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The multiple usages of Notch signaling in development, cell differentiation and cancer

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Notch is a well-conserved signaling pathway all through evolution that is crucial to specify different cell fates. Although there is a strong context dependent component in each decision, the basic mechanisms that originate from the interplay among ligands and receptors is greatly preserved. In this review we will cover the latest findings on the different mechanisms for Notch activation and signaling. The regulation of this pathway is essential to understand development, cell differentiation and disease.

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Introduction

Notch signals are key players in cell fate decisions that involve cell–cell interactions. The Notch system is a widely used mechanism to generate cell diversity and maintain tissue integrity [1]. Through the expression levels of ligands and receptors present in nearby equipotent cells, Notch response is able to provide directional signals into one of the cells that affect the outcome of that cell and its neighbors. This is important for tissue patterning and can be induced by upregulation of a particular Notch ligand in one or a group of cells, thus creating a cascade of signals in adjacent cells. Lateral inhibition and lateral induction are the most common models that have been proposed to explain how Notch signals are propagated in nearby cells. Since Notch pathway is highly conserved all along the evolution, invertebrate model organisms such as *Caenorhabditis elegans* and *Drosophila* have been used to study the mechanism underlying Notch regulation and function.

Under physiological conditions, Notch signaling is working essentially in all different tissues both during embryonic development and in the adulthood. The versatility of this pathway is what provides the enormous diversity of outcomes that are required to generate cell and organisms diversification during evolutionary adaptation. However, uncontrolled Notch regulation can also result in undesired life-threatening effects, including different types of cancer.

In this review, we will summarize the most recent data on the mechanisms used by the Notch pathway to regulate cell decisions, tissue homeostasis and cancer. We will also discuss the therapeutic options that are currently being exploited in this field.

Recent discoveries about Notch signaling

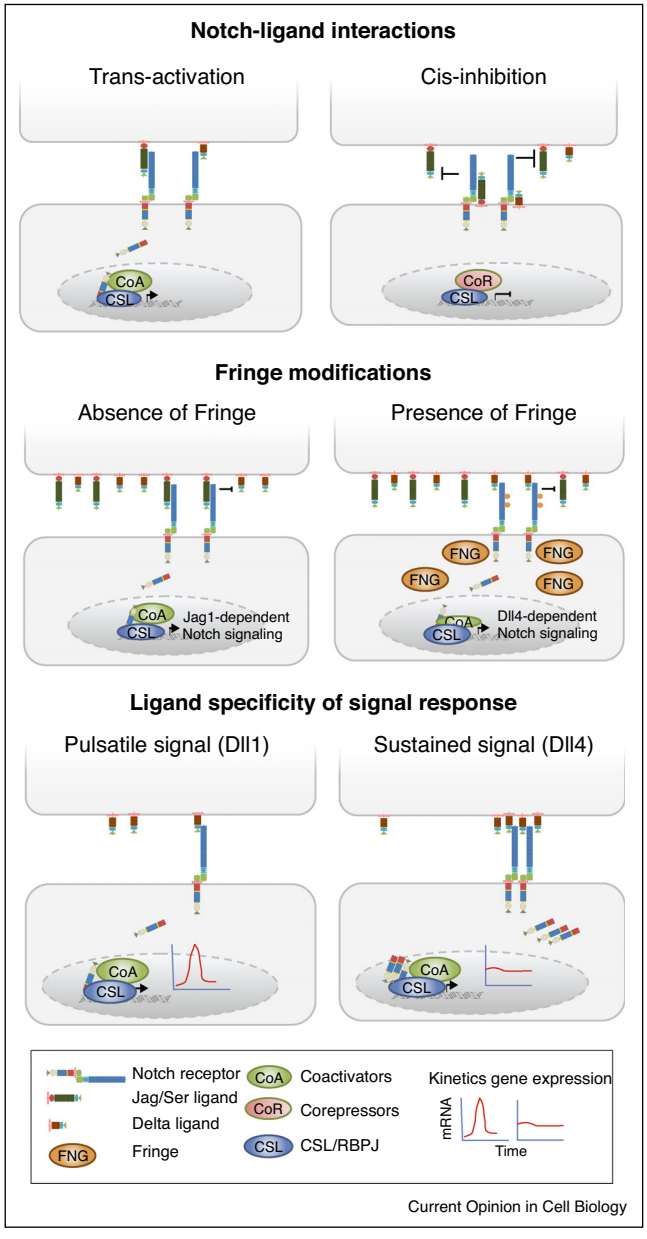
Genetic screenings in *Drosophila* carried by Morgan and colleagues at the beginning of the twentieth century identified a genetic alteration that induced notches in the wing margin. This particular Notch phenotype is what gave name to all Notch receptors and the whole signaling pathway. Years later, Notch, the gene responsible for this phenotype, was cloned in *Drosophila*, followed by the identification of its orthologue genes in different types of vertebrate organisms including humans. The high conservation of this family of receptors all through evolution rapidly suggested the relevance of their function.

The Notch protein is a cell receptor that suffers several modifications before it is presented in the cellular membrane in its functional conformation. In its mature form, Notch contains an extracellular domain, a single transmembrane domain and an intracellular domain. Receptor activation takes place by interaction of the extracellular domain of Notch with ligands of either the Jagged/Serrate or Delta family that are present in the neighboring cells. This interaction results in Notch cleavage at several sites by the action of different enzymatic activities, leading to the release of the intracellular Notch (NIC) fragment and subsequent translocation into the nucleus. Once in the nucleus, NIC participates in gene transcription in association with its DNA binding partner CSL/RBPj. Despite the great variety of Notch targets, the more common and best characterized are the Hes/Enhancer of Split family of transcriptional repressors.

The elements described above are considered the core of the Notch signaling cascade and have been investigated and revised for many years. However, new ideas are

originating from crystallographic studies, which will be covered in this review, together with other aspects of Notch signaling that are receiving most attention in the last years (Figure 1).

Figure 1



Notch response depends on the type of receptor–ligand interaction. **(a)** Ligand in a neighbouring cell activates the receptor in the adjacent cell. Instead, a ligand presented in the same cell as the receptor will prevent the receptor to respond to other ligands in trans and avoid signaling. **(b)** The absence of fringe modification in the Notch receptor favors Jagged interaction while the presence of fringe favors the interaction with Delta ligands. **(c)** Ligands induce specific intensity and duration of Notch signaling mainly due to the capacity of aggregation and recruitment of receptor molecules. Dll1 is described as an inducer of pulsatile signal, while Dll4 aggregates and induces the activation of several Notch molecules.

Notch molecules and ligands: lessons from crystallography

It was in 2006 when the crystal structure of the Notch1 activating complex with CSL/RBPj and Mastermind (Mam) was reported [2,3]. These studies were critical to understand how Notch works at the chromatin level, and how we can interfere with its function if required. In brief, the NIC domain contains several functional elements including seven ankyrin repeats that are crucial for the interaction with other intracellular and nuclear elements. The interaction domain of NIC with CSL/RBPj expands from repeats two to seven, being the seventh repeat specifically responsible for the interaction with the Notch co-activator Mam. Recruitment of the acetyl transferase p300 is indirect and requires de presence of Mam. The detailed structural characterization of the Notch transcriptional complex led to the development of specific Mam peptides that act as Notch inhibitors by binding to the interaction site of NIC and Mam. Using these peptides, it was demonstrated that Notch inhibition is a plausible strategy to combat T-cell acute lymphoblastic leukemia (T-ALL) [4].

The structural characterization of the Notch ligands interaction with the extracellular domain of Notch has been harder to obtain, and only recently Notch1–Dll4 and Notch1–Jag1 crystal structures have been published. The Garcia's lab developed a strategy to select for mutant ligands with higher affinity for Notch1, thus allowing the crystallization of a more stable complex that permitted the analysis. From these studies, we learnt that Delta-like 4 binds a glycosylated Notch1 receptor with O-glucose and O-fucose modifications of EGF repeats 11–13 acting as surrogate aminoacids [5]. These modifications would likely interfere with specific antibodies targeting this particular Notch interaction sites, thus suggesting that other molecules that bind either Jag or Dll ligands with high affinity (thus competing Notch binding) may be a better therapeutic strategy. Antibodies targeting Dll4 have indeed shown to be efficient in specifically inhibiting Dll4-delivered signals [6] and are promising tools for angiogenesis-based anticancer therapy. Several antibodies targeting Jag1 have also been developed and are currently being tested for their therapeutic applications [7,8•]. Instead, specific Notch blocking antibodies are directed to the negative regulatory region (NRR) of the receptor, the exposure of which is required for the S2 cleavage accomplished by the ADAM10 metalloprotease [9].

Recently, the crystal structure of the Notch1–Jagged1 complex was also resolved [10•] showing that Jagged1 binds to Notch1 receptor when specifically fucosylated on the EGF repeats 8 and 12. Association between Jagged1 and Notch exhibits a catch bond behavior that prolongs their interactions in the range of cellular forces. As a result, higher tension is required to dissociate Notch from

Jagged1 than from Delta-like 4, thus providing an additional mechanism to discriminate between signals derived from different Notch ligands (Figure 1).

Fringe modifications and other posttranscriptional modifications of Notch receptor

As mentioned in the previous section, modifications of Notch imposed by specific glycosyltransferases are at the base of its selective activation by Jagged or Delta ligands. In addition to the sugar moieties that pofut (O-fucose) and poglut (O-Glucose) enzymes deliver, the fringe glycosyltransferases are responsible to elongate the O-fucose residues by adding N-acetylglucosamine (GlcNAc) to the EGF repeats of Notch. There are three fringe proteins in mammals: Lunatic, Manic and Radical that display different enzymatic and functional properties. Although Lunatic and Manic primarily inhibit Notch1 activation by Jagged1 and enhance activation by Delta, Radical fringe enhances activation by both ligands. Only recently, the specific modifications imposed by particular fringe proteins on the Notch1 receptor have been reported. By mass spectrometry analysis, it was found that Lunatic and Manic, but not Radical fringe, induce similar modifications of Notch1 at the EGF repeats 6 and 3, thus inhibiting Notch1 activation by Jagged1 [11•]. It can easily be anticipated that the functional consequences and therapeutic implications of Fringe-mediated modifications are enormous, as they will determine the capacity of Notch to respond to specific ligands under particular scenarios including cancer. Other post-translational modifications of either Notch or associated factors have also been shown to regulate Notch signaling. This is the case of Notch SUMOylation that attenuates its activity [12•], Notch acetylation leading to stabilization of the NIC fragment in endothelial cells lacking SIRT1 [13] GSK3-mediated Notch2 phosphorylation in hematopoietic progenitor cells [14] or acetylation of Maml1 by p300 that is required for initiating Notch-driven transcription [15].

Functional implications of endocytosis in Notch regulation

Endocytosis is an important regulatory mechanism of the Notch pathway. Ligands and receptors are both susceptible of being internalized in different types of endocytic vesicles thus adjusting the amount of ligand and receptors that are available in the cell membrane. As a result, the strength and nature of ligand/receptor interactions that in turn translates into the amount of NIC that is produced and enters the nucleus to activate transcription (Figure 2).

It is not only the amount of ligands and receptors what is regulated by this process, but once Notch is present at the cellular membrane and after ligand binding endocytosis of the latter in the sending cell exerts a pulling force on the extracellular part of the receptor. In particular, this force is responsible for disruption of the NRR leading to exposure of the domain containing the S2 cleavage site and the subsequent conformational change that will allow the processing of the intracellular domain by the γ-secretase/presenilin complex in S3 site (reviewed in [16]).

The endocytic machinery required for a proper Notch function shares some elements with the general endocytic pathway. However some elements are quite specific although not exclusive. For example, the E-Ring ubiquitin ligases Neuralized and Mindbomb are key regulators of Notch ligands ubiquitination, which eventually determines their functionality [17]. Recently, it was demonstrated that the Epsin adaptor protein is essential to facilitate ligand endocytosis by the signal-sending cell after Notch cleavage thus providing the sufficient pulling force required for S2 exposure and Notch signaling. Otherwise, ligand is transendocytosed by the Notch-expressing cell and the Notch signal is aborted [18].

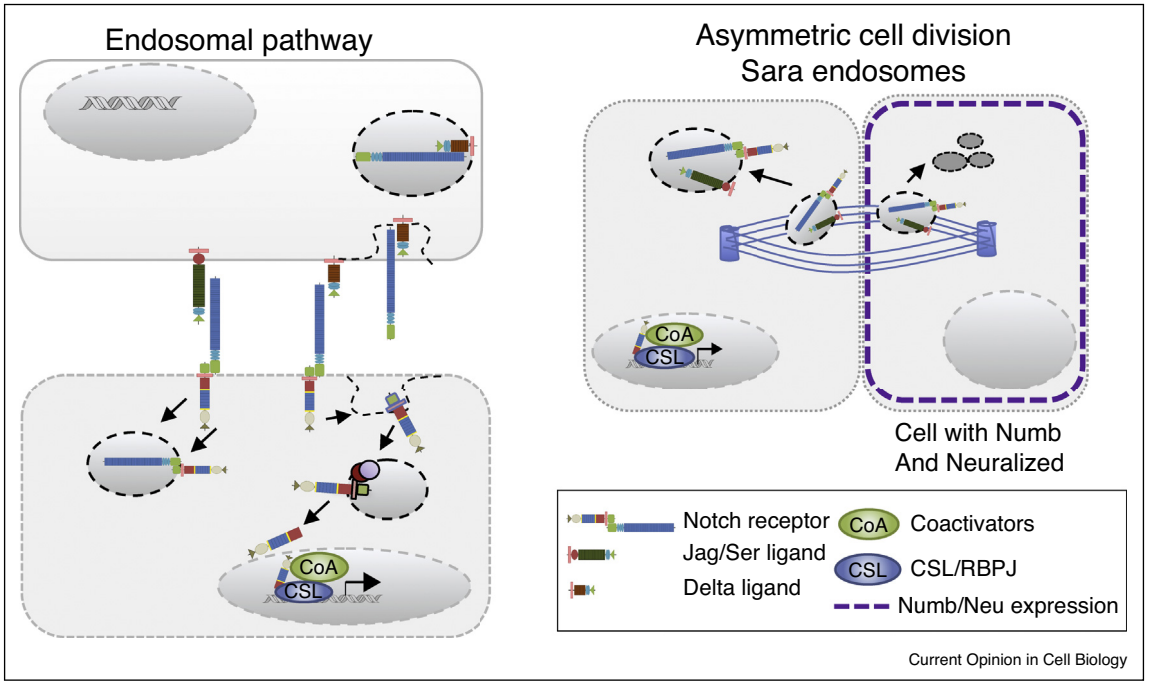
The proper control of the endocytic machinery is also responsible for delivering polarized/unequal Notch signals at a single cell level, thus providing the base for asymmetric cell division (reviewed by [19]).

Non-conventional mechanisms for Notch signal regulation

The canonical mechanism that leads to Notch pathway activation is based on the interactions of Notch receptor with its ligand presented in a neighboring cell, cleavage of the Notch receptor in two different sites (S2 and S3) resulting in NIC release, NIC nuclear translocation and activation of specific gene transcription. Although most of our understanding of Notch functions is related to this type of activation, there are multiple exceptions to this linear regulation of the receptor. For example, *Drosophila* Deltex was found to promote ligand independent activation of Notch in the endosomal compartment of the cells in a presenilin-dependent manner [20]. In the same animal model, the group of González-Gaitán proposed a mechanism in which ligand–receptor binding takes place within multivesicular endosomes of single cells, which express the Sara protein, and drive directional Notch activation during asymmetric cell division [21]. This binding occurs in an anti-parallel configuration (the same that is found in the canonical trans-interaction), as opposed to the parallel binding that is commonly associated to cis-interactions (between receptors and ligands present in the membrane of the same cell) that are believed to prevent signaling (known as cis-inhibition). Interestingly, differential inheritance of the Sara endosomes in the intestinal stem cell compartment leading to asymmetric cell division compartment is required to maintain the homeostasis of the intestinal lineages in *Drosophila* [22] (Figure 2).

As suggested from structural studies, different levels of Notch activation may depend on the pulling force that is

Figure 2



Endosomal regulation of Notch signaling. **(a)** Ubiquitination of the ligand and the presence of Epsin and clathrin endocytic vesicles will produce a pulling force that will activate the Notch receptor when they bind. Instead the absence of the clathrin and Epsin endosomes will result in the endocytosis of the receptor with the bound ligand in the receiving cell. The absence of pulling will block the activation of the receptor and the signal will be aborted and the molecules will degrade. Alternatively, the ubiquitination of the receptor may lead to recycling to the membrane. **(b)** Asymmetric cell division will produce asymmetric Notch signal by the inheritance of SARA endosomes with Notch and ligand that will allow the activation of Notch within the endosome. If the cell that inherits the SARA endosomes has Numb and Neuralized, the endosomes will be degraded.

provided from the ligand. In a recent study, the Ellowitz lab shed some light into the question of how different is Notch signaling depending on the ligand involved. In particular, they demonstrated that Notch signal activation is pulsatile and sustained depending on the type of ligand and the way it aggregates in the cell membrane [23••]. By measuring Hes1 transcriptional activity as a surrogate for Notch activation, the authors demonstrate that pulsatile signaling involves clustering of Dll1 while sustained signaling imposed by Dll4 is ligand clustering-independent. Thus, a general conclusion would be that different levels and amplitude of Notch signal, which are achieved by the combinatory effect of ligand and receptor availability, Fringe-mediated modifications leading to ligand discrimination, differences in the endocytic pathway, cis-inhibition or trans-inhibition and intracellular signaling, can provide a comprehensive explanation to most of the reported observations on the diverse role of Notch in cell fate decisions such as those involved in hematopoietic stem cell determination [24•], tissue and stem cell homeostasis [25] and oncogenic transformation [26].

Apart from its intracellular function as transcription factor in association with CSL/RBPj and Mam, several reports have described interactions with other transcription factors and regulators. Thus, Notch can interact with the IKK kinases to regulate transcription in cervical cancer cells [27] and T-ALL [28]. In endothelial cells, shear stress is sufficient to promote Dll4-dependent Notch cleavage to expose the Notch1 transmembrane domain, which mediates the establishment of the endothelial barrier in a transcriptional-independent manner [29].

Notch in cancer

Notch activity has been extensively linked to cancer where it can exert both tumor suppressor and promoter activity depending on the cellular context. One of the best-characterized roles of Notch as tumor suppressor was found in the mouse skin. In particular, Notch activity is required in keratinocytes to maintain the skin barrier integrity. In the absence of epidermal Notch, an inflammatory response is initiated leading to induction of thymic stromal lymphopoietin (TSLP) that acts as a tumor suppressor. Loss of TSLP receptor in the context of Notch deficient skin results in skin carcinogenesis [30,31]. By contrast intestinal Notch1 acts as tumor promoter [26,32] by blocking differentiation of adenoma cells into the secretory pathway [33]. Interestingly enough, in APC-mutated adenoma cells, activation of

Notch is dependent on the signal provided by Jagged1 that is transcriptionally induced downstream of beta-catenin [26], one of the main tumor promoters in the intestine.

Notch1 activation is also a pivotal tumor driver in human T-ALL. Therefore, 50% of these tumors carry mutations in either the Notch molecule itself [34] or in elements of the Notch pathway leading to aberrant activation of the pathway [35]. Interestingly, Notch activation in transformed T cells directly impacts in another relevant cancer drivers such as NF-κB pathway [36]. Since T-ALL has been an important disease model to study Notch regulations, many interactors have been identified in these leukemic cells. For example Vav1 regulation of Notch stability [37••] and β-catenin collaboration in transcriptional activity [38], among others. Mutations in Notch1, Notch2 and Fbw7 have also been found in chronic lymphocytic leukemia (CLL) [39] and in diffuse large B-cell lymphoma [40].

Activation of different Notch homologues was also found associated with malignancy in multiple solid cancer types such as squamous cell carcinoma [41], thyroid carcinoma [42], melanoma [43], cutaneous T-cell lymphoma and mycosis fungoides [44] but the molecular mechanisms underlying this association are not well-established. Directly related with breast cancer therapy, it was found that Jagged1 is induced by chemotherapy in the osteoblast thus promoting bone metastasis of breast cancer cells through Notch activation. In this system, combination therapy involving anti-Jagged1 blocking antibodies prevented tumor metastasis and reduced already established lesions [45••]. Recently, activated Notch3 was also found to be particularly relevant in several types of cancer including liver cancer [46], basal breast cancer [47] and melanoma [48].

Concluding remarks

The Notch signaling pathway is a robust cell fate regulator, crucial to generate cellular diversification during morphogenesis and essential in the adulthood for stem cell and tissue maintenance. To accomplish this essential mission in multiple cell types and tissues, Notch activation displays exquisite ligand selectivity that is particularly relevant when it discriminates between physiologic and pathologic conditions. Thus, much work should be done to better understand the mechanisms underlying ligand-dependent Notch activation and the downstream elements that are responsible for the plethora of outcomes that Notch accomplishes. Dissecting the mechanisms leading to Notch function selectivity will offer a unique possibility to manipulate the pathway for therapeutic purposes.

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