Notch signalling: a simple pathway becomes complex

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Abstract | A small number of signalling pathways are used iteratively to regulate cell fates, cell proliferation and cell death in development. Notch is the receptor in one such pathway, and is unusual in that most of its ligands are also transmembrane proteins; therefore signalling is restricted to neighbouring cells. Although the intracellular transduction of the Notch signal is remarkably simple, with no secondary messengers, this pathway functions in an enormous diversity of developmental processes and its dysfunction is implicated in many cancers.

ADAM-family metalloproteases
Transmembrane disintegrins and metalloproteases that proteolytically cleave the juxtamembrane region of cellular transmembrane proteins and detach their extracellular regions — this process is known as ectodomain shedding.

γ-secretase complex
Presenilin, a multispan membrane protein, is the catalytic subunit, and the transmembrane proteins nicastrin and APH1 stabilize the presenilin holoprotein. PEN2, a two-pass transmembrane protein, induces endoproteolysis of presenilin and maturation of the γ-secretase complex.

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doi:10.1038/nrm2009

Notch signalling has a simple framework that is highly conserved throughout the animal kingdom[1–3] (FIG. 1). Both the Notch receptor and its ligands, Delta and Serrate (known as Jagged in mammals), are transmembrane proteins with large extracellular domains that consist primarily of epidermal growth factor (EGF)-like repeats (BOX 1). Ligand binding promotes two proteolytic cleavage events in the Notch receptor (FIG. 1). The first cleavage is catalysed by ADAM-family metalloproteases, whereas the second is mediated by γ-secretase, an enzyme complex that contains presenilin, nicastrin, PEN2 and APH1 (REFS 4–6). The second cleavage releases the Notch intracellular domain (Nicd), which then translocates to the nucleus and cooperates with the DNA-binding protein CSL (named after CBF1, Su(H) and LAG-1) and its co-activator Mastermind (Mam) to promote transcription (FIG. 1; BOX 1). The precise numbers of Notch paralogues differ between species (TABLE 1) — for example, there are four Notch receptors in mammals (Notch1–4), two in *Caenorhabditis elegans* (LIN-12 and GLP-1) and one in *Drosophila melanogaster* (Notch) — but the basic paradigm is common throughout[1–3].

The Notch pathway functions during diverse developmental and physiological processes, which can broadly be subdivided into three categories (BOX 2). The first functions of Notch to be well characterized were those affecting neurogenesis in flies and vertebrates[1]. From these studies it became evident that Notch acts at different stages of development even within one tissue. For example, Notch first regulates the number of cells that acquire neural potential (lateral inhibition; BOX 2), and subsequently it determines whether progeny will adopt neural or glial fates[7] (lineage decisions; BOX 2). Notch inhibits neural differentiation in many lineages, but recently Notch was shown to promote neural fates in mouse and human embryonic stem cells,[8] underscoring the importance of the cellular context in the determination of the outcome of signalling. Iterative activation of Notch has now been detected in multiple lineages. A recent example is the midgut progenitor cells in both mammals and *D. melanogaster,* in which Notch maintains proliferating progenitor cells and regulates binary cell-fate decisions in the stem cell progeny[9–12]. In addition to the ever-increasing examples of biologically important roles for the Notch pathway during development, Notch activity also emerges as a contributory factor to many cancers[3]. For example, mutations in Notch1 were detected in more than 50% of T-cell acute lymphoblastic leukaemias[13].

The basic core Notch-transduction pathway is the same in most Notch-dependent processes. However, the mechanisms that regulate the pathway are different. Although it is still unclear how the interaction between a ligand and the Notch extracellular domain (ECD) results in the activation of the receptor, many factors are emerging that influence whether or not a productive ligand–receptor interaction occurs, including the precise location of the receptor in the cell. This review aims to summarize our current understanding of the mechanisms that function on the core Notch pathway (CSL-independent signalling and crosstalk with other pathways are beyond the scope of this article). One challenging question is, what determines in which cells the ligands and the receptor are active? Often, dramatic differences in signalling and signal reception between cells do not correlate with obvious differences in the expression levels of the ligands or the receptor. It has recently become evident that post-translational modifications and trafficking of the Notch ligands and receptor affect the activation of the pathway. Another important

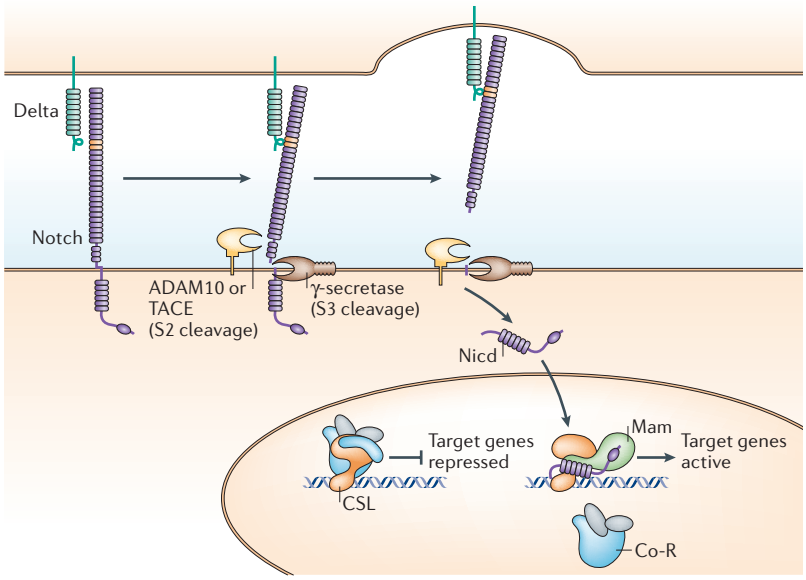


Figure 1 | **The core Notch pathway.** Binding of the Delta ligand (green) on one cell to the Notch receptor (purple) on another cell results in two proteolytic cleavages of the receptor. The ADAM10 or TACE (TNF-α-converting enzyme; also known as ADAM17) metalloprotease (yellow) catalyses the S2 cleavage, generating a substrate for S3 cleavage by the γ-secretase complex (brown). This proteolytic processing mediates release of the Notch intracellular domain (Nicd), which enters the nucleus and interacts with the DNA-binding CSL (CBF1, Su(H) and LAG-1) protein (orange). The co-activator Mastermind (Mam; green) and other transcription factors (see also FIG. 4) are recruited to the CSL complex, whereas co-repressors (Co-R; blue and grey) are released.

question is, what happens once Nicd enters the nucleus? Both the duration of signalling and the identity of target genes have impacts on the output of Notch activation, so their regulation is of major importance. Together, the different mechanisms give a perspective on how this simple pathway can be manipulated, but they also show that we are still just beginning to understand the full complexities of Notch regulation.

Regulation of Notch-ligand activity

Expression of Notch ligands during development is quite dynamic and contributes significantly to differential activity of the pathway. In some developmental contexts, the ligand is produced by a distinct population of cells (boundaries/inductive signalling; BOX 2). However, under many circumstances, differential ligand transcription is not sufficient to explain why certain cells become the signal-sending cells. Other post-transcriptional mechanisms are clearly at work.

Ubiquitylation and ligand activity. The identification of the E3 ubiquitin ligases, Neuralized (Neur) and Mind bomb (Mib), that interact directly with Notch ligands and are required for ligand activation (FIG. 2) was a striking and surprising recent discovery[14,15]. Loss of Neur in *D. melanogaster* or *Xenopus laevis* and of Mib1 in zebrafish results in neurogenic phenotypes[16–19]. In *D. melanogaster*, *mib1* mutants have a later defect of arrested appendage (imaginal disc) development (possibly due to persistence of maternal protein or redundancy with MIB2). The MIB1-associated defects can be rescued by expression of Neur, which indicates that these two proteins — although they share few structural similarities apart from RING domains — can perform the same function[20–22]. Much of the difference between these two E3 families might be attributed to their expression patterns and to their regulation (see below), although it remains possible that they preferentially interact with different Notch ligands.

In normal cells, the extensive trafficking of Notch ligands through the cell is evident from intracellular puncta that are detected in different tissues and animals. This trafficking is compromised in the absence of Neur or Mib, as ligands accumulate at the cell surface but are inactive[18,21]. This surprising observation indicates that regulation of ligand activity by Neur and Mib is intimately associated with endocytosis (FIG. 2) and it requires the ubiquitin-binding protein Epsin[23–25] and probably the J-domain-containing protein auxilin (which can disassemble clathrin coats)[26].

Different models have been proposed to explain the link between ubiquitylation, endocytosis and ligand activity[14,15,23]. For example, ligand endocytosis could generate a 'pulling force' on a bound receptor that causes a conformational change in the juxtamembrane region[27]. Another possibility is that ubiquitylation promotes ligand clustering. Indeed, Notch activation is more effective if soluble ligands are clustered through fusion to an Fc moiety or through immobilization on plastic[28,29]. A third possibility is that ubiquitylation permits trafficking into an endocytic compartment, which enables ligand modification or results in re-insertion of the ligand into specific membrane domains. Two observations support this model. Segregation of RAB11, a component of the recycling endosome, influences signalling in the *D. melanogaster* sensory organ precursors (SOP). Furthermore, mutations in an exocyst component, SEC15, compromise SOP Notch signalling[30,31].

Paradoxically, some functional ligands in *C. elegans* are secreted (for example DSL-1; REF. 32) and so would presumably not be ubiquitylated. However, the ubiquitin-binding protein Epsin is also required for Notch (LIN-12) signalling activity in this animal, implying that mechanisms of ligand activation are conserved[33]. Whatever the mechanism for ligand activation, regulation of E3 ligases is potentially one significant strategy for controlling the activity of the Notch pathway, as exemplified by the Bearded-related family of small inhibitory polypeptides[34,35] (BOX 3). Therefore, elucidating the mechanism of ligand activation is of prime importance.

Ligand localization. The localization of ligands within the cell is important for effective signalling and might be influenced by other proteins. For example, Echinoid, an immunoglobulin C2-type cell-adhesion molecule, colocalizes with Notch and Delta at adherens junctions in *D. melanogaster*.

Genetic interactions indicate that Echinoid functions as a positive regulator to promote Notch signalling[36]. Echinoid colocalizes with Delta in endocytic vesicles, and Echinoid overexpression depletes Delta from the membrane. Therefore, it is possible that Echinoid

E3 ubiquitin ligase
An adaptor protein that links ubiquitin-conjugating E2 enzymes with substrates and contributes to the catalytic transfer of ubiquitin onto the substrate.

Epsin
A clathrin and phosphatidylinositol-4,5-bisphosphate-binding protein that contains ubiquitin-interaction motifs. It is thought to facilitate endocytosis of ubiquitylated cargo proteins.

Auxilin
A J-domain-containing protein that is implicated in the disassembly of clathrin from clathrin-coated vesicles.

Recycling endosome
A compartment that sorts transmembrane proteins that are recycled to the plasma membrane following endocytosis.

Exocyst
A heteromeric protein complex that is required for polarized exocytosis of post-Golgi secretory vesicles.

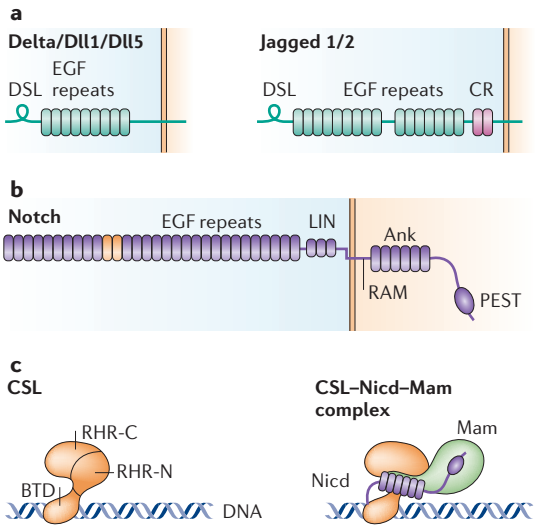
Box 1 | **The Notch-pathway players**

DSL ligands

Notch ligands (**a**) are transmembrane proteins that are characterized by an N-terminal DSL (Delta, Serrate and LAG-2) domain that is essential for interactions with the Notch receptor. The extracellular domains of the ligands contain varying numbers of epidermal growth factor (EGF)-repeats. The ligands are subdivided into two classes, Delta or Delta-like (Dll) and Serrate (Jagged in mammals), depending on the presence or absence of a cysteine rich (CR) domain.

Notch receptors

The mature Notch receptor (**b**) is produced through a furin cleavage during biosynthesis (see FIG. 3). Notch extracellular domains contain 29–36 EGF repeats, 3 cysteine rich LIN repeats and a region that links to the transmembrane and intracellular fragment. This linker region is important in preventing premature activation of the receptor and is altered in 26% of activating mutations that are associated with T-cell acute lymphoblastic leukaemia[13]. EGF-repeats 11 and 12 (orange) are essential for ligand binding. The intracellular portion consists of a RAM domain, six ankyrin (Ank) repeats and a C-terminal PEST domain. It also contains nuclear localization signals. Individual types of Notch receptor have additional protein–protein interaction motifs.



Nuclear effectors

The key transducer of the Notch-signalling pathway is a DNA-binding protein, CSL (CBF1, Su(H) and LAG-1) (**c**). CSL is similar to the Rel family of transcription factors. However, CSL differs from Rel in the insertion of a central modified β-trefoil domain (BTD) between the two Rel-homology regions (RHR-N, RHR-C)[93]. DNA contacts are predominantly made through the RHR-N and BTD domains. The BTD domain contains a hydrophobic pocket that is thought to mediate the interaction with the Notch intracellular domain (Nicd). To activate transcription, the co-activator Mastermind (Mam) is required. Mam proteins from different species share little sequence homology apart from an N-terminal region that forms an extended α-helical domain that contacts the RHR-N and RHR-C domains of CSL and the Ank domain of Nicd in a trimeric complex[94,95].

promotes endocytic activation of Delta. Alternatively, Echinoid-mediated adhesion could favour Notch–Delta interactions. Consistent with this notion, it has been shown that altered cytoarchitecture of cells can affect their signalling potential[37]. Furthermore, the intracellular domains of some Notch ligands contain protein–protein interaction motifs (for example, PDZ-binding motifs) that can bind to intracellular scaffolding proteins[38–40]. Deletion of the cytoplasmic PDZ-binding motif in Delta had minimal effects on most functions of Notch signalling[38]. Nevertheless, some subtle neuronal defects were observed[38]. So, sequences within the ligand intracellular domain might modulate activity, affect localization or mediate an independent reverse-signalling activity.

Ligand processing and soluble ligands. Structurally, the ligands share many characteristics with Notch itself and are prone to similar modifications (see below) including proteolytic processing[41,42]. However, the purpose of ligand cleavage remains unclear. One suggestion is that proteolytic processing of the ligand contributes to ligand downregulation[43]. For example, loss of the metalloprotease Kuzbanian-like, which has been shown to cleave Delta, results in ectopic Notch signalling in certain locations[44]. Another suggestion is that cleaved or secreted ligands antagonize Notch signalling, because, under most circumstances, soluble ligand fragments inhibit receptor signalling[28,43,45]. However, several members of the Notch-ligand family in *C. elegans* have no transmembrane domains, but their expression can rescue worms that lack more conventional ligands, indicating that the secreted ligands retain signalling potential[32]. It is also possible that cleavage of transmembrane ligands could transmit an intracellular signal through activities that are associated with the ligand's intracellular domains — that is, reverse signalling. Further investigations are needed to identify all of the functional consequences of ligand proteolysis on Notch signalling *in vivo*.

Tuning of Notch-receptor activation

Notch receptors have broad expression patterns in many tissues, but analyses of where cleavage occurs or where target genes are expressed reveal a limited profile of activation. Furthermore the ability to respond to a specific ligand is spatially regulated in the chick inner ear or the *D. melanogaster* wing[46,47]. These observations indicate that the activity of the receptor must also be regulated through post-transcriptional mechanisms (FIG. 3).

Role of glycosylation. Notch proteins have a large ECD that consists of multiple EGF-like repeats, which are sites for glycosylation[48]. The enzyme *O*-fucosyl transferase (*O*-Fut) adds the first fucose and is essential for the generation of a functional receptor[49–51]. Depletion of *O*-Fut in *D. melanogaster* and mice results in phenotypes that resemble those associated with lack-of-Notch signalling

Bearded-related proteins
Short polypeptides that were identified in insects and contain an N-terminal amphipathic helix and 2 or 3 conserved motifs.

Adherens junction
A cell–cell junction that mediates adhesion through cadherins and regulates and/or links to the actin cytoskeleton.

PDZ-binding motif
A motif at the C terminus of a protein that is recognized by a PDZ-domain-containing protein. PDZ-domains are conserved 80–90-residue domains that fold into a β-sandwich and are found in many scaffold and signalling proteins.

Table 1 | **Notch-pathway components and auxiliary factors in different species**

Component type	*Drosophila melanogaster*	Vertebrates and mammals	*Caenorhabditis elegans*
Receptor	Notch	Notch1–4	LIN-12, GLP-1
Ligand	Delta, Serrate	Delta1–4/A–D, Serrate, Jagged1–2	APX-1, LAG-2, ARG-1, DSL-1
CSL DNA-binding protein	Su(H)	CBF1/RBPkJ	LAG-1
Co-activator	Mastermind	Mastermind1–3	LAG-3
Co-repressor	Hairless, SMRTR*	SMRT	
γ-secretase complex	Presenilin, nicastrin, APH1, PEN2	Presenilin1–2, nicastrin, APH1, PEN2	SEL-12/presenilin, APH-2/nicastrin, APH-1, PEN-2
Glycosyl transferase	Fringe	Lunatic Fringe, Radical Fringe, Manic Fringe	
Metalloprotease, receptor cleavage	Kuzbanian, Tace CG7908*	ADAM10, TACE/ADAM17	SUP-17/Kuzbanian, ADM-4/TACE
Metalloprotease, receptor cleavage	Kuzbanian-like		
Ring finger E3 (ligand regulation)	Mind bomb 1	Mind bomb 1–2	
Ring finger E3 (ligand regulation)	Neuralized	Neuralized1–2	F10D7.5*
Ring finger E3 (receptor regulation)	Deltex	Deltex	
HECT domain E3 (receptor regulation)	Su(dx), NEDD4	Itch, NEDD4*	WWP-1
F-box E3 (nuclear)	Archipelago*	FBW7/SEL10	SEL-10
Numb, cytoplasmic Notch inhibitor	Numb	Numb, Numb-like	
Numb-associated kinase	Numb-associated kinase	AP2-associated kinase	SEL-5
4-pass transmembrane protein, positive regulator	Sanpodo		
Immunoglobulin C2-type cell-adhesion molecule	Echinoid		IGMC-1*
bHLH repessors, target genes	E(spl)bHLH	HES/ESR/HEY	REF-1
Neuralized E3 inhibitors	Bearded, Tom, M4		

*Protein homologues that have not yet been tested for roles in Notch signalling. Where no protein is listed, relatives have not been identified. AP2, adaptor protein-2; CSL, CBF1, Su(H) and LAG-1; E3, ubiquitin ligase; bHLH, basic-helix–loop–helix.

(for example, presenilin mutants). Not only is the enzymatic activity important, *O*-Fut also functions as a chaperone to promote the folding and transport of Notch from the endoplasmic reticulum to the cell membrane[52]. *O*-Fut that has lost the capability to glycosylate, because of mutations in the active site, retains the capability to chaperone Notch proteins to the cell-surface. This finding shows that the chaperone function of *O*-Fut is not a secondary effect of glycosylation.

It is possible that *O*-Fut might contribute to the spatial regulation of Notch activity, as its expression pattern is not uniform[49–51]. Furthermore, a *Notch* mutation that introduces a fucosylation site into EGF-repeat 14 results in ectopic receptor activity in a subset of neural cells in *D. melanogaster*, which indicates that differences in primary fucosylation could contribute to differential activity of the receptor in different cell types[53].

After the addition of the first fucose, the carbohydrate chains can subsequently be extended by other glycosyl transferases, such as those of the Fringe family[48]. Multiple EGF repeats in Notch have the potential to be modified and, therefore, a large repertoire of differentially modified receptors could be generated. It has been shown that these glycosyl-modifications alter the capability of ligands to activate Notch. For example, in dorsal cells of the *D. melanogaster* wing, Fringe potentiates activation by Delta and renders Notch resistant to activation by Serrate[48].

Using soluble ligand or receptor fragments these differences can be correlated with effects on binding affinities in cell-culture assays[54,55]. For example, Serrate binds with higher affinity to Notch fragments that have been fucosylated and with lower affinity to fragments that have been further modified by Fringe[56]. Mutation

Fringe
A Golgi-resident glycosyl-transferase that was first identified in *D. melanogaster* and has three homologues in mammals: Lunatic Fringe, Radical Fringe and Manic Fringe.

Box 2 | **Different modes of Notch action**

Lateral inhibition

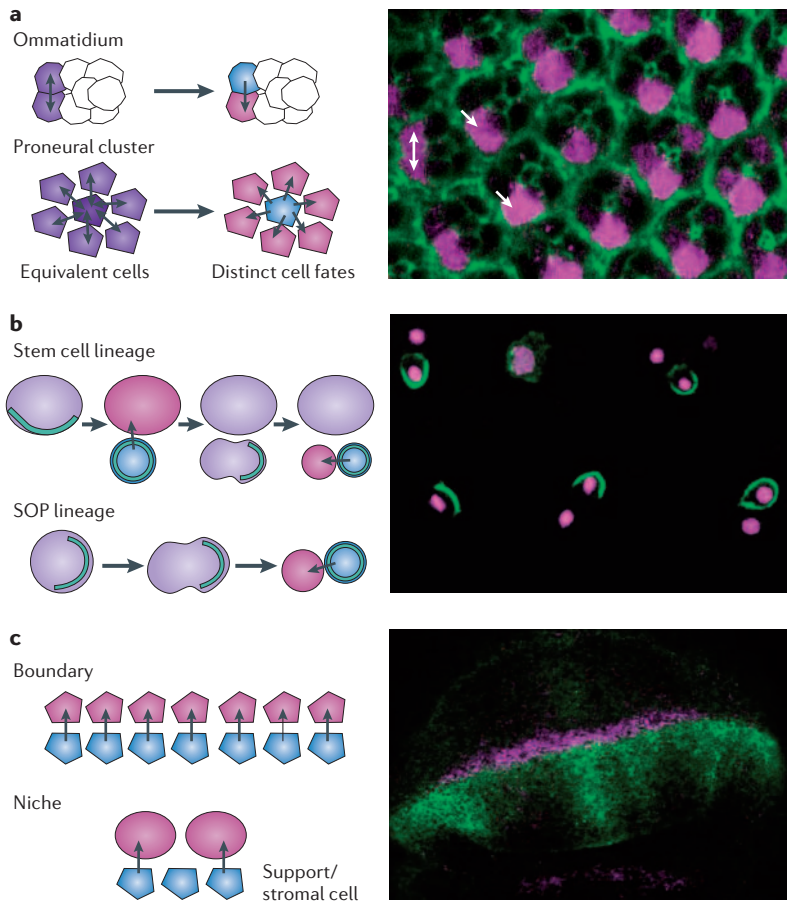
Notch signalling amplifies small or weak differences within roughly equivalent populations of cells. The diagrams (**a**) represent Notch signalling (black arrows) in ommatidia (upper) and neural preclusters (lower) that resolves equivalent (purple) cells into distinct fates (blue and pink; cells with the highest Notch activity are coloured pink). A confocal image shows Notch activity (E(spl) mδ0.5 expression; pink) in developing ommatidia of a fly eye (the green staining marks cell membranes and the thin peripheral cells demarcate each ommatidium). At early stages, mδ0.5 expression is sometimes detected in both posterior photoreceptors (see double arrow) before signalling is refined, and then it is only detected in one (R4) cell (see single arrows).

Lineage decisions

Notch signalling between two daughter cells is dependent on asymmetrical inheritance of Notch regulators (for example, Numb). Diagrams (**b**) illustrate segregation of regulators (green) in progeny from a hypothetical stem cell lineage (upper) and the *Drosophila melanogaster* sensory organ precursors (SOP) lineage (lower). Thin black arrows indicate the direction of Notch signalling, pink cells acquire highest Notch activation. The confocal image shows Numb distribution (green, by Partner of Numb (PON)-GFP) in SOP lineages (nuclei are pink). Confocal bottom row left; A Numb crescent is evident prior to division. Confocal bottom row right; Numb is present in one of two daughter cells. Image courtesy F. Wirtz-Peitz and J. Knoblich, both at the University of Vienna, Austria.

Boundaries/inductive

Notch signalling occurs between two populations of cells and can establish an organizer and/or segregate the two groups. Diagrams (**c**) of signalling at a boundary (upper) or between stromal and progenitor cells (lower). Black arrows indicate the direction of Notch signalling, pink cells have Notch activation. The confocal image is of the fly wing primordium, in which Notch activity, as measured by Wg expression (pink), is detected at the boundary of Serrate-expressing cells (green).



of a glycosylation site in EGF-repeat 12, a crucial repeat for ligand binding, allows activation of Notch by Serrate even in the presence of Fringe, which indicates that this is a key site for modification[57]. The results are therefore most simply reconciled with a model in which Fringe glycosylation affects binding affinities between ligands and specific EGF-repeats. Under some conditions, Jagged still binds to Notch in the presence of Fringe, although the receptor is not activated, raising the possibility that the stability and/or the duration of interactions are important[58]. Furthermore, Lunatic Fringe, a mammalian homologue of Fringe, potentiates Delta binding in *in vitro* studies and promotes Notch activity at the somite clefts, but behaves as a Notch inhibitor within the Delta-driven oscillatory somite clock in some species[59,60]. These observations indicate that glycosylation patterns might do more than producing an all-or-none effect on different ligands.

Proteolytic cleavage of Notch. The discovery that Notch activation entails proteolytic cleavage (S3 cleavage) that is mediated by γ-secretase was an important breakthrough[4–6] (FIG. 1). However, this finding raised some perplexing questions, such as where in the cell does S3 cleavage occur and what renders Notch into a substrate?

Truncation of the Notch ECD stimulates S3 cleavage, and it seems that the efficiency of cleavage correlates with the length of ECD[61]. This explains the constitutive activity that is observed in the human TAN1 (also known as Notch1) and INT3 (also known as Notch4) oncoproteins for which chromosomal rearrangements have truncated the ECD[3,6]. *In vivo*, the S3 cleavage occurs in response to a prior (S2) cleavage that is mediated by ADAM metalloproteases within the ECD, and is elicited by productive ligand binding[6,62–64]. Two metalloproteases have been implicated in the S2 cleavage, ADAM10 (also known as Kuzbanian; Kuz) and tumour-necrosis factor-α (TNFα)-converting enzyme (TACE; also known as ADAM17), and evidence indicates that these have partially redundant roles[63–65]. The S2 protease cleavage remains an important aspect for investigation, particularly because studies of metalloproteases reveal the potential for regulation by external factors, membrane environment and intracellular signalling pathways[66].

There is also potential for regulation of γ-secretase. Ubiquitylation on a juxtamembrane lysine residue was necessary for S3 cleavage in mammalian cell assays, indicating that cleavage occurred after Notch endocytosis[67]. Another recent study proposed that the transmembrane protein Crumbs, a regulator of epithelial polarity, mediates a negative feedback on γ-secretase activity[68]. Further studies are needed to determine the importance and diversity of γ-secretase regulation *in vivo*. However, presenilin is already being investigated as an important target for drug interventions — presenilin-mediated cleavage of amyloid precursor protein is associated with the accumulation of plaques in Alzheimer's disease. Drugs are now being tested in clinical trials for selected types of cancer in which mutations in Notch contribute to the pathology, including T-cell acute lymphoblastic leukaemias[69].

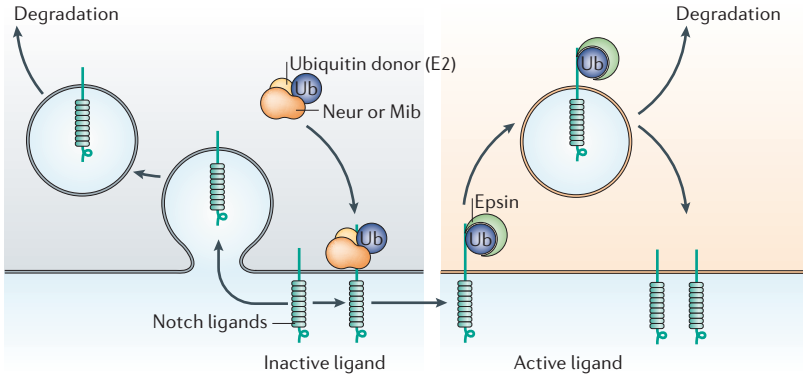


Figure 2 | **Ligand activation entails ubiquitylation.** The E3 ubiquitin (Ub) ligases Neuralized (Neur) and Mind bomb (Mib) interact directly with Notch ligands. Prior to modification by Neur or Mib, ligands are inactive, and can be endocytosed and degraded. Neur- or Mib-mediated ubiquitylation of Notch ligands is required for Epsin-mediated endocytosis. Ligands (in the light orange area) are then competent to signal either because endocytosis is directly associated with receptor activation or because it allows entry into a specific compartment or membrane domain that renders ligands active. They can also be targeted for degradation. E2, ubiquitin-conjugating enzyme.

Notch endocytosis and trafficking. Notch is a cell-surface receptor, so its expected location is the plasma membrane. However, a substantial amount of Notch is targeted for degradation and a large fraction of Notch is detected in the cytoplasm in compartments of the endocytic pathway. Studies in *D. melanogaster* have shown that Notch colocalizes with the small RAB GTPases RAB5 and RAB7, which are both markers of the endocytic pathway. Moreover, Notch accumulates in intracellular structures when the endocytic progression is perturbed[70,71]. Mutations in several endocytic components in *D. melanogaster* (for example, HRS/VPS27, Syntaxin I and β-arrestin) result in elevated Notch protein levels, without affecting the activity of the Notch pathway[71–73]. By contrast, other mutations that compromise sorting of ubiquitylated membrane proteins (most notably mutations in the ESCRT components VPS25 and TSG101 (also known as VPS23)), result in dramatic hyperplasia that is due to overactivation of the Notch pathway[74–76].

But why does a block in one step in trafficking have such profound effects on activity, whereas a block in a different step does not? This is a puzzling question, especially because mutations in *vps25* and *tsg101/vps23* perturb a later step than those in *syntaxin I* and *hrs*. One suggestion is that Notch colocalizes with ligands and/or γ-secretase only in certain compartments. Another possibility is that the physiological composition of certain endocytic compartments favours ectodomain shedding, a step that mimics ligand activation[67]. Activity of the trapped Notch seems to be dependent on presenilin activity because elevated Notch activity in cells that were treated with RNA interference against ESCRT components is sensitive to a γ-secretase inhibitor[74]. However, the contribution of ligands to the activation has not been fully assessed, and further studies are needed to ascertain why certain endocytic sorting mutants result in such potent Notch activation and whether this activation is relevant to signalling under normal circumstances.

The activity of Numb, a well characterized Notch inhibitor, also involves endocytosis. Numb is asymmetrically segregated into one of two daughter cells in several lineages, and a search for mutants giving *numb*-related phenotypes identified α-adaptin, a component of the adaptor protein-2 (AP2) complex that links cargoes to clathrin coats of transport vesicles[77]. Numb interacts with the ear domain of α-adaptin and with Notch, so it could directly recruit Notch into endocytic vesicles. Furthermore, mammalian Numb promotes Notch ubiquitylation[78]. However, in *D. melanogaster*, Numb associates with the 4-pass transmembrane protein Sanpodo, which performs an unknown but essential role in Notch signalling wherever Numb-mediated regulation is crucial[79,80]. Plasma-membrane accumulation of Sanpodo is reduced by Numb in circumstances in which no detectable change in Notch accumulation is observed, which indicates that Sanpodo could be a primary endocytic target.

A further link between Numb, Notch and endocytosis comes from the identification of Numb-associated kinase (NAK)[81], which is related to AP2-associated kinases and to SEL-5, a suppressor of dominant *lin-12* phenotypes in *C. elegans*[82]. However, partial rescue of Numb phenotype is observed with Numb proteins that lack the α-adaptin-interaction domain, which is indicative of alternative mechanisms of Numb-mediated antagonism[83]. Further studies are required to determine more fully the mechanism of Numb action and the role of Sanpodo in the regulation of Notch activity.

Ubiquitylation and Notch trafficking. Entry into the endosomal and multivesicular-body-sorting pathway is thought to be intimately linked with ubiquitylation of transmembrane proteins. Several E3 ligases that target Notch have already been identified[84]. The Itch/NEDD4/Su(dx) family of HECT domain E3 ligases are predominantly negative regulators of signalling, which indicates that by modifying Notch they target it for degradation. Studies in mammalian cells mapped the interaction domain of Itch to the RAM–ankyrin repeat region of Nicd[85], which correlates with a 'down-regulation targeting signal' that has been identified in *C. elegans* LIN-12 (REF. 86). The targeting signal was subsequently shown to interact with ALX-1, the homologue of Bro1 — a *Saccharomyces cerevisiae* protein that is involved in a late step in multivesicular-body sorting — and WWP-1, the homologue of Itch/NEDD4/Su(dx)[87]. However, there might be more than one Notch motif associated with recognition by this E3-ligase family, as a more C-terminal PPXY motif affected the capability of NEDD4 to promote Notch degradation in *D. melanogaster*[88].

The subtle phenotypes caused by mutations in the HECT E3 ligases indicate that they do not make a crucial contribution to Notch signalling. However, whether this is indeed the case will remain an open question until the consequences of eliminating all of the members of this family are analysed. Nevertheless, by modulating turnover, Itch/NEDD4/Su(dx) could regulate the amount of Notch that is available to interact with ligands. We do not as yet know the extent to which these enzymes can them-

RAB GTPases
Members of the Ras superfamily of small GTPases, RAB proteins regulate vesicle budding, fusion and motility.

HRS/VPS27
A protein that contains ubiquitin-interaction motifs and is important for sorting ubiquitylated endosomal cargoes.

Syntaxin I
Integral membrane protein with sequence similarity to t-SNAREs that is involved in vesicle docking and vesicle fusion.

β-arrestin
Also known as non-visual arrestin, it is a cytoplasmic protein that promotes endocytosis of G-protein-coupled receptors and is present in coated vesicles.

ESCRT
(Endosomal sorting complex required for transport). Three heteromeric protein complexes, ESCRTI, ESCRTII and ESCRTIII, function sequentially in the sorting of membrane proteins into the multivesicular body.

Box 3 | **Making a difference**

Several mechanisms are used in different Notch-dependent processes to regulate ligand and receptor activities.

Lateral inhibition

The following mechanisms could contribute:

- Receptor turnover — destabilization of Notch in the cell that will become the signal-sending cell (for example, through NEDD4-family E3 ligases (WWP-1, Itch, NEDD4, Su(dx))[87].
- Regulation of Neuralized (Neur) E3-ligase activity — Neur inhibitors, encoded by the Bearded family, are expressed in the cells in which Notch is activated[34,35].
- E(spl)/HES bHLH (basic-helix–loop–helix) and REF-1 repressors — target genes that are upregulated in response to Notch activation encode repressor proteins that inhibit cell-fate promoting genes[107,135].

Together these three mechanisms amplify small differences in signalling activity between cells. However, they do not explain how a difference arises in the first place.

Lineage decisions

The following regulators are asymmetrically segregated into one of two daughter cells in the *Drosophila melanogaster* sensory organ precursors (SOP) lineage to regulate Notch signalling.

- Numb — inhibits Notch receptor through a mechanism that involves endocytosis and another transmembrane protein, Sanpodo[80].
- Neur E3-ligase activity — Neur is asymmetrically segregated into the same daughter cell as Numb, favouring ligand activation[136].
- Ligand trafficking — the recycling endosome (marked by RAB11) is asymmetrically segregated with Neur and favours ligand activity[31]. Mutations in an exocyst component, SEC15, also perturb asymmetrical trafficking[30].

Together these mechanisms result in different signalling capabilities in two daughter cells. The asymmetrical segregation is dependent on the apical basal and planar cell-polarity machinery.

Boundaries/inductive signalling

The following intrinsic differences between two populations of cells translate into distinct signalling populations[46,47,130,137].

- Restricted expression of ligands.
- Restricted expression of Fringe glycosyl transferases — results in modifications to Notch that alter its capability to respond to ligands[47,137].
- Feedback regulation of Fringe expression — can be positive (for example, rhombomere boundaries[137]) or negative (for example, chick somites)[130].

selves be regulated, although it has been shown that Itch is a substrate for the mitogen-activated protein kinase (MAPK) JNK1 in mammalian cells[89] and that the EGF-receptor pathway regulates turnover through ALX-1 and/or WWP-1 in *C. elegans* vulval precursors[87].

A second E3 ligase that binds to Nicd, within the ankyrin repeats, is the RING finger protein Deltex[90]. Increased expression of Deltex in the *D. melanogaster* wing promotes Notch signalling, antagonizes the negative effects of Su(dx), and results in increased Notch accumulation in endocytic vesicles[90,91]. In this tissue, Deltex has also been found to interact with the β-arrestin Kurz, which might therefore mediate the internalization of the Notch–Deltex complex[73]. In agreement with this notion, loss of *kurz* results in elevated membrane levels of Notch, whereas Deltex overexpression has the opposite effect[73,91]. Although *kurz* mutants produce phenotypes of elevated Notch signalling in the wing, *deltex* mutants produce the converse, so the precise relationship is complex. Intriguingly, in several mammalian cells, including lymphoid cells and neurons, Deltex antagonizes Notch, further complicating the picture[16,92]. Perhaps the precise balance of different E3-ligase activities dictates the outcome on Notch localization and activity. These ubiquitin modifications could potentially influence the length of time that the receptor is located on the surface, its accessibility to ligands, or its capability to interact with γ-secretase[67].

It is evident that Notch is subject to different types of post-transcriptional regulation. Glycosylation and proteolytic processing steps have a crucial influence on receptor activity, and are potentially important steps for drug intervention. Ubiquitylation and endocytic trafficking can modulate the amount of receptor that is available for signalling and could therefore provide powerful mechanisms to tune the activity of the pathway.

Differing nuclear landscapes

Following Notch activation, Nicd enters the nucleus and directly regulates the expression of target genes. Among the Notch targets, the best characterized are the bHLH (basic-helix–loop–helix) genes of the E(spl)/HES class. However, the response to Notch differs greatly between cell types — for example, Notch promotes cell proliferation in some contexts and apoptosis in others[3]. The capability to elicit different responses might partly arise from crosstalk with other pathways. It also depends on the enhancers that are responsive to Notch regulation in a given cell.

CSL proteins: pivotal in the switch. CSL proteins are the essential effectors of the Notch pathway (FIG. 4). These DNA-binding proteins have been highly conserved throughout evolution (for example, there is 84% identity between human and *D. melanogaster* proteins). The crystal structure of the DNA-binding domain revealed a striking similarity with the Rel family of transcription factors[93] (BOX 1). Nicd forms a trimeric complex with CSL and the co-activator Mam, which is essential for Nicd-dependent transcription *in vitro* and *in vivo*[94–97]. Another Nicd-interacting protein, SKIP (Ski-interacting protein), a transcriptional coregulator and component of spliceosomes, is also recruited to promoters at the same time[98,99]. Mam in turn recruits the histone acetylase p300, which promotes assembly of initiation and elongation complexes[100].

The assembly of the co-activator complex not only promotes transcription, but also results in turnover of Nicd. The rapidly changing levels of pathway activity require that the nuclear effectors do not have a long half-life. This is achieved by recruitment of factors such as cyclin-dependent kinase-8 (CDK8), which phosphorylates Nicd, rendering it into a substrate for the nuclear ubiquitin ligase SEL10 (REFS 98,101). In mammalian cells, SEL10 preferentially interacts with a phosphorylated form of Nicd and the expression of a dominant negative SEL10 leads to increased expression of Notch targets[102–104]. This interaction requires the C-terminal PEST region, consistent with observations that *Notch* with C-terminal truncations behave as gain-of-function alleles, and in humans they contribute to

AP2 complex
A heterotetrameric trafficking adaptor complex that comprises two large, one medium and one small subunit. It interacts with clathrin and also, through the appendage domain of a large subunit, binds to accessory proteins, including Epsins.

Rel family
Also known as nuclear factor (NF)-κB proteins, this family of transcription factors contains a conserved domain (the Rel-homology domain (RHD)) that is required for DNA binding and dimerization. These proteins are important in defence against infectious diseases and cellular stress.

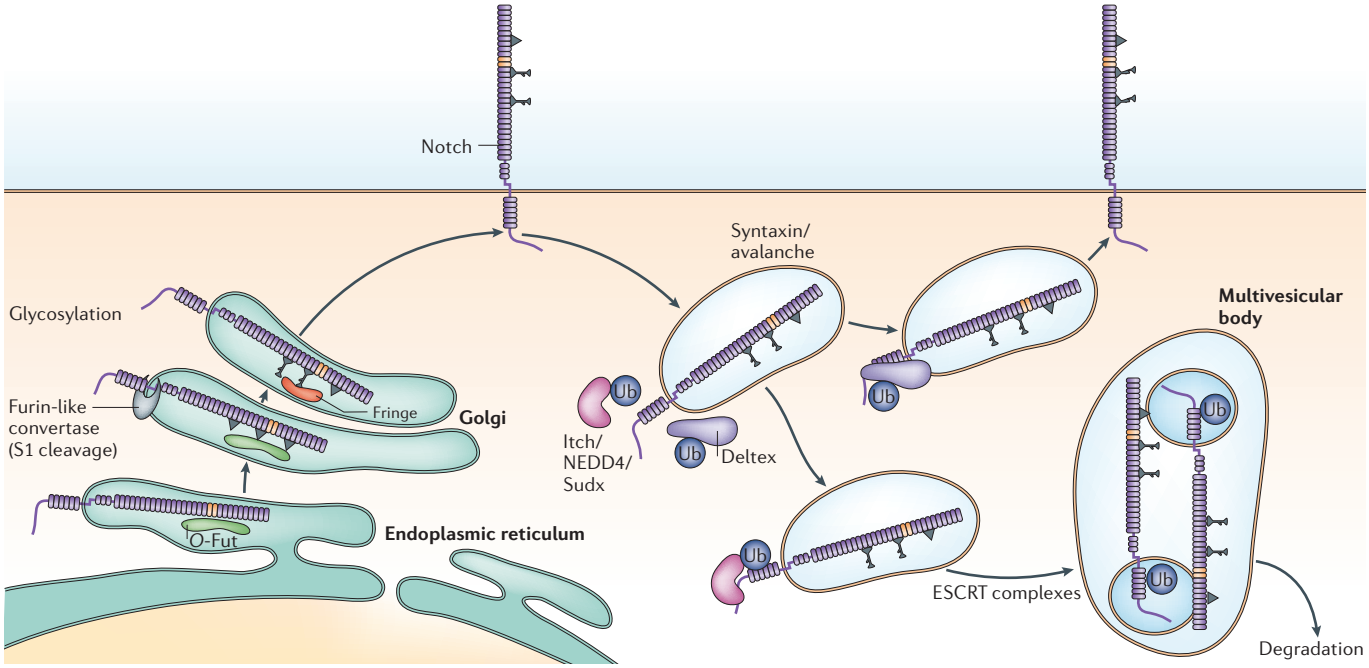


Figure 3 | **Processing and trafficking regulate Notch-receptor activity.** Notch (purple) is produced in the endoplasmic reticulum where it interacts with the *O*-fucosyl transferase (*O*-Fut; green) and is transported to the Golgi. In the Golgi, it is processed by Furin-like convertase (grey, S1 cleavage) and glycosylated (shown as dark grey protrusion from Notch) by *O*-Fut and other glycosyltransferases (for example, Fringe; red) before export to the cell surface. Notch that is endocytosed from the cell surface can be recycled or degraded through the multivesicular-body pathway. Actions of the ubiquitin ligases Deltex (purple) and Itch/NEDD4/Su(dx) (pink) regulate trafficking, although their precise roles are not yet clear. Other proteins (syntaxin, ESCRT complexes) that affect trafficking are indicated, but their sites of action are hypothetical and remain to be fully clarified. Ub, ubiquitin.

oncogenicity[13]. Destruction of Nicd would result in the dissociation of Mam and other co-activators, but it is unclear whether CSL proteins would also be affected or whether they remain intact on the DNA. Simple models predict that they remain on the DNA, and CSL is detected, by chromatin immunoprecipitation assays, at the HES1 promoter after Nicd has dissociated[98].

In the absence of Notch activity, CSL proteins recruit co-repressors. In *D. melanogaster*, the adaptor Hairless tethers the more global repressors Groucho and CtBP, which recruit histone deacetylases[105–107]. So far, no homologue of Hairless has been identified and, in mammalian cells, CSL co-repressors include SMRT (also known as NcoR) and SHARP (also known as MINT/SPEN)[108,109], which in turn recruit CtBP or other global co-repressors. Two other CSL-interacting proteins, SKIP and CIR (CBF1-interacting co-repressor), are also part of the repression complex[99,110]. Homologues of these mammalian proteins exist in *D. melanogaster* and have been linked with CSL or Notch signalling. For example, the SMRT homologue SMRTER is implicated in CSL-dependent repression of Delta expression in the *D. melanogaster* eye and the SHARP homologue Spen affects CSL protein levels in the embryo[111,112]. However, the relationship between the different co-repressors is still unclear; are they all recruited simultaneously or are there different co-repressor complexes, potentially with spatial and temporal differences?

In mice and *D. melanogaster*, the phenotypes that are produced by depleting the single CSL are similar but not identical to loss-of-Notch function. Initially, these differences led to the speculation about CSL-independent Notch signalling. Subsequently, it became clear that many differences could be explained by derepression of target genes, as these CSL mutations also affect the repressive function of CSL[113–115]. It is notable that derepression is modest and is only detected in a small number of cells[113], although it is sufficient to partially rescue the wing primordium defects in *D. melanogaster* presenilin mutants[116]. One example of an essential role for CSL repression is in the SOP lineage, in which it is required for cell-fate specification and later physiological function[107,117,118]. The fact that more global derepression is not observed in the absence of CSL indicates that Nicd and Mam supply essential co-activator function and/or that CSL-repressor complexes are only essential for a small component of target-gene repression.

Epigenetic regulators. The precise mechanisms that are involved in Notch-dependent transcription are not yet known, although studies in mammalian cells have revealed a number of recruited cofactors[98,119]. These include the histone acetyl transferase GCN5 (REF. 120) as well as the SWI/SNF chromatin-remodelling enzyme Brahma (also known as BRM), which interacts directly with CSL proteins in co-immunoprecipitation assays[119].

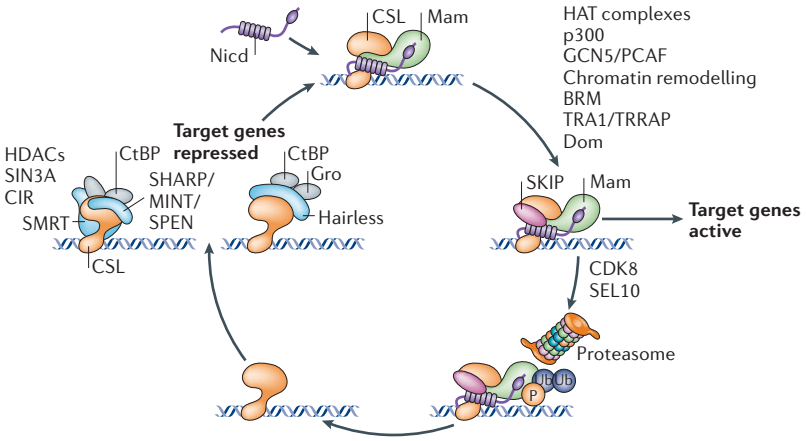


Figure 4 | **Nuclear cycle of CSL.** The Notch intracellular domain (Nicd, purple) forms a trimeric complex with the DNA-binding protein CSL (CBF1, Su(H) and LAG-1; orange) and the co-activator Mastermind (Mam, green). SKIP (Ski-interacting protein), a protein that interacts with the ankyrin repeat domain of Nicd and with CSL is also present. Histone acetyl transferases (HATs; p300 and/or PCAF/GCN5) and chromatin-remodelling complexes (BRM, TRA1/TRRAP and Dom) are recruited and contribute to activation and elongation of transcription at target genes. Kinases such as cyclin-dependent kinase-8 (CDK8) and the SEL10 E3 ligase modify Nicd, making it a substrate for proteosomal degradation. In the absence of Nicd, CSL is associated with co-repressors, the precise composition of which might vary according to species and cell type. Two putative co-repressor complexes are illustrated: a mammalian complex containing SMRT, SHARP (also known as MINT and SPEN) and CtBP, and a *Drosophila melanogaster* complex containing Hairless, CtBP and Groucho (Gro). Although not depicted, SKIP might also be part of the repression complex. The co-repressors recruit histone deacetylases (HDACs; such as HDAC1, HDAC3, HDAC4 and RPD3) and other cofactors (SIN3A and CIR). Target genes are repressed until more Nicd is produced to re-initiate the cycle. P, phosphate; Ub, ubiquitin.

Consistent with these biochemical interactions, the defects caused by reduced Brahma function (using a dominant negative) in *D. melanogaster* are enhanced by mutations that reduce the activity of the Notch pathway[121]. Similarly, mutations in TRA1 (also known as TRAPP; involved in the recruitment of the chromatin modification complexes SAGA and TIP60) and in Domino (a histone exchange protein in the TIP60 complex) enhance the phenotypes of some *Notch* and *mam* alleles[122].

The emerging picture is that Notch signalling requires recruitment of histone acetylase complexes and exchange of histone variants to activate transcription. In addition, BRE1, a homologue of the yeast histone 2B ubiquitin ligase, is crucial for Notch function *in vivo* and stimulates Notch-dependent transcription in a transient transfection assay[123]. Sumoylation might also regulate the activity of key nuclear components[124]. Together the data show that Notch activity is highly sensitive to chromatin modifications and histone re-arrangements that could contribute to target-gene specificity. Furthermore, overexpression of two Polycomb group epigenetic silencers enhances Notch-induced over-proliferation and also causes hypermethylation of a tumour suppressor gene[125], indicating further mechanisms that could constrain the accessibility of enhancers and cooperate with Notch to confer different programmes of gene expression.

Cooperation with tissue-specific activators. CSL sites alone are often poor at mediating activation *in vivo*, indicating that Nicd functions in combination with tissue-specific factors[126]. The best-characterized examples are the proneural bHLH proteins, which synergize with Nicd in *D. melanogaster*; there is as yet no evidence for them having a similar role in vertebrates[107,114,127]. During neurogenesis, the proneural proteins are expressed in groups of cells in which they promote neural development. Activation of Notch in a subset of cells within each group results in expression of target genes such as those of the *E(spl)* family. Analysis of the regulatory sequences from these genes revealed both CSL- and proneural-protein-binding sites[114]. Mutations in the proneural-protein-binding sites eliminate activation of the target genes[107]. Therefore, binding of tissue-specific activators contributes to robust target-gene expression, and can explain the specificity of Notch responses in different cell types. In some cases, the precise arrangement of binding sites influences the cooperation between Notch and other DNA-bound activators[128,129]. Also, during lateral inhibition, and in the somite clock, the targets themselves feedback to inhibit their own transcription, thereby ensuring a transient burst of transcription in response to Notch activation[130]. However, barely a handful of tissue-specific activators that work in cooperation with Notch have been identified, so the extent and diversity of such factors is still unclear.

Making a difference

How are these different regulatory mechanisms deployed in different Notch-dependent processes? Examples are given in BOX 3. Here I would like to highlight two aspects of this deployment. First, the activity of the receptor and its ligands can be controlled in various ways. Different mechanisms might be harnessed to establish or amplify differences between cells in specific contexts. Much of this regulation affects the subcellular distribution of the proteins, most particularly their trafficking in the endocytic pathway and/or their post-translational modification, such as glycosylation, ubiquitylation and most probably phosphorylation. Furthermore, several mechanisms are frequently employed together to enhance the efficiency of signalling. For example, there might be positive regulation of the ligand and downregulation of the receptor in the same cell, making for a more robust mechanism[32,86,131]. Furthermore, these mechanisms can be targeted in feedback loops, as is exemplified by the polypeptide inhibitors of Neur[34,35] (BOX 3).

Second, if the pathway responds to dynamic fluctuations in its environment and/or if it is used iteratively, there must be mechanisms to ensure that the response is short-lived. This is particularly important in processes such as the somite clock, in which Notch activity oscillates — autoinhibitory feedback of the E(spl)/HES Notch targets and the regulation of Lunatic Fringe are two mechanisms that contribute to this oscillatory Notch activity[59,130]. In addition to negative-feedback mechanisms, transient signalling is favoured by destruction of Nicd after recruitment of transcription initiation factors on target enhancers[98,101]. Furthermore, the

Somitogenesis
The development of somites, the segmental blocks of mesoderm that give rise to the axial skeleton, muscles and dermis.

proteins and mRNAs from target genes are, in many cases, rapidly degraded. For example, mutations that increased the half-life of *Hes7* mRNA disrupted somitogenesis, which highlights the importance of rapid turnover[132]. The short half-life of many target-gene mRNAs can be explained by the fact that they are subject to regulation by microRNAs (miRNAs), as seen with the *E(spl)/HES* genes[133,134]. Mutations that affect miRNA target sites or miRNAs themselves can result in phenotypes resembling increased Notch activity, underscoring the importance of this regulation.

An important area that has not been discussed in this review because of space limitations is the crosstalk between Notch and other signalling pathways. For example, what are the mechanisms that control the regulators of the Notch pathway? And, to what extent can the core components of the Notch pathway be modified by or interact directly with components from other pathways? These additional levels of regulation are likely to contribute to Notch-pathway activity in development and disease.

Future Directions

What emerges from much of the recent literature is that the precise location of the Notch ligand and the receptor in the cell can have profound effects on signalling. Currently it is very difficult to determine what the different routes of trafficking are and how exactly they impact on activity. Other obvious holes in our understanding are; how does a ligand interaction promote the second cleavage of Notch, the key first step towards ligand–receptor interaction; and what ubiquitin-dependent step creates a productive ligand? More sophisticated *in vivo* imaging with methods to allow tracking of proteins in different states (for example after a specific ubiquitylation) is required to unravel these mechanisms. We also need to know how these modifications impact on the length of time Notch and its ligands are actually at the cell surface and able to interact and how they impact on the stability of Notch following activation. It is evident, therefore, that methods for monitoring activity in real time are needed to understand the dynamics of signalling in each developmental process.

Understanding how and why different target genes are activated according to cell type and time are another set of important issues. Currently, the characterized direct targets of Notch activity can be counted on a few fingers, but even in those cases we have little knowledge about what makes a gene a target in specific cells. Furthermore, with the few identified targets, it is difficult to explain the varied consequences of Notch activation. Genome-wide studies are likely to increase the spectrum of targets and allow the development of a systematic approach for understanding the different responses.

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Acknowledgements
My apologies to all those colleagues whose important contributions have not been acknowledged due to the space constraints of this Review. My thanks to A. Krejci, M. Glittenberg, A. Djiane and the reviewers for helpful comments, and to F. Wirtz-Peitz and J. Knoblich (Institute of Molecular Biotechnology, Vienna, Austria) for the beautiful image of SOPs in BOX 2. Work on Notch signalling in my laboratory is currently supported by grants from the Medical Research Council, the Wellcome Trust and the Association for International Cancer Research.

Competing interests statement
The author declares no competing financial interests.

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