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Proteolytic Cleavage of Notch: "HIT and RUN"

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Abstract

The Notch pathway is a highly conserved signaling pathway in multicellular eukaryotes essential in controlling spatial patterning, morphogenesis and homeostasis in embryonic and adult tissues. Notch proteins coordinate cell-cell communication through receptor-ligand interactions between adjacent cells. Notch signaling is frequently deregulated by oncogenic mutation or overexpression in many cancer types. Notch activity is controlled by three sequential cleavage steps leading to ectodomain shedding and transcriptional activation. Here we review the key regulatory steps in the activation of Notch, from receptor maturation to receptor activation (HIT) via a rate-limiting proteolytic cascade (RUN) in the context of species-specific differences.

Keywords

Notch cleavage; γ-secretase; ADAM metalloprotease; furin; presenilin; cancer; GSI (γ-secretase inhibitor)

INTRODUCTION

The Notch pathway is a highly conserved signaling pathway in multicellular eukaryotes essential in controlling spatial patterning, morphogenesis and homeostasis in embryonic and adult tissues [1, 2]. Notch proteins coordinate tissue homeostasis through receptor-ligand interactions on adjacent cells [3]. Disruption of this homeostatic control by deregulated Notch signaling underlies developmental defects and neoplastic transformation [4-6]. Notch receptors are Type I transmembrane glycoproteins that transduce signals by binding to membrane bound ligands (*i.e.* Delta, Jagged) on adjacent cells (Fig. **1**). In *Drosophila melanogaster*, a single prototypical Notch protein (N) is found together with two ligands, Delta (Dl) and Serrate (Ser, Jagged in mammals). In the nematode *Caenorhabditis elegans*, two Notch-like receptors, LIN-12 and GLP-1 [7], and several Delta-Jagged type ligands (LAG-2/APX-2) are found. In mammals four Notch genes (Notch1-4) and five ligands:

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Jagged1-2, Delta-like ((Dll) 1, 3 and 4) are known. These ligands are collectively called DSL ligands (**D**elta and **S**errate/in *Drosophila* and **L**AG-2 in *C. elegans*) [8]. Notch ligand-receptor interactions are regulated by O-linked glycosylation/fucosylation of EGF-like repeats in the Notch receptor and DSL ligands by Fringe proteins during ER-Golgi transport [9-11]. Glycosylated Notch receptors have a higher affinity for Delta than for Jagged type of ligands [12]. Upon ligand binding, Notch receptors undergo two successive proteolytic cleavages: an ectodomain cleavage followed by intramembranous cleavage. This two-step activation mechanism termed Regulated Intramembrane Proteolysis (RIP) is central to physiological and pathological Notch signaling and is known as the canonical Notch pathway [8]. RIP releases the Notch intracellular domain (NICD), which translocates to the nucleus and binds the transcription factor CBF1/Su(H)/Lag-1 or CSL (RBP-κ in mice) participating in a complex activating its target genes [13-17]. In the absence of ligand the Notch juxtamembrane localized heterodimerization domain (HD) inhibits extracellular proteolysis and activation [18, 19]. The Notch signaling cascade is deregulated in many human cancers and in human T-cell leukemia oncogenic mutations in *NOTCH1* mapping to the HD and PEST domain are frequently found [20], making Notch proteolysis an attractive therapeutic target. Here we review the proteolytic events that govern Notch receptor maturation and activation under physiological and non-physiological conditions with an emphasis on differences and similarities between mammals flies and worms.

I. FURIN S1 CLEAVAGE

During maturation in the *trans*-Golgi network, Notch precursors are cleaved at Site-1 (S1) by a furin-like convertase producing a heterodimeric receptor with the Notch extracellular domain (NECD) non-covalently bound to a transmembrane/intracellular fragment (TMIC) [21-23].

Early studies had already detected smaller fragments of Notch in mammals and worms [24, 25]. Lower molecular weight species of Notch2 were observed in cell lines as well as in rat embryo and human tissue extracts [21], all indicative of receptor cleavage. The intracellular portion of fragmented Notch is found to be associated with membranes, showing the cleavage occurs in the Notch extracellular domain (NECD). Inhibiting trafficking between the ER and Golgi with Brefeldin A, or decreasing the incubation temperature blocked the cleavage of full-length Notch, demonstrating processing occurs in the *trans*-Golgi network. Notch2 is expressed at the cell surface as a heterodimer, whereas the unprocessed full-length Notch2 does not reach the plasma membrane. The interaction of the ligand Delta with the heterodimeric receptor showed that furin cleaved Notch is capable of binding ligand [21]. Like Notch2, Notch1 is also heterodimer at the cell surface [23]. Previous observations of artificially truncated Notch molecules (LNR) lacking the EGF-repeats of the Notch extracellular domain showed a cleavage of Notch1 at site RQRR [22], reminiscent of a consensus furin cleavage site (Fig. **2**). In fact, inhibiting or knocking out furin activity strongly decreased Notch1 cleavage. Mutating the furin-cleavage site RQRR severely attenuated Notch1 processing. Mutating two neighboring dibasic furin sites (RK:1627-1628 and KR:1632-1633) completely impaired Notch signaling [23].

These data show furin processing is required for the proper cell surface expression and signaling of Notch. Both in mammalian as well as fly systems evidence for furin-independent Notch signaling has been reported as well [26, 27]. Surface biotinylation in mammalian cells shows that ~95% of heterodimeric Notch1 is expressed at the cell surface, estimating that ~5% of Notch1 at the cell surface is unprocessed. Furin inhibition decreases heterodimeric Notch1 receptors on the cell membrane, as a result a relative increase of unprocessed Notch1 molecules on the cell surface and a concomitant decrease in CSL-dependent Hes1-reporter activity is observed. Yet, in a Notch1-dependent myogenic differentiation model, the stimulated unprocessed Notch1 receptor, unable to signal via ~CSL, could suppress myogenesis. Although the contribution of low endogenous levels of Notch could not be excluded, similar results were obtained using the overexpression of the furin-cleavage resistant triple mutant of Notch1 in the myogenic differentiation assay [26], pointing to a role of furin-independent Notch signaling. At present it is not known how wide-spread these non-canonical functions of Notch are in mammalian tissues.

Similar results were obtained in *Drosophila*, where Notch immunopreciptations from larvae demonstrate that uncleaved full-length receptors are the predominant form on the cell surface [27]. *In vivo* overexpression of Notch molecules lacking the S1 cleavage domain (N^{BC}) could partially rescue the Notch loss-of-function phenotype in *Notch* null transgenic fly embryos. Stimulated N^{BC} even interacted with CSL/Su(H), demonstrating Notch activity in the absence of furin cleavage. Strikingly, *in vivo* expression of hybrid Notch molecules (*i.e. Drosophila* Notch containing a mouse furin site) demonstrates the bulk of Notch reaches the cell surface as a heterodimer, and could suppress the zygotic *Notch* phenotype. This indicates that dFurin is able to efficiently process the mouse S1 site in contrast to the *Drosophila* S1 site. Interestingly, the converse hybrid murine Notch receptors carrying the *Drosophila* S1 cleavage region expressed in human cells show the opposite; only unprocessed full-length receptors reach the cell surface. Thus, mFurin is not able to process the *Drosphila* S1 site due to a lack of the minimal furin cleavage consensus site. Apparently furin cleavage is not a prerequisite for Notch transport to the cell surface in *Drosophila* [27]. Structural and functional analyses have recently demonstrated furin cleavage in *Drosophila* [28]. Purified Notch fragments were analyzed using mass spectrometry, which identified two putative furin cleavage sites, F1 (RKNK) and F2 (RLKK). Interestingly, only the F2 site mutation showed a dramatic decrease in the production of heterodimeric receptors. Yet, either the F1 site mutant or a S1 cleavage loop deletion containing the furin consensus site appeared to be processed normally, indicative of a non-furin-dependent cleavage [28, 29]. In addition, only expression of the F1 mutant in *Notch* null embryos could rescue the Notch loss-of-function phenotype. Cell surface expression could only be shown for the F1 mutant, explaining the lack of Notch activity in the F2 mutant. Reducing or inhibiting the two furin convertases present in *Drosophila* did not affect Notch processing or activity *in vitro* and *in vivo*, indicating that either i) furin processing is not important in *Drosophila* or ii) a distinct enzyme processes Notch [28].

Mutations or deletions introduced in proteins can result in differences in charge leading to alteration of conformation or protein folding. This complicates the functional analysis of furin activity in Notch S1 cleavage. Structural prediction and crystallographic studies show

that removal of the furin loop from Notch1 and Notch2 receptors does not lead to major structural changes in the conformation of the extracellular domain, in particular the negative regulatory region (NRR). Yet, between Notch receptors major differences exist; whereas removal of the S1-loop from Notch2 leads to normal trafficking, cell surface expression and ligand-dependent processing, furin cleavage loop-mutants of Notch1 are severely attenuated in cell surface expression and transcriptional activity. Despite being uncleaved, S1-deficient Notch receptors retain the ability to signal in ligand-dependent and independent manner [29]. These data argue that whereas S1 cleavage is common it is not required for Notch1 and Notch2 receptor signaling in mammalian cells.

Recently a potential enhancer of S1 cleavage was identified, Cripto-1, which binds only the non-furin cleaved form of Notch [30]. Roles of Cripto-1 in both trafficking and maturation of the receptor are dose-dependent; increasing the S1 cleaved Notch receptor localization in lipid rafts, which serve as organizing centres for the assembly of signaling molecules. *Cripto-1* null cells show a slight accumulation of the full-length non-furin processed Notch1 receptor, leading to a decrease in both cell surface expression and activity reflected in reduced activation of Notch transcriptional targets [30]. This indicates a role for Cripto-1 in the maturation of Notch through an enhancement of furin-dependent S1 cleavage during Notch receptor trafficking.

Whether Notch furin cleavage is solely a requirement or has an additional regulatory function in regulating Notch activity in tissues remains to be established in mammalian cells but these results point to a potential novel mode of Notch regulation of the duration or amplitude of Notch signaling via ~regulation of S1 cleavage.

II. JUXTAMEMBRANE S2 CLEAVAGE

Following S1 cleavage mature Notch receptors can interact with membrane bounds ligands expressed on adjacent cells. During physiological signaling ligand binding is thought to induce a conformational change enabling access of a membrane-bound ADAM metalloprotease to cleave a scissile bond in the Notch extracellular domain [31-33]. ADAMs (a disintegrin and metalloprotease) are membrane bound zinc-dependent metalloproteases that function as signaling scissors regulating the activity of transmembrane signaling molecules. For substrate proteolysis the Zn^{2+} ion is essential and Zn^{2+}-chelating drugs such as hydroxamate-type inhibitors inhibit most ADAMs, although these are mostly broad-spectrum inhibitors [34]. There is ample experimental evidence from flies, worms, and mammals that implicate the ADAM sheddases in Notch receptor cleavage and activation.

ADAM-dependent cleavage, also termed S2 cleavage, occurs in the juxtamembrane extracellular domain proximal to the Notch transmembrane domain. S2 cleaved Notch fragments are extremely short-lived and hence can only be observed when blocking downstream processing or by hyper-activation of metalloprotease activity [19, 32, 35]. Genetic studies in *Drosophila* and *C. elegans* demonstrated that ADAM10 (Kuz/SUP-17) plays a critical role in Notch/LIN-12 signaling upstream ~of γ-secretase cleavage [36-38], that is executed by the aspartyl protease presenilin (PS) which cleaves the Notch transmembrane domain at Site-3 (S3). Early fly studies had identified the metalloprotease

Kuz as a neurogenic gene pointing to a role for Kuz in Notch signaling *in vivo*. The *kuz* mutant phenotypes showed similarity with the Notch receptor loss-of-function phenotype, such as central nervous system hyperplasia and a multiple bristle phenotype [39]. Maternal and zygotic *kuz* mutant flies have a more severe phenotype than *Notch* mutant flies suggesting that Kuz has other substrates as well. For instance, Kuz plays an important role in neuronal guidance by cleavage of Eph/Ephrin receptors [40, 41]. Several groups proposed that Kuz was the protease responsible for furin cleavage of Notch [21, 36, 38]. A comprehensive analysis on the role of Kuz in the regulation of Notch receptor signaling in flies showed that Kuz could cleave Notch [36]. Expression of a dominant-negative Kuz (KuzDN), lacking the protease domain, resembled Kuz loss-of-function phenotypes, implying the protease domain is essential to its biological activity. Kuz loss-of-function phenotypes can be rescued in flies by expression of an activated form of Notch (NICD), indicating Kuz acts upstream of the intramembranous cleavage by presenilin (PS). Flies expressing KuzDN showed a loss of Notch cleavage, demonstrating Kuz also processes Notch *in vivo* [36]. Similar studies in flies and worms show that Kuz acts cell autonomously and is needed during ligand interaction or subsequent steps in activation, upstream of γ-secretase cleavage [37, 38]. Reducing Kuz/SUP-17 activity in worms does not lead to suppression of *NICD*/*lin12-intra* phenotypes, whereas it could suppress membrane bound Notch mutants. Apparently, SUP-17 ~needs the extracellular domain to facilitate the Notch/LIN-12 signaling. Although loss of *sup-17* lowers LIN-12 activity, it does not abolish it. This could be due to redundance of the closely related protease TNF-α-converting enzyme (TACE/ADAM17) [38, 42].

Although a cell autonomous function has been attributed to Kuz in the Notch expressing cell, the Notch ligand Dl is also processed by Kuz [43], making it difficult to attribute *kuz* phenotypes to a defect in Notch cleavage, Dl cleavage or both. Expression of KuzDN in flies displays both *Notch* null and *Dl* null phenotypes. Surprisingly, this phenotype can be rescued by additional copies of *Dl*, while extra copies of *Notch* showed negligible suppression of the phenotype. This indicates that high concentrations of Dl could counteract KuzDN, suggesting that Notch stimulated by Dl can possibly be cleaved by other proteases. Experiments expressing Dl in *Drosophila* S2 demonstrate an extracellular cleavage fragment of Dl, Dl^{EC}, which is released in the culture medium. This cleavage increases upon co-expression of wildtype Kuz, whereas KuzDN co-expression inhibits this cleavage [43]. *In vivo* Dl^{EC} is not detected in *kuz* null embryos, while it is produced in $kuz^{-/+}$ flies. The soluble Dl^{EC} appears to be inactive and is only produced in order to downregulate Dl from the membrane [44]. Coculture of Kuz expressing cells with Dl expressing cells did not reveal an increase of cleavage, indicating Kuz does not cleave Dl in *trans*-orientation [45]. In addition, mammalian studies indicate that the ligands Dll1 [46, 47] and Jagged1 [46] are processed to some extent by Kuz/ADAM10 and/or other ADAM proteases. This probably acts to relieve *cis*-inhibition of Notch, caused by ligand binding to Notch on the signal-receiving cell maintaining the receptor in a conformational state unable to receive ligand binding in *trans* [48]. Interestingly, in *Drosophila* a Kuz related protein, Kuz-like (Kul), is the protease dedicated for Dl cleavage on the signaling cell [49], preserving the asymmetric distribution of Dl in order to maintain unidirectional Notch signaling. To date a homolog for

Kul has not been identified in mammals yet. Altogether, Kuz/Kul and TACE/ADAM17 are capable of cleaving Dl [49].

Studies in mammalian cells have demonstrated that the ectodomain cleavage of Notch1 receptor is regulated by ligand binding and is distinct from furin/S1 cleavage during receptor maturation [32]. Using murine Notch carrying fly gain-of-function mutations a novel cleavage product, migrating between the S1 and S3 fragments, was identified. This product was termed Notch extracellular truncation (NEXT), the cleavage was coined S2 (Site-2). N-terminal sequencing revealed the amino terminal cleavage residue to be between residues Ala1710 and Val1711 of murine Notch1 [31, 32] (Fig. **2**). This finding was later confirmed on endogenous Notch1 signaling as well [19]. Intriguingly, by biochemical purification ADAM17/TACE was shown to be required for Notch1 cleavage *in vitro* excluding Kuz/ADAM10 [31]. Mutation of the Val1711 cleavage site abrogated *in vitro* cleavage but not cleavage in cells [19, 31]. Forced ectodomain oligomerization using hybrid CD4-Notch receptors results in a defect in the S2 cleavage step suggesting that the conformation of the extracellular domain may act as a negative regulator of S2. Indeed non-oligomerizing CD4-Notch fusions are a substrate for S2 and active in signaling [50]. In an effort to inhibit S2 cleavage, a series of protease inhibitors were tested, of which only the broad-spectrum metalloprotease inhibitors (Zn^{2+}-chelators) 1,10-o-phenanthroline and TAPI/IC-3 [31, 32] showed a robust decrease in S2 cleavage pointing to a role for metalloproteases in this process. Incubation of Notch with crude cell extracts from *ADAM17*−/− fibroblasts did not exhibit S2 cleavage activity while extracts from wild-type cells did [31] demonstrating ADAM17 is the activity responsible for Notch1 S2 cleavage. To address this involvement, expression of gain-of-function Notch in *ADAM10* null cells did not show a defect in Notch processing [32].

Experiments in *Drosophila* revealed a direct role for Kuz in extracellular cleavage of Notch [51]. Notch physically interacts with Kuz and KuzDN indicating that the protease domain is dispensable for binding [51]. Gain-of-function mutants, lacking parts of the repressive LNR repeats, are ligand independent Notch receptors that function in a *Dl* null background. However, they still require Kuz for their activation, since they could not fully rescue *kuz* null phenotypes. Completely ligand independent mutants lacking, in addition to the LNR repeats also EGF 1-18, show an almost exclusive requirement of Kuz for activation *in vivo*. This indicates that a distinct protease, possibly TACE/ADAM17, is capable of processing Notch in the absence of Kuz independent of stimulation. An accumulation of the S2 cleavage fragment could be visualized by *in vitro* knockdown of presenilin (PS). However, knocking down both Kuz and PS does not result in a S2 cleavage band or any other cleavage product. Interestingly, TACE/ADAM17 overexpression could only partially rescue the S2 cleavage in *kuz/PS* null cells, yet this fragment does not seem to be processed very efficiently [51], indicating TACE/ADAM17 is sufficient but not necessary.

In addition, knockout studies in mice do not support the *in vitro* observation that TACE/ADAM17 is the most important Notch S2 protease. Whereas *Notch1*−/− mice die around embryonic day E9.5 with severe defects in haematopoiesis and neurogenesis [52-55], *ADAM17*- deficient mice show lethality at embryonic day E17.5 [56], not resembling the *Notch1* phenotype. Possibly TACE/ADAM17 is either redundant with another protease or

has a more spatial or temporal restricted role in Notch signaling [51, 57]. For example TACE/ADAM17 may have a specific role during differentiation of monocyte progenitors into macrophages [31]. The phenotype of *ADAM10*−/− mice however, does phenocopy the *Notch* phenotype [57]. These mice die around embryonic day E9.5 showing disturbed somitogenesis, defects in brain and heart development, the latter mimicking that of an E7.5 stage. Notch target genes *Hes5* and *Dll1* showed disturbed expression *in vivo* [57]. In contrast, some tissues, which are known to require Notch signaling, like the otic vesicles, appear to develop normally in the absence of *ADAM10*. Although a detailed analysis of the effects of ADAM10 on the Notch pathway in various tissues is still lacking the embryonic lethality is similar to *Notch1/Notch4* and *PS1/PS2* knockout mice. Moreover, T-cell-specific deletion/disruption of *ADAM10 in vivo* phenocopied the *Notch1* null phenotype during thymocyte development [55, 58, 59]. More recent studies using a conditional *ADAM10* knockout investigated the role of ADAM10 in the hematopoietic system [59, 60] and during brain development [61]. Notch signaling plays essential roles in the development of T-lymphocytes. Specific *ADAM10* knockout in thymic T-cells leads to impaired thymocyte development, decreasing thymic cellularity by 2- to 3-fold. Thymocytes lacking ADAM10 showed attenuated NICD production and activation of target genes like *Hes1* and *Deltex-1* [59]. ADAM10 has also been implicated in marginal zone B-cell development; a Notch2-dependent process. In naive B-cells expressing Notch2, activation of Notch2 is required for the development of the marginal zone B-cell (MZB) lineage at the expense of follicular B-cells. Specific knockout of *ADAM10* in B-cells using *CD19-Cre* leads to a complete block in splenic MZB cell production and a concomitant increase in follicular B-cells levels. Notch2 specific and other general Notch targets show significantly lower expression in *ADAM10*-deficient B-cells [60]. These studies indicate ADAM10 plays a critical role in activation of Notch receptors in the haematopoietic system. In the developing brain *Nestin-Cre* driven *ADAM10* loss leads to late embryonic and perinatal lethality with intracranial haemorrhages [61]. In *ADAM10*-deficient brains premature differentiation into postmitotic neurons at the expense of neural progenitor cells is observed. Furthermore, *in vitro ADAM10*-deficient progenitors show a substantial loss in neurosphere formation indicating an important role for ADAM10 in neural stem cell maintenance. In brains of *ADAM10* knockout mice NICD1 production was impaired and a reduction in Notch target gene *Hes1/5* expression was observed suggesting that most of the phenotypes are a consequence of impaired Notch signaling [61]. These results demonstrate an important role of ADAM10 as the S2 protease for Notch signaling *in vivo*.

Loss-of-function studies in worms, flies and mammals indicate that Kuz/ADAM10 is probably not the sole S2 protease and that TACE/ADAM17 could act redundantly with ADAM10 [31, 42, 51]. In worms inactivation of either *kuz/sup-17* or *TACE/adm-4* alone does not cause reduced Notch/LIN-12 or GLP-1 signaling [42]. In contrast, *sup-17/adm-4* double mutant worms ~lacking vulval precursor cells show delayed ovulation and sterility, reminiscent of *lin-12* or *glp-1* loss-of-function phenotype. This synthetic sterility could be rescued by ectopic expression of a LIN-12(E) construct, not dependent anymore on S2 cleavage demonstrating that SUP-17/ADM-4 protease acts upstream of S2 cleavage. However, depletion of both *adm-4* and *sup-17* does not display a general failure of LIN-12 or GLP-1 signaling, indicating that in *C. elegans* possibly more ADAM10/17 related

proteases could provide redundant activity in the absence of *adm-4* and *sup-17* [42]. Moreover, Kuz/ADAM10 or TACE/ADAM17 can activate Notch in the absence of ligand stimulation, a process that may be important in tumors overexpressing ADAM proteins [51, 62]. In fly neural cells ectopic expression of either TACE/ADAM17 or Kuz can activate Notch cleavage and activity in a ligand independent manner. Knocking down Kuz in cells overexpressing TACE/ADAM17 ~does not affect the constitutive activation of Notch, indicating TACE/ADAM17 can fully compensate for loss of Kuz [63]. This suggests that deregulated expression of ADAM10 and/or ADAM17 in cancers may induce Notch shedding in a ligand-independent manner. Interestingly ADAM10 is also a γ-secretase substrate suggesting that in addition to its role in receptor shedding membrane cleavage leads to ADAM10 accumulation in the nucleus [64]. Although evidence that cleaved nuclear ADAM10 has any signaling activity is lacking, it uncovers a potentially novel role for ADAM proteins as dual address proteins as well.

The role that ligand binding plays on Notch S2 cleavage remains a subject of debate. Two models prevail; the *pulling force model* and the *lift and cut* model. The first model suggests dissociation of the Notch heterodimerization domain, after which S2 cleavage takes place at the newly exposed N-terminus [65, 66]. Ligand-receptor binding induces a pulling force due to ligand transendocytosis [33] causing dissociation of the NECD-TMIC Notch heterodimer, of which the membrane bound TMIC fragment is subsequently processed by S2 and S3 cleavage. Inhibiting metalloproteases by broad spectrum inhibitors shows the dissociation and transendocytosis itself is not dependent on metalloprotease activity. However, a prerequisite for this process is S1 cleavage [65]. Although an endocytosis defective mutant displays Notch phenotypes in *Drososphila* neurogenesis [67], this ligand endocytosis based dissociation model of Notch is not exclusive. Studies using immobilized Dl ligand or paraformaldehyde fixed cells expressing Dl, show that these ligands are still capable of activating Notch *in vitro* [44, 45], suggesting that ligand transendocytosis may not always be required for Notch activation. A different way of Notch activation could be explained by the *lift-and-cut model*. Of note, this model does not exclude a role of ligand transendocytosis. Several lines of evidence argue that the ectodomain of Notch contains negative regulatory properties. Intramembranous cleavage of Notch is induced by the removal of its NECD [22, 68, 69]. Moreover, by systematically shortening extracellular sequences of a Notch activity reporter protein a gradual increase in Notch activity was observed [66]. It is believed that in the absence of ligand mature Notch receptors are held into an inactive “proteolysis resistant” or closed state because the Negative Regulatory Region (NRR) composed of the HD domain and the globular Lin12/Notch repeats (LNR) inhibits Notch activation. Crystallographic studies revealed a globular structure of the Notch negative regulatory region (NRR) comprising the heterodimerization domain and the three LNR modules folding over the S2 cleavage site like a three leaf clover protecting it from cleavage. Ligand binding to Notch receptors unfolds the NRR permitting cleavage by a metalloprotease at a site close to the membrane (S2) [18, 70]. A substantial conformational change is needed to access the S2 site since mutants lacking the most N-terminal LNR-A domain are in a ‘closed’ conformation and inactive. Only ΔEGF-mutants lacking both LNR-A and LNR-B show Notch activity in reporter assays, which is further enhanced upon removal of LNR-C [18].

In almost 25% of patients suffering from T-cell acute lymphocytic leukaemia (T-ALL) activating mutations have been identified in the *NOTCH1* gene, within the heterodimerization (HD) domain [20]. HD mutations lead to unfolding of the NRR domain that normally keeps the receptor in a proteolysis resistant or auto-inhibited state in the absence of ligand [70, 71]. Most T-ALL mutations tested act ligand independent. The mutations in the Notch HD domain can be classified in three types of activating mutations; Class 1 that show spontaneous dissociation of the heterodimer and Class 2 mutations are insertions in the extracellular domain of Notch, displacing the S2 cleavage site away from the protective HD-NRR structure [71]. A related class of mutations is formed by the juxtamembrane expansions (JME), caused by a tandem duplication of a part of exon 28 distal to the S2 site, however, they do not affect stability of the NRR *per se*. Yet, they do show increased S2 cleavage leading to Notch activation, most likely by providing an unprotected (novel) S2 site [72].

Recently, we and others have shown that ligand-dependent Notch activation requires ADAM10, not TACE/ADAM17, whereas ligand independent signaling is mediated by multiple proteases [19, 73]. Furthermore, using an S2 specific antibody it was shown that loss of *ADAM10* impairs specific cleavage at Val1711, the major mNotch1 S2 cleavage site, and loss of transcriptional reporter activity [19]. Re-expression of ADAM10 could rescue Notch1 activity again, while ADAM17 could not [73]. Furthermore, depletion of *ADAM9*, *ADAM12*, and *ADAM15*, which have been implicated in Notch signaling [64, 74, 75], did not affect Notch1 S2 cleavage or activity [19]. ADAM15 has also been implicated in the shedding of ADAM10 which is also a γ-secretase substrate. However, *ADAM15*-deficient cells do not show a defect in Notch1 Val1711 cleavage [19]. The significance of this remains to be established in the context of Notch1 signaling. Although ligand-dependent Notch signaling in the absence of ADAM17/TACE does not influence cleavage or activity some residual S2 cleavage was observed in *ADAM10*−/− cells indicating an alternative protease is capable of cleaving Notch1 in the absence of ADAM10 [19]. Yet, this does not seem to be ADAM17 [73]. Possibly this residual activity could be due to the action of MMP-7, which has been shown to act upstream of γ-secretase induced Notch activation in pancreas, and is able to cleave Notch *in vitro* [76]. Altogether, ligand-dependent Notch signaling relies almost only on ADAM10. Interestingly, in accordance with previous studies in flies, overexpression of ADAM17/TACE resulted in ligand independent activation of wildtype Notch [51, 63]. In addition to this, ligand independent fly mutations in Notch do not depend on ADAM10, instead this S2 cleavage is ADAM17-dependent [31, 32, 73]. Importantly, all ligand independent T-ALL mutations (type I) tested are all cleaved at Val1711 [19] and rely on the concerted action of multiple proteases, since cells lacking *ADAM10* and *ADAM17* still allow Notch activation [73]. These results and the observation that under high concentrations of broad spectrum metalloprotease inhibitor Notch proteins are still active argues for the involvement of another class of proteases in Notch activation or of an S2-independent step in Notch activation [19]. An interesting point brought up by Bozkulak *et al*. is that ADAM17 induced cleavage does not involve ectodomain shedding, indicating this cleavage does not take place at the cell surface [73]. However, using immunofluorescence we showed that this fly-derived Notch1 mutant, fully dependent on ADAM17 cleavage [73], is cleaved at the cell surface [19], making ADAM17-dependent

ectodomain shedding plausible. Furthermore, some yet to be further characterized internalized structures could be observed. Outstanding questions are whether these vesicles contain γ-secretase competent Notch S2 cleaved molecules and whether these are recycled to the plasma membrane or follow other intracellular routes for activation or degradation.

Although it appears that all Notch homologues are processed in a similar manner [77], currently only for Notch1 and Notch3 a clear S2 cleavage fragment is observed [78]. It will be of interest to determine if all four mammalian Notch homologues rely on S2 cleavage for their activation and if this is mediated by the same or similar proteases.

III. γ-SECRETASE S3 CLEAVAGE

Following S2 cleavage, NEXT proteins are cleaved within their transmembrane domain at Site-3 (S3) by the aspartyl protease presenilin, part of a multi-protein complex termed γ-secretase, releasing the Notch Intracellular Domain (NICD) the final step in the generation of an activated Notch molecule. Whereas flies have a single presenilin gene (PS), worms and mammals have two presenilin-1 and presenilin-2 (PS1 and PS2) that are highly similar but mutually exclusive in the γ-secretase complex. NICD which has two nuclear localization signals translocates to the nucleus where it binds to the DNA-bound protein CSL (an acronym for vertebrate CBF/RBP-κ, *Drosophila* Su(H), and *C. elegans* Lag-1) together with co-activator protein Mastermind (MAM) [79], leading to target gene activation [80, 81]. In the absence of NICD, CSL is associated with ubiquitous co-repressors SKIP, SMRT and MINT to repress transcription of target genes [82, 83]. Through structural and biophysical analysis the composition of the CSL/NICD complex has been identified at the atomic level [84, 85]. From these studies it is now clear that the Notch ANK repeats are essential together with CSL for binding to co-activator proteins such as Mastermind whereas the N-terminal RAM domain of NICD is required for NICD binding to CSL/RBP-κ as well as inducing derepression of target genes [80, 81]. The expression of NICD consisting of only the CDC10/Ankyrin repeats, responsible for CSL binding [84], can activate Notch signaling in the absence of a RAM domain [86-89]. Yet, it is still dependent on nuclear access, since CDC10 repeats coupled to membrane localization signals are unable to signal [89]. While virtually all Notch signaling in all species is thought to rely on CSL, there are examples of non-canonical Notch signaling not requiring CSL/RBP-κ [90].

Nuclear Access of Notch

Early studies had already reported the presence of nuclear Notch [69, 91, 92]. However, the first demonstration of a role for nuclear Notch was reported by Kopan *et al.* who demonstrated a requirement for nuclear translocation of Notch1 in suppressing myogenic differentiation [93]. Besides NICD also membrane-bound Notch forms lacking the extracellular domain (N E) produced activated Notch phenotypes in frogs [94] and in flies [91, 95] demonstrating that like NICD, N E also acts as a constitutively active protein. Cell culture-based experiments further demonstrated that N E and NICD activated target reporter constructs, inhibited myogenic and neurogenic differentiation, and impacted T-cell development in a similar manner [24, 81, 93, 96, 97]. Further characterization of these membrane bound forms of Notch revealed that the receptor was subject to cleavage at the membrane, releasing the Notch intracellular domain (NICD), which subsequently

translocates to the nucleus [22]. Isolation and sequencing of cleaved products identified the S3/γ-secretase cleavage site, between Gly1743 and Val1744 of mouse Notch1 [16] (Fig. **2**). Val1744 is also required for Notch1 processing and homozygous Val1744 loss-of-function mutation in mice leads to an almost complete abrogation of Notch1 processing with similar phenotypes to that of *Notch1* null mice [52-54]. Concurrent with these observations, experiments in transgenic *Drosophila* flies expressing Notch-GAL4VP16 fusion proteins, demonstrated Notch cleavage and activity *in vivo* [89, 98]. Modulating expression levels of the Notch ligand Delta led to corresponding changes in nuclear Notch accumulation [99]. Constitutively active Notch E-GV16 (lacking NECD) molecules could activate the reporter, whereas full-length Notch-GV16 molecules could not, showing that transcriptional activity depended on ligand-receptor interactions [89] and later that cleavage required presenilins [100]. Interestingly, in a *CSL/Su(H)* null background NICD-GV16 did not display any UAS-driven *LacZ* reporter gene expression, indicating that Su(H) somehow stabilizes Notch in order to function [98].

Furthermore, by using NICD fusion proteins with transcriptional activators (VP16) or repressors (Engrailed, Hairy) these studies demonstrated the direct involvement of Notch in transcriptional activation [89]. The development of antibodies and protocols recognizing the cleaved neo-epitope of Notch1 are now able to also detect the endogenous NICD fragment *in situ* [101-103]. All four Notch receptors are processed through a conserved Valine within the transmembrane domain of Notch 1-4 (Fig. **2**) and produce NICD species with different transcriptional activities [77, 104]. Within Notch1, Val1744 is only required for Notch processing by γ-secretase, but not for transcriptional regulation [16]. Mutagenesis of the conserved Valine has established that Val1744 is critical for Notch activity. Different mutations have different effects on the efficiency of cleavage reflected in transcriptional output [16, 50]. Other experiments have also revealed that NICD species with distinct N-termini (*e.g.* Gly or Leu) are also subject to N-terminal end rule of proteosomal degradation [105]. This underscores the need for careful analysis of unnatural substrate variants by mutagenesis as differences in both structure, stability or both may underlie defects in substrate activity [19, 106, 107]. Interestingly, neither the extracellular domain nor the Notch intracellular domain seems to be required for Notch1 S3 cleavage in mammalian cells [50, 66].

Ligand receptor interactions are often regulated by dimerization. Experiments determining the oligomerization state of Notch and its activity revealed that both active and inactive signaling molecules are mostly monomeric when at the cell surface [50]. Receptor dimerization can occur via ~the EGF repeats however, this occurs mostly intracellularly [50]. Notch ectodomain oligomerization has now been confirmed by cryo-electron microscopy [108]. Transgenic flies ectopically expressing TMD-Notch fusions have shown that it is mostly secondary/tertiary structure that PS recognizes rather than a specific primary sequence. Also forced ectodomain and transmembrane domain dimerization (using the mammalian Glycophorin A TMD) inhibited Notch cleavage suggesting γ-secretase substrates are mostly monomeric [66]. Dimerization experiments of both wildtype and mutated (cleavage-resistant) TMD of Notch1-4 and a distinct γ-secretase substrate Amyloid β Precursor Protein (APP) showed they were capable of forming dimers [50]. In contrast, the

APP TMD has a GXXXD dimerization motif that is a target for γ-secretase inhibiting drugs, suggesting that this γ-secretase substrate is a dimer [109].

Interestingly, dimerization of NICD is important for a subset of targets in Notch driven T-cell leukemia whereas for other transcriptional targets dimerization is not required [110]. All four murine Notch homologs are similarly processed by PS and produce NICDs that translocate to the nucleus binding CSL and activate transcription [77, 104, 111].

γ-Secretase Genetics

Genetic mutagenesis screens in worms identified a suppressor of LIN-12 ~activity, *sel-12*, causing an egg-laying defect [112]. *sel-12* mutations enhance the phenotypes of *lin-12* and *glp-1* hypomorphic alleles, whereas it suppresses *lin-12* hypermorphic alleles. Cell ablation experiments show SEL-12 acts cell autonomously since cells with reduced *sel-12* function carrying *lin-12* hypermorphic alleles suppress *lin-12* activity, adopting a *lin-12* independent cell fate [112]. In addition to SEL-12 ~a related protein was found based on similarity; HOP-1, which rescues *sel-12* related phenotypes. Reducing HOP-1 ~activity in a *sel-12* mutant background displays phenotypes associated with *lin-12* and *glp-1* loss, indicating HOP-1 and SEL-12 ~function redundantly [113]. Since NICD rescues *sel-12* phenotypes this indicated SEL-12 acts upstream or parallel at generation of NICD at the membrane. SEL-12, which ~has 7 predicted TMD domains, ~is thought to function in the membrane. It is observed in the perinuclear region, probably ER/Golgi, as well as in the plasma membrane. Decreasing SEL-12 activity leads to a reduction in accumulation of LIN-12 on the cell surface, whereas expression in the ER/Golgi is unaltered, suggesting that SEL-12 may also ~function in LIN-12 trafficking [114].

The homolog of *sel-12* in mammals is *presenilin-1*, or *PS1*. Knockout mice lacking *PS1* die at birth [115, 116]. The most prominent phenotypes are observed in the axial skeleton [116] and central nervous system [115] similar to *Notch1*−/− mice [52, 54]. Whereas mice lacking PS1 are embryonic lethal, mice lacking the PS1 homolog PS2 are viable with relatively minor defects [117]. Cells lacking both PS1 and PS2 are devoid of γ-secretase activity, suggesting that at least *in vitro* no additional compensatory activities exist that could explain PS1/2 independent cleavage of Notch or APP [118]. Mice expressing hypomorphic *PS1* alleles with severe γ-secretase deficiency survive into adulthood with severe skeletal abnormalities however, with apparently normal brain development. Although these results support the notion that PS1 may be the most important player in γ-secretase, PS2 may provide an important tissue-specific redundant function [119]. Increasing evidence suggest that slight modifications within the γ-secretase complex are responsible for altering substrate specificity. For example certain mutations within the γ-secretase subunit Nicastrin affect Notch-S3 cleavage but leave APP cleavage intact [120, 121]. One of the most striking examples is the finding that the Abelson tyrosine kinase inhibitor Gleevec/Imatinib specifically targets γ-secretase activating protein (GSAP), which only affects APP but not Notch cleavage [122, 123]. A different modifier of γ-secretase specifically acting on APP but not on Notch cleavage is the non-steroid anti-inflammatory drug (NSAID) Sulindac sulfide [124, 125]. Clearly such Notch-sparing γ-secretase inhibitors may find better clinical

application in Alzheimer's treatment and will provide further insight into the complexity of γ-secretase activity.

γ-Secretase Complex

Deficiency of PS1/2 inhibits the normal cleavage of APP, Notch and other RIP substrates indicating its key importance for γ-secretase activity [126, 127]. Immunoprecipitating PS1 and its homolog PS2 in mammalian cells revealed a physical interaction with both the membrane and precursor forms of Notch1 [128], supporting its role in trafficking [114]. Expressing Notch in *PS1*−/− cells *in vitro* shows that the NICD variant can translocate to the nucleus, whereas membrane bound ΔE does not, suggesting a direct role for PS1 in membrane processing [126]. An accumulation of an S2 cleavage fragment is seen in a *PS1* null background, which is greatly diminished by expressing a dominant negative ADAM10, KuzDN, demonstrating that PS acts downstream of ligand binding and ADAM10 cleavage [50, 100, 129].

Mutations in PS1 and PS2 cause early-onset familial Alzheimer's disease and are found throughout the protein coding sequence. Two conserved aspartate residues Asp257 and Asp385 that lie within the two anti-parallel transmembrane domains constitute the active site of γ-secretase and are essential for PS activity [130]. Consistently, overexpression PS1 Asp mutants in *PS1*−/− cells cannot rescue NICD cleavage, whereas very low levels of wildtype PS1 are sufficient to promote NΔE cleavage in *PS1* null cells [131]. To become active, PS must undergo endoproteolysis, which generates a N- and C-terminal fragment (NTF and CTF) [132, 133]. Asp mutants are also defective in endoproteolysis [130], and therefore phenotypes associated with these mutations are hard to interpret since they could either result from insufficient cell surface expression due to defective autoproteolysis or from loss of substrate cleavage. Loss of endoproteolysis affects the topology of the two Asp residues resulting in a cleavage defective presenilin. Expression of either NTF or CTF alone is not sufficient to rescue the *sel-12* phenotype in worms, however expression of both NTF/CTF rescues the *sel-12* phenotype. The combination of the wildtype NTF with an Asp mutant CTF (and *vice versa*) is unable to rescue the *sel-12* phenotype indicating that loss of PS1 activity is not due to endoproteolysis, but due to cleavage of its substrates. Precipitation of both NTF- and CTF-fragments allows the formation of an active γ-secretase activity [134]. In addition to either PS1 or PS2, three other transmembrane proteins form the catalytically active γ-secretase complex; Nicastrin (Nct) [135, 136], Aph-1 [137] and PEN-2 [138-142]. The transmembrane protein Nct contains a large ectodomain, which is believed to be used for substrate recognition and docking. Both the mature and immature form of Nct can bind to the extracellular domain of Notch, suggesting that Nct like PS is already bound to Notch during maturation [143]. In contrast to these reports, a recent study describes a functional γ-secretase in *Nct*-deficient cells albeit less active [144]. The other two co-factors of the presenilin-dependent γ-secretase complex show more apparent roles. Two *Aph-1* alleles (Aph-1A & B) have been described contributing to the heterogeneity of the γ-secretase complex [137, 145]. In addition, rodents have a duplication of the gene, resulting in Aph-1C, which upon loss does not display an obvious phenotype [146]. Although Aph-1A functions in stabilizing the complex during assembly, its homolog Aph-1B does not have comparable stabilizing activity. The Aph-1A containing γ-secretase complex is essential for Notch

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signaling in development [147, 148]. Compositions of the γ-secretase complexes might differ depending on the affinities of its various substrates [137]. The subunit PEN-2 triggers endoproteolyis of presenilin and stabilizes the PS fragments [149, 150]. A recent report using *in vitro* recombinant PEN-2 and PS1, demonstrates PEN-2 alone is capable of inducing endoproteolysis and catalytic activation of PS1 [151]. Both Aph-1 and PEN-2 are necessary and sufficient for assembly and correct trafficking of stable mature γ-secretase complex to the cell surface and to endocytic compartments exerting its activity [152].

Mouse models expressing the Notch V1744G S3 cleavage mutant are embryonically lethal. Although Notch V1744G mRNA and protein were produced, no NICD generation can be detected, implying this variant is unable to be processed by PS. These mice resemble a *Notch1* null and *PS1/2* null phenotype, showing that Notch Val1744/S3 processing is required for Notch function and viability. However, the somites in Val1744G homozygotes are unaffected, indicating very low levels of NICD is sufficient for their development [53]. Since no NICD could be detected in Val1744G mice this indicates a PS-independent activation of Notch. Lack of cleavage but retention of transcriptional activity was also observed in *PS1/2* double knock-out (DKO) cells [153]. The membrane tethered NotchΔE shows higher *Hes1* activation levels than in untransfected *PS1/2* DKO cells. Moreover, ligand stimulation of endogenous Notch in *PS1/2* null cells displayed a CSL-dependent activation of the Hes1 reporter. Interestingly, ΔE-GV16 could not activate an UAS-reporter, indicating the Notch-dependent activation does not rely on nuclear access [153]. Although loss of *PS1/2* in mice shows defects in somitogenesis [116], *PS1/2* DKO/*Notch V1744G* mice developed normal looking somites. This implies that there is some activation of Notch V1744G independent of presenilin resulting in sufficient levels of Notch activity for somite formation. Yet, this PS-independent activity is not able to reach these same activation levels in mice expressing wildtype Notch1. Experiments in *PS1/2* DKO cells confirmed a two-fold increase in activation of Notch1ΔE V1744G compared to wildtype Notch1ΔE, however, no Val1744 cleaved NICD was produced in these cells [154]. Whether similar PS-independent somite-specific cleavage occurs on other Notch variants or other substrates is not known but could also be an artefact of the introduced mutation. These data also support the notion of a cleavage-independent function of PS1/2 in somitogenesis and that in their absence, other protease-like activities are capable of Notch activation. However, whether they have physiological importance in Notch activation is not known.

Similar to the processing of APP, Notch is also cleaved at multiple positions within the TMD. In cell culture medium small cleavage products of Notch can be detected, termed Nβ in analogy of Aβ peptides derived from APP cleavage. Mass spectrometric analyses revealed Nβ peptides are the result of an additional cleavage (S4) in the centre of the transmembrane domain, varying in position but occurring mainly at Ala1731 (Fig. **2**). Moreover, PS1 mutations in familial Alzheimer's disease show, similar to Aβ, a shift in Nβ cleavage [155]. In addition, upon multiple Alanine into Valine substitutions in the transmembrane of Notch1 no shift in the major cleavage site was observed, indicating that Valine residues *per se* do not play a crucial role in PS-dependent intramembranous cleavage [156]. In an effort to determine the order of the intramembrane cleavage events, mutations were introduced in the S3 site and secretion of Nβ was analyzed. As expected S3 mutations reduced γ-secretase

production of NICD, but also reduced S4 cleavage. To determine the order of cleavages extracts were immunodepleted by specific S3-NICD precipitations and the N-termini of the Myc precipitates were sequenced. This did not result in the detection of S4-NICD, indicating S3 cleavage occurs prior or simultaneously with S4 cleavage [107].

Endocytosis

As described above endocytosis plays a major role in both ligand and receptor expressing cells. For example Dynamin - critical for formation of clathrin coated vesicles - is required in both ligand and receptor expressing cells for effective Notch signaling [67]. Although ligand endocytosis is well documented in receptor activation by transendocytosis [33, 65], the role of endocytosis on γ-secretase cleavage of Notch is poorly understood. Inhibition of endocytosis through expression of a dominant-negative Eps15 (Eps15DN), leads to an exclusion of Notch from the nucleus. These experiments identified a monoubiquitinated Lysine K1749 distal to the transmembrane domain residue critical for ligand mediated Notch cleavage and activity. Mutating the K1749 into Arginine results in loss of Notch endocytosis and severely decreased γ-secretase mediated NICD cleavage [157], demonstrating the impact of endocytosis on Notch signaling. Experiments using cell-free assays demonstrate part of Notch γ-secretase-dependent processing occurs in endocytosed vesicles, where variations in S3 cleavage site are found. Analyses on total levels of NICD1 demonstrate it is most efficiently cleaved at Ser1747, just outside the inner membrane leaflet. According to the N-terminal end rule [105] S1747 is less stable and therefore induces a much weaker activation of Notch target genes. NEXT isolation from different subcellular fractions revealed distinct NICD species in different locations. Plasma membrane tethered Notch is primarily cleaved at V1744, while in endosomal fractions the receptor is cleaved at S1747. Moreover, decreasing endocytosis rates by expressing Dynamin mutants showed a shift in cleavage from S1747 to V1744. One prediction from these results is that blocking endocytosis in receptor expressing cells, thereby enriching for the more stable and robust Val1744-NICD, causes a gain-of-function phenotype. Interestingly, Notch molecules harbouring S3 cleavage site mutations showed a different cleavage pattern; the previously described unprocessed V1744G mutant [53] appears to be cleaved mainly at L1745, leading to very weak Notch signaling. Importantly, the ubiquitination and endocytosis defective Notch1 K1749R mutant [157] is mainly cleaved at L1746, S1747, and at R1749. This in part explains the results obtained previously, the lack of Val1744-NICD detection in the Notch1 K1749R mutant, and the decrease observed in transcriptional activity of both Notch1 V1744G and K1749R mutants [158].

CONCLUSIONS AND PERSPECTIVES

The highly conserved Notch signaling pathway controls developmental patterning and homeostasis in animal tissues. The last decades of research in the Notch signaling pathway has provided a general consensus about receptor activation. Three evolutionary conserved cleavage events control the proper assembly and activation of the receptor leading to signaling.

The maturated Notch receptor is cleaved at S1 by furin-like convertases. Although there is some debate about activation of unprocessed Notch, the heterodimeric receptor appears to form the majority of the cell surface receptor molecules in most organisms. Possibly during embryogenesis unprocessed CSL-independent Notch signaling can be of importance in certain stages of development for a defined period. Unfortunately, no information is available on the requirement of furin for Notch3 and Notch4 signaling although they are likely processed in a similar manner [29].

The S2 cleavage appears to be necessary for Notch activation in all organisms but has still some unanswered questions. Both ADAM10 and ADAM17 have roles in this cleavage event, probably acting distinct in Notch S2 cleavage depending on structural differences. However, in the absence of both ADAM10 and 17 residual activity is observed, indicating a different, unknown protease activity. Future research should address the role of the contribution of the unknown protease in Notch activation.

The identification of the presenilin-dependent γ-secretase leading to the release of NICD by an intramembrane cleavage event seems to be unique, no other protease is capable of fully taking over Notch activation. However, some Notch activating processes are observed in the absence of both mammalian PS1 and PS2. Possibly this could be the same activation of non-furin processed Notch described above, not depending on any cleavage at all. Hence the furin- and PS-independent activation of Notch remains an obscure and therefore interesting question to be answered. In addition, the composition of the γ-secretase complex is a subject of thorough investigation. Perhaps different γ-secretase compositions act in different subcellular locations cleaving Notch at various positions, creating a large variety of short- and long-lasting Notch signals during several stages of development. The identification of these substrate and cell type specific activities will have profound influence on targeting γ-secretase to specific substrates in diseases.

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REFERENCES

[1]. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science. 1999; 284:770–6. [PubMed: 10221902]

[2]. Gazave E, Lapebie P, Richards GS, et al. Origin and evolution of the Notch signalling pathway: an overview from eukaryotic genomes. BMC Evol Biol. 2009; 9:249. [PubMed: 19825158]

[3]. Wilson A, Radtke F. Multiple functions of Notch signaling in self-renewing organs and cancer. FEBS Lett. 2006; 580:2860–8. [PubMed: 16574107]

[4]. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer. 2003; 3:756–67. [PubMed: 14570040]

[5]. van Es JH, van Gijn ME, Riccio O, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature. 2005; 435:959–63. [PubMed: 15959515]

[6]. Vooijs M, Ong CT, Hadland B, et al. Mapping the consequence of Notch1 proteolysis *in vivo* with NIP-CRE. Development. 2007; 134:535–44. [PubMed: 17215306]

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[7]. Kimble J, Simpson P. The LIN-12/Notch signaling pathway and its regulation. Annu Rev Cell Dev Biol. 1997; 13:333–61. [PubMed: 9442877]

[8]. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell. 2009; 137:216–33. [PubMed: 19379690]

[9]. Moloney DJ, Panin VM, Johnston SH, et al. Fringe is a glycosyltransferase that modifies Notch. Nature. 2000; 406:369–75. [PubMed: 10935626]

[10]. Okajima T, Irvine KD. Regulation of notch signaling by o-linked fucose. Cell. 2002; 111:893–904. [PubMed: 12526814]

[11]. Panin VM, Papayannopoulos V, Wilson R, Irvine KD. Fringe modulates Notch-ligand interactions. Nature. 1997; 387:908–12. [PubMed: 9202123]

[12]. Haines N, Irvine KD. Glycosylation regulates Notch signalling. Nat Rev Mol Cell Biol. 2003; 4:786–97. [PubMed: 14570055]

[13]. Christensen S, Kodoyianni V, Bosenberg M, Friedman L, Kimble J. lag-1, a gene required for lin-12 and glp-1 signaling in Caenorhabditis elegans, is homologous to human CBF1 and Drosophila Su(H). Development. 1996; 122:1373–83. [PubMed: 8625826]

[14]. Fortini ME, Artavanis-Tsakonas S. The suppressor of hairless protein participates in notch receptor signaling. Cell. 1994; 79:273–82. [PubMed: 7954795]

[15]. Fryer CJ, Lamar E, Turbachova I, Kintner C, Jones KA. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. Genes Dev. 2002; 16:1397–411. [PubMed: 12050117]

[16]. Schroeter EH, Kisslinger JA, Kopan R. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature. 1998; 393:382–6. [PubMed: 9620803]

[17]. Stifani S, Blaumueller CM, Redhead NJ, Hill RE, Artavanis-Tsakonas S. Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. Nat Genet. 1992; 2:343. [PubMed: 1303292]

[18]. Gordon WR, Vardar-Ulu D, Histen G, et al. Structural basis for autoinhibition of Notch. Nat Struct Mol Biol. 2007; 14:295–300. [PubMed: 17401372]

[19]. van Tetering G, van Diest P, Verlaan I, et al. Metalloprotease ADAM10 is required for Notch1 site 2 cleavage. J Biol Chem. 2009; 284:31018–27. [PubMed: 19726682]

[20]. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004; 306:269–71. [PubMed: 15472075]

[21]. Blaumueller CM, Qi H, Zagouras P, Artavanis-Tsakonas S. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell. 1997; 90:281–91. [PubMed: 9244302]

[22]. Kopan R, Schroeter EH, Weintraub H, Nye JS. Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. Proc Natl Acad Sci USA. 1996; 93:1683–8. [PubMed: 8643690]

[23]. Logeat F, Bessia C, Brou C, et al. The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc Natl Acad Sci USA. 1998; 95:8108–12. [PubMed: 9653148]

[24]. Aster J, Pear W, Hasserjian R, et al. Functional analysis of the TAN-1 gene, a human homolog of Drosophila notch. Cold Spring Harb Symp Quant Biol. 1994; 59:125–36. [PubMed: 7587062]

[25]. Crittenden SL, Troemel ER, Evans TC, Kimble J. GLP-1 is localized to the mitotic region of the C. elegans germ line. Development. 1994; 120:2901–11. [PubMed: 7607080]

[26]. Bush G, diSibio G, Miyamoto A, et al. Ligand-induced signaling in the absence of furin processing of Notch1. Dev Biol. 2001; 229:494–502. [PubMed: 11150244]

[27]. Kidd S, Lieber T. Furin cleavage is not a requirement for Drosophila Notch function. Mech Dev. 2002; 115:41–51. [PubMed: 12049766]

[28]. Lake RJ, Grimm LM, Veraksa A, Banos A, Artavanis-Tsakonas S. *In vivo* analysis of the Notch receptor S1 cleavage. PLoS One. 2009; 4:e6728. [PubMed: 19701455]

[29]. Gordon WR, Vardar-Ulu D, L'Heureux S, et al. Effects of S1 cleavage on the structure, surface export, and signaling activity of human Notch1 and Notch2. PLoS One. 2009; 4:e6613. [PubMed: 19701457]

[30]. Watanabe K, Nagaoka T, Lee JM, et al. Enhancement of Notch receptor maturation and signaling sensitivity by Cripto-1. J Cell Biol. 2009; 187:343–53. [PubMed: 19948478]

[31]. Brou C, Logeat F, Gupta N, et al. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. Mol Cell. 2000; 5:207–16. [PubMed: 10882063]

[32]. Mumm JS, Schroeter EH, Saxena MT, et al. A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. Mol Cell. 2000; 5:197–206. [PubMed: 10882062]

[33]. Parks AL, Klueg KM, Stout JR, Muskavitch MA. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development. 2000; 127:1373–85. [PubMed: 10704384]

[34]. Georgiadis D, Yiotakis A. Specific targeting of metzincin family members with small-molecule inhibitors: progress toward a multifarious challenge. Bioorg Med Chem. 2008; 16:8781–94. [PubMed: 18790648]

[35]. Gomez-del Arco P, Kashiwagi M, Jackson AF, et al. Alternative promoter usage at the Notch1 locus supports ligand-independent signaling in T cell development and leukemogenesis. Immunity. 2010; 33:685–98. [PubMed: 21093322]

[36]. Pan D, Rubin GM. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis. Cell. 1997; 90:271–80. [PubMed: 9244301]

[37]. Sotillos S, Roch F, Campuzano S. The metalloprotease-disintegrin Kuzbanian participates in Notch activation during growth and patterning of Drosophila imaginal discs. Development. 1997; 124:4769–79. [PubMed: 9428413]

[38]. Wen C, Metzstein MM, Greenwald I. SUP-17, a Caenorhabditis elegans ADAM protein related to Drosophila KUZBANIAN, and its role in LIN-12/NOTCH signalling. Development. 1997; 124:4759–67. [PubMed: 9428412]

[39]. Rooke J, Pan D, Xu T, Rubin GM. KUZ, a conserved metalloprotease-disintegrin protein with two roles in Drosophila neurogenesis. Science. 1996; 273:1227–31. [PubMed: 8703057]

[40]. Fambrough D, Pan D, Rubin GM, Goodman CS. The cell surface metalloprotease/disintegrin Kuzbanian is required for axonal extension in Drosophila. Proc Natl Acad Sci USA. 1996; 93:13233–8. [PubMed: 8917574]

[41]. Hattori M, Osterfield M, Flanagan JG. Regulated cleavage of a contact-mediated axon repellent. Science. 2000; 289:1360–5. [PubMed: 10958785]

[42]. Jarriault S, Greenwald I. Evidence for functional redundancy between C. elegans ADAM proteins SUP-17/Kuzbanian and ADM-4/TACE. Dev Biol. 2005; 287:1–10. [PubMed: 16197940]

[43]. Qi H, Rand MD, Wu X, et al. Processing of the notch ligand delta by the metalloprotease Kuzbanian. Science. 1999; 283:91–4. [PubMed: 9872749]

[44]. Varnum-Finney B, Wu L, Yu M, et al. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. J Cell Sci. 2000; 113(Pt 23):4313–8. [PubMed: 11069775]

[45]. Mishra-Gorur K, Rand MD, Perez-Villamil B, Artavanis-Tsakonas S. Down-regulation of Delta by proteolytic processing. J Cell Biol. 2002; 159:313–24. [PubMed: 12403816]

[46]. LaVoie MJ, Selkoe DJ. The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. J Biol Chem. 2003; 278:34427–37. [PubMed: 12826675]

[47]. Six E, Ndiaye D, Laabi Y, et al. The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. Proc Natl Acad Sci USA. 2003; 100:7638–43. [PubMed: 12794186]

[48]. Fiuza UM, Klein T, Martinez Arias A, Hayward P. Mechanisms of ligand-mediated inhibition in Notch signaling activity in Drosophila. Dev Dyn. 2010; 239:798–805. [PubMed: 20063416]

[49]. Sapir A, Assa-Kunik E, Tsruya R, Schejter E, Shilo BZ. Unidirectional Notch signaling depends on continuous cleavage of Delta. Development. 2005; 132:123–32. [PubMed: 15576412]

[50]. Vooijs M, Schroeter EH, Pan Y, Blandford M, Kopan R. Ectodomain shedding and intramembrane cleavage of mammalian Notch proteins is not regulated through oligomerization. J Biol Chem. 2004; 279:50864–73. [PubMed: 15448134]

[51]. Lieber T, Kidd S, Young MW. kuzbanian-mediated cleavage of Drosophila Notch. Genes Dev. 2002; 16:209–21. [PubMed: 11799064]

[52]. Conlon RA, Reaume AG, Rossant J. Notch1 is required for the coordinate segmentation of somites. Development. 1995; 121:1533–45. [PubMed: 7789282]

[53]. Huppert SS, Le A, Schroeter EH, et al. Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. Nature. 2000; 405:966–70. [PubMed: 10879540]

[54]. Swiatek PJ, Lindsell CE, del Amo FF, Weinmaster G, Gridley T. Notch1 is essential for postimplantation development in mice. Genes Dev. 1994; 8:707–19. [PubMed: 7926761]

[55]. Radtke F, Wilson A, Stark G, et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity. 1999; 10:547–58. [PubMed: 10367900]

[56]. Peschon JJ, Slack JL, Reddy P, et al. An essential role for ectodomain shedding in mammalian development. Science. 1998; 282:1281–4. [PubMed: 9812885]

[57]. Hartmann D, de Strooper B, Serneels L, et al. The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum Mol Genet. 2002; 11:2615–24. [PubMed: 12354787]

[58]. Manilay JO, Anderson AC, Kang C, Robey EA. Impairment of thymocyte development by dominant-negative Kuzbanian (ADAM-10) is rescued by the Notch ligand, delta-1. J Immunol. 2005; 174:6732–41. [PubMed: 15905513]

[59]. Tian L, Wu X, Chi C, et al. ADAM10 is essential for proteolytic activation of Notch during thymocyte development. Int Immunol. 2008; 20:1181–7. [PubMed: 18635581]

[60]. Gibb DR, El Shikh M, Kang DJ, et al. ADAM10 is essential for Notch2-dependent marginal zone B cell development and CD23 cleavage *in vivo*. J Exp Med. 2010; 207:623–35. [PubMed: 20156974]

[61]. Jorissen E, Prox J, Bernreuther C, et al. The disintegrin/metalloproteinase ADAM10 is essential for the establishment of the brain cortex. J Neurosci. 2010; 30:4833–44. [PubMed: 20371803]

[62]. Borrell-Pages M, Rojo F, Albanell J, Baselga J, Arribas J. TACE is required for the activation of the EGFR by TGF-alpha in tumors. EMBO J. 2003; 22:1114–24. [PubMed: 12606576]

[63]. Delwig A, Rand MD. Kuz and TACE can activate Notch independent of ligand. Cell Mol Life Sci. 2008; 65:2232–43. [PubMed: 18535782]

[64]. Tousseyn T, Thathiah A, Jorissen E, et al. ADAM10, the rate-limiting protease of regulated intramembrane proteolysis of Notch and other proteins, is processed by ADAMS-9, ADAMS-15, and the gamma-secretase. J Biol Chem. 2009; 284:11738–47. [PubMed: 19213735]

[65]. Nichols JT, Miyamoto A, Olsen SL, et al. DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. J Cell Biol. 2007; 176:445–58. [PubMed: 17296795]

[66]. Struhl G, Adachi A. Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. Mol Cell. 2000; 6:625–36. [PubMed: 11030342]

[67]. Seugnet L, Simpson P, Haenlin M. Requirement for dynamin during Notch signaling in Drosophila neurogenesis. Dev Biol. 1997; 192:585–98. [PubMed: 9441691]

[68]. Greenwald I. Structure/function studies of lin-12/Notch proteins. Curr Opin Genet Dev. 1994; 4:556–62. [PubMed: 7950324]

[69]. Lieber T, Kidd S, Alcamo E, Corbin V, Young MW. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. Genes Dev. 1993; 7:1949–65. [PubMed: 8406001]

[70]. Sanchez-Irizarry C, Carpenter AC, Weng AP, et al. Notch subunit heterodimerization and prevention of ligand-independent proteolytic activation depend, respectively, on a novel domain and the LNR repeats. Mol Cell Biol. 2004; 24:9265–73. [PubMed: 15485896]

[71]. Malecki MJ, Sanchez-Irizarry C, Mitchell JL, et al. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. Mol Cell Biol. 2006; 26:4642–51. [PubMed: 16738328]

[72]. Sulis ML, Williams O, Palomero T, et al. NOTCH1 extracellular juxtamembrane expansion mutations in T-ALL. Blood. 2008; 112:733–40. [PubMed: 18411416]

[73]. Bozkulak EC, Weinmaster G. Selective use of ADAM10 and ADAM17 in activation of Notch1 signaling. Mol Cell Biol. 2009; 29:5679–95. [PubMed: 19704010]

[74]. Asai M, Hattori C, Szabo B, et al. Putative function of ADAM9, ADAM10, and ADAM17 as APP alpha-secretase. Biochem Biophys Res Commun. 2003; 301:231–5. [PubMed: 12535668]

[75]. Dyczynska E, Sun D, Yi H, et al. Proteolytic processing of delta-like 1 by ADAM proteases. J Biol Chem. 2007; 282:436–44. [PubMed: 17107962]

[76]. Sawey ET, Johnson JA, Crawford HC. Matrix metalloproteinase 7 controls pancreatic acinar cell transdifferentiation by activating the Notch signaling pathway. Proc Natl Acad Sci USA. 2007; 104:19327–32. [PubMed: 18042722]

[77]. Saxena MT, Schroeter EH, Mumm JS, Kopan R. Murine notch homologs (N1-4) undergo presenilin-dependent proteolysis. J Biol Chem. 2001; 276:40268–73. [PubMed: 11518718]

[78]. Li K, Li Y, Wu W, et al. Modulation of Notch signaling by antibodies specific for the extracellular negative regulatory region of NOTCH3. J Biol Chem. 2008; 283:8046–54. [PubMed: 18182388]

[79]. Petcherski AG, Kimble J. Mastermind is a putative activator for Notch. Curr Biol. 2000; 10:R471–3. [PubMed: 10898989]

[80]. Tamura K, Taniguchi Y, Minoguchi S, et al. Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). Curr Biol. 1995; 5:1416–23. [PubMed: 8749394]

[81]. Jarriault S, Brou C, Logeat F, et al. Signalling downstream of activated mammalian Notch. Nature. 1995; 377:355–8. [PubMed: 7566092]

[82]. Barrick D, Kopan R. The Notch transcription activation complex makes its move. Cell. 2006; 124:883–5. [PubMed: 16530033]

[83]. Lubman OY, Korolev SV, Kopan R. Anchoring notch genetics and biochemistry; structural analysis of the ankyrin domain sheds light on existing data. Mol Cell. 2004; 13:619–26. [PubMed: 15023333]

[84]. Wilson JJ, Kovall RA. Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA. Cell. 2006; 124:985–96. [PubMed: 16530045]

[85]. Nam Y, Sliz P, Song L, Aster JC, Blacklow SC. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. Cell. 2006; 124:973–83. [PubMed: 16530044]

[86]. Kurooka H, Kuroda K, Honjo T. Roles of the ankyrin repeats and C-terminal region of the mouse notch1 intracellular region. Nucleic Acids Res. 1998; 26:5448–55. [PubMed: 9826771]

[87]. Kato H, Taniguchi Y, Kurooka H, et al. Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. Development. 1997; 124:4133–41. [PubMed: 9374409]

[88]. Aster JC, Xu L, Karnell FG, et al. Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1. Mol Cell Biol. 2000; 20:7505–15. [PubMed: 11003647]

[89]. Struhl G, Adachi A. Nuclear access and action of notch *in vivo*. Cell. 1998; 93:649–60. [PubMed: 9604939]

[90]. Sanalkumar R, Dhanesh SB, James J. Non-canonical activation of Notch signaling/target genes in vertebrates. Cell Mol Life Sci. 2010; 67:2957–68. [PubMed: 20458516]

[91]. Fortini ME, Rebay I, Caron LA, Artavanis-Tsakonas S. An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. Nature. 1993; 365:555–7. [PubMed: 8413612]

[92]. Struhl G, Fitzgerald K, Greenwald I. Intrinsic activity of the Lin-12 and Notch intracellular domains *in vivo*. Cell. 1993; 74:331–45. [PubMed: 8343960]

[93]. Kopan R, Nye JS, Weintraub H. The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development. 1994; 120:2385–96. [PubMed: 7956819]

[94]. Coffman CR, Skoglund P, Harris WA, Kintner CR. Expression of an extracellular deletion of Xotch diverts cell fate in Xenopus embryos. Cell. 1993; 73:659–71. [PubMed: 8500162]

Europe PMC Funders Author Manuscripts

[95]. Rebay I, Fehon RG, Artavanis-Tsakonas S. Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell. 1993; 74:319–29. [PubMed: 8343959]

[96]. Pear WS, Aster JC, Scott ML, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J Exp Med. 1996; 183:2283–91. [PubMed: 8642337]

[97]. Shawber C, Nofziger D, Hsieh JJ, et al. Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. Development. 1996; 122:3765–73. [PubMed: 9012498]

[98]. Lecourtois M, Schweisguth F. Indirect evidence for Delta-dependent intracellular processing of notch in Drosophila embryos. Curr Biol. 1998; 8:771–4. [PubMed: 9651681]

[99]. Kidd S, Lieber T, Young MW. Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos. Genes Dev. 1998; 12:3728–40. [PubMed: 9851979]

[100]. Struhl G, Greenwald I. Presenilin is required for activity and nuclear access of Notch in Drosophila. Nature. 1999; 398:522–5. [PubMed: 10206646]

[101]. Tokunaga A, Kohyama J, Yoshida T, et al. Mapping spatio-temporal activation of Notch signaling during neurogenesis and gliogenesis in the developing mouse brain. J Neurochem. 2004; 90:142–54. [PubMed: 15198674]

[102]. Cheng HT, Kim M, Valerius MT, et al. Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. Development. 2007; 134:801–11. [PubMed: 17229764]

[103]. Pan Y, Lin MH, Tian X, et al. gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. Dev Cell. 2004; 7:731–43. [PubMed: 15525534]

[104]. Ong CT, Cheng HT, Chang LW, et al. Target selectivity of vertebrate notch proteins. Collaboration between discrete domains and CSL-binding site architecture determines activation probability. J Biol Chem. 2006; 281:5106–19. [PubMed: 16365048]

[105]. Varshavsky A. The N-end rule pathway of protein degradation. Genes Cells. 1997; 2:13–28. [PubMed: 9112437]

[106]. Blat Y, Meredith JE, Wang Q, et al. Mutations at the P1’ position of Notch1 decrease intracellular domain stability rather than cleavage by gamma-secretase. Biochem Biophys Res Commun. 2002; 299:569–73. [PubMed: 12459176]

[107]. Chandu D, Huppert SS, Kopan R. Analysis of transmembrane domain mutants is consistent with sequential cleavage of Notch by gamma-secretase. J Neurochem. 2006; 96:228–35. [PubMed: 16300632]

[108]. Kelly DF, Lake RJ, Middelkoop TC, et al. Molecular structure and dimeric organization of the Notch extracellular domain as revealed by electron microscopy. PLoS One. 2010; 5:e10532. [PubMed: 20479883]

[109]. Richter L, Munter LM, Ness J, et al. Amyloid beta 42 peptide (Abeta42)-lowering compounds directly bind to Abeta and interfere with amyloid precursor protein (APP) transmembrane dimerization. Proc Natl Acad Sci USA. 2010; 107:14597–602. [PubMed: 20679249]

[110]. Liu H, Chi AW, Arnett KL, et al. Notch dimerization is required for leukemogenesis and T-cell development. Genes Dev. 2010; 24:2395–407. [PubMed: 20935071]

[111]. Mizutani T, Taniguchi Y, Aoki T, Hashimoto N, Honjo T. Conservation of the biochemical mechanisms of signal transduction among mammalian Notch family members. Proc Natl Acad Sci USA. 2001; 98:9026–31. [PubMed: 11459941]

[112]. Levitan D, Greenwald I. Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer’s disease gene. Nature. 1995; 377:351–4. [PubMed: 7566091]

[113]. Li X, Greenwald I. HOP-1, a Caenorhabditis elegans presenilin, appears to be functionally redundant with SEL-12 presenilin and to facilitate LIN-12 and GLP-1 signaling. Proc Natl Acad Sci USA. 1997; 94:12204–9. [PubMed: 9342387]

[114]. Levitan D, Greenwald I. Effects of SEL-12 presenilin on LIN-12 localization and function in Caenorhabditis elegans. Development. 1998; 125:3599–606. [PubMed: 9716525]

[115]. Shen J, Bronson RT, Chen DF, et al. Skeletal and CNS defects in Presenilin-1-deficient mice. Cell. 1997; 89:629–39. [PubMed: 9160754]

Europe PMC Funders Author Manuscripts

[116]. Wong PC, Zheng H, Chen H, et al. Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. Nature. 1997; 387:288–92. [PubMed: 9153393]

[117]. Herreman A, Hartmann D, Annaert W, et al. Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. Proc Natl Acad Sci USA. 1999; 96:11872–7. [PubMed: 10518543]

[118]. Herreman A, Serneels L, Annaert W, et al. Total inactivation of gamma-secretase activity in presenilin-deficient embryonic stem cells. Nat Cell Biol. 2000; 2:461–2. [PubMed: 10878813]

[119]. Rozmahel R, Huang J, Chen F, et al. Normal brain development in PS1 hypomorphic mice with markedly reduced gamma-secretase cleavage of betaAPP. Neurobiol Aging. 2002; 23:187–94. [PubMed: 11804702]

[120]. Rozmahel R, Mount HT, Chen F, et al. Alleles at the Nicastrin locus modify presenilin 1-deficiency phenotype. Proc Natl Acad Sci USA. 2002; 99:14452–7. [PubMed: 12388777]

[121]. Mastrangelo P, Mathews PM, Chishti MA, et al. Dissociated phenotypes in presenilin transgenic mice define functionally distinct gamma-secretases. Proc Natl Acad Sci USA. 2005; 102:8972–7. [PubMed: 15951428]

[122]. Netzer WJ, Dou F, Cai D, et al. Gleevec inhibits beta-amyloid production but not Notch cleavage. Proc Natl Acad Sci USA. 2003; 100:12444–9. [PubMed: 14523244]

[123]. He G, Luo W, Li P, et al. Gamma-secretase activating protein is a therapeutic target for Alzheimer's disease. Nature. 2010; 467:95–8. [PubMed: 20811458]

[124]. Takahashi Y, Hayashi I, Tominari Y, et al. Sulindac sulfide is a noncompetitive gamma-secretase inhibitor that preferentially reduces Abeta 42 generation. J Biol Chem. 2003; 278:18664–70. [PubMed: 12637581]

[125]. Weggen S, Eriksen JL, Das P, et al. A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature. 2001; 414:212–6. [PubMed: 11700559]

[126]. De Strooper B, Annaert W, Cupers P, et al. A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature. 1999; 398:518–22. [PubMed: 10206645]

[127]. De Strooper B, Saftig P, Craessaerts K, et al. Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature. 1998; 391:387–90. [PubMed: 9450754]

[128]. Ray WJ, Yao M, Nowotny P, et al. Evidence for a physical interaction between presenilin and Notch. Proc Natl Acad Sci USA. 1999; 96:3263–8. [PubMed: 10077672]

[129]. Ye Y, Lukinova N, Fortini ME. Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants. Nature. 1999; 398:525–9. [PubMed: 10206647]

[130]. Wolfe MS, Xia W, Ostaszewski BL, et al. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature. 1999; 398:513–7. [PubMed: 10206644]

[131]. Ray WJ, Yao M, Mumm J, et al. Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch. J Biol Chem. 1999; 274:36801–7. [PubMed: 10593990]

[132]. Podlisny MB, Citron M, Amarante P, et al. Presenilin proteins undergo heterogeneous endoproteolysis between Thr291 and Ala299 and occur as stable N- and C-terminal fragments in normal and Alzheimer brain tissue. Neurobiol Dis. 1997; 3:325–37. [PubMed: 9173929]

[133]. Thinakaran G, Borchelt DR, Lee MK, et al. Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. Neuron. 1996; 17:181–90. [PubMed: 8755489]

[134]. Levitan D, Lee J, Song L, et al. PS1 N- and C-terminal fragments form a complex that functions in APP processing and Notch signaling. Proc Natl Acad Sci USA. 2001; 98:12186–90. [PubMed: 11593035]

[135]. Yu G, Nishimura M, Arawaka S, et al. Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. Nature. 2000; 407:48–54. [PubMed: 10993067]

[136]. Chen F, Yu G, Arawaka S, et al. Nicastrin binds to membrane-tethered Notch. Nat Cell Biol. 2001; 3:751–4. [PubMed: 11483961]

[137]. Shirotani K, Edbauer D, Prokop S, Haass C, Steiner H. Identification of distinct gamma-secretase complexes with different APH-1 variants. J Biol Chem. 2004; 279:41340–5. [PubMed: 15286082]

Europe PMC Funders Author Manuscripts

[138]. Goutte C, Tsunozaki M, Hale VA, Priess JR. APH-1 is a multipass membrane protein essential for the Notch signaling pathway in Caenorhabditis elegans embryos. Proc Natl Acad Sci USA. 2002; 99:775–9. [PubMed: 11792846]

[139]. Francis R, McGrath G, Zhang J, et al. aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. Dev Cell. 2002; 3:85–97. [PubMed: 12110170]

[140]. Esler WP, Kimberly WT, Ostaszewski BL, et al. Activity-dependent isolation of the presenilin-gamma -secretase complex reveals nicastrin and a gamma substrate. Proc Natl Acad Sci USA. 2002; 99:2720–5. [PubMed: 11867728]

[141]. Edbauer D, Winkler E, Regula JT, et al. Reconstitution of gamma-secretase activity. Nat Cell Biol. 2003; 5:486–8. [PubMed: 12679784]

[142]. Kimberly WT, LaVoie MJ, Ostaszewski BL, et al. Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. Proc Natl Acad Sci USA. 2003; 100:6382–7. [PubMed: 12740439]

[143]. Shah S, Lee SF, Tabuchi K, et al. Nicastrin functions as a gamma-secretase-substrate receptor. Cell. 2005; 122:435–47. [PubMed: 16096062]

[144]. Zhao G, Liu Z, Ilagan MX, Kopan R. Gamma-secretase composed of PS1/Pen2/Aph1a can cleave notch and amyloid precursor protein in the absence of nicastrin. J Neurosci. 2010; 30:1648–56. [PubMed: 20130175]

[145]. Hebert SS, Serneels L, Dejaegere T, et al. Coordinated and widespread expression of gamma-secretase *in vivo*: evidence for size and molecular heterogeneity. Neurobiol Dis. 2004; 17:260–72. [PubMed: 15474363]

[146]. Dejaegere T, Serneels L, Schafer MK, et al. Deficiency of Aph1B/C-gamma-secretase disturbs Nrg1 cleavage and sensorimotor gating that can be reversed with antipsychotic treatment. Proc Natl Acad Sci USA. 2008; 105:9775–80. [PubMed: 18626010]

[147]. Ma G, Li T, Price DL, Wong PC. APH-1a is the principal mammalian APH-1 isoform present in gamma-secretase complexes during embryonic development. J Neurosci. 2005; 25:192–8. [PubMed: 15634781]

[148]. Serneels L, Dejaegere T, Craessaerts K, et al. Differential contribution of the three Aph1 genes to gamma-secretase activity *in vivo*. Proc Natl Acad Sci USA. 2005; 102:1719–24. [PubMed: 15665098]

[149]. Sato T, Diehl TS, Narayanan S, et al. Active gamma-secretase complexes contain only one of each component. J Biol Chem. 2007; 282:33985–93. [PubMed: 17911105]

[150]. Takasugi N, Tomita T, Hayashi I, et al. The role of presenilin cofactors in the gamma-secretase complex. Nature. 2003; 422:438–41. [PubMed: 12660785]

[151]. Ahn K, Shelton CC, Tian Y, et al. Activation and intrinsic {gamma}-secretase activity of presenilin 1. Proc Natl Acad Sci USA. 2010; 107:21435–40. [PubMed: 21115843]

[152]. De Strooper B, Annaert W. Novel research horizons for presenilins and gamma-secretases in cell biology and disease. Annu Rev Cell Dev Biol. 2010; 26:235–60. [PubMed: 20604710]

[153]. Berechid BE, Kitzmann M, Foltz DR, et al. Identification and characterization of presenilin-independent Notch signaling. J Biol Chem. 2002; 277:8154–65. [PubMed: 11756438]

[154]. Huppert SS, Ilagan MX, De Strooper B, Kopan R. Analysis of Notch function in presomitic mesoderm suggests a gamma-secretase-independent role for presenilins in somite differentiation. Dev Cell. 2005; 8:677–88. [PubMed: 15866159]

[155]. Okochi M, Steiner H, Fukumori A, et al. Presenilins mediate a dual intramembranous gamma-secretase cleavage of Notch-1. EMBO J. 2002; 21:5408–16. [PubMed: 12374741]

[156]. Tanii H, Jiang J, Fukumori A, et al. Effect of valine on the efficiency and precision at S4 cleavage of the Notch-1 transmembrane domain. J Neurosci Res. 2006; 84:918–25. [PubMed: 16802341]

[157]. Gupta-Rossi N, Six E, LeBail O, et al. Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. J Cell Biol. 2004; 166:73–83. [PubMed: 15240571]

[158]. Tagami S, Okochi M, Yanagida K, et al. Regulation of Notch signaling by dynamic changes in the precision of S3 cleavage of Notch-1. Mol Cell Biol. 2008; 28:165–76. [PubMed: 17967888]

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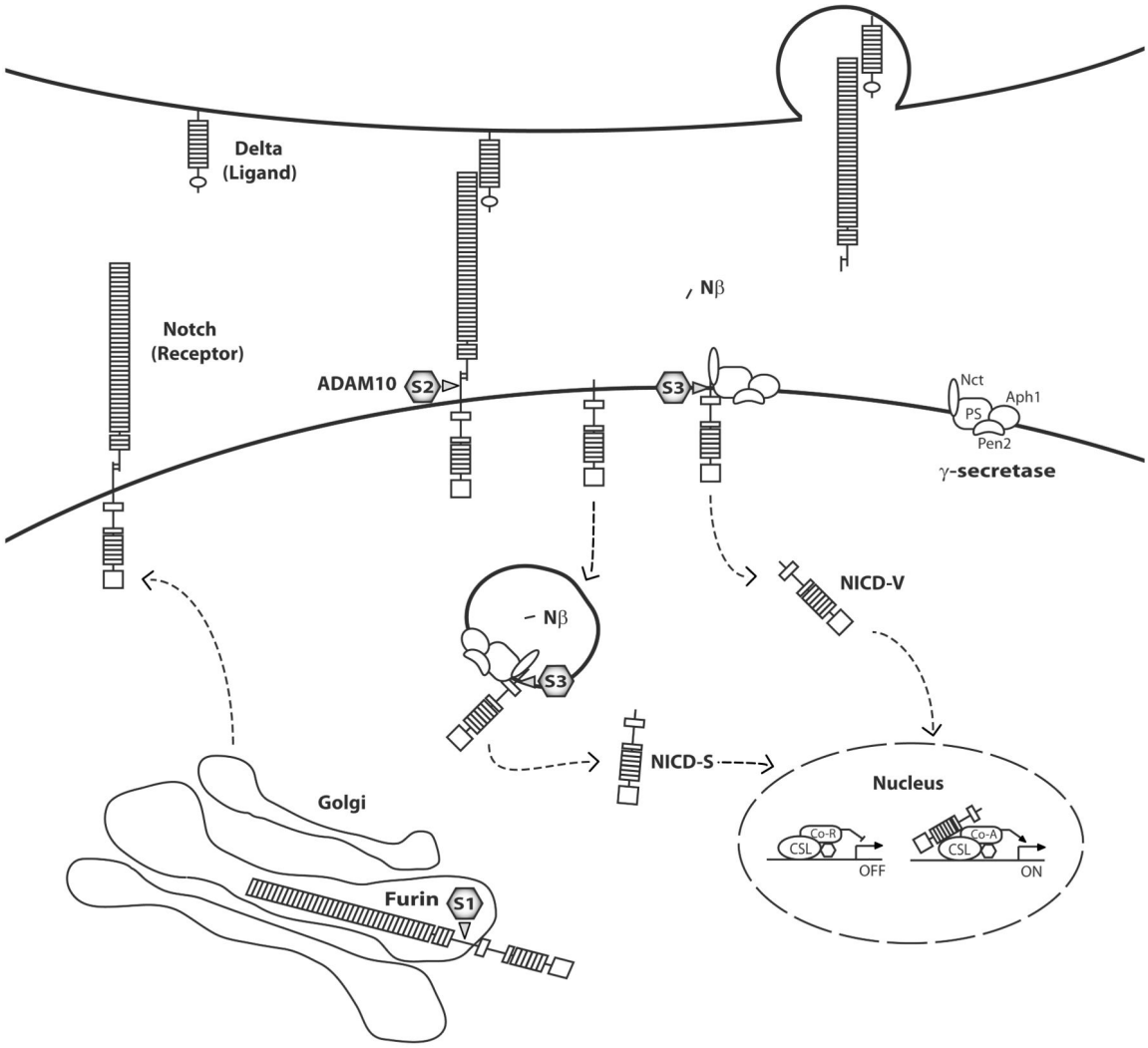

Fig. (1). Overview of Notch proteolysis

A schematic overview of the Notch signaling pathway showing the proteolytic events. During maturation and transport of the receptor to the membrane Notch gets cleaved in the Golgi system at Site-1 (S1) by a furin-like convertase, resulting in a heterodimeric transmembrane receptor. At the cell surface in the absence of ligand the receptor is proteolysis-resistant. Only upon binding of ligand, inducing a substantial conformational change, the metalloprotease ADAM10 is able to cleave Notch at the newly exposed Site-2 (S2). This sheds off the NECD which can be transendocytosed into the ligand-expressing cell. On the receptor-expressing cell S2 cleavage results in a NEXT fragment that either is directly cleaved at the membrane at Val1744 (NICD-V) by the γ-secretase complex, or NEXT is internalized and processed in endocytic compartments by γ-secretase at Ser1747 (NICD-S). NICD translocates to the nucleus where it participates in a co-activator complex binding to CSL and starts target gene transcription. An additional cleavage at Site-4 (S4) is mediated by the γ-secretase in the centre of the transmembrane domain resulting in the N fragment, probably meant to clear residual Notch fragments from the membrane.

Fig. (2). Partial alignments of the different cleavage regions

Sequences from Notch receptors of fly, worm, rodent and human are shown. *Upper panel:* The S1 cleavage region shows low conservation. Yet, in every species possible furin sites could be identified. *Middle panel:* The juxtamembrane region containing the S2 cleavage site shows a high conservation among mammalian receptors that all show Valine residues on the proposed S2 cleavages sites. *Lower panel:* Transmembrane domains of the various receptors show high conservation, in particular mammalian receptors that all show a Valine residue close to the inner leaflet of the plasma membrane. Yet, also worm and fly Notch show conservation of this Valine. Underlined sequences are previously described cleavage sites.