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Mechanism and Significance of *cis*-Inhibition in Notch Signalling

Review

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Notch receptors in a given cell are activated by cell surface ligands in neighbouring cells but can also be inhibited by the ligands present within the same cell. This process is known as *cis*-inhibition of Notch. Additionally, reciprocal *cis*-inhibition of the ligands by Notch has also been observed, albeit to a limited extent. Here, we review the mechanisms, functional relevance and potential implications of these *cis*-inhibitory interactions for Notch-mediated fate decisions.

Introduction

Notch receptors are key components of a highly conserved signalling cassette with pleiotropic effects during development and tissue homeostasis in metazoans. They are involved in a wide variety of processes, generally controlling binary fate decisions between neighbouring cells as Notch signalling requires direct contact between signal-sending and signal-receiving cells [1]. Notch proteins are receptors for ligands of the DSL family (Delta-like/Jagged in mammals, Delta/Serrate in *Drosophila melanogaster*, Lag-2 in *Caenorhabditis elegans*). Both receptors and ligands are transmembrane proteins present at the cell surface. Ligand–receptor *trans*-interaction, i.e. between neighbouring cells, results in Notch *trans*-activation (Figure 1A). However, studies — for the most part in *Drosophila*, but also in vertebrates — have indicated that ligand–receptor interactions can also take place within the same cell. These *cis*-interactions reduce the ability of a cell to receive the signal from neighbouring cells by a process called '*cis*-inhibition' of the receptor by the ligand (Figure 1B). Here, we review the functional significance and molecular mechanisms of this *cis*-inhibition in Notch signalling. We also discuss recent data suggesting that ligand-receptor *cis*-interactions may also result in the reciprocal *cis*-inhibition of the ligand by the receptor in certain contexts (Figure 1C,D).

Notch Signalling

Notch activity is regulated through a series of proteolytic steps. Upon binding, DSL ligands elicit a proteolytic cleavage in the extracellular domain of Notch mediated by metalloproteases (ADAM/TACE in mammals, Kuzbanian in *Drosophila*). This ligand-dependent cleavage generates a form of Notch that lacks the extracellular domain and is the substrate of the intramembrane γ-secretase complex for constitutive proteolytic release of the intracellular domain of Notch (NICD). The latter translocates to the nucleus where it associates with the CSL (CBF-1 in mammals, Su(H) in *Drosophila*, Lag-1 in *C. elegans*) and Mastermind proteins to activate transcription (reviewed in [2,3]).

Although simple at first glance, Notch signalling is very complex when its regulation is considered. In particular, glycosylation of the extracellular domain is required for proper folding and trafficking to the plasma membrane. These modifications also modulate Notch interactions with the ligands [4]. Also, ubiquitination of the ligands by the evolutionarily conserved E3-ubiquitin ligases Neuralized (Neur) and Mindbomb1 (Mib1) is required for receptor activation and, concomitantly, causes a reduction of the amount of ligands present at the plasma membrane through endocytosis and degradation. Similarly, Notch endocytosis and trafficking to endosomes also affect the amount of Notch at the cell surface as well as the formation of NICD. Several endocytic routes have been proposed, including Notch removal from the membrane to avoid spurious or excessive activation (reviewed in [5]). Finally, control of NICD turnover is critical to avoid sustained activation (see [2,3] for a detailed description of Notch regulation).

cis-Inhibition of the Receptor by the Ligands

Early genetic experiments in *Drosophila* uncovered intriguing relationships between *Notch* and its ligand *Delta (Dl)*. In addition to many allele-specific interactions, several unexpected gene-dosage interactions were noted. In certain contexts, double loss-of-function conditions for ligand and receptor suppressed the individual phenotypes of each other while gene duplication of the ligand enhanced receptor loss of function phenotypes and vice versa [6–8]. These interactions suggested that the ratio between the ligand and the receptor concentrations can influence the signalling outcome. In 1997, two seminal papers [9,10] demonstrated for the first time that ligands actually display a *cis*-inhibitory effect on Notch in order to properly specify the wing margin.

During early stages of wing development, Notch is expressed ubiquitously while Delta and the second ligand Serrate (Ser) are expressed in the ventral and dorsal compartments, respectively. Glycosylation of Notch in the dorsal compartment by the glycosyltransferase Fringe (Fng), which is expressed dorsally, increases its affinity for Delta but reduces it for Ser. Thus, Delta signals from the ventral cells to glycosylated Notch in the dorsal cells while Ser signals from the dorsal cells to unmodified Notch in the ventral cells, allowing bidirectional signalling across the compartment border [4,11]. This mechanism leads to strong and robust activation of Notch in the cells immediately abutting the dorsoventral compartment border, which in turn controls the expression of the Notch ligands as well as of several genes required for wing growth and patterning [12–14]. Evidence for *cis*-inhibition of Notch by its ligands came first from over-expression experiments: over-expression of Delta in dorsal cells resulted in ectopic activation of Notch targets, whereas over-expressed Ser only triggered Notch activation in ventral cells [10,12–14]. Unexpectedly, however, dorsal cells over-expressing Delta did not show any activation of Notch but were only able to activate Notch in the surrounding adjacent wild-type cells (Figure 2).

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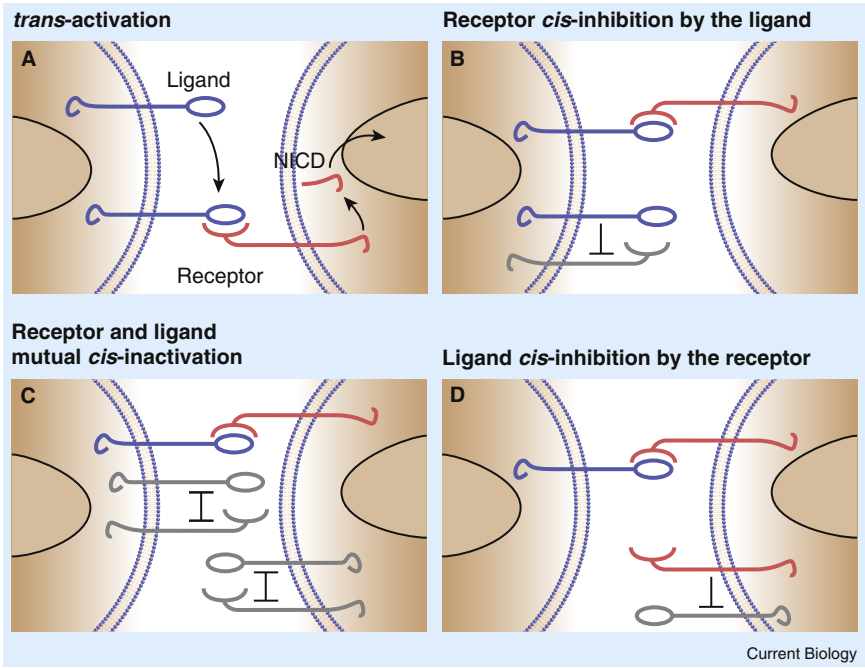

Figure 1. Possible *cis*- and *trans*-interactions between Notch and its ligands.

(A) *trans*-activation. The ligand (blue) at the surface of the signal-sending cell interacts with Notch (red) at the surface of the neighbouring signal-receiving cell. *Trans*-activated Notch is cleaved to generate NICD. (B–D) *cis*-interactions. The presence of both the ligand and the receptor in the same cell leads to the inhibition of the receptor by the ligand (B), the mutual inactivation of the receptor and the ligand (C) or the inhibition of the ligand by the receptor (D). The extent to which *cis*-inhibition of the receptor by the ligand exists independently of *cis*-inhibition of the ligand by the receptor is not known. Indeed, ligand-receptor *cis*-interactions may result in the titration and inhibition of both ligands and receptors. Inactive ligand and receptor are shown in grey.

Reciprocal observations were made for Ser over-expressing cells in the ventral compartment. Moreover, when cells at the dorsoventral border, where Notch is endogenously active, were included within the group of cells that over-express either Delta or Ser, Notch activity was abolished. These results led to the conclusion that high levels of the ligands can repress Notch activity in a cell-autonomous manner, in *cis* [10,15,16]. Inhibition of Notch signalling by high levels of ligands was further observed in other tissues. For instance, over-expression of Delta induced sensory cell fate changes that were dependent on endogenous Delta dosage: triploidy for endogenous Delta suppressed the phenotype, while haploidy enhanced cell fate change frequency [17].

In other developmental contexts, however, Notch signalling seems to be more insensitive to ligand over-expression. Sensory organ precursor cells (SOPs), which give rise to sensory bristles, are selected from groups of cells (proneural clusters) with similar developmental potential by a Notch-mediated patterning process called 'lateral inhibition' [18]. Lateral inhibition is also used during eye development to select the ommatidial founder photoreceptor R8 from a group of equipotent cells [19]. Reduction of Notch activity in these clusters of equipotent cells leads to supernumerary SOPs or R8 cells and over-activation of Notch leads to a reduction in their number. While the first explicit model of *cis*-inhibition was proposed to interpret genetic data on the role of Notch in SOP specification [20], Delta over-expression [21,22] and changes in *Delta* gene copy number [23] had little effect on SOP specification, and almost no effect in R8 specification [22]. These observations may be interpreted such that either *cis*-inhibition plays no major role in these patterning processes or that *cis*-inhibition can operate to regulate patterning over a wide range of Delta concentrations. However, over-expression of Ser appeared to inhibit Notch despite the fact that endogenous Ser has no major role in SOP or R8 specification [22]. A stronger inhibitory effect of Ser compared to that of Delta has also been observed in other contexts, although the reasons for this are unclear [8,16,22].

Importantly, there is one caveat with over-expression experiments: the observed effects depend on abnormally high, non-physiological levels of the ligands. There are, however, two instances where a role for ligand *cis*-inhibition

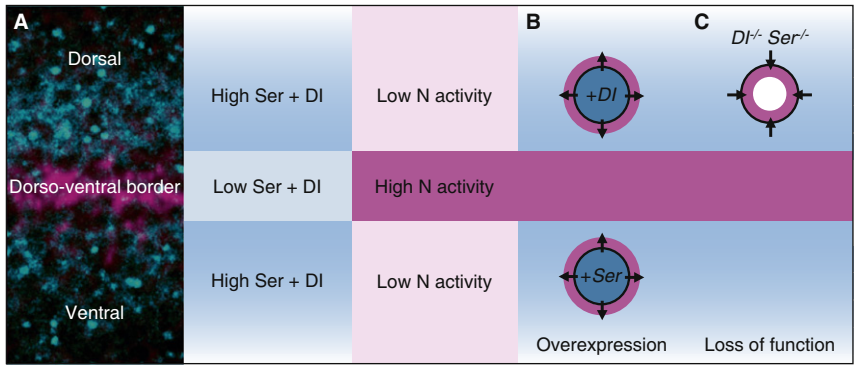

Figure 2. *cis*-Inhibition of the Notch receptor in the *Drosophila* wing imaginal disc.

(A) Distribution of the ligands and the receptor. Left panel shows a magnification of the central region of a late third instar larval disc including the dorsoventral border. Delta expression in turquoise and that of the Notch target Wingless is in magenta. Ser expression is similar to that of Delta at this stage. Note that cells expressing high levels of the ligands activate Notch in cells with low levels. (B,C) Clonal analysis experiments demonstrating *cis*-inhibition in the wing margin. The clone border is marked in black. (B) Delta over-expression activates Notch in the surrounding wild-type dorsal neighbouring cells while the cells that over-express Delta do not activate Notch. This mimics the endogenous situation described in (A). Ser causes the same effects in the ventral compartment. (C) *Ser* and *Delta* loss of function clones cause ectopic activation of Notch, but only in the mutant cells at the border of the clone, in contact with the wild-type neighbours.

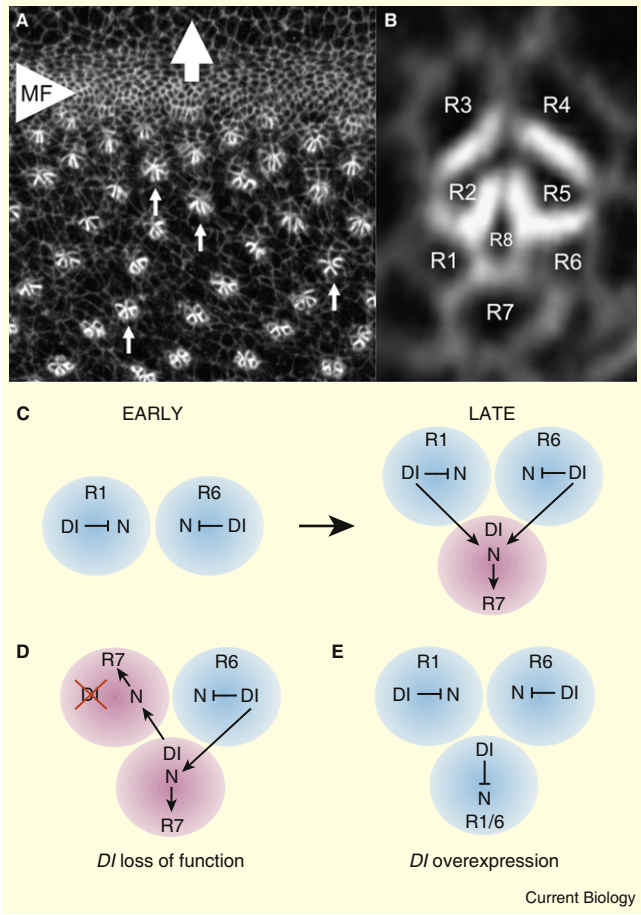

Figure 3. *cis*-Inhibition in the *Drosophila* eye.

(A) DE-Cadherin staining of the developing eye imaginal disc in *Drosophila*. The morphogenetic furrow (MF, arrowhead) moves towards the anterior, leaving an evenly spaced array of ommatidial clusters (small arrows). (B) An ommatidial pre-cluster with the eight photoreceptors (R1–R8). R8 is specified first, followed by R2/R5, R3/R4 and R1/R6. The last photoreceptor to be recruited is R7, which receives the Delta signal from R1 and R6 to properly acquire its fate. (C) The proposed model based on *cis*-inhibition states that, following their specification, the R1/R6 cells start expressing Delta and that this early expression *cis*-inhibits Notch. When the R7 precursor is recruited into the cluster, Delta present in the R1/R6 pair activates Notch in R7, leading to Delta expression in R7. When Delta expression starts in R7, this cell has already acquired its fate whereas the R1/R6 pair cannot receive the signal from R7 due to *cis*-inhibition of Notch by Delta. (D,E) Key experiments supporting the model. (D) When R1 is mutant for *Dl*, the R7 precursor receives Delta signalling from R6 and properly adopts its fate. However, Delta in R7 is now able to activate Notch in R1, thereby producing a cell fate transformation from R1 to R7. In contrast, Delta from R7 has no effect on R6, indicating that Delta in this cell is able to *cis*-inhibit Notch. (E) When Delta is over-expressed in the R1, R6 and R7 precursors before they are recruited, the R7 precursor adopts the R1/R6 fate, suggesting that early presence of Delta blocks Notch in the R7 precursor as it does in R1 and R6. Thus, although Delta can *cis*-inhibit Notch in R7, it is not normally present at the time when the R7 precursor is recruited so that Notch can receive the Delta signal from R1 and R6.

of Notch has been demonstrated by loss-of-function experiments. First, in the wing imaginal disc in *Drosophila*, Ser and Delta expression patterns are initially compartment-specific (see above), while at later stages Ser, and Delta are expressed in both dorsal and ventral cells flanking the dorsoventral border (Figure 2A) [9,10]. Interestingly, these cells show no activation of Notch. When clones of cells double mutant for *Delta* and *Ser* were generated in these regions, ectopic activation of Notch was detected in the mutant cells at the border of the clone, in contact with the wild-type *Ser* and *Delta* expressing cells (Figure 2) [9,24]. Clones mutant for either *Delta* or *Ser*, but not both, caused only minor or no effects, suggesting that the presence of only one of the ligands may be sufficient for Notch *cis*-inhibition [9]. This indicates that Notch is normally *cis*-inhibited by its ligands in cells flanking the dorsoventral border and could only be activated in these cells upon loss of both ligands. Additionally, the relative levels of *trans*-activating versus *cis*-inhibiting ligands may be relevant for Notch activity since ectopic activation of Notch was observed in cells in which Notch is normally *cis*-inhibited upon over-expression of either *Delta* or *Ser* in neighbouring cells [10].

The second example of a physiological role of Delta in *cis*-inhibition, this time at the single cell level, comes from the *Drosophila* eye. There, ommatidial development starts with the specification by lateral inhibition of the ommatidium founder cell R8, which in turn begins the recruitment of the rest of the 7 photoreceptors (R1–7) (Figure 3A,B) [25]. R1, R6 and R7 are the last photoreceptors to be recruited into the ommatidial cluster. R1 and R6 are recruited first and start expressing Delta. Delta signals redundantly from R1 and R6 to activate the R7 fate in the last photoreceptor to be recruited [26]. These three cells (R1, R6 and R7) form an equivalence group as any cell with active Notch will adopt the R7 fate while any cell with inactive Notch will adopt the R1/R6 fate [26]. In this context, when only one of the cells of the R1/R6 pair is mutant for *Delta*, there was no effect on the fate of R7 as the wild-type cell can still signal, but it unexpectedly caused the mutant cell to become R7 (Figure 3D). This phenotype is not consistent with Delta acting in *trans* to activate Notch but rather suggests that loss of *Delta* resulted in Notch activation. This fate transformation is dependent on Delta activity in the R7 precursor: when R7 is also mutant for *Delta*, no fate transformation was observed in the mutant cell of the R1/6 pair. This important result led to the conclusion that Delta *cis*-inhibits Notch in R1/R6 and protects these cells from the Delta signal emanating from R7. By the time R7 starts expressing *Dl*, this cell has already received the signal from R1 and R6, and cannot signal back due to the *cis*-inhibition of Notch by Delta in R1 and R6 (Figure 3) [27]. Early expression of Delta in the R1, R6 and R7 precursors causes the three of them to acquire the R1/6 fate, suggesting that Delta-mediated Notch *cis*-inhibition is taking place now in the R7 precursor in addition to R1 and R6 (Figure 3E) [27].

Whereas only two cases of developmental decisions involving *cis*-inhibition in physiological conditions have been uncovered so far, other developmental processes relying on *cis*-inhibition may exist as suggested by over-expression experiments. However, as loss-of-function approaches disrupt both *trans*-activation and *cis*-inhibition, it has proven difficult to reveal a possible role of *cis*-inhibition in developmental contexts where *trans*-activation and *cis*-inhibition cannot be analyzed separately [28].

cis-Inhibition in Vertebrates

Evidence for *cis*-inhibition of vertebrate Notch receptors by their Delta-like and Ser-like (Jagged) ligands is so far mostly based on over-expression studies. Co-transfection experiments in cell culture have shown *cis*-inhibitory effects by

using Notch reporters or by analyzing specific Notch-dependent cellular phenotypes. For instance, chick Delta-1 and chick Ser-1 have been shown to significantly reduce the activity of mouse Notch-1 when co-expressed, as monitored by a HES5-luciferase reporter assay [29]. Similar cell culture experiments showed that *Xenopus laevis* Delta-1 is able to *cis*-inhibit Notch-1 transcriptional activity [30]. Studies of cellular phenotypes point in the same direction. For example, mouse Notch-1 regulates neurite outgrowth in postmitotic neurons. Expression of its intracellular constitutively active fragment in N2a neuroblastoma cells, a model for committed neurons in cell culture, resulted in significant neurite shortening. Neurite shortening was also observed when N2a cells were co-cultured with mouse Delta-1-transfected fibroblasts, consistent with activation in *trans*. Conversely, a dominant-negative version of mouse Delta-1 caused extensive neurite outgrowth. However, when mouse Delta-1 was transfected into N2a cells, a phenotype similar to that of dominant negative mouse Delta-1 was observed, suggesting that mouse Delta-1 can inhibit mouse Notch-1 signalling in *cis* [31].

These results are further supported by *in vivo* experiments: a truncated form of *Xenopus* Delta-1 lacking the intracellular domain showed dominant negative effects on Notch signalling in *Xenopus* embryos [32]. The inhibitory effect was later demonstrated to be cell-autonomous in chick and mouse embryos [33,34], suggesting that this truncated version of Delta may therefore inhibit Notch by a mechanism similar to the *cis*-inhibition mechanism elicited by the wild-type version of Delta. A role for *cis*-inhibition has been proposed for the maintenance of stem cells in the mouse and human epidermis. In this context, Delta-like 1 (Dll1) is expressed at high levels in stem cell clusters in the basal layer [35]. Cell culture and *in vivo* experiments have suggested that Dll1 may act both by *cis*-inhibiting Notch to promote stem cell state maintenance inside these clusters and by signalling to the surrounding cells to promote their differentiation [35,36].

Interestingly, mammalian genomes encode a variant of Delta, Dll3, that *cis*-inhibits Notch but appears unable to *trans*-activate Notch, possibly because its intracellular tail lacks lysine residues and, therefore, cannot be ubiquitinated by Mib [37]. In mice, Dll3 is required for proper somitogenesis but it is not yet clear whether Dll3 acts by *cis*-inhibiting Notch in this context. Indeed, increasing the levels of Dll3 relative to Dll1, the main Notch activating ligand in this process, did not reveal antagonistic relationships between Dll1 and Dll3 [38].

In summary, *cis*-inhibition appears to be a property of the Notch signalling pathway that is conserved between vertebrates and invertebrates, but further studies are needed to fully understand which ligands and receptors are involved and in which cases this regulation takes place *in vivo*.

Molecular Interactions Underlying *cis*-Inhibition

We are only beginning to understand the molecular mechanism underlying *cis*-inhibition of Notch by its ligands. Several lines of evidence indicate that it involves direct ligand–receptor interaction. Early experiments in *Drosophila* S2 cells showed Notch–Delta co-clustering that was independent of the intracellular domain of Notch, suggesting that Notch and Delta might interact via their extracellular domains [39]. More direct evidence came from *in vivo* structure–function studies in *Drosophila* showing that the receptor-binding domain of Ser is required for *cis*-inhibition [15]. Additionally, co-immunoprecipitation experiments indicated that chick Delta-1 and Ser-1 physically interact with mouse Notch-1 in transfected cells cultured at low density (to limit cell–cell interactions, hence ligand-receptor *trans*-interactions) [29]. Formation of these heterodimers required the extracellular but not the intracellular domain of mouse Notch-1. Together, these data indicate that *cis*-inhibition involves direct interaction between the EGF repeats of Notch and the DSL domain of the ligands.

A recent structural analysis further suggested that Notch may interact with the DSL domain of its ligands via two modes of interaction, referred to as parallel and antiparallel binding: interaction of Ser with Notch in *trans* would follow antiparallel binding, leading to activation of Notch, whereas interaction in *cis* would be mediated by parallel binding, resulting in inhibition of Notch [40]. Interaction of Notch with its ligands in *cis* may inhibit signalling either by forming complexes that are inactive for signal reception, thereby titrating Notch, or by regulating a catalytic process that results in Notch inhibition, for instance by promoting the internalization and degradation of Notch whereas ligands are recycled. A recent study [41] has provided support for a titration-based mechanism. The activity of a Notch reporter-construct in response to various concentrations of Delta in *trans* and/or in *cis* was precisely measured using a cell-based assay (Figure 4A) [41]. Two key observations were reported. First, Notch activity gradually increased with increasing concentrations of Delta in *trans* (Figure 4B). A graded response to Delta in *trans* was observed independently of Delta levels in *cis* (below a threshold level). Second, upon decreasing concentration of Delta in *cis*, a Notch response to Delta in *trans* was observed below a threshold level of Delta in *cis*. The steepness of this Notch response depended on the levels of Delta in *trans*. However, the threshold level of Delta in *cis* below which Notch is activated was independent of Delta levels in *trans* (Figure 4C). These observations were best explained by a model based on titration [41]. Indeed, inactivation through titration has been shown in other contexts to lead to a sharp response of receptors to ligand concentration in *cis* [42]. By contrast, models based on a catalytic inactivation of Notch by Delta in *cis* indicated that the level of Delta in *cis* for which a significant Notch response is predicted varied with the levels of Delta in *trans*, which do not fit the observed results [41]. Thus, these studies indicate that ligands have the ability to titrate Notch in *cis*.

One issue raised by this titration-based mechanism concerns the ability of ligands that interact with Notch in *cis* to signal. Indeed, whether the same molecule can simultaneously interact in *cis* and in *trans* with Notch is not known. In case ligands *cis*-interacting with Notch are inactive, then receptors reciprocally *cis*-inhibit ligands. Accordingly, *cis*-inhibition of the receptor by the ligand (Figure 1B) would be mechanistically coupled to *cis*-inhibition of the ligand by the receptor (Figure 1C) in a mutual inhibition process (Figure 1D). This important issue needs to be explored.

Molecular Output of *cis*-Interactions

The titration model implies that Notch can no longer signal when bound in *cis* by its ligand but does not provide a mechanistic explanation for this phenomenon. A recent study in *Drosophila* S2 cells shows that ligands can *cis*-inhibit Notch signalling when activated by EDTA [24]. EDTA is known to activate Notch, in a ligand-independent-manner,

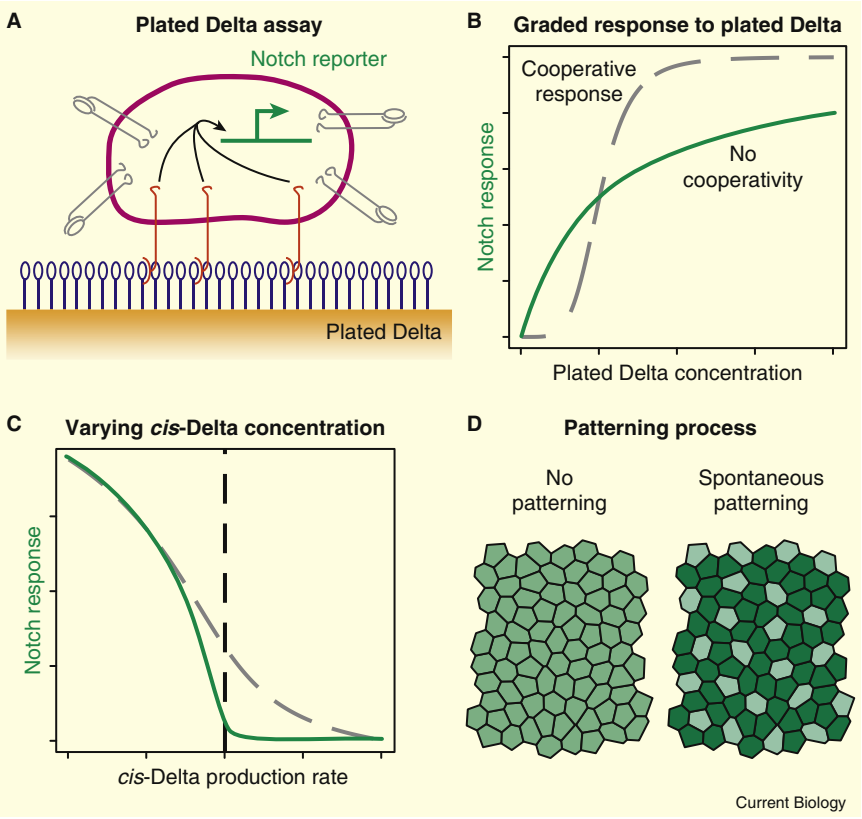

Figure 4. Properties of *cis*-interactions and their significance for patterning.

(A) Experimental assay used in [41] to measure *trans*-activation and *cis*-inhibition properties. Cells expressing a modified form of Notch and varying concentration of *cis*-Delta are grown on Delta-coated plates. Notch is activated by plate-bound Delta (*trans*-Dl). Notch activity is measured as a function of *trans*-Delta and *cis*-Delta concentrations using a reporter gene assay. (B) Graph representing Notch activity as a function of plate-bound Delta. As Delta concentration increases, Notch response increases gradually (solid line), i.e. non-cooperatively. The dotted line represents a typical cooperative response, which is not observed experimentally. (C) Graph representing Notch activity as a function of *cis*-Delta production rate. In the actual experiments, plate-bound Delta and Notch concentrations are kept constant while *cis*-Delta concentration decays over time. A sharp activation of Notch is observed at a *cis*-Delta concentration that does not depend on *trans*-Delta levels. This response fits a model whereby Notch and Delta mutually inactivate each other in *cis*. In the model proposed by [41], the affinity of Notch to *cis*-Delta needs to be much higher than that to *trans*-Delta to explain the sharp response of Notch to varying *cis*-Delta concentration independently of *trans*-Delta levels. A curve similar to the one shown in grey is predicted if the affinities of Notch for *cis*-Delta and *trans*-Delta were within the same range. (D) Patterning of a two-dimensional epithelium by lateral inhibition. No spontaneous patterning occurs when lateral inhibition involves only non-cooperative Notch-mediated transcription-based inhibition of the ligand (left). Adding mutual inactivation to this model results in spontaneous patterning (right).

presumably by chelating divalent ions that are required to stabilize the extracellular domain of Notch, which includes the S2 site [43]. Destabilization of this region by EDTA would unmask the S2 cleavage site in the absence of *trans*-activating ligands. The observation that ligands antagonize in *cis* the activation of Notch by EDTA suggests that ligands do not simply *cis*-inhibit Notch by interfering with ligand-receptor *trans*-interactions (Figure 5). Consistent with this, *cis*-inhibition of Notch by Delta in cultured cells did not prevent interaction with cells expressing Delta only [24].

Alternatively, ligands have been proposed to *cis*-inhibit Notch by interacting with the receptor in the secretory pathway, hence blocking its expression at the cell surface [29] (Figure 5). Heteromeric complexes of mN1 with cSer1 and cDl1 were indeed retained intracellularly [29]. This model, however, remains controversial. First, cell surface biotin labelling experiments suggest that expression of cDl1, mDll1 and mDll3 did not significantly affect the amount of surface-exposed Notch receptors [29,37]. Second, while ectopic expression of Ser in imaginal discs reduced Notch levels in *Drosophila*, over-expression of mutant forms of Ser that accumulate at the plasma membrane causes *cis*-inhibition without altering Notch distribution [15]. These results suggest that, at least in *Drosophila*, ligands can modulate the level of Notch at the cell surface but this regulation may not be necessary for *cis*-inhibition. Consistent with this, no changes in Notch levels or distribution are detected upon loss of *cis*-inhibition in *Delta Ser* double mutant wing imaginal disc cells [24,44].

Another possibility is that the *cis*-ligand interferes with the ADAM/TACE metalloprotease-dependent S2 or the γ-secretase-dependent S3 cleavages (Figure 5). For instance, interaction in *cis* might stabilize the structure of the LNR repeats of Notch, thereby preventing access of the metalloprotease to the S2 cleavage site. Alternatively, since Delta can also be subjected to S2 and S3 cleavages in a manner very similar to Notch [45], Delta and Notch might be competing in *cis* for the protease activities required for Notch signalling. These hypotheses remain, to our knowledge, so far untested. Interestingly, several ligand-independent constitutively active forms of Notch causing acute lymphoblastic leukaemia in humans bear mutations in the heterodimerization region of Notch [46]. Structural analysis of this region raises the possibility that these mutations may destabilize this region, therefore exposing the S2 cleavage site in the absence of a *trans*-activating ligand [47]. Whether ligands can *cis*-inhibit this form of Notch or not has not been analyzed, but it could provide very useful information regarding the molecular basis of *cis*-inhibition.

Regulation of the *cis*-Inhibition of Notch

Regulation of *cis*-inhibition likely involves processes that can modulate receptor–ligand interaction, such as the sugar modification of the extracellular domain of either Notch or its ligands. In one study, expression of the vertebrate homolog of *Drosophila Fringe*, Lunatic Fringe (LuFng), was found to reduce the amount of receptor–ligand complexes in mammalian cells grown at low density. This effect

correlated with reduced *cis*-inhibition of Notch by Delta [29]. In another study, expression of LnFng did not change the amount of Notch–Dll3 complexes and did not reduce *cis*-inhibition of Notch by Dll3 [37]. Consistent with these latter results, Fringe was found to be dispensable for *cis*-inhibition in a *Drosophila* over-expression assay [15]. However, as Fringe proteins regulate ligand affinity for the receptor, differences in expression levels and/or assays may account for this discrepancy. Thus, *in vivo* loss-of-function assays would best address the possible role of Fringe in *cis*-inhibition vs. *trans*-activation.

Additionally, while ligand ubiquitination is essential for *trans*-activation, several lines of evidence indicate that this regulation does not play a significant role in *cis*-inhibition. First, ligand ubiquitination might not be essential for *cis*-inhibition, as truncated forms of Delta that lack the intracellular domain, as well as mammalian Dll3, which lacks intracellular lysine residues and cannot be ubiquitinated, are able to inhibit Notch in *cis*. Second, the E3 ubiquitin ligase Neur is required for *trans*-activation but dispensable for *cis*-inhibition. In the R1/R6/R7 system, removal of *neur* activity from R1 and R6 not only disrupts the ability of Delta to signal and causes transformation of R7 into an R1/R6 fate but, more interestingly, in contrast to the removal of *Dl*, has no effect on the fate of the R1/R6 cells mutant for *neur*. This suggests that, in the absence of Neur, Delta can no longer signal, i.e. *trans*-activate, but can still *cis*-inhibit and block the Delta signal coming from the R7 precursor [27]. Consistent with this, a mutant form of Ser that does not interact with Neur and Mib-1 could not *trans*-activate Notch but retained its *cis*-inhibitory activity in the wing [15]. Additionally, cell culture experiments have also shown that RNAi-mediated inactivation of Neur and Mib-1 had no effect on the ability of Delta and Ser to *cis*-inhibit Notch [24] and that increasing levels of Mib1 did not antagonize the *cis*-inhibiting activity of XDl-1 on N1 [30].

Finally, while ligand ubiquitination appears to be dispensable for the *cis*-inhibition of Notch by its ligands, it is conceivable that the loss of E3 ubiquitin ligase activity leads not only to decreased *trans*-activation of Notch but also to increased *cis*-inhibition to the accumulation of ligands at the cell surface.

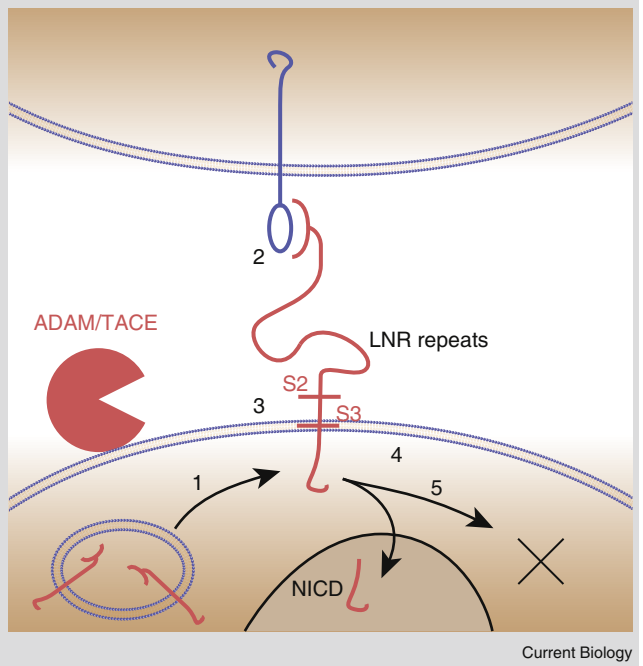

Figure 5. Possible molecular mechanisms underlying *cis*-inhibition of Notch.

Down-regulation of the amount of receptor (red) at the cell surface can be achieved by inhibiting its trafficking and folding (1) or promoting its degradation (5). Alternatively, ligands in *cis* and in *trans* may compete with each other to bind the receptor at the cell surface (2). Another possibility is that *cis*-ligands may stabilize the Lin-Notch repeats (LNR), blocking the access of the ADAM/TACE metalloproteases to the S2 cleavage site, or they may even compete with Notch for the metalloprotease activity (3). The same argument applies regarding S3 cleavage (4), which directly leads to a reduced production of the active form of Notch (NICD).

Role of *cis*-Inhibition and Mutual Inactivation in Patterning

A recent important paper [41] has provided experimental evidence suggesting that *cis*-interactions might have a much stronger influence on the ability of Notch to generate patterns than previously thought. One prominent role of Notch during development is its ability to pattern through lateral inhibition an extended field of equipotent, undifferentiated cells (Figure 4D). This process leads to the specification of two cell types at regular spatial intervals and underlies, for instance, the patterning of neuroepithelia in vertebrates and *Drosophila*. In lateral inhibition, equipotent cells have initially similar levels of Notch and Delta activities and become specified into cells with either high Delta and low Notch or low Delta and high Notch [18,48]. Classic models have considered that this patterning involves trans-activation of Notch by Delta and occurs through a regulatory feedback loop whereby high Notch activity in a given cell results in low Delta activity in the same cell. Since Notch regulates gene transcription, it is commonly accepted that this feedback loop involves a transcription-based mechanism that reduces ligand activity in response to receptor activation [49,50]. This feedback loop was proposed to operate by amplifying small stochastic differences between neighbouring cells [51]. Modelling studies suggest that this feedback loop based on transcriptional regulation of Delta activity by activated Notch, directly or indirectly, can account for the observed patterns, although patterning can only be achieved with a significant level of cooperativity in the signal transduction pathway [52,53]. Cooperativity could be achieved at any level in the signal transduction pathway. For instance, cooperativity might result from interaction between signalling components or at the level of gene regulation. Moreover, bi-dimensional patterning, as it occurs in neuroepithelia patterned by Notch-mediated lateral inhibition, requires a higher level of cooperativity than one-dimensional patterning [41]. A recent study [41] has, however, suggested that *trans*-activation of Notch by Delta, as measured using an artificial reporter assay, is a non-cooperative process (Figure 4B). This result may imply that models based only on transcriptional feedback loops are not sufficient for proper patterning.

Theoretical studies have demonstrated that *cis*-inhibition of Notch by Delta, together with a regulatory feed-back loop, facilitates patterning via lateral inhibition [53]. In brief, fluctuations that increase the level of Delta in a given cell have two effects: they inhibit Delta activity in the neighbouring cells, via a feedback loop as described above, and also

make this given cell less receptive to the Delta signal produced by its neighbours. These two effects are combined to efficiently amplify small differences in Notch activity between cells. Cooperativity was actually shown to be no longer necessary for patterning when *cis*-inactivation of Notch by Delta occurs by titration and is accompanied by reciprocal *cis*-inactivation of Delta by Notch (Figure 1D), resulting in mutual inactivation (Figure 1C) [41]. In support of this view, a sharp response of Notch to Delta in *cis* was observed in cell-culture studies, which is best interpreted by a mutual-inactivation model. Moreover, theoretical studies demonstrate that mutual inactivation, together with a non-cooperative feed-back loop, can efficiently generate patterning upon minor variations in ligand–receptor ratios [41]. In addition, a recent study [54] argues that mutual inactivation shortens the time delay between signal emission in one cell and the response to this signal in neighbouring cells and that this increases accuracy in patterning.

While mutual inactivation facilitates patterning by lateral inhibition, it does not seem to be sufficient for patterning. In all models proposed, a regulatory feedback loop is included. Additionally, while this feedback loop is often considered to link Notch activation to Delta inhibition, this feedback may also take other forms like, for instance, a Notch auto-regulatory loop whereby *trans*-activated Notch stimulates Notch synthesis [55].

Finally, normal patterns can probably be generated over a wide range of Notch and Delta concentrations, provided that the starting point is the same for all the cells in the cluster, with spatial irregularities in the pattern occurring only where cells with different Notch or Delta levels are juxtaposed [51]. Thus, uniform over-expression of the ligand may not necessarily result in phenotypes associated with low Notch signalling. This in turn suggests that over-expression might not be a useful assay to reveal the possible existence of *cis*-inhibition. These considerations underscore the importance of combining modelling studies with quantitative analysis of emerging patterns *in vivo* to identify *cis*-inhibitory interactions and characterize their role in patterning.

cis-Inhibition of the Ligands by the Receptor

The titration model mentioned above suggests the possibility of reciprocal *cis*-inhibition of the ligand by the receptor in a mutual inactivation process (Figure 1C). Although limited, some instances of *cis*-inhibition of the ligands have indeed been observed. A study in cell culture has shown that co-expression of Notch and Delta reduces the ability of Delta to signal to cells expressing Notch only [41]. In *C. elegans*, down-regulation of DSL signalling activity by the Notch receptor LIN-12 in the P6.p vulva precursor cell is required to properly pattern the vulva. This down-regulation requires the extracellular domain of LIN-12 but not its transcriptional activity, suggesting that *cis*-inhibitory interactions may occur between Notch and its ligands although no mechanism has been proposed [56]. In zebrafish, morpholino experiments have indicated that Notch is required for the endocytosis and degradation of DeltaD and DeltaA. Transplantation experiments further demonstrated that Delta internalization depends on the presence of Notch both in *cis* and in *trans*, supporting the idea of *cis*-regulation of the ligand by the receptor [57]. Similar observations have recently been reported for *Drosophila* Ser. Clones of wing imaginal cells mutant for Notch showed higher levels of Ser than surrounding wild-type cells. Notch is able to cause Mib1-independent Ser endocytosis and in the absence of Notch, Ser accumulates at the plasma membrane, causing non-autonomous activation of Notch in surrounding cells. Again, Ser *cis*-inhibition appeared to be independent of Notch transcriptional activity and dependent on the Notch extracellular domain [44]. This *cis*-inhibition of the ligand by Notch has so far been only reported in the wing and only affects Ser — Delta expression, localization and activity were not detectably affected by the presence or absence of Notch in the same cell [44].

Thus, only a limited number of studies have so far provided some experimental evidence for *cis*-inhibition of the ligands by the receptor. Additionally, whether *cis*-inhibition of the ligand by the receptor is accompanied by the reciprocal *cis*-inhibition of the receptor by the ligand remains to be explored. Thus, the general significance of the proposed 'mutual-inactivation-by-titration model' remains to be established.

Conclusions

cis-Inhibitory interactions have emerged as key regulatory mechanisms for Notch signalling in both vertebrates and invertebrates. Although the basis underlying *cis*-inhibition, as well as the consequences of these *cis*-interactions, still remain to be elucidated at the molecular level, a growing body of evidence indicates that ligands and receptors directly interact, leading to receptor inactivation. We have also emphasized here the possible contribution of *cis*-inhibition of Notch and of mutual inactivation for patterning by lateral inhibition. A major challenge is to now examine to what extent these models apply *in vivo*. Analyzing the dynamics of cell fate decisions based on *cis*-inhibition would greatly benefit from quantitative 4D imaging of receptors and ligands. It will also be important to develop genetic approaches and imaging tools to discriminate between *cis*- and *trans*-interactions *in vivo*. Addressing these issues in the case of Notch receptors may have a broad impact for our system-level understanding of signal processing by the cell. Indeed, several other signalling pathways may have possibly evolved similar forms of ligand-receptor *cis*-inhibition. These include the axon guidance molecules Eph receptors and their ephrin ligands [58] and Semaphorin6A and its receptor Plexin-4A [59] as well as the MHC class I receptors and their ligands [60]. Whether these signalling pathways take any advantage from the properties of *cis*-inhibition in order to generate stable cellular states, as may be the case for Notch in lateral inhibition, is an exciting topic to be explored. In any case, finding *cis*-inhibition in several independent transduction pathways reinforces the idea that *cis*-inhibition has intrinsically valuable properties that still need to be fully deciphered in dynamic and *in vivo* studies.

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