Notch signaling as a therapeutic target

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Signals transduced by Notch receptors influence differentiation and proliferation in a wide variety of cell types. Activation of a Notch signal by one of several ligands triggers a series of proteolytic cleavages that release the intracellular region of Notch from the membrane, allowing it ultimately to translocate to the nucleus and activate the transcription of downstream target genes. Recent studies have elucidated the roles of several key proteins that participate in and modulate these central events in Notch signal transduction. These advances offer a variety of potential avenues to manipulate Notch signaling for therapeutic purposes in the treatment of cancer and in stem cell maintenance.

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Abbreviations

APP	β-amyloid precursor protein
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
DSL	Delta, Serrate, LAG-2
ECN	extracellular Notch subunit
EGF	epidermal growth factor
HES	Hairy/Enhancer of Split
ICN	intracellular Notch
Maml1	Mastermind-like protein 1
NTM	Notch transmembrane subunit
PS	presenilin
T-ALL	T-cell acute lymphoblastic leukemias

Introduction

Genes of the *Notch* receptor family encode a class of evolutionarily conserved transmembrane receptors that transmit signals affecting development in organisms ranging from sea urchins to humans. Evolutionary divergence of invertebrates and vertebrates has been accompanied by at least two rounds of gene duplication: flies possess a single *Notch* gene, worms two (*glp-1* and *lin-12*) and mammals four (*NOTCH1–4*).

Because Notch receptors and their ligands are both single-pass transmembrane proteins expressed on the cell surface, they are capable of communicating signals between adjacent cells. Signals transmitted through Notch receptors control cell fate decisions in a wide array of developmental processes from neurogenesis to oogenesis [1]. Experimental increases or decreases in *Notch* gene dosage typically result in an increased abundance of cells adopting one fate at the expense of a second alternative fate. In specific contexts, *Notch* also influences apoptosis, cellular proliferation, and the organization of tissue boundaries, activities that further contribute to its broad role in morphogenesis (for a review, see [2]).

Here, we will summarize current understanding of the molecular events that participate in Notch signal transduction, focusing on potential avenues for therapeutic intervention. First, we highlight the molecular events required to communicate an activating signal via Notch receptors and describe cellular mechanisms that modulate Notch signaling. Then, we address the potential for treating certain cancers by inhibiting Notch signal transduction. Finally, we discuss the potential for *ex vivo* tissue engineering by using activating ligands to maintain stem cells in culture.

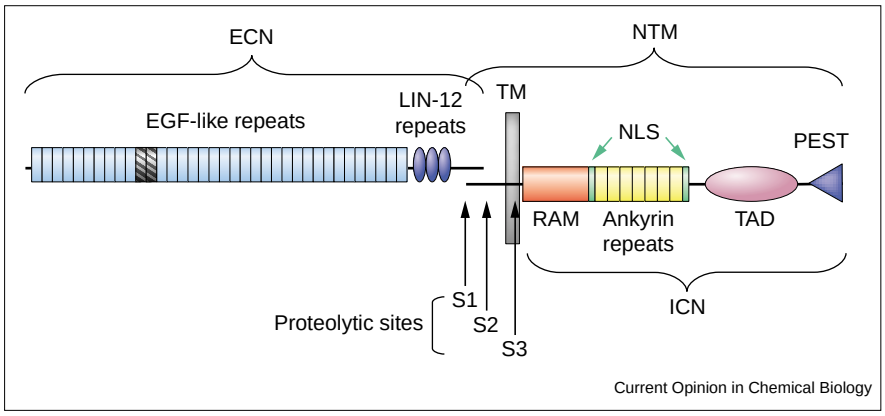
Notch and human disease

Validation of the Notch signaling pathway as a potential therapeutic target has emerged from the demonstration that a variety of human diseases result from mutations in genes encoding Notch receptors or their ligands. These diseases range from cancer to neurodegenerative disorders, reflecting the diversity of processes regulated by Notch signaling. The connection between unrestrained Notch signaling and malignancy was first recognized when a recurrent t(7;9)(q34;q34.3) chromosomal translocation, which creates a truncated, constitutively active variant of human Notch1, was identified in a subset of human acute T-cell acute lymphoblastic leukemias (T-ALL) [3]. This observation led to studies using mouse models which revealed that Notch1 signaling is essential for T cell development [4], that Notch1-mediated signals promote T cell development at the expense of B cell development [5], and that excess Notch signaling during development leads to T cell neoplasia [6,7].

Mutations in human Notch3 lead to the development of the hereditary disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). The vast majority of CADASIL patients harbor point substitutions of residues within the cysteine-rich epidermal growth factor (EGF)-like modules of the extracellular domain, resulting in addition or a deletion of a single cysteine residue [8]. Because formation of disulfide bonds between conserved pairs of cysteine residues is probably required for proper folding of EGF-like modules, CADASIL may represent a loss of function phenotype; on the other hand, accumulation of the Notch3 extracellular domain in CADASIL patients also suggests the potential for toxic gain of function [9].

Human diseases have also been attributed to defects in two of the five known Notch ligands. Alagille's syndrome, an autosomal dominant disease, results from frameshift mutations of the ligand Jagged-1 [10–13] that lead to

Figure 1



Domain organization of Notch receptors. This cartoon uses human Notch1 as a prototype. Proteolytic cleavage by furin at site S1 produces two subunits, ECN and NTM, which remain non-covalently associated at the cell surface. EGF-like modules 11 and 12, implicated in ligand binding by *Drosophila* Notch, are shaded. Sites S2 and S3 identify the sites of proteolytic cleavage induced upon activation by ligand. ICN, intracellular domain of Notch; NLS, nuclear localization signal; PEST, proline, glutamate, serine, threonine-rich sequence; TAD, transactivation domain; TM, transmembrane.

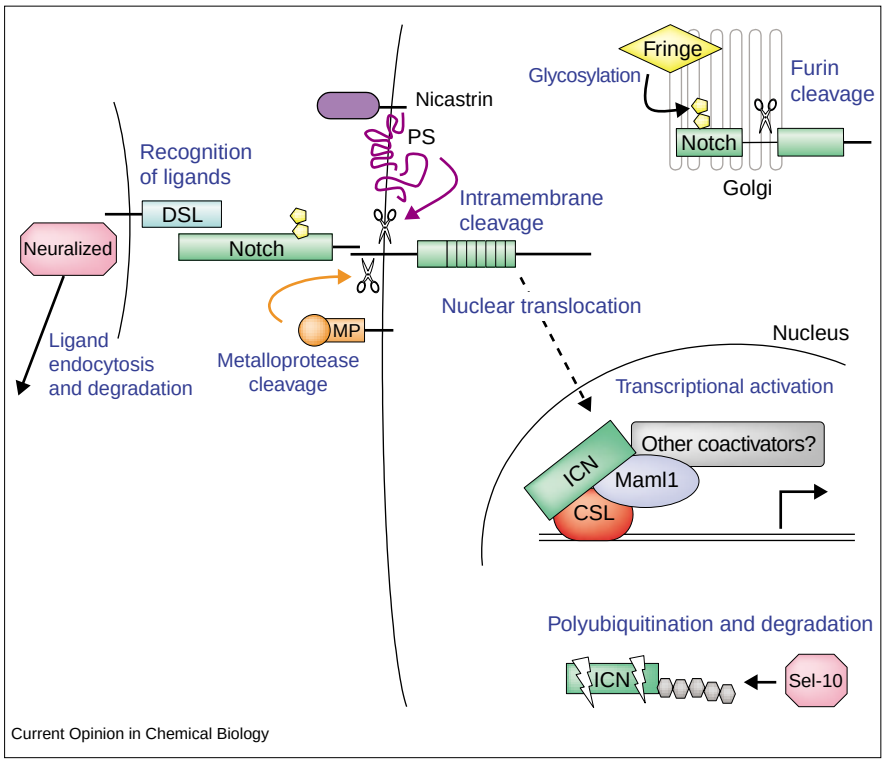
developmental defects in several organ systems including liver, heart, eye, skeleton and/or kidney. Homozygous mutations in the ligand Delta-like3 have been linked to a developmental defect of the axial skeleton called spondylocostal dysostosis; a similar phenotype is observed in *pudgy* mice, which lack a functional Delta-like3 gene product [14•].

Domain organization of Notch receptors

NOTCH genes encode single-pass transmembrane glycoprotein receptors with a modular organization that assembles several sets of structural motifs in a highly conserved arrangement (Figure 1). Notch receptors are proteolytically processed during transport to the cell surface by a furin-like protease at a site about 70 amino acids external to the transmembrane domain (S1 in Figure 1) [15], producing an extracellular Notch (ECN) subunit and a Notch transmembrane (NTM) subunit. These two subunits, which remain non-covalently associated, constitute the mature heterodimeric cell-surface receptor [16].

Notch ECN subunits contain 29–36 N-terminal EGF-like repeats (36 in human Notch1), followed by three tandemly

Figure 2



Central biochemical events involved in transducing and modulating Notch signals. Each step represented in the figure is discussed in the text.

repeated LIN-12 modules that precede the S1 site by about 100 residues. Within the EGF repeat region lie binding sites for the activating ligands [17]. The LIN-12 modules, which comprise a unique domain of Notch receptors, participate in maintaining Notch in a resting conformation before ligand-induced activation [18–20]. Indeed, disruption of the structural integrity of the LIN-12 repeats by chelation of calcium, which is required for maintaining the native structure of LIN-12 repeats [21], leads to Notch activation in cell lines [22].

The short extracellular region of the NTM subunit includes a pair of conserved cysteine residues. The intracellular region of NTM includes a novel domain of about 100 residues termed RAM [23], which is followed by seven ankyrin/CDC10 repeats [24,25] flanked by two nuclear localization signals, a less-highly conserved region with varying transactivating activity, and a C-terminal PEST sequence (denoting a sequence rich in the amino acids Pro, Glu, Ser and Thr).

Central events of Notch signaling

The central events that occur during Notch signal transduction are highly conserved from flies to humans (Figure 2). Activation by ligand triggers a series of proteo-lytic cleavages of NTM that release intracellular Notch (ICN) from the membrane. ICN then translocates to the cell nucleus, where it binds to a highly conserved downstream transcription factor, called CSL in mammals (suppressor of hairless in flies). Binding of ICN to CSL is accompanied by the activation of transcription of target genes, which include the Hairy/Enhancer of Split (HES) family of helix–loop–helix transcription factors. The molecular events that carry out each of these core steps in Notch signaling, with recent advances highlighted, are summarized below.

Five Notch ligands have now been identified in humans: Jagged1, Jagged2, Delta-like1, Delta-like3 [14•,26] and Delta-like4 [27–29]. Each of the ligands is a single-pass transmembrane protein with a conserved N-terminal Delta, Serrate, LAG-2 (DSL) motif essential for binding to Notch. A series of EGF-like modules C-terminal to the DSL motif precede the membrane-spanning segment; in contrast to Notch receptors, the ligands possess short cytoplasmic tails of 70–215 amino acids at their C-termini. Because both the four Notch receptors and their five ligands are expressed in a wide range of tissue types in mammals, cell fate decisions may integrate signals based on the temporal and spatial patterns of expression of different ligand–receptor pairs.

Components of the endocytic machinery are essential for transduction of a Notch signal once ligand binding has occurred [30,31]. In flies, ligand endocytosis promotes dissociation of the Notch heterodimer with accompanying trans-endocytosis of ECN into the delta-expressing cells [32]. Rubin's group has shown that the activity of Neuralized, a ubiquitin E3 ligase [33•], is required both for endocytosis of Delta, and for its subsequent degradation [34••]. Although the cell-autonomy of Neuralized function can vary in different systems, homozygous loss-of-function of Neuralized in flies always leads to loss of Notch signaling [35–38].

Upon perturbation of the interface between the two non-covalently associated Notch subunits by events associated with ligand binding, the NTM subunit becomes sensitive to at least two sequential proteolytic cleavages (at sites S2 and S3; Figure 1) that release its intracellular portion (ICN) from the membrane. This 'regulated intramembrane proteolysis' (see [39••] for a review) of Notch is highly analogous to the processing of β-amyloid precursor protein (APP). Ligand-dependent cleavage of human Notch1 at site S2 is probably mediated by a member of the disintegrin and metalloprotease (ADAM) family of proteases, either TNF-α converting enzyme (TACE) or Kuzbanian (Kuz). *In vitro*, TACE can carry out proteolysis at the S2 site of hNotch1 [40,41]; alternatively, Kuz has been genetically and biochemically linked to activation of *Drosophila* Notch [42–44].

After cleavage has occurred at site S2, presenilin(s) (PS) and its cofactor(s) catalyse subsequent proteolysis within the transmembrane region at the S3 site. Membrane-bound forms of Notch require PS for nuclear localization and downstream signaling, whereas free intracellular Notch does not [45–47]. PSs are likely to be novel aspartyl proteases, as two well-conserved aspartate residues are required for function [48]. In addition, transition state analogues of aspartyl proteases inhibit proteolytic activity [49,50], and photoaffinity versions of two specific compounds selectively modify PS1 [51••]. Loss of function of the novel protein nicastrin shows loss-of-function Notch-like phenotypes in worms [52••,53,54] and flies [55,56], and membrane-tethered Notch, PSs, and nicastrin participate in a multiprotein complex [57•]. These findings support a model in which PSs and nicastrin cooperate to establish the catalytic activity and substrate specificity required for proteolysis of Notch and other cleavage substrates.

When ICN is released from the membrane, it translocates to the nucleus to activate expression of downstream genes [58–60], which include the HES family of basic helix–loop–helix transcription factors [61–63]. The primary nuclear target of ICN is a ubiquitous DNA-binding transcription factor called CSL [61,64,65]. ICN uses both its RAM and ankyrin repeat regions to bind CSL, transforming CSL from a repressor into an activator. Activation by ICN appears to be potentiated by recruitment of co-activators, such as Mastermind-like protein 1 (Maml1), which forms a stable multiprotein–DNA complex with ICNs from hNotch1-4 and CSL both *in vitro* and in cells [66•] (see also Update). Certain histone acetylases (PCAF and GCN5) also associate with ICN [67].

Modulation of Notch signaling

Known modulators of Notch signaling act by regulating ligand responsiveness, controlling ICN turnover, and by other less well-characterized mechanisms. Current evidence convincingly argues that controlled post-translational variation of the glycosylation patterns of Notch ECN subunits by Fringe glycosyltransferases can alter the responsiveness of Notch receptors to different ligands, either by directly masking important ligand contacts, or by interfering with steps in signal transduction following ligand binding. Glycosylation of the ECN subunit of hNotch1 with an uncommon tetrasaccharide, Sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc-α1-O-Ser/Thr [68], takes place during maturation in the Golgi apparatus [69••,70•]. A family of glycosyltransferases, which includes *Drosophila* Fringe [71] and its three orthologues in mammals, called Radical, Manic and Lunatic Fringe [72,73], catalyse transfer of the second sugar, *N*-acetylglucosamine, onto fucose [69••,70•]. Many of the potential *O*-fucose modification sites fall within Notch EGF repeats affected by Abruptex mutations, which perturb Notch signaling in flies [74,75], and variation in the extent and pattern of Notch glycosylation by Fringe in flies differentially influence the ability of Notch to respond to signals from Serrate and Delta [71]. The rates with which different Fringe enzymes modify Notch receptors vary [69••], suggesting the possibility that each Fringe protein might preferentially glycosylate different sites on the four receptors [76]. Although the consequences of Fringe-mediated glycosylation upon ligand sensitivity is complex in mammals, and may differ depending on which Notch receptor (N1–N4) is modified [77], glycosylation by Fringe proteins also appears to render mammalian Notch receptors resistant to certain ligands while maintaining competence for activation by others.

Rapid degradation of ICN after nuclear translocation attenuates Notch signaling, and may explain why detection of endogenous forms of activated Notch in the nucleus has remained so elusive [58,60]. Recent studies have revealed that ubiquitination of ICN by human Sel-10 [78], an F-box protein that forms an SCF-like E3 ubiquitin ligase, can target ICN for degradation [79•]. Phosphorylation of ICN, which has been consistently observed [80–83], appears to be necessary for the destruction of ICN [84]. The functional connection between phosphorylation and Notch signaling activity has been demonstrated in a myeloid differentiation assay using 32D cells, which exhibit an increased Notch2 signal and a block in differentiation upon inhibition of phosphorylation [85].

Although CSL is clearly a primary downstream target of activated Notch, some studies suggest that CSL-independent events also result from activation of Notch (e.g. [86]). Although the molecular mechanisms underlying CSL-independent signaling are not yet clear, proteins reported to associate with the ankyrin repeats of ICN include Deltex [87], Numb [88,89], and NFκB [90,91], any or all of which may contribute to CSL-independent Notch signaling.

Notch as a therapeutic target

Inhibition of signaling

Although the causative role of activated Notch in human carcinogenesis has only been demonstrated explicitly for hNotch1 in some cases of T-ALL, Notch receptors are expressed in a wide range of cancers and in tumour-derived cell lines (see also Update). For example, Notch is highly expressed in neoplastic lesions in human cervix [92] as well as in human renal cell carcinoma cells [93]. Multiple lines of investigation in animal models have reinforced the idea that increased Notch signaling can be oncogenic. Enforced expression of ICN2 causes T lymphoblastic lymphoma in the mouse, transgenic mice expressing lck promoter-drivien ICN3 develop T-cell lymphomas [94], and ICN4 promotes the development of murine breast carcinomas [95]. Moreover, weakly activated Notch alleles (stabilized through deletion of the PEST sequences) can act synergistically with other oncogenes such as myc and E2A-Pbx1 to accelerate and exacerbate lymphomagenesis [96•,97•]. Enforced expression of the ligand Delta-like4 has also been linked to murine lymphomagenesis [98]. Furthermore, ICN1 and ICN2 transform baby hamster kidney cells *in vitro* [99].

Even with a normal complement of Notch signaling components, certain cancers may exploit downstream targets of activated Notch to maintain the transformed phenotype. Epstein-Barr virus, which commonly drives B-cell lymphomas in immunocompromised individuals, produces a protein called Epstein-Barr nuclear antigen-2, which mimics ICN by binding to CSL and activating transcription to send a Notch-like signal downstream [100,101]. More generally, just as Notch signaling has been implicated in the self-renewal of normal stem cells, it is plausible that the self-renewal of clonogenic neoplastic stem cells will also require some level of Notch activation. If so, interruption of Notch signaling might limit the proliferation capacity of neoplasms, through either differentiation-induced cell-cycle arrest and/or programmed cell death.

Notch signaling can be inhibited in principle by blocking ligand binding, preventing ligand endocytosis, inhibiting regulated intramembranous proteolysis, or by interfering directly with the action of ICN. Although Notch ECN subunits contain 29–36 EGF-like repeats, recombinant fragments containing as few as two of these modules can associate with Notch ligands and act as competitive inhibitors for binding to full-length Notch [102]. Inactivation of most forms of membrane-tethered Notch might also be achieved by inhibition of either the S2 metalloproteases, which have a broad range of cellular targets, or the S3 nicastrin/PS protease complex, which has only been implicated in cleavage of three different classes of substrates. Because certain mutations [103,104] or inhibitors [105••] have been found to differentially affect APP processing and Notch cleavage, it appears reasonable to search for γ-secretase inhibitors that selectively prevent cleavage of membrane-tethered Notch.

Direct inhibition of ICN activity may be the most promising approach, however, because naturally occurring constitutively active truncations of Notch result in free, active ICN. Such a strategy would require inhibiting the formation of CSL–ICN complexes or interference with the recruitment of co-activators to the CSL–ICN complex to prevent expression of downstream target genes. The Maml1-binding site on ICN appears to be essential for transcriptional activation, because truncated forms of Maml1 that retain affinity for ICN but lack the activator domain act as dominant-negative proteins in ICN-dependent CSL-responsive reporter assays [66•,106]. A more detailed knowledge of the biochemistry and structure of ICN-dependent transcriptional activation complexes should facilitate a search for small molecules that interfere with their assembly (see also Update).

Activation of signaling

Because Notch receptors participate in the decision between self-renewal and differentiation for many different types of cells, the potential to maintain stem cells in culture using activated Notch raises various opportunities for development of therapeutics in stem cell biology and tissue engineering. Strategies for activation or potentiation of a Notch signal have included growth of primary cells in culture in the presence of soluble ligand [107•,108] or with ligand conjugated to a solid support, which may better mimic the *in vivo* situation in which membrane-bound ligands activate Notch proteins [109–111]. Other pharmacologic approaches that might increase Notch activity would include (i) inhibition of Fringe (or other enzymes involved in Notch glycosylation [112–114]), thereby modulating the responsiveness of Notch to its ligands, (ii) inhibiting Notch degradation by preventing phosphorylation and subsequent Sel-10-dependent ubiquitination, or (iii) de-repressing or directly activating transcription of CSL-dependent target genes.

The strategy of harnessing Notch activation for the purpose of stem-cell self-renewal in culture has advanced most rapidly with hematopoetic stem cells. Jagged-1 expressed on stromal cells seems to activate Notch on haematopoietic stem cells *in vivo* [108,115], with addition of certain growth factors also necessary to induce proliferation [116]. Although the use of Notch signaling for stem cell maintenance and tissue engineering may be practically difficult, because each cell type may require unique culture conditions containing additives such as appropriate cytokines and growth factors, haematopoietic stem cells have been successfully immortalized by constitutive activation of Notch signaling [117••].

Conclusions

Notch signaling controls differentiation and proliferation in a variety of different cellular contexts. Whereas inactivation of Notch signals may block cell proliferation in certain tumors, enhanced activation may help maintain stem cell viability and pluripotency. Recent advances have clarified many of the molecular events involved in Notch signaling, yielding a range of potential targets for both inhibition and activation of Notch, each of which may have therapeutic benefit. These new insights provide opportunities for manipulation of Notch signaling *in vivo* and *ex vivo*, possibly allowing for rational control of stem cell proliferation and differentiation. Chemical screens for Notch signal modifiers should produce lead compounds of great interest to basic scientists and clinicians alike.

Update

Recent evidence from two groups lends additional strong support to the physiologic and pathophysiologic relevance of Maml1 recruitment to complexes containing CSL and ICN on DNA. First, Capobianco's group identified high molecular weight (>1 MDa) nuclear complexes containing ICN1, Maml1 and CSL in a human cell line derived from a T-ALL associated with a chromosomal translocation involving *NOTCH1*. Furthermore, they also showed that the ability of ICN1 and ICN2 to transform RKE cells correlates tightly with entry into complexes with CSL and Maml1 [118]. Secondly, the groups of Kintner and Jones addressed one potential mechanism of transcriptional activation by Maml1. In a purified system, they showed that Maml1 recruits the transcriptional co-activator p300 to ICN1/CSL complexes, and that this activity is essential for CSL-dependent transcription from chromatinized templates [119]. Recent work has also shown that Notch1 is highly expressed in the tumor cells of Hodgkin and anaplastic large cell lymphoma, and that the Notch ligand Jagged-1 drives the proliferation of cell lines derived from these tumors *in vitro* [120].

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