Annual Review of Biophysics

Biophysics of Notch Signaling

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Annu. Rev. Biophys. 2021. 50:157–89

First published as a Review in Advance on February 3, 2021

The *Annual Review of Biophysics* is online at biophys.annualreviews.org

https://doi.org/10.1146/annurev-biophys-101920-082204

Keywords

Notch signaling, signal transduction, mechanotransduction, structural biology, transcription, intramembrane proteolysis

Abstract

Notch signaling is a conserved system of communication between adjacent cells, influencing numerous cell fate decisions in the development of multicellular organisms. Aberrant signaling is also implicated in many human pathologies. At its core, Notch has a mechanotransduction module that decodes receptor–ligand engagement at the cell surface under force to permit proteolytic cleavage of the receptor, leading to the release of the Notch intracellular domain (NICD). NICD enters the nucleus and acts as a transcriptional effector to regulate expression of Notch-responsive genes. In this article, we review and integrate current understanding of the detailed molecular basis for Notch signal transduction, highlighting quantitative, structural, and dynamic features of this developmentally central signaling mechanism. We discuss the implications of this mechanistic understanding for the functionality of the signaling pathway in different molecular and cellular contexts.

Contents

1. INTRODUCTION

Notch signaling is the primary juxtacrine signaling pathway used for direct cell-to-cell communication between neighboring cells during development. At its core, the Notch pathway is a mechanotransduction pathway involving the direct interaction between receptors and ligands that conveys information between adjacent sender and receiver cells. During development it is used for coordinating differentiation by directing lateral inhibition, boundary formation, asymmetric cell divisions, and several additional processes (5, 15). Mutations in this pathway are associated with multiple genetic disorders and cancer, both as oncogenes and as tumor suppressors (8).

Over the past two decades, and more intensively in the past few years, quantitative information on the structural, biochemical, and biophysical aspects of this pathway has emerged. These data provide a detailed mechanistic understanding of how signaling is transduced, starting from the binding interaction between receptors and ligands, going through the mechanotransduction process, and ending in a quantitative transcriptional response. In this review, we aim to provide an up-to-date picture of how Notch signaling is transduced at the molecular and cellular levels, with emphasis on the quantitative understanding of the relevant structural details, the mechanical forces

applied during activation, and the length and timescales involved in each step of the process. This review is therefore focused on the molecular and mechanistic aspects of Notch signaling, rather than the processes that it regulates.

1.1. Overview of the Receptors and Ligands

In this section, we describe the main components involved in the Notch signaling pathway and broadly map the sequence of events leading from the initial receptor–ligand interaction to the downstream transcriptional response. In the following sections, we describe the mechanistic and biophysical processes underlying each of these events.

The two main players in the Notch pathway are the Notch receptors and the Notch ligands of the Delta/Serrate/Lag-2 (DSL) family (**Figure 1**). The receptors and ligands are highly conserved across metazoans, but the number of homologs among species varies—*Drosophila* has one Notch receptor (Notch) and two DSL ligands (Delta, Serrate), while mammals have four Notch receptors (Notch1–4) and five DSL ligands (Delta-like 1,3,4 and Jagged 1,2). Both the Notch receptors and the DSL ligands are single transmembrane proteins that contain long chains of epidermal growth factor (EGF) repeats. The *Drosophila* Notch extracellular domain (NECD) contains 36 EGF repeats that would extend over 120 nm in length if fully elongated. The DSL ligands contain an N-terminal MNNL domain (also known as C2) and DSL domain, followed by 8–16 EGF repeats, which would themselves span roughly 30–65 nm in an extended conformation.

1.2. Overall Sequence of Events in Signal Transduction

Notch signals are initiated when DSL ligands on the signal-sending cell bind to Notch receptors on signal-receiving cells (**Figures 1** and **2*a***). The working model for signaling posits that ligand ubiquitylation by the ubiquitin ligase Mindbomb (MIB) triggers clathrin-mediated endocytosis (CME) in the signal-sending cell. Endocytosis is thought to generate a pulling force on the bound Notch receptors (**Figure 2*b*,*c***), leading to opening up of the negative regulatory region (NRR) domain of Notch and subsequent cleavage of the NECD by a disintegrin and metalloproteinase domain–containing protein 10 (ADAM10) (**Figure 2c**). While the cleaved NECD undergoes trans-endocytosis into the signal-sending cell, the remaining portion of Notch is processed again by the γ-secretase protease complex, leading to the release of the Notch intracellular domain (NICD) (**Figure 2*d***). Finally, the NICD translocates to the nucleus, where it forms a Notch transcriptional activation complex (NTC) with the DNA-binding protein RBPJ and a cofactor of the mastermind family (MAM in flies, MAML in mammals) (**Figure 1**, *inset*). Transcriptional activation of Notch targets is also regulated by a repression complex formed by RBPJ [Suppressor of Hairless, Su(H)] bound to corepressors [Hairless in flies; SMRT/HDAC1-associated repressor protein (SHARP) and four and a half LIM domains (FHL1) in mammals]. Prior to activation, transcription from target genes is repressed by the repression complexes (**Figure 2*e***). When signaling is induced, NTCs are formed, allowing transcription from Notch target genes. The strength of the Notch response is likely modulated by the relative abundance of activation and repression complexes and competition among them (**Figure 2*f***).

2. RECEPTOR–LIGAND INTERACTIONS

2.1. Identification of the Receptor–Ligand Interaction Region

Although genetic analyses suggested that the protein products of *Drosophila* Notch and Delta might interact, evidence supporting this idea did not emerge until experiments carried out in

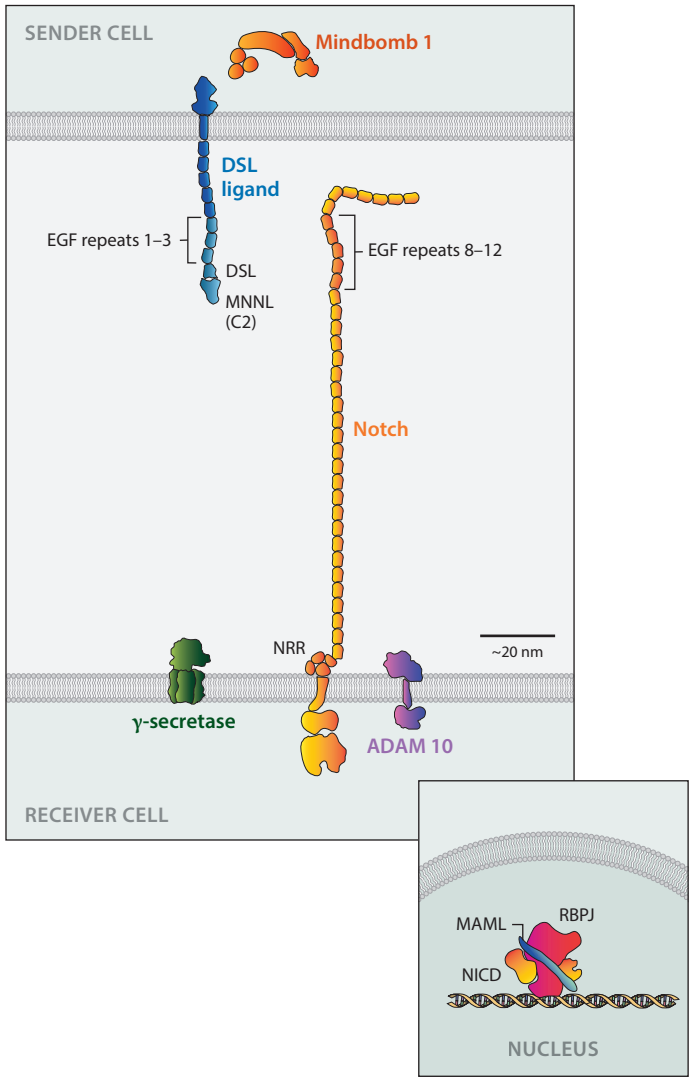
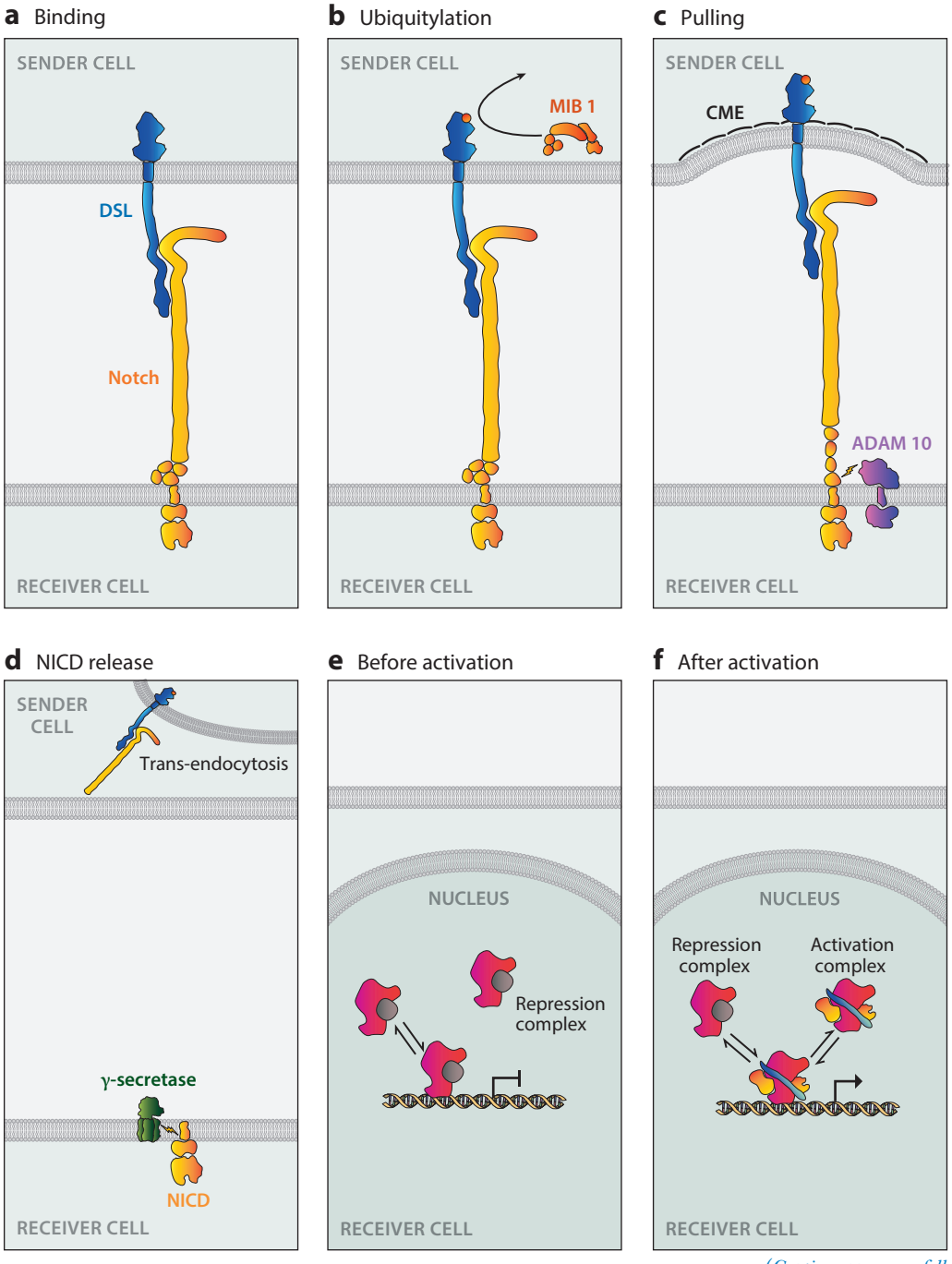


Figure 1

The main protein components of the Notch signaling pathway. The central part of the figure shows a schematic of the main components in the sender and receiver cells. The interaction domains within the Notch receptors and Notch ligands are highlighted. The inset shows a schematic of the activation complex in the nucleus. The schematic is drawn approximately to scale (see scale bar), partially based on current structural knowledge. Some structural aspects are hypothesized (including the extended conformation of the receptors and ligands). Abbreviations: ADAM 10, a disintegrin and metalloproteinase domain-containing protein 10; DSL, Delta/Serrate/Lag-2; NRR, negative regulatory region.

transfected *Drosophila* S2 cells in culture provided evidence that Notch receptors bound to both Delta and Serrate ligands (36). In these studies, Notch-transfected cells aggregated into clusters with Delta-transfected cells but not with each other or with untransfected cells. The modularity of the Notch protein then made it possible to use this cell aggregation assay to deduce the ligand-binding region within the NECD. EGF repeats 11–12 were found to be the smallest region sufficient to permit aggregation of Notch-expressing cells with Delta- or Serrate-expressing



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Schematic of the sequence of events during canonical Notch signaling. The main events at the cell boundary are: (*a*) Notch receptors bind to DSL ligands; (*b*) ligand is ubiquitylated by MIB1; (*c*) a ligand undergoing CME pulls on the Notch receptor, opening the NRR domain and allowing cleavage by ADAM10 (*lightning symbol*); and (*d*) a second intramembrane cleavage releases the NICD that translocates to the nucleus. The Notch extracellular domain remains bound to the ligand and trans-endocytoses into the signal-sending cell. (*e*, *f*) The nuclear repression complexes (containing RBPJ and corepressor) and activation complexes (containing NICD, RBPJ, and MAML) (*e*) before and (*f*) after Notch activation. Abbreviations: ADAM10, a disintegrin and metalloproteinase domain–containing protein 10; CME, clathrin-mediated endocytosis; DSL, Delta/Serrate/Lag-2; MIB1, Mindbomb1; NICD, Notch intracellular domain; NRR, negative regulatory region.

cells (113). Moreover, the formation of Notch–ligand complexes was dependent on the presence of Ca^{++} (36). Several years later, a solid phase-binding assay was used to demonstrate binding between purified ligand and receptor protein fragments. These studies detected binding of a purified Jag1-Fc fusion protein to purified N-terminal fragments of murine Notch1, Notch2, and Notch3 encompassing the first 14 (Notch3) or 15 (Notch1, Notch2) EGF-like repeats (127).

Other early studies identified several O-linked sugar modifications that functionally modify the responsiveness of Notch receptors to Delta and Serrate/Jagged family ligands (for a recent review, see 103). The EGF repeats of Notch proteins are O-fucosylated at a series of consensus sites distributed across much of the ligand-binding domain by the O-fucosyltransferase enzymes O-fut1 in flies (100) and its homolog POFUT1 in mammals (152). These O-fucose modifications can be extended by Fringe enzymes, which are a family of β1,3-N-acetylglucosmine (GlcNac) glycosyltransferases (19, 91). After GlcNac modification, these modified sites can be additionally extended with galactose and sialic acid moieties to produce a fully mature tetrasaccharide chain (47). Consensus sites for glucosylation of Notch proteins by Rumi (1) and its mammalian homolog POGLUT1 (137) also reside on the opposite face of the EGF repeats, and these sites are further modified by xylosyltransferase enzymes (77, 121, 122).

Alongside these reports, additional studies using purified ligand and receptor fragments have zeroed in on the minimum-length region of the ligands sufficient for inducing a Notch response in the immobilized ligand assay and for formation of complexes with various EGF-repeat regions of different mammalian Notch receptors. Signaling assays using immobilized ligand fragments have shown that the N-terminal ligand region from the MNNL (C2) domain through EGF repeat 3 (MNNL-EGF3) is needed to produce full-strength signal-sending activity for human Dll1, Dll4 (4), and Jag1 (24, 84). Serial truncations of the EGF repeats from human Notch1 also showed that responsiveness to Dll1 and Dll4 in coculture assays also required EGF repeats 8–10, extending the zone of functional importance beyond the minimal region required for ligand binding mapped in cell aggregation assays with *Drosophila* Notch. These functional mapping studies are in line with cell-based binding studies showing that *Drosophila* Notch proteins spanning EGF repeats 6–36 bind substantially more strongly to Delta-expressing S2 cells than do proteins spanning EGF repeats 10–36 (158). The *jigsaw* phenotype in flies, which results from a V361M mutation in EGF repeat 8 of *Drosophila* Notch, leads to selective loss of function in response to Serrate and reduced binding of Serrate in a cell-based capture assay, confirming the functional importance of EGF8 in ligand binding and in tuning differential ligand responsiveness across species (161).

2.2. Differential Activity Driven by Diversity of Notch Receptors and Ligands

The diversity of Notch receptors and ligands, particularly in more advanced organisms, raises the question of how downstream signaling is affected by the types of receptors and ligands, as well as by modulating proteins. Cellular context and tissue distribution are added layers of in vivo complexity that further contribute to differences in activity and functional output. Early work in *Drosophila*

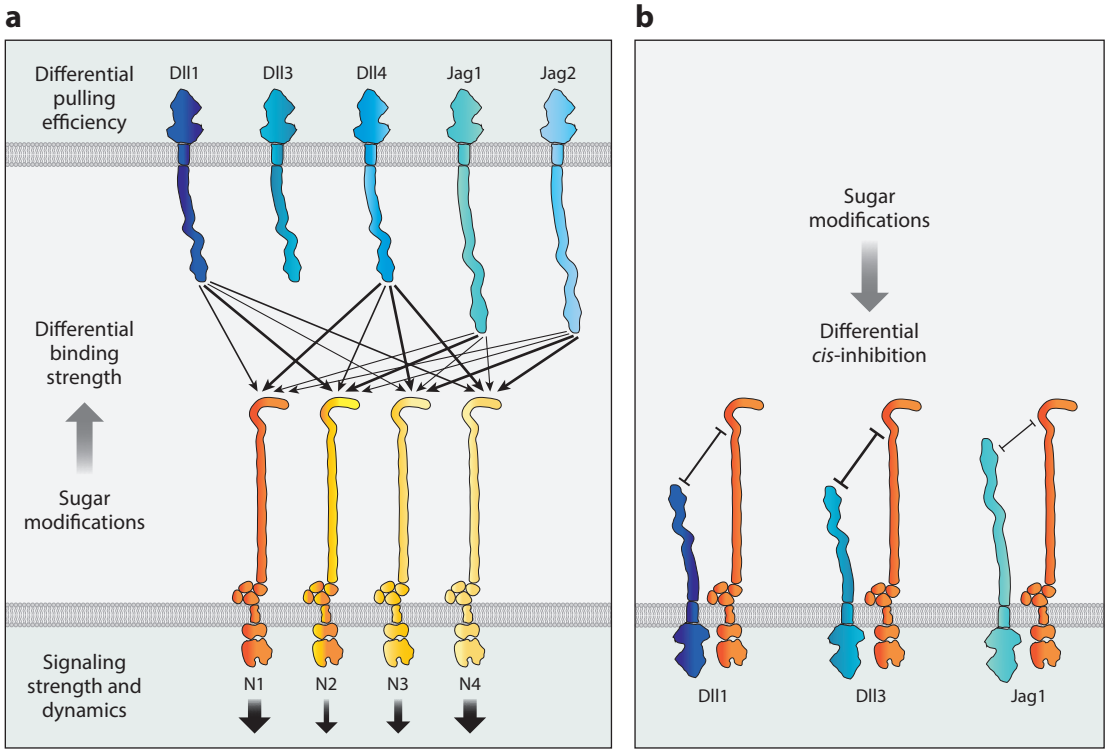


Figure 3

Diversity of receptor–ligand interactions. (*a*) A schematic showing the factors affecting the strength of Notch signaling for different receptors, ligands, and regulator modifications. Differential binding is depicted by arrows, where the thickness of the arrows represents the binding strength (the thicknesses drawn are only schematic and do not necessarily represent actual binding strength). The binding strength can depend on sugar modifications in the Notch receptors. Note that Dll3 does not trans-activate Notch receptors. Additional factors affecting signaling strength are differential pulling efficiency by the ligands and the efficiency of NICD cleavage and its stability. (*b*) A schematic of *cis*-inhibition between Notch receptors and ligands. Interaction between receptors and ligands in the same cell mutually inhibits the activity of both receptors and ligands. Sugar modification can affect *cis*-interactions as well. Abbreviation: NICD, Notch intracellular domain.

showed that the *Drosophila* ligands Delta and Serrate exhibit differential activity regulated by the glycosyltransferase Fringe (37, 59, 104). Expression of Fringe in a signal-receiving cell leads to glycosylation and elongation of the sugar residue chains on the EGF repeats of the NECD (91), which preferentially enhance the binding of Delta to Notch and inhibit the binding of Serrate to Notch. An example of this type of preferential ligand activity is the process of defining boundary cells in the wing margin in *Drosophila*. Cells on both sides of the dorsal–ventral boundary, but nowhere else, are activated because Serrate expressed on the dorsal side activates nonglycosylated Notch on the ventral side, where Fringe is not expressed, while Delta on the ventral side activates Notch glycosylated by Fringe expressed in the dorsal side. Thus, differential activity of the ligands can be utilized to define different cellular domains.

Similarly, the mammalian homologs of Fringe, Lunatic fringe (Lfng), Manic fringe (Mfng), and Radical fringe (Rfng), can differentially modulate the interaction between the Notch receptors and ligands (55, 56) (see **Figure 3*a***). Similar to the role of Fringe in *Drosophila*, Lfng and Mfng in mammals enhance activation by Dll1 and suppress activation by Jag1 (55). In contrast, Rfng does not differentiate between Dll1 and Jag1, enhancing signaling from both ligands (55).

Several studies have attempted to quantify the affinity of various mammalian Notch receptors for Delta-like and Jagged ectodomain fragments using a variety of biochemical methods (**Table 1**). Although the use of different-length protein fragments, different expression systems for protein production, and different experimental methods makes it difficult to compare results across studies, important conclusions have emerged from these binding measurements. First, there are intrinsic differences in the affinity of different Notch receptors for the two major Delta-like ligands, Dll1 and Dll4, with Notch1 favoring Dll4 by an order of magnitude and Notch2 favoring Dll1 by roughly threefold (4, 145). These studies used a Notch1 protein that was O-fucosylated but was not elaborated by the Fringe glycosyltransferases. Second, the intrinsic affinity of Notch1 for both Dll1 and Jag1 increases upon O-fucosylation and subsequent Fringe modification of T466 of Notch1 (139). Remarkably, however, the binding affinity of Notch1 for Dll4 did not seem to be substantially affected upon O-fucosylation or Fringe modification, and glucose modification did not detectably affect the affinity of Notch1 for any of the ligands tested in these studies (139).

The intrinsic differences between the activity of Notch receptors and ligands in mammals has attracted significant attention in recent years, complementing the detailed biochemical analyses described above. Potential differences in activity may come from the observed differences in binding affinities between receptors and ligands, as well as from other differences on the ligand side, for example, modulation of ubiquitylation and endocytosis, and on the receptor side, for example, modulation of the release of NICD (**Figure 3**).

Several studies have shown activity differences between Dll4 and Dll1 in mouse models. Dll4 activates T cells at a lower surface concentration than Dll1, consistent with the affinity differences noted above (90). Work performed in transgenic mice showed that Dll4 cannot fully rescue Dll1 deletion and that such replacement exhibits a severe somitogenesis phenotype, suggesting that the two ligands have different activities in vivo (110). A follow-up study used chimeric ligands of Dll4 and Dll1 to identify which domains encode the differences between the two ligands (145). This work first showed that the extracellular domain (ECD), and not the intracellular domain (ICD), encodes the differences. Interestingly, replacing the specific contact residues in the Dll1 ECD with those from the Dll4 ECD did not disturb the functionality of Dll1, despite enhancing the binding affinity to Notch1, suggesting that the known contact interface visualized in the X-ray structure of the Dll4–Notch1 complex is not the sole determinant of the differences between the two ligands. Finally, cell-based and biochemical analysis showed that Dll1 and Dll4 differentially activate Notch1 and Notch2, with Notch1 activated more strongly by Dll4 and Notch2 activated more strongly by Dll1.

Another recent study investigated the differences in activity between Dll1 and Dll4 using live cell imaging to track the dynamics of a Notch1 transcriptional reporter in response to activation by sender cells containing either Dll1 or Dll4 (98). Interestingly, this study found that activation by Dll1-expressing cells led to a pulsed transcriptional response, while activation by Dll4 led to a sustained transcriptional response in the receiving cells. In contrast to the results described above, this study found that the differential activity was encoded by the ICD of the ligands and suggested that clustering of Dll1, but not of Dll4, may underlie the pulsatile versus sustained response in Dll1 and Dll4, respectively.

Similar questions regarding differential activity were also applied to the receiving side. Two recent works asked whether differences between Notch1 and Notch2 were encoded in the ECDs or ICDs of the receptors. Chimeras in which NICD1 was switched with NICD2, and vice versa, in mouse showed that the two ICDs are interchangeable in multiple tissues but that the ECD of Notch2 is more efficient in releasing its ICD, potentially because of more efficient surface clustering (80, 81). Differences in phenotypes in gene dose–sensitive tissues (i.e., tissues where heterozygous mutants had phenotypes) were associated with cell-specific differences in NICD

Table 1 Notch–ligand quantitative measurements of binding

Receptor	Ligand	Notch glycosylation	Method	K_d	Reference
Human Notch1(11–13)	Human Jag1(DSL-EGF3)	None	SPR	ND	27
Human Notch1(11–14)	Human Dll1(N-EGF3)	None	SPR	130 ± 14 μM	28
Human Notch1(10–14)	Human Dll1(N-EGF3)	None	SPR	201 ± 22 μM	28
Human Notch1(6–15)-biotin-His6	Human Dll1(N-EGF5)-His6	Fucosylated	SPR	3.4 ± 0.5 μM	4
Human Notch1(6–15)-biotin-His6	Human Dll4(N-EGF5)-His6	Fucosylated	SPR	0.27 ± 0.07 μM	4
Human Notch1(6–15)-biotin-His6	Human Dll1(N-EGF5)-His6	Fucosylated	BLI	1.6 ± 0.2 μM	145
Human Notch1(6–15)-biotin-His6	Human Dll4(N-EGF5)-His6	Fucosylated	BLI	0.43 ± 0.05 μM	145
Human Notch1(1–14)-Fc*	Human Jag1(N-EGF3)	Not determined	SPR	7.1 ± 0.1 μM	139
Rat Notch1(1–14)-biotin-His8	Rat Dll4(N-EGF2)	Fucosylated	SPR	12.7 μM	83
Rat Notch1(10–14)-biotin-his8	Rat Dll4(N-EGF2)	Fucosylated	SPR	8.63 μM	83
Rat Notch1 EGF(11–13)-biotin-his8	Rat Dll4(N-EGF2)	Fucosylated	SPR	7.51 μM	83
Rat Notch1(1–14)-biotin-his8	Rat Dll4(N-EGF2)-E2**	Fucosylated	SPR	0.06 μM	83
Rat Notch1(10–14)-biotin-his8	Rat Dll4(N-EGF2)-E2**	Fucosylated	SPR	0.07 μM	83
Rat Notch1 EGF(11–13)-biotin-his8	Rat Dll4(N-EGF2)-E2**	Fucosylated	SPR	0.06 μM	83
Rat Notch1 EGF(8–12)	Rat Jag1(N-EGF3)	Fucosylated	SPR	ND	84
Rat Notch1 EGF(11–12)	Rat Jag1(N-EGF3)	Fucosylated	SPR	ND	84
Rat Notch1 EGF(8–12)	Rat Jag1-V1(N-EGF3)**	Fucosylated	SPR	0.81 μM	84
Rat Notch1 EGF(11–12)	Rat Jag1-V1(N-EGF3)**	Fucosylated	SPR	5.4 μM	84
Rat Notch1 EGF(8–12)	Rat Dll4(N-EGF3)	Fucosylated	SPR	9.7 μM	84
Rat Notch1 EGF(11–12)	Rat Dll4(N-EGF3)	Fucosylated	SPR	12.8 μM	84
Mouse Notch2(1–15)-FLAG-His6	Mouse Jag1-Fc***	Not known	ELISA	0.7 nM	127
Mouse Notch2(1–15)-FLAG-His6	Mouse Jag1-Fc***	Not known	Flow	0.4 nM	127
Mouse Notch2(1–15)-Fc*	Dll1(N-EGF5)-His6	Not known	BLI	0.36 ± 0.11 μM	145
Mouse Notch2(1–15)-Fc*	Dll4(N-EGF5)-His6	ND	BLI	1.3 ± 0.2 μM	145
Fly Notch(11–20)-V5_his6	Fly Delta-Fc***	Natural S2-cell glycosylation	Solid phase assay	1.2 nM	107
Fly Notch(11–14)-V5-his6	Fly Delta-Fc***	Natural S2-cell glycosylation	Solid phase assay	16.6 nM	107
Fly Notch(11–20)-V5-His6	Notch(21–30)-Fc***	Natural S2-cell glycosylation	Solid phase assay	27.5 nM	107
Fly Notch(11–14)-V5-his6	Notch(21–30)-Fc***	Natural glycosylation	Solid phase assay	21.2 nM	107

*represents the dimeric receptor; **represents the enhanced affinity ligand; ***represents the dimeric ligand.
Abbreviations: BLI, biolayer interferometry; EGF, epidermal growth factor; ELISA, enzyme-linked immunoabsorbent assay; ND, not defined; SPR, surface plasmon resonance.

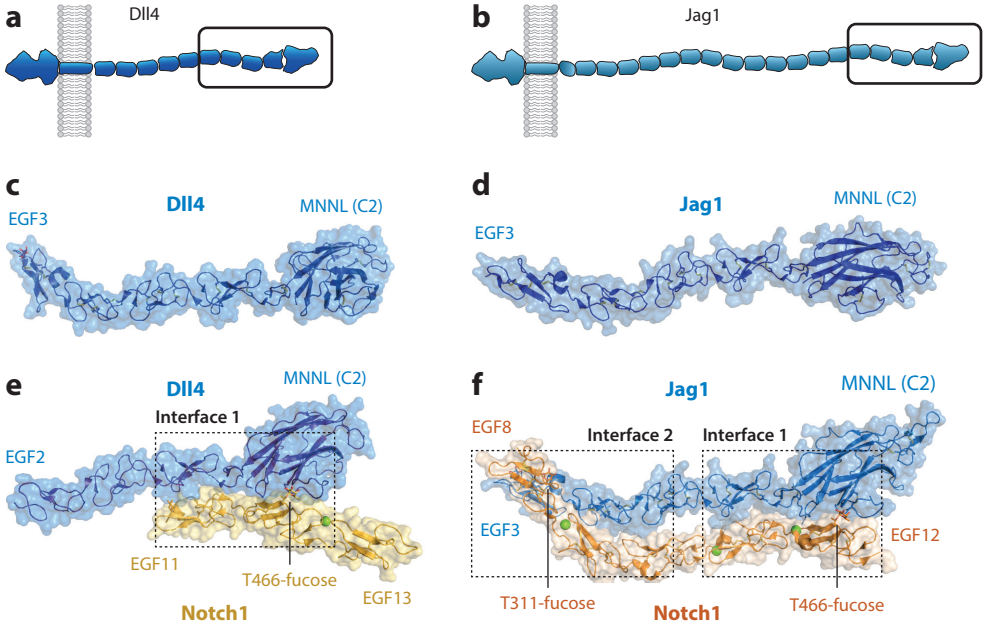


Figure 4

Structures of Notch–ligand complexes. (*a*,*b*) Schematics of (*a*) Dll4 and (*b*) Jag1. The MNNL-EGF3 regions of each ligand are shown in boxes. (*c*,*d*) Structures of the MNNL-EGF3 fragments of (*c*) Dll4 (PDB ID 5MVX) and (*d*) Jag1 (PDB ID 4CC0), each rendered as a schematic with a transparent surface. (*e*) Structure of a Dll4–Notch1 complex, with each protein rendered as a schematic with a transparent surface (PDB ID 4XLW). Dll4 is blue, and Notch1 is gold. Disulfide bonds and T466 are shown as sticks, and the fucose modification of T466, also rendered with sticks, is indicated. Calcium ions bound to the Notch1 fragment are shown as green spheres. (*f*) Structure of a Jag1–Notch1 complex, with each protein rendered as a schematic with a transparent surface (PDB ID 5UK5). Jag1 is blue, and Notch1 is orange. Disulfide bonds, T311, and T466 are shown as sticks, and the fucose modifications of T311 and T466, also rendered with sticks, are indicated. Calcium ions bound to the Notch1 fragment are shown as green spheres. Abbreviations: EGF, epidermal growth factor; PDB, Protein Data Bank.

stability. Overall, regulation of Notch concentration and distribution at the cell surface and stability of NICD seem to be important contributors to the in vivo differences observed between Notch1 and Notch2.

2.3. Structural Basis for Notch–Ligand Interactions

Crystallographic and nuclear magnetic resonance (NMR) studies of isolated ligand and receptor fragments, in combination with structures of high-affinity evolved Dll4 and Jag1 ligands bound to Notch1 fragments, make it possible to put these biochemical and activity findings into a structural context. The structure of the Notch1 EGF11–13 region was first determined by NMR spectroscopy (46); subsequently, structures of both the Notch1 and Notch2 EGF11–13 fragments were also solved by X-ray crystallography (27, 133). X-ray structures show that these EGF repeats depend on ligation of a calcium ion at the interdomain interface to stabilize an extended conformation, providing an explanation for prior observations that ligand binding is calcium ion dependent (4, 28, 36). Structures of receptor binding Delta-like and Jagged ligand fragments, as well as the complete Dll1 ectodomain, were also determined crystallographically (24, 27, 57, 133, 139). These structures show that both the Delta-like and Jagged ligands also adopt an extended conformation (**Figure 4*a–d***) and reveal that the N-terminal MNNL domain adopts the same

fold as a C2 domain, such as those found in synaptotagmin and other proteins. There is crystallographic evidence that the Jag1 and Jag2 MNNL (C2) domains can bind calcium at high ion concentrations, and MNNL-containing regions of both Jagged and Delta-like Notch ligands can bind liposomes (24, 133), but whether the MNNL domain is used functionally to recognize lipids in a calcium-dependent manner in vivo remains a controversial unresolved question in the Notch field.

The secret sauce that finally allowed determination of the structures of Notch receptor–ligand complexes was the selection of high-affinity ligand variants from a yeast display library (83, 84). The high-affinity ligands could be readily purified in complex with Notch1 binding regions using size-exclusion chromatography, enabling crystallization and structure determination. The first structures solved were complexes of rat Notch1 EGF11–13 with high-affinity SLP (serine, leucine, and proline, the three mutated residues) variants of rat Dll4 (MNNL-EGF1 and MNNL-EGF2). The Notch1 fragment contains two post-translational modifications, an O-linked fucose on Thr466 and an O-linked glucose residue attached to Ser435. The binding interface between Notch1 and Dll4 encompasses two discrete regions of contact, with the two proteins aligning in an antiparallel arrangement (**Figure 4*e***). The first site is between the MNNL domain of Dll4 and EGF repeat 12 of Notch1, and the second is between the Dll4 DSL domain and EGF repeat 11 of Notch1. A key interaction at the contact interface is between the O-fucose moiety attached to T466 on EGF12 of Notch1 and His64 and Tyr65 of the MNNL domain (**Figure 4*e***). When the structure of the complex is compared with unliganded Notch1, the conformation of Notch1 is not substantially altered. In contrast, the MNNL domain of Dll1 undergoes an approximately 25-degree rotation relative to the DSL domain upon complexation, indicating the existence of a flexible hinge between the MNNL and DSL domains. Remarkably, none of the three SLP mutations of Dll4 responsible for imparting high Notch1 affinity lie at the contact interface. Instead, two lie at the MNNL-DSL intramolecular interface, and the third is in the core of the DSL domain, suggesting that the selected mutations enhance affinity by favoring the binding-active conformation.

The Jag1–Notch1 complex includes EGF domains 8–12 from Notch1 and a high-affinity Jag1 (MNNL-EGF3) variant, also selected from a yeast display library (84). The Notch1 EGF domains have O-linked fucose, glucose, and N-acetylglucosamine modifications, and calcium ions are bound to EGF9, EGF11, and EGF12 (84). As in the shorter Notch1–Dll4 complex, the two proteins are oriented antiparallel to each other; in this complex, the interface extends over a distance of 120 Å (84), with participation of all domains from both proteins. Notch1 EGF12 and EGF11 interact with the Jag1 MNNL and DSL domains in a manner analogous to the interface visualized in the Notch1–Dll4 structure (interface 1; **Figure 4*f***). A second interfacial region derives from EGF repeats 10–8 of Notch1 in contact with EGF repeats 1–3 of Jag1, respectively (interface 2; **Figure 4*f***). In the Jag1–Notch1 complex, there is a second protein–sugar interaction between an O-fucose modification on T311 of EGF8 from Notch1 and EGF3 of the Jag1 fragment. Another important distinction between the Notch1–Dll4 complex and the Notch1–Jag1 complex is that the binding energy for the interaction of Notch1 with Dll4 appears to rely exclusively on interface 1 for its binding energy, whereas in the interaction with Jag1, both interface 1 and interface 2 contribute to the measured affinity of the interaction (84; for a review, see 14).

Together, these structural studies have clarified the overall extended architecture of the isolated receptors and ligands. They have established that receptor and ligand molecules are oriented antiparallel in the complexes, shown how the various N-terminal modules of the ligands engage the ligand-binding EGF repeats of Notch, and identified subtle differences that distinguish recognition of Jagged and Delta-like ligands. Most importantly, they reveal a critical role for O-linked fucose modifications of the receptor at its interfaces with both classes of ligands,

providing a molecular explanation for the importance of these sugar modifications in Notch signal transduction.

2.4. Regulation of Signaling by *cis*-Inhibition Between Receptors and Ligands

Early studies in flies identified an inhibitory effect of Notch ligands expressed in the same cells as Notch receptors (30, 60). This effect, termed *cis*-inhibition, has since been observed in many other tissues, as well as in other organisms (10). Quantitative analysis in a cell-culture live imaging assay, as well as mathematical modeling, showed that, depending on the relative levels of Notch receptors and ligands, *cis*-inhibition can cause cells to be in either a sender mode, in which cells can send signals but cannot receive them, or a receiver mode, in which cells can receive signals but cannot send them (128, 129). This sharp switch between senders and receivers is important in defining sharp boundaries, as well as in driving the Notch-mediated lateral inhibition process.

Later work expanded this analysis to situations involving multiple ligands and fringe proteins (75). Similar to its role in trans-activation, modification of Notch1 by Lfng and Mfng enhances *cis*-inhibition of Delta-like ligands and inhibits *cis*-inhibition by Jag1 and Jag2. This modulation by Fringe expands the potential states in which a cell can be found (e.g., sender or receiver). For example, a cell expressing Notch1, Lfng, and both Dll1 and Jag1 can be a receiver for Dll1 signals from some neighboring cells (assuming it has more Notch1 than Dll1) and a sender of Jag1 signals to other cells, since glycosylated Notch1 *cis*-interacts more poorly with Jag1. Thus, the combinatorial action of multiple receptors and ligands can diversify the potential signaling states of cells.

It is interesting to note that some ligands have only a *cis*-inhibitory effect. In mammals, Dll3 cannot *trans*-activate Notch receptors but can *cis*-inhibit Notch1 (71). In fact, *cis*-inhibition of Notch1 by Dll3 is essential for proper somitogenesis in mice (22, 120).

Why does binding in *cis* promote inhibition rather than activation of the receptors? First, it has been suggested that the interactions responsible for *cis*-inhibition may occur before the ligands and receptors reach the cell surface. For example, the interaction between Dll3 and Notch1 was shown to be restricted to the Golgi and the late endosomes, suggesting that Dll3 prevents the localization of Notch1 at the cell surface (22). However, for other receptors and ligands, both the receptors and the ligands do get to the cell surface but are not able to interact in *trans* when *cis*-inhibited (75). Second, it has been suggested that *cis*-inhibition results from the inability of ligands to exert pulling forces when bound to the receptors in *cis*. This is supported at the structural level, since receptors and ligands likely bind in *cis* in the same antiparallel direction as when they bind in *trans*. Surprisingly, however, Jagged1, Dll1, and Dll4 have also been shown to induce *cis*-activation of both Notch1 and Notch2 in isolated cells expressing ligand, Notch, and Rfng (97). It would be interesting to examine whether such *cis*-activation indeed occurs in vivo, and what is the mechanism of activation in this case.

3. REGULATED INTRAMEMBRANE PROTEOLYSIS AS THE ACTIVATION STEP

Notch proteins are normally quiescent in the absence of ligand and undergo activation in response to ligand stimulation by regulated intramembrane proteolysis (RIP). RIP, which was first described in the case of proteolytic release of the steroid response element–binding protein from the endoplasmic reticulum membrane (18, 112, 116), enables the release of NICD to the nucleus. For Notch, this multistep event involves a first cleavage by an ADAM protease in the NRR region and a second cleavage by γ-secretase in the intramembrane region. Recent structural studies have elucidated many of the molecular details of this process.

3.1. The Notch Negative Regulatory Region and Receptor Quiescence

Notch receptors are held in their proteolytically resistant off state by the NRR that immediately precedes the plasma membrane. The NRR consists of three LIN12/Notch repeats (LNRs) and a juxtamembrane heterodimerization domain (HD). Paradoxically, the HD actually undergoes constitutive cleavage by a furin-like protease during transit to the cell surface at a site called S1 (82), yet the two subunits remain noncovalently associated with each other (111, 117), explaining the origin of its name. Nevertheless, the mature Notch receptor at the cell surface is resistant to further proteolysis until binding of Notch to a ligand releases the autoinhibition imposed by the NRR and allows ADAM metalloproteases to cleave just external to the membrane at site S2 (17, 94), enabling subsequent γ-secretase cleavage near the inner membrane leaflet at site S3 (31, 132, 163).

Evidence that the NRR is the autoregulatory switch that controls Notch activity dates to early genetic studies in *Caenorhabditis elegans*, in which mutations in the NRR domain led to autonomous activity of the Notch homolog Lin12 (45). Several additional studies in worms and flies also indicated that the NRR plays an important autoregulatory role in suppressing premature receptor activation. A mutation in the NRR of the worm receptor Glp1 (S642N) also results in an autonomous gain-of-function phenotype, as do point mutations in the *Drosophila* NRR (16, 85), the mouse Notch1 NRR (94), and the human Notch1 NRR, which is frequently mutated within the HD in human T cell acute lymphoblastic leukemia (T-ALL) (42, 154). Furthermore, truncations of mammalian Notch receptors that lack the EGF repeats but that retain the full NRR are constitutively inactive (64, 117), whereas further truncations that remove the LNR repeats are constitutively active (43, 117), indicating that the integrity of the NRR is both necessary and sufficient to maintain quiescence.

X-ray crystal structures of the NRR regions of human Notch1 (42), Notch2 (43), and Notch3 (160) revealed the underlying molecular basis for Notch autoinhibition (**Figure 5*a***) shows the structure of the Notch1 NRR as a representative example). Each of the three NRRs adopts a conformation that resembles a head of cauliflower, in which the HD domain is the stalk and the LNR modules are the three florets, enveloping the HD domain in a closed, compact conformation. Interactions between residues in the linker connecting the first and second LNR repeats and the terminal beta-strand that contains the S2 site conceal the metalloprotease cleavage site, resulting in autoinhibition (**Figure 5*a***).

The structure of the NRR shows that each LNR is stabilized by a bound calcium ion. This observation suggests that the reason treatment of Notch-expressing cells with ethylenediaminetetraacetic acid (EDTA) activates receptors in the absence of ligand is that chelation disrupts the structural integrity of the LNR domains, relaxes the interdomain interface, and exposes the metalloprotease cleavage site (66, 111). Indeed, hydrogen–deuterium exchange experiments performed on Notch1 and Notch3 NRRs showed directly that EDTA treatment relaxes the structure around the LNR domain and the HD domain at the S2 cleavage site, showing that chelators such as EDTA result in ligand-independent exposure of the S2 site and activation (141, 142).

The structure of the Notch1 NRR also clarifies why leukemia-associated mutations result in ligand-independent proteolysis of the receptor. The vast majority of leukemogenic mutations lie within the hydrophobic core of the HD (**Figure 5*b***). Disruption of hydrophobic residues within the interior of the protein by the leukemia-associated mutations, which are often nonconservative substitutions, destabilizes the closed state of the protein and thereby permits access of the metalloprotease to the S2 cleavage site in the absence of ligand (87).

Just as it is possible for point mutations, such as those found in oncogenic forms of Notch1, to destabilize the NRR, it is possible to destabilize the closed conformation of the NRR with

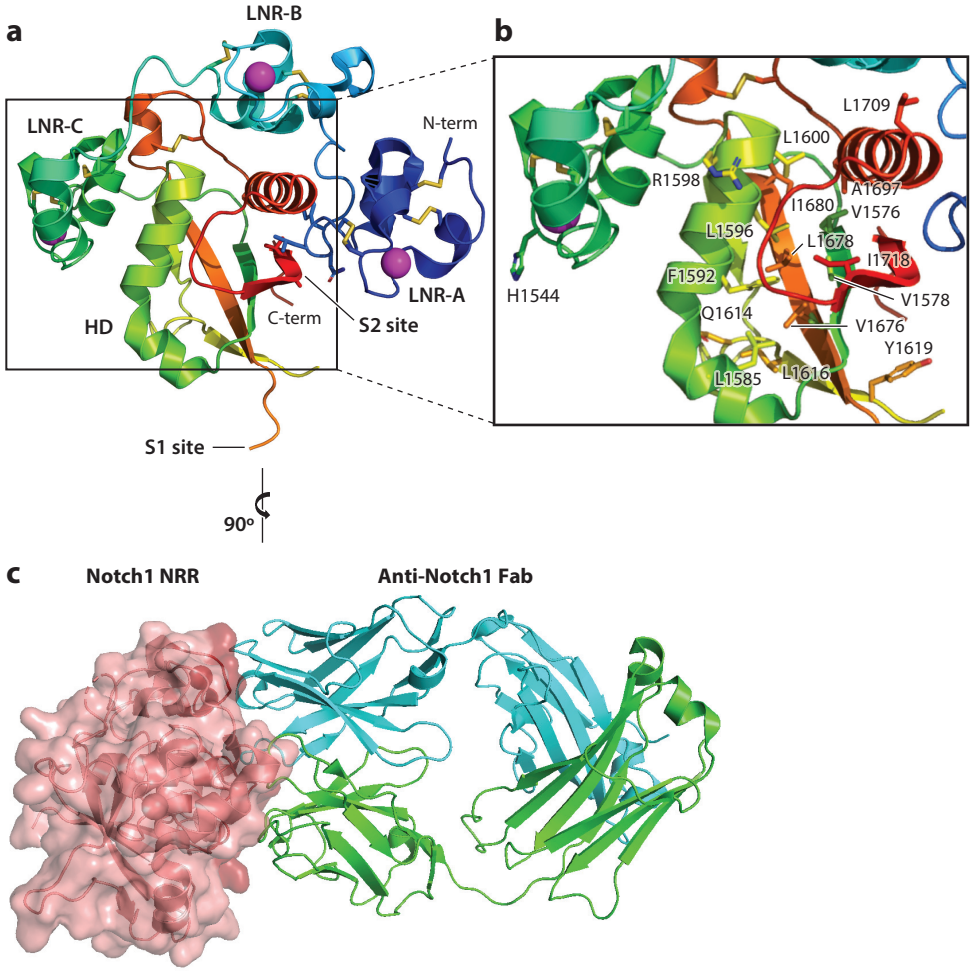


Figure 5

Notch1 negative regulatory region (NRR) structure and antibody allosteric inhibition. (*a*) Ribbon representation of the Notch1 NRR structure [Protein Data Bank (PDB) ID 3IO8]. The protein is colored across the colors of the spectrum from blue at the N terminus to red at the C terminus. Disulfide bonds are shown in yellow, and bound calcium ions are shown as purple spheres. Side chains that mask the S2 cleavage site at the intramolecular interface between the LIN12/Notch repeat (LNR) A-B linker and the heterodimerization domain are shown as sticks. (*b*) Zoomed-in view of the Notch1 NRR highlighting the residues mutated in human T cell acute lymphoblastic leukemia (T-ALL). Mutated residues are shown as sticks. (*c*) The Fab fragment of a Notch1 allosteric inhibitory antibody in complex with the Notch1 NRR (PDB ID 3L95). The Fab is rendered in schematic form, with the heavy chain in cyan and the light chain in green. The NRR is rendered as a transparent pink surface over a schematic representation, with residues at the contact interface in a darker shade.

an antibody selective for the open conformation or to stabilize the closed conformation of the NRR with an antibody selective for the autoinhibited conformation. The only activating antibody that has been reported is directed against an epitope on the LNR-A domain of human Notch3 (78). In contrast, several different research groups have developed anti-NRR inhibitory antibodies directed at three of the human Notch receptors: Notch1 (2, 6, 157), Notch2 (157), and Notch3

(12, 78). Unlike the Notch3-activating antibody, which only contacts a single LNR-A domain, all of the inhibitory antibodies recognize discontinuous epitopes that bridge from the exterior LNR domains (the molecular cap) to the HD domain stem, acting as clamps that favor the closed conformation of the NRR (**Figure 5*c***). Because the antibodies are allosteric and do not compete with ligand binding, they broadly inhibit signal transduction in response to all activating ligands. In addition to inhibiting normal ligand-dependent Notch signaling in cells, anti-Notch1 allosteric inhibitory antibodies also suppress ligand-independent signals resulting from activating mutations seen in T-ALL (6, 157). In effect, the destabilization of the NRR due to the leukemogenic mutation is functionally offset by the stabilizing effect of binding to the allosteric antibody.

The large distance separating the ligand-binding site from the intramolecular autoregulatory interface within the NRR led to the proposal of a mechanotransduction model for signaling, in which exposure of S2 is the molecular output resulting from capture of the ligand at the receptor binding site and delivery of mechanical force by the bound ligand (43). Experimental work focused on investigating this mechanotransduction model is discussed in Section 5.

3.2. Activating Proteolysis by a Disintegrin and Metalloproteinase Domain–Containing Protein 10 and γ-Secretase

Recent works on the structures of ADAM10 and γ-secretase reveal the enzymatic side of how the proteolytic cleavages of Notch are regulated. On the one hand, access of the ADAM10 metalloprotease to its preferred cleavage site in the Notch ectodomain is regulated by the opening of the NRR (i.e., substrate conformation). On the other hand, proteolysis of substrates by ADAM10 is also regulated by enzyme maturation, leading to release of an inhibitory prodomain and the conformation of the mature enzyme. The X-ray crystal structure of the complete ADAM10 ectodomain showed an unexpected overall enzyme architecture, in which the catalytic domain abuts disintegrin- and cysteine-rich domains that immediately precede the transmembrane region of the protein (118). These domains exhibit an unanticipated autoregulatory function by partially occluding the active site, even after the prodomain has been released (**Figure 6*a*,*b***).

Inspection of the structure of the γ-secretase holoenzyme, determined with a catalytically inactive presenilin mutant and a Notch transmembrane polypeptide covalently tethered to the presenilin subunit using a disulfide bond (162), shows why metalloprotease cleavage is required to render Notch sensitive to proteolysis by the γ-secretase complex (**Figure 6*c***). Before ADAM10 cleavage, Notch would be excluded from the γ-secretase active site by a steric clash between the NRR and the nicastrin subunit of the enzyme. Once the Notch ectodomain is released by ADAM10 cleavage, there is no steric hindrance preventing access of the truncated transmembrane fragment of Notch to the γ-secretase active site, and intramembrane cleavage can then take place without interference (**Figure 6*d***).

4. SENDER CELL REQUIREMENTS

Activation of Notch by DSL ligands is also actively regulated within the sender cells. Early work in *Drosophila* and *Xenopus* identified Neuralized as an E3 ubiquitin ligase of Delta (32, 72, 106, 164) that promotes ligand endocytosis and is required for ligand activity in various tissues. A couple of years later, a second E3 ubiquitin ligase, Mib, was identified, first in zebrafish (53) and later in *Drosophila*, where it has functions that are distinct from those of Neuralized (74).

The mammalian homolog MIB1 is the major E3 ubiquitin ligase in mammals. It interacts with all of the mammalian ligands and is required in almost all of the major Notch activities in vivo (62). The mammalian homologs of Neuralized, Neur1 and Neur2, in contrast, do not seem to have

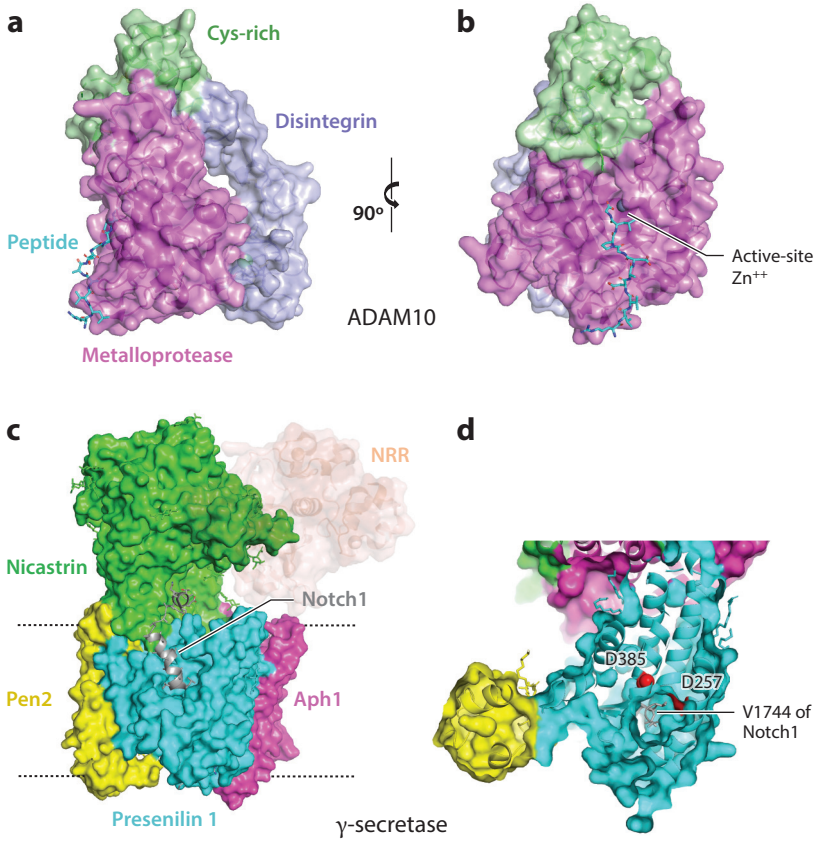


Figure 6

Structures of the proteases responsible for activating cleavage of Notch. (*a*,*b*) Structure of the ADAM10 ectodomain, shown in surface representation and colored by domain (PDB ID 6BE6). The C-terminal segment of a neighboring subunit, bound in the active site of the enzyme, is shown with cyan sticks. The Zn^{++} ion at the active site is shown as a gray sphere. (*c*) Structure of the γ-secretase complex, with a Notch1 substrate covalently tethered in the active site (PDB ID 6IDF). γ-Secretase is shown in surface representation, colored by domain, and the Notch1 substrate is shown as a gray schematic with side chains rendered as sticks. The structure of the NRR is also included as a transparent surface overlapping the nicastrin subunit of γ-secretase to show that nicastrin sterically precludes access of γ-secretase to its cleavage site until ADAM10 has cut Notch near the C-terminal end of the NRR. (*d*) Zoomed-in view of the presenilin active site. The positions of the catalytic aspartate residues on the enzyme are shown with red spheres. In the structure shown, D358 of the presenilin subunit was mutated to alanine to render the enzyme inactive and permit structure determination. Abbreviations: ADAM10, a disintegrin and metalloproteinase domain–containing protein 10; NRR, negative regulatory region; PDB, Protein Data Bank.

strong functionality in the Notch pathway, as double homozygous mutants have weak phenotypes that seem unrelated to Notch activity (63). More recent work showed that MIB1 activity blocks the *cis*-inhibition of Notch by Dll1 during neurogenesis, suggesting a role for ubiquitylation of Dll1 in *cis*-inhibition as well as *trans*-activation (13).

Mammalian MIB1 is a large multidomain E3 ligase that contains an N-terminal MZM region consisting of two Mib/Herc2 domains flanking a ZZ-type zinc finger, a REP region with two mib repeats, an ankyrin repeat domain, and a C-terminal trio of RING finger domains (**Figure 7*a***). MIB1 relies on a bipartite strategy to recognize the cytoplasmic tails of both Delta-like and Jagged

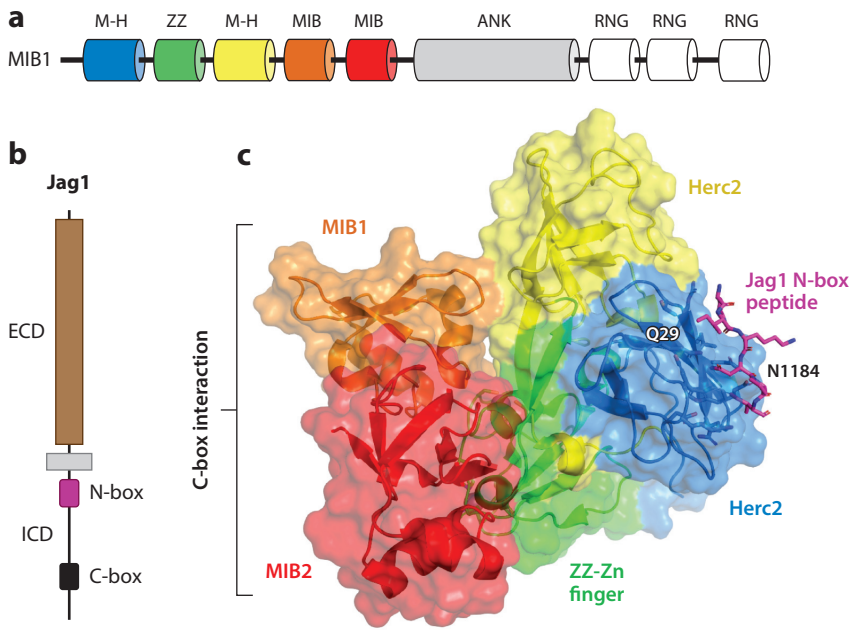


Figure 7

Recognition of Jag1 by MIB1. (*a*) Domain organization of MIB1. (*b*) Schematic of Jag1, highlighting the positions of the N-box (*purple*) and C-box (*black*). (*c*) Structure of the MIB1–Jag1 complex (PDB ID 4XI7). MIB1 is rendered in schematic form, colored by domain with a transparent surface, and the Jag1 N-box peptide is shown in purple sticks. The anchoring interaction between Q29 of MIB1 and N1184 of Jag1 is indicated. Abbreviations: ANK, ankyrin repeat region; M-H, Mib/Herc2; MIB, Mindbomb; PDB, Protein Data Bank; RNG, ring finger domain; ZZ, ZZ-type zinc finger.

ligands (88), in which the MZM region uses its first Mib/Herc2 domain to bind one target site on the ligand tail, termed the N-box (**Figure 7*b*,*c***), and the REP region uses its mib repeats to recognize a second recognition element on the ligand tail, termed the C-box. This bipartite recognition mode may promote extrusion of the loop between the binding sites to facilitate ligand ubiquitination; alternatively, it may facilitate clustering of ligands by recognizing N- and C-boxes in *trans* on adjacent ligand molecules, either in a 2:2 complex or in larger, daisy-chained assemblies.

Ligand ubiquitylation was shown to recruit the endocytic adapter protein Epsin, which then induces CME of the DSL ligands (149, 150). Since it was shown that neither ligand ubiquitylation nor Epsin is required for general endocytosis and recycling of Notch ligands, it was soon proposed that DSL ligands can go through two types of endocytic processes—bulk endocytosis, which is not involved in Notch activation, and ubiquitylation and Epsin-dependent endocytosis, which is involved in activation (40, 89, 150). These observations gave rise to two models for the role of endocytosis—the ligand recycling model and the pulling force model. The ligand recycling model suggests that recycling is required for some modifications of the ligands that are necessary for their activity (50). The pulling force model suggests that endocytosis is required for generating a pulling force on the receptor. In recent years, strong evidence has accumulated in favor of the pulling force model. The pulling force model is discussed in detail in Section 5.

It is interesting to note that, although the canonical pathway of activation through ubiquitylation and endocytosis seems to be quite general, there is some evidence for alternative activation pathways. In recent work in *Drosophila*, it was shown that even in the absence of ubiquitylation

or E3 ubiquitin ligases, Delta has residual activity, and that this ubiquitin-independent activity of Delta is relevant in vivo (13). Moreover, in some cases, the binding of Neuralized is sufficient to activate Delta with no lysines (and thus no ubiquitylation), suggesting that Neuralized binding has an ubiquitin-independent function. In addition, work with synthetic Notch (115) (see Section 7) has shown that synthetic Notch ligands lacking a natural, ligand-derived ICD can be functional.

In addition to recycling, Notch ligands can also diffuse on the cell membrane. Recent work used fluorescence recovery after photobleaching (FRAP) to estimate the diffusion coefficient and the recycling rate of Dll1 in mammalian cells (58). Measured diffusion coefficients of Dll1 ($\sim 0.01-0.1\ \mu m^2/s$) enabled estimation of the diffusion length scale, namely, the typical distance that Dll1 travels before endocytosing, which was estimated to be $\sim 1\ \mu m$. Interestingly, this means that the Notch ligands can diffuse over relatively short distances before endocytosing.

These dynamics have implications for signaling strength, as they affect the number of receptor–ligand pairs that form at the contact area between cells. Mathematical modeling showed that, for contact diameters larger than the diffusion length scale (i.e., $> 1\ \mu m$), the signaling should be proportional to the contact area (58). Experiments performed using micropatterned devices, in which the contact area between senders and receivers could be controlled, indeed showed that signaling strength was correlated with the contact area (125). Interestingly, this dependence on contact area can lead to bias of cell fate decisions according to cell size.

5. PULLING IT ALL TOGETHER: A MECHANOTRANSDUCTION MODEL FOR RECEPTOR ACTIVATION

Early studies in flies led to the initial proposal that bound ligand might exert a pulling force on the receptor to trigger cleavage. Genetic studies showed that soluble ligand ectodomains or ligands lacking a cytoplasmic tail were signaling-inactive and dominant negative inhibitors of signaling (134, 135). Additionally, genetic studies in flies showed that loss of dynamin activity also led to a Notch loss-of-function phenotype (123). Recognition that receptor extracellular domains can be observed in the ligand cell after activation then led to the proposal that receptor activation was triggered by mechanical strain exerted on the receptor by ligand endocytosis (99, 105).

Combined with the structural, biochemical, and molecular findings outlined above, this work made it increasingly clear that activation of Notch may require mechanical pulling to expose the protease site, and that the NRR may function as the mechanosensor. In this model, force may be exerted by ligand endocytosis, opening the NRR to permit subsequent cleavage events. It remained unclear, however, what magnitude of force might be required to expose the cleavage site to protease, what the source of this force might be, and whether delivery of force by bound ligand would be necessary for cleavage.

Several groups have used single-molecule approaches to investigate the force required to break ligand–receptor bonds, the force required to allow receptor cleavage in vitro, and the force required to induce signal activation in cells and in vivo (**Table 2**). One study pulled on receptor–ligand complexes of Notch1-Fc fragments bound to Dll1-expressing cells with an optical trap, estimating a Notch1–Dll1 rupture force of 19 pN (126) and an endocytosis stall force for Dll1 of between 2 and 5 pN (89).

Atomic force microscopy was used to analyze unfolding transitions of the Notch2 NRR and to show that forced unfolding would allow proteolytic cleavage of the purified Notch2 NRR (130). The force required to permit metalloprotease cleavage of the Notch1 NRR was then measured directly using magnetic tweezers, which showed that the Notch1 NRR was cleaved by metalloprotease at 5.4 pN of force but not at 3.5 pN (44). A variety of different assays also investigated the force required to stimulate Notch signaling in cells, including tension-gated tethers in which the ECD of Dll1 was attached to the surface with a DNA zipper (151); a DNA unspooling assay

Table 2 Relevant force scales in Notch signaling

Force description	Values	Reference	Comment
Forces sufficient for activating Notch1 in cells (Dll1)	F < 12 pN	151	Used DNA-tethered ligands
Forces sufficient for activating Notch1 in cells (Dll1)	4 pN < F < 12 pN	25	Improved DNA-tethered ligands
Forces sufficient for activating Notch1 in cells (Dll4)	F ~ 2pN	44	Magnetic beads attached to ligands
Forces sufficient for activating Notch1 in cells (beads covalently attached)	1 pN < F < 9 pN	119	Magnetic nanoparticles covalently attached to Notch1 receptors
Forces sufficient for activating Notch1 in cells	Jag1: 4 pN < F < 12 pN Dll4: F < 4 pN	84	Improved DNA-tethered ligands
Forces sufficient for proteolytic cleavage of NRR	3.5 pN < F < 5.4 pN	44	Magnetic beads attached to NRR
Rupture forces for receptor–ligand interactions	~19 pN	126	Optical tweezers pulling on Notch1-Fc
Stall force opposing Dll1 endocytosis	2–5 pN	89	Optical trap bound to Notch1-Fc opposing endocytosis
Adhesion forces between sender and receiver cells	~14 nN	3	AFM on S2 cells
Forces required for invagination via clathrin-mediated endocytosis	~100–200 pN ~10s pN with clathrin/bar	Reviewed in 70	NA

Abbreviations: AFM, atomic force microscopy; NA, not applicable; NRR, negative regulatory region.

called nano yoyo (25); and two cell-based magnetic tweezers assays, one using ligand-coated magnetic beads and another using monovalent magnetic nanoparticles (119). These assays established that applying force to receptor-bound soluble ligand can activate Notch (44), and that the force required lies in a physiologically accessible force regime, likely between 2 and 10 pN. In addition, studies using a biomembrane force-probe arrangement were used to show that the lifetime of Notch–ligand complexes demonstrates catch bond behavior (**Table 3**), increasing as a function of increasing force to reach a maximum at roughly 10 pN of force (84). This catch-bond behavior of the ligands helps explain how a relatively weak (low μM) monovalent affinity between ligand and receptor is nevertheless capable of resisting rupture and of delivering sufficient force to open up the NRR and allow proteolytic cleavage to take place. This body of cell-based and in vitro studies was nicely complemented by in vivo studies in the fly, in which the natural NRR mechanosensor was replaced with the von Willebrand factor A2 domain (VWF-A2) mechanosensor and with various VWF-A2 mutants of varying mechanosensitivity to show that delivery of mechanical force by bound ligand is also necessary for signaling in flies (73).

6. TRANSCRIPTIONAL REGULATION BY RBPJ AND NOTCH NUCLEAR COMPLEXES

Upon its release from the cell membrane, Notch gains access to the nucleus and stimulates transcription through effector complexes that contain the transcription factor RBPJ [Su(H) in flies] and a coactivator of the mastermind family (108, 109, 156). The one-to-one stoichiometry between a single receptor–ligand complex and a single transcriptional activator molecule contrasts with the biochemistry of many other signaling pathways, which rely on a series of enzymatic amplification steps to communicate the signal. Recent studies have both elucidated the structural

Table 3 Timescales and diffusion coefficients of Notch-relevant events

Timescale, diffusion coefficient	Details	Time	Reference(s)	Comments
Bond lifetime Notch1-Jag1, 0 pN	In vitro Notch1(8–12)-Jag(N-3)	0.2 s	84	Using BFP force clamp spectroscopy
Bond lifetime Notch1-Jag1, 9 pN	In vitro Notch1(8–12)-Jag(N-3)	~0.5 s	84	Using BFP force clamp spectroscopy
Bond lifetime Notch1-Jag1V, 0 pN	In vitro Notch1(8–12)-Jag_JV1(N-3)	~0.9 s	84	High-affinity Jag1 variant using BFP force clamp spectroscopy
Bond lifetime Notch1-Dll4, 0 pN	In vitro Notch1(8–12)-Dll4(N-3)	~0.15 s	84	Using BFP force clamp spectroscopy
Bond lifetime Notch1-Dll4, 9 pN	In vitro Notch1(8–12)-Dll4(N-3)	~0.4 s	84	Using BFP force clamp spectroscopy
Clathrin-mediated endocytosis	Mammalian cells	~30 s	Reviewed in 70	NA
Processing of Notch receptors	Mammalian cells	15–30 s	119	After applying 9 pN using nanoparticle
Appearance of NICD in the nucleus	*Drosophila*	~10 min	29	Live imaging of Notch-GFP after asymmetric cell division
Appearance of NICD–RBPJ complexes in the nucleus	Mammalian cells	5–10 min	52	Split luciferase system
NICD half-life	Mammalian cells	90–180 min	52, 67	Split luciferase system
Residence time of Su(H) on E(spl) site in Notch-OFF	*Drosophila* larval salivary glands	~0.1–4 sec	41	FRAP on E(spl) locus
Residence time of Su(H) on E(spl) site in Notch-ON	*Drosophila* larval salivary glands	~8–15 sec	41	FRAP on E(spl) locus
Transcriptional burst duration	*Caenorhabditis elegans*	5–40 min	76	Live imaging using MS2-GFP; ON times vary with signal strength
Transcriptional burst duration	*Drosophila* embryo	1–10 min	35	Live imaging using MS2-GFP; ON times vary with signal strength
Diffusion rate of Dll1 on cell membrane	Mammalian cells	0.01–0.1 $\mu m^2/s$	58	FRAP-TIRF imaging
Diffusion of Su(H) in the nucleus	*Drosophila* salivary gland	0.1–2 $\mu m^2/s$	41	Single particle tracking

Abbreviations: BFP, biomembrane force probe; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; NA, not applicable; NICD, Notch intracellular domain; Su(H), Suppressor of Hairless; TIRF, total internal reflection.

basis of this response and highlighted its quantitative nature through detailed investigation of the transcriptional regulation in the nucleus.

6.1. Repression Complexes

In the absence of nuclear Notch, RBPJ functions as a transcriptional repressor. Repressor activity is derived from the ability of RBPJ to associate with several different nuclear corepressor

proteins, including SHARP (68, 102) and FHL1 (138) in mammals and the Hairless protein in flies (69, 86, 92). The methyl-lysine reader protein L3MBTL3 is also reported to interact with the Notch coactivator RBPJ, and this complex is thought to repress transcription by recruiting the repressive histone demethylase KDM1A (159). In addition, the RBPJ-interacting protein called RBPJ-interacting and tubulin-associated (RITA1) has been implicated in the repression of Notch signaling by facilitating export of RBPJ out of the nucleus (136).

A series of biochemical and structural studies have shown that transcriptional repression by FHL1 and SHARP relies on direct binding of the repressor proteins to RBPJ when bound to DNA. RBPJ contains three structural domains: N- and C-terminal Rel-homology domains that flank an interior beta-trefoil domain, with DNA recognition achieved by the N-terminal and beta-trefoil domains (65). Structures of FHL1 and SHARP complexes that contain RBPJ and cognate DNA have been solved by X-ray crystallography (26, 167). FHL1 uses a hydrophobic sequence to bind in a groove on the beta-trefoil domain (**Figure 8*a***). In contrast, SHARP recognizes RBPJ through a bipartite recognition mechanism, with one site of contact overlapping the FHL1 binding site and a second contact site residing on the N-terminal Rel-homology domain (**Figure 8*b***). Although the detailed interactions differ, this binding site on the Rel-homology domain is also used in the fly for binding of its repressor protein Hairless (166), which induces a larger-scale conformational change in Su(H) by wedging a third beta strand into the binding site.

6.2. Activation Complexes

Entry of Notch into the nucleus is thought to depend on the canonical α/β1 importin pathway (110, 111). Upon nuclear entry, NICD assembles into an NTC with MAML and RBPJ on DNA (although binding to RBPJ may occur before entrance into the nucleus). NICD contains a RAM region followed by an ankyrin repeat domain; a variable transactivation domain; and a proline, glutamic acid, serine, and threonine (PEST) sequence at the C-terminal end. Although the RAM region of NICD binds to RBPJ with submicromolar affinity, formation of NTCs on DNA only requires the ankyrin repeat domain of Notch and a segment from the N-terminal end of MAML, even though the ankyrin domain does not itself form stable RBPJ complexes with measurable binding affinity (33). In the human RBPJ–NICD1–MAML1–DNA complex (96), this remarkable cooperativity in assembly depends on the creation of a composite MAML1 binding interface derived from the ankyrin repeat domain of Notch in contact with the Rel-homology domains of RBPJ (**Figure 8*c***). The ability of RBPJ to recruit NICD into NTCs is greatly enhanced by the presence of the RAM region of Notch, which uses a hydrophobic WFP sequence to bind in the same groove of the beta-trefoil domain that is occupied in the transcriptional repressor complexes (155) (**Figure 8*c***). In studies using protein binding microarrays with purified proteins, accrual of NICD and MAML proteins into the complex does not alter the DNA binding site preferences of RBPJ (34).

The use of a shared binding site on RBPJ for repressors and for the RAM region of NICD raises the question of how NICD accesses its binding site on RBPJ to convert it from a repressor into a transcriptional activator. Given the binding affinity of repressors for RBPJ, and the cooperativity in assembly of transcription complexes on DNA, it is unlikely that NICD simply displaces bound corepressors to flip a transcriptional switch. A more likely scenario, consistent with several studies investigating the genomic landscape of RBPJ and Notch on DNA as a function of signal activation (21, 66, 148) and showing that chromatin immunoprecipitation of RBPJ is greatly enhanced in the presence of NICD, is that repressor complexes unload from DNA, and new NTCs assemble on DNA, to induce a transcriptional response.

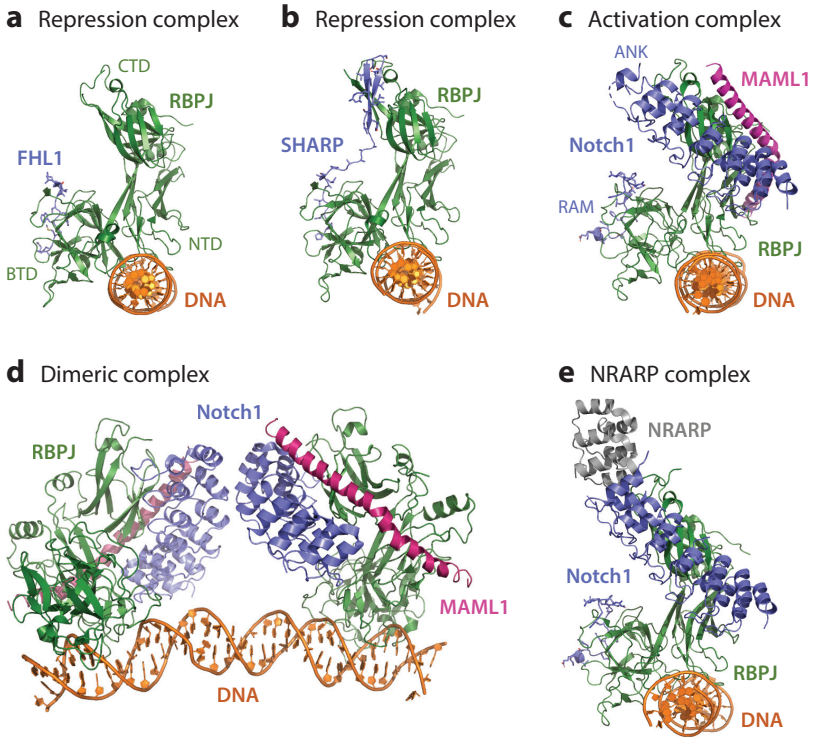


Figure 8

Structures of RBPJ repressor and Notch nuclear complexes. (*a*) The structure of the RBPJ–FHL1–DNA repressor complex, shown as a ribbon, looking down the axis of the DNA double helix (PDB ID 4J2X). The NTD and the BTD of RBJ make direct contact with the DNA. The FHL1 peptide binds to a groove on the BTD of RBPJ and does not contact the DNA. (*b*) The structure of the RBPJ–SHARP–DNA repressor complex, rendered as a ribbon and shown in the same orientation as in panel *a*. SHARP contacts both the BTD and CTD of RBPJ (PDB ID 6DKS). (*c*) Structure of a human Notch1–MAML1–RBPJ–DNA transcriptional activation complex (PDB ID 3V79), shown as a ribbon and in the same orientation as in panel *a*. The RAM region of Notch binds to the BTD at the same site as the repressors. The ANK domain binds to the NTD and CTD of RBPJ, and the N-terminal segment of MAML1 recognizes a composite surface derived from the ANK domain of Notch1 and the Rel-homology domains of RBPJ. (*d*) Structure of a dimeric Notch1 transcriptional activation complex (PDB ID 3NBN), shown in an orientation perpendicular to the helical axis of the DNA. The interface between the two Notch1 subunits of the complex is on the convex face of the ANK domains. (*e*) Structure of a NRARP–Notch1–RBPJ–DNA complex (PDB ID 6PY8) shown as a ribbon and in the same orientation along the DNA as in panels *a–c*. Note that the binding sites for NRARP and MAML1 are nonoverlapping and compatible with simultaneous binding of both proteins. Abbreviations: ANK, ankyrin repeat; BTD, beta-trefoil domain; CTD, C-terminal Rel-homology domain; FLH1, four and a half LIM domains; NRARP, Notch-regulated ankyrin repeat protein; NTD, N-terminal Rel-homology domain; PDB, Protein Data Bank; SHARP, SMRT/HDAC1-associated repressor protein.

6.3. Activation Complex Dimerization

Researchers in the Notch field have appreciated for over 25 years that there are paired RBPJ binding sites found in the regulatory elements that control the expression of Notch-responsive genes. These so-called sequence-paired sites (SPSs) (11) are oriented head to head and have a typical spacing of 15–17 base pairs between the RBPJ binding sites. The core NTC is also capable of cooperatively forming dimers on these paired RBPJ binding sites (11, 79, 95). Cooperative

assembly of NTC dimers on SPSs requires the presence of all three components of the core complex: Notch, RBPJ, and MAML. The ability to form these dimeric NTC complexes relies on a small protein–protein interface between the convex face of the Notch ankyrin repeat domains, with a conserved arginine residue (R1984 in human Notch1) playing a critical role in driving cooperative loading of the two NTCs (**Figure 8*d***). Genome-wide studies in leukemic cells suggested that up to 20% of Notch binding sites are associated with loading of dimeric complexes (124), and reconstitution of a split DNA adenine methyl transferase using a technique called split-DAM ID also suggests that loading of dimers onto paired sites is a prevalent occurrence (48). In mammalian systems, dimerization appears to have more of a fine-tuning than a central role in the transcriptional response. In contrast to wild-type NICD1, which, when transduced into murine hematopoetic cells, induces T-ALL in a bone marrow transplantation assay in all mice within 12 weeks, introduction of an R1984A transgene in the same assay does not produce leukemia (79). When a dimerization-deficient mutation is knocked into both murine Notch1 and Notch2, the exogenous stress of fur mite infestation was required to elicit cardiac developmental and gut defects in the mutant mice (61), a hypomorphic phenotype that is far from the embryonic lethality of a Notch1 (or Notch2)-only knockout.

6.4. Feedback Inhibition by the Notch-Regulated Ankyrin Repeat Protein

The small Notch-regulated ankyrin repeat protein (NRARP) is a canonical target of activated Notch in a variety of different contexts. Functional studies in knockout mice implicate it as a negative feedback regulator that modulates the Notch response in the skeletal system and in vascular development. At a biochemical and structural level, feedback regulation by NRARP also relies on direct association of NRARP with the core NTC (54). Binding of NRARP to Notch–RBPJ complexes is independent of DNA or MAML binding and also uses a composite surface derived from the ankyrin repeat domain of Notch and the C-terminal Rel-homology domain of RBPJ (**Figure 8*e***). How NRARP downregulates the Notch response is less clear, but there is some evidence that the binding of NRARP promotes the degradation of NICD complexes, leading to termination of transcriptional induction by NTCs.

6.5. Kinetics of RBPJ Complexes on the Chromosome

How do activation and repression complexes compete for binding at the target gene promoter? Recent work used live imaging in *Drosophila* to track endogenously tagged Su(H)–green fluorescent protein (GFP) and Hairless-GFP dynamics in the nucleus (41). The authors of this work used FRAP and single-molecule tracking to extract recruitment rates, dwell times, and diffusion coefficients of the complexes on a highly active locus [the E(spl) locus] in the presence and absence of NICD (**Table 3**). Their studies showed that the presence of NICD not only enhances recruitment of activation complexes to the E(spl) locus, but also enhances recruitment of repression complexes. They found that typical dwell times of the activation complex on the locus in the presence of NICD is on the order of approximately 10 s, compared with approximately 1 s for Su(H) in the absence of NICD or in a repression complex. Their analysis suggests an assisted-loading model in which activation complexes recruited to the locus further open the chromatin and increase accessibility to both activation and repression complexes. This work further strengthens the idea that the switch between activation and repression is not binary, but instead depends on a balance between repressors and activators.

6.6. Dynamics of Transcriptional Response

To investigate how the transcriptional response varies as a function of the concentration of NICD, three recent papers used the MS2-MCP system to perform live imaging of nascent transcripts

from Notch responsive loci in *Drosophila melanogaster* and *C. elegans* (35, 76, 146). These studies showed that the transcription from Notch target genes exhibits stochastic bursts; that the burst ON-times, but not the burst OFF-times (i.e., frequency), vary with Notch signaling strength (i.e., amount of NICD); and that the burst can be sustained for up to 60 min (**Table 3**). Moreover, it seems that the initiation of the transcriptional response depends on a threshold amount of NICD, and that this value changes from cell to cell (35, 146) and depends on other transcription factors regulating the responsive genes (35). Interestingly, cooperative SPS binding motifs can enhance transcriptional burst size but do not change the threshold.

6.7. Notch Intracellular Domain Turnover: A Bind and Discard Mechanism

Given the importance of temporal control in the response to a Notch signal, it is not surprising that the turnover of NICD in the nucleus is highly regulated. Like many other unstable proteins, the NICD contains an unstructured PEST-rich C-terminal domain. Early work showed that the formation of an active complex with RBPJ and Mastermind on DNA stimulates recruitment of the mediator complex, containing the Cyclin C/CDK8 kinase, which leads to phosphorylation of the PEST domain (38, 39). The phosphorylated PEST domain is then identified by the E3 ubiquitin ligase, FBXW7, which ubiquitylates the NICD in certain contexts and marks it for proteasomal degradation (51, 144). Additional kinases that target NICD have also been identified. Some, like CDK8, are associated with NICD degradation, while others are associated with other functions of NICD (20, 23).

Recent work in *Drosophila* showed that NICD binding to SPSs stimulates mediator complex–dependent phosphorylation and subsequent degradation of NICD (67). This bind and discard mechanism therefore ensures that NICD molecules are first utilized for activation of transcription before being targeted for degradation. Mathematical modeling also showed that this mechanism is sensitive to the duration of Notch activation, namely, that active degradation of NICD affects long (>1 h) but not short (>30 min) duration processes (67). The importance of NICD turnover regulation is highlighted by the fact that a large fraction of T-ALL patients carry stabilizing PEST mutations (154), as well as occasional FBXW7 loss-of-function mutations (101, 140).

7. SYNTHETIC NOTCH

The insights obtained from the mechanistic understanding of Notch activation allowed, in recent years, exploitation of the modular structure of the Notch receptors and ligands to develop synthetic variants of Notch where both the binding domains and the transcriptional response can be exchanged with synthetic alternatives (**Figure 9**). These variants have been used to understand the mechanism of activation, track Notch-induced activity in vivo, and generate variants for synthetic biology applications.

Early work replaced the NICD in flies with the Gal4-VP16 activator to provide evidence for NICD translocation to the nucleus following Notch activation (131). Similar Notch variants were used in mammalian systems to identify the domains required for activation of Notch1 (9) and to characterize *cis*-inhibition (129). In mice, Notch1 with its ICD replaced by Cre was used to induce recombination in tissues where Notch1 is active (147).

More recently, synthetic Notch variants in which the ECD and ICD were replaced by dimerizing FKBP-FRB domains and Gal4, respectively, were used to measure the pulling force required for Notch proteolysis (44). This work reported the use of human synthetic Notch receptors in which the FK506 binding domain (FRB) replaced EGF repeats 1–23 in Notch1, FKBP replaced

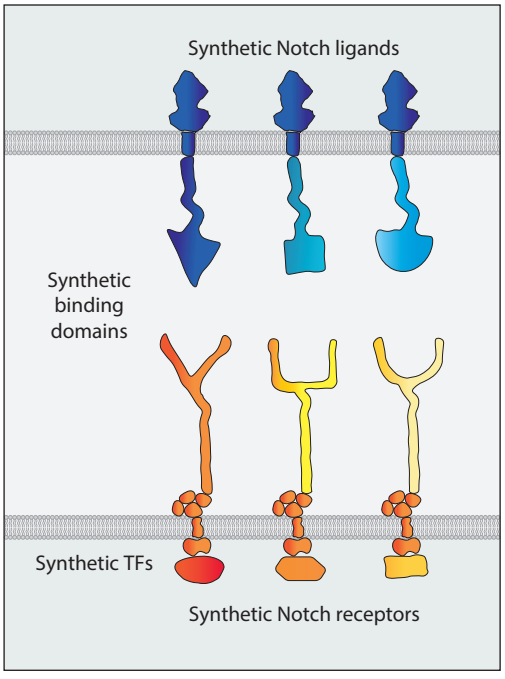


Figure 9

Schematic of the concept of synthetic Notch. In synthetic Notch systems, the binding domains of the receptors and ligands are replaced by synthetic dimerizing domains (e.g., FKBP:FRB + rapamycin, GFP:α-GFP). The intracellular domains are replaced by synthetic TFs (e.g., Gal4-VP16, TetR-VP16, Gal4-KRAB) or other effector domains. The endogenous NRR and transmembrane domains of Notch are retained, maintaining the mechanotransducing functionality of these synthetic receptors. Abbreviations: GFP, green fluorescent protein; NRR, negative regulatory region; TF, transcription factor.

the DSL and MNNL domains in Dll4, and the intracellular domain contained a Gal4 DNA binding domain to report on the Gal4-response element. It also reported the use of a receiver–sender system in which the ECD of the ligand was completely replaced with GFP, and all 36 EGF repeats of the receptor were replaced with α-GFP. Crucially, the NRR and transmembrane domains of Notch were left intact, retaining the core mechanotransduction functionality of the constructs. This idea of exploiting the modularity of Notch was further expanded in several recent works in which the ECD and ICD of Notch were replaced by a variety of dimerizing domains (e.g., CD19:α-CD19, GFP:α-GFP) and transcription factors (e.g., Gal4-VP16, tTA, Gal4-KRAB) (93). These synNotch receptors were used to establish synthetic morphogenesis (93, 143), as well as to reprogram T-cells to respond to specific presented antigens (114, 115) and detect angiogenesis (153). Finally, a synthetic Notch approach used the modularity of Notch to develop an assay to identify other surface proteins containing putative proteolytic switches that can functionally replace the NRR (49). In this system, various domains with potential proteolytic sensitivity were substituted in place of the NRR for evaluation in a reporter gene assay, leading to the identification of a variety of domains that may have mechanoresponsive properties capable of regulating protease access. Synthetic sender–receiver systems exploiting the modularity and molecular mechanism of Notch signaling are clearly emerging as an exciting frontier for molecular engineering, capable of providing a large number of programmed functionalities in vitro and in vivo.

8. CONCLUDING REMARKS

This review focuses on providing a molecular mechanistic and biophysical picture of the sequence of events leading to signal transduction in the Notch pathway. The knowledge obtained in recent years has honed our understanding of the details at each step of a signaling event, starting with receptor–ligand binding, through tripping of the NRR mechanosensor, and ending in a quantitative transcriptional response. General features of signaling revealed by elucidating these steps in Notch activation have relevance for other signaling pathways that rely on force for activation or depend on assembly of transcriptional activation complexes. The regulation of proteolysis by force-induced unfolding of the NRR is a paradigm for other force-regulated processes, and the assembly of the transcriptional activation complex is likewise representative of cooperative events that regulate a transcriptional response, one that, in this case, also involves competition between coactivators and corepressors vying for the same DNA binding sites.

The deep understanding of the Notch pathway that has now emerged also provides a molecular roadmap for its manipulation for therapeutic purposes. Suppression of oncogenic signaling by ligand-blocking and anti-NRR antibodies, as well as inhibition of γ-secretase in Notch-driven cancer, are both strategies that have been pursued clinically (2, 7, 165), and the molecular engineering of synthetic Notch receptors holds future promise for cell-based therapies and other synthetic biology applications.

Naturally, there are still many open questions that have yet to be addressed. One area for future study will be determining how signaling outputs are converted into specific and distinct transcriptional responses in different cells. The sequence of steps that take place at the cell membrane and the timescales and the dynamics of these events also remain poorly understood. Finally, new advances using correlated light and electron microscopy should eventually make it possible to visualize Notch–ligand complexes directly in the context of a cell.

DISCLOSURE STATEMENT

S.C.B. receives sponsored research funding from Novartis and Erasca, Inc. He is on the scientific advisory board for Erasca, Inc.; an advisor to MPM Capital; and a consultant for Ayala Pharmaceutical, Scorpion Therapeutics, and IFM. D.S. is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Maya Sprinzak for help with figure design. We are also grateful to our many colleagues in the Notch field who have contributed to current understanding of this major developmental signaling pathway and ask for forbearance about any references that were not included due to space limitations. This work was supported by National Institutes of Health award R01 CA220340 (to S.C.B.); the US-Israel Binational Science Foundation award 2017245 (to S.C.B. and D.S.); and the European Research Council under the European Union's Horizon 2020 research and innovation programme, grant agreement 682161 (to D.S.).

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Volume 50, 2021

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Errata

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