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Review article

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Modes of Notch signalling in development and disease

Sarah J. Bray [1] & Anna Bigas [2,3]

Abstract

Many different animal developmental and homeostatic processes rely on signalling via the highly conserved Notch pathway. Often Notch signalling has iterative roles during cell specification and differentiation, controlling not only the state of progenitor cells but also the fate and function of their progeny. Its roles continue throughout the lifespan of the organism, regulating normal tissue maintenance, as well as operating in response to damage. Consistent with such fundamental roles, the pathway has been associated with numerous diseases, including cancers. Understanding how Notch signalling is orchestrated to bring about different outcomes is challenging, given that it has many diverse functions. Classic models proposed that stochastic differences in cell states were important to polarise signalling during cell fate decisions. Subsequently, the importance of oscillatory Notch signalling was uncovered, and it became clear that it operates in different modalities depending on the regulatory inputs. With the advent of ever-more-sensitive live-imaging and quantitative approaches, it is becoming evident that differences in the dynamics, levels and architectures of Notch signalling are critical in shaping and maintaining tissues. This Review focuses on the cellular and molecular mechanisms involved in conferring different modalities on Notch pathway operations and how these enable different types of functional outcomes from pathway activation. We also discuss their dysregulation in cancer.

Sections

[1]Department of Physiology Development and Neuroscience, University of Cambridge, Cambridge, UK. [2]Program in Cancer Research, Institut Hospital del Mar d'Investigacions Mèdiques, CIBERONC, Barcelona, Spain. [3]Josep Carreras Leukaemia Research Institute, Barcelona, Spain. e-mail: sjb32@cam.ac.uk

Introduction

Notch signalling has a pivotal role in many cell fate and tissue patterning decisions during animal development, and it continues to be involved in tissue homeostasis and regeneration throughout life[1–4]. Excessive or compromised Notch activity contributes to diseases, including cancers[4–6]. Like the other handful of developmentally important signalling pathways, the Notch pathway is highly conserved, with homologues present in species throughout the animal kingdom. Over a century of research has unveiled the full complexities of its regulation[7–9], but at its heart there is a simple Notch signalling pathway (Fig. 1), which will be the focus of our Review.

Transmembrane Notch proteins, the receptors in this pathway, are activated by transmembrane ligands of the Delta or Serrate/Jagged families that are present on the surface of cells in contact[9,10] (Fig. 1 and Box 1). This interaction results in the proteolytic release of the Notch intracellular domain (NICD), which drives the transcriptional activation of target genes in the nucleus by forming a complex with the DNA binding protein CSL (CBF1, Suppressor of Hairless, Lag1), also known as RBPj[11], and co-activators of the Mastermind family (MAM)[12–14].

Insightful structural, biochemical and genetic studies have revealed that Notch activation involves a mechanoallosteric change in the receptor[9,10]. A mechanical force, usually generated by endocytosis of the ligand, opens up a juxtamembrane domain in Notch — the negative regulatory region (NRR) — exposing a cleavage site for Adam proteases[10,15–17]. This cleavage releases the Notch extracellular domain, which is *trans*-endocytosed into the ligand expressing cell, and leaves the NICD anchored via a transmembrane domain. This fragment in turn, with its short residual exposed extracellular sequence, is a substrate for the γ-secretase complex that liberates NICD[18–20] (Fig. 1).

Given that Notch activity is fundamental for fate decisions, differentiation, proliferation and patterning of highly organized tissues, how can this simple transduction mechanism translate into a diversity of functions? Part of the answer lies in the context provided by other intercellular signals and cell-type specific transcription factors. But, importantly, the pathway also functions in different modes, depending on the downstream nuclear circuitry, the deployment of ligands, the presence of modulatory factors and the architecture of tissues (Table 1). Notably, it is becoming evident that these vary temporally and spatially to confer different levels and patterns of signalling that, in turn, generate the appropriate cell types essential for tissue development and maintenance[21,22]. Furthermore, live imaging is revealing how different modes of signalling even evolve during the timescale of a cell fate decision[23,24].

In this Review, we describe the different modes of Notch signalling and the molecular and cellular mechanisms that lead to these different signalling modes. To do so we discuss Notch signalling at multiple levels, including spatiotemporal organization, dynamics and feedback mechanisms, and how these different adaptations are suited for different types of cellular decision-making. Conversely, the normal modes are disrupted in cancer and the nature of those disruptions has implications for therapeutic interventions.

Stochastic Notch signalling and lateral inhibition

In developing tissues, different cell types are frequently generated through lateral inhibition Competitive interactions between equivalent competent cells lead to singling out of a precursor cell (for example, a neural precursor) that inhibits the neighbouring cells from following the same fate[25,26]. This core principle of cell patterning is driven by Notch signalling (Fig. 2).

At the heart of lateral inhibition is a negative feedback loop that amplifies small initial differences in Notch ligand and receptor activity among neighbours. The cell that gains higher ligand expression or activity (for example, Delta) can more effectively activate Notch in its neighbours, driving them towards the opposite fate (high Notch, low Delta). Original models proposed that a noisy fluctuation of sufficient

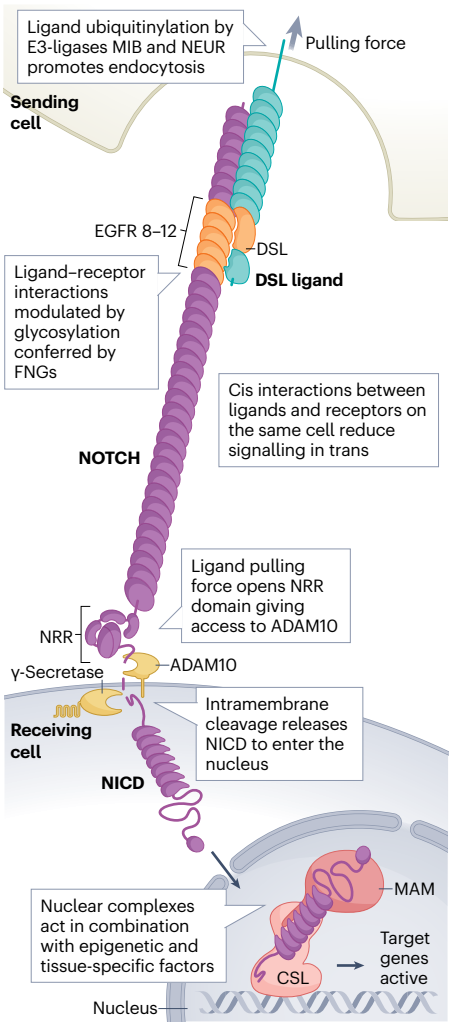


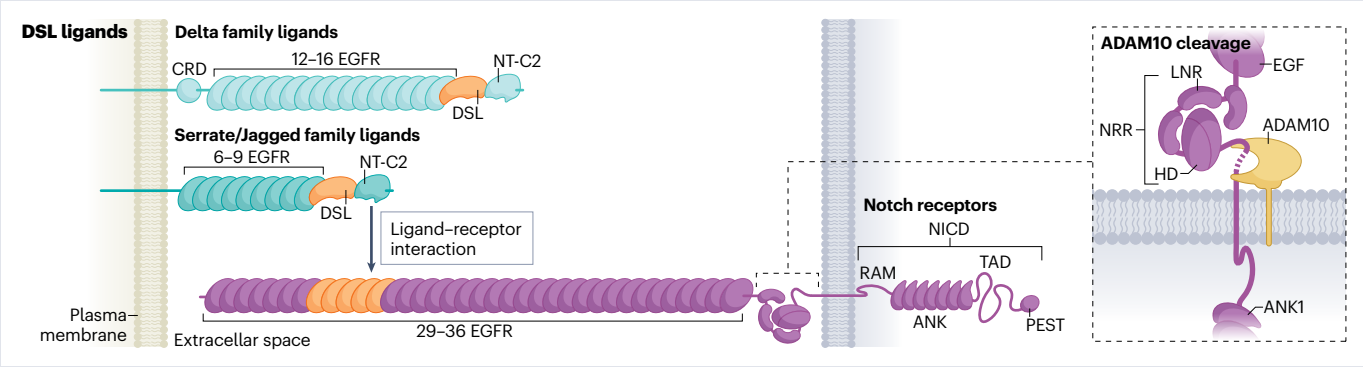
Fig. 1 | Overview of the core Notch pathway. Transmembrane ligand (turquoise) expressed on the signal sending cell interacts via its DSL (orange) and N-terminal C2 domain with the transmembrane NOTCH receptor (purple) on adjacent cells, directly contacting EGFR 8-12 (orange). Some EGFR in the ligands are also involved in receptor binding and activation[160] (Box 1). A pulling force, usually driven by ligand endocytosis and requiring ubiquitination of ligand intracellular domain[17], brings about a change in conformation of the juxtamembrane negative regulatory region (NRR), exposing a site for cleavage by ADAM metalloproteases, (brown), predominantly ADAM10 in physiological conditions[15]. Other sources of force or ligand patterning have also been suggested to promote activation[161–163]. The residual transmembrane moiety is a substrate for γ-secretase (yellow) and the intramembranous cleavages releases Notch intracellular domain (NICD). NICD forms a tripartite nuclear complex with the DNA-binding protein CSL (CBF1, Suppressor of Hairless, Lag1; light pink) and the coactivator MAM (Mastermind; dark pink)[12,13,164] that promotes transcription of target genes, in collaboration with other factors. DSL, Delta, Serrate and Lag2; FNG, Fringe; MIB, Mindbomb; NEUR, Neuralized.

Box 1 | Diagram summarizing properties of Notch receptors and ligands

In flies and vertebrates, there are two families of ligands (turquoise) — Delta/Delta-like and Serrate/Jagged — that have slightly different structures. The different structures confer different affinities for Notch receptors to the ligand, and varying degrees of susceptibility to modulation by receptor glycosylation[165,166]. They all contain an amino-terminal C2-like domain (NT-C2), a Delta, Serrate and Lag2 (DSL) domain and a sequence of EGF-like repeats (EGFR). Members of the Serrate/Jagged family also contain a cysteine-rich (CRD) domain. Ligands in *Caenorhabditis.elegans* contain EGFR but are otherwise quite divergent.

All Notch receptors (purple) share the same basic structure, with a large extracellular domain comprised of 29–36 EGFRs and an intracellular domain with a structured core of ankyrin repeats (ANK), flanked by intrinsically disordered regions, RAM and transactivation domain (TAD)[10,165]. The negative regulatory region (NRR) region in the extracellular domain (encompassing three LIN-12/Notch repeats (LNR) and the juxtamembrane heterodimerization domain (HD)) is configured so that a conserved hydrophobic plug sterically obstructs the ADAM metalloproteinase cleavage site[128]. RAM and ANK are essential for nuclear complex formation, both binding directly to CSL. The interaction of ANK with CSL creates a 'groove' into which the N-terminal helix of Mastermind sits[12,13]. A serine–threonine rich PEST domain is a site for post-translational modifications that regulate NICD stability[129]. Many EGFRs in the Notch extracellular domain can be modified with sugars, including those mediated by Fringe glycosyltransferases that regulate Notch–ligand interactions[61].

Drosophila melanogaster contains one Notch receptor, whereas mammals possess four Notch receptors (Notch1–4), which differ in the numbers of EGFR (36 in Notch1,2; 34 in Notch3; 29 in Notch4) and in the disordered regions at the C-terminus (TADs have only been defined in Notch1 and Notch2). Lin-12 and GLP-1, the receptors in *C. elegans* contain 14 and 11 EGFR, respectively. Differences in the intracellular domain of Notch paralogues influence their transcriptional potency[145].



magnitude between cells expressing similar levels of Notch and Delta would create an initial difference that would be amplified by a negative feedback loop. This is the 'classic' model of lateral inhibition[27] (Fig. 2).

The concept that subtle differences between cells are sufficient to generate a template for lateral inhibition arose in part from experiments with genetically mosaic tissues. The simplest scenario involved two apparently equivalent cells in *Caenorhabditis elegans* that take on different roles in vulval development: one becomes an anchor cell (AC), which is required for vulva induction, whereas the other becomes a ventral uterine precursor cell (VU). On the basis of ablation experiments, each have an equal chance of becoming an AC and the outcome relies on signalling via the *C. elegans* Notch, LIN-12. In genetic mosaic experiments, whichever cell lacked LIN-12 activity always became an AC, and it was proposed that initial signalling differences would be reinforced by a feedback loop to drive a stable fate decision[28,29].

Similar results emerged from experiments following sensory organ development in flies, in which Notch signalling is required for a single sensory organ precursor (SOP) to be selected from a group of cells with proneural potential (neural fate being repressed by Notch). In genetically mosaic flies in which patches of cells with different gene dosage for Notch or Delta were juxtaposed, the position of sensory organs along the mosaic boundaries was biased, preferentially forming in cells that had lower Notch or higher Delta[30]. This argued that SOP selection depended on the relative levels of Delta or Notch, and it was proposed that, if activation of Notch diminished the ability to produce active Delta, it would give rise to a feedback loop that amplified differences between adjacent cells as in worm AC and VU cells. Subsequent experiments probing roles of proneural (*Achaete-scute*) and HES (*Hairy* and *Enhancer of split*) gene families argued that these factors are involved in the regulatory loop between Notch and Delta[31–34] (Fig. 2). Regulatory nodes affecting ligand activity and stability may also contribute.

Many different biological contexts exhibit properties like those established for vulval precursors and SOPs, indicating that they rely on this mode of Notch signalling[2]. Initial models posited that the lateral inhibition feedback loop could generate spatial patterns from random stochastic fluctuations among equivalent cells, provided that the feedback loop was strong enough[27]. Probing into molecular requirements for its robustness has highlighted additional aspects of regulation[35]. Furthermore, a synthetic circuit mimicking the transcriptional feedback was not, on its own, sufficient to generate cell fate bifurcation from genetically identical cells. A modulatory circuit reinforcing Notch activation, involving the Fringe (FNG) glycosyl transferase which modifies EGF repeats in the Notch extracellular domain (Box 1) was required to robustly generate two cell fates[36]. Thus, although an attractive model, it is unclear whether situations exist in vivo in which stochastic differences between ligand and receptor

levels are sufficient to set in motion the lateral inhibition circuit and drive cell fate decisions.

Indeed, more recent studies suggest that initial differences in the activity of Notch ligand and receptor between *C. elegans* AC/VU cells are not, as originally envisaged, due to stochastic noise. Instead, they are prefigured by the relative time when a key transcription factor (HLH-2) regulating LIN-12/Notch expression is first expressed[37]. This in turn is biased by the relative birth order of the two cells. Thus, events in ancestors create an initial difference that is then reinforced via LIN-12, Notch signals to resolve their fates[37]. Recent live-imaging studies also suggest a much more dynamic restriction of cell competence among Drosophila SOPs that involves different phases, and only transitions into strong lateral inhibition once the neural precursors start to emerge[23].

In both these scenarios the landscape of the ligand and receptor expression is not totally uniform. Subtle differences between cells create an initial imbalance in signalling that is reinforced by lateral inhibition, although the tissue retains the ability to accommodate last minute rearrangements or cell losses. Another type of imbalance that could bias signalling is the relative size and/or contact surfaces between cells. A model of lateral inhibition that incorporated a variable from contact area predicted that smaller cells would be more likely to adopt precursor 'Notch OFF' state. Indeed, chick inner ear precursor cells had, on average, a smaller apical area than other neuroectodermal cells, but whether this was a cause or consequence of them acquiring precursor fate was not tested and no systematic size bias was detected in a another context of proneural lateral inhibition[38–40].

The concept that ancestry or cell shape can pre-figure the bias in lateral inhibition means that stochastic or random fluctuations in Notch may have only a minor role in most patterning decisions. But, even if it predominantly operates in an already uneven landscape, the lateral

Table 1 | Summary of Notch signalling modes

Signalling mode and its features	Examples of cells and processes	Organism	Representative refs.
Stochastic Signalling between equivalent cells Inhibitory feedback loop	Neurogenesis	Multiple	25,26
	Gonad and vulval precursors	*Caenorhabditis elegans*	28,37,159
	Sensory organ precursor	*Drosophila melanogaster*	23,30,32
	Inner ear precursor cell	Chick	38,39
Oscillatory Direct autorepression of HES/Her genes Coupled by Notch signalling	Somitogenesis	Multiple	44–48,50,53,54
	Neural stem cells	Mouse	65,67
	Muscle satellite cells	Multiple	66
	Spinal cord precursors	Mouse	69
	Pancreatic progenitors	Mouse	22,72
Sustained Distinct signal-producing population No negative feedback regulation May involve positive feedback or stabilization Notch intracellular domain	Maintenance of intestinal stem cells	Mouse	84
	Maintenance of lung club cells	Mouse	85,86
	Wing dorsal–ventral boundary	Drosophila	78,87,89
	Germ layer boundary	*Xenopus laevis*	90
	Striatal astrocytes	Mouse	91
Digital (ON/OFF) Binary ON/OFF decision Frequently involves segregation of pathway inhibitors (e.g., Numb) or activators (e.g., Mindbomb, Neuralized)	Neural lineages	*D. melanogaster*	100,101
	Sensory organ lineages	*D. melanogaster*	98,102,103
	Radial glia	Mouse	104
	Lineage segregations	Multiple	105,106
	Mammary stem cell potential	Mouse	107
Analogue (graded) Graded levels or duration of signalling May involve deployment of different ligands, Fringes and/or *cis* inhibition May involve variations in cell contacts and processes	Haematopoietic commitment	Mouse	110–112
	Angiogenesis	Multiple	11,113,114
	Mucociliary cell fates	*X. laevis*	117
	Floorplate progenitors	*Zebrafish*	97
	Brain neural progenitor fates	Mouse	71,116
	Neural Stem cell quiescence	*D. melanogaster*	119
Pathogenic Activating or inhibitory mutations in Notch receptors Aberrant expression of ligands	T cell acute lymphoblastic leukaemia-activating mutations in Notch1,2	Human, mouse	121,127,132–134
	Colorectal cancer	Human, mouse	137
	Mammary gland luminal tumours	Human, mouse	139
	Tumour promoter in solid tumours	Human	140,141
	Skin tumour suppressor	Human, mouse	136

inhibitory Notch signalling mode, with its inherent negative feedback, is fundamental to many cell-fate decisions and is deployed in multiple contexts throughout tissue development and homeostasis.

Oscillatory Notch signalling

Although lateral inhibition is a powerful Notch signalling mode to generate alternate cell states, formation of regular sized somites and maintenance of many stem and progenitor cells requires oscillatory Notch activity (Table 1). Such oscillations are, in essence, a time-keeping mechanism that can be harnessed either to form precise spatial patterns, as in somitogenesis, or to generate a cycle of transition opportunities, as in progenitor cells. In the latter, each oscillation creates a brief window of opportunity for cells to transition into a more differentiated state. Any coupling between oscillating cells generates a higher order of patterning, with cells oscillating in phase or antiphase depending on the nature and strength of coupling[41–43].

A key factor in establishing oscillations is direct autorepression of the HES genes (also known as HES-related (Her) in fish) by their own protein products. This autoregulation generates an intrinsic intracellular oscillator. When coupled to Notch signalling, a tissue-level regulation

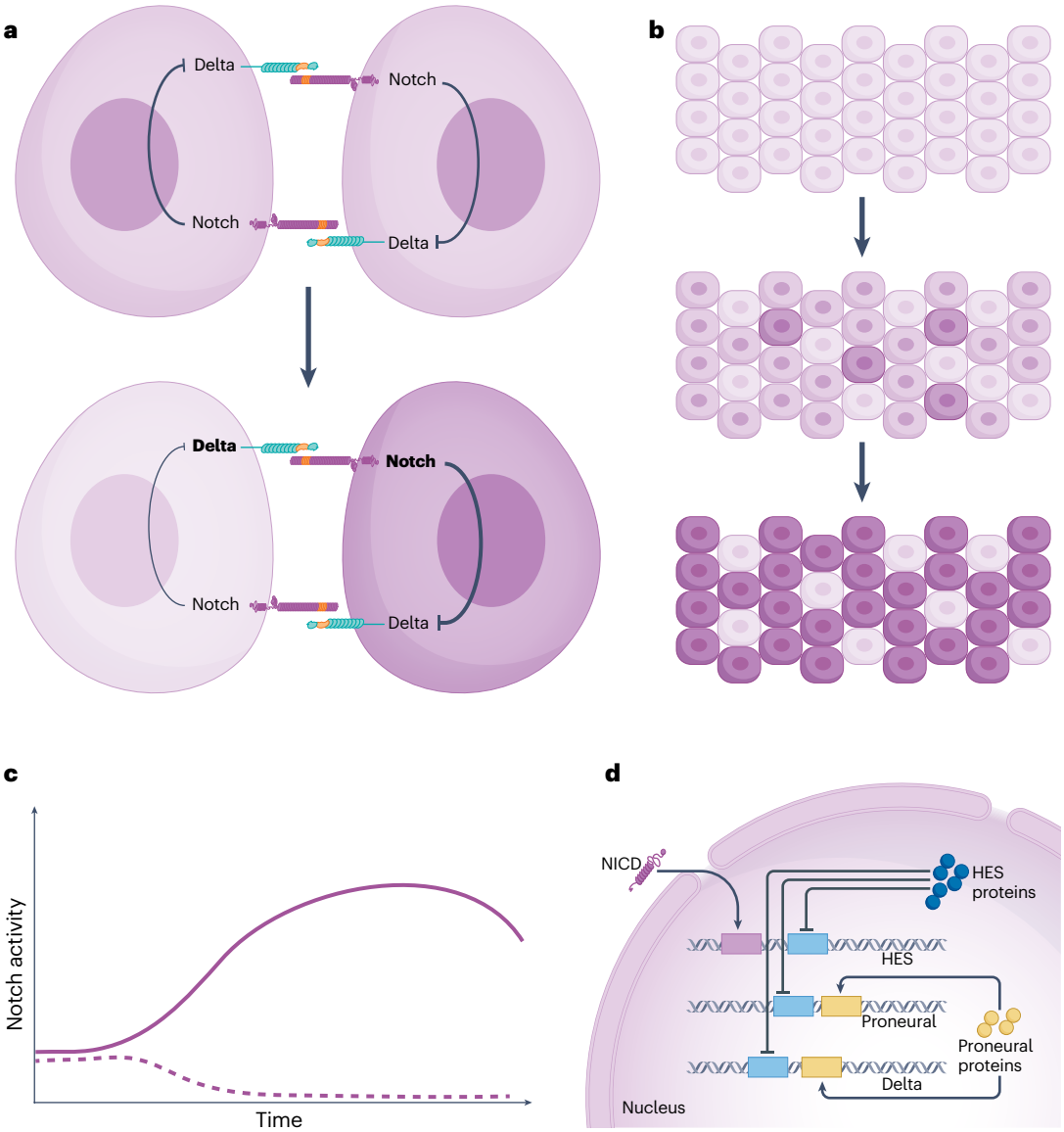


Fig. 2 | Stochastic Notch signalling and lateral inhibition. a,**b**, Cartoons illustrating signalling between two equivalent cells resulting in stochastic bias (**a**) and consequences in a cell field (**b**). Notch activity indicated in purple shaded according to activity levels. **c**, A graph that illustrates the hypothetical Notch activity levels in the two cells (the solid line shows the cell acquiring higher Notch and the dashed line shows the cell acquiring higher Delta). **d**, A schematic summarizing the feedback mechanisms involved in classic lateral inhibition. Direct target genes of Notch intracellular domain (NICD), the genes *Hairy* and *Enhancer of split* (HES), produce HES proteins that inhibit proneural and Delta genes. No HES is produced in the absence of NICD, allowing proneural proteins to accumulate and upregulate Delta expression (modified from ref. 120).

is imposed (Fig. 3). As activation of Notch stimulates expression of HES/Her genes, their transcription will be governed both by positive regulation from activated Notch and by autoinhibition from HES/Her proteins. The balance in these two inputs, and their modulation, will shape the oscillatory profile.

Rhythmic oscillations of HES/Her gene activity are responsible for the sequential division of the paraxial mesoderm into somites[44,45]. Inhibiting Notch activity, for example, by treating zebrafish embryos with a γ-secretase inhibitor, leads to a loss of coherence in the oscillating gene expression and often to a decrease in amplitude, but does not abolish it[46]. At the same time, isolated paraxial mesoderm cells exhibit oscillatory HES/Her gene expression but the cycles are heterogeneous and unsynchronized[47,48]. Such oscillating paraxial mesoderm single cells can however self-organize to generate waves in gene expression through Notch-dependent synchronization[49,50]. When precursors cycling with different phases were mixed, strikingly they all acquired the phase of the more advanced phase in a 'winner-takes-all' synchronization[51]. These experiments illustrate the importance of cell–cell Notch signalling in coordinating cell-intrinsic HES oscillations. It may also be important for maintaining the amplitude of oscillations over time[52].

Early models predicted that stable oscillations of Notch signalling rest on three key principles: (1) negative feedback regulating transcription, (2) short half-lives of oscillating mRNAs and proteins, and (3) delay times between transcription and protein production. The delay was a key feature in explaining how direct autorepression of HES/Her genes can produce an intracellular oscillator. In a similar manner, the time taken to implement the Notch cell–cell signalling (time delay) is important in establishing the phase of oscillations when cells are coupled by Notch activity[42,53,54] (Fig. 3).

Two interesting properties emerged when parameters were adjusted in models. First, the Notch signalling control circuit could generate oscillations, independent of a HES/Her autorepression loop. Second, linking the two via HES/Her-mediated repression of ligand expression, the mechanism invoked for lateral inhibition, also resulted in oscillations. How can the same regulatory loop generate such different outcomes? Varying relative contributions from the two regulatory loops and the degree of 'noise' in the system, was sufficient to switch from oscillatory to ON/OFF steady states[55] (Fig. 3). In agreement, variations in fundamental characteristics, such as protein stability, levels of microRNAs and transcription noise within these loops, can dramatically change the outcome from Notch signalling[56–58].

The presence of different modulators is important in phasing Notch oscillations. These include the lunatic fringe (LFNG) glycosyl transferase that modifies EGF repeats in Notch receptors and ligands[59–62]. Oscillatory expression of LFNG results in periodic glycosylation of receptor and ligands at specific stages of the clock. Recent studies suggest that, in mice, LFNG prevents inhibitory *cis*-interactions (between Notch and Dll3) yielding higher proportions of the active cell surface receptor when it is present[63]. As knockout of LFNG altered the amplitude of HES7 oscillations in receiving cells in cell-mixing experiments but not the intrinsic HES7 oscillations in isolated cells, it was proposed that LFNG adjusts the coupling delay to favour robust oscillations[48].

In many tissue types, dynamic HES autorepression is important in maintaining undifferentiated progenitors[64]. For example, in mouse embryonic neural stem cells, HES1 oscillations periodically repress expression of proneural genes (*Ascl1*) and ligands (*Dll1*) so that these genes also oscillate, and a salt-and-pepper expression pattern is

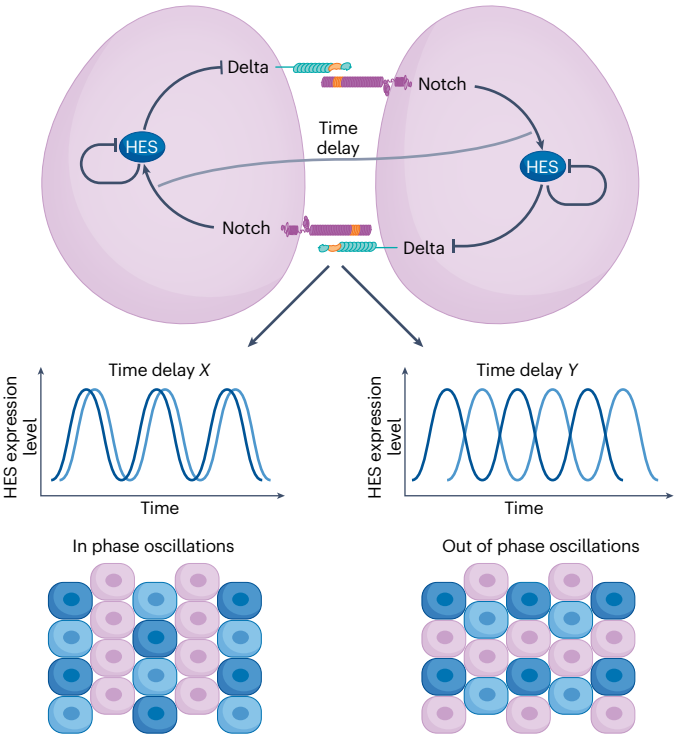


Fig. 3 | Oscillatory Notch signalling. Cartoon illustrating the feedback loops in two cells that leads to oscillatory HES (Hairy and Enhancer of split) expression. The time delay of signalling is influenced by factors, including the length and intron number of the specific HES genes upregulated and/or the presence of Fringe glycosyltransferases, which glycosylate EGF-like repeats and modify ligand–receptor interactions. As a consequence, oscillations in neighbouring cells may be in phase or out of phase as illustrated by the graphs, with outcomes on HES expression levels (darker and lighter blue shading) in cell fields as below.

detected in fixed tissues[65]. Similar oscillations occur in muscle satellite cells[66]. This dynamic oscillatory HES activity regulates quiescence and cell cycle entry and prevents stable Ascl or MyoD expression and differentiation[67].

What is the role of Notch signalling in these oscillating precursors? It was proposed that neighbouring cells can mutually activate Notch signalling via their Dll1 oscillations, leading to neighbouring progenitors having antiphase Notch oscillations[68]. In contrast, in the spinal cord region, recent live imaging of HES5 expression revealed microclusters of cells with positively correlated HES5 expression. This argues for a mode of coupling through Delta–Notch signalling more akin to that during somitogenesis, and models that invoked a weak coupling strength along with an intercellular time delay mimicked the emergence of dynamic microclusters[69].

Clearly, if oscillatory patterns are shaped by an intercellular time delay (Fig. 3), they will be influenced by parameters that include the time required for ligand synthesis. In agreement, changing the *Dll1* gene length, by deleting introns, dampened HES1 oscillations, impairing neural stem cell maintenance and proliferation, leading to accelerated neurogenesis and microcephaly[70]. Similarly, when Dll1 oscillations in myogenic stem cells were perturbed (for example, by a mutation in the regulatory enhancer) this accelerated the timing

of myogenic differentiation, precluding self-renewal[71]. Disparities between HES1 oscillations in prenural tube cells and in neighbouring presomitic mesoderm cells, which both originate from the same neuromesodermal progenitors, are ascribed to differences in ligand expression, with only the presomitic mesoderm exhibiting high levels of oscillatory Dll1 (ref. 24).

Dynamics and patterns of oscillations are also modulated via *cis* inhibition or via deployment of different ligands. Oscillating expression of HES1 in pancreatic progenitor cells involves a combination of ligand-mediated *trans* activation from neighbouring cells and indirect ligand–receptor *cis* interactions[72]. An interplay between Dll1 and Jag1 ligands occurs; Dll1 is required for the periodicity of oscillations that maintain progenitors, Jag1 *cis* inhibition facilitates cell fate segregation. It is argued that different types of neighbouring cell (epithelial and mesenchymal) will contribute different amounts of Dll1 and Jag1 ligands compared with their epithelial neighbours. In this manner, the 3D architecture influences the balance of signalling and, in turn, the proportions of progenitors and differentiating cells[22].

Sustained Notch signalling

Rather than a finite or oscillatory signal, the maintenance of progenitors and the establishment of stable boundaries between cell populations frequently require prolonged or sustained Notch signalling. Three features help to generate periods of sustained Notch activity. First, signalling is uncoupled from the negative feedback regulation, which is a signature of lateral inhibition and oscillatory modes. Second, a distinct signal-producing population is involved, as exemplified in several progenitor types in which sustained Notch activity is maintained by ligands produced from a niche (Fig. 4). Third, conditions that prolong or stabilize NICD increase the tendency for sustained signalling. For example, the prolyl-isomerase Pin1 modifies the intracellular domains of Notch1 and Notch4 to prevent their ubiquitylation and proteasomal degradation[73,74].

The absence of negative feedback relies on the upregulation of target genes with different properties. For example, a slowly dividing subpopulation of embryonic neural progenitors that includes the precursors of adult neural stem cells, is explained by their expression

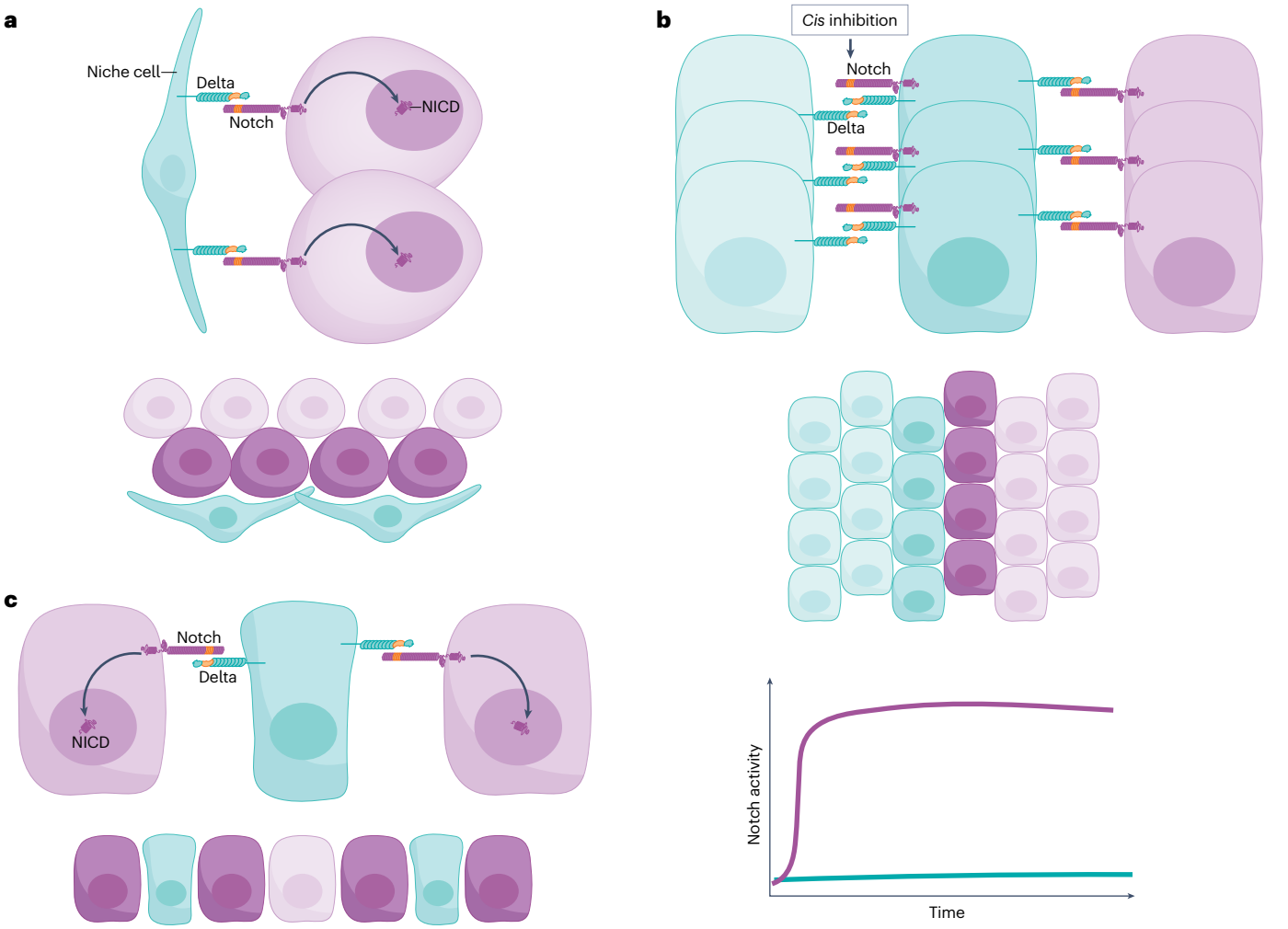


Fig. 4 | Sustained Notch signalling. a–**c**, Cartoons illustrating three cell arrangements leading to sustained signalling and consequences in the cell field: DSL ligands provided by niche cells (**a**), boundary cells of adjacent domain (turquoise) (**b**) or tissue-specialized cells (turquoise) (**c**) (as in paneth cells of the intestine) activate Notch receptors (purple) on neighbouring cells. Notch activity is indicated by purple shading of the cells with graph depicting hypothetical profile of Notch activity in Notch active cell (purple line) and ligand-producing cell (turquoise line). NICD, Notch intracellular domain.

of Hey1 rather than HES5. As Hey1 mRNA has a longer half-life compared with HES5 and its promoter does not manifest the same negative regulation, Notch activity will be maintained without promoting oscillations[75]. Likewise, in *D. melanogaster* embryos, Notch activity induces sustained HES expression through cooperation with regional specific transcription factors[76]. In the pituitary gland, where Notch signalling has an essential role in controlling the lineage commitment of Pit1$^+$ precursors, it does so by directly regulating expression of a distinct target gene, *Prop1*, that makes them competent to follow a later-born cell fate[77]. In these contexts, the genes that are turned on do not attenuate signalling.

Additionally, in some contexts, direct targets include genes encoding Notch ligands. This will tend to intensify and perpetuate ligand expression and to sharpen boundaries between regions. Examples include the dorsal–ventral boundary of *D. melanogaster* wing imaginal discs[62,78], the prosensory field in the chick inner ear[79] and the differentiation of cardiac neural crest cells into smooth muscle cells[80,81]. One question is what prevents these systems spiralling out of control? It has been postulated that, once ligands exceed a certain threshold, they will become *cis* inhibitory so that signalling is self-limiting[78,82]. Additionally, the presence of LFNG and MFNG can modulate activity of Dll-type and Jag-type ligands by altering their capacity for *cis* inhibition, as occurs in the cochlea, where they also render the Notch receptor less sensitive to Jag1 signalling from neighbouring cells[83].

A second feature enabling sustained signalling is a distinct ligand-producing source (Fig. 4). In many instances, neighbouring cells with a different identity or specialization provide the ligand, a signalling mode sometimes referred to as lateral induction when it promotes an alternate fate. In intestinal crypts, differentiated Paneth cells constitute the niche for Lgr5 stem cells, acting as a source for ligands, including Dll4 (ref. 84). Likewise, in mouse lungs, club cells are maintained through Jag1 expression on adjacent ciliated cells, which interacts with Notch2 receptors present on club cells to prevent their differentiation into ciliated cells. Jag1 inhibition in adult mice induced direct *trans* differentiation of one cell type to another, without cell division[85]. In airways, the source of ligand is different. The parent cells serve as the 'niche' and continuously supply a Notch ligand to their daughter cells, the secretory progenitor cells, which show continuous Notch2 activity at steady state[86].

In a similar manner, signalling at boundaries involves the establishment of a ligand source (Fig. 4), positioned by the actions of locally expressed signals and transcription factors. In *D. melanogaster* wing imaginal discs, expression of Serrate is restricted to one side of the boundary by the dorsal domain-specific transcription factor Apterous[87]. Misexpression of Serrate is sufficient to create an ectopic boundary[78,87–89]. Similarly, Notch1 signalling is involved in neuroectoderm segregation by refining germ layer boundaries during *Xenopus laevis* gastrulation. Here, a domain of ligand expression is established by a combination of positive (Nodal) and negative (Churchill1) regulators. Together these position Dll1, giving rise to a delimited 'marginal zone' of signalling separating mesoderm and neuroectoderm[90].

In many tissues in which sustained Notch signalling is important in maintaining regenerative potential, the source of ligands remains to be established. A population of striatal astrocytes is kept in a latent neurogenic state by Notch activity[91]. NICD is present in astrocyte nuclei and is lost after injury, allowing them to produce new neurons in injured brains[92]. However, which cells supply the ligand to sustain Notch activity in these astrocytes is not well defined. Indeed, it is possible that low-level Notch activity is ligand independent, elicited through processing in the endocytic pathway[17,93,94]. How injury affects the activity is unclear in either case.

Sustained signalling is usually coupled with a mechanism that ultimately limits the response. Sometimes this is due to availability of ligand, which may decrease or be eliminated over time as neighbours differentiate. Other mechanisms involve changes in chromatin to adjust the nuclear response. For example, cochlea supporting cells change their ability to re-enter the hair-cell programme postnatally. Initially they retain plasticity and *trans* differentiate into hair cells when Notch activity is inhibited[95]. At later stages, their ability to *trans* differentiate is lost in a manner that correlates with enhancer decommissioning at many hair-cell genes, highlighting the role of epigenetics in shaping the outcome from signalling[96]. In other contexts, differentiation depends on the attenuation of Notch signalling. For example, Notch signalling is required for neural progenitor maintenance in the developing spinal cord and the time at which signalling is attenuated defines the fate of the postmitotic progeny[97].

Sustained Notch activity thus frequently results from ligands produced by a distinct Notch-independent source to decouple it from a signalling feedback loop. Many examples are emerging in which the resulting continuous, often low-level, Notch activity enables regenerative or plastic cell states to be maintained in tissues and to be unleashed when tissue damage disrupts the ligand source.

Digital (ON/OFF) Notch activity

Using digital Notch signals implies that once a critical threshold of Notch activation is surpassed, cells will adopt the 'ON' fate. Below that threshold cells will adopt the 'OFF' fate. Although most Notch signalling events may ultimately result in bifurcated fate outcomes due to negative feedback, additional mechanisms ensure that progeny in a cell lineage adopt a binary ON or OFF state. Frequently these rely on segregation of key factors during cell division[98] (Fig. 5).

Binary Notch ON/OFF states are established in the progeny of SOP and neural stem cells in *D. melanogaster*, where they are essential for generating distinct identities. In these lineages, an oriented division generates a Notch ON cell and a Notch OFF cell[99–101] (Fig. 5) through the differential segregation of several factors during mitosis. In SOPs, the Notch inhibitor Numb is asymmetrically segregated into one cell in which it interacts with endocytic components, including the adaptor protein complexes AP-1 and AP-2, to regulate endocytosis and recycling of Notch, leading to depletion from the membrane. At the same time, the E3-ligase Neuralized is segregated into the same anterior cell, in which it positively promotes ligand activity along with other elements of the endocytic apparatus that also become differentially segregated[98,102].

Likewise, two alternate intermediary cell types in fly motion circuit T4 and T5 lineage arise through asymmetrical segregation of cortically associated proteins, including Numb[101,103]. A second Notch ON/OFF decision then confers appropriate identity (T4 versus T5) on sister neuronal progeny, important for the organization and correct orientation of their dendritic processes[101]. Whether asymmetric Numb or an alternate mechanism is involved in the second binary choice, and whether other regulators are asymmetrically segregated as in SOPs, remains to be established.

A similar unequal segregation of the E3-ligase Mindbomb determines cell-fate decisions in asymmetrically dividing radial glia (Fig. 5). Mindbomb is preferentially segregated into one daughter cell, which thus becomes the ligand source to maintain higher Notch activity in

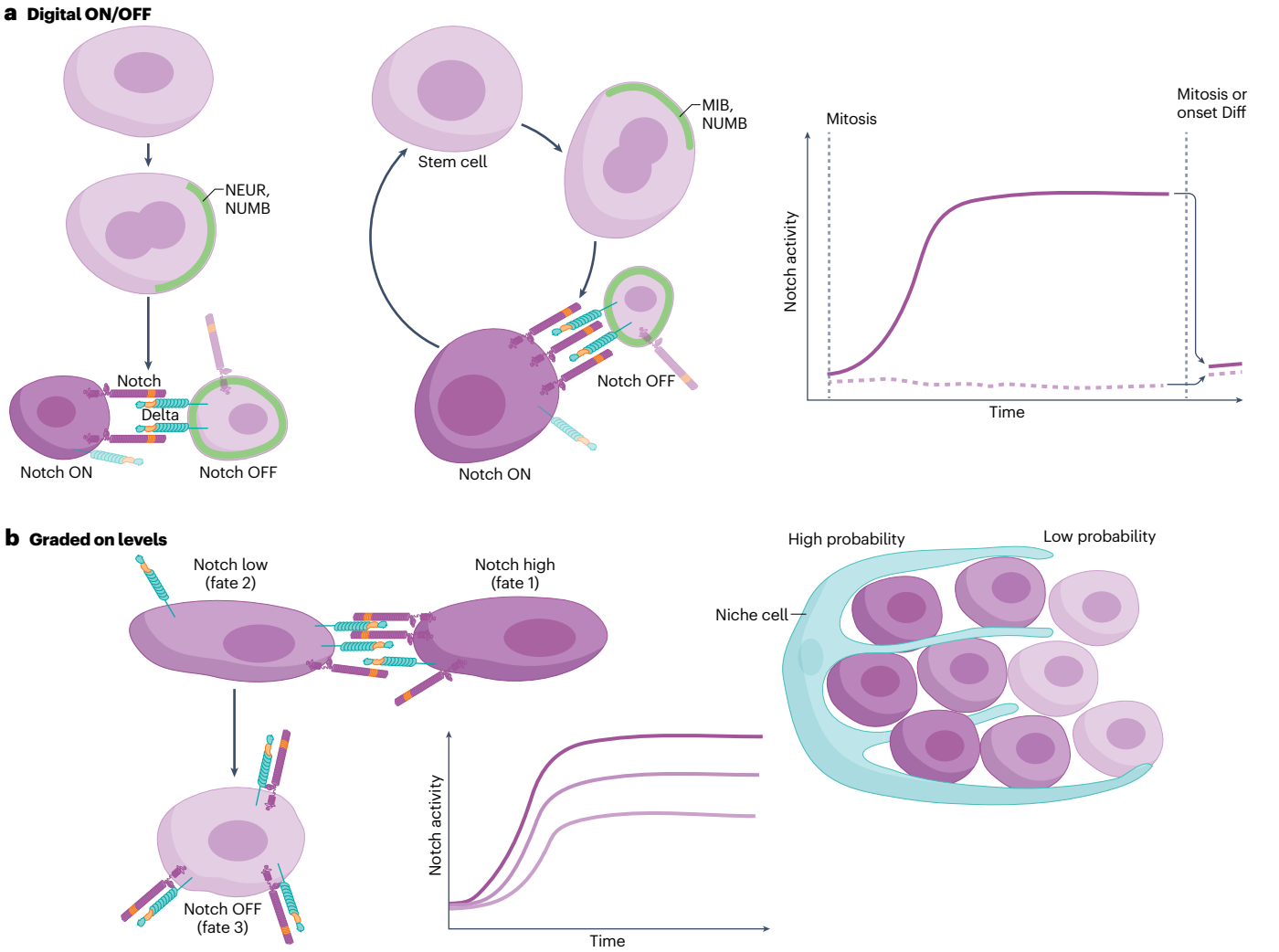


Fig. 5 | Digital and graded Notch signalling. a, The asymmetrical segregation of factors (green) — such as Numb (NUMB), Neuralized (NEUR) and Mindbomb (MIB) — results in reduced activity of Notch or ligands indicated by light shading of the icons, to create distinct ON and OFF states in the progeny. Different levels of Notch activity are indicated by the magenta shading; and the graph depicts hypothetical activity profiles that are reset by mitosis or differentiation onset (diff). **b**, Differential levels of ligands and receptors (left, an example from haematopoietic stem cell emergence) and/or extent of contacts with a niche (right, example from *Caenorhabditis elegans* gonad) result in different levels of Notch activity, indicated by purple shading; the graph depicts hypothetical profiles of Notch activity.

the other daughter cell[104]. As a consequence, the basal cell retains self-renewal potential. The tissue-intrinsic apical–basal polarity and planar polarity orchestrates the unequal sorting of the critical Notch regulators in SOPs and radial glia to direct the alternate fate potential of the progeny. This unequal sorting of key components during mitosis is thus a key mechanism that yields a binary ON/OFF Notch decision.

Other levels of regulation that can promote binary outcomes include differential expression of long non-coding RNAs and/or microRNAs that target different components in the pathway. For example, Notch controls the decision between mesodermal (cardiomyocyte; Notch OFF) and neuroectodermal (Notch ON) lineages in differentiating embryonic stem cells[105]. Expression of long non-coding RNAs helps differentiate signalling by promoting Notch OFF cardiomyocyte fates. One such long non-coding RNA is CARMA, which indirectly regulates the amount of CSL translation via its effects on mir133a2 levels[106]. This is one of the few examples were levels of CSL, the DNA-binding component of the pathway, is the primary cause of distinct fates.

Despite the identification of several mechanisms ensuring a discrete ON/OFF threshold, there are many examples in which this has not yet been deciphered. One example is how Notch restricts the potential of mammary stem cells to luminal (Notch ON) or basal (Notch OFF) unipotency. Notably, the activation of Notch in postnatal mammary glands can induce a luminal fate in basally committed cells[107]. Understanding the mechanisms that confer binary outcomes has special relevance in cancer, in which reactivation of embryonic programmes is commonly used for cells to gain plasticity.

Analogue (graded) Notch activity

Graded Notch activity, in terms of levels (Fig. 5) or duration of signalling, is emerging as an underappreciated strategy for cell fate diversity across a wide range of different processes. Contributory mechanisms for generating graded activities include the deployment of distinct ligand levels or combinations of ligands, the presence of FNGs that modify the receptors to either enhance or diminish ligand activity, and conditions yielding differing levels of *cis* inhibition. A further, less explored, possibility is that different numbers or sizes of cell contacts could have a role[38]. A graded probability of Notch (GLP-1) dependent transcription in the *C. elegans* gonad is probably the consequence of proximity and contacts with the distal tip cell niche[108] (Fig. 5).

Both ligand levels and *cis* inhibition contribute to neural prepatterning of the small sensory bristles in *D. melanogaster*. These are arranged in several rows and each bristle emerges from a sensory precursor. Careful imaging of HES fluorescent reporters and mathematical modelling led to the conclusion that proneural stripes arise as a consequence of a bimodal gradient of Delta expression and *cis* inhibition between Delta and Notch. Together these bring about stripes of Notch activity in regions of intermediate Delta levels, in part because Notch is suppressed by cis-inhibition where ligand levels are highest. In this region of low Notch activity, cells seem to acquire a bivalent state from which some subsequently escape and go on to become sensory precursors, inhibiting their close neighbours via classic lateral inhibition[23].

In vertebrates, expression of different combinations of Notch receptors and ligands enables graded levels of Notch activity. An example is the endothelium of the embryonic aorta, where specification of the precursor for haematopoietic stem cells (HSCs) requires a lower level of Notch activity than that required to specify arterial endothelial precursors (Fig. 5). The different signalling levels were detected using mouse lines in which the intracellular domain of Notch1 was replaced with Cre proteins (NIP-Cre)[109]. Two distinct lines were generated using Cre proteins of different stability: NIP-Cre^{LOW}, labelling cells with low and high activity; and NIP-Cre^{HIGH}, labelling cells with high activity only. Although arterial cells were labelled by both lines, only the most efficient NIP-Cre^{LOW} line labelled HSCs. This implies that HSC precursors experience a low level of Notch activity not captured by NIP-cre^{HIGH} (ref. 110). The activity levels correlate with expression of two ligands, Dll4 and Jag1. Neutralization of Dll4 via antibody drove the cells to differentiate into HSCs[111], thus high Notch activity appears to rely on Dll4. Conversely, Jag1 is important for low-level Notch signalling to specify HSC fates. It achieves this, at least in part, through *cis* inhibition of Notch1 in the precursors[112]. Thus, coincident expression of a combination of ligands, with different propensity for *cis* inhibition, sets thresholds of Notch activity necessary to direct HSC versus arterial outcomes.

Similarly, differential signalling via Dll4 and Jag1 regulate vascular branching during angiogenesis. Upregulation of Dll4 in distal tip cells activates Notch in neighbouring trailing stalk cells, suppressing tip cell behaviour. In contrast, Jag1 is expressed mainly in stalk cells, where it antagonizes Dll4–Notch signalling. Fringe-mediated glycosylation of Notch contributes to this gradation by potentiating Dll4 and impairing Jag1 signalling[11]. Branching is highly dynamic, and cells can switch fates when levels of Notch activity fall below a threshold. Thus, the levels of Notch activity differ depending on the balance of ligands present at any one time, leading to a dynamic process of tip cell fate selection during sprouting angiogenesis[113]. Reversible acetylation of N1ICD may also contribute to the fine tuning of the endothelial Notch responses[114].

Notch activity levels often distinguish progenitor cells from their differentiated daughter cells, as occurs during mammalian brain neurogenesis. Single-cell RNA profiling experiments distinguished three levels of Notch activity among the daughter cells: high levels corresponded to those adopting embryonic cerebellar progenitor fate, intermediate levels to inhibitory fates and lowest levels became excitatory neurons. In this context, the expression of different levels of Dll1 and Dll3 is a contributory factor in determining different levels of Notch activity and, consequentially, specific cell fates. Dll3 levels are highest in the low Notch activity cells (excitatory neurons)[115], in agreement with a *cis*-inhibitory function of this ligand[116].

In contrast, a temporal rather than spatial gradient of Dll1 activity regulates mucociliary cell fates in *X. laevis*[117]. Four mucociliary cell types are generated sequentially, each expressing a different HES gene in a manner dependent on levels of Notch signalling. Modifying Dll1 levels was sufficient to coordinately shift cell type ratios, arguing it is the main driver[117]. Similarly in zebrafish neural tube, the maintenance of floorplate progenitors and the number and types of different interneurons produced were sensitive to signalling durations. Broadly, the shortest duration promoted early born fates and the longer duration later-born fates. What regulates signalling duration to give rise to different interneuron fates has not been established, but expression of Jag2b appears to sustain signalling in the long-term progenitors[97].

The regulation of cell quiescence, cell proliferation and cell differentiation by graded levels of Notch activity may occur in many tissue types. This regulation of cell behaviour is illustrated by zebrafish pancreas, whereby high Notch activity induces quiescence and low levels promote endocrine progenitor amplification in the intrapancreatic duct[118]. Similarly, changes in Notch activity levels occur during maturation of *D. melanogaster* neuroblasts, in which Notch activation is required for quiescence, whereas maintenance and exit from quiescence is achieved with low Notch levels[119].

How different levels of Notch activity are generated remains to be established in many contexts, as well as how they promote or inhibit different outcomes. However, it is becoming clear that different levels of signalling, rather than an all or none outcome, shape and maintain stem cell compartments[120], as well as the architecture of many tissues.

Pathogenic Notch activity in cancer

The discovery of a translocation involving NOTCH1 in T cell acute lymphoblastic leukaemia (T-ALL) hinted at Notch's potential as an oncogene[121]. T-ALL remains the prime example of Notch-dependent cell transformation and has been important for unravelling downstream transcriptional targets, including c-Myc[122] and IL-7R[123], as well as the crosstalk with other pathways via β-catenin[124], NFκB[125] and phosphatidylinositol 3-kinase (PI3K)[126].

Mutations in a majority of T-ALL patients map to two hot spots in NOTCH1, the NRR and PEST regions, a discovery that was pivotal in recognizing NOTCH1 as a key driver in T-ALL[127] (Fig. 6 and Box 1). Mutations in NRR destabilize its structure to favour cleavage and spontaneous activation of the receptor. Even mutations causing mild destabilization result in increased ligand-independent NOTCH1 activation[128]. Mutations in the PEST region increase stability of NICD, by interfering with phosphorylation sites contained within this region[129,130]. For example, PEST phosphorylation by the mediator-associated kinase CDK8 can trigger degradation of NICD1 (ref. 131). Thus, both types of T-ALL mutations are positioned to increase Notch activity in tumour cells, providing them with a growth and survival advantage. Mouse models featuring constitutively active Notch in haematopoietic cell progenitors

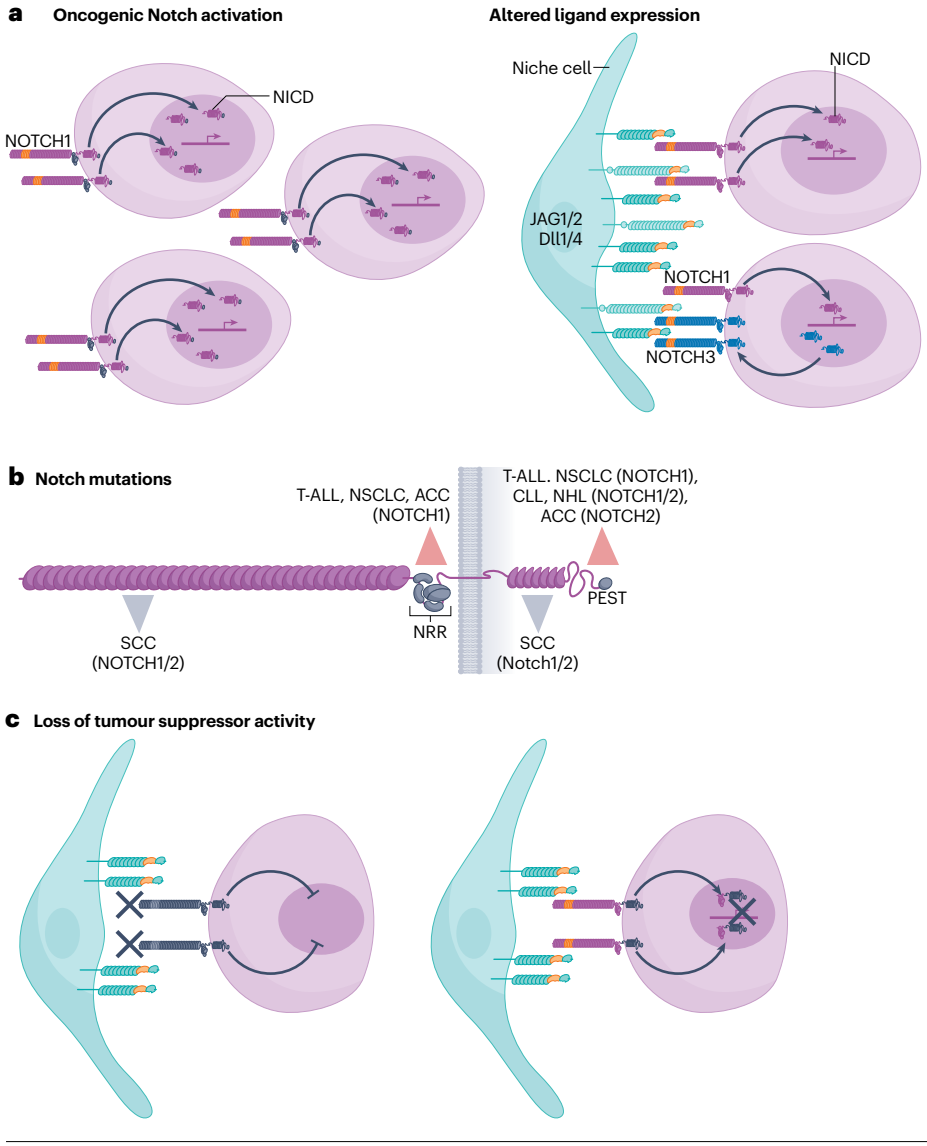


Fig. 6 | Notch oncogenic modes of action. **a**, Oncogenic Notch activation: constitutive or overactive NOTCH1 arising due to mutations (grey) in the Notch receptor that facilitate activation (NRR) or increase stability (PEST) (see **b**). Oncogenic activity can also be driven by overexpression of ligands in niche cells. In cell types in which *NOTCH3* is a downstream target of NOTCH1, it is often overexpressed or activated reinforcing the overall levels of activity. **b**, Notch mutations: regions (grey) with activating (top, pink arrows) and inactivating (bottom, grey arrows) mutations are diagrammed in relation to a stylized NOTCH protein. **c**, Loss of tumour suppressor activity: in tissues in which Notch activity suppresses tumorigenesis, tumours arise due to mutations that inactivate of Notch by frame shifts or deletions that disrupt the protein (grey). Inactivating mutations can disrupt the frame of the receptor (left) or just the intracellular domain and prevent transcriptional activity (right). ACC, adenoid cystic carcinoma; CLL, chronic lymphocytic leukaemia; NHL, non-Hodgkin lymphoma; NICD, Notch intracellular domain; NRR, negative regulatory region; NSCLC, non-small-cell lung cancer; SCC, squamous cell carcinoma; T-ALL, T cell acute lymphoblastic leukaemia.

faithfully reproduce aggressive T cell leukaemia[132]. However, equivalent mutations to those found in the human disease state are insufficient to induce leukaemia in mice and collaboration with other signals or mutations is required for leukaemogenesis[133,134].

Following the advent of large-scale sequencing, numerous tumours were discovered to harbour mutations in similar hot spots to those in T-ALL. Tumour types that exploit Notch as a tumour promoter, include breast, lung, glioblastoma, pancreas, CLL, T cell lymphomas and colorectal cancer[4,5]. In contrast, the presence of inactivating Notch mutations in certain epithelial tumours (for example, squamous cell carcinoma and basal cell carcinoma), in renal cell carcinomas and in cervical cancers indicate the ability of Notch to function as a tumour suppressor in those contexts[4,5,135,136] (Fig. 6).

Besides mutations in Notch receptors, several cancers are linked to misexpression of Notch pathway members. In colorectal adenoma cells, β-catenin promotes transcription of JAG1, which leads to high Notch activation (Fig. 6). An absence of MFNG glycosyl transferase also alters signalling by preventing the normal interaction of DLL1 with NOTCH1, instead favouring JAG1 (ref. 137). The tumour microenvironment, including released exosomes, may also generate a source of JAG1 or other ligands as detected in hepatic cancer cells grown on stiff extracellular matrix[138].

Recently, NOTCH3 has attracted the attention in cancer research because elevated NOTCH3 levels have been linked with tumour promotion, high invasiveness, chemotherapy resistance and metastasis in numerous cancer types[139–142]. NOTCH3 can be upregulated as a consequence of NOTCH1 activation, resulting in a positive cycle that reinforces levels of Notch activity[143,144] (Fig. 6). NOTCH3 differs from NOTCH1/2 structurally, having fewer EGF repeats, differing RAM and lacking a defined TAD. These differences affect ligand preferences and the transcriptional activity of NOTCH3 (ref. 145) with cancer-specific transcriptional targets and protein interactions[146,147]. Tumour suppressor functions of NOTCH3 in breast cancer have also been reported[148]. A prolonged overall survival in tumours expressing NOTCH3 was

observed in patients, probably through transactivation of PTEN[149]. In agreement with that observation, *Notch3*-deficient mice developed tumours in parous mammary glands[150], although forced activation induced luminal-like tumours in a similar model[151]. These complex oncogenic conditions probably reflect the cell and context-specific outcomes from Notch signalling, as well as varied effects from the tumour microenvironment, key factors for tumour progression[152,153].

To overcome immunosuppressive tumour environments, novel engineered strategies have been developed using synthetic Notch receptors (SynNotch) in T cells. SynNotch consists of a Notch molecule with antibody chains recognizing a tumour antigen in the extracellular domain. After encountering the tumour, SynNotch T cells activate Notch signalling, leading to Notch-dependent production of cytokines (such as IL-2), promoting immune infiltration in the tumours[154]. Such strategies illustrate the potential for therapeutics that manipulate Notch activity.

The different pathological conditions in cancer underscore the importance of finely regulating the Notch pathway to sustain healthy cells and tissues. Precise comprehension of its specific demands in various contexts could enable us to manipulate the pathway to clinical advantage.

Conclusions and perspectives

We have illustrated how qualitatively different modes of Notch signalling are established by variations in key parameters and in the relationships between the interacting cells. The characteristics of each signalling mode are instrumental in conferring outcomes that are critical to shape and maintain tissues, as with oscillations in progenitors

Glossary

Adaptor protein complexes
Multiprotein complexes that mediate vesicle formation and trafficking by linking cargo proteins to the vesicle coat.

β-Catenin
A key intracellular component in the Wnt signalling pathway. Wnt binding to its receptor prevents β-catenin degradation, allowing it to regulate gene expression.

Cell transformation
The process whereby normal cells become cancerous acquiring uncontrolled growth and division.

Club cells
Non-ciliated epithelial cells in the airway that play a role in detoxification, secretion of protective proteins and airway regeneration.

E3-ligase
An enzyme that tags proteins with ubiquitin altering their interactions with other adaptor proteins in the cell and in some cases targeting them for degradation by the proteasome.

Enhancer decommissioning
The process of shutting down active enhancers, often by removing transcriptional machinery of histone modifications, to regulate gene expression.

Exosomes
Small extracelluar vesicles released by cells to carry proteins, lipids and nucleic acids, important for cell communication in processes such as immune response and tumour progression.

Extracellular matrix
A network of proteins and polysaccharides surrounding cells, providing structural support and signals for cell adhesion, migration or differentiation.

Genetically mosaic tissues
Tissues composed of cells with different genetic makeups, often resulting from mutations, genetic recombination or experimental manipulation.

IL-7R
Receptor protein for the cytokine interleukin 7 (IL-7). Binding of IL-7 activates other downstream pathways and regulates the development, proliferation and survival of immune T and B lymphocytes.

Immunosuppressive tumour environments
A complex networks of cancer cells, immune cells and signalling molecules that suppresses immune activity, allowing tumours to evade immune. destruction. Key contributions include regulatory T cells, myeloid-derived suppressor cells and immunosuppressive cytokines.

Lateral inhibition
A process in which a cell becomes selected from a group of equivalent cells and inhibits its neighbouring cells from adopting the same fate. Promotes the formation of distinct cell types in tissues.

Mediator-associated kinase CDK8
(cyclin-dependent kinase 8). Regulatory subunit of the Mediator complex that regulates gene expression by phosphorylating transcription factors and RNA polymerase II. Linked to cancer progression.

Mucociliary cell
Ciliated epithelial cells in the respiratory track that secrete mucus aiding in the clearance of particles and pathogens.

Muscle satellite cells
Stem cells located beneath the basal lamina of muscle fibres, responsible for muscle growth, repair and regeneration.

NFκB
(nuclear factor kappa-light-chain-enhancer of activated B cells) pathway. Controls immune responses, inflammation, cell survival and proliferation. Activation leads to NFκB moving to the nucleus to induce inflammatory and survival genes. Chronic activation is linked to disease.

Paraxial mesoderm
A region of the mesoderm located on either side of the neural tube that forms somites and contributes to the musculoskeletal system.

PI3K
(phosphatidylinositol 3-kinase). Kinase enzyme that regulates cell growth, survival and metabolism. PI3K activation triggers protein kinase B (PKB, or Akt) signalling, which promotes cell survival and inhibits apoptosis. Dysregulated in cancer and metabolic diseases.

Prolyl-isomerase Pin1
Peptidyl-prolyl isomerases are enzymes that regulate the stability, localization and activity of proteins. Pin1 recognizes and isomerises the phosphorylated serine/threonine–proline (pSer/Thr–Pro) motif.

Somites
Segmented structures from the paraxial mesoderm that give rise to the vertebrae, skeletal, muscles and dermis.

Translocation
Movement of a chromosome segment to a new location in the genome, within the same or a different chromosome, may be associated with cancer.

Wing imaginal discs
The wing primordia, epithelial structures in insect larvae that develop into adult wings during metamorphosis.

versus binary ON/OFF in their daughter cells. Converting an oscillatory signal to a sustained one, by expressing constitutively active Notch, results in premature differentiation and depletion of progenitors, underscoring the importance of levels and modes of Notch signalling. The various models illustrate how sometimes quite subtle changes in relative protein levels or transcriptional feedback can have profound consequences on the outcome from signalling.

Although there are data to support the different modes, in many cases the regulatory networks remain somewhat hypothetical. Furthermore, in many contexts in which Notch activity is known to regulate cell fates or the maintenance of cell identities, little is known about the source or nature of the signal. Indeed, could different types of ligand and/or receptor favour different modalities? There are suggestions that the levels and dynamics of signalling differ according to the ligand[155], but it is hard to evaluate this contribution in vivo. Likewise, what role *cis* inhibition has and how this is balanced with activation in vivo is just starting to be addressed[156]. Indeed, some scenarios may rely on ligand-independent signalling, with Notch becoming activated as a consequence of its routing within the endocytic pathway after internalization[93,94]. Teasing out these different contributions will be important both for understanding the mechanisms of signalling and for manipulating them for therapeutic or regenerative purposes.

The availability of more sophisticated quantitative methods will make it possible to fill some of these gaps in our knowledge to distinguish how and where different Notch signalling modalities are deployed. For example, by tagging endogenous proteins and target genes to track them in real time, the true dynamics and profiles of signalling are being elucidated[76,157,158]. Single-cell transcriptomics, coupled with spatial information and single-cell proteomics will help to decipher whether there are distinct ligand presenting cells and what their origins are. It will also tease apart the types and relative levels of the different ligands and receptors that are present. Armed with more quantitative data, it will be possible to refine and add to the models to gain a more profound understanding of the rules that govern the different signalling modes and their importance for generating different developmental, physiological and disease outcomes.

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