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Notch signaling: the demise of elegant simplicity

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Notch signaling can be viewed as an elegantly simple pathway that begins when the Notch receptor binds ligand, and ends when the Notch intracellular domain enters the nucleus and activates transcription. However, it is becoming increasingly clear that this core pathway is subject to a wide array of regulatory influences, from those that affect ligand–receptor interactions to those that govern the choice of Notch target genes. Even Notch ligands are now being scrutinized with respect to the possibility that they, too, function in the nucleus. A complete understanding of Notch signaling therefore requires us to look well beyond the core pathway.

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Abbreviations

BMP	bone morphogenetic protein
CSL	CBF-1, suppressor of hairless, LAG-1 (also known as RBP-Jκ)
HES	hairy enhancer of split
HRT	hairy related transcription factor (also known as HERP, HESR and Hey)
MeCP2	methyl-cytosine binding protein-2
MINT	Msx2-interacting nuclear target protein
N^{IC}	Notch intracellular domain
NIC	Notch intracellular domain
NICD	Notch intracellular domain
NRARP	Notch regulated ankyrin repeat protein
SMRT	silencing mediator for retinoid and thyroid-hormone receptors
TGF	transforming growth factor

Introduction

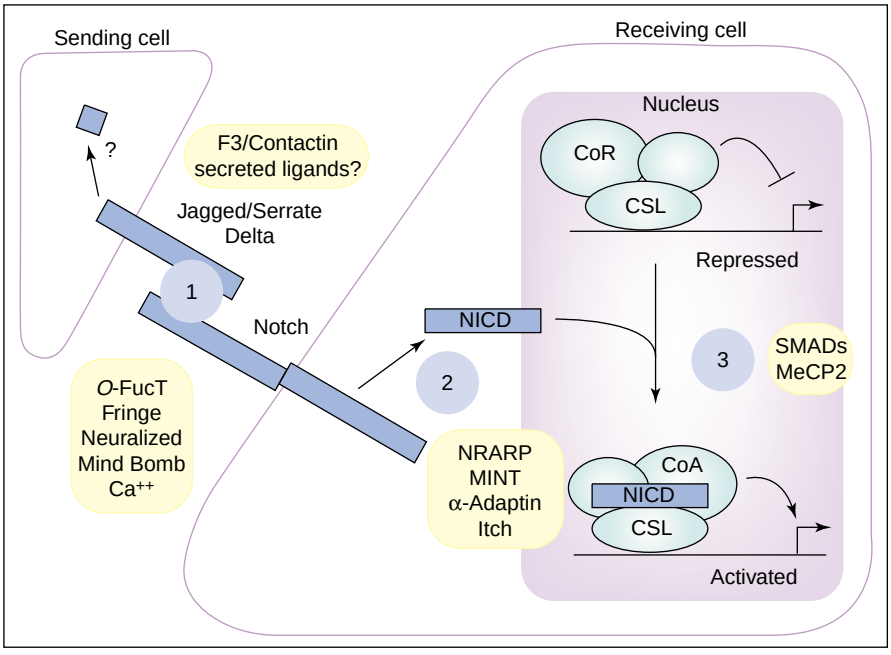
The Notch proteins are cell-surface receptors, the signaling activities of which regulate a variety of developmental processes. Identified initially in *Drosophila* — where the first mutant allele gave rise to a notched wing — Notch proteins have since been found in virtually all metazoans and have been studied extensively in flies, worms and mammals. Notch's role in lateral inhibition during *Drosophila* neurogenesis gave rise to the view that Notch signaling typically regulates binary cell-fate choices, relegating cells to 'default' developmental pathways. Although there are many additional examples in which Notch signaling functions to restrict particular fate choices, Notch signaling is now known to promote the development and/or proliferation of some cell types and to influence multiple developmental steps within a given lineage. As with most signaling pathways, the effects of Notch signaling are exquisitely context and cell type-dependent. Depending on the cell, Notch can act as an oncogene or function as a tumor suppressor. The pathway has been the subject of several excellent reviews covering its various roles in development and cancer [1–6]. This review will focus on our understanding of the mechanisms by which activity of the core signaling pathway is modulated.

The core pathway

The Notch proteins (Notch1–Notch4 in vertebrates) are single-pass receptors that are activated by the Delta (or Delta-like) and Jagged/Serrate families of membrane-bound ligands. They are transported to the plasma membrane as cleaved, but otherwise intact polypeptides. Interaction with ligand leads to two additional proteolytic cleavages that liberate the Notch intracellular domain (NICD, ICD, N^{IC} or NIC) from the plasma membrane [7,8]. NICD enters the nucleus, where it interacts with the DNA binding protein CSL [CBF (C-promoter binding factor)-1, Suppressor of Hairless, LAG-1; also known as RBP-Jκ (recombination signal binding protein Jκ) [9]]. In the absence of NICD, CSL represses transcription through interactions with a co-repressor complex, containing a histone deacetylase [10–12]. Upon entering the nucleus, NICD displaces the co-repressor complex from CSL and replaces it with a transcriptional activation complex that includes NICD, Mastermind, the histone acetyltransferase p300 and, possibly, PCAF p300/CBP [CREB (cyclic AMB response element binding protein) binding protein]-associated factor [13–17]. Notch signaling, thus, converts CSL from a repressor to an activator, leading to the transcription of target genes. The target genes include members of the Hes and HRT/HERP/Hey families of transcriptional repressors, therefore Notch signaling is often viewed as a transcription cascade.

The core pathway can be divided into three basic steps: activation of the receptor, generation of active NICD, and stimulation of target gene transcription (Figure 1). This review will focus on recent advances that have elucidated how the activity of each step of the core pathway can be regulated.

Figure 1



The core Notch signaling pathway. The three basic steps include: (1) receptor activation, (2) the generation of active NICD and (3) the activation of downstream targets. The proteins and molecules that are now known t o regulate each step are shown in the boxes.

Step one: regulating receptor activation

The most common, and conceptually, the simplest level of control is through the cell type-specific expression of Notch ligands. Irrespective of whether or not a cell expresses Notch proteins, signaling should not occur if a neighboring cell does not express ligand. With the exception of Jagged1 in vertebrates [18,19], little is known about the signals and transcription factors that regulate ligand transcription; however, simple ligand expression is not the only determinant of Notch signal strength. Recent experiments have improved our understanding of the importance of ligand ubiquitylation and Notch glycosylation in modulating the level of receptor activation. Notch activation through atypical ligands has also been described.

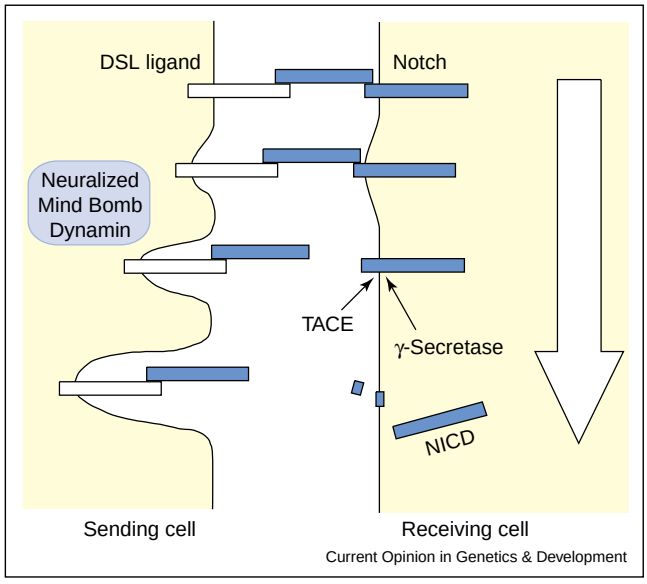
Ligand internalization

The activity of Notch ligands can be influenced by proteins that regulate their internalization and degradation. *Neuralized*, a gene that promotes Notch activity, encodes an E3 ubiquitin ligase that promotes the internalization and degradation of Delta [20–22]. A similar activity has now been ascribed to the protein encoded by Zebra fish *mind bomb* [23]. The idea that ligand internalization stimulates Notch signaling was provided initially by the analysis of Drosophila *shibire* mutants, which phenocopy certain Notch loss-of-function mutations [24]. *Shibire* encodes the fly homologue of dynamin, a protein that helps to process endocytic vesicles, and thus links ligand activity to ligand endocytosis. Together, these and other observations [25] support a model in which ligand internalization promotes the shedding of the Notch extracellular domain from a neighboring cell, thereby promoting the subsequent proteolytic cleavages that generate NICD (Figure 2). Asymmetric cell divisions can lead to the unequal distribution of Neuralized protein (in addition to the Notch inhibitor Numb) in daughter cells, amplifying the differences in those cells' abilities to send and receive a Notch signal [26]. The molecular details that link the ubiquitin ligase activity of Neuralized and Mind Bomb to ligand internalization are yet to be defined.

Notch glycosylation

Notch signaling is also influenced by receptor glycosylation [27]. The fringe genes (lunatic fringe, manic fringe and radical fringe, in vertebrates) encode glycosyltransferases (specifically, β1,3 N-acetylglucosaminyltransferases) that modulate Notch's response to its ligands [28]. The glycosylation of *Drosophila* Notch or vertebrate Notch1 by Fringe leads to differential activation by the two classes of ligand —inhibition of Serrate/Jagged-mediated signaling and activation of signaling through Delta [29]. The actions of Fringe depend on prior *O*-fucosylation (by *O*-FucT-1 in mammals and Nti/OFUT1 in flies), a modification that is absolutely required for all Notch signaling [30–32]. Thus, while the initial modification of Notch by fucose is necessary for all ligand-induced signaling, subsequent modifications by the Fringe

Figure 2



A model for the generation of active NICD. In this model, the internalization of ligand serves to physically pull the Notch extracellular domain from the surface of the receiving cell. This allows access of the ADAM family protease TACE and subsequent cleavage by the γ-secretase complex, liberating NICD from the plasma membrane.

proteins modulate Notch's response to individual ligands. These modifications directly influence Notch-ligand interactions. Although most of the EGF repeats contain consensus sites for *O*-FucT-1modification, recent work has shown that the glycosylation of one particular repeat (EGF12 in *Drosophila* Notch) is necessary for the inhibitory effect of Fringe on Serrate–Notch signaling [33].

Calcium

Notch signaling also responds to levels of extracellular calcium. It has been known for some time that the EGF repeats found in the extracellular domains of both Notch and its ligands bind calcium ions. Indeed, depletion of calcium from the medium of tissue-culture cells leads to a potent ligand-independent activation of the Notch receptor, presumably by altering the structure of the extracellular domain [34]. Work in the chick has now implicated Notch in the establishment of left–right asymmetry during development [35••]. In this case, the penultimate signal appears to be asymmetrical activation of the H^+/K^+-ATPase, which, in turn, establishes an asymmetric gradient of calcium ions to which Notch responds. The strength of the Notch signal correlates positively with the anticipated concentration of extracellular calcium, a correlation that that can be recapitulated with cultured cells [35••].

F3/Contactin

The GPI-linked neural cell recognition molecule F3/Contactin is not a member of the DSL (Delta/Serrate/Lag-2) family of Notch ligands. Nevertheless, it has been shown recently to be a functional ligand for Notch and to mediate certain aspects of Notch-mediated promotion of oligodendrocyte differentiation [36••]. NB-3, an F3/Contactin related molecule that is expressed by neurons, can similarly promote oligodendrocyte differentiation [37]. Interestingly, although γ-Secretase inhibitors and dominant-negative forms of Notch (and of Deltex) can inhibit the activity of F3/Contactin, dominant-negative forms of CSL cannot. This raises the possibility that F3/Contactin, although capable of generating nuclear NICD, works through an additional, novel pathway that may or may not collaborate with the core NICD/CSL pathway. Although CSL-independent pathways have been proposed for other developmental effects of Notch [38,39], a molecular definition of such pathways remains elusive.

Proteolyzed ligands

Drosophila Delta can be proteolytically cleaved by the ADAM (A disintegrin and A metalloprotease) family protease Kuzbanian, leading to its release from the plasma membrane. Genetic studies indicate that Kuzbanian enhances Notch signaling in flies, suggesting that both membrane-bound and cleaved Delta can activate the pathway [40,41]. Lending support to this idea, studies with *C. elegans* indicate that DSL-1, a protein that lacks a predicted transmembrane domain, can substitute for the typical membrane-bound Notch ligand LAG-2 in specifying vulval precursor cells [42••]. Although the extracellular portion of *Drosophila* Delta can mimic membrane-bound Delta in a neurite outgrowth assay using cultured cells [40], subsequent experiments have shown that extracellular forms of Notch ligands typically antagonize signaling [43]. The reason why soluble ligands promote signaling in some contexts but not others remains to be determined. Recent studies indicate that Notch ligands can be processed further by the presenilin–γ-secretase complex, resulting in a portion of the intracellular domain ending up in the nucleus, possibly affecting transcription [44•,45]. Hence, Notch signaling might have to be viewed, not just from the point of view of the cell that activates the core pathway, but also from the point of view of events that may occur in the ligand-expressing cell.

Step two: regulating the level or activity of NICD

When ligand has rendered the Notch receptor susceptible to cleavage, initially by TACE (TNF-α converting enzyme) and then by the γ-Secretase complex, NICD is generated [7,8]. Although individual roles for the components of the γ-Secretase complex have now been described [46,47], it is not known if the complex is subject to active regulation *per se*. The same is true for TACE. Nevertheless, the level and activity of NICD can be modulated through protein–protein interactions and by proteosome-mediated degradation.

MINT

MINT (Msx2-interacting nuclear target protein) was identified in a two-hybrid screen using CSL (RBP-J) as bait [48••]. MINT interferes with the ability of NICD to bind CSL *in vitro* and is able to inhibit NICD-mediated transcriptional activation in cultured cells, suggesting that MINT is an inhibitor of Notch signaling *in vivo*. It remains to be determined whether MINT functions like Hairless, a *Drosophila* protein that recruits co-repressors to Su(H), the fly homologue of CSL [49]. MINT expression is found in the testis, brain, spleen, lung, liver and kidney, and mouse embryos lacking the gene die at around embryonic day 12, displaying a variety of developmental defects. Fetal liver cells from both wild type and $MINT^{-/-}$ embryos can generate both B and T cells in irradiated mice. However, $MINT^{-/-}$ cells generate higher numbers of marginal zone B cells at the expense of follicular B cells in the recipient mice. This is consistent with the idea that Notch signaling regulates the follicular- versus marginal-zone B-cell fate choice and that MINT is a negative regulator of Notch in this setting. The roles of MINT in other cell types are yet to be demonstrated.

NRARP

NRARP (Notch regulated ankyrin repeat protein) was identified in a screen of RNAs induced by Notch [50,51]. The protein contains two ankyrin repeats and inhibits Notch activity, either through the formation of an inhibitory complex with CSL–NICD and/or by destabilizing NICD. Forced expression of NRARP in hematopoietic progenitors blocks T-cell development, consistent with it being an inhibitor of Notch signaling *in vivo* [52].

Numb

Numb has long been known to bind Notch and to antagonize Notch signaling cell autonomously [53–55], yet the precise mechanism by which Numb functions has been elusive. Recent studies have invoked the involvement of α-Adaptin [56], a protein that promotes endocytosis, and the E3 ubiquitin ligase Itch [57]. The requirement of α-Adaptin and its physical interaction with Numb suggest a mechanism whereby Numb targets the intact Notch receptor for endocytosis, thus reducing its concentration at the plasma membrane. By contrast, the involvement of Itch and its physical interaction with Numb suggests a distinct mechanism, whereby Numb targets Notch for proteolysis. The association with Itch is also consistent with the observation that Numb can inhibit the activity of NICD, which is not found on the plasma membrane.

Step three: regulating downstream targets and effectors

Once in the nucleus and able to bind CSL, NICD directs the formation of a multi-protein complex that activates transcription. Although several putative direct target genes have been identified, many important questions are still unanswered. For example, transcriptional activation by NICD is cell type-dependent, such that only a subset of Notch's primary targets may be activated when cells encounter ligand [58]. The mechanisms that control this selectivity are unknown. In addition, although the half-life of nuclear NICD is relatively short (1–1.5 h), due to the action of the ubiquitin ligase Sel-10 [59], there appears to be a link between NICD phosphorylation and degradation [14]. The details of this link also remain obscure. Finally, NICD may activate transcription of several genes, but little is known about which of those genes is/are responsible for the phenotypic effects of Notch signaling. In the case of Notch inhibiting neuronal development, it appears that the target genes Hes1 and Hes5 mediate many and perhaps all of the Notch effects [60]. Knockout studies have suggested additional links between Notch and known targets in the development of a variety of tissues and cell types. These include links between Notch and HES-1 in pancreatic development [61–63], Notch and its target Hey2 in cardiac development [64–66], and Notch and the targets Hey1 and Hey2 in vascular development [67]. Notch's ability to block adipogenesis in a cell culture model can be mimicked by HES-1, suggesting that maintenance of HES-1 expression might account for most of Notch's activity in that particular setting [68]. The IL-4 gene has been reported to be a direct target of Notch, which may explain Notch's ability to promote the formation of TH2 helper T cells [69•]. The direct targets that mediate Notch's other effects on T-cell development and its ability to inhibit muscle development remain unknown.

The molecular components of the CSL–co-repressor complex [10–12] and the CSL–NICD-co-activator complex [13–17] have been relatively well defined. Mastermind, a component of the co-activator complex, binds the p300 histone acetyltransferase and, somehow, also promotes NICD phosphorylation and degradation [14]. It remains to be determined to what extent the co-repressor and co-activator complexes cooperate with additional DNA binding proteins to affect the overall activity of Notch target genes *in vivo*. Indeed, studies of T cells that are devoid of CSL (RBP-J) argue that CSL does not appreciably repress transcription in the absence of NICD [70•].

MeCP2

MeCP2 (Methyl-Cytosine Binding Protein) is a protein that binds methylated CpG islands and recruits co-repressors to DNA. In *Xenopus*, MeCP2 interacts with the co-repressor SMRT (silencing mediator for retinoid and thyroid-hormone receptors) to regulate xHairy2a, a primary target of NICD [71••]. Notch signaling leads to the release of MeCP2 from the xHairy2a promoter, possibly by displacing SMRT from CSL and weakening the interactions of the MeCP2–SMRT complex with the promoter. Paradoxically, a mutant form of *Xenopus*

MeCP2 that cannot bind SMRT (this mutation is analogous to that found in certain Rett Syndrome patients) is not released upon Notch signaling, and this correlates with diminished induction of xHairy2a transcription. The functional interaction between MeCP2 and Notch is not likely to be applicable to all promoters that bind CSL, butt this study illustrates the complexity of interactions that might occur at Notch-responsive promoters and how other pathways may converge to affect Notch phenotypes.

SMADs

Two recent reports have provided a molecular explanation for at least some of the crosstalk between the Notch and TGF-β signaling pathways. One showed that a low level of Notch signaling is required for BMP4-mediated inhibition of myogenesis [72•]. BMP4 is able to activate transcription of the Notch target gene *Hey1*, partly through an ability of CSL to recruit activated SMAD1 to the promoter via NICD. The other report showed that the same is also true for TGF-β, which activates the Hes-1 promoter through the CSL-dependent recruitment of SMAD3 [73]. Thus, BMP and TGF-β can feed into the Notch pathway by augmenting the transcription of Notch target genes.

Perspectives

Our understanding of the Notch signaling pathway has grown enormously over the past few years. In fact, a *bona fide* role for Notch cleavage and for nuclear NICD was not fully appreciated until 1998 [74–76]. In a relatively short time period, the proteins that define the core pathway have been identified, along with additional proteins that modulate the pathway. Major issues that have yet to be fully explored include: the precise mechanism by which ligand internalization and ubiquitylation promotes receptor activation; the potential role of ligand cleavage in affecting gene expression in ligand expressing cells and; the identification of the transcriptional events downstream of Notch that govern phenotype. Although several Notch target genes have been identified, most of these have yet to be linked to Notch's specific effects on differentiation and tumorigenesis. Secondary Notch targets, such as those regulated by the Hes and HRT/Herp/Hey families of transcriptional repressors [77], are even less well characterized. The challenge over the coming years will be to extend Notch signaling from the core pathway, downstream towards the proteins that directly elicit a cell response. Given that the effects of Notch can be profoundly cell type-dependent, it is likely that the downstream pathway will not be universal, but will, instead, be defined by branches and tributaries that might flow in particular cell types, but not others.

Acknowledgements

Due to space constraints I was not able to reference much of the work that has led to our current understanding of the Notch signaling pathway. For those whose work was not cited, please accept my apologies. T Kadesch is supported by grants from the Department of Defense and the National Institutes of Health.

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