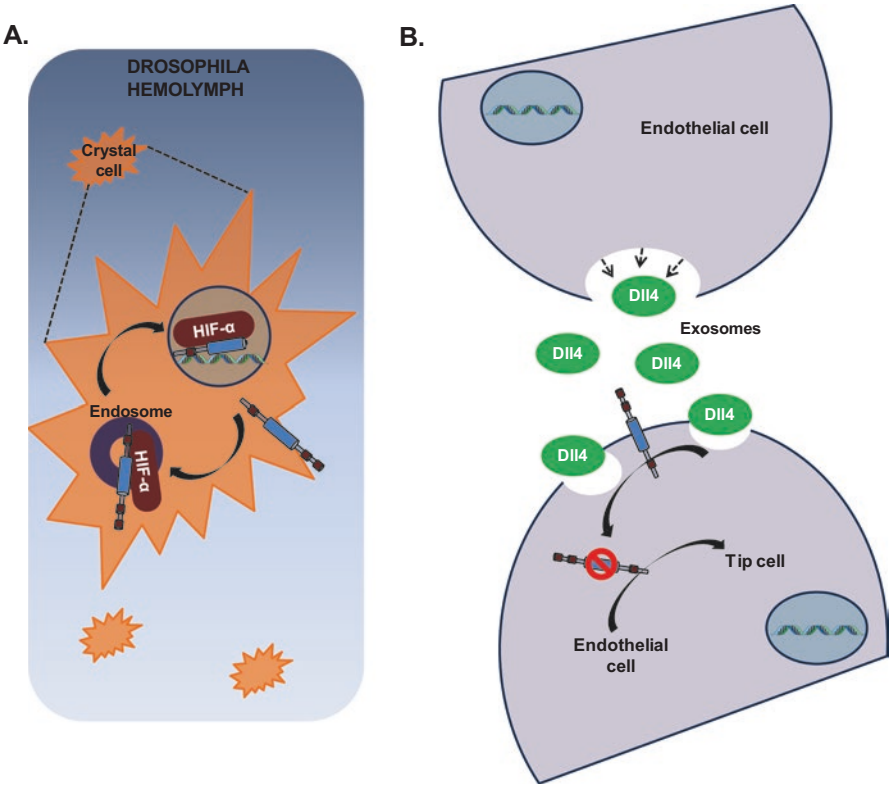

Fig. 2.2 Other forms of noncanonical Notch signaling. (**a**) Notch and HIF-1α interaction in *Drosophila* crystal cells. (**b**) Effect of Dll4 containing exosomes on Notch signaling

the past 3 decades. Canonical Notch signaling is known to impact multiple cell fate decisions. However, noncanonical Notch signaling has only recently been recognized with reports of signaling events through Notch that are RBPJ-independent. Moreover, with the growing number of studies, it is increasingly realized that noncanonical Notch signaling can occur in the nucleus as well as in nonnuclear environments. In this chapter, we have attempted to describe the various nuclear and nonnuclear interactions of Notch that contribute to its noncanonical role (summarized in Fig. 2.2). However, there is still much to be understood in terms of the molecular partners of Notch in this noncanonical pathway and the biological consequences of these interactions. Future studies in this regard will not only help reveal the multiple roles played by Notch through its noncanonical interactions but also provide a basis to explore and develop novel therapeutic strategies that influence Notch signaling in unique contexts. The ability to influence Notch signaling in situations where Notch expression drives disease (such as cancer and various autoimmune diseases) while leaving Notch signaling required for cell or tissue homeostasis (such as replacement of the epithelia in the intestine) intact could prove of great value in the clinic.

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