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Notch Signaling in Cell–Cell Communication Pathways

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Abstract The Notch signaling pathway is a well-conserved molecular mechanism that serves the purpose of coordinating cellular events occurring within a group of neighboring cells. The interplay between ligands and receptors of the Notch family is crucial in determining Notch status and the outcome of any specific cellular event often triggered by other signaling pathways. However, different characteristics of the cells involved can also determine their capacity to send or receive specific signals. We have revised how Notch integrates some of the instructive signaling pathways to govern cell fate. Understanding how cells communicate and whether Notch positively or negatively regulates this process is critical for better understanding the mechanisms of cell and tissue homeostasis, which is relevant for regenerative medicine.

Keywords Notch · Wnt · HH · TGF · NFkB · Hippo

Introduction: Basics of Notch Signaling

The Notch pathway has been extensively studied over the past 20 years, since the Notch receptor was cloned in *Drosophila* [1] and identified in human T-cell acute lymphoblastic leukemia (T-ALL) cells [2].

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The Notch pathway is conserved from invertebrates to mammals. It signals by cell–cell interactions in which a sending cell expressing a ligand interacts with a receiving cell expressing the receptor. Receptors and ligands are usually present in different neighboring cells and they signal in trans, but it is well documented that they can be co-expressed in the same cell and signal in cis, which is associated with the inhibition of the pathway (or cis-inhibition) (Fig. 1).

Signaling in trans is the best-known Notch signal, and requires the appropriate ligand to find the right receptor in a neighboring cell to trigger the intracellular signaling cascade. This process involves two proteolytic cleavages that result in the release of the Notch intracellular fragment (also known as N1IC). The N1IC will then translocate to the nucleus, where it will interact with transcription factors, chromatin proteins and coactivators to regulate transcription (reviewed in [3] and Fig. 1). Although many aspects of Notch signaling have been determined, several issues are still unknown. One of the remaining questions is how Notch serves so many pleiotropic functions using the same intracellular elements, even in cells or tissues that may be simultaneously receiving different Notch signals. One possible mechanism underlying this signaling diversity would rely on the number of Notch molecules that penetrate the nucleus. Thus, a low concentration of Notch molecules in the nucleus would preferentially activate genes containing high-affinity binding domains or specific promoters containing either paired binding sites for Notch homodimer binding [4] or adjacent binding sites favoring the association of heterodimeric complexes (i.e. Notch plus a second transcription factor). Another mechanism to explain the heterogeneous Notch signal outputs found in different cell types would involve crosstalk between different pathways and at different levels, which may impose specific transcriptional programs depending on particular combinations (Fig. 2).

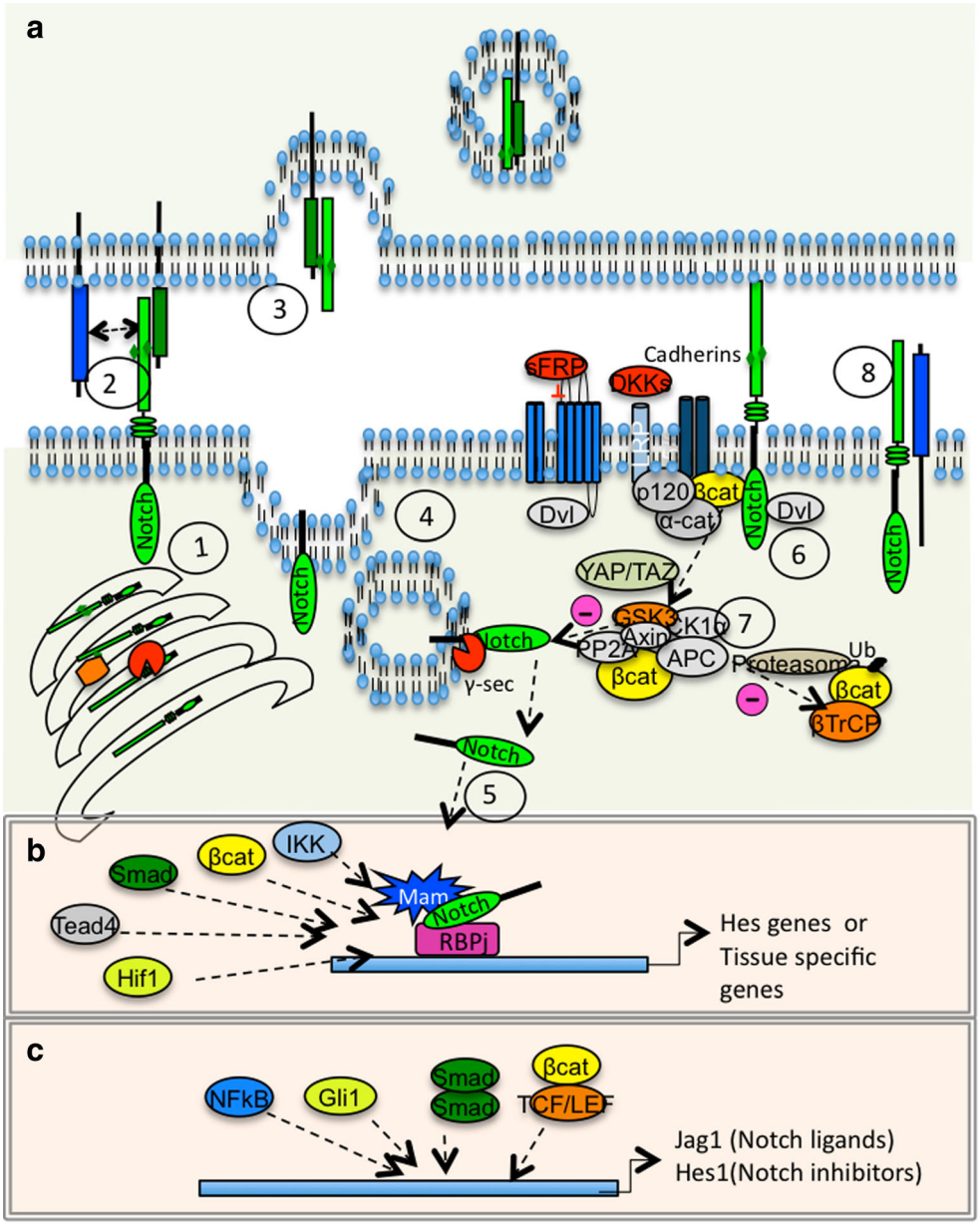

Fig. 1 Basic elements involved in the Notch pathway and interactions with other signaling pathways. (**a**) 1) Modifications of the Notch receptor in the Golgi apparatus by Kuzbanian, Pofut, Poglut and Fringe; 2) productive Fringe-modified Notch interaction with the Delta ligand and inhibition of the Jagged ligand; 3) endosomal internalization of ligand and extracellular Notch after signaling; 4) γ-secretase cleavage of Notch receptor and translocation to the nucleus; 5) translocation of Notch to the nucleus for transcriptional regulation. Alternative scenario: 6) Notch interaction with β-catenin on the cellular membrane; 7) β-catenin destruction complex in the absence of Wnt ligand. Other possibility: 8) cis-inhibition of the Notch receptor by a ligand. (**b**) Core elements of the Notch transcriptional complex (Notch-IC, RBPJ and Mam) and nuclear interactors. (**c**) Transcription factors that regulate the Notch pathway through Notch-independent transcriptional activation of Notch ligands or Notch inhibitors

In this review, we will revise the best-documented connections of Notch with other pathways.

Ligands and Receptors

In mammals, there are four different receptors (Notch1–4), and all are able to respond to the four different ligands: Jagged (Jag)1-2, and Delta-like (Dll) 1, 4 [5]. These ligands generally interact with neighboring cells (in trans), but can also inhibit Notch in cis. A fifth ligand, Dll3, appears to act exclusively in cis-inhibitory functions [6].

Although there is a great overlap in the expression patterns of ligands and receptors, genetic analysis of mutant animals has revealed specific non-redundant functions for each.

The different Notch ligands share several structural elements, such as EGF repeats and the DSL (for Delta-Serrate-

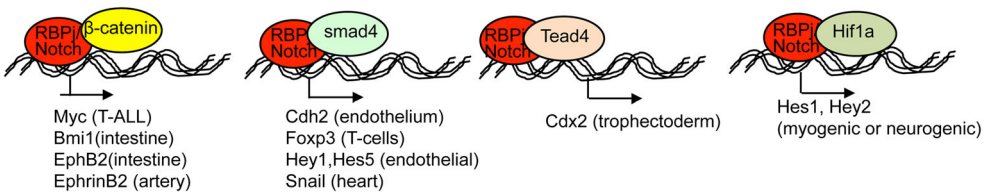

Fig. 2 Integration of Notch with other transcription factors that converge at the DNA level, on enhancers or promoters to regulate gene transcription

Lag2); however, it is only recently that crystallographic studies have begun to decipher particularities for each one [7]. The Notch receptors also share a basic structure and mechanism of activation. In brief, the association of Notch with its ligand originates a pulling force that sequentially exposes the Notch/Lin repeats (NLR) region, contained in the negative regulatory region (NRR), that enables the ADAM metalloprotease cleavage at a juxtamembrane site. This is followed by an intramembrane cleavage catalyzed by the γ-secretase complex. Because this last cleavage is totally conserved among all Notch receptors, inhibitors targeting Notch proteases (the majority developed against the γ-secretase complex) are commonly used to target general Notch activity. Alternatively, the NRR region is unique for each receptor, and it has been successfully used to target specific Notch homolog functions with blocking antibodies [8].

Trafficking of Notch receptors and ligands by endocytosis establishes an additional level of control that can determine the output of the signal. Since endocytosis of ligands and receptors is mainly ubiquitin-mediated, the ubiquitin ligases targeting Notch ligands (such as Mind bomb or Neuralized) and Notch receptors (such as Deltex, Suppressor of Deltex, Numb or Itch) are crucial regulatory elements in Notch activity (reviewed in [9]).

Nuclear Signaling and Chromatin Regulation

Canonical Notch signaling converges towards a single transduction cascade (see Fig. 1), which involves the interaction of N-IC with the DNA binding factor RBPJ and the coactivator Mastermind (Mam). Other elements are also found in this complex, including the histone acetyl transferases p300 or scaffold proteins such as SHARP.

The classical view of Notch transcriptional activity presumes that RBPJ is constitutively bound to the DNA, recruiting repressor elements such as NCoRs and HDACs in the absence of N-IC, while exchanging repressors for activators in its presence. However, recent ChIP-sequencing data reveal a more complex scenario, with different types of NOTCH and RBPJ target genes, likely influenced by other tissue-specific transcription factors. In muscle cells, dynamic binding of RBPJ is observed in response to Notch activation, accompanied of N1-IC, p300 recruitment and acetylated H3K27. In contrast, few consensus sites are statically occupied by RBPJ devoid of recruitment of N1-IC [10•]. The dynamic nature of Notch/RBPJ sites has been corroborated by ChIP-Seq studies in *Drosophila* [11], but also with independent methods (SpDamID) in kidney cells [12]). In addition to H3K27 acetylation, H3K56 acetylation, a core histone modification that affects nucleosome stability, has been associated with N-IC recruitment [13•].

In T-ALL cells that contain high levels of basal NOTCH activity, only 10 % of the NOTCH/RBPJ sites are dynamically responding to NOTCH inhibition-activation by a simultaneous increase of both NOTCH and RBPJ recruitment. Dynamic NOTCH complexes are found mainly associated with enhancers or super-enhancers in different cell types [14•]. These sites are closely associated with RUNX1 binding sites, an association previously observed in *Drosophila* [15]. The biological meaning of Notch binding to the super-enhancers is as yet unknown, but it could have a role as an integrator of signaling pathways [16••]. Interestingly, super-enhancers are regulatory regions with a critical function in development and disease [17••], processes that are highly linked to Notch activity.

Signal Crosstalk in Development, Inflammation and Stress Response

Multicellular organisms require coordinated cellular interactions for their development. The study of this development in diverse model organisms has led to the surprising observation that only a few signaling pathways control the key steps of cell fate decisions and tissue formation. These roughly fall into a small group that includes Notch, Wnt, Hedgehog (HH), transforming growth factor β (TGFβ)/bone morphogenetic proteins (BMP), Hippopotamus-like phenotype (Hippo) pathway, fibroblast growth factor (FGF), epidermal growth factor (EGF), non-receptor tyrosine kinase JAK-STAT (Janus kinase/signal transducers and activators of transcription), nuclear factor-kappa B (NFkB) and retinoic acid receptor (RAR). With the exception of Notch and Hippo and possibly Wnt, which require cell–cell contact, these pathways work in a paracrine fashion involving secreted diffusible growth factors. All these pathways converge in a small number of transcription factors that regulate the transcriptional output of these signals. Our current knowledge on how Notch signals are integrated with each of these signals to coordinate cell fate will be reviewed below (Fig. 2).

Wnt and Notch Signaling

The Wnt factors can trigger three main responses: canonical/β-catenin pathway, planar polarity (Junk/Rac) pathway and Ca++ response (PKC, CAMKII). While these are all important in development, the canonical/β-catenin pathway is the main contributor to Notch signaling, and vice versa. Activation of this pathway is largely restricted by the amount of nuclear β-CATENIN, which is a function of the activity of a β-CATENIN destruction complex that contains ADENOMATOUS POLYPOSIS COLI (APC), CASEIN KINASE 1 (CK1) and GLYCOGEN-SYNTHASE-KINASE 3β (GSK3β), among others. The presence of Wnt factors leads to the inhibition of GSK3β, thus preventing the degradation of β-CATENIN and consequently allowing its nuclear accumulation. In the nucleus, β-CATENIN interacts with TCF/LEF transcription factors to activate transcription (reviewed in [18]).

Genetic and biochemical interactions between Notch and β-catenin have been widely observed in different animal and cellular models. In general, these interactions involve the cross-transcriptional activation of ligands, receptors and inhibitors, creating feedback loops that will impact on the strength, the time, or the field of activation (reviewed in [19]). The biological significance of this crosstalk is context-dependent. There are well-documented examples supporting an inhibitory role of one pathway over the other, but also numerous examples of synergistic interaction where the two activities collaborate in gene transcription. Many examples have been reported in *Drosophila* for both types of interactions. In the *Drosophila* wing, Notch activity induces wingless (the only *Drosophila* Wnt) in the dorsal-ventral boundary; subsequently, wingless signals to surrounding cells to increase expression of Notch ligands, which signal back to the boundary to maintain wingless activity [20]. On the other hand, both Notch and β-catenin collaborate at the transcriptional level to activate the wing master regulator, vestigial [21]. In vertebrates, similar interactions between Notch and Wnt have been reported in both physiological and pathological conditions. For example, Notch, through its target gene Hes1, regulates intestinal homeostasis by the inhibition of Math1, a master regulator of the secretory lineage [22], thus preventing a massive mucosecretory differentiation of the stem and progenitor cell compartments. However, genetic data are now challenged by the use of specific blocking antibodies against Notch receptors, which results in a depression of Wnt activity and activation of secretory genes, indicating that the Notch pathway is required to maintain appropriate levels of Wnt activity in the intestinal crypt [23]. This inhibitory mechanism is also proposed to function in regulating the levels of Wnt3a in intestinal stem cells (ISC), which are known to depend on both Wnt/β-catenin and Notch activity. A Notch-dependent inhibitory mechanism for β-catenin activity has also been shown in the skin [24]. In addition, both Notch and β-catenin are involved in transcriptional activation of ISC genes such as olfm4, Ascl2, c-myc, EpHB2 or Bmi1 [25••, 26••]. The most plausible scenario is that different networks of positive and negative interactions result in specific outcomes leading to stem cell self-renewal or cell differentiation into a particular lineage. In contrast, the adult hematopoietic system seems to be less dependent on these pathways, at least under physiological conditions [27, 28]. In addition to gene co-regulation, other biochemical interactions generate relevant feedback between Notch and Wnt pathways. For example, GSK3β kinase can simultaneously regulate β-catenin degradation and inactivate Notch by phosphorylation [29]. In addition, the association of Notch with β-catenin at the cellular membrane may suggest a role for adherens junctions in regulating the levels of both active proteins [30, 31]. Finally, Notch and β-catenin can interact in the nucleus in accordance with their function in co-regulating gene transcription in a context-dependent manner.

Hedgehog (HH)

Signaling of the HH pathway is initiated when secreted HH binds PATCHED (Ptc) at the cell surface, relieving inhibition of the transmembrane G protein-coupled receptor SMOOTHENED (SMO), and ultimately triggering the activation of the GLI (glioblastoma-associated oncogene) transcription factor (reviewed in [32, 33]. GLI-1 and GLI-2 mainly act as transcriptional activators, while GLI-3 generates a repressor form (GLI3R) in the absence of HH signaling or following its inhibition [34–36].

Although HH is a master regulator of development, its function in the central nervous system is better understood (reviewed in [37]). In this system, it is sonic hedgehog (SHH) that initially acts as a morphogen to pattern the dorsal-ventral axis of the neural tube and to establish the distinct ventral neuron populations in a concentration-dependent manner (reviewed in [38]). However, mutants of the different elements of the pathway display broad developmental defects, indicating a general function for HH in multiple tissues. In the adult, the HH pathway is largely restricted to the regulation of stem cell homeostasis.

Crosstalk between Notch and HH pathways seems to involve transcription of *HES1*, Notch ligands and/or *GLI* factors, but it is unclear whether these are cell type-specific mechanisms. In this sense, it has been reported that HH can regulate *HES1* transcription in a Notch-independent manner in the sarcoma cell line 10 T1/2 [39], as well as in retinal progenitor cells [40]. Activation of GLI1 has also been shown to induce *NOTCH1* and *JAGGED1* expression in different regions of the brain [41], whereas HH activates *JAG2* expression in motor neuron progenitors [42••]. Conversely, HES1 represses *GLI1* expression in glioblastoma cells [43], which may generate a feed-forward regulatory loop. Together, these data reinforce the concept that signaling pathway coordination is required for the proper regulation of cellular decisions in different stem cell systems.

TGFβ/BMP

The TGFβ/BMP molecules act as paracrine signals that interact with specific receptors to activate the intracellular Smad effectors. In general, SMAD1/5/8 are phosphorylated in response to BMP, whereas SMAD2/3 are phosphorylated downstream of TGFβ or activin-like signals. Phosphorylated SMADs can then interact with their common mediator SMAD4 and translocate to the nucleus to regulate transcription.

Researchers have demonstrated that the TGFβ/BMP pathway can directly regulate *JAG1* transcription through the SMAD1/5 factors in endothelial cells [44]. A similar process was reported for mesenchymal stem cells, which allowed their differentiation into smooth muscle cells [45]. In addition, different Notch target genes can be cooperatively regulated by

Notch and Smad factors at the promoter level, as is the case with *Hes1* or *Cdh2* (N-cadherin) [46, 47].

Interactions between TGFβ/BMP and Notch pathways appear to be extremely important in the epithelial-to-mesenchymal transition processes that take place during heart development. In this system, integration of Notch1 and BMP2 positively affects *Snail* expression that is crucial for endocardial cell invasiveness and cardiac valve formation [48].

There are also models in which TGFβ/BMP can simultaneously promote and antagonize Notch signaling in the same cell type to generate a non-synchronized oscillatory gene expression that involves Hes/Hey proteins. In the case of endothelial lineage, this type of interaction results in the establishment of the sharp tip and stalk cell boundaries (reviewed in [49]).

Growth Control and Pluripotency: Hippo/Yap

Mutations in components of this pathway result in the overgrowth of tissue structures (hippopotamus-like phenotype) and tumor formation. The primary downstream Hippo effectors are YAP and its paralog TAZ, and the kinases MST1/2 and LATS1/2. HYPPO signaling results in the inhibition of YAP and TAZ phosphorylation, leading to their nuclear translocation and transcriptional activation of pro-proliferative genes and TEAD transcription factors [50]. Activation of this pathway in fibroblasts facilitates induced pluripotent stem cell (iPS) production in response to the pluripotency factors. In the first embryonic decisions, Notch and Hippo (through Tead4) converge in the regulation of *Cdx2* to specify the trophectoderm lineage [51••]. In this sense, differences in YAP subcellular localization and Hippo activation are important in the pluripotency of embryonic stem cells (ESC) [52].

In somatic stem cells, the Hippo pathway controls the populations of intestinal [53] and neuronal progenitors [54] in coordination with other stem cell pathways. Moreover, compelling biochemical data show that YAP/TAZ can participate in the β-catenin destruction complex, thus creating another important level of regulation between signaling pathways [55].

Signals for Stress, Hypoxia, Inflammation

Adult tissues are exposed to various perturbations, including oxygen radicals, pathogen invasion and inflammation, which activate or repress signaling pathways regulating tissue homeostasis. Thus, stem cells and progenitors need to integrate these signals in an exquisite manner to maintain the integrity of the tissues under adverse conditions. It is not surprising, then, that inflammatory mediators have also been found to be important in tissue development and function in coordination with other canonical developmental pathways. For example, inflammatory signals were recently identified as crucial regulators of hematopoietic stem cell specification [56, 57, 58••]. The pathways downstream of these inflammatory cytokines include NFκB (for TNFα), Stat3 and/or IRF2 (for IFNγ). Downstream of TNFα, NFκB can induce upregulation of the Jag1 ligand, which is required for proper Notch activation in the developing hematopoietic stem cells (HSCs) [58••]. Similarly, TNFα can activate Notch in the bone marrow HSCs through upregulation of endothelial Jagged2 [59]. Interactions between the Notch and NFκB pathways do modulate Notch transcriptional output in different contexts, and they involve other upstream NFκB elements such as the inhibitor of NFκB, IκBα or the IKK kinase complex rather than the transcription factor p65-NFκB per se [60, 61]. In addition, IκBα was recently identified as a regulator of transcriptional repressor complexes targeting Hes1, among other relevant developmental-related genes [62].

Other interactions of Notch with the Jak/Stat pathway downstream of cytokine signaling or with Ras/MAPK downstream of receptor tyrosine kinases (RTK) have been described [63]. These pathways are the main regulators of proliferation and cell survival. Similarly, Notch in association with the PTEN/PI3K/Akt pathway (upstream of mTOR) participates in the regulation of growth. Different genetic and biochemical interactions of Notch with elements of the above-mentioned pathways have been described (for review [64]).

Finally, hypoxia is also an important element in the regulation of stem cells and cell differentiation. The interaction of NOTCH with HIF1 (the principal factor induced in response to hypoxia) was previously identified in mammalian cells [65], and more recently confirmed in *Drosophila* [66], and might be particularly relevant for understanding how Notch regulates stem cell programs in the hypoxic niches.

Conclusion

As this review has illustrated, a handful of signaling pathways are known to control stem cell specification and tissue development. However, understanding the level of integration between their different upstream and downstream elements has just begun. Theoretically, there is an infinite possible combination of interactions including activators and inhibitors that can determine the outcome of each signal. These circuits can further regulate chromatin modifiers, thus adding another layer of complexity. Furthermore, the different signaling pathways are not only in an ON/OFF state, but there are also oscillatory feedback loops and intensity effects that determine their final outcome. Genomic studies in *Drosophila* have shown that Hes repressors are at the base of the oscillatory feedback loops observed in the Notch pathway [67], an observation that has been confirmed in several mammalian systems. In the embryonic aorta, when HSCs are formed, Notch

signaling leads to the transcriptional activation of both *Hes1* and the hematopoietic gene *Gata2*. Subsequently, accumulation of the Hes1 protein at certain levels inhibits both *Gata2* and *Hes1* transcription. This type of regulation, called 'incoherent feed-forward loop', is what allows the strict regulation of Gata2 expression in a particular cell population [68••].

In conclusion, further investigation of the signals regulating stem cell homeostasis and the integration and interpretation of these signals within the specific cellular context is critically important for gaining a better understanding of the complexity of these systems and the ability to modulate them for therapeutic applications.

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Compliance with Ethical Standards

Conflict of Interest Anna Bigas and Lluis Espinosa declare that they have no conflict of interest.

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- •• Of major importance

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