

A Notch updated

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Cell-cell signaling mediated by the Notch receptor is iteratively involved in numerous developmental contexts, and its dysregulation has been associated with inherited genetic disorders and cancers. The core components of the signaling pathway have been identified for some time, but the study of the modulation of the pathway in different cellular contexts has revealed many layers of regulation. These include complex sugar modifications in the extracellular domain as well as transit of Notch through defined cellular compartments, including specific endosomes.

Notch signaling is an evolutionary conserved signaling pathway that is involved in a wide variety of developmental processes, including adult homeostasis and stem cell maintenance (Artavanis-Tsakonas et al., 1999; Lai, 2004; Le Borgne et al., 2005a; Bray, 2006). Loss of function of components of this pathway causes inherited genetic diseases such as Alagille syndrome, spondylocostal dysostosis (SCD), and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; Gridley, 2003), whereas up-regulation of Notch activity has been associated with T cell acute lymphatic leukemia (T-ALL; Jundt et al., 2008). Signaling occurs when the ligands DSL (Delta, Serrate, Lag2) bind and interact with Notch, thereby inducing a series of cleavages named S2, S3, and S4 (Fig. 1). The S2 cleavage is mediated by ADAM/TACE metalloproteases (Brou et al., 2000; Lieber et al., 2002), whereas the S3/4 cleavage is an intramembranous cleavage mediated by the presenilin-dependent γ -secretase (De Strooper et al., 1999; Struhl and Greenwald, 1999; Okochi et al., 2002), resulting in the translocation of the intracellular domain of Notch (NICD) into the nucleus. Nuclear NICD then interacts with a transcriptional factor CSL (CBF1/RBPJK in mammals, Su(H) in flies, and LAG-1 in worms) to activate downstream target genes

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Abbreviations used in this paper: Avl, Avalanche; Bib, big brain; CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; Dx, Deltex; ECD, extracellular domain; EE, early endosome; GOF, gain of function; HD, heterodimer domain; Lfng, Lunatic fringe; Lgd, lethal giant discs; MVB, multivesicular body; NECD, Notch extracellular domain; NEXT, Notch external truncation; NICD, Notch intracellular domain; SCD, spondylocostal dysostosis; T-ALL, T cell acute lymphatic leukemia.

(Artavanis-Tsakonas et al., 1999; Lai, 2004; Le Borgne et al., 2005a; Bray, 2006). In mammals, there are four Notch proteins (Notch 1–4), three Delta-like proteins (Dll 1, 2 and 4), and two Jagged proteins (Serrate in fly). Both the Delta-like and Jagged proteins have a DSL domain and EGF repeats. In addition, Jagged proteins have a cysteine-rich domain (Kiyota and Kinoshita, 2002). The Notch protein consists of an extracellular domain (ECD) with 36 EGF domains, a heterodimer domain (HD), and three LNR (Lin-12, Notch repeats) domains, followed by transmembrane domain, ankyrin repeats, and a PEST motif (Fig. 1 A). These domains of Notch provide a platform for modifications and specific regulatory events.

Notch signaling is an unusual signaling pathway, whose activity does not rely on secondary messengers for amplification. Rather, Notch signaling is modulated by glycosylation, differential intracellular trafficking, and ubiquitin-dependent degradation. In this review, we will discuss current findings related to the tuning of Notch activity at three different levels: the glycosylation events and their impact on Notch; the cleavage events required for ligand-dependent Notch activation and the mechanisms to prevent ligand-independent activation; and, finally, endosomal trafficking of Notch to regulate protein sorting, recycling, and degradation. The regulation of the DSL ligands through endocytosis has been recently summarized in other reviews (Le Borgne et al., 2005a; Nichols et al., 2007; D'Souza et al., 2008).

The disulfide bonds and glycosylation events influence Notch activity

The ECD of Notch modulates the ligand-dependent signaling potential of Notch, and some of the current research on the ECD can be divided into two main topics: (1) ligand binding and glycosylation, and (2) negative regulation of Notch signaling via the three LNRs and HD. Binding of Notch to its DSL ligands was first demonstrated by their ability to cause aggregation of *Drosophila melanogaster* S2 cells (Rebay et al., 1991). Based on deletion analyses and aggregation assays, EGF repeats 11–12 in Notch were shown to be necessary and sufficient to mediate DSL binding (Fig. 1). More recent studies invoke the help of EGF repeats 5–9 and 25–36 for this receptor–ligand interaction (Xu et al., 2005). The EGF-like domain contains six characteristic

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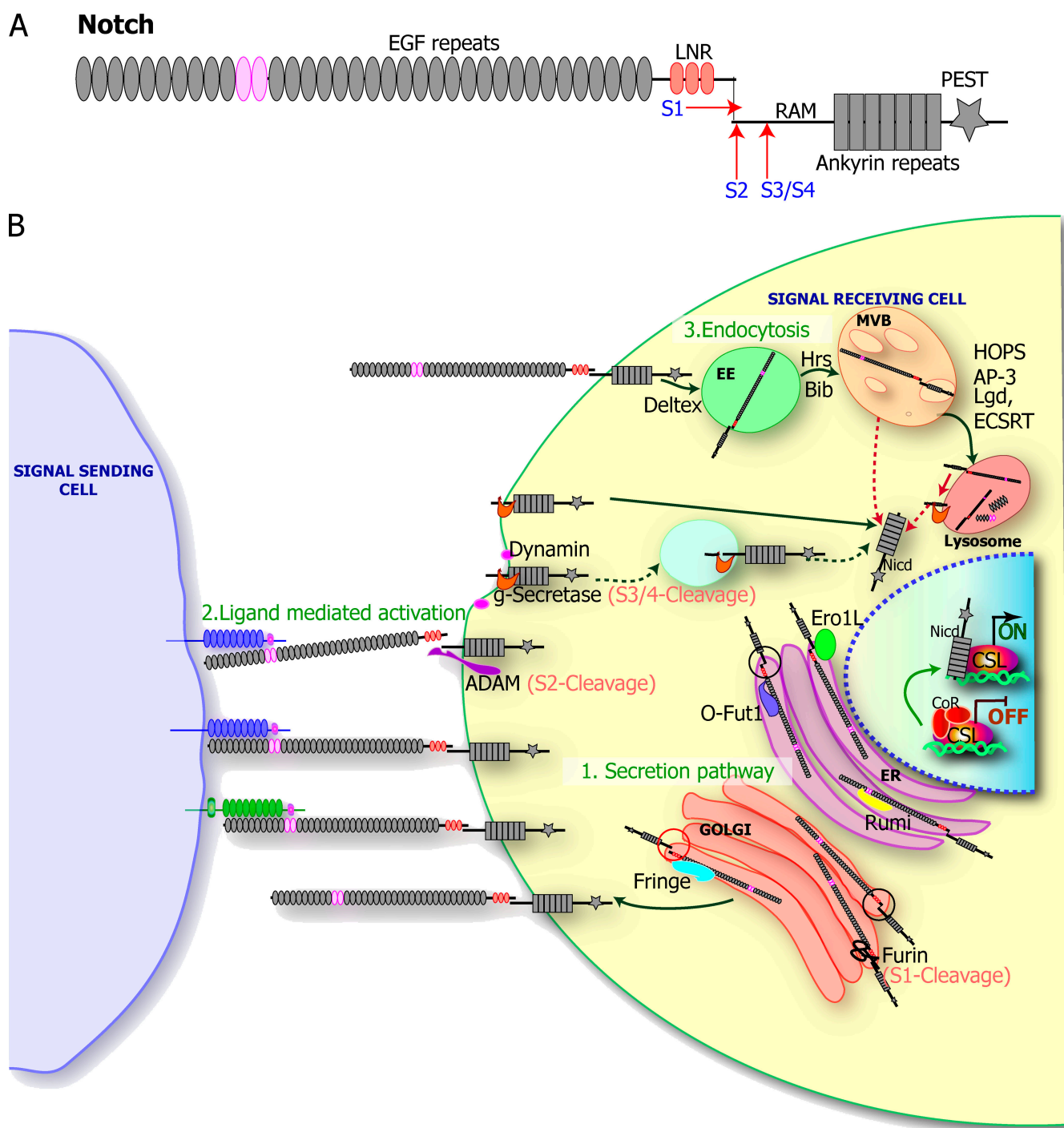


Figure 1. Schematic illustration of Notch and the pathway. (A) The N-terminal part of the Notch ectodomain consists of 36 EGF-like repeats (gray ovals) and three LNRs (lin-12 Notch repeat; orange ovals). EGF repeats 11 and 12 interact with the ligands (pink ovals). NICD consists of an N-terminal RAM (recombination binding protein-J-associated molecule) domain, an ankyrin (ANK) domain (gray rectangles), and less-conserved regions, including a variable transactivation domain and a C-terminal PEST sequence (gray star). The red arrows indicate the cleavage sites: S1 (Furin), S2 (ADAM metalloprotease), and S3/S4 (γ -secretase). (B, 1) Secretory pathway: the modifications during the secretion of Notch to the membrane in the ER (purple) and the Golgi (orange). Notch is translated inside ER, where it is glycosylated by an *O*-fucosyltransferase *O*-Fut1 (light purple) and *O*-glucosyltransferase Rumi (yellow). Note the black circle on the Notch molecule in the ER; because Notch is not cleaved, the extracellular and intracellular domains are physically linked. Notch is then translocated into Golgi, where it is cleaved by Furin protease (scissors) at the S1 site and further modified by the *N*-acetylglucosaminyltransferase, Fringe. Note the red circle on the Notch molecule in the Golgi after S1 cleavage; the extracellular and intracellular domains are not physically linked. (2) Ligand-mediated activation: Notch (gray) interacts with the DSL ligands, Delta (blue) and Serrate (green), resulting in a series of proteolytic cleavage events induced by ligand binding. The S2 cleavage is mediated by ADAM protease (purple), whereas the S3/4 cleavage event is mediated by γ -secretase (g-secretase, in orange). Several studies also suggest that γ -secretase-mediated cleavage can occur inside endocytic compartment (shown in the light blue circle). (3) The endocytic regulation of the Notch receptor: full-length Notch can undergo endocytosis, leading to translocation of Notch into EEs, MVB, and lysosomes. From genetic data, several proteins have been identified to modulate this process, including Hrs and Bib, possibly during the EE-to-MVB transition

cysteine residues that form three pairs of disulfide bonds (Fleming, 1998). The structural role of disulfide bonds in EGF repeats of Notch has been further highlighted because a human disease known as CADASIL has been associated with impaired disulfide bond formation in the EGF repeat of the NOTCH3 protein (Joutel et al., 1996; Gridley, 2003). CADASIL is an inherited dominant disease that is often associated with arteriopathy, subcortical ischemic strokes, and disability due to impairment in vascular smooth muscle function (Tournier-Lasserre et al., 1991). Most CADASIL patients have either a gain or a loss of 1–3 cysteine residues in the EGF repeats of NOTCH3 (Joutel et al., 1996), resulting in a gain or a loss of specific disulfide bonds. However recent studies have uncovered rare *NOTCH3* mutations that do not seem to involve cysteine residues in patients with CADASIL-like symptoms (Mizuno et al., 2008). Although many mutations have been documented, the molecular mechanism underlying the disease is poorly defined, and there is a debate as to whether CADASIL results from aberrant Notch signaling or protein accumulation (for reviews see Spinner, 2000; Fryxell et al., 2001).

In flies, there are a set of Notch mutations known as Abruptex alleles that are semidominant and are also associated with a gain or a loss of disulfide bond formation (Kelley et al., 1987; de Celis and Garcia-Bellido, 1994; Fryxell et al., 2001). Flies with Abruptex alleles often exhibit loss of wing veins and loss of bristles, phenotypes often associated with a gain of function of Notch activity (de Celis and Garcia-Bellido, 1994). How these alleles result in a hyperactivity of Notch is still under investigation. The mutations in the Abruptex alleles have been mapped to the EGF repeats 24–29 of Notch (Hartley et al., 1987; Kelley et al., 1987; de Celis and Bray, 2000). Interestingly, it has been recently shown that EGF 22–27 binds to the EGF 11–14 (Pei and Baker, 2008). Because EGF 11–14 can bind to both the ligands and EGF 22–27 intramolecularly, it is reasonable to suggest that EGF 22–27 normally interferes and competes with ligand binding, providing an added layer of regulation (Pei and Baker, 2008). Both CADASIL and the Abruptex alleles are specifically caused by unpaired cysteines within the EGF repeats of Notch. Hence, it has been suggested that *D. melanogaster* Abruptex mutations may serve as a model for studying CADASIL (for review see Fryxell et al., 2001), though this link needs to be interpreted with caution (for review see Louvi et al., 2006). Based on gain-of-function (GOF) phenotypes observed in Abruptex mutants, it is tempting to speculate that CADASIL mutations in human *NOTCH3* might result in a similar gain of function of NOTCH3. This hypothesis still awaits experimental validation.

The Notch extracellular domain (NECD) is heavily glycosylated, and the biological consequences of these modifications are slowly being unraveled. EGF repeats with the C²-X₄₋₅-T/S-C³ (Haines and Irvine, 2003) consensus sequence can be modified by *O*-fucosyltransferase, which adds fucose to the serine or threonine site. The *O*-fucose site of NECD can be further modified with *N*-glycans mediated by a β 1, 3-*N*-acetylglucosaminyl-

transferase, Fringe (Bruckner et al., 2000; Ju et al., 2000). During fly wing development, loss-of-function analyses of Fringe indicate that the sugar modification activity of Fringe promotes Notch's ability to bind Delta and negatively affects its interaction with Serrate (Panin et al., 1997). This finding is further strengthened by biochemical assays showing that Fringe-modified Notch exhibits higher binding affinity for Delta than Serrate (Lei et al., 2003). In addition, an evolutionary conserved *O*-fucose site at the Notch EGF repeat 12 contributes to decreasing the Notch–ligand interaction in both flies and mice (Lei et al., 2003; Ge and Stanley, 2008). In flies, a Notch protein with a mutation at this *O*-fucose site is ectopically activated by Serrate and overrides the regulation of Fringe in vivo, and this mutant also binds Serrate efficiently in biochemical assays in the presence of Fringe (Lei et al., 2003). Furthermore, a specific knockin mouse carrying an *O*-fucose mutant in *Notch1* EGF repeat 12 (*Notch1*^{12f}) has been created (Ge and Stanley, 2008). Transheterozygous mice carrying a *Notch1*^{12f} allele and a *Notch1* null allele exhibit embryonic lethality and defects similar to *Notch1* homozygous null embryos. However, homozygous *Notch1*^{12f} mice are viable with defects in T cell specification and functions. Importantly, thymocytes from these mutant mice show decreased binding capacity to Delta1-expressing cells. (Ge and Stanley, 2008). In addition, because other EGF repeats promote Notch–ligand interaction, other *O*-fucosylation sites on EGF repeats 26 and 27 have been reported to modulate the binding affinity of Notch for its ligands (Xu et al., 2005). It is thought that *O*-fucosylation of multiple EGFs may affect the local folding of EGFs and contribute to ligand binding. In addition to Fringe-dependent glycosylation, modifications by other glycosyltransferases after adding the *N*-glycan at the *O*-fucose sites have been identified (Moloney et al., 2000). However, in vitro purified Notch without these modifications is still able to bind their ligands very efficiently (Xu et al., 2007), which suggests that these modifications contribute minimally to modulating Notch–ligand interaction.

The role of *O*-fucosylation and Fringe proteins in mammals is less well defined and seems to contradict to some extent the data acquired in flies. Three mammalian fringes have been identified: Lunatic fringe (Lfng), Radical fringe (Rfng), and Manic fringe (Mfng; Moran et al., 1999). It has been suggested that Fringe proteins can either affect the binding affinity between Notch and its ligands or enhance S2 cleavage of Notch (Yang et al., 2005). Although the three Fringe homologues are capable of carrying out glycosylation reactions (Rampal et al., 2005), a role of Lfng has only been clearly demonstrated in somitogenesis (Evrard et al., 1998; Zhang and Gridley, 1998) and T cell formation (Visan et al., 2006). Interestingly, and contrary to the fly data, experiments in mammals indicate that Lfng inhibits the Delta–Notch interaction during somitogenesis (Dale et al., 2003; Morimoto et al., 2005). Moreover, the knockout of *rfrng* displays no obvious phenotype (Zhang et al., 2002), and a

of Notch, Lgd, and ESCRT complex, or during the MVB-to-lysosomes transition. These proteins further modulate Notch activity as described in the text. The dotted red arrow shows that in mutants that affect trafficking from the MVB to the lysosome, or if Notch is not trafficked to the lumen of the lysosome, Notch can undergo γ -secretase cleavage, resulting in a Notch GOF phenotypes.

knockout of *mfng* has yet to be documented. However, mutations in *LFNG* have been identified in patients with SCDs (Sparrow et al., 2006), a disease associated with vertebral segmentation defects. The other genes associated with SCDs are *Dll3* and *MESP2*, a bHLH protein regulating somitogenesis by inducing the expression of *Lfng* (Morimoto et al., 2005).

Given the important role of Fringe-dependent glycosylation of Notch, the enzyme mediating the *O*-fucosylation of the *O*-fucose sites in the EGF repeats must play a role before the action of Fringe. This enzymatic property has been shown in biochemical assays to be mediated by *O*-fucosyltransferase (*O*-fut1 in mammals and *Ofut1* in flies). Flies and mice with a loss of function of *O*-*fut1* exhibit severe Notch-related defects (Okajima et al., 2003; Shi and Stanley, 2003), providing strong evidence that *O*-fut1 is indeed involved in modulating Notch activity. Because only the *O*-fucose sites of Notch are further modified by Fringe, one would assume that *O*-fucosylation is only required for Fringe-dependent processes. However, *Ofut1* has recently been shown to have other functions that are independent of its enzymatic activity (Okajima et al., 2005, 2008; Sasamura et al., 2007). In flies, the nonenzymatic functions of *Ofut1* have been reported to affect folding of Notch in the ER (Okajima et al., 2005), and *Ofut1* has also been proposed to have an extracellular function that modulates Notch endosomal trafficking (Sasamura et al., 2007). In *Ofut1*-deficient fly cells, the majority of Notch is misfolded and trapped in the ER, which suggests that *Ofut1* is required for Notch folding. Interestingly, this phenotype can be rescued by overexpressing the enzymatically inactive form of the fucosyltransferase *Ofut1*^{R275A} (Okajima et al., 2005, 2008), which indicates that *Ofut1* functions as a chaperone of Notch. However, this enzymatic defective *Ofut1* does not rescue the wing defects typically associated with loss of *fringe*. Hence, the chaperone activity is required for both Fringe-dependent and -independent processes of Notch and plays a crucial role in the quality control of Notch in the ER. Sasamura et al. (2007) have also proposed an extracellular function for *Ofut1*. However, the fact that *Ofut1* is required in a cell-autonomous fashion and the data presented by Okajima et al. (2008) indicate that a chaperone function for *Ofut1* is quite likely. Interested readers are referred to a review by Vodovar and Schweisguth (2008).

In mammals, the precise role of *O*-fucosylation in Notch signaling remains to be understood: i.e., it is unknown if *O*-fut1 plays a role as a chaperone. Similar to flies that lack *Ofut1*, loss of *O*-fut1 in mice leads to embryonic lethality and causes numerous developmental defects similar to those that have been described in *CBF1* (the downstream transcription factor of Notch, named *Su(H)* in flies)-deficient mice; Shi and Stanley, 2003). Recent studies in embryonic stem cells in which *O*-fut1 was removed, and Chinese hamster ovary cell lines in which *O*-fut1 was knocked down, show that Notch–ligand interaction and ligand-induced Notch activation are impaired in biochemical assays, highlighting the role of *O*-fucosylation in optimizing the Notch–ligand binding (Stahl et al., 2008). However, in these mutant cells, the levels of Notch on the membrane are normal compared with the wild type, which suggests that mammalian *O*-fut1 may not be required for Notch folding (Stahl et al., 2008). Further analyses will be required to dissect the role of

O-fucosylation in specific developmental contexts using conditional knockout techniques.

In addition to *O*-fucosylation, Notch is also modified by *O*-linked glucosylation. Although *O*-glucosylation of Notch had been established some time ago on the basis of biochemical assays (Moloney et al., 2000), the functional significance of this modification and the protein responsible for this modification were unknown until recently. The *O*-glucosyltransferase is encoded by the *rumi* gene in *D. melanogaster* (Acar et al., 2008). *Rumi* is an ER protein that adds glucose to residues in numerous Notch EGF repeats with the consensus C¹-X-S-X-P-C² sequence (Moloney et al., 2000). In *rumi* mutant flies, Notch signaling is severely affected at 28°C but only mildly at 18°C, which suggests that glucosylation of Notch is required at the restrictive temperature to either stabilize its folding or trafficking (Acar et al., 2008). Indeed, Notch trafficking seems to be impaired in cells that lack *Rumi* at the restrictive temperature, but Notch with impaired or no glucosyl residues still traffics to the cell membrane, where it seems to bind *Delta* properly on the basis of biochemical assays. However, the S2 cleavage of Notch is severely impaired based on a Western blotting assay. The precise mechanism as to why Notch accumulates at the membrane at the restrictive temperature and how the S2 cleavage is impaired remains to be established. In addition, although 18 EGFs can be potential targets for *O*-glucosylation, which glucosylation site within NECD is important to convey this temperature-sensitive phenotype remains unknown. There are three homologues of *Rumi* in mammals; however, their roles in Notch signaling remain unknown.

The cleavage events required for ligand-dependent Notch activation and the mechanisms to prevent ligand-independent activation

After Notch enters the Golgi, it is cleaved by *Furin*, a cleavage event that has been termed S1 cleavage (Logeat et al., 1998). Although the S1 cleavage of Notch can be observed in both fly and mammalian cell culture, whether it is essential for Notch activity is still a matter of debate. In flies, the majority of Notch protein does not undergo S1 cleavage, and mutations in *furin 1* (CG10772) do not cause Notch signaling defects (Kidd and Lieber, 2002). However, a second *furin*-like paralogue (*furin 2* [CG18743]) exists in flies, and its function is unknown. Because *furin2* may play a redundant role with *furin 1*, a double mutant of both genes would be required to assess if S1 cleavage is indeed required in fly. In mammals, although small deletion mutants of *Notch* removing the S1 cleavage site are defective in signal transduction, these mutants may not only affect S1 cleavage but also S2 cleavage. Obviously, mutations affecting only the S1 cleavage site would be valuable for understanding its role in Notch signaling.

Notch is kept in an inactive state before ligand binding through a tight interaction between the LNRs and the HD (Fig. 2, top). From structural analyses, it appears that the S2 cleavage site in the HD is embedded and protected by the three LNRs (Gordon et al., 2007). The current hypothesis is that the pulling force generated by endocytosis of the ligand would weaken the

interaction between the LNRs and the HD, thereby freeing the S2 cleavage and allowing access to the S2 site for the ADAM proteases (Fig. 2, bottom). Interestingly, in T-ALL patients, ~30% of the mutations in NOTCH1 have been mapped to the interface of LNRs and the HD (Weng et al., 2004; Gordon et al., 2007). These mutations are thought to be GOF mutations causing a constitutive activation of Notch signaling. However, when these mutant proteins are overexpressed in mouse hematopoietic precursors, they fail to initiate leukemia on their own, although ectopic T cell development is observed (Chiang et al., 2008). However, these GOF alleles can accelerate the onset of leukemia initiation mediated by K-ras overexpression, which suggests that cooperation of the activated Notch and other oncogenes may underlie leukemia (Chiang et al., 2008). Although there are many mutations identified in the HD region of NOTCH1 in T-ALL patients, only one mutation was identified in the LNR region (Mansour et al., 2007), which suggests that there is a stringent

control in the cell to monitor correct folding of LNRs. This hypothesis is strengthened by recent findings on Ero1L in the fly. Ero1L is an ER-associated thiol oxidase required for disulfide bond formation. Although it is presumably required for most if not all disulfide bond formation based on yeast data (Tu et al., 2000), the loss-of-function phenotypes of Ero1L in flies indicate a role in lateral inhibition during peripheral nervous system development and inductive signaling during adult wing formation (Tien et al., 2008), two hallmarks of Notch-related developmental contexts. Although both the DSL ligands and Notch contain numerous disulfide bonds in their ECD, Ero1L-deficient cells show a prominent accumulation of Notch inside the ER without affecting DSL localization and function, which suggests that the problem may lie in the LNRs of Notch. Note that the LNRs are only found in Notch in flies, although there are mammalian LNR-containing proteins termed pregnancy-associated plasma protein-A (PAPP-A, pappalysin-1) and PAPP-A2 (Boldt et al., 2004), which have no homologues in *D. melanogaster*. Biochemical data further indicate that disulfide bonds in LNRs of Notch are targets of Ero1L (Tien et al., 2008). Therefore, it is reasonable to suggest that a stringent control of disulfide bond formation of the LNRs in the ER ensures the proper folding of LNRs (Tien et al., 2008).

After Notch is cleaved by a metalloprotease at the S2 site, the Notch external truncation (NEXT) domain is ready to be cleaved by the γ -secretase complex, a four-protein complex with enzymatic activity to cleave peptides within membranes (Okochi et al., 2002). Although the cleavage site and the protein complex mediating the cleavage have been identified (Okochi et al., 2002), the cellular compartment in which the cleavage event occurs is yet to be fully elucidated. The γ -secretase complex has been detected on the cell membrane as well as in endocytic compartments (Gupta-Rossi et al., 2004), and recent work shows that the S3/4 cleavage can happen on the membrane as well as in endocytic compartments (detailed in next section). In addition, the activity of γ -secretase can also be regulated to affect Notch signaling. For instance, loss of a regulator of epithelial polarity Crumbs in the fly increases γ -secretase activity, which leads to an up-regulation of Notch activity (Herranz et al., 2006), and provides an example in which γ -secretase activity can be regulated to modulate Notch activity.

Endocytic trafficking of Notch affects its activity

Endocytic trafficking affects the activity of several important signaling pathways (Seto et al., 2002). During endocytosis, a variety of molecules such as membrane components, receptors, and in some cases their associated ligands, are delivered to the endosomal pathway. In the classical endocytic pathway, cargo is internalized by clathrin-mediated endocytosis (Mills, 2007), and the clathrin-coated vesicles are pinched off from the membrane by the dynamin GTPase (Baba et al., 1995). The cargo in the clathrin-coated vesicles is then delivered to early endosomes (EEs) that in turn deliver endocytic cargo to late endosomes/multivesicular bodies (MVBs) or target it for recycling. Recycling endosomes are believed to bud off from EE membranes and then fuse with plasma membrane endosomes (Seto et al., 2002).

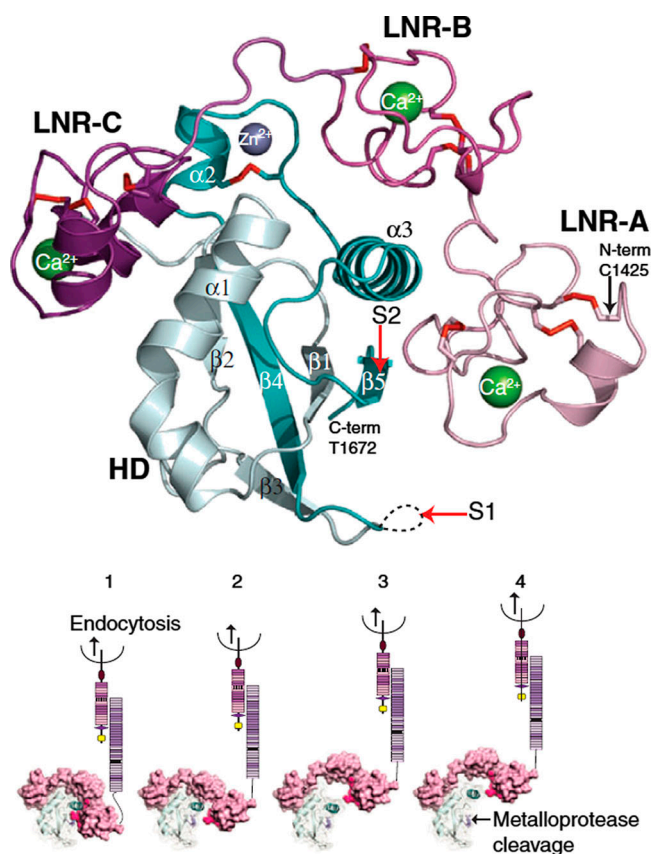


Figure 2. X-ray structure of the human NOTCH2 negative regulatory region (NRR) in its autoinhibited conformation, and models for signal activation. The top panel shows a ribbon representation of the NOTCH2 NRR. The LNR modules are colored in different shades of pink and purple, and the HD is in white and green. The three bound Ca^{2+} ions are shown in green, the bound Zn^{2+} ion is purple, and the disulfide bonds are red. The positions of S1 and S2 cleavage are indicated with red arrows. The bottom panel shows the model for activation by mechanical force driven by the DSL endocytosis. For unbound Notch, the LNRs (pink structure in 1–4) protect the S2 cleavage site in HD (white structure in 1–4). When Notch binds to DSL, which then undergoes endocytosis (1 and 2), this generates a mechanical force for disengaging LNRs from the HD (shown in 1–3). This relaxation between LNRs–HD interaction allows the metalloprotease to access the S2 cleavage site (shown in 4), leading to Notch activation. The figure is adapted from Gordon et al. (2007, 2008).

The MVBs sort the cargo into internal vesicles and eventually deliver them to the lysosomes, where they are degraded (Saksena et al., 2007).

Numerous studies have validated an important role for endocytosis and recycling of DSL ligands in the signal-sending cell (Emery and Knoblich, 2006; Le Borgne, 2006). However, a role for endocytosis in the signal-receiving cell in regulating Notch activity is still ill defined. The first piece of evidence showing that Notch activity is subject to endocytic regulation came from studies in a temperature-sensitive *dynammin* mutant (*shibire*) in the fly (Seugnet et al., 1997). It was shown that both the signal sending and receiving activity of Notch signaling likely depends on dynamin-dependent endocytosis (Seugnet et al., 1997). However, overexpression of NEXT does not lose its activity even when endocytosis is blocked in *shibire^{ts}* mutants. The later observation argues that in the overexpression scenario, a large quantity of NEXT can bypass the requirement of endocytosis in cells receiving the signal.

Recent work, in which different endocytic mutants in *D. melanogaster* were systematically analyzed for Notch localization, processing, and signaling, lends further credence to the idea that the endocytosis of the Notch receptor is crucial for its signaling ability (Vaccari et al., 2008). Genes involved in EE fusion events such as GTPase Rab5, and the syntaxin Avalanche (Avl; Wucherpfennig et al., 2003; Lu and Bilder, 2005) are indeed required for endogenous activity of Notch. It was shown that S3/S4 cleavage of Notch is much reduced when trafficking to the EE is impaired, which suggests that at least part of the NEXT is cleaved upon endocytosis (Vaccari et al., 2008). Consistent with the idea that NEXT may be cleaved mostly in the late endosomes, a new study indicates that an aquaporin (*Big brain [bib]* in *D. melanogaster*) that is involved in endosomal maturation is also required for optimal Notch activity. In the absence of *bib*, EE arrests to form abnormal clusters, and Notch accumulates in these abnormal endosomes, resulting in a Notch partial loss-of-function phenotype (Kanwar and Fortini, 2008). However, mutants affecting protein sorting in the late endosomes or MVB result in *Notch* GOF phenotypes, not loss-of-function phenotypes. In clones of *lethal giant discs (lgd)*, which encodes a C2 domain-containing protein that binds phospholipids, Notch exhibits a ligand-independent GOF phenotype. In *lgd* mutant cells, MVBs are enlarged, and Notch accumulates in these abnormal MVBs (Childress et al., 2006; Gallagher and Knoblich, 2006; Sevier and Kaiser, 2006). Another example of a Notch GOF resulting from abnormal MVB maturation defects is observed in mutations of *vps25*, a component of the ESCRT-II complex involved in sorting MVB cargo into lysosomes (Saksena et al., 2007). In *vps25* mutant cells, there is a ligand-independent Notch GOF phenotype (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Herz et al., 2006). It has therefore been proposed that when there is an MVB-to-lysosome maturation defect, there might be an increase in γ -secretase cleavage of NEXT, resulting in a ligand-independent Notch GOF phenotype. This is consistent with data that suggest that γ -secretase can act in endosomes and has optimal activity at low pH (Lah and Levey, 2000; Pasternak et al., 2003; Gupta-Rossi et al., 2004). Together, these data suggest a model in which both

full-length Notch and NEXT are endocytosed and degraded in lysosomes.

This model is further supported by studies on another ligand-independent regulator of Notch endocytosis and activation called Deltex (Dx). Dx encodes a ring finger E3 ubiquitin ligase (Hori et al., 2004). Overexpressing Dx leads to the expression of Notch downstream genes in a Su(H)- and ligand-independent manner in the wing pouch of the third-instar wing disc. Overexpression of Dx also results in the translocation of Notch from the apical cell surface into the late endosome, where it accumulates stably and colocalizes with Dx. It has been reported that Dx interacts with a nonvisual β -arrestin Kurtz, and that together they form a trimeric protein complex with Notch (Mukherjee et al., 2005). Functional assay indicates that loss of Kurtz results in elevated levels of Notch, which is consistent with its role in regulating Notch stability (Mukherjee et al., 2005). Furthermore, when Notch trafficking is blocked using a dominant-negative form of Rab5, Dx-mediated activation of Notch signaling is inhibited, suggesting that the accumulation of Notch in the late endosome is required for Dx-dependent Notch activity (Hori et al., 2004). It was therefore proposed that Dx protects Notch from entering the degradative pathway, but how this is regulated is unclear. In a suppressor screen for the Dx overexpression phenotype, it was found that *D. melanogaster* homologues of the HOPS complex, which mediate late endosomal maturation and lysosomal fusion events (Warner et al., 1998; Seals et al., 2000; Wurmser et al., 2000; Rink et al., 2005), and components of the AP-3 complex, which traffics cargo to the lysosome-limiting membrane (Peden et al., 2004; Theos et al., 2005), are required for Dx-induced Notch endocytosis and activation (Wilkin et al., 2008). Dx activity opposes trafficking of Notch into the lumen of the lysosomes, where it is degraded and instead limits Notch to the limiting membrane of the lysosome membrane. This in turn results in Notch ectodomain shedding by lysosomal proteases. The truncated NEXT is then proposed to be cleaved by the γ -secretase to release the NICD (Wilkin et al., 2008). This work provides further support to the model that lack of maturation of MVB/late endosomes into lysosomes results in ectopic γ -secretase-mediated cleavage of Notch, leading to a GOF phenotype. In addition to Dx, the stability of the Notch receptor and other components of the pathway such as Numb are regulated by proteins that play a role in the ubiquitination pathway such as Su(Dx)/itchy (Fostier et al., 1998; Qiu et al., 2000), Sel-10 (Hubbard et al., 1997; Wu et al., 2001), Nedd4 (Sakata et al., 2004; Wilkin et al., 2004), Neuralized, Mindbomb (Lai and Rubin, 2001; Le Borgne and Schweisguth, 2003; Le Borgne et al., 2005b), and LNX (Nie et al., 2002). We refer the reader to reviews on the role of E3 ligases in the Notch pathway for further details (Lai, 2002; Le Borgne, 2006).

It is important to note that there might be different routes for Notch activation either on the membrane or inside endosomal compartments. For instance, in animals mutant for *hrs* (*hepatocyte growth factor-regulated tyrosine kinase substrate*), which encodes a protein involved in MVB formation (Lloyd et al., 2002), Notch accumulates in intracellular compartments without affecting its signaling activity. Although this may suggest that

Hrs is not required for Notch signaling, removing *hrs* in an *lgd* background can inhibit its Notch GOF phenotypes, suggesting that Lgd acts in an Hrs-dependent pathway to down-regulate Notch (Childress et al., 2006; Gallagher and Knoblich, 2006; Sevier and Kaiser, 2006). Furthermore, when trafficking through the EE and MVB are disrupted, such as in a double mutant of *avl* (a syntaxin that regulates the trafficking to the EE), *tsg101* (tumor suppressor gene 101, which encodes a component of the ESCRT-I complex that regulates MVB sorting), Notch activity is still slightly increased (Vaccari et al., 2008). However, based on prior data, the prediction was that Notch activity would be completely blocked because of the defect in EE formation. As this is not the case, alternative routes of Notch trafficking must exist that still need to be characterized. Also, the possibility of whether the localization and the activity of the γ -secretase complex in the *avl tsg101* double mutant background is altered has not been explored. Finally, although a critical role for ligand recycling in the signal-sending cell has been well characterized (Emery et al., 2005; Jafar-Nejad et al., 2005; Emery and Knoblich, 2006) a crucial role for the recycling of Notch receptors in the signal reception has not yet been uncovered (discussed in Le Borgne, 2006).

In summary, Notch is modified at different levels in the secretory pathway by glycosyltransferases during ligand activation by proteases, and recent evidence suggests that several players ensure that Notch does not undergo ligand independent-activation during endocytosis. These insights can be harnessed to manage Notch-related disease conditions and provide us interesting glimpses into the strategies used by cells to manage this critical signaling pathway.

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