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Review

The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism

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Notch signaling regulates many aspects of metazoan development and tissue renewal. Accordingly, the misregulation or loss of Notch signaling underlies a wide range of human disorders, from developmental syndromes to adult-onset diseases and cancer. Notch signaling is remarkably robust in most tissues even though each Notch molecule is irreversibly activated by proteolysis and signals only once without amplification by secondary messenger cascades. In this Review, we highlight recent studies in Notch signaling that reveal new molecular details about the regulation of ligand-mediated receptor activation, receptor proteolysis, and target selection.

Diversity among different species and cell types appears to have evolved through the combinatorial use of a relatively small number of conserved signaling pathways. Among these, the Notch signaling pathway of metazoans enables short-range communication between cells. Indeed, transmission of Notch signals requires physical contact between cells under most circumstances. Notch signals are often used to select between preexisting developmental programs. In a context-dependent manner, Notch signals can promote or suppress cell proliferation, cell death, acquisition of specific cell fates, or activation of differentiation programs. This occurs in cells throughout development of the organism and during the maintenance of self-renewing adult tissues. Because Notch plays a critical role in many fundamental processes and in a wide range of tissues, it is not surprising that aberrant gain or loss of Notch signaling components has been directly linked to multiple human disorders. These disorders include developmental syndromes (for example, Alagille syndrome, Tetralogy of Fallot, syndactyly, spondylocostal dysostosis, familial aortic valve disease; Garg et al., 2005; Gridley, 2003), adult-onset diseases (such as CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; Louvi et al., 2006), and cancer. Notch signaling has emerged as a specific therapeutic target for T cell acute lymphoblastic leukemia (Weng et al., 2004) and colon cancer (van Es et al., 2005), as well as a potential target in the effort to curb tumor angiogenesis (Noguera-Troise et al., 2006; Ridgway et al., 2006). In addition to its importance in disease, any meaningful manipulation of embryonic or adult stem cells will also require the development of receptor-specific antagonists and agonists of Notch signaling. As a consequence, research that examines the finer mechanistic details of Notch activation and nuclear activity is of growing clinical and commercial relevance.

A hallmark of Notch signaling that sets it apart from other conserved signaling pathways is its mechanism of signal transduction. Notch signaling relies on the ability of a ligand to bring about receptor proteolysis, resulting in the release of an active Notch fragment. A second unusual feature of Notch signaling is that intramembrane proteolysis is involved in receptor activation. After its release by proteolysis from a membrane tether, the Notch intracellular domain (NICD) travels to the nucleus. There, the NICD associates with a DNA binding protein to assemble a transcription complex that activates downstream target genes (Figure 1). This core signal transduction pathway is used in most Notch-dependent processes and is known as the "canonical" pathway. However, depending on the cellular context, the amplitude and duration of Notch activity can be further regulated at various points in the pathway. Here, we summarize our current understanding of canonical Notch signaling mechanisms and highlight recent studies that bring new insights into the molecular and biochemical events that occur during ligand-receptor recognition, receptor activation, intramembrane proteolysis, and target gene selection.

A Growing Roster of Core Pathway Components

Notch receptors are large single-pass type I transmembrane proteins (see Figure 2A for domain organization). Whereas the fly *Drosophila melanogaster* possesses only one Notch receptor and the worm *Caenorhabditis elegans* possesses two redundant Notch receptors, mammals have four Notch paralogs that display both redundant and unique functions (Table 1). The extracellular domain of all Notch proteins contains 29–36 tandem epidermal growth factor (EGF)-like repeats, some of which mediate interactions with the Notch ligand (Figure 2A). Productive interactions with ligands presented by neighboring cells (*trans* interactions) require repeats 11–12. In contrast, *cis*-inhibition by ligands expressed in the same cell depend on an activity that maps to the *Abruptex* region of *Drosophila* Notch, which includes EGF repeats 24–29. Many EGF repeats bind to calcium ions, which play an important role in determining the structure and affinity of Notch in ligand binding (Cordle et al., 2008b) and can affect signaling efficiency (Raya et al., 2004). Within the extracellular domain structure, the EGF repeats are followed by a

unique negative regulatory region (NRR), which is composed of three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (Figure 2A). The negative regulatory region plays a critical role in preventing receptor activation in the absence of ligands and will be discussed in detail later. Most surface Notch proteins are cleaved by furin-like convertases within the secretory pathway (Figure 1) at site 1 (S1) located within an unstructured loop that protrudes from the heterodimerization domain. This cleavage event converts the Notch polypeptide into an NECD-NTMIC (Notch extracellular domain-Notch transmembrane and intracellular domain) heterodimer held together by noncovalent interactions between the N- and C-terminal halves of the heterodimerization domain (Figures 1 and 2A).

The single transmembrane domain of the Notch receptor ends with a C-terminal "stop translocation" signal comprised of 3–4 arginine/lysine (Arg/Lys) residues. Intracellularly, the RAM (RBPjκ association module) domain forms a high-affinity binding module of 12–20 amino acids centered on a conserved WxP (tryptophan-any amino acid-proline) motif. A long, unstructured linker containing a nuclear localization sequence links the RAM domain to seven ankyrin repeats (ANK domain). Following the ANK domain is an additional bipartite nuclear localization sequence and a loosely defined and evolutionarily divergent transactivation domain. The very C terminus of the receptor contains conserved proline/glutamic acid/serine/threonine-rich motifs (PEST) that harbor degradation signals (degrons), which regulate the stability of NICD. *Drosophila* Notch also contains a glutamine-rich repeat (OPA) (Figure 2A).

Our understanding of Notch ligands is rapidly evolving. Most Notch ligands are themselves type I transmembrane proteins (Figure 2B) (reviewed in D'Souza et al., 2008). Recent studies have refined our understanding of their structure and function (Cordle et al., 2008a; Komatsu et al., 2008). The largest class of Notch ligands is characterized by three related structural motifs: an N-terminal DSL (Delta/Serrate/LAG-2) motif, specialized tandem EGF repeats called the DOS (Delta and OSM-11-like proteins) domain (Komatsu et al., 2008), and EGF-like repeats (both calcium binding and non-calcium binding) (Figure 2B). DSL ligands can be classified on the basis of the presence or absence of a cysteine-rich domain (Jagged/Serrate or Delta, respectively) and the presence or absence of a DOS domain (Figure 2B). As we will detail later, both the DSL and DOS domains are involved in receptor binding. Additional proteins lacking DSL and DOS domains have been reported to act as noncanonical ligands for Notch receptors in the central nervous system and in cultured cells (for example, F3/Contactin1, NB-3/Contactin6, DNER, MAGP1, and MAGP2) (Figure 2B, Table 1) (reviewed in D'Souza et al., 2008). However, these activities have been largely unexplored, and the physiological functions for these proteins in the Notch pathway remain to be elucidated.

It is now well established that Notch receptor activation is mediated by a sequence of proteolytic events (Figures 1 and 2A) (reviewed in Bray, 2006). Ligand binding leads to the cleavage of Notch by ADAM (a disintegrin and metalloprotease) proteases at site 2 (S2), located ~12 amino acids before the transmembrane domain and deeply buried within the negative regulatory region (Figures 2 and 3A). Site 2 cleavage is a key regulatory step in Notch activation, but some ambiguity still exists regarding the enzymes that mediate cleavage. Indeed, only ADAM17/TACE (tumor

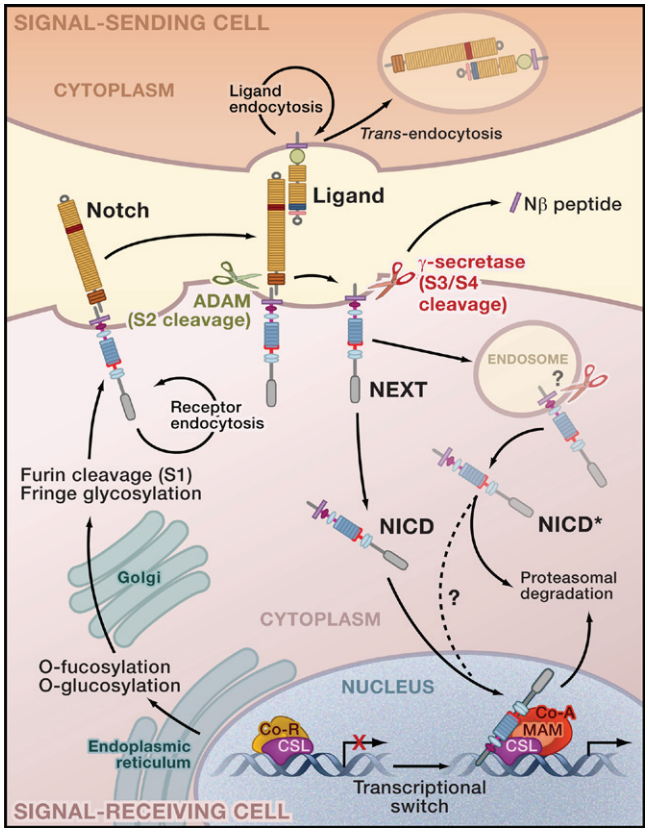

Figure 1. The Core Notch Signaling Pathway Is Mediated by Regulated Proteolysis

The newly translated Notch receptor protein is glycosylated by the enzymes O-fut and Rumi, which are essential for the production of a fully functional receptor. The mature receptor is produced after proteolytic cleavage by PC5/furin at site 1 (S1). It is then targeted to the cell surface as a heterodimer that is held together by noncovalent interactions. In cells expressing the glycosyltransferase Fringe, the O-fucose is extended by Fringe enzymatic activity, thereby altering the ability of specific ligands to activate Notch. The Notch receptor is activated by binding to a ligand presented by a neighboring cell. Endocytosis and membrane trafficking regulate ligand and receptor availability at the cell surface. Ligand endocytosis is also thought to generate mechanical force to promote a conformational change in the bound Notch receptor. This conformational change exposes site 2 (S2) in Notch for cleavage by ADAM metalloproteases (perhaps after heterodimer dissociation at site 1). Juxtamembrane Notch cleavage at site 2 generates the membrane-anchored Notch extracellular truncation (NEXT) fragment, a substrate for the γ-secretase complex. γ-Secretase then cleaves the Notch transmembrane domain in NEXT progressively from site 3 (S3) to site 4 (S4) to release the Notch intracellular domain (NICD) and Nβ peptide. γ-Secretase cleavage can occur at the cell surface or in endosomal compartments, but cleavage at the membrane favors the production of a more stable form of NICD. NICD then enters the nucleus where it associates with the DNA-binding protein CSL (CBF1/RBPjκ/Su(H)/Lag-1). In the absence of NICD, CSL may associate with ubiquitous corepressor (Co-R) proteins and histone deacetylases (HDACs) to repress transcription of some target genes. Upon NICD binding, allosteric changes may occur in CSL that facilitate displacement of transcriptional repressors. The transcriptional coactivator Mastermind (MAM) then recognizes the NICD/CSL interface, and this triprotein complex recruits additional coactivators (Co-A) to activate transcription.

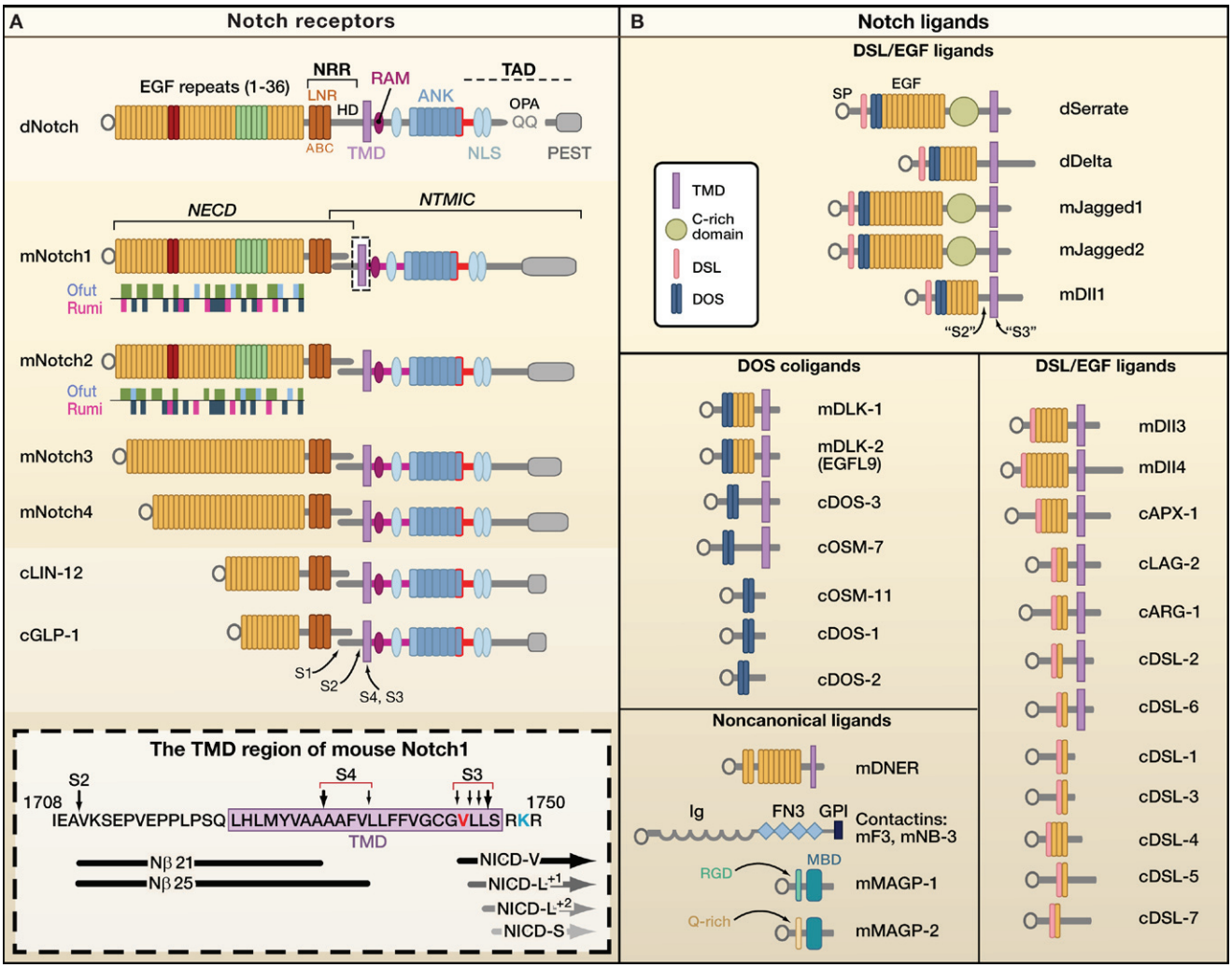

Figure 2. Domain Organization of Notch Pathway Receptors, Ligands, and Coligands

(A) Notch receptors are large type I proteins that contain multiple extracellular EGF-like repeats. *Drosophila* Notch (dNotch) and the four mammalian Notch paralogs (mNotch1–4) differ in the number of repeats (29–36) but all are much longer than the *C. elegans* Notch proteins (cLIN-12 and cGLP-1). EGF repeats 11–12 (red) and 24–29 (green) mediate ligand interactions. EGF repeats may contain consensus motifs for fucosylation by O-Fut1 and glucosylation by Rumi; the putative distribution of fucosylation sites (common, green; unique, light blue) and glucosylation sites (common, dark blue; unique, magenta) are shown for mNotch1 and mNotch2. Note that the ligand-binding regions differ in their modification patterns. EGF repeats are followed by the negative regulatory region (NRR), which is composed of three cysteine-rich Lin12-Notch repeats (LNR-A, -B, and -C) and a heterodimerization domain (HD). Notch also contains a transmembrane domain (TMD), a RAM (RBPjκ association module) domain, nuclear localization sequences (NLSs), a seven ankyrin repeats (ANK) domain, and a transactivation domain (TAD) that habors conserved proline/glutamic acid/serine/threonine-rich motifs (PEST). The transactivation domain in *Drosophila* Notch also has a glutamine-rich repeat (OPA). In contrast to *Drosophila* Notch, mammalian Notch proteins are cleaved by furin-like convertases at site 1 (S1), which converts the Notch polypeptide into an NECD-NTMIC (Notch extracellular domain-Notch transmembrane and intracellular domain) heterodimer that is held together by noncovalent interactions between the N- and C-terminal halves of the heterodimerization domain. (Inset) Details of the mouse Notch1 transmembrane domain (TMD) (purple box) and flanking residues showing the Notch cleavage sites and corresponding cleavage products. After ligand binding, Notch is cleaved at site 2 by metalloproteases. γ-Secretase can cleave multiple scissile bonds at site 3 (arrows), but only NICD molecules initiating at valine 1744 (V1744) (NICD-V) evade N-end rule degradation. Cleavage then proceeds toward site 4 until the short Nß peptides (most are 21 amino acids) can escape the membrane lipid bilayer. Amino acid substitution of V1744 to glycine (V1744G, red) and lysine 1749 to arginine (K1749R, blue) shift the cleavage site.

(B) Known ligands and putative ligands of Notch receptors can be divided into several groups on the basis of their domain composition. Classical DSL ligands (DSL/DOS/EGF ligands) contain the DSL (Delta/Serrate/LAG-2), DOS (Delta and OSM-11-like proteins), and EGF (epidermal growth factor) motifs and are not found in *C. elegans*. *C. elegans* and mammalian DSL-only ligands lacking the DOS motif (DSL/EGF ligands) are a subtype of DSL ligands that may act alone (for example, mDLL4) or in combination with DOS coligands (for example, cDSL-1 and possibly Dll3). This subfamily includes soluble/diffusible ligands. Noncanonical ligands lacking DSL and DOS domains have been reported to activate Notch in some contexts.

necrosis factor α converting enzyme) is able to cleave Notch substrates in vitro, and yet TACE null mice do not have as severe a phenotype as Notch null mice. In contrast, the Kuzbanian/ADAM10/Sup-17 metalloprotease is essential for Notch activity in all phyla (Deuss et al., 2008).

The shedding of the Notch ectodomain creates a membrane-tethered intermediate called Notch extracellular truncation (NEXT) that is a substrate for γ-secretase, a multicomponent member of a growing family of intramembrane cleaving proteases (I-CLiPs) (reviewed in Selkoe and Wolfe,

Table 1. Core Components and Modifiers of the Notch Pathway

Component Function	Type	*Drosophila*	*Caenorhabditis elegans*	Mammals
Receptor		Notch	LIN-12, GLP-1	Notch 1–4
Ligand	DSL/DOS	Delta, Serrate		Dll1, Jagged1 and 2
	DSL only		APX-1, LAG-2, ARG-2, DSL1–7	Dll3 and 4
	DOS Coligands		DOS1–3, OSM7 and 11	DLK-1, DLK-2/EGFL9
	Noncanonical			DNER, MAGP-1 and -2, F3/Contactin1, NB-3/Contactin6
Nuclear Effectors	CSL DNA-binding transcription factor	Su(H)	LAG-1	RBPjκ/CBF-1
	Transcriptional Coactivator	Mastermind	LAG-3	MAML1-3
	Transcriptional Corepressors	Hairless, SMRTR		Mint/Sharp/SPEN, NCoR/SMRT, KyoT2
Receptor Proteolysis	Furin convertase (site 1 cleavage)	?	?	PC5/6, Furin
	Metalloprotease (site 2 cleavage)	Kuzbanian, Kuzbanian-like, TACE	SUP-17/Kuzbanian, ADM-4/TACE	ADAM10/Kuzbanian, ADAM17/TACE
	γ-secretase (site 3/site 4 cleavage)	Presenilin, Nicastrin, APH-1, PEN-2	SEL-12, APH-1, APH-2, PEN-2	Presenilin 1 and 2, Nicastrin, APH-1a-c, PEN-2
Glycosyltransferase modifiers	O-fucosyl-transferase	OFUT-1	OFUT-1	POFUT-1
	O-glucosyl-transferase	RUMI		
	β1,3-GlcNAc-transferase	Fringe		Lunatic, Manic, and Radical Fringe
Endosomal Sorting/Membrane Trafficking Regulators	Ring Finger E3 Ubiquitin ligase (ligand endocytosis)	Mindbomb 1–2, Neuralized		Mindbomb, Skeletrophin, Neuralized 1–2
	Ring Finger E3 Ubiquitin ligase (receptor endocytosis)	Deltex		Deltex 1–4
	HECT Domain E3 Ubiquitin ligase (receptor endocytosis)	Nedd4, Su(Dx)	WWP-1	Nedd4, Itch/AIP4
	Negative regulator	Numb		Numb, Numb-like, ACBD3
	Neuralized Inhibitors	Bearded, Tom, M4		
	Other endocytic modifiers	sanpodo		
NICD Degradation	F-Box Ubiquitin ligase	Archipelago	SEL-10	Fbw-7/SEL-10
Canonical Target bHLH Repressor Genes		*E(spl)*	*REF-1*	*HES/ESR/HEY*

The Notch family of receptors mediates short-range communication between cells throughout development and during adult tissue renewal. During maturation and trafficking to the cell surface, Notch receptors undergo furin processing and glycosylation, which can impact their responsiveness to their ligands. The activity and availability of Notch receptors and ligands are also regulated by endocytic trafficking, which can be modulated by the activity of different ubiquitin ligases. A wide range of Notch ligands can bind and activate the Notch receptor, inducing further proteolytic processing of the receptor by specific proteases. The Notch intracellular domain (NICD), the end product of Notch receptor activation, localizes to the nucleus, where it associates with the DNA binding protein CSL [CBF1/RBPjk/Su(H)/Lag-1] and other transcriptional coactivators to bind and activate target genes. In the absence of NICD, CSL can also function in conjunction with transcriptional corepressor proteins to repress gene expression. DSL, Delta/Serrate/LAG-2 motif; DOS, Delta and OSM-11-like proteins motif.

2007; Wolfe and Kopan, 2004). γ-Secretase cleaves NEXT progressively within the transmembrane domain, most likely starting near the inner plasma membrane leaflet at site 3 (S3) and ending near the middle of the transmembrane domain at site 4 (S4) (Figure 2A). Only after γ-secretase cleavage is the NICD free to translocate to the nucleus. There, NICD interacts with the DNA-binding protein CSL [CBF1/RBPjκ/Su(H)/Lag-1] first through its RAM domain. The ANK domain of NICD then associates with CSL to help recruit the coactivator Mastermind/Lag-3, which in turn recruits the MED8 mediator transcription activation complex, thereby inducing upregulation of downstream target genes (reviewed in Kovall, 2008) (Figure 1). Additional proteins can also modify the output from Notch receptors (listed in Table 1 and presented in Ilagan and Kopan, 2007).

Regulation of Ligand-Receptor Interactions

Given that each Notch molecule undergoes proteolysis to generate a signal and thus can only signal once, regulation of either ligand or receptor availability at the cell surface is key to controlling Notch activation. One simple way of regulating availability is to restrict ligand or receptor expression spatially and temporally. Indeed, different ligands and receptors can have overlapping as well as distinct expression patterns dur-

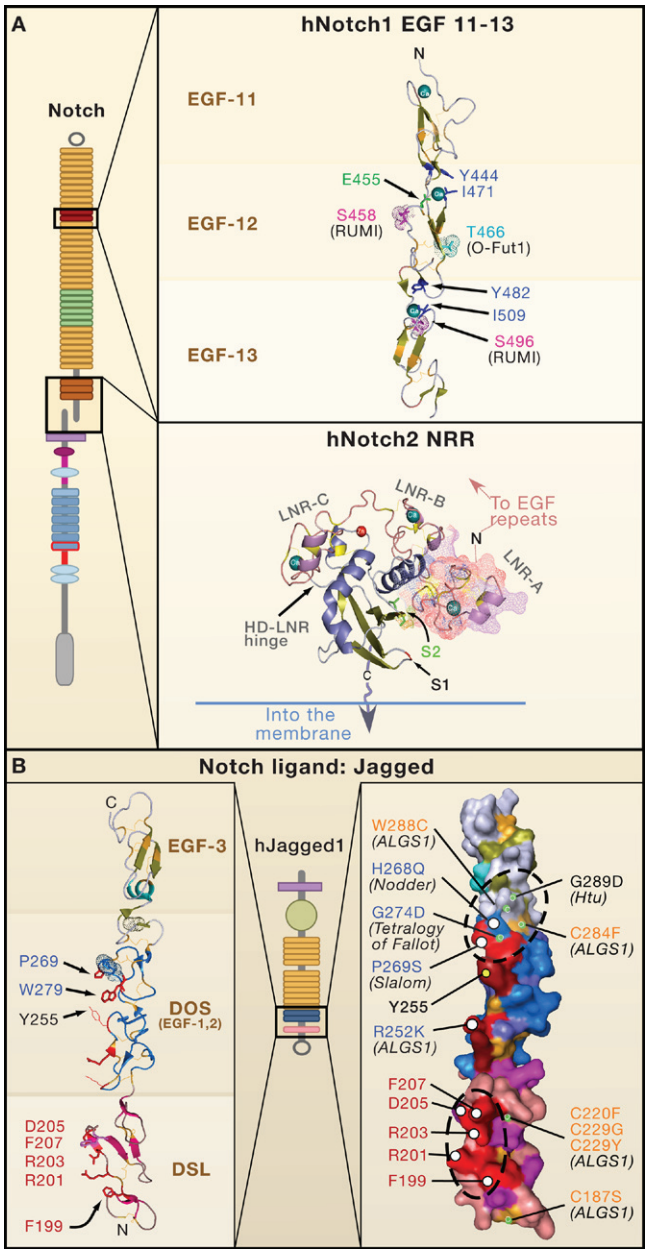

Figure 3. Atomic Resolution of Domains in Human Notch1, Notch2, and Jagged1

(A) (Top) The ligand-binding domain (EGF 11–13) from the crystal structure of human Notch1 (PDB:2VJ3). The ligand-binding domain of Notch1 is centered on EGF repeat 12, which contains residues that coordinate Ca^{2+} binding (blue), as well as O-glucosylation (serine 458, serine 496) and O-fucosylation (threonine 466) sites. Mutation of glutamic acid 455 to valine (E455V) abolishes ligand binding in *Drosophila*. This region in human Notch1 (hNotch1) was suggested to interact with human Jagged1 (hJagged1) DSL ligand on the basis of computational docking models (Cordle et al., 2008a). However, glycosylation at serine 458 may block access to this site. Threonine 466 is essential for productive Notch activation in the mouse but not in the fly. (Bottom) The negative regulatory region from the crystal structure of human Notch2 (PDB:2OO4). The negative regulatory region folds to protect the S2 cleavage site (green), which is located in a pocket protected by LNR-A, the HD-C helix, and the LNR-A/B linker. The furin cleavage site (S1) lies within an unstructured loop that was removed to facilitate crystallization. LNR repeats bind to calcium ions; chelation of Ca^{2+} leads to negative regulatory region dissociation and Notch activation.

(B) The crystal structure of the hJagged1 Notch-binding domain (PDB:2VJ2) containing the DSL, DOS, and EGF repeat 3 (EGF-3) regions. (Left) The hJagged1 ribbon structure.The DSL fold is distinct from the EGF fold; DSL amino acids required for interaction with Notch are labeled in red (Cordle et al., 2008a). The phenylalanine 207 to alanine (F207A) substitution generates a null protein. In contrast, arginine 203 to alanine (R203A) and phenylalanine 199 to alanine (F199A) substitutions ablate binding of ligand presented from another cell (*trans*) but not *cis* binding of ligand present in the same cell. Aspartic acid 205 to alanine (D205A) and arginine 201 to alanine (R201A) substitutions are hypomorphic. The DOS domain contains two conserved atypical EGF repeats (defined by the presence of the conserved amino acids in blue) (Komatsu et al., 2008). Tyrosine 255 (Y255) is characteristic of Jagged DSL ligands and is replaced by a small hydrophobic amino acid in Delta-like ligands (this residue may be involved in defining sensitivity to Fringe glycosyltransferase activity). (Right) Surface rendering of the hJagged1 ribbon structure. The structure is rotated so that the Notch-binding interface (dotted circles) is facing the reader. Surface exposed residues are indicated by white circles, whereas buried residues are indicated by green circles. Mapping of known Jagged mutations in humans and mouse to the structure indicates that the DOS domain could form part of the Notch-binding interface. Human Alagille syndrome (ALGS)-associated missense mutations (orange) are likely to affect disulfide bonds (and thus the structural integrity of these domains). A positively charged cluster of highly conserved surface-exposed residues within the DSL domain (red) identifies a putative Notch-binding surface. Interestingly, missense mutations (blue) associated with Tetralogy of Fallot in humans and autosomal dominant inner ear malformations in mice (*headturner*, *slalom*, and *Nodder*) cluster near a common DOS region. Mutations in *headturner* and Tetralogy of Fallot affect amino acids buried under the surface defined by *slalom* and *Nodder* mutations and may impact the structure of the potential Notch binding site within DOS. Note that arginine 252 (mutated to lysine in ALGS1) and tyrosine 255 (Y255), which is unique to Jagged, are also aligned with the putative Notch binding surfaces on the DSL and DOS domains. The exact topology of the Notch/ligand interface remains to be explored by cocrystallization. Structures were generated with MacPymol (http://www.pymol.org).

ing development and are subject to regulation by other signaling pathways (reviewed in Wu and Bresnick, 2007). Though important, differential expression patterns of the ligands and receptors are not enough to explain the observed differences in signaling activity. The regulation of trafficking and post-translational modifications have emerged as important mechanisms that control ligand or receptor availability and productive ligand-receptor interactions.

Ligand and Receptor Endocytosis and Trafficking

Endocytic trafficking of the DSL ligands plays a critical role in enhancing their signaling activity. This has been a subject of many excellent recent reviews (Le Borgne, 2006; Nichols et al., 2007b). Therefore, we will only summarize a few pertinent points here and discuss other aspects of endocytosis that are more directly related to the receptor activation process later in the review. Ligand endocytosis is triggered by monoubiquitination that is mediated by the E3 ubiquitin ligases Neuralized and Mindbomb. After endocytosis, a poorly characterized process occurs to produce a more active cell surface ligand. Current models for the nature of ligand modification include clustering of the ligand, posttranslational modifications to the ligand, and recycling of the ligand into specific membrane microdomains (Le Borgne, 2006; Nichols et al., 2007b). Interestingly, *bearded* protein family members, which negatively regulate Neuralized activity and thus reduce the efficiency of Notch activation by Delta (Bardin and Schweisguth, 2006), are themselves Notch target genes (Lai et al., 2000). This regulation of Bearded proteins by Notch constitutes a negative feedback loop that is

further fine-tuned by miRNAs that target *bearded* and *E(spl)* mRNAs (Lai et al., 2005; Stark et al., 2003), reducing their half life and mitigating their impact on Neuralized. In addition to regulating this aspect of endocytosis, miRNAs can also regulate Delta expression (Kwon et al., 2005).

As with the DSL ligands, several mechanisms control the steady-state levels of the Notch receptors at the cell surface and therefore regulate their availability for ligand binding. For example, several E3 ubiquitin ligases—Deltex, Nedd4, Su(Dx)/Itch, Cbl—can direct Notch receptor trafficking toward lysosomal degradation or toward recycling, thereby impacting receptor half life (reviewed in Bray, 2006; Le Borgne, 2006; Nichols et al., 2007b). Numb, in cooperation with the AP2 component α-adaptin and the AP2- or Numb-associated kinase (NAK), may promote Notch degradation in daughters of an asymmetrically dividing cell. In the majority of systems, Numb acts as a Notch antagonist, although in at least one context, Numb can act synergistically with Notch (Range et al., 2008). Numb is not active when lateral Notch signaling occurs between resting cells. Recent studies in vertebrates suggest that the restriction of Numb action to dividing cells reflects its need for the protein ACBD3, a partner that is trapped in the Golgi (Zhou et al., 2007). Strikingly, physical separation between Numb (a cytosolic protein) and ACBD3 (a Golgi protein) prevents Numb from affecting Notch in resting cells. During mitosis, however, Golgi fragmentation allows ACBD3/Numb complexes to form, thereby activating Numb to antagonize Notch activity via an unknown mechanism that is independent of Numb concentration (W. Zhong, personal communication) and thus may be catalytic. Notably, although mechanistic details of the Numb inhibitory mechanism remain to be discovered, this mechanism can be activated in all cells by the expression of a myristoylated form of ACBD3 that can constitutively associate with Numb. The Numb/ACBD3 complex inhibits lateral signaling even in cases where Notch activation results from contact between unrelated cells (Zhou et al., 2007). Thus, the coupling of ACBD3 retention in the Golgi with asymmetric segregation of Numb to one daughter cell is a unique way to take advantage of Golgi fragmentation during mitosis to ensure that Notch activity is regulated in a precise spatial pattern (Zhou et al., 2007). As with Neuralized and Bearded, Notch can feed back and regulate Numb levels (Chapman et al., 2006), sustaining the signal in cells that attained high levels of Notch activation.

Receptor Glycosylation

Notch receptors are large glycoproteins: many of their EGF repeats can be modified by two forms of O-glycosylation, O-fucose and O-glucose (reviewed in Haines and Irvine, 2003; Rampal et al., 2007; Stanley, 2007; Vodovar and Schweisguth, 2008). After translation, the Notch protein is fucosylated on EGF repeats containing the consensus sequence $C_2XXX(A/G/S)(S/T)C_3$ (C, cysteine; X, any amino acid; A, alanine; G, glycine; S, serine; T, threonine) by the GDP fucose protein O-fucosyltransferase (O-fut1 in *Drosophila*; Pofut1 in mammals). Although this modification was initially thought to be essential to produce a functional receptor, later studies in *Drosophila* demonstrated that the requirement was actually for Ofut's fucosylation-independent ER chaperone activity. Indeed, nonfucosylated Notch receptors are able to reach the cell surface, bind to ligands, and transduce signals (Okajima et al., 2008; Rampal et al., 2007; Stanley, 2007; Vodovar and Schweisguth, 2008).

Although it is still possible that O-fucosylation can facilitate proper Notch folding in the ER, fucosylation does appear to be essential for Notch signaling events that require regulation by Fringe glycosyltransferases. Fringe proteins extend O-fucose by adding more sugar moieties. This modification in the Notch ligand binding domain can determine which ligands can bind to activate the receptor. Fringe-mediated addition of a single N-acetylglucosamine on EGF repeat 12 in *Drosophila* Notch is sufficient to enhance receptor binding to Delta but reduce receptor binding to Serrate in vivo and in vitro (Xu et al., 2007). Although glycosyltransferase enzymes have been conserved between flies and mice, the consequences of Notch glycosylation and fucosylation in flies are not always mirrored in mammals (a role for Notch glycosylation has yet to be determined in *C. elegans*). Elimination of the fucosylation site on EGF repeat 12 of fly Notch led to a hyperactive response to Serrate (Jagged) even in the presence of Fringe, but did not affect the response to Delta (Lei et al., 2003). However, a substitution in an analogous position on EGF repeat 12 of mouse Notch1 (Threonine 466) generated a hypomorphic allele unable to support T cell differentiation (a Delta-dependent process) in homozygous animals. When in combination with a null allele, it is embryonic lethal (Ge and Stanley, 2008). Mammalian cells (Stahl et al., 2008) or animals (Ge and Stanley, 2008; Zhou et al., 2008) defective in fucosylation display a profound reduction in Notch signaling that extends beyond fringe-dependent processes. In contrast to *Drosophila*, surface Notch3 levels were not reduced in mouse *Pofut1*$^{-/-}$ embryonic stem cells relative to wild-type controls (Stahl et al., 2008). Binding of monoclonal antibodies suggested that proper folding of the receptors occurred. However, ligand binding was completely abolished, consistent with a subtle change in receptor folding (Stahl et al., 2008). Indeed, ligand binding in *Pofut1*-deficient cells can be rescued by overexpressing an inactive, unrelated protein (α-glycosydase I). Overexpression of α-glycosydase I seems to activate structurally unrelated chaperones that refold the Notch ECD and rescue ligand binding in *Pofut1*$^{-/-}$ cells (Stahl et al., 2008). This observation supports the general model that fucose is not required for ligand binding and suggests that it is a global upregulation in chaperone activity and not the dedicated chaperone activity of Ofut/Pofut1 that rescues Notch folding in mammals. Further support for ligand binding to "sugarless" Notch is provided by in vitro binding studies with unmodified receptor and ligand-interacting domains expressed and purified from bacteria (Cordle et al., 2008a; Cordle et al., 2008b).

In mammals, elucidation of the effects of Fringe on Notch activity is complicated by the presence of multiple receptors, ligands, and Fringe proteins (Lfng, Lunatic fringe; Mfng, Manic fringe; and Rfng, Radical fringe). Moreover, vertebrate glycosyltransferases appear to make a mechanistic contribution to Notch biology that is different from that in flies. Lfng modifies Notch in T cells in a manner that enhances Delta-to-Notch signaling and limits Jagged-to-Notch signaling (Visan et al., 2006). In contrast, Lfng inhibits Delta-to-Notch signaling in the somite (Dale et al., 2003). More puzzling still, fringe-modified Notch2

retains its ability to respond to Jagged1, whereas fringe-modified Notch1 does not (Hicks et al., 2000). The distribution of consensus fucosylation and glycosylation sites on mouse Notch1 and Notch2 reveals a glycosylation pattern that is largely conserved between the two paralogs (Figure 2A). However, distinct paralog-specific distribution of glycosylation sites is apparent within the ligand-binding domain, which may contribute to some of the observed receptor-specific responses to ligands (Hicks et al., 2000). Further modifications of Notch receptors by β1,4-galactosyltransferases (and possibly sialyltransferases) may also play a modulatory role in mammalian receptor function in certain contexts (Chen et al., 2001).

The glycosyltransferase RUMI, recently identified in *Drosophila*, is an enzyme that adds O-glucose to serine residues in the Notch consensus sequence C_1XSXPC_2 (Acar et al., 2008). Loss of Rumi leads to impaired Notch signaling in a variety of contexts, indicating that it is a general regulator of Notch signaling. Unlike Ofut1, Rumi's function resides mainly in its glucosyltransferase activity. Notably, Rumi's contributions to Notch signaling are temperature sensitive (even flies lacking the gene encoding Rumi are normal at lower temperatures). Consistent with this observation, Notch receptors still reach the cell surface and bind to ligands in Rumi-deficient cells. Although it cannot be ruled out that Rumi regulates subtle aspects of Notch receptor folding, stability, or proper maturation, it is possible that O-glucosylation also contributes to the receptor activation process. Notably, O-glucose can be further extended by additional modifications (possibly by xylosyltransferases), but the importance of such modifications to Notch biology remains to be demonstrated. Delta and Serrate/Jagged ligands also contain consensus glycosylation sites and can be substrates for both O-fucosylation and Fringe modification. As Ofut1 and Rumi both appear to function cell autonomously in signal-receiving cells, the biological function of glycosylation in the Notch pathway is largely centered on receptor modulation (Haines and Irvine, 2003; Stanley, 2007).

The generation of glycosylation-deficient Notch alleles in vertebrates, coupled with the development of improved methods to detect the glycosylation status of receptors in various in vivo contexts, will undoubtedly continue to make important contributions to the understanding of Notch signaling. However, the exact mechanistic contribution of sugars to Notch signaling remains a mystery. Glycans appear to play a minor part in the ligand/receptor recognition mechanism (Cordle et al., 2008a; Cordle et al., 2008b). They may also contribute to the mature conformation of the Notch extracellular domain, thereby modulating the receptor activation process. Although a full molecular explanation for the differential effects of glycosylation may have to wait for the crystal structure of the respective receptor/ligand complexes, the data we summarized above are consistent with the hypothesis that fucosylation and glycosylation at critical residues define the strength of receptor-ligand interactions, altering the probability of activation and consequently modulating signal strength. Mammalian and fly Notch proteins lacking serine in their 12th EGF repeat may behave differently due to variations in the distribution of fucose and glucose on their surface.

Receptor Activation

The key to Notch receptor activation is the regulation of ectodomain shedding. A crucial regulatory point in Notch signal transduction is ligand-induced and ADAM metalloprotease-mediated Notch receptor cleavage to release the ectodomain (Brou et al., 2000; Mumm et al., 2000). The cleavage site S2 for metalloproteases resides within the negative regulatory region of Notch, which encompasses the Lin12-Notch repeat (LNR) modules and the heterodimerization domain. The negative regulatory region functions to prevent Notch proteolysis in the absence of ligand. "Leaky" Notch signaling occurs when point mutations (Weng et al., 2004) or viral integration (Girard et al., 1996) disrupt the negative regulatory region, causing T cell acute lymphoblastic leukemia in humans and lymphomas in mice, respectively. Mutations in the linker between the LNR domains also result in activated Notch phenotypes in *C. elegans* (Greenwald and Seydoux, 1990), further underscoring the conserved nature of the mechanism that keeps Notch "off" in the absence of ligands.

How does the negative regulatory region protect the receptor from cleavage by ADAM metalloproteases, and how can the Notch ligand reverse this block? Early models attempting to explain the function of the negative regulatory region postulated that receptor oligomers were resistant to proteolysis and that ligand binding generated monomeric Notch molecules sensitive to protease cleavage (Kopan et al., 1996; Struhl and Adachi, 2000). However, biochemical measurement of the oligomeric state of wild-type and mutation-activated Notch proteins at the cell surface revealed that the oligomerization status of Notch did not correlate with its activity (Vooijs et al., 2004). Importantly, Notch dimerization was mediated by the EGF repeats and not by the negative regulatory region, leaving the negative regulatory region function unexplained (Vooijs et al., 2004). Although it is unlikely that oligomerization plays a major role in negative regulatory region function, changes in oligomerization status may still be important for optimal ligand-receptor binding, the stoichiometry of which remains to be determined. Ligand and receptor oligomerization plays an important role in several other signaling pathways (such as signaling pathways mediated by G protein-coupled receptors, receptor tyrosine kinases, or integrins), and it will be interesting to see whether this will also apply to Notch. Hints that oligomerization could be involved emerge from the ability of receptors and ligands to form homodimers via their EGF repeats (Vooijs et al., 2004), the clustering of cell surface Notch receptors at sites of contact with Delta-expressing cells (Luty et al., 2007; Nichols et al., 2007a), and the requirement for soluble (i.e., non-membrane associated) DSL ligands to be preclustered before they can activate Notch receptors on the cell surface (Hicks et al., 2002; Varnum-Finney et al., 2000). Receptor and ligand oligomerization may enhance receptor-ligand binding affinity and could explain the strong adhesion forces between Delta- and Notch-expressing cells as determined by atomic force microscopy (Ahimou et al., 2004).

An alternative mechanism for Notch activation was inspired by the observation that in *Drosophila*, the Notch ectodomain is *trans*-endocytosed by ligand-presenting/signal-sending cells, whereas the Notch intracellular domain is localized to signal-

receiving cells (Parks et al., 2000) (Figure 1). A genetic link between endocytosis and Notch signaling was strengthened by the characterization of the dynamin homolog in *Drosophila*, *shibire* (*shi*). Dynamin, a pleckstrin homology repeat containing GTPase, is necessary for pinching off clathrin coated pits from the plasma membrane for vesicle formation during endocytosis. *shi* mutants show strikingly similar phenotypes to Notch loss-of-function alleles during several developmental processes in *Drosophila*. Genetic analyses during peripheral nervous system development indicated that NEXT-like molecules lacking the negative regulatory region are properly processed at site 3 in *shi* mutants (Struhl and Adachi, 2000), demonstrating that endocytosis is only required for negative regulatory region-containing molecules. It was proposed that the mechanical strain generated by receptor *trans*-endocytosis somehow exposes site 2 in Notch for protease cleavage (Parks et al., 2000). Although this mechanotransduction model of Notch activation cannot explain how site 2 cleavage can be blocked by the negative regulatory region, it does propose a mechanism for how the inhibitory effects of the negative regulatory region on site 2 cleavage can be nullified.

Seeing Is Believing: Structural Insights

A recent high-resolution structure of the negative regulatory region has provided molecular details regarding its function (Gordon et al., 2007). The heterodimerization domain (consisting of HD-N and HD-C) forms a globular folded domain that makes extensive contacts with the three calcium-binding LNR modules (LNR-A, -B, and -C) (Figure 3A, lower panel). Site 1 is located within an unstructured loop that does not contribute to the stability of the heterodimerization domain (Gordon et al., 2008b; Gordon et al., 2007; Malecki et al., 2006). Conversely, site 2 is located in a β strand buried within an inaccessible pocket in the receptor (Figure 3A, lower panel). Direct steric occlusion (by the LNR-AB linker) and global domain stabilization (by interactions between LNR-B and the HD-C helix) both prevent premature cleavage of the receptor in the absence of ligands. Indeed, to produce a Notch receptor with constitutive signaling activity, LNR-A, the LNR-AB linker region, and LNR-B must all be removed.

The structure of the negative regulatory region clearly defines the "off" state of the receptor, confirms that autoinhibition is intrinsic to monomeric Notch molecules, and physically delineates the domain genetically defined to keep the receptor inactive. The structure also provides the molecular logic for the requirement of a large-scale conformational movement to expose the metalloprotease cleavage site (site 2), a prerequisite for Notch activation. However, precisely how this conformational change in the negative regulatory region occurs is still controversial. As discussed above, many studies suggest that a mechanical force is involved in Notch activation (the mechanotransduction model). Given the deep active site pocket in the metalloprotease ADAM17/TACE, it has been suggested that not only does the receptor activation mechanism forcibly lift at least two of the three LNR repeats, but that the process must also disengage the stabilizing helix within the heterodimerization domain from the site 2 containing strand, perhaps by partially unfolding the helix (Gordon et al., 2007). This would then allow the metalloprotease access to the scissile bond at site 2 (a model dubbed "lift and cut") (Gordon et al., 2007). Alternatively, because Notch molecules engaged by ligands have already been cleaved at site 1 by a protein convertase, the mechanical force generated by *trans*-endocytosis could simply be facilitating NECD/NTMIC heterodimer dissociation followed by exposure of site 2 for cleavage (Nichols et al., 2007a). Notably, *Drosophila* Notch proteins cannot be cleaved by protein convertases (Kidd and Lieber, 2002), and yet they are still activated by cell-bound ligands and inhibited by soluble ligands lacking the ability to "lift." Thus, nonenzymatic dissociation may not be a critical intermediate step required for the activation of all Notch receptors.

It is also possible that ligand binding leads to an allosteric change in the negative regulatory region structure from a protease-resistant to a protease-sensitive conformation (the allosteric model). Allostery does not require force and is more likely to occur in receptors with shorter extracellular domains, such as the *C. elegans* Notch receptors Lin-12 and Glp-1. Indeed, the allosteric model could provide a possible explanation for the ability of soluble DSL ligands to activate Notch in *C. elegans* but inhibit Notch in flies and vertebrates (but see discussion on DOS coligands below). In this light, it is interesting to note that unlike in flies and vertebrates, in *C. elegans* Notch glycosyltransferases are not known to play an important role in receptor activation. This is consistent with the notion that glycans can participate in mechanotransduction-mediated receptor activation, (perhaps by contributing to optimal adhesion between glycosylated receptors and their ligands), a mechanism that worms would not need if they activate their Notch receptor via an allosteric mechanism. According to this view, Rumi and Fringe, which do not affect binding (Acar et al., 2008; Hicks et al., 2000), could modify the separation forces involved in resolving ligand-receptor complexes and/or in the transmission of such forces to trigger unfolding of the negative regulatory region, a prerequisite for site 2 cleavage and subsequent receptor activation. It remains to be determined whether differential glycosylation regulates the adhesion strength between Notch and its ligands. Crystallization of ligand/receptor complexes and measurement of the force within individual units of receptor-ligand interactions in living cells will help address these questions. Additional studies are required to distinguish which activation mechanism is occurring in various in vivo contexts.

Further support for the importance of the negative regulatory region in regulating site 2 accessibility was recently provided by a consortium effort to develop functional agonistic and antagonistic antibodies against Notch3 (Li et al., 2008). Two high-affinity antagonists and one agonist were identified by the study and found to bind to adjacent epitopes within the Notch3 negative regulatory region (Li et al., 2008). The agonist increased site 2 cleavage and ectodomain shedding in a receptor-specific and metalloprotease-sensitive manner, whereas the antagonists blocked site 2 cleavage in response to ligands. Interestingly, the antagonists formed a "lock" by binding to an epitope comprising amino acids in both the LNR-A and HD-C regions of Notch3, likely increasing the energy required to expose site 2. In contrast, the agonist bound to an epitope in the LNR-A region, most likely interfering with the interaction

between the LNR-A region and the heterodimerization domain. That the binding of an antibody to LNR-A can result in increased site 2 cleavage and Notch activation suggests that the negative regulatory region structure is dynamic, alternating between a "closed" (as observed in the crystal structure) and a hypothetical "open" state. This dynamic structure could potentially even allow proteases access to site 2 without ligand binding at some low probability, which would provide a possible explanation for the recent report of ligand-independent cleavage of full-length (i.e., negative regulatory region-containing) Notch receptors by ADAM metalloproteases (Delwig and Rand, 2008).

Collectively, these results are consistent with the view that limiting accessibility to site 2 is the key function of the negative regulatory region. They also support the notion that mechanical force is likely involved in receptor activation and that activating or cancer-causing mutations in the negative regulatory region shift the equilibrium to an "open" state. Similarly, agonistic antibodies and high concentrations of ADAM metalloproteases may be trapping or exploiting the "open" conformation to activate Notch in a ligand-independent manner. Turning Notch signaling "off" pharmacologically via γ-secretase inhibition has become a common experimental tool. Now, a deeper understanding of the site 2 control switch makes it possible to transiently turn endogenous Notch signaling "on" whenever needed for therapeutic or tissue-engineering purposes. The agonistic antibody can thus be viewed as the first truly soluble Notch ligand that binds to the negative regulatory region instead of the EGF repeats.

Diffusible Ligands: May the Force be with You

Multiple lines of evidence support the idea that force-generating mechanical leverage is important in ligand-mediated activation of Notch and that soluble (and thus diffusible) ligands can act as dominant negative proteins to block Notch signaling (D'Souza et al., 2008). However, this view of Notch activation has been predominantly challenged by the fact that five of the ten *C. elegans* DSL ligands are soluble (Chen and Greenwald, 2004; Komatsu et al., 2008). If the Notch activation mechanism is conserved and requires in all species the unfolding and dissociation of the negative regulatory region, how can diffusible DSL ligands like DSL1 in *C. elegans* activate Notch? One possible explanation discussed above is that perhaps shorter Notch receptors, such as those in *C. elegans*, allow site 2 exposure to proteases by allostery instead of requiring force. However, the recent discovery and characterization of five *C. elegans* coligands suggest an alternative possibility that may move us closer to solving this mystery (Komatsu et al., 2008). The authors of this recent study noticed that all *C. elegans* DSL ligands lacked the DOS domain that is present in most DSL ligands from other phyla (Figure 2B). Remarkably, genetic evidence suggests that in *C. elegans*, DSL ligands lacking the DOS domain cooperate with the soluble DOS domain-containing protein OSM-11 (and possibly with other DOS proteins) to stimulate Notch activation in a subset of developmental contexts (Komatsu et al., 2008). These observations have led to a model proposing that secreted and membrane-bound DOS proteins work with membrane-bound and secreted DSL ligands, respectively, to gain sufficient mechanical leverage for receptor activation. This bipartite ligand binding remains to be confirmed biochemically. However, the importance of the DOS domain for receptor binding is supported by positive interactions between LIN-12 and OSM-11 in yeast two-hybrid assays (Komatsu et al., 2008) and by previous biochemical studies showing that the first two EGF repeats of Jagged1 containing the DOS domain were critical for high-affinity binding to cell surface Notch receptors (Shimizu et al., 1999). Interestingly, DOS domain-only proteins—Delta-like1 (DLK1) and DLK2/EGFL9—are also found in mammalian cells. Mammalian DLK1, when expressed in worms, can compensate for the loss of OSM-11 (Komatsu et al., 2008), raising the possibility that DLK1 and possibly DLK2 may have similar functions in mammalian cells. It is possible that the two proteins also enhance Notch activation by mammalian DSL ligands lacking DOS domains (Dll3, Dll4) in certain physiological contexts and compete with DOS domain-containing DSL ligands (Dll1, Jag1, and Jag2) in other contexts. It should be noted that Dll3 is unable to replace Dll1 in vivo (Geffers et al., 2007) and is unable to activate Notch in cultured cells (Ladi et al., 2005). However, these experiments in cultured cells may need to be repeated in the presence of DLK1 or DLK2 to decisively rule out Dll3 as a Notch activator. An additional possibility for the mode of action of the secreted DOS proteins and noncanonical ligands is that they may interact with extracellular matrix proteins to provide sufficient leverage to unfold/dissociate the negative regulatory region and activate Notch.

Further support for the importance of the DOS domain stems from the crystal structure of human Jagged1 (the first crystal structure of a mammalian Notch ligand fragment) (Cordle et al., 2008a) and from mapping known human and mouse mutations in *Jagged1* onto the crystal structure (Figure 3B). The crystallized Jagged1 fragment, which contained the DSL domain and the first three EGF repeats, has an extended rod-like structure. The DSL domain has a distinct organization that bears some structural similarities to an EGF repeat (Figure 3B). A positively charged cluster of highly conserved residues within the DSL domain constitutes a Notch-binding surface, the importance of which was confirmed by mutagenesis, in vitro binding assays, and in vivo functional assays (Figure 3B) (Cordle et al., 2008a). The DOS domain encompasses EGF repeats 1 and 2, which exhibit atypical secondary structures but still form the classical EGF disulfide bond pattern, therefore defining a distinct functional domain. This domain is structurally similar to a variant EGF-like repeat from the stalk region of integrin β2 (Beglova et al., 2002). Mutations linked in humans to the genetic disorder Alagille syndrome and the congenital heart disorder Tetralogy of Fallot and in mice to the autosomal dominant inner ear malformations *headturner*, (Kiernan et al., 2001), *slalom* (Tsai et al., 2001), and *Nodder* (Ingenium Corporation; personal communication) cluster near a conserved region in the DOS domain (Figure 3B). Together, these amino acids define another potential receptor-binding surface that is contiguous with the one identified in the DSL domain. Mutations in *headturner* mice and human patients of Tetralogy of Fallot affected amino acids buried under a protein surface defined by the location of *slalom* and *nodder* mutations and may impact the structure of the putative Notch binding site within the DOS domain (Figure 3B). Although many independent observations confirm that the 12th EGF repeat in Notch is critical for ligand binding, the interaction

domains within Jagged1 span an area greater than EGF repeat 12 alone (Figures 3A and 3B, all domains at the same scale). Elucidation of how the DOS and DSL domains simultaneously engage the Notch receptor requires the crystallization of the relevant ligand domains (DSL, DOS, and EGF) with the appropriate interacting domain from the Notch receptor. Moreover, as the sites for modification by the glycosyltransferase Rumi and the O-fucosyltransferase Pofut1 are present in repeat 12 (Figure 3A), it will be interesting to see how sugar moieties affect ligand binding.

Together, these recent studies further emphasize the importance of mechanical leverage in vertebrate Notch activation. Therefore, significant concerns arise regarding the interpretation and physiological relevance of observations based on Notch activation mediated solely by diffusible ligand fragments, by synthetic DSL peptides, or by bacterially produced DSL ligands. Additional studies using ligand molecules that harbor mutations in the Notch-binding DOS and DSL motifs, as defined by the studies summarized above, are needed to establish whether these nonphysiological ligands are truly exerting their apparent biological effects via the Notch pathway.

NEXT Up, Intramembrane Notch Proteolysis

Even though all membrane-tethered forms of Notch can interact with γ-secretase within the secretory pathway, only molecules with a free N terminus become substrates for intramembrane proteolysis by γ-secretase (Shah et al., 2005). The length of this extracellular N terminus determines how efficient γ-secretase cleavage is; longer regions are less efficiently cleaved. This explains why inhibition of metalloproteases, despite dissociation of the NECD-NTMIC heterodimer at S1 after ligand binding, still results in a marked reduction in Notch cleavage at site 3 by γ-secretase and thus target activation (Nichols et al., 2007a). This is likely also the explanation for why NEXT (Notch extracellular truncation) molecules, the substrates of γ-secretases, show decreased cleavage if they are elongated by the fusion of an extracellular dimerization domain (Mumm et al., 2000; Struhl and Adachi, 2000).

Once NEXT enters the active site of γ-secretase, its transmembrane domain is sequentially cleaved starting near the cytosolic plasma membrane leaflet (Qi-Takahara et al., 2005; Zhao et al., 2005). This initial cleavage at site 3 releases NICD, whereas the last cleavage at site 4 releases the Nß peptide (named after the Aß peptide, which is released from another γ-secretase substrate, amyloid precursor protein, which is associated with Alzheimer's disease; Okochi et al., 2002) (Figures 1 and 2A). Immunoprecipitation and Edman sequencing of mouse Notch1 C-terminal fragments identified a single NICD species starting at valine 1744 (V1744) (Schroeter et al., 1998). More recently, mass spectrometric analysis of cleavage products from an in vitro assay using NEXT-like substrates identified NICD variants with diverse N termini (NICD-V starting with V1744; NICD-L molecules starting with lysine 1745 or 1746; NICD-S starting with serine 1747) (Figure 2A) (Tagami et al., 2008). Quantification of the variants in a reconstituted system and in cells treated with proteasome inhibitors showed that the predominant scissile bond lies between lysine 1746 (L1746) and serine 1747 (S1747), and not between glycine 1745 (G1745) and V1744 as previously thought. Importantly, these NICD variants were also produced from full-length Notch activated in cocultures of receptor-producing cells and ligand-expressing cells, as well as in embryonic and adult mouse tissues. Thus, it is highly likely that these variants occur in vivo. As expected from the N-end rule in protein degradation, NICD-S and NICD-L are rapidly degraded by the 26S proteasome, making them extremely short-lived in cultured cells (Blat et al., 2002; Tagami et al., 2008; Varshavsky, 1996). Although we cannot rule out a biological role for these short-lived products, NICD-V likely mediates the bulk of Notch1 signals because of its stability. The genetic evidence supporting this conclusion comes from reanalysis of mice homozygous for the Notch V1744G allele (Huppert et al., 2000), which were originally thought to express receptors that are highly resistant to γ-secretase cleavage. Instead, this amino acid substitution from valine to glycine shifted the γ-secretase cleavage site to generate more of the labile NICD-L (Tagami et al., 2008). The subsequent reduction in NICD stability proves detrimental to Notch signaling in vivo (Huppert et al., 2000), providing evidence that the labile NICD molecules are insufficient to compensate for the loss of NICD-V.

Location, Location, Location

In addition to regulating receptor maturation and cell surface levels, endosomal sorting has an important role in preventing improper ligand-independent Notch receptor activation. Mutations in ESCRT complex proteins vps25 or erupted/Tsg101/vps23 lead to accumulation of Notch in late endosomal vesicles that permit ectopic activation of Notch via γ-secretase-dependent proteolysis (Vaccari et al., 2008). Because Notch ligand also accumulates in these vesicles, it is unclear whether the ectopic activation process is due to receptor stimulation by ligands in *cis* or an ectodomain shedding-independent activity of γ-secretase. Another protein, Lethal (2) Giant Discs (LGD) is also required to maintain Notch in an "off" state. When the levels of LGD protein are altered by either gene loss or overexpression, ligand-independent Notch activation is observed (Nichols et al., 2007b). It remains to be seen whether ADAM metalloproteases and γ-secretase are involved in this ligand-independent receptor activation. Notably, loss of hrs, an FYVE finger domain-containing endocytic protein, leads to Notch accumulation in an early endosomal compartment upstream of the ESCRT or LGD proteins but does not lead to ectopic activation. Therefore, the ESCRT complexes and LGD are likely to be normally involved in Notch downregulation, indicating that endosomal sorting acts to restrict receptor activation at or near the cell surface. Mistrafficking of Notch may place it in a compartment where proteolysis is less constrained, perhaps because the negative regulatory region shifts to an "open" conformation at a lower pH. Thus, defects in endosomal sorting of Notch may contribute to pathogenesis in different cellular contexts.

The identification of the subcellular location where cleavage of Notch at site 3 and site 4 occurs during the normal ligand-activation process has been controversial. Dynamin/shi, Rab5, and the endocytic syntaxin avl, which are all involved in early endosome formation, are required in signal-receiving cells. Although it has been demonstrated that

endocytosis is not required for NEXT cleavage in vivo (Struhl and Adachi, 2000), it was suggested that monoubiquitination and endocytosis of Notch are required to target the Notch/γ-secretase complex into an endocytic vesicle where efficient site 3 cleavage occurs. Indeed, the substitution of lysine 1749 with arginine (K1749R) in Notch simultaneously abolished monoubiquitination, endocytosis, and NICD accumulation (Gupta-Rossi et al., 2004). However, an alternative explanation for this result emerged from analysis of the K1749R transmembrane domain mutant for scissile bond selection by γ-secretase. Like V1744G, the K1749R substitution caused a shift in scissile bond preference, producing labile NICD species instead of NICD-V and thus leading to a loss of Notch activity. In addition to transmembrane domain composition, scissile bond selection was also strongly influenced by the subcellular localization of the γ-secretase/substrate complex during cleavage (Figure 1) (Tagami et al., 2008). At the plasma membrane, the bond between G1743 and V1744 is preferentially cleaved to generate the stable NICD-V species. However, in endosomes, γ-secretase preferentially cleaves the bond between L1746 and S1747, generating labile NICD-L and NICD-S species. This change in bond preference may be due to a lower pH in endosomes or due to differences in lipid environments, which may not only impact the specific activity of γ-secretase (Osenkowski et al., 2008) but also alter the positioning of the substrate transmembrane domain and thus cleavage site selection. The notion that stable NICD-V is generated at the plasma membrane or in the earliest vesicles to pinch off from the plasma membrane is consistent with the observation that non-cell-permeable γ-secretase inhibitors can still block Notch proteolysis (Tarassishin et al., 2004). Therefore, although γ-secretase is active in many cellular membranes and its proteolytic activity is independent of the composition of the Notch transmembrane domain, scissile bond selection and, consequently, the stability of NICD are highly dependent on both receptor cellular location and transmembrane domain composition (Tagami et al., 2008). It is clear that future efforts to correlate Notch activity, endosomal location, and proteolysis will have to take NICD species and half-life into consideration in order to properly assess the presence or absence of NICD/Notch activity. It is worth noting in this context that the apical polarity protein *Crumbs* was proposed to restrict the activity of γ-secretase and thus to limit the extent of Notch activation (Herranz et al., 2006). This too needs to be reevaluated as *Crumbs* may instead impact scissile bond selection by γ-secretase.

So what is the role of endocytosis in Notch activation? The important observation that NICD-V is produced before or during budding of the endocytic vesicle led us to revisit the hypothesis that translocation into a "cleavage endosome" is an important step in Notch activation. An alternative explanation for the phenotypes associated with the loss of dynamin/shi, Rab5, or syntaxin avl in signal-receiving cells (Vaccari et al., 2008) is that these protein deficiencies lower the forces generated by *trans*-endocytosis of ligand, thereby reducing ligand-induced negative regulatory region dissociation and subsequent receptor activation. If this interpretation is correct, the expression of receptors containing point mutations in the negative regulatory region that affect domain folding but remain ligand-dependent should suppress *Rab5*, *shi*, or *avl* mutations.

Interestingly, it was recently reported that the loss of the aquaporin-related channel *Big Brain* (*bib*), one of the earliest identified neurogenic genes in *Drosophila*, impaired endosomal maturation and, consequently, reduced Notch signaling during neurogenesis. Loss of *bib* suppressed ectopic Notch activation in ESCRT mutants (Kanwar and Fortini, 2008). Surprisingly, Bib exerts its effects on Notch signaling not by preventing Notch from entering into a "cleavage endosome" but by acting downstream of cleavage at site 3 (Kanwar and Fortini, 2008). Truncated, NEXT-like Notch molecules were cleaved in $bib^{-/-}$ cells to form a NICD-like fragment, but ectopic Notch activity was not detected, indicating that nuclear entry or target activation was impaired (Kanwar and Fortini, 2008). However, the $bib^{-/-}$ phenotypes do not indicate a general deficiency in nuclear entry of key cytosolic proteins (for example, the transcription activators SMAD and Armadillo). The effects of NICD overexpression also displayed no requirement for *bib*, establishing that loss of Bib does not compromise the NICD nuclear translocation machinery, CSL availability, or target gene activation. Instead, the generated NICD appears to remain associated with endosomes. Acidification of endosomes fails to occur in $bib^{-/-}$ cells. Indeed, Bib proteins that lack ion channel activity mimic *bib* loss of function (Kanwar and Fortini, 2008). How the release of NICD could be affected by the acidity of endocytic compartments in specific developmental contexts remains to be elucidated. It has been proposed that the defect might reflect impaired association of the endosome with cytoskeletal or cytoplasmic transport factors (Kanwar and Fortini, 2008). An alternative explanation for these observations is that in $bib^{-/-}$ cells, scissile bond selection by γ-secretase is altered such that cleavage occurred closer to the middle of the transmembrane domain to produce NICD molecules with longer, more lipophilic N termini that keep NICD anchored to the membrane. As the resolution of western blots is not sufficient to compare the composition of NICD produced by wild-type and $bib^{-/-}$ cells, testing this hypothesis will require mass spectrographic analysis of the NICD species produced in $bib^{-/-}$ endosomes.

γ-Secretase, Adding to the Complexity

The cleavage of Notch by γ-secretase was traditionally thought of as a constitutive proteolytic event, with the critical regulatory steps occurring either upstream (i.e., during ligand binding and ectodomain shedding) or downstream (i.e., NICD degradation, as discussed below) of intramembrane receptor cleavage. However, as γ-secretase activity and function have been further characterized, we have come to realize that intramembrane proteolysis can also be regulated by a variety of factors (Parks and Curtis, 2007). γ-Secretase is composed of four membrane proteins in a 1:1:1:1 stoichiometry (Sato et al., 2007). These four proteins—the catalytic component presenilin and three limiting cofactors, NCT (Nicastrin), Pen2, and Aph1—are necessary and sufficient to reconstitute enzymatic activity in cells lacking the activity (yeast, Sf9 cells). Because mammals have two presenilin isoforms and at least two APH isoforms (three in mice), mammalian cells can have at least four different γ-secretase enzyme complexes with differing biochemical properties and

protein interactions. Indeed, presenilin1-containing complexes exhibit specific activities distinct from presenilin2-containing complexes; γ-secretases containing different Aph1 isoforms also make different contributions in vivo (Tolia and De Strooper, 2008). Although the relevance of γ-secretase composition to Notch biology is still unexplored, some studies suggest that different Aph1 complexes might contribute differentially to Notch signaling (Tolia and De Strooper, 2008). Some of the remaining key questions are whether different γ-secretase complexes reside in different subcellular locations, whether they have different requirements for enzymatic activity (such as optimal pH and membrane lipid composition), and, most importantly, whether they can provide a biochemical basis for scissile site selection in Notch.

Notably, like many type I transmembrane proteins, Notch ligands are also subject to extracellular cleavage by ADAM proteases followed by transmembrane domain cleavage by γ-secretase (D'Souza et al., 2008). Ligand processing may be important to prevent any antagonistic effects of the active ligand on the ligand-producing cell, to limiting active ligand availability, or to promote efficient membrane clearance for ligand presentation to Notch-signal receiving cells. Although ligand processing could, in principle, generate biologically active fragments, no physiological evidence has yet emerged to support bidirectional signaling by the ligand intracellular domains that result from cleavage events.

Transcriptional Regulation

Once NICD is released by γ-secretase, it translocates into the nucleus. The processes and proteins that regulate nuclear translocation are still unclear. In the nucleus, NICD is unable to bind DNA on its own, but it acts to affect transcription with the help of its partner, a CSL protein. CSL directs NICD to specific target genes, the recognition of which appears to be independent of Notch. NICD/CSL could also affect nuclear events by competing with other proteins for the transcription coactivator Mastermind (MAM; MAML in mammals). The nuclear milieu that exists before the arrival of NICD will dictate which targets are available to CSL and thus can be activated by Notch (reviewed in Bray, 2006). Recent studies have begun to explore this regulation in greater detail.

CSL as a Repressor

Studies in *Drosophila* indicate that in the absence of NICD, the CSL protein Su(H) actively represses its target promoters. Loss of *Su(H)* in flies also lacking the γ-secretase component presenilin results in transient activation of Notch target genes (Koelzer and Klein, 2006). Su(H) mediates transcriptional repression by recruiting the transcriptional corepressor proteins SKIP, hairless/CtBP, and Gro/TLE (reviewed in Bray, 2006). In addition, Su(H) can silence transcription at multiple sites via recruitment of Asf1, a histone chaperone involved in nucleosome assembly (Goodfellow et al., 2007). Interestingly, modulating Asf1 levels does not impact gene targets of the Wnt, sonic hedgehog homolog (SHH), transforming growth factor (TGF), or epidermal growth factor (EGF) pathways (Goodfellow et al., 2007), implying that Asf1 has a specific role in repressing Notch targets. In cultured *Drosophila* S2-N cells, SKIP associates with Su(H) in the repressor complex. Importantly, whereas knockdown of *Su(H)* in S2-N cells derepressed only the two genes regulated by Notch, *M3* and *Mß* (Krejci and Bray, 2007), knockdown of *Asf1* derepressed additional genes within the *E(spl)* complex, a cluster of linked genes encoding bHLH proteins. Curiously, Asf1-repressed genes were not randomly distributed but rather were all located centromeric to *M3* (Goodfellow et al., 2007), thus dividing the *E(spl)* locus in two domains of transcription activation and repression. Asf1 must remain associated with these promoters to maintain a stable nucleosome complex for transcriptional repression in the absence of Su(H), which is required for the inititial recruitment of Asf1 to all these sites but is only required for Asf1 retention telomeric to *M3*. By inference, Su(H)/SKIP/hairless/Asf1 complexes must transiently bind to such sites, delivering Asf1 to an unknown partner, which helps in its retention in regions centromeric to *M3*. In the mammalian nucleus, although the CSL protein RBPjκ can form complexes with many ubiquitous corepressor proteins, such as CIR, FLH1C/KyoT2, and NCoR/SMRT (reviewed in Bray, 2006), it is SHARP/MINT/SPEN that has emerged as the critical repressor of Notch target genes in vivo (Oswald et al., 2005; Tsuji et al., 2007).

It is important to note that target repression by CSL proteins is not the rule. In contrast to the case in *Drosophila*, the loss of the CSL protein LAG-1 in *C. elegans* does not result in phenotypes characteristic of a gain-of-Notch function (but see Ghai and Gaudet, 2008). In addition, expression of *ref-1*, a gene target in many Notch-mediated decisions, is not elevated when CSL binding sites are mutated (Neves et al., 2007). Therefore, in nematodes, Notch targets are already poised for transcription and are not actively repressed by LAG-1. In mammalian skin, the phenotype of RBPjκ loss is not as severe as that seen when multiple Notch receptors or γ-secretase is lost, an observation that could be consistent with derepression of Notch targets in RBPjκ null animals (Demehri et al., 2008). Surprisingly, removal of RBPjκ in Notch or presenilin mutants did not alleviate their phenotype, suggesting that in the skin, target repression does not play an important role and raising the possibility that Notch signals in that tissue in a poorly defined, RBPjκ-independent manner (Demehri et al., 2008). Similarly, target derepression was not observed during the differentiation of T helper cells (Ong et al., 2008).

The lack of derepression is consistent with the recent findings that CSL occupancy of many Notch target promoters in *Drosophila* is transient. In *Drosophila* cells, silenced regions in the promoters of genes encoded within the *E(spl)* complex are rarely occupied by Su(H) (Krejci and Bray, 2007). However, in cultured human cells, CSL can be continuously detected at the Hes1 promoter in the absence of NICD (Fryer et al., 2004; Krejci and Bray, 2007). Notably, in both *Drosophila* and human cells, CSL occupancy on the promoter is always enhanced when NICD is present. Taken together, these studies demonstrate that CSL occupancy on the target promoters can be a dynamic process. Whether different Notch targets distinguished by differential CSL occupancy will have different activation kinetics is an interesting point for future investigation.

Transcriptional Activation and Target Selection

Whether there is active repression or not, the binding of NICD to CSL mediates the "transcriptional switch" to activate gene expression from the target promoter. The crystal structures of

Notch, CSL, and the Notch/CSL/MAM nuclear complexes from multiple organisms provide details of this transcriptional activation process at atomic resolution (reviewed in Gordon et al., 2008a; Kovall, 2008). These structures confirm that the activation complex forms in a stepwise manner and give insights with regard to the molecular changes likely to facilitate switching from repression to activation (reviewed in Barrick and Kopan, 2006; Gordon et al., 2008a; Kovall, 2008). The high-affinity binding of the RAM domain to CSL increases the local concentration of ANK domains, thereby permitting it to bind to RBPjκ and promote dissociation of transcriptional repressors (Del Bianco et al., 2008; Friedmann et al., 2008; Lubman et al., 2007). The ANK/CSL interface is then recognized by the transcriptional coactivator Mastermind/LAG-3 (Nam et al., 2006; Petcherski and Kimble, 2000; Wilson and Kovall, 2006). This ternary complex recruits histone acetyltansferases, chromatin remodeling factors, and the Mediator complex (Fryer et al., 2004) to assemble an active transcription complex on target promoters. Importantly, the 74 amino acid domain of mammalian Mastermind (MAML1), which binds to ANK/RBPjk, is a potent, global, and apparently specific inhibitor of Notch signaling (for example, see Maillard et al., 2008).

When Notch target promoters contain optimally spaced head-to-head sites [Su(H) paired sites], cooperative binding between two CSL/NICD/MAM complexes mediated by ANK/ANK domain interactions is observed in vitro (Nam et al., 2007). Though cooperativity explains why binding site orientation is important (Cave et al., 2005; Ong et al., 2006), it is unclear whether such cooperatively bound complexes are important in vivo and whether they also form on promoters in which spacing is suboptimal. Notably, the amino acids mediating these protein interactions are conserved on all four vertebrate Notch paralogs, allowing one to speculate that heterotypic interactions between different Notch paralogs may refine the regulation of transcription by Notch proteins (Nam et al., 2007). These specific amino acids are not conserved in *C. elegans* Notch proteins, but interestingly, Su(H) paired sites are also not conserved in the genomes of different nematode species (Neves and Priess, 2005). It is important to note that in vertebrates, multimerization of CSL binding sites (with head-to-tail orientation) is sufficient to elicit Notch-dependent activation in vivo in some but not all tissues responsive to Notch signaling (Mizutani et al., 2007; Souilhol et al., 2006).

It is tempting to describe Su(H) paired site-containing genes as high-affinity Notch targets. However, it is clear that even Hes1, the archetypical Su(H) paired site-containing Notch target, is not always responsive to Notch1 (Lee et al., 2007) and that many genes that contain Su(H) paired sites in their promoters do not respond to Notch signaling (Neves and Priess, 2005). Moreover, many characterized Notch-responsive enhancers are combinatorial. In *C. elegans*, Notch (LIN-12) cooperates with a GATA related protein to regulate *ref-1* expression in the endoderm, but cooperates with an NK-class factor to drive *ref-1* expression in the mesoderm (Neves et al., 2007). In *Drosophila*, Notch cooperates with the bHLH protein daughterless (Cave et al., 2005) or the transcription factor grainyhead (Furriols and Bray, 2001) to activate Su(H) paired site-containing promoters of the *E(spl)* complex genes. It is conceivable that tissue-specific target gene expression is controlled by the ability of different Notch paralogs to synergize or physically interact with diverse transcription factors bound on neighboring enhancers. Evidence for a qualitative difference among Notch paralogs was recently shown in vitro, where Notch3 seemed best equipped among the paralogs to cooperate with a nearby zinc finger protein (Ong et al., 2006).

Additional Partners in the Nucleus?

Several studies have shown that when overexpressed, NICD can interact with different transcriptional cofactors from multiple signaling pathways (for example, SMADs, NFκB, and HIF1α) to impact transcription from their target promoters (Kluppel and Wrana, 2005; Poellinger and Lendahl, 2008). These interactions likely reflect binding of adjacent enhancer-associated complexes by transcription activation complexes containing NICD. Given what is known about NICD, it is less likely that these observations reflect the distribution of NICD molecules among new transcription cofactor partners according to binding affinity and the local concentrations of such partners. As mentioned earlier, NICD interacts with CSL through a conserved WxP motif in its RAM domain with a small contribution from its ANK domain. Despite some sequence divergence, all four mammalian NICD RAM domains interact with the CSL protein RBPjκ with a similar affinity of ~200 nM (Del Bianco et al., 2008; Friedmann et al., 2008; Lubman et al., 2007). Although this affinity is not high enough to exclude the possibility that NICD associates with other proteins, it is important to note that free NICD is not detected at equilibrium in vitro when RBPjκ is in stoichiometric excess (Lubman et al., 2007). Therefore, under physiological conditions in the nucleus where a high concentration of RBPjκ is coupled with a low concentration of NICD, it is unlikely that there will be many free NICD molecules available to associate with other partners. However, it cannot be ruled out that when the concentration of RBPjκ proteins is limiting, some NICD molecules could associate with other factors such as SMAD, HIF1α, or NFκB. This remains to be demonstrated with physiological concentrations of NICD. Notably, RBPjκ can associate with at least one partner other than Notch—the bHLH protein p48/PTF1a (Beres et al., 2006; Hori et al., 2008; Masui et al., 2007). Thus, perhaps some of the effects of NICD overexpression could merely be the result of NICD titrating RBPjκ away from other binding partners.

This titration effect by NICD may explain some observations made in mammalian cells. As is the case with RBPjκ, MAM proteins can also associate with other transcription factors such as β-catenin, Mef2c, and p53 (reviewed in McElhinny et al., 2008). In mammalian cells, competition between NICD and the myogenic factor Mef2c for binding of MAML1 may offer a long-sought-after mechanism for the inhibition of myogenesis by truncated Notch ANK domains. Even though the NICD ANK domain alone cannot activate Notch targets, it could still associate with RBPjκ when overexpressed and thus titrate MAM away from Mef2C. In summary, it appears that both the coactivators and corepressors that act in the Notch signaling pathway are shared with other pathways, thus providing an alternative explanation for why overexpression of NICD can impact transcription of genes regulated by proteins outside of the Notch pathway.

All Good Things Must Pass: Signal Downregulation

Activation of Notch receptors releases a quantum of signal in the form of NICD. Given what we know about Notch biology, sustained NICD accumulation can be deleterious. Highlighting the importance of NICD turnover is the observation that deletion of the Notch receptor C-terminal or PEST domain, or mutations that stabilize NICD, can cause T cell acute lymphoblastic leukemia in humans (Weng et al., 2004). Thus, in addition to the above-mentioned mechanisms that primarily control NICD production, optimal signal strength is regulated in most cells by ensuring that NICD half-life is short. Most Notch-mediated processes require a transient pulse of activity that in some cases lasts only as long as a fraction of the cell cycle (Ambros, 1999). Even the few processes that require prolonged activation still seem to modulate activation strength. One such regulation point occurs during the transcriptional activation process, wherein NICD is phosphorylated within the PEST domain by the CDK8 kinase and targeted for proteasomal degradation by E3 ubiquitin ligases that include Sel10/Fbw7 (Fryer et al., 2004; O'Neil et al., 2007; Thompson et al., 2007; Tsunematsu et al., 2004). This process eliminates NICD, disassembles the transcription activation ternary complex, and resets the cell for the next round of signaling. It remains to be established whether CDK8 and Fbw7 are general mediators of NICD degradation. Other kinases and E3 ubiquitin ligases are likely to also participate in NICD regulation in context-dependent circumstances. Indeed, analysis of T cell acute lymphoblastic leukemia-associated Notch deletion alleles identified a conserved regulatory phosphorylation site (WSSSSP) in Notch proteins that is disrupted in patients of the disease (Chiang et al., 2006). The kinases, phosphatases, and ubiquitin ligases that target this site remain to be identified and characterized.

Conclusions and Perspectives

The efforts to understand the role developmental pathways play in adult tissue homeostasis and disease requires detailed knowledge of how the "on" and "off" states of such pathways are brought about and what mechanisms ensure their robustness. This knowledge should reveal vulnerabilities in the pathways that lead to disease and should provide insights into how to control or restore the balance to achieve a desired biological outcome. In the three decades following the cloning of Notch, a significant body of work has provided detailed mechanistic understanding of Notch activation and signal transduction. These efforts have provided new tools with which to inhibit or activate Notch signals. They have further allowed for atomic-level resolution of key structural elements involved in Notch receptor activation and transcription complex assembly. We now await structural analysis of ligand-receptor complexes and direct measurements of forces involved in Notch activation. These will provide information necessary to bridge the major gaps in our understanding of this unique ligand-mediated receptor activation mechanism. Better understanding of the growing array of combinatorial possibilities of DOS protein-DSL ligand interactions that could enable fine control over forces exerted by ligands on Notch (and, hence, activation probability) will also provide insights into Notch activation mechanisms. CSL-corepressor complexes also need to be examined at the atomic level to allow for better elucidation of the transcriptional switch. Together with ongoing efforts to identify and characterize cellular activities that enable other signaling pathways to control the output from Notch proteins, these areas of inquiry offer the promise of research tools to better our understanding of the pleiotropic effects of Notch signaling in development and disease as well as additional potential therapeutic avenues.

Recent studies have also led to a new appreciation of the underlying complexities of Notch proteolytic activation. Scissile bond selection, the impact of N-end rule degradation, and the dependence of both on the subcellular location of the cleavage activity will necessitate redesigning of experiments seeking to measure NICD production. Further developments in mass spectrometry may one day enable analysis of peptides isolated from small biological samples, thereby improving investigation into the function of the various endocytic trafficking modulators of Notch. Improvements in chromatin immunoprecipitation (ChIP) technology that will allow for the detection of NICD at physiological levels (currently not feasible) will undoubtedly uncover new nuclear partners and help to complete the story of target selection by different Notch paralogs in different cellular contexts (see Krejci et al., 2009).

Another major hurdle yet to be addressed relates to the issue of receptor redundancy. In which processes do Notch paralogs have specific or redundant functions and what are the underlying molecular mechanisms governing these differences? Do heterotypic NICD interactions occur at target promoters, and do they have a biological function? Developmental syndromes associated with Notch loss will benefit from receptor-specific agonists or activation of paralog-specific targets, if present. Receptor-specific antagonists (for example, Notch1 inhibition in T cell acute lymphoblastic leukemia) are predicted to work better than γ-secretase inhibitors if redundancy with other Notch paralogs will alleviate the toxicity associated with general Notch pathway inhibition. Related to this issue are mechanistic questions regarding how target selection and activation by different Notch paralogs is achieved, as well as what the functional thresholds attained by different concentrations of NICD are. Despite some progress in tools for monitoring Notch pathway activity (for example, see Ohtsuka et al., 2006; Souilhol et al., 2006; Vooijs et al., 2007), the field will benefit greatly from improvements in the methods used (for example, cleavage-specific antibodies, reporter strains, noninvasive imaging approaches) to identify cells engaged in Notch signaling, to quantify the levels of all four NICD proteins, to monitor target activation, and to record its biological consequences. Finally, despite genetic confirmation that noncanonical γ-secretase-dependent but RBPjk-independent Notch signaling occurs in flies and mammals, its mechanism remains as obscure as ever, presenting another interesting challenge to the field.

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