

# Notch signal transduction: a real Rip and more

## Gerry Weinmaster

The Notch signaling pathway functions in a wide variety of processes that regulate tissue patterning and morphogenesis in developing vertebrates and invertebrates. Research on the mechanism of ligand-induced Notch signal transduction has revealed a novel and essential element in the signal cascade. Some recent findings support a model in which sequential proteolytic cleavage serves to regulate Notch signal transduction.

### Addresses

Department of Biological Chemistry, UCLA School of Medicine, 33–257 Center for Health Sciences, Box 951737, Los Angeles, California 90095-1737, USA; e-mail: gweinmaster@mednet.ucla.edu

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### Abbreviations

<b>ADAM</b>	a Disintegrin and Metalloproteinase
<b>APP</b>	$\beta$ -amyloid precursor protein
<b>CBF1</b>	C promoter binding factor 1
<b>CSL</b>	CBF1/Su(H)/Lag-1
<b>E(spl)</b>	Enhancer of split
<b>HES</b>	Hairy and E(spl)
<b>NICD</b>	Notch intracellular cytoplasmic domain
<b>PS</b>	presenilin
<b>Rip</b>	regulated intramembrane proteolysis
<b>Su(H)</b>	Suppressor of hairless
<b>TACE</b>	tumor necrosis factor- $\alpha$ converting enzyme

### Introduction

Cellular specification, differentiation, proliferation, and survival are among the repertoire of complex and hierarchical processes that rely on proper Notch signal transduction [1]. With many eucaryotic cell surface receptors, ligand binding begins a cascade of events which culminates in altered patterns of gene expression. The receptors themselves, however, remain firmly in the plasma membrane. The first clue that Notch might function somewhat differently came from studies using constitutively active forms of this protein. Surprisingly, cells expressing this form of Notch showed both cell-surface and nuclear localization of the protein. From this (and many other studies), a model has emerged in which Notch seems to act both as a ligand-binding receptor and as a direct modulator of gene expression. Of course, this duality of function presents something of a dilemma: subsequent to ligand-binding, how does the transmembrane protein Notch get to the nucleus to modify the transcriptional machinery? A model for Notch signaling has been proposed in which ligand-binding induces proteolysis of Notch to liberate an intracellular cleavage fragment that translocates to the nucleus to regulate transcription of Notch-responsive genes [2,3].

In support of this model, two nuclear localization signals have been identified in the Notch intracellular domain and Notch itself has been shown to physically interact with

DNA-binding proteins — known as CSL after mammalian CBF1, *Drosophila* Su(H) and *Caenorhabditis elegans* Lag-1 [4]. Importantly, the Notch/CSL complex directly activates transcription of Notch target genes such as E(spl)/HES. Moreover, proteolytic processing of membrane-bound forms of Notch that signal constitutively correlates with a signal for Notch in the nucleus as well as activation of gene expression and changes in cell phenotype [5]. Nonetheless, convincing data to support that Notch signaling under normal conditions is regulated through ligand-induced proteolytic release of its intracellular domain have been difficult to obtain. The inability to detect Notch proteolytic fragments in the nucleus following ligand activation has hampered the general acceptance of the ‘nuclear access’ model of Notch signaling but recent genetic and biochemical studies using invertebrate and mammalian systems have provided additional data which support this model.

### Ligand-induced proteolytic cleavage and nuclear localization of Notch

If ligand-induced Notch signaling requires nuclear translocation, then it was argued that the levels of Notch in the nucleus must be sufficient to drive gene expression yet too low for direct detection by conventional means [6]. In fact, transfection of Notch DNA in mammalian cells at concentrations that activate gene transcription does not allow direct detection of nuclear Notch protein but protein is detected in the nucleus when higher concentrations of DNA are transfected [7]. Moreover, the *Drosophila* UAS–Gal4–lacZ genetic system has provided evidence for Notch activity in the nucleus in response to ligand in the absence of visual detection of nuclear Notch protein [6,8]. In this system ligand-dependent lacZ activity is only detected with full-length Notch constructs in which the GAL4 sequences are inserted in the intracellular portion of Notch and not when they are inserted in the extracellular sequences, suggesting that cleavage, release, and translocation of the Notch cytoplasmic domain to the nucleus must account for the observed lacZ expression. Direct demonstration of ligand-induced proteolysis of Notch, however, was not reported in these studies.

Such genetic experiments complement biochemical studies in mammalian tissue culture systems in which proteolysis of Notch has been more fully characterized. Evidence that Notch can be cleaved at the cell surface was first obtained through pulse-chase analysis of a constitutively active, membrane-bound form of Notch1 (N<sup>AE</sup>) lacking most of the extracellular domain [5]. A Notch-derived peptide containing the intracellular domain (NICD) can be detected in N<sup>AE</sup>-expressing cells and the appearance of NICD is blocked by protease inhibitors as well as drugs that disrupt protein trafficking. This key finding suggests that NICD is produced from

membrane-bound N<sup>AE</sup> through proteolysis at a site near the cell surface. Importantly, the appearance of NICD was correlated with the appearance of Notch in the nucleus.

Sequencing of the NICD cleavage fragment identified a conserved valine residue within the transmembrane (TM) domain as a putative cleavage site [7]. Mutation of this valine in N<sup>AE</sup> resulted in diminished levels of Notch protein in the nucleus, and attenuated N<sup>AE</sup>-mediated transcription, suggesting that proteolytic cleavage might regulate ligand-induced Notch signal transduction [7]. Moreover, a fragment of Notch similar to NICD is generated from wild-type Notch1 when coexpressed with the Notch ligand Jagged1, and mutation of the TM valine prevented the appearance of this cleavage fragment [7]. Although these data are interpreted to reflect the dependence of Notch signaling on ligand-induced proteolytic processing of full-length Notch, neither ligand-induced signaling nor biological changes dependent on ligand binding have been correlated with Notch cleavage in a single cell type.

### Presenilins function in proteolytic cleavage of Notch

The protease responsible for NICD generation has not yet been definitively identified but a number of findings suggest that presenilins participate in this event. The human presenilin genes *PS1* and *PS2* were first identified in an inherited, early onset form of Alzheimer's disease [9]. Neurodegeneration characteristic of Alzheimer's Disease is associated with the accumulation of amyloid peptides (A $\beta$ ) produced through aberrant proteolysis of  $\beta$ -amyloid precursor protein (APP). APP is a type 1 transmembrane protein of unknown function that is proteolytically processed by three proteases known as  $\alpha$ -,  $\beta$ - and  $\gamma$ -secretases but only the  $\beta$ - and  $\gamma$ -secretases produce neurotoxic A $\beta$  peptides. Mutations in human PS proteins result in aberrant processing of APP within its single TM domain, producing increases in pathogenic A $\beta$  peptides, and loss of either PS1 or PS2 activity in mice impairs the intramembrane cleavages of APP [10–12]. Based on mutations of two conserved transmembrane aspartate residues in PS1 that abolish both APP cleavage and endoproteolysis of PS1, it has been proposed that PS proteins are autoactivated intramembraneous aspartyl proteases [13]. Taken together, the data have to date suggested that PS proteins function as  $\gamma$ -secretases or cofactors of this protease.

Homology between PS1 and Sel-12, a *C. elegans* protein that facilitates signaling by the Notch homolog LIN-12, provided the first connection between PS proteins and Notch/LIN-12 signaling [14,15]. Corroborating this notion, PS-deficient *Drosophila* mutants have phenotypes similar to those produced through loss of Notch activity [16•,17•]. Importantly, PS activity is required for nuclear access and signaling by wild-type Notch and a ligand-independent membrane-bound form of Notch (similar in structure to N<sup>AE</sup>) [16•], whereas the activity of NICD-like, constitutively active forms of Notch are completely

unaffected by losses in PS [16•,17•]. A relationship between Notch and PS activity is similarly evident in 'knockout' studies of transgenic mice where losses in PS activity resemble losses in Notch1 function [18–20]. Furthermore, NICD levels are greatly reduced when N<sup>AE</sup> is expressed in cells obtained from PS1 or PS2 knockout mice, and the Notch processing defect can be rescued through expression of wild-type PS1 [21•,22•]. The dependence of Notch processing on PS has also been demonstrated through use of  $\gamma$ -secretase inhibitors as well as a dominant negative form of PS1 [21•,23]. Dominant negative PS1 impairs Notch cleavage without altering cell-surface expression of Notch, suggesting that PS1 is not required for Notch stability or targeting to the plasma membrane. Taken together, these studies argue that PS proteins function in the proteolytic processing of Notch and that Notch function is critically dependent on proteolytic cleavage and subsequent transport to the nucleus.

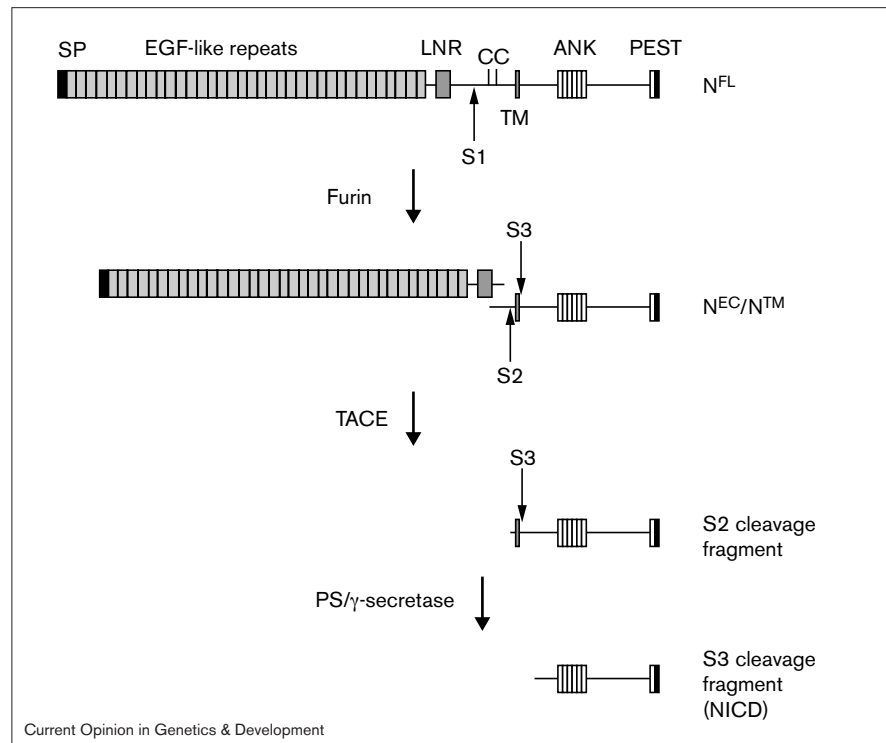
Decreased NICD production in PS-deficient cells also correlates with loss of nuclear Notch localization and diminished transactivation of a HES-1-reporter [21•,22•]. Interestingly, different mutant human PS1 proteins have been tested and not all rescue Notch cleavage to the same degree as wild-type PS1 [22•]. Moreover, mutant human proteins have reduced ability to rescue the *sel-12* mutant phenotype [15]. This is surprising because a mutant human *PS1* gene rescues the *PS1* embryonic lethality in mice [24,25]. Mutations in *PS1* are associated with aberrant cleavage of APP at the  $\gamma$ -secretase site with enhanced production of APP cleavage products but these mutant PS1 proteins appear less efficient at cleaving Notch. In addition, the pharmacological profiles for generation of A $\beta$  and NICD from APP and Notch, respectively, in the presence of  $\gamma$ -secretase inhibitors are very different [26]. Whether these differences in APP and Notch processing represent differences in regulation of PS-dependent  $\gamma$ -secretase proteolysis is unclear but they may allow treatment of AD with drugs that target this protease without compromising Notch function. Consistent with the idea that PS is involved in the intramembrane cleavage of Notch that generates NICD, coexpression and cell-surface complexes of Notch and PS have been demonstrated both in *Drosophila* and mammalian cells [23•,27,28].

### Multiple proteases regulate Notch signaling

Establishing a role for ligand-induced proteolysis of Notch in signal transduction has been technically challenging and somewhat controversial but there is now considerable support for this idea. The full complement of factors and precise sequence of events that regulate ligand-dependent, intramembrane proteolysis of Notch remain to be defined. As with other signal-transduction schemes, the final pathway is likely to be considerably more complex than current models predict. Indeed, several additional enzymatic steps are necessary for NICD production and at least four proteases have now been implicated in Notch signaling. A model for sequential proteolytic events regulating the

**Figure 1**

Schematic representation of proteolytic cleavage sites identified in Notch1, putative proteases involved and the resultant proteolytic cleavage fragments. Unprocessed Notch1 ( $N^{FL}$ ) contains an amino-terminal signal peptide (SP), followed by two cysteine-rich domains; the first contains 36 tandem repeats related to epidermal growth factor (EGF-like repeats) and the second is a repeat conserved within the LIN-12/Notch/GLP-1 family (LNR). S1 represents the proteolytic cleavage site recognized by furin (RXRR) ~75 residues upstream of the single transmembrane domain (TM). Cleavage at S1 generates an amino-terminal fragment that contains most of the extracellular sequences ( $N^{EC}$ ) and a carboxy-terminal fragment ( $N^{TM}$ ) that contains a pair of conserved cysteines (CC), the TM and cytoplasmic sequences with ankyrin repeats (ANK) and a PEST domain. S2 in  $N^{TM}$  represents the putative TACE cleavage site 12 residues upstream of the TM domain (AV) that, in response to ligand binding, would produce a membrane-associated S2 cleavage fragment. Cleavage at S2 facilitates S3 cleavage within the TM domain near the cytoplasmic face (GV) that is dependent on PS/ $\gamma$ -secretase activity. Cleavage at S3 releases the Notch intracellular domain (NICD) from the membrane.



release of Notch in a ligand-dependent manner has been proposed [29<sup>•</sup>,30<sup>•</sup>]. To date, three proteolytic cleavage sites have been mapped in the Notch1 protein and these are depicted in Figure 1 (Site1 [S1], Site 2 [S2], and Site 3 [S3]).

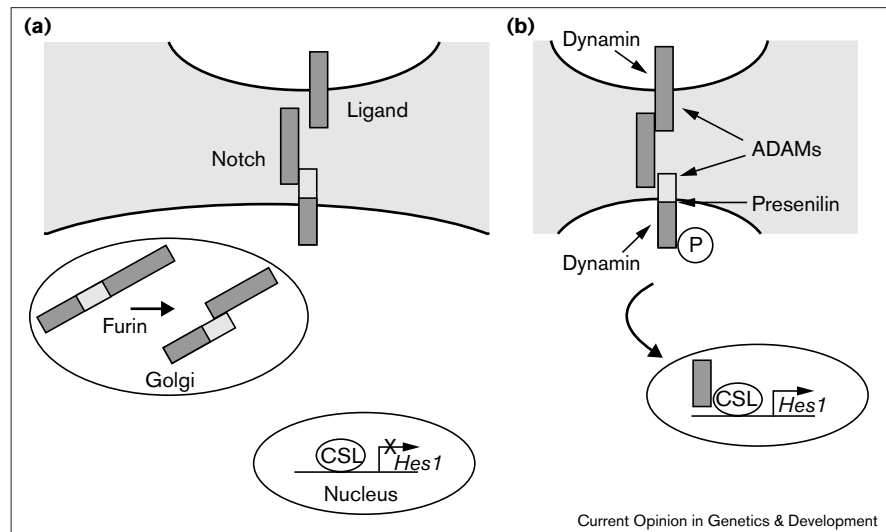
The mature Notch receptor is a heterodimer composed of amino-terminal 180–200 kDa ( $N^{EC}$ ) and carboxy-terminal 120 kDa ( $N^{TM}$ ) fragments which are generated via proteolytic cleavage of the primary translational product of the Notch gene [31–33,34<sup>•</sup>]. This proteolysis occurs in the secretory pathway by a furin-like convertase that cleaves full-length Notch ( $N^{FL}$ ) at a site ~75 amino acids amino-terminal of the TM domain (Figure 1). Mutation of the Notch1 furin recognition site (or addition of furin inhibitors) prevents the generation of  $N^{EC}$  and  $N^{TM}$  from  $N^{FL}$  ([33]; G Bush, G Weinmaster, unpublished data). Loss of heterodimeric Notch correlates well with a loss in ligand-induced activation of CSL-reporters, suggesting that heterodimeric Notch is required for signaling ([35]; G Bush, G Weinmaster, unpublished data). It has been proposed that two conserved cysteine residues stabilize the Notch heterodimer, although  $N^{EC}$  and  $N^{TM}$  fragments remain associated via  $Ca^{2+}$ -dependent, noncovalent interactions [34<sup>•</sup>]. Several EGF-like repeats have been proposed to bind  $Ca^{2+}$  but deletion analysis indicates that these repeats do not participate in heterodimeric stabilization but rather the LNR motif (just upstream of the furin-cleavage site) is essential for stable  $N^{EC}$ - $N^{TM}$  heterodimers. Interestingly, treatment of Notch1-expressing cells with  $Ca^{2+}$  chelators leads to release of  $N^{EC}$  into the

culture medium, generation of an NICD-like fragment that localizes to the nucleus, and CSL-dependent transcription [34<sup>•</sup>]. As  $N^{TM}$  is similar in size to  $N^{AE}$ , it is proposed that  $Ca^{2+}$  depletion promotes dissociation of the Notch heterodimer which leads to proteolysis of membrane-bound  $N^{TM}$  and generation of NICD. The NICD transits to the nucleus to activate transcription.

Furin cleavage of Notch1 at S1 does not require ligand, whereas cleavage at S2 and S3 sites only occurs following ligand binding. According to the proteolysis model of Notch activation, ligand binding to Notch induces a conformational change(s) that is necessary for cleavage at S2 which, in turn, facilitates cleavage at S3 [9,36]. Recently, work from two groups [29<sup>•</sup>,30<sup>•</sup>] has identified a putative S2 cleavage site at a conserved valine located 12 amino acids amino-terminal of the TM domain, placing it upstream of the S3 intramembrane cleavage site regulated by PS/ $\gamma$ -secretase (Figure 1). Together, these studies suggest a proteolytic cascade in which cleavage at S3 requires prior cleavage at S2, inhibition at S3 leads to accumulation of the S2 cleavage fragment, and inhibition of S2 precludes generation of the S3 cleavage fragment. Mutagenesis of the valine at S2 leads to decreases in both S2 and S3 cleavage fragments, confirming the identity of the S2 cleavage site and its obligate role in S3 cleavage. Purification of the S2 proteolytic activity has identified a protease with characteristics of a metalloprotease known as TACE for its proteolysis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$  converting enzyme) [29<sup>•</sup>]. TACE is an ADAM (a Disintegrin and Metalloproteinase) with known 'shedase' activity that

Figure 2

A current model for Notch signaling involves a series of proteolytic cleavage events, the first of which (a) results in cleavage of the primary Notch translation product by furin in the secretory pathway to produce the mature heterodimeric form of Notch that is expressed on the cell surface where it interacts with ligand. (b) Following ligand binding to Notch, at least two different ADAM proteases (ADAM 10 [Kuz] and ADAM 17 [TACE]) and a PS-dependent  $\gamma$ -secretase have been implicated in Notch signaling. Ligand-binding to Notch may allow access of TACE to the Notch extracellular domain for cleavage at this site. Kuz may function to cleave the Notch ligand to reduce conformational stress induced through ligand-binding to Notch. Cleavage by these ADAMs might facilitate a subsequent cleavage within the transmembrane domain by PS- $\gamma$ -secretase to release the intracellular domain of Notch from the plasma membrane. In the nucleus, the Notch intracellular domain functions with CSL to direct transcription of Notch-



responsive genes such as *Hes1*. Endocytosis regulated by dynamin as well as phosphorylation of the Notch intracellular

domain (indicated by 'P') may provide additional modes of regulation in Notch signal transduction.

functions in the generation (as well as downregulation) of ligands for receptors through cleavage at the cell surface [37]. Although originally thought to be specific for TNF- $\alpha$  recent analyses have indicated that a number of different cell-surface proteins, including APP, are cleaved by TACE [38,39].

Genetic studies in *Drosophila* and *C. elegans* support a role for additional ADAM family members, Kuzbanian (Kuz, ADAM10) and Sup-17, respectively in Notch/LIN-12 signaling [40–42]. Transgenic mice deficient in either Kuz or Notch1 display similar phenotypes [30<sup>\*</sup>] but the exact role of Kuz in Notch signaling remains unclear. A role in heterodimeric Notch formation as well as regulation of ligand-dependent cleavage of Notch has been proposed for Kuz, although direct demonstration of Notch proteolysis by Kuz has been difficult [33,40]. Even though TACE can cleave truncated, chimeric Notch proteins *in vitro* [29<sup>\*</sup>], data supporting a role for TACE in Notch signaling are not as convincing as data reported for Kuz. The phenotype of TACE knockout mice [38] is very different than that described for mice deficient in Notch1 [19,20]; however, given the large number of TACE substrates, this is probably not surprising. That TACE (rather than Kuz) cleaves at S2 is supported by the observation that Kuz protein does not copurify with S2 proteolytic activity and Notch processing at S2 occurs in cells deficient in Kuz, but not in cells lacking TACE [29<sup>\*</sup>,30<sup>\*</sup>]. As both TACE and Kuz have been reported to cleave APP at the  $\alpha$ -secretase site [39,43], the possibility remains that both of these ADAMs cleave Notch at S2.

The ligands that bind and activate Notch are also type 1 transmembrane proteins [44] and recent evidence indicates that Kuz can cleave Delta to generate an active

soluble ligand, suggesting yet another function for this protease in Notch signaling [45<sup>\*</sup>]. Although Delta cleavage fragments have been identified *in vivo* and *in vitro* [45<sup>\*</sup>,46] and soluble forms of Delta can activate Notch signaling *in vitro* [45<sup>\*</sup>,47,48], genetic studies indicate a requirement for direct cell–cell interactions in Notch signaling. Kuz activity is required for ligand-induced Notch signaling but not for cleavage or activity mediated by constitutively active forms of Notch ([35]; G Bush, G Weinmaster, unpublished data). Perhaps Kuz cleaves Delta after it binds to Notch-expressing cells and relieves ‘conformational stress’ induced by ligand binding which would otherwise prevent the S2 and S3 cleavages required for signaling.

### Proteolytic cleavage of Notch: not so novel after all?

Notch proteolysis and translocation to the nucleus seems, at first glance, to be a novel signal transduction mechanism. However, a number of recent reports describe similar regulated intramembrane proteolysis (Rip) in species from bacteria to man, which serve to regulate fundamental homeostatic processes such as cellular differentiation, lipid metabolism, and response to unfolded proteins [49<sup>\*\*</sup>]. In all cases of Rip, membrane proteins are cleaved at multiple sites in response to specific stimuli with the final cleavage site residing within the TM domain. A number of studies suggest that intramembrane cleavage can not take place until obligatory cleavage shortens the extracellular sequence to fewer than 30 residues. Consistent with these Rip requirements, S3 intramembrane cleavage of both APP and Notch are regulated by PS/ $\gamma$ -secretase that requires prior cleavage by TACE at an S2 site 12 residues upstream of the TM domains in both APP and Notch [29<sup>\*</sup>,39].

## Is there more?

Although considerable progress has been made towards defining the molecular mechanisms that regulate ligand-induced Notch signaling, several aspects are still uncertain. For example, does the intramembrane cleavage of Notch occur at the plasma membrane, or within intracellular vesicles? The *Drosophila* homolog of dynamin functions in endocytosis and is required for Notch signaling [50,51\*]; moreover, intracellular ligand-receptor complexes have been detected in both flies [51\*,52,53] and worms [54]. What part does internalization play in downstream signaling? Roles for endocytosis in the ligand-presenting as well as the Notch-receiving cell have been proposed. In fact, it has been suggested that ligand-induced endocytosis of Notch may lead to dissociation of heterodimeric Notch and subsequent activation of the signaling cascade [51\*].

It is believed that Notch translocates to the nucleus where it interacts with CSL; however, the subcellular compartment where these complexes form is not well characterized. CSL proteins have been shown to co-immunoprecipitate with unprocessed Notch, heterodimeric Notch and Notch cleavage fragments [7,30\*,55]. What about phosphorylation of Notch or its ligands? There is evidence for Notch phosphorylation [55,56\*\*] but neither the protein kinases nor the functional consequences of such phosphorylation are known. Perhaps phosphorylation regulates endocytosis, interactions with CSL, release of the Notch intracellular domain or even turnover of this fragment in the nucleus. Recent evidence suggests that Notch cleavage fragments may be regulated by proteasome-directed proteolysis, which may account for the widespread difficulty in detecting nuclear Notch [21\*\*,57\*]. Moreover, genes encoding proteins involved in ubiquitinated-targeting of proteins to the proteasome have been identified through genetic interactions with Notch and LIN-12 [58,59]. Curiously, a strong signal for Notch in the nucleus has been detected in post-mitotic neurons; however, their neural progenitors do not show a signal for Notch in the nucleus despite high levels of cell surface Notch expression [56\*,60\*\*]. What accounts for these differences in nuclear Notch expression during neurogenesis and development of neural connections in the brain?

## Conclusions

A proteolytic cascade appears to regulate CSL-dependent Notch signaling (Figure 2). The first proteolytic cleavage of Notch occurs by furin to produce a mature heterodimeric cell surface receptor capable of interacting with ligand [33,35]. Following ligand binding to Notch, at least two different membrane metalloproteinases (ADAM 10 [Kuz] and ADAM 17 [TACE]) and a presenilin-dependent  $\gamma$ -secretase have been implicated in Notch signaling [21\*\*,22\*\*,29\*,30\*,45\*]. Kuz and TACE may function to cleave either the Notch ligand or Notch itself at sites in their extracellular domains. Cleavage in the Notch extracellular domain may facilitate a subsequent intramembrane cleavage by presenilin-dependent  $\gamma$ -secretase to release the cytoplasmic domain of Notch from the plasma membrane, which in

turn functions with CSL in the nucleus to direct transcription of Notch-responsive genes such as *HES1*. Proteolytic cleavage appears to be critical to CSL-dependent Notch signaling; however, considerable evidence suggests that Notch signaling occurs independently of this transcription factor [31,61–65]. Does proteolysis also regulate CSL-independent Notch signaling? The answers to these and many other questions will no doubt continue to reveal additional surprising aspects of Notch signal transduction.

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Expression of PS1 mutated at either of two conserved aspartate residues impairs APP processing at the  $\gamma$ -secretase site and prevents normal processing of PS1. Proteolytic processing was affected without changes in the subcellular distribution of PS1 suggesting that PS1 does not function in

protein transport consistent with results from cell-free microsomes which imply that it plays a direct role in  $\gamma$ -secretase cleavage either as the secretase or as a cofactor for the secretase. That the mutant PS1 inhibits proteolysis in the presence of endogenous PS1 suggests that this mutant has dominant negative activity.

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Western-blot analysis of *Drosophila* larval extracts detects aberrant processing of Notch in mutants indicating that PS is required for some aspect of Notch processing. The increase in higher molecular weight cleavage fragments of Notch in the absence of PS may reflect the accumulation of an extracellular cleavage product in the absence of PS activity. The Notch subcellular distribution in PS mutants appears normal suggesting that protein trafficking is not affected by loss in PS activity. In contrast to other reports, a ligand-independent membrane-bound form of Notch lacking most of the extracellular domain functions in the absence of PS. Moreover, a Notch mutant that retains the extracellular sequences thought to negatively regulate Notch is also active and its activity does not require PS. The reason for these differences is not clear.

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Cells from mice deficient in PS1 are used to demonstrate that proteolytic release of the intracellular domain from a membrane-bound form of Notch1 (N<sup>AE</sup>) and its appearance in the nucleus are impaired in the absence of PS1; however, expression of a soluble intracellular form of Notch in the nucleus is unaffected.  $\gamma$ -secretase inhibitors also prevent processing of N<sup>AE</sup>, suggesting that a presenilin-dependent  $\gamma$ -secretase may function in the proteolytic release of the Notch intracellular domain.

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NICD generation from membrane-bound forms of Notch is decreased in cells deficient in PS1 and this processing defect is rescued by the expression of PS1. Interestingly, PS1 mutant proteins associated with Alzheimer's disease are impaired in their ability to rescue the defect in NICD production detected in PS1-deficient cells. Ligand-independent membrane bound forms of Notch1 are processed to different levels depending on either the presence or absence of sequences downstream of the ankyrin repeats, suggesting that these sequences may be involved either in the generation or turnover of NICD.

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Expression of D267A mutant PS1 prevents the generation of NICD from N<sup>AE</sup> as previously found with PS1-deficient cells and inhibitors of  $\gamma$ -secretase consistent with the dominant negative effects described for this mutant PS1 in APP processing. The D267A PS1 mutant is unable to rescue the

loss of N<sup>AE</sup> processing in cells lacking PS1, indicating that the mutant PS1 is inactive. Expression of the D267A PS1 mutant does not disrupt N<sup>AE</sup>-PS1 complex formation or trafficking of N<sup>AE</sup> to the cell surface. Biotinylation experiments provide evidence that PS1 is expressed at the cell surface in a complex with N<sup>AE</sup> where it could function in proteolytic release of NICD. Biotin-labeled unprocessed Notch and heterodimeric Notch coimmunoprecipitate with PS1, indicating that both forms of Notch are complexed with PS1 at the cell surface.

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