The molecular logic of Notch signaling – a structural and biochemical perspective

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Summary
The Notch signaling pathway constitutes an ancient and conserved mechanism for cell-cell communication in metazoan organisms, and has a central role both in development and in adult tissue homeostasis. Here, we summarize structural and biochemical advances that contribute new insights into three central facets of canonical Notch signal transduction: (1) ligand recognition, (2) autoinhibition and the switch from protease resistance to protease sensitivity, and (3) the mechanism of nuclear-complex assembly and the induction of target-gene transcription. These advances set the stage for future mechanistic studies investigating ligand-dependent activation of Notch receptors, and serve as a foundation for the development of mechanism-based inhibitors of signaling in the treatment of cancer and other diseases.

Key words: Biological signaling, Protein biochemistry, Receptor, Regulated intramembrane proteolysis, Transcription

Introduction
Notch receptors are modular, single-pass transmembrane proteins that receive signals from transmembrane ligands that are expressed on neighboring cells. The signals that are transduced by these receptors have a central role in cell-fate decisions, both during embryonic development and in adult tissue homeostasis. The essential role of Notch signaling during development is evident from the embryonic lethality that is associated with deficiencies in Notch signaling in various model organisms, including worms, flies and mice. Notch signals are used iteratively at different decision points to guide functional outcomes that depend heavily on gene dose and signaling context. Significantly, both deficiencies in and abnormal increases of Notch signaling are also associated with human developmental anomalies and cancer, again emphasizing the importance of precisely regulating the intensity and duration of Notch signals (for reviews, see Aster et al., 2008; Bray, 2006).

The core protein components of the Notch signaling circuit are present in metazoan organisms ranging from sea urchins to humans (Fig. 1A). Canonical Notch signaling begins when a ligand of the Delta/Serrate/LAG-2 (DSL) family binds to Notch at the cell surface (Fehon et al., 1990). Ligand engagement initiates a process called regulated intramembrane proteolysis (RIP), in which the Notch receptor is first cleaved at a juxtamembrane extracellular site (S2) by a metalloprotease of the ADAM (a disintegrin and metalloprotease) family (Brou et al., 2000; Mumm et al., 2000). This ligand-dependent cleavage step renders the metalloprotease-sensitive N-terminus of the receptor to the intramembrane proteolysis substrate, and the receptor is further processed by γ-secretase at a cryptic site within the juxtamembrane region of the Notch intracellular domain (NICD) to allow it to translocate into the nucleus, where it assembles into a transcriptional activation complex (Jarriault et al., 1995). The core components of this complex include the DNA-binding transcription factor CSL {C-promoter-binding factor (CBF-1 in mammals; also known as RBP-J)/Suppressor of hairless [Su(H) in Drosophila melanogaster]/LAG-1 (in Caenorhabditis elegans)} (Fortini and Artavanis-Tsakonas, 1994; Jarriault et al., 1995; Tamura et al., 1995), the ICN and a co-activator protein of the Mastermind (MAM)/LAG-3 family (Petcherski and Kimble, 2000a; Petcherski and Kimble, 2000b; Wu et al., 2000). Activation of transcription at CSL-binding sites also appears to depend on the recruitment of additional co-activators, such as p300 (E1A binding protein p300; EP300) or CREB-binding protein (CBP) (Fryer et al., 2002; Wallberg et al., 2002); these coactivators might constitute the main link between the core Notch-containing complex and the general transcription machinery.

A number of different proteins are known to modulate Notch signal transduction. The induction of Notch cleavage by ligands relies on E3 ligases – such as Neuralized (Deblandre et al., 2001; Lai et al., 2001; Pavlopolous et al., 2001; Yeh et al., 2001) and Mindbomb (Itoh et al., 2003) – which facilitate epsin-dependent endocytosis of ligands in the ligand-expressing cells (Lai et al., 2005; Le Borgne et al., 2005a; Le Borgne et al., 2005b; Overstreet et al., 2004; Wang and Struhl, 2004; Wang and Struhl, 2005). Other modulators of signaling exert their effects by regulating ligand responsiveness [e.g. Fringe and Rumi glycosyltransferases (Acar et al., 2008; Bruckner et al., 2000; Cohen et al., 1997; Johnston et al., 1997; Moloney et al., 2000; Panin et al., 1997)], by controlling ligand and ICN turnover [e.g. SEL-10 and related F-box proteins (Gupta-Rossi et al., 2001; Hubbard et al., 1997; Mao et al., 2004; Oberg et al., 2001; Tsunematsu et al., 2004; Wu et al., 2001)], and by other less-well-characterized mechanisms [e.g. Deltex-1 (Busseau et al., 1994; Diederich et al., 1994; Matsuno et al., 1995; Matsuno et al., 1998; Mukherjee et al., 2005)]. The importance of the C-terminal PEST domain in regulating ICN turnover has also taken on far greater significance in light of newly identified mutations in human T-cell acute leukemias that delete the PEST region of the ICN of Notch1 (ICN1) (Weng et al., 2004).
Bray, 2006), here we will apply a structural and biochemical perspective to summarize progress towards understanding three central facets of canonical Notch signal transduction: (1) ligand recognition, (2) autoinhibition and the switch from protease resistance to protease sensitivity, and (3) the mechanism of nuclear-complex assembly and the induction of target-gene transcription. A summary of structures that contain components of the Notch signaling pathway and their complexes is presented in Table 1.

**Ligand recognition**

**Domain organization**

The N-terminal part of the Notch ectodomain consists of a series of epidermal growth factor (EGF)-like repeats that are responsible for ligand binding (Fig. 1B). Each EGF-like repeat is about 40 residues long and contains six cysteine residues that form three disulfide bonds with a characteristic pairing. The number of EGF-like repeats varies among receptors from different species. The two Notch receptors in the worm, LIN-12 and GLP-1, have 14 and 11 N-terminal EGF-like repeats, respectively, whereas the Notch receptor in the fly and the four mammalian Notch receptors are much larger, having 29-36 EGF-like repeats (Fleming, 1998).

The extracellular domains of Notch ligands of the DSL family also have a modular architecture (Fig. 1C). The N-terminal region of the ligands, with the exception of the worm proteins (which are the most divergent among the group), contains a conserved ~100 residue MNNL module (at the N-terminus of Notch ligands) domain (Fig. 1C). All ligands then contain a distinct cysteine-rich module called a DSL domain near the N-terminus, followed by a series of iterated EGF-like repeats that precede the transmembrane segment. Serrate and Jagged ligands also contain a cysteine-rich domain that bears some sequence similarity to von Willebrand factor C domains between the EGF-like repeats and the transmembrane domain, whereas the Delta class of ligands does not (Fig. 1C).

**Notch-ligand interactions**

Early studies that investigated the binding of the fly Notch receptor to its ligands used cell-aggregation assays to detect an interaction between cells that expressed the receptor Notch and those that expressed the ligand Delta (Fehon et al., 1990). This approach was then used to show that the minimal region of Notch that is necessary and sufficient for association with Delta comprises a pair of EGF-like repeats (11 and 12) (Rebay et al., 1991). In a different assay,

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**Table 1. Structures of Notch-pathway-related proteins and their complexes**

| Description | PDB ID code | Species | Method | Reference |
|-------------|-------------|---------|--------|-----------|
| Extracellular Notch | | | |
| LNR-A domain (NOTCH1) | 1pb5 | Human | NMR | (Vardar et al., 2003) |
| Ligand-binding, EGF-like repeats 11-13 (NOTCH1) | 1toz | Human | NMR | (Hambleton et al., 2004) |
| LNR-HD (NOTCH2) | 2oo4 | Human | X-ray | (Cordle et al., 2008a) |
| Ligand-binding, EGF-like repeats 11-13 (NOTCH1) | 2vj3 | Human | X-ray | (Cordle et al., 2008a) |
| Notch ligands | | | | |
| JAGGED1, DSL domain and EGF-like repeats 1-3 | 2vj2 | Human | X-ray | (Cordle et al., 2008a) |
| Intracellular Notch | | | | |
| ANK (Notch) | 1ot8 | Fly | X-ray | (Zweifel et al., 2003) |
| CSL(LAG-1)-DNA | 1ttu | Worm | X-ray | (Kovall and Hendrickson, 2004) |
| Ankyrin repeats 3-7 (Notch1) | 1ymp | Mouse | X-ray | (Lubman et al., 2005) |
| ANK (NOTCH1) | 1uyh | Human | X-ray | (Ehebauer et al., 2005) |
| ANK(NOTCH1)-MAML1-CSL(RBP-J)-DNA | 2v8y | Human | X-ray | (Nam et al., 2006) |
| RAMANK(LIN-12)-MAM(SEL-8)-CSL(LAG-1)-DNA | 2v8y | Human | X-ray | (Nam et al., 2006) |
| CSL(Rbp-j)-DNA | 2y6l | Worm | X-ray | (Wilson and Kovall, 2006) |
| Hydroxylated ANK (Notch1) | 2c9q | Mouse | X-ray | (Cordle et al., 2008a) |
| RAM(LIN-12)-CSL(LAG-1)-DNA | 3bzd | Worm | X-ray | (Cordle et al., 2008a) |
| RAM(LIN-12)-CSL(LAG-1)-DNA | 3bzd | Worm | X-ray | (Cordle et al., 2008a) |
| CSL(RBP-J)-DNA | 3bzd | Worm | X-ray | (Cordle et al., 2008a) |
| Extracellular Notch-associated molecules | | | | |
| TACE | 1bkc | Human | X-ray | (Maskos et al., 1998) |
| Manic fringe | 2g0a | Mouse | X-ray | (Jinek et al., 2006) |
| Manic fringe + UDP/Mn | 2jtb | Mouse | X-ray | (Jinek et al., 2006) |
| γ-Secretase | – | Human | EM | (Ogura et al., 2006) |
| γ-Secretase | – | Human | EM | (Lazarov et al., 2006) |
| Intracellular Notch-associated molecules | Suppressor of Deltex [Su(dx)] WW domains 3-4 | 1tk7 | Fly | NMR | (Fedoroff et al., 2004) |
| Deltex ring-H2 finger | 1v87 | Mouse | NMR | (Miyamoto et al., 2004) |
| Deltex WW domain | 2a90 | Fly | X-ray | (Zweifel et al., 2005) |
| Su(dx) WW domain 4/phosphorylated Notch peptide | 2jmk | Fly | NMR | (Jennings et al., 2007) |
| S-phase kinase-associated protein 1 (SKP1)-F-box and WD repeat domain-containing 7 (FBW7) | 2vpp | Human | X-ray | (Hao et al., 2007) |
| SKP1/FB7/cyclin-E C-terminal degron | 2vov | Human | X-ray | (Hao et al., 2007) |
| SKP1/FB7/cyclin-E N-terminal degron | 2vov | Human | X-ray | (Hao et al., 2007) |
| Factor Inhibiting HIF-1 (FIH)/Notch1 peptide | N/A | Mouse | X-ray | (Coleman et al., 2007) |
| FIH/Notch1 peptide | N/A | Mouse | X-ray | (Coleman et al., 2007) |

PDB, protein data bank. Note that, in this table, ANK refers to the ankyrin domain.
Irvine and colleagues constructed a series of fusions between ectodomain fragments of the fly Notch receptor and alkaline phosphatase to assess the binding of these molecules to S2 cells that expressed either the Delta or Serrate ligands (Xu et al., 2005). These studies confirmed the essential importance of EGF-like repeats 11 and 12 for binding to both ligands, and pointed out that additional repeats between 6-36 also contribute to the strength of binding in this cell-based assay. These studies also investigated the influence of modification of the Notch receptor by the N-GlcNAc transferase Fringe in modulating binding, showing that Fringe modification confers competence for the binding of Delta, but interferes with binding to Serrate. As far as the ligands are concerned, the conserved N-terminal MNNL and DSL domains both appear to participate in optimal binding to Notch (Parks et al., 2006; Shimizu et al., 1999).

We are aware of three published reports of binding assays that were performed in vitro with isolated receptor and ligand ectodomain fragments to quantify binding affinities. The Hirai group used an ELISA assay to detect the binding of soluble mouse Jagged1-Fc fusions to His-tagged mouse Notch2 ectodomain fragments that were immobilized to a surface (Shimizu et al., 1999). They determined a binding affinity of approximately 0.7 nM for the in vitro interaction between Jagged1 and an N-terminal fragment of Notch2 spanning EGF-like repeats 1-15. The Baker group also used an ELISA assay to assess interactions between secreted ectodomains of fly Notch EGF-like repeats 11-20 and Delta,
estimating a dissociation constant ($K_d$) of 1.87 nM for this interaction from their solid-phase binding assays. Interestingly, they also detected an association between a Notch polypeptide containing EGF-like repeats 11-20 and a separate Notch fragment comprising EGF-like repeats 21-30 (Pei and Baker, 2008). This latter region overlaps with the part of Notch that contains the Abruptex class of alleles, a genetically defined, complex group of dominant Notch mutations that appear to result in enhanced Notch signaling. Finally, binding interactions between minimally interacting regions of receptor and ligand were examined by surface plasmon resonance, and a $K_d$ of 130 M was reported for the binding of bacterially expressed, biotinylated NOTCH1 EGF-like repeats 11-14 to a Delta-like 1 (DLL1) fragment consisting of the N-terminal domain, the DSL domain and the first three EGF-like repeats. In this study, site-directed mutagenesis of a key Ca$^{2+}$-coordinating residue in EGF-like repeat 12 was then used to show that the structural integrity of the Ca$^{2+}$-binding site in this repeat is required for specific binding (Cordle et al., 2008b).

Despite the accruing evidence that multiple regions of both Notch receptors and ligands are involved in the binding interaction that leads to a signal, there is limited structural information available to guide a mechanistic understanding of ligand recognition. Handford and colleagues have reported nuclear magnetic resonance (NMR) and X-ray structures of a polypeptide comprising EGF-like repeats 11-13 from human NOTCH1 (Cordle et al., 2008a; Hambleton et al., 2004), and an X-ray structure of a region of JAGGED1 consisting of the DSL domain and EGF-like repeats 1-3 (Cordle et al., 2008a). In the structure of the NOTCH1 polypeptide, each EGF-like repeat adopts a characteristic EGF fold. This fold consists of a core two-stranded, antiparallel $\beta$-sheet, which is oriented parallel to the axis between its N- and C-termini, and three disulfide bonds. In the X-ray structure, the coordination of Ca$^{2+}$ fixes the orientation between adjacent repeats, creating a gently curving, rod-like structure (Cordle et al., 2008a). By contrast, in the NMR structure, the position of EGF-like repeat 13 is less well defined with respect to repeat 12, suggesting that the interdomain relationship between EGF-like repeats 12 and 13 is more dynamic, and that the position of repeat 13 is not rigidly fixed with respect to repeat 12 (Fig. 2A). On the basis of the structure of these three Ca$^{2+}$-binding EGF modules, the authors proposed that the ectodomain of Notch is in an extended conformation, with consecutive Ca$^{2+}$-binding EGF-like repeats that form rigid rod-like structures, which are perhaps interrupted by non-Ca$^{2+}$-binding repeats that impart some flexibility (Fig. 2B). To account for the results from their studies, Irvine and colleagues proposed an alternative model, in which the Notch molecule adopts a compact global conformation for optimal interaction with DSL ligands (Xu et al., 2005) (Fig. 2C). Their model postulates that the Notch ectodomain contains several potential hinge points, allowing the domain to fold onto itself to form a triple-stranded structure upon binding to ligand to afford extra avidity. The hinge points would presumably be derived from interdomain flexibility in the linkers that connect non-Ca$^{2+}$-binding EGF-like repeats. The ability of EGF-like repeats 11-20 of the Drosophila Notch receptor to bind in trans to repeats 21-30 in solid-phase binding assays is consistent with this idea. The compact-conformation model (Fig. 2C) also rationalizes, at least in part, why there are so many EGF-like repeats in extracellular Notch, and creates a model for how variation in the glycosylation state of the receptor might modulate the affinity for different ligands by changing the flexibility of the hinges.

The X-ray structure of the JAGGED1 polypeptide (residues 187-335, corresponding to the DSL domain and the first three EGF-like repeats) represents the first glimpse of a Notch ligand at high resolution (Cordle et al., 2008a). This ligand-derived polypeptide adopts an extended structure that is reminiscent of the elongated conformation seen in EGF-like repeats 11-13 of NOTCH1 itself (Fig. 2D). The structure of the DSL domain resembles an EGF-like fold with an N-terminal extension, but the disulfide-bonding pattern is different, because one of the canonical disulfides from the EGF-like core is missing and a new disulfide bond is located within the N-terminal appendage. Accompanying pull-down experiments and functional studies that were conducted in flies support the idea that a key contribution to Notch binding is derived from conserved residues that are exposed along one face of the DSL domain...
(Fig. 2D), pinpointing for the first time a potential receptor-binding interface on a Notch ligand.

Unanswered questions in ligand recognition
Despite recent progress, the unresolved issue of ligand recognition means that a persistent gap remains in the current understanding of Notch signaling, and many questions are still unanswered. First, how do the receptors actually recognize ligands, and what is the stoichiometry of ligand-receptor complexes once they are formed? How much do the binding affinities vary for different ligand-receptor pairs, and how much do regions that flank the key interacting domains contribute to binding affinity and specificity? Lastly, how do post-translational glycosylation events modulate ligand recognition and signaling? The low affinity (130 M) of EGF-like repeats 11-14 for DLL1 fragments (Cordle et al., 2008b) suggests that additional EGF-like repeats might be required on the Notch-receptor side, the ligand side, or on both sides to increase avidity, as the work of Irvine and colleagues suggests. In addition, clustering of ligand or Notch receptor might be necessary to mediate adhesive interactions in a recognition synapse between communicating cells. This idea is supported by atomic-force microscopy studies that measured strong adhesion forces between cells that expressed Notch and those that expressed ligand (Ahimou et al., 2004). Once ligand-receptor complexes are formed, the next step in receptor activation relies on proteolytic release of the intracellular portion of Notch from the membrane. Thus, we next focus on how the protease resistance of Notch is maintained in the absence of ligand and how it might be released upon ligand engagement.

Activation switch
The negative regulatory region (NRR) of the Notch receptor, which is sandwiched between the ligand-binding and transmembrane domains (Fig. 1B), harbors the structural machinery that is necessary to maintain metalloprotease resistance in the absence of ligand. The NRR contains three cysteine-rich LIN-12–Notch repeats (LNRs) and a ‘heterodimerization domain’ (HD), which immediately precedes the membrane and contains both the furin (S1) and metalloprotease (S2) cleavage sites. Several lines of evidence gathered over many years led to the conclusion that the NRR is the ‘activation switch’ of the receptor. Receptors that lack the EGF-like repeats are functionally inert (Kopan et al., 1996; Lieber et al., 1993; Rebay et al., 1993; Struhl and Adachi, 1998). By contrast, deletion of the LNR modules or point mutations of key residues away from the HD domain might include localized movement or melting of the HD domain might include localized movement or melting of the HD domain, or might even cause complete unfolding of the HD domain with accompanying subunit dissociation (Nichols et al., 2007).

In either an allosteric or mechanotransduction model for Notch activation, it is unlikely that the activating metalloproteases can gain access to the Notch S2 site after mere stripping of the LNR modules away from the HD domain, because the active sites of metalloproteases, such as tumor-necrosis factor α (TNFα)-converting enzyme (TACE, also known as ADAM17) lie in a deep cleft (Fig. 3D). Thus, we would expect that certain key secondary structural elements of the HD domain also unravel after displacement of the LNR repeats, generating an ‘open’ conformation that renders the S2 site accessible to the protease. Such conformational changes in the HD domain might include localized movement or melting of helix3, which is anchored above the cleavage site (Fig. 3B), to release the strand that contains the S2 site, or might even cause complete unfolding of the HD domain with accompanying subunit dissociation (Nichols et al., 2007).

The discovery that mutations are frequently found in the HD domain of NOTCH1 in human T-ALL patients moved NOTCH1 to the forefront in understanding disease pathogenesis and also pointed to the NOTCH1 NRR as a mechanism-based therapeutic target. These mutations, which map primarily to the highly conserved hydrophobic interior of the HD, lead to ligand-independent increases in signaling, suggesting that domain destabilization facilitates ligand-independent S2 cleavage and subsequent receptor activation (Malecki et al., 2006;
Weng et al., 2004). Recently, inhibitory and activating antibodies against the NRR from human NOTCH3 were reported (Li et al., 2008) – the epitope of the inhibitory antibody includes residues from both the LNR-A and HD domains, consistent with the notion that it clamps the NRR in its metalloprotease-resistant conformation. Importantly, the existence of modulatory antibodies constitutes proof of principle, showing that it is possible to identify mechanism-based therapeutics that turn Notch signaling on or off via the NRR.

Additional studies to define the structural characteristics of the ‘on’ state of the NRR in normal and disease-associated signaling should also help to guide the development of mechanism-based modulators of signaling.

Nuclear translocation and transcriptional regulation

The ICN, which is released from the membrane upon γ-secretase cleavage, is a potent inducer of target-gene transcription. In canonical Notch signaling, transcriptional activation depends on the formation of a core protein-DNA complex that includes the ICN, the CSL transcription factor and a protein of the MAM family [MAM-like (MAML) in mammals].

The ICN comprises several functional regions, including an N-terminal RAM (recombination binding protein-J-associated molecule) domain (Tamura et al., 1995), an ankyrin (ANK) domain and less-conserved regions, including a variable transactivation domain (Kurooka et al., 1998) and a C-terminal PEST sequence (Fig. 1A). The ANK domain, which comprises seven ANK repeats, is the most conserved region of the ICN and is essential for Notch-receptor function. Each ANK repeat is about 33 residues long and typically folds into a pair of antiparallel helices followed by a β-hairpin that connects to the next repeat; together, the repeats stack to form a curved, elongated domain (Mosavi et al., 2004). In X-ray structures of isolated ANK domains from the fly Notch receptor and human NOTCH1, ANK repeats 2-7 are ordered, whereas the first repeat remains disordered (Ehebauer et al., 2005; Nam et al., 2006; Zweifel et al., 2003).

The structure of worm CSL (LAG-1), bound to a 13-mer DNA duplex corresponding to a CSL-binding site in the proximal promoter of the hes1 gene (a well-characterized human target of activated Notch), revealed an unpredicted domain organization for CSL (Kovall and Hendrickson, 2004). The ANK domain, which comprises seven ANK repeats, is the most conserved region of the ICN and is essential for Notch-receptor function. Each ANK repeat is about 33 residues long and typically folds into a pair of antiparallel helices followed by a β-hairpin that connects to the next repeat; together, the repeats stack to form a curved, elongated domain (Mosavi et al., 2004). In X-ray structures of isolated ANK domains from the fly Notch receptor and human NOTCH1, ANK repeats 2-7 are ordered, whereas the first repeat remains disordered (Ehebauer et al., 2005; Nam et al., 2006; Zweifel et al., 2003).

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Structural insights into Notch signaling

atop the RHR-N. The DNA is bound by the BTD and the RHR-N and makes no contacts with the distal RHR-C domain. A large surface is buried at the protein-DNA interface, with extensive non-specific contacts between the highly conserved and basic CSL face and the phosphate backbone, and base-specific contacts with six of the eight base pairs of the core CSL-binding consensus.

Genetic, molecular and biochemical studies of the interaction between CSL and ICN established that binding of ICN to CSL is bipartite, through a higher-affinity stable interaction with the RAM region (Kovall and Hendrickson, 2004; Lubman et al., 2007; Tamura et al., 1995) and a much weaker, but detectable, interaction with the ANK domain (Aster et al., 1997; Tamura et al., 1995). By contrast, binding of the third key component for transcriptional regulation, MAML1, to ICN-CSL complexes on DNA occurs independently of the RAM domain, whereas inclusion of the ANK domain of ICN is necessary and sufficient for recruitment of MAML1 to the CSL-ICN complexes (Nam et al., 2003). The assembly of this ternary complex only requires a short N-terminal region of MAML1, which acts as a potent dominant-negative inhibitor of Notch signaling in functional assays because it lacks the part of the protein that normally recruits the transcriptional machinery (Weng et al., 2003). Neither CSL nor ICN1 alone binds detectably to MAML1, but together the two proteins cooperate to bind MAML1 with high affinity. The cooperative assembly of the MAML1-ANK-CSL-DNA complex suggests that a primary function of the ANK domain of ICN1 is to render CSL competent for MAM loading, for which there are two possible recruitment mechanisms: allosteric exposure of a cryptic binding site on one partner of the ICN-CSL complex or binding to a composite surface that is created upon complexation.

Insights from structures of Notch transcription complexes

X-ray crystal structures of human and worm Notch ternary complexes (NTCs), which include portions of intracellular Notch, CSL and MAM bound to DNA, provide the structural basis for cooperativity in the recruitment of MAM to NTCs (Nam et al., 2006; Wilson and Kovall, 2006). In both the human and worm complexes, the concave face of the Notch ANK domain packs against the two
RHR domains of CSL, with most of the contact interface derived from RHR-C. The MAM polypeptide, which is disordered in solution in the case of human MAML1 (Nam et al., 2006), adopts a helical conformation in both complexes, and makes extensive contacts with a composite surface that is derived from the interface between the ANK and RHR-C domains. A kink in the MAM helix also allows it to wrap further around CSL to contact the RHR-N domain in both cases (Fig. 4A).

In several studies of isolated Notch ANK domains, the first ANK repeat does not adopt a typical ANK fold, and is at least partially disordered (Ehebauer et al., 2005; Nam et al., 2006; Zweifel et al., 2003). However, this first repeat is ordered in both the human (Nam et al., 2006) and worm (Wilson and Kovall, 2006) NTC structures. The worm NTC structure, which includes both RAM and ANK regions, also has electron density from two short parts of the N- and C-termini of RAM, but the remainder of RAM is not visible in the structure, an observation that is consistent with disorder in the intervening region. A 20-residue sequence at the N-terminus of RAM, which includes a conserved WxP motif that has been implicated in the binding of RAM to the BTD of CSL in previous studies, binds to a groove in the BTD of CSL at a site that is distant from the CSL interface with the ANK domain and MAM. The C-terminal part of the RAM region adopts an ANK-like fold, which caps the N-terminus of the ANK domain and might help to stabilize the first ANK repeat in the complex. Similar to MAM and the first ANK repeat of Notch, the N-terminal cap also appears to become ordered only upon complex formation.

When the CSL-DNA and ternary complexes from worm are directly compared, it is evident that CSL adopts a more closed conformation in the complex with RAMANK (constructs of Notch that encompass both the RAM and ANK domains) and MAM than it does alone (Kovall and Hendrickson, 2004; Wilson and Kovall, 2006). Areas of the RHR-N and BTD that are near the DNA interface superimpose well, but the RHR-C and parts of the BTD of CSL in the ternary complex are closer to each other, filling more of the cavity in the U-shaped CSL molecule. By contrast, the CSL molecule in the human NTC complex, which only includes the ANK domain of Notch, remains in a more open conformation that more closely resembles that of the worm CSL-DNA complex.

Role of the RAM region in transcription-complex assembly

It has been speculated that this difference between the human and worm ternary complexes was due to the binding of the N-terminal RAM peptide in the worm structure, which was not present in the human structure (Barrick and Kopan, 2006). Alternatively, the conformational differences between the complexes could also be accounted for by species-specific differences in the RAM and ANK regions of the various proteins, or by intrinsic flexibility of the CSL protein (Fig. 4). Three recently reported structures, including two worm CSL-RAM-DNA complexes in different crystallographic space groups and a mouse CSL-DNA complex, further clarify the influence of RAM on the conformation of CSL (Friedmann et al., 2008). A significant finding from these studies is that the RAM region does not induce global closure of CSL upon binding, indicating that the primary structural differences between the two ternary complexes are a consequence of interspecies variation or conformational flexibility.

One consistent conformational difference noted among different classes of CSL-containing structures is in the position of a loop (connecting strands C and C') of the RHR-N domain of CSL; this loop is located directly below the C-terminal portion of MAM (Friedmann et al., 2008). In all structures that contain either the ANK or RAM domain (or both domains, as in the case of the worm ternary complex), this loop is in an ‘open’ conformation, with the loop flipped away from MAM, whereas the loop is in a ‘closed’ conformation in the two CSL-DNA structures that were solved in isolation from worm and mouse (Fig. 4B). Because a side chain from the loop would clash with one of the MAM side chains when the loop is in the closed position, the authors propose that the binding of RAM to the BTD of CSL might trigger opening of the loop in the RHR-N domain, but this possibility has not yet been tested directly. Examination of the structures suggests that a simple side-chain rotation around the β-carbon atom of the loop residue might prevent the clash, but perhaps a movement of the entire loop is the most energetically favored way to accommodate RAM.

Recent biochemical studies, which complement these crystal structures, suggest that the primary role of the RAM region is to recruit intracellular Notch to CSL. Biophysical studies of the complete RAM region in isolation and in the context of RAM-ANK polypeptides have shown that RAM is natively unstructured (Nam et al., 2003). In addition, the 70 residues linking the part of RAM bound to the β-trefoil domain of CSL and the N-terminal cap of the ANK domain remains disordered in the worm transcription complex (Wilson and Kovall, 2006). Modeling of the unstructured region between the bound RAM peptide and the N-terminal end of the ANK domain as a worm-like chain reveals that the most probable distance between the two CSL-binding domains is about 50 Å (Bertagna et al., 2008), which is in very good agreement with the distance between the binding sites in the ternary-complex structures. The affinity of RAM peptides for various CSL proteins from different species, which has been determined in several different studies (Del Bianco et al., 2008; Friedmann et al., 2008; Lubman et al., 2007), ranges from 0.022 to 2 M. By contrast, the affinity of the ANK domain for CSL is low (Nam et al., 2003), and has been estimated to be greater than 20 M (Del Bianco et al., 2008). Above the concentrations that are needed for saturated binding of RAM peptide to CSL, the worm-like-chain approximation predicts an effective concentration for the ANK domain of around 0.5 mM (Bertagna et al., 2008), which is consistent with fluorescence-transfer experiments suggesting that recruitment via RAM leads to docking of the ANK domain onto its cognate site on CSL (Del Bianco et al., 2008). Together, the structural and biochemical studies best support a model for complex assembly in which the high-affinity RAM peptide interacts first with its binding site on CSL, increasing the effective local concentration of the ANK domain and allowing this domain to nestle onto the RHR part of CSL to create the composite interface for MAM recruitment.

Despite the structural and biochemical information that is now accumulating about the RAM and ANK domains of intracellular Notch, less is known about the overall structure of the entire ICN molecule that is released from the membrane upon γ-secretase cleavage. Recent single-particle electron-microscopy analysis of ICN from the fly indicates that intact ICN can adopt either an extended or a compact conformation (Kelly et al., 2007). At low ionic strength, the compact form is favored, whereas increasing Ca2+ concentrations induce a shift from a compact ICN form that cannot bind CSL to an extended form that binds CSL, and ultimately to a fibrous aggregated form that can no longer bind CSL (Kelly et al., 2007). Together, the biochemistry and electron-microscopy data suggest that modulation of the conformation of ICN regulates its activity. However, given that the Ca2+ concentrations used in these studies are unlikely to be
physiological, the triggers that might be responsible for such conformational rearrangements remain unknown.

**Formation of higher-order complexes on specialized promoter elements**

A remaining challenge in understanding how ICN acts as an accessory transcription factor is to elucidate the transcriptional codes that constitute Notch response elements in different biological signaling contexts. One small step in that direction emerged from the consideration of the packing arrangement of symmetry-related complexes in the structure of the human NTC on an 18-mer CSL consensus binding site from the *hes1* promoter region (Nam et al., 2007; Nam et al., 2006). In a number of well-characterized Notch-responsive genes in flies and mammals, including *hes1* (Hairy and enhancer of split 1), the proximal promoter contains dual Su(H)-paired sites or ‘sequence-paired’ binding sites (SPSs). These elements consist of two CSL-binding sites that are oriented head to head and that are typically separated by 16 or 17 nucleotides (Bailey and Posakony, 1995; Nellesen et al., 1999). Previous studies of genes containing SPS elements have shown that the integrity of the SPS is needed for proper Notch-dependent gene transcription in cell-based assays and transgenic flies (Cave et al., 2005; Ong et al., 2006).

In the structure of the human NTC, crystal contacts between two copies of the ANK domain that are related by a two-fold symmetry axis orient the two 18-mer *hes1* DNA duplexes in a near-linear orientation that mimics an inverted-repeat pair of sites. In the crystal lattice, the two CSL-binding sites are about 65 Å apart, corresponding approximately to the distance spanned by 19 base pairs of B-form DNA (Fig. 5). Biochemical studies revealed that residues that are engaged in these crystal contacts help to guide cooperative dimerization of NTCs on the SPS from the *hes1*-promoter region and that disruption of the putative dimer interface within the ANK domain prevents the induction of a luciferase reporter gene under the control of the same promoter region (Nam et al., 2007). The existence of clustered CSL sites in many other Notch-responsive genes also raises the possibility that the assembly of higher-order Notch complexes might represent a more general mechanism for regulating target-gene transcription. Taken together with the structural and biochemical studies of complexes that are assembled on single sites, the potential for dimerization of NTCs leads to a new working model for the assembly of various Notch nuclear complexes (Fig. 6).

**Conclusion and perspectives**

A number of significant structural and biochemical advances over the past several years have enhanced the current understanding of the molecular logic of Notch signaling, but the picture is by no means complete. There are representative structures of a ligand-binding fragment of human NOTCH1 and of a minimal Notch-binding fragment of human JAGGED1, yet the three-dimensional organizations of the complete Notch and ligand ectodomains remain unknown, as does the structural basis for ligand recognition. Similarly, the X-ray structure of the activation switch of human NOTCH2 has revealed the molecular basis for autoinhibition, but a clear picture of how ligand stimulation relieves this autoinhibition has yet to emerge.

With regards to Notch transcription complexes, structural and biochemical studies of the core assemblies from human and worm provide substantial insight into how ICN and CSL combine to recruit MAM proteins. However, eukaryotic gene transcription usually requires the concerted binding of multiple DNA-binding transcription factors, and Notch-responsive genes are no exception. Understanding the structural basis for cooperativity between the various transcription factors is a challenge that remains to be fully addressed.
Notch transcription complex and other DNA-binding transcription factors is likely to be one future research frontier. Another might be how the core transcription complexes engage other key transcriptional regulators, such as histone acetyltransferases and chromatin-remodeling complexes, to activate gene transcription. Deciphering the additional complexity that results from post-translational modifications, such as phosphorylation and hydroxylation, represents yet another future challenge. These problems, along with other unresolved issues in the biochemistry and signaling of the Notch pathway, should keep researchers busy for several years to come.

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