Notch/LIN-12 signaling: transduction by regulated protein slicing

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Intercellular signaling through the Notch/LIN-12 transmembrane receptors regulates growth and differentiation during animal development. Moreover, defects in the conserved Notch/LIN-12 pathway are linked to human diseases. Here, we review models for two key steps in Notch/LIN-12 signaling: ligand-mediated activation of the receptor and receptor-mediated activation of transcription. Ligand binding appears to permit proteolysis of the receptor; as a result, the receptor's intracellular domain can enter the nucleus and function as a transcriptional co-activator.

Notch/LIN-12 SIGNALING regulates an enormous array of cell interactions during animal development, thereby controlling growth, differentiation and patterning in many tissues[1]. In nematodes, for example, the specification of blastomere fates during early embryogenesis, proliferation of germline tissue and vulval development, all rely on this pathway. In *Drosophila melanogaster* and vertebrates, the pathway controls the cell-fate decision between epidermis and neuron, as well as a multitude of other fate decisions in nearly all tissues. Consistent with its key role in development, dysfunction of the Notch/LIN-12 pathway has been implicated in numerous human diseases, including leukemia, cervical and colon carcinomas, Alzheimer's, stroke (CADASIL) and Alagille syndrome[2]. The central role played by the Notch/LIN-12 pathway in development and disease makes a detailed understanding of its signaling mechanism a high priority. In this review, we focus on current models for that mechanism. We apologize in advance to those not cited and refer readers to other reviews for more-comprehensive referencing[1–4].

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Core components of the Notch/LIN-12 pathway

The heart of the Notch/LIN-12 pathway consists of the DSL ligand (for Delta, Serrate, LAG-2), the Notch/LIN-12 receptor and the CSL downstream transcription factor (for CBF1, Su(H), LAG-1). Table I shows this basic pathway and provides the names of individual family members. These components and their essential molecular functions have been conserved throughout the animal kingdom, although the ligands and receptors appear to be larger and more complex in *Drosophila* and vertebrates than in *Caenorhabditis elegans*. The existence of multiple ligands and receptors in an individual organism (Table I) can be explained, at least in part, by the need to impose precise spatial and temporal regulation upon their expression.

In this review, we focus our discussion on the three-component, stripped-down version of the Notch/LIN-12 signaling pathway. Although these three components might not be essential for all Notch/LIN-12 signaling, they appear to be critical for the vast majority of signaling by these receptors. In addition to these core components, other factors and target genes clearly play key roles. Of particular note are *Drosophila kuzbanian* and its *C. elegans* homolog *sup-17* (which encode ADAM proteases that affect signaling)[5,6], *C. elegans* SEL-12 and its human homologs, presenilins PS1 or PS2 (which can regulate LIN-12/GLP-1 signaling)[7], and *fringe* (which encodes a secreted regulator that differentially affects Delta and Serrate ligands)[8]. A detailed consideration of these and other regulators and target genes is beyond the scope of this review.

How is Notch/LIN-12 signaling achieved? Genetic analyses have defined a pathway of control: a DSL ligand activates the Notch/LIN-12 receptor and the receptor, in turn, activates the CSL transcription factor (Table I). In addition, feedback loops within the pathway reinforce and amplify signaling[1,3]. A biochemical understanding of Notch/LIN-12 signaling, however, is only just emerging and many details remain poorly understood.

Signaling by DSL ligands

DSL ligands are transmembrane proteins that possess a conserved architecture and contain the following elements: a signal sequence; a non-conserved N-terminal region of approximately 100 amino acid residues; a family-specific DSL domain; a variable number of epidermal-growth-factor-like (EGFL) repeats; a single membrane-spanning domain (TM); and a non-conserved intracellular (IC) domain (Fig. 1). A systematic structure–function analysis has been accomplished for the smallest member of the family, the *C. elegans* LAG-2 protein[9–11]. These studies demonstrate that the first 166 amino acids of

Table I. The Notch/LIN-12 signaling pathway

	DSL ligand	Receptor activation →	Notch/LIN-12 receptor	Transcription-factor activation →	CSL effector
Caenorhabditis elegans	LAG-2, APX-1		LIN-12, GLP-1		LAG-1
Drosophila melanogaster	Delta, Serrate		Notch		Su(H)
Vertebrates	Delta 1 and 2; Jagged 1 and 2		Notch 1–4		CBF1[a]

Negative regulators, positive regulators, downstream target genes and regulatory loops have been removed from the pathway for simplicity. [a]CBF1 is also known as RBP-Jκ and KBF2.

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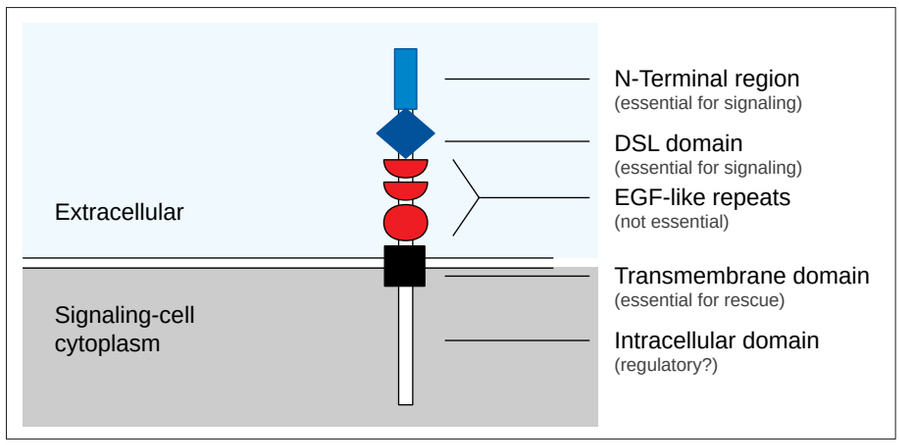

Figure 1

The LAG-2 DSL ligand and its functional domains. The following parts of the protein are thought to be extracellular: a non-conserved N-terminal region (bright blue bar); the DSL, family-specific domain that is a variant of epidermal growth factor (EGF)-like repeats (dark-blue diamond); and EGF-like repeats (red circles). LAG-2 also possesses two potential half-EGFL repeats (red semicircles). The transmembrane domain (TM; black square) is predicted to span the membrane once. The IC, non-conserved intracellular region (open bar) is predicted to be in the cytoplasm of the signaling-cell. All DSL ligands have all these regions in the same order, but others have more EGF-like repeats; in addition to the domains shown, Jagged and Serrate receptors of *Drosophila melanogaster* and vertebrates possess a conserved cysteine-rich region located between the EGFL and TM. The proposed functions of each region are shown in brackets.

LAG-2, which include the signal sequence, and the N-terminal and DSL regions, are sufficient for unregulated signaling but not for mutant rescue. This small fragment can induce excess germline proliferation in an otherwise-wild-type background, but it cannot rescue a null mutant. To achieve mutant rescue, membrane association is required[10]. This suggests that the N-terminal and DSL regions must be localized or concentrated to achieve precise signaling. If LAG-2 carries a deletion of either the N-terminal region or the DSL domain, it cannot signal, suggesting that both portions are critical for signaling[10].

Unlike the N-terminal and DSL regions of the ligand, neither the EGFL repeats nor the IC region is essential[10]. However, mutations in the EGFL repeats can influence ligand activity[12–14] and, because these repeats are present in all DSL proteins, it seems unlikely that they have no function. Perhaps they play an ancillary role, such as stabilization of the ligand–receptor complex. Alternatively, they might play a negative regulatory role, such as competing with the receptor for binding to the DSL domain, in which case their removal would not be expected to reduce signaling. The role of the IC region is similarly enigmatic: in *C. elegans*, its removal increases LAG-2 activity[10], while in *Drosophila* and vertebrates, a similar deletion has a dominant negative effect[15,16]. These two contrasting results suggest that the IC region of the ligand plays a regulatory role that has diverged during evolution.

The specificity of DSL–receptor interactions has been examined by constructing chimeric transgenes that place the coding region for one ligand under the control of regulatory sequences of another. LAG-2 and APX-1 were found to be interchangeable in *C. elegans*[11,17]; similarly, either Delta or Serrate could support the decision between neural and epidermal fates in the *Drosophila* embryo[18]. However, Delta and Serrate are not always equivalent, and their differences appear to map to their N-terminal and DSL domains[19].

Ligand activation of the receptor

How does a DSL ligand bind its receptor and activate signaling? The mature receptor is composed of two polypeptides that are covalently linked. One of the subunits is entirely extracellular (EC); the other, which we call TM–IC (for transmembrane–intracellular region), includes some extracellular amino acid residues, the transmembrane domain and the intracellular region (Fig. 2)[20–22]. Generation of the EC and TM–IC subunits occurs by proteolysis of the full-length receptor within the trans-Golgi network of the cell[22]. The identity of the protease responsible for this cleavage is controversial: one group suggests that the ADAM protease called *kuzbanian* is required[5], while another group demonstrates a furin-like cleavage at this site[23].

The EC fragment of the receptor is composed of EGF-like repeats and the three family-specific LNG repeats (Fig. 2). Truncated receptors lacking this EC portion are constitutively active[4], suggesting that the EC fragment inhibits receptor signaling and that binding of receptor by ligand frees it from that inhibition. Both the EGF-like and LNG repeats are essential for ligand-mediated regulation of the receptor[4,24,25]. Missense mutations in either group of motifs can eliminate signaling, and a small in-frame deletion of the LNG repeats leads to a null phenotype, although mutant proteins remain present (Refs 24, 25; S. Crittenden and J. Kimble, unpublished). These EGFL and LNG mutations might inactivate the receptor by reducing ligand binding or by disrupting some other step in signal transduction (e.g. release from EC inhibition). In *Drosophila* Notch, the *Abruptex* missense mutations reside in a small cluster of EGFL repeats; these mutations increase receptor activity, suggesting that the group of EGFL repeats identified by *Abruptex* mutations normally plays a negative regulatory role in signaling[26–28]. While vertebrate Notch/LIN-12 receptors also have Abruptex-like repeats, no Abruptex-like cluster is apparent in the smaller nematode receptors. The Abruptex EGFL repeats probably play a regulatory role and are not central to signaling by all Notch/LIN-12 receptors.

The ligand-binding site within the EC fragment has been explored in tissue-culture cells by assaying the aggregation of Delta- and Notch-expressing cells[29]. These studies suggest that two Ca^{2+}-binding repeats (EGFL11, EGFL12) in *Drosophila* Notch are critical for a Ca^{2+}-dependent association with Delta. Missense mutations in EGFL7 of *C. elegans* GLP-1 (Ref. 24) and EGFL12 of *Drosophil*a Notch[25] can abolish receptor activity; however, the nematode EGFL7 repeat does not bear a Ca^{2+}-binding repeat signature, and the significance of these mutations for ligand binding has not been tested. Therefore, binding studies will be essential to show definitively which EGFL repeats are critical for mediating the ligand–receptor interaction.

The LNG repeats, as well as the region between those repeats and the transmembrane domain, are not only required for receptor function but also have been implicated in receptor regulation. Thus, certain missense mutations in either the LNG repeats themselves or in the LNG –TM interval can activate the receptor and render it ligand-independent[30–32].

A reasonable interpretation is that these mutations affect amino acid residues that are critical for the proposed EC inhibition and, therefore, release the receptor from that inhibition.

Figure 3 presents two models for activation of the receptor by the ligand. In the first model, binding of the ligand to the receptor's EGFL repeats induces receptor dimerization, thereby activating the receptor (Fig. 3a). In support of this idea, the ligand appears to bind the EGFL repeats (see above), and receptor dimerization has been suggested by genetic studies[28,30]. In the second model, the ligand interacts with the LNG repeats and disrupts an inhibitory interaction between the EC and TM–IC fragments of the receptor (Fig. 3b). Dimerization of either ligand or receptor can be included in this second model but, for simplicity, is not illustrated. This second model is suggested by the influence of the LNG repeats and the LNG–TM interval on ligand-regulated repression of the receptor (see above). Clearly, both models are speculative, and neither is probably correct in detail. Nonetheless, the mechanism probably involves the EGFL and the LNG repeats, because both are critical for receptor activity. Perhaps the actual mechanism combines features of the two models in Fig. 3. For example, the ligand could dock on the EGFL repeats, which, in turn, might lead to an interaction with, or conformational change within, the LNG repeats that results in release from EC inhibition. One approach to understanding this step in the signaling mechanism is to compare, at a biochemical level, the wild-type receptor, which is repressed unless bound by ligand, with mutant ligand-independent receptors, which are de-repressed. Such a comparison could shed light on how the transition from the inactive to the active state of the receptor occurs.

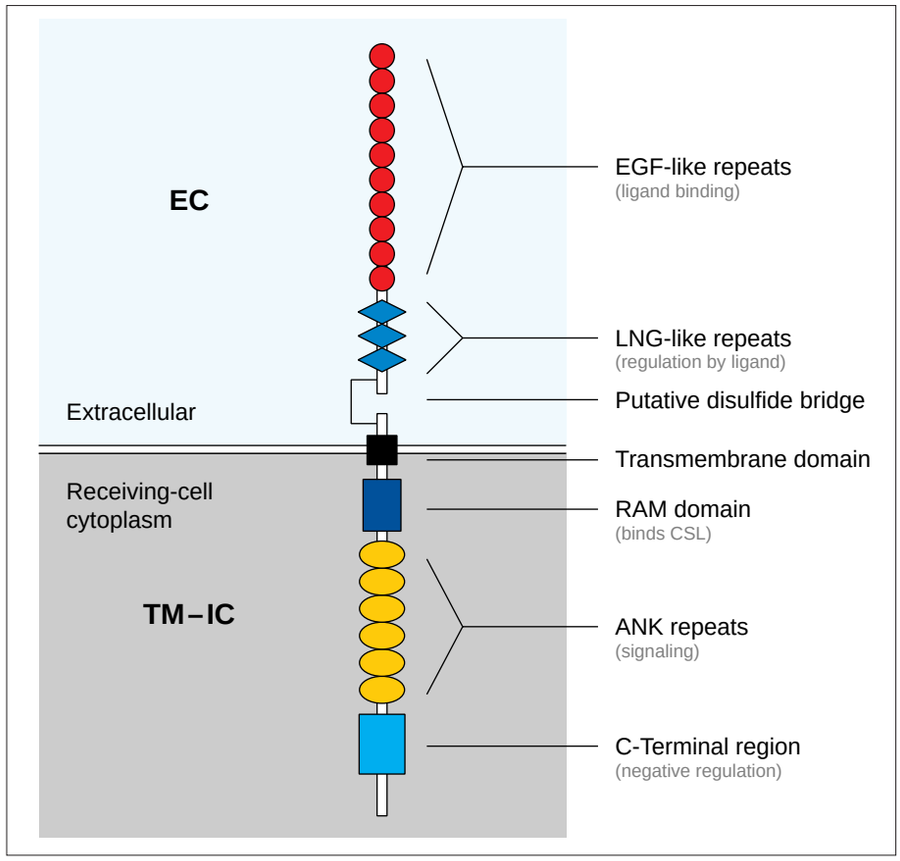

Figure 2

The GLP-1 receptor and its functional domains. Mature receptors are composed of two polypeptides: EC, which bears most of the extracellular domain; and transmembrane–intracellular region (TM–IC), which bears a short stretch of extracellular amino acids, the transmembrane domain and the intracellular region. These two subunits are probably linked by a disulfide bond[22]. Individual motifs include: epidermal growth factor (EGF)-like repeats (red circles); LNG, receptor-specific, cysteine-rich repeats (bright blue diamonds); a transmembrane domain (black square); the RAM domain (also known as RAM23; dark-blue rectangle); and ANK, ankyrin or *cdc10/SWI6* repeats (yellow ovals). All Notch/LIN-12 receptors have all these regions in the same order. However, LIN-12 and GLP-1 have fewer EGFL repeats than other receptors; one cysteine-rich region present in GLP-1 and LIN-12 but not in other Notch/LIN-12 receptors (the T+Y domain) is omitted for simplicity. The proposed function of each region is shown in brackets.

Receptor activation of transcription

How does signaling activate the CSL transcription factor? Unlike many receptors, the Notch/LIN-12 receptor appears to possess no enzymatic activity of its own. Nonetheless, functions can be ascribed to three distinct regions within the intracellular (IC) region of the receptor (Fig. 2). Of most importance is the ANK-repeat region. Missense mutations in the ANK repeats eliminate the activity of both normal and constitutively activated receptors[24,30,33,34]. Moreover, overexpression of a small fragment containing the ANK repeats (plus approximately 30 amino acid residues at either end) activates the pathway in a fashion that is dependent on the ANK repeats (Refs 33, 35; V. Kodoyianni and J. Kimble, unpublished). Furthermore, the ANK-repeat region can bind to the CSL transcription factor, although nonspecifically[36], and can also dramatically enhance transcription by the CSL transcription factor in tissue-culture cells[34,37]. Together, these data support the idea that the ANK-repeat region can act in the nucleus as a transcriptional co-activator. The RAM domain, which lies just inside the transmembrane domain, binds strongly to the CSL transcription factor *in vitro* and *in vivo*[36–38]. However, overexpression of the RAM domain neither activates the pathway nor interferes with endogenous signaling[36]. Finally, the C-terminal region negatively regulates receptor activity by poorly understood mechanisms that appear to be species specific[39,40].

Two models to explain CSL activation by the receptor have been suggested. The first proposes regulated transport of the CSL transcription factor from the cytoplasm to the nucleus (Fig. 4a); the second proposes regulated proteolytic cleavage of the receptor, followed by entry of the receptor IC into the nucleus (Fig. 4b). In this second model, the receptor IC cleavage product is predicted to act as a transcriptional co-activator when bound to the CSL protein. Evidence for each model has been obtained using overexpressed and, in some cases, mutant proteins[34,41–43]. However, both models predict a change in the subcellular distribution of either the receptor IC domain or the CSL transcription factor, but no such change has been observed *in vivo* by careful examination of the endogenous proteins (Refs 21, 44, 45; V. Kodoyianni and J. Kimble, unpublished).

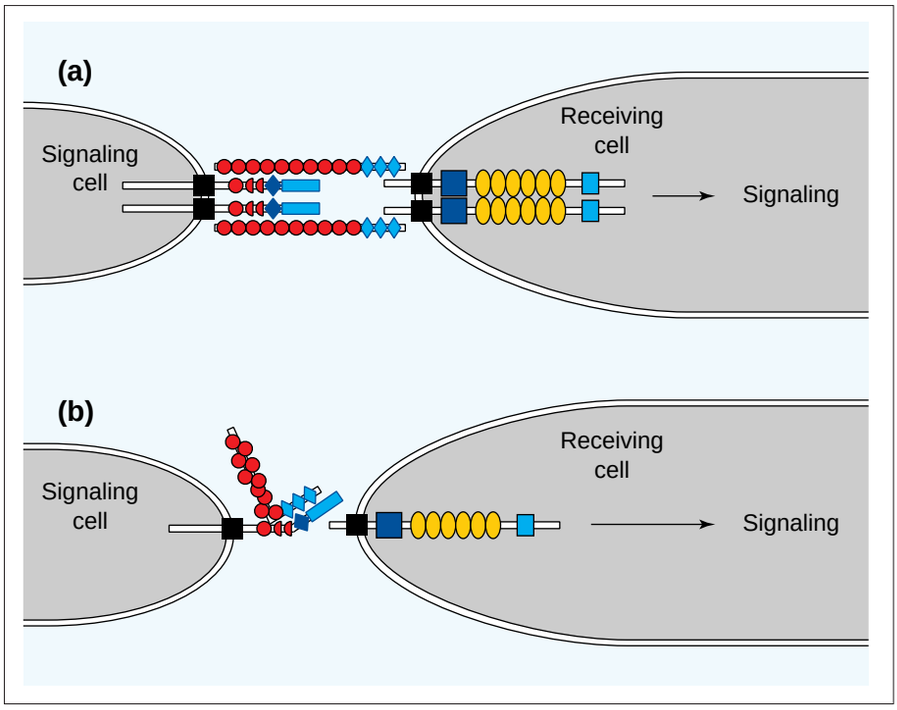

Figure 3

Molecular models for receptor activation by ligand binding. **(a)** Ligand binding induces receptor dimerization, which, in turn, activates the receptor. **(b)** Ligand binding disrupts heterodimer association in the receptor and releases the transmembrane–intracellular region from inhibition, thereby activating the receptor.

Recently, a second proteolytic-cleavage site has been identified in the Notch1 receptor of vertebrates; in this case, the receptor IC domain is released by proteolysis[46]. This cleavage site, which is distinct from the maturation cleavage in the LNG–TM interval, is in the receptor's transmembrane domain at its C-terminal border[46]. Of particular significance is that ligand binding induces this cleavage, and mutations of the cleavage site dramatically reduce receptor activity and signaling[46]. The fragment generated by this cleavage is called the Notch1 intracellular domain (NICD)[46]. Cleavage might release either the NICD alone (Fig. 4b), or an IC–CSL complex for entry into the nucleus (Fig. 4c). Intriguingly, signaling by the NICD in tissue-culture cells occurred at concentrations that were not detectable by immunocytochemistry[46], which might explain why the NICD has not been observed in the nucleus during development.

A similar mechanism (i.e. one in which proteolytic cleavage releases a transcription factor) has been demonstrated for activation of the sterol regulatory element binding protein (SREBP)[47]. In this case, ligand-induced proteolysis releases the SREBP transcription factor from a membrane-bound form in the endoplasmic reticulum. One minor difference is that SREBP is a DNA-binding protein, whereas the NICD appears to be a transcriptional co-activator that does not itself bind to DNA[34]. However, in both cases, the linkage between ligand binding and transcriptional regulation is remarkably simple and direct.

Conclusions and future prospects

The Notch/LIN-12 signaling pathway is a ubiquitous pathway that controls a variety of processes during development. The signal-transduction mechanism that is emerging involves at least two steps: (1) ligand-induced activation of the receptor, perhaps by release from EC inhibition; and (2) receptor-induced activation of the CSL DNA-binding protein, perhaps by generation of an IC proteolytic fragment that acts in the nucleus with the CSL protein to promote transcription.

Although these broad strokes paint a reasonable picture, many questions remain. How does the receptor EC domain inhibit receptor activity, and how does ligand binding relieve such inhibition? Does ligand binding induce dimerization and thereby generate a substrate for the protease, or does ligand binding permit the transmembrane domain to readjust its position and expose a site for proteolytic cleavage? What role do the presenilins play in signaling? As mentioned at the beginning of this review, the presenilins can regulate LIN-12/GLP-1 signaling in nematodes[7]. Recent work supports a role for presenilins in the facilitated cleavage of APP (Ref. 48). Do the presenilins also facilitate cleavage of Notch/LIN-12 receptors? What is the role of the ADAM proteases in signaling? Do they mediate the newly identified ligand-induced cleavage? Does the NICD join the CSL protein in the nucleus, or is a complex formed with the CSL at the membrane, so that the two are released together for entry into the nucleus? Finally, can the receptor act through mechanisms other than those outlined

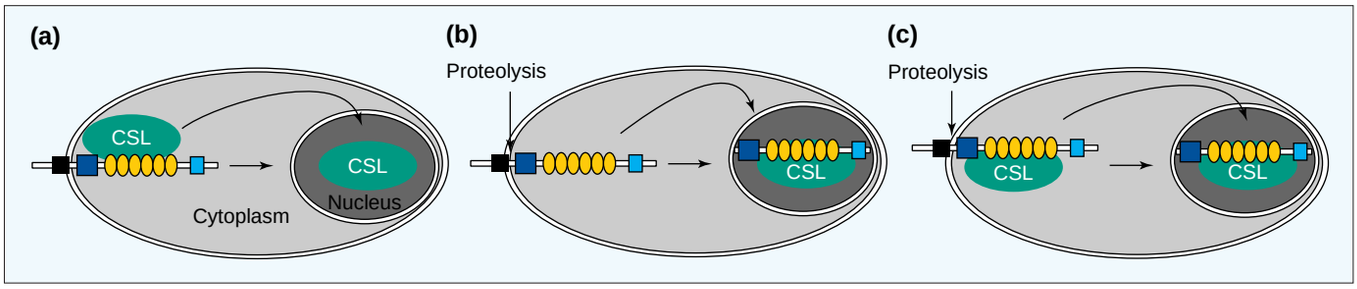

Figure 4

Molecular models for CSL activation by receptor. **(a)** Regulated translocation of the CSL protein to the nucleus. **(b)** Regulated proteolysis of the receptor leads to translocation of the receptor intracellular region (IC) to join CSL in nucleus, where they act together to activate transcription. **(c)** Regulated proteolysis of the receptor leads to translocation of both receptor IC and CSL protein to nucleus, where they act together to activate transcription.

here? Although our understanding of the signaling mechanism used by Notch/LIN-12 receptors remains limited, major inroads have clearly been made. Challenges for the future include the analysis of the signaling mechanism in biochemical detail and the generation of reagents to intervene in the pathway for therapeutic uses.

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