

# The roles of receptor and ligand endocytosis in regulating Notch signaling

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Development 132, 1751-1762

Published by The Company of Biologists 2005

doi:10.1242/dev.01789

## Summary

Cell-cell signaling is a central process in the formation of multicellular organisms. Notch (N) is the receptor of a conserved signaling pathway that regulates numerous developmental decisions, and the misregulation of N has been linked to various physiological and developmental

disorders. The endocytosis of N and its ligands is a key mechanism by which N-mediated cell-cell signaling is developmentally regulated. We review here the recent findings that have highlighted the importance and complexity of this regulation.

## Introduction

Signal transduction by many surface receptors is tightly connected to membrane trafficking. For many years, the internalization of receptors by endocytosis was mostly thought to be associated with signal attenuation and with the downregulation of cell-cell signaling. Indeed, endocytosis regulates the steady-state level of receptors, transmembrane ligands and associated factors at the cell surface, and can also target activated receptors for lysosomal degradation. However, over the past decade, intensive research in the field has provided strong evidence that endocytosis and endosomal sorting (see Box 1) may also play an essential role in signal transduction. For example, endocytosis may serve to bring ligand-bound receptors to signal-transducing machinery that is localized to specific intracellular compartments, or may regulate the transport of active ligands from cell to cell (for a review, see Seto et al., 2002).

Signaling by Notch (N) receptors has multiple and essential roles in many cell fate decisions and in patterning events from worms to humans (Lai, 2004). N receptor signaling is regulated at multiple levels (Schweisguth, 2004). One of the first indications that endocytosis plays a key role in N signaling came from the analysis of a *Drosophila* temperature-sensitive mutation called *shibire<sup>ts</sup>* (*shi<sup>ts</sup>*), which was later shown to encode dynamin, a GTPase required for the pinching off of endocytic vesicles from the plasma membrane (Chen et al., 1991; van der Blik and Meyerowitz, 1991) (see Box 1). Mutant embryos raised at the restrictive temperature have a *Notch*-like neurogenic phenotype, characterized by hypertrophy of the nervous system at the expense of the ventral epidermis (Poodry, 1990). Although coated pits formed normally in this mutant, vesicles fail to pinch off from the cell surface. This correlation led C. Poodry to ask: 'could a block in endocytosis account for an interruption in the communication necessary for normal epidermal and neural differentiation?' (Poodry, 1990). Fifteen years of research later, the answer is clearly yes. However, the detailed molecular consequences of a complete block of endocytosis on N signaling are not as simple as this answer may suggest. Here,

we review and discuss recent findings on the roles of endocytosis in the regulation of N receptor signaling.

## N receptor signaling: an overview

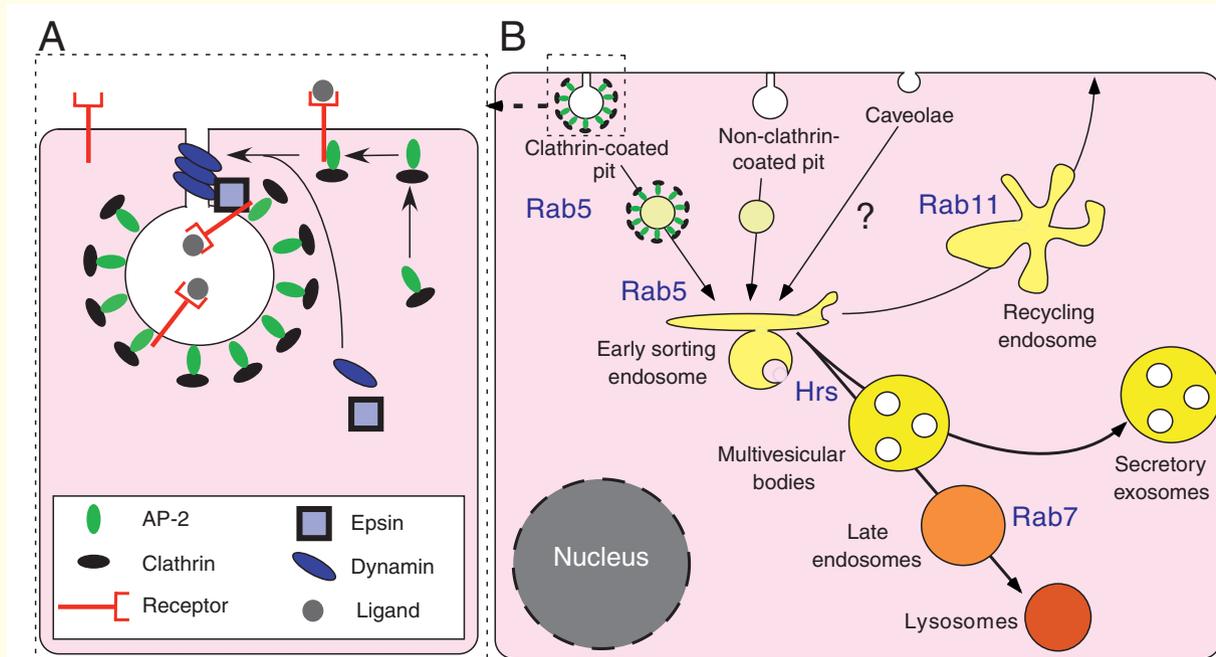
N receptors are type I transmembrane proteins that are present at the plasma membrane as heterodimers. They consist of an ectodomain called NECD (for Notch Extracellular Domain) and a membrane-tethered intracellular domain (Fig. 1). The extracellular part of N contains a variable number of Epidermal Growth Factor (EGF)-like repeats, which are involved in ligand binding. Upon ligand binding, N undergoes two successive proteolytic cleavages. The first cleavage at the extracellular S2 site is ligand-induced and is mediated by extracellular proteases of the ADAM/TACE family. S2 cleavage of N generates an activated membrane-bound form of N. In the absence of ligand binding, the extracellular LIN-12/Notch Repeats (LNRs) prevent S2 cleavage. The S2-cleaved, membrane-bound form of N is further processed at the endomembrane S3 site by the  $\gamma$ -secretase complex. This leads to the cytoplasmic release of the intracellular domain of N, called the NICD (Notch Intracellular Domain). The NICD localizes to the nucleus and associates with a DNA-binding protein called CSL (for human, CBF1; *Drosophila*, Suppressor of Hairless; *C. elegans*, Lag-1) to regulate the expression of its target genes. This CSL-dependent signaling pathway is called the canonical N pathway (Kopan, 2002).

N receptors are activated by transmembrane ligands of the DSL family (for Delta and Serrate from *Drosophila* and Lag-2 from *C. elegans*; we refer to these N ligands as the DSL ligands) (Fleming, 1998). Delta (Dl) and Serrate (Ser) are the only two known N ligands in *Drosophila*. In mammals, five DSL ligands have been identified: three are structurally related to Dl (Dl1, 2 and 4; these are also called Dl-like or Dll), and two are more similar to Ser (Jagged1 and 2). These DSL ligands exhibit significant variations in specific domain size and composition. Their intracellular tails are also poorly conserved. However, they all have in their extracellular domain a DSL motif involved in N binding and a variable number of EGF repeats. Biochemical analyses in *Drosophila* and

mammals have indicated that, similarly to N, DSL ligands are cleaved sequentially to release extracellular and intracellular fragments (Bland et al., 2003; Ikeuchi and Sisodia, 2003; Klueg et al., 1998; LaVoie and Selkoe, 2003; Mishra-Gorur et al., 2002; Qi et al., 1999; Sapir et al., 2005; Six et al., 2003) (Fig. 1). By analogy with N receptor signaling, it has been suggested that the intracellular domains of D1 and Jagged may signal intracellularly, raising the possibility that DSL proteins are involved in bidirectional signaling (Bland et al., 2003;

Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six et al., 2003). Further studies are required to test this possibility and to establish the signaling activity of the intracellular domains of the DSL ligands. Ligand processing also generates soluble, extracellular forms that can bind N receptors, raising the possibility that DSL ligands act as secreted ligands. However, a careful examination of the biological activity of purified, soluble D1 produced from *Drosophila* cells has suggested that the cleavage of D1 is associated with its degradation (Mishra-

### Box 1. Endocytosis and endosomal sorting



Endocytosis is an essential cell-surface membrane trafficking event that delivers soluble molecules, membrane components or receptors (and possibly their associated ligands) to the endocytic pathway. There are at least two basic endocytosis mechanisms (see figure): (1) clathrin-dependent endocytosis, in which receptors and their bound ligands are internalized by clathrin-coated pits; and (2) clathrin-independent endocytosis, which generally depends on cholesterol-rich membrane domains and includes caveolae-mediated endocytosis, which partly functions in the endocytosis of ubiquitinated cargos (Chen and De Camilli, 2005; Sigismund et al., 2005).

In clathrin-dependent endocytosis, vesicle coats consist of clathrin and the heterotetramer Assembly Protein 2 (AP2); the AP2 complex links (directly or indirectly) receptors and clathrin. The receptors entering clathrin-coated vesicles (CCVs, see figure) contain sorting signals in their cytosolic domains. These signals can be intrinsic to the receptor or added post-translationally, such as mono-ubiquitination, which has been recently shown to be an endocytic signal. The pinching off of the clathrin-coated domain requires accessory proteins, such as epsin, which link receptors to coat components and contribute to the bending of the lipid bilayer (Ford et al., 2002; Wendland, 2002). Epsin also functions as an adaptor that binds ubiquitinated cargos and promotes their endocytosis in a clathrin-independent manner (Chen and De Camilli, 2005; Sigismund et al., 2005; Aguilar and Wendland, 2005). Finally, in clathrin-dependent and clathrin-independent endocytosis, Dynamin is required for vesicle fission from the donor membrane. After CCV fission from the plasma membrane (see figure), the clathrin coat is removed and recycled for another round of transport. Uncoated vesicles fuse together to form new endosomes, or fuse with pre-existing early (sorting) endosomes (Maxfield and McGraw, 2004) (see figure), a process that is partly controlled by the small GTPase Rab5. Following endocytosis and transportation to sorting endosomes, many receptor-bound ligands are transported to and degraded in late endosomes (a Rab7-positive compartment) or lysosomes (see figure). By contrast, many receptors, such as the Transferrin receptor, are re-used several hundred times, whereas others, such as EGFR, are targeted for degradation to prevent extended signaling. Some receptors, like N, are activated by proteolytic cleavages, possibly during internalization, and cannot be recycled. Transport through the recycling endosome is essential for returning important molecules to the cell surface. Endosomal compartments also function as signaling compartment(s), whereas multivesicular bodies (MVBs) are probably en route to degradation. MVBs or endosomal carrier vesicles (ECVs) – intermediate compartments between sorting and late endosomes (Gruenberg and Stenmark, 2004) – form on early endosomal membranes and are characterized by the budding of small vesicles inside their lumen, an event requiring *hrs* (hepatocyte growth factor receptor tyrosine kinase substrate) activity. In antigen presenting cells and melanocytes, MVBs can fuse back to the cell surface as secretory exosomes (see figure). The endocytosis of N and its ligands is dynamin-dependent but whether N endocytosis is clathrin-dependent is not known. The Numb-dependent inhibition of N signaling requires  $\alpha$ -adaptin activity, an AP2 subunit, indicating that N probably follows the clathrin-dependent endocytosis pathway. Genetic studies also suggest that D1 endocytosis probably does involve clathrin (Cadavid et al., 2000).

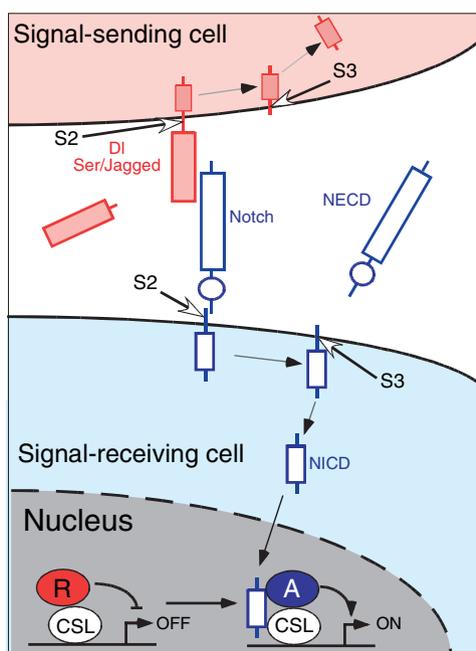
Gorur et al., 2002). Thus, ligands of the DI and Ser/Jagged families appear to signal as cell-surface transmembrane proteins.

### Ligand endocytosis promotes N activation

Several lines of evidence indicate that the endocytosis of DSL ligands is essential for N receptor activation. First, the clonal analysis of the conditional *shi<sup>ts</sup>* mutation in *Drosophila* demonstrated that dynamin-dependent endocytosis is required in both signal-receiving cells and in signal-sending cells to promote DI-dependent N activation (Seugnet et al., 1997). DI and Ser colocalize both at the cell surface and in the intracellular vesicles in *Drosophila* (Kooch et al., 1993; Parks et al., 1995). These vesicles were first suggested to be endocytic in nature because they were not detected in *shibire* mutant cells (Kramer and Phistry, 1996). Moreover, antibody uptake assays in living *Drosophila* tissues have shown that DI and Ser are rapidly endocytosed (Le Borgne et al., 2005; Le Borgne and Schweisguth, 2003b). In contrast with these studies, the subcellular localization of DSL ligands remains largely unexplored in vertebrates. In one study, zebrafish DeltaD was shown to predominantly localize to endocytic vesicles in neuroepithelial cells (Itoh et al., 2003). Importantly, the localization of DI to endocytic vesicles correlates well with

DI signaling in many different developmental contexts, and endocytosis-defective DI proteins have reduced signaling capacity in *Drosophila* (Parks et al., 2000).

Additional evidence for the role of endocytosis in N signaling regulation has come from genetic screens in *Drosophila* and zebrafish. These screens identified epsin and two E3 ubiquitin ligases, Neuralized (Neur) and Mind bomb (Mib in zebrafish; Dmib in *Drosophila*) (see Fig. 2), as being key regulators of ligand signaling activity. Loss of *neur*, *mib/Dmib* or *lqf* (*liquid facet*, the *Drosophila* epsin gene) activity results in phenotypes that are associated with loss of N signaling. The accumulation of DI at the surface of *lqf* mutant cells in *Drosophila* probably occurs because of reduced levels of DI endocytosis (Overstreet et al., 2004; Tian et al., 2004; Wang and Struhl, 2004). Neur associates with DI and promotes DI ubiquitination and endocytosis in both *Drosophila* and *Xenopus* (Deblandre et al., 2001; Lai et al., 2001; Le Borgne and Schweisguth, 2003b; Pavlopoulos et al., 2001; Yeh et al., 2001). The Neur-dependent internalization of DI is observed in *N* mutant *Drosophila* embryos (Morel et al., 2003), indicating that the DI-N interaction is not required for the endocytosis of DI. Dmib associates both with DI and Ser, and promotes DI and Ser endocytosis (Le Borgne et al., 2005; Lai et al., 2005), whereas Zebrafish Mib has thus far been shown to only physically associate with and regulate the endocytosis of DI (Chen and Casey Corliss, 2004; Itoh et al., 2003). Interestingly, *neur* and *Dmib* are required for distinct subsets of N signaling events in *Drosophila*, indicating that these two E3 ubiquitin ligases have complementary functions. Moreover, loss of *Dmib* activity can be compensated for by ectopic Neur expression, indicating that Neur and Dmib have related molecular activities (Le Borgne et al., 2005). As the regulated trafficking of DSL ligands is likely to be governed by ubiquitin-dependent molecular interactions, it will be important to determine whether Neur and Mib/Dmib ubiquitinate DSL ligands at common or distinct sites, and whether they regulate the mono-, multi- and/or poly-ubiquitination of DSL ligands (Box 2). Interestingly, Neur regulates not only the internalization of DI but also its degradation (Deblandre et al., 2001; Lai et al., 2001), indicating that the ubiquitination of DI may have a dual antagonistic role in promoting and downregulating DI signaling activity. These two distinct outcomes may result from the same ubiquitin modifications. Alternatively, the rate of degradation following endocytosis may depend on the number, or type, of ubiquitination events (mono versus polyubiquitination) that are catalyzed by Neur and Mib/Dmib. A biochemical analysis should resolve these issues. Finally, and most importantly, clonal analysis in *Drosophila* and transplantation studies in zebrafish have indicated that *neur*, *Mib/Dmib* and *lqf* act non-autonomously to promote N activation (Bingham et al., 2003; Itoh et al., 2003; Le Borgne et al., 2005; Le Borgne and Schweisguth, 2003a; Le Borgne and Schweisguth, 2003b; Li and Baker, 2004; Overstreet et al., 2004; Pavlopoulos et al., 2001; Tian et al., 2004; Wang and Struhl, 2004). Thus, these data suggest that the endocytosis of DI and Ser in signal-sending cells is strictly required for N activation in many, if not all, N-mediated decisions in *Drosophila*. Whether this applies to other organisms is discussed below.



**Fig. 1.** Notch signaling involves regulated-intramembrane proteolysis. Upon ligand binding, N undergoes two successive proteolytic cleavages (Brown et al., 2000). The first cleavage at the S2 site is ligand induced and generates an activated membrane-bound form of N (blue) that is further processed at the S3 site by the  $\gamma$ -secretase complex. This leads to the release of the NICD. The association of the NICD with DNA-bound CSL factors disrupts CSL-co-repressor (R) complexes and promotes the assembly of CSL-co-activator (A) complexes, thereby mediating a transcriptional switch. DSL ligands (pink) can be similarly processed, first at extracellular sites (S2) and then at an intramembrane S3 site by the  $\gamma$ -secretase complex (see text for details). CSL, CBF1, Suppressor of Hairless, Lag-1; DI, Delta; N, Notch; NECD, Notch extracellular domain; NICD, N intracellular domain; Ser, Serrate.

## How does endocytosis promote ligand signaling activity?

The observation that the endocytosis of a transmembrane ligand in a signal-sending cell is associated with receptor activation in a signal-receiving cell is seemingly paradoxical. Indeed, endocytosis removes the ligand from the cell surface where it interacts with its receptor. Several models have been proposed to resolve this paradox (Fig. 3).

One model postulates that the S2 cleavage site of ligand-bound N becomes unmasked when the formation of clathrin-coated pits in signal-sending cells induces pulling forces on the NECD (Parks et al., 2000) (see Fig. 3). In support of this model, the dynamin-dependent endocytosis of DI appears to be required for the trans-endocytosis of the NECD in *Drosophila* (Parks et al., 2000). However, it is not known whether N receptor activation requires the trans-endocytosis of extracellular N.

Other models postulate that the DSL ligands are produced as inactive or poorly active ligands and that endocytosis is a prerequisite for the surface expression of active DSL ligands.

One such model is based on the observation that DI and Ser accumulate in large endocytic vesicles that may correspond to multi-vesicular bodies (MVBs). In this model, endocytosis is required to produce DI-containing exosomes – extracellular vesicles that are produced from the fusion of a MVB with the plasma membrane (Le Borgne and Schweisguth, 2003a). These vesicles would be the active form of the DSL ligand. Consistent with this model, an active form of DI that co-eluted with full-length DI was detected in the culture medium of DI-expressing S2 cells (Mishra-Gorur et al., 2002). This model is, however, not supported by the observation that loss of *hrs* activity, which inhibits the formation of MVBs, does not significantly impair DI signaling activity (Jekely and Rorth, 2003).

Another model proposes that endocytosis allows the DSL ligands to undergo post-translational modification in the sorting and/or recycling endosomes, to produce fully active ligands (Wang and Struhl, 2004). This model is supported by the analysis of chimeric DI proteins. It had been previously shown that a truncated DI lacking its intracellular domain (ICD) cannot signal (Sun and Artavanis-Tsakonas, 1996).

Drosophila	Mammals	Attributed function in N signaling
D-mib  1226	Mind-bomb	Ubiquitination of Ser and DI; endocytosis of Ser and DI; activation of N signaling (Itoh et al., 2003; Le Borgne et al., 2005)
Mind-bomb related  1050	Skeletrophin	Unknown
Neuralized  755	Neuralized 1-2	Ubiquitination of DI (and Ser?); endocytosis of DI (and Ser?); activation of N signaling (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001)
Deltex  738	Deltex 1-4	Ubiquitination of N?; endocytosis and endosomal sorting of N towards Rab11-positive compartment; CSL-independent activation of N? (Hori et al., 2004; Wilkin et al., 2004)
Su(dx)  950	Itch/AIP4	Ubiquitination of N?; endocytosis and endosomal sorting of N towards Rab7-positive compartment; degradation of N in lysosomes (Qiu et al., 2000; Sakata et al., 2004; Wilkin et al., 2004)
Nedd4  1007	Nedd4	
D-smurf  1062	Smurf	Unknown
Cbl  879	Cbl	Binds to Notch1; lysosomal degradation of N; binds to AIP4/Itch (Jehn et al., 2002)

 Ring Finger	 Mib HERC2	 ZnF ZZ	 Ankyrin repeat	 Neuralized homology	 HECT	 WWE	 C2
 WW	 SH2 SH2	 N Cbl_N	 N2 Cbl_N2	 UBA			

**Fig. 2.** Structure and function of E3 ubiquitin ligases involved in N signaling. Four families of E3 ubiquitin ligases have been described: the Ring finger family; the HECT (homologous to E6AP COOH terminus) family; the F-box and multi-subunit E3 ubiquitin ligases (SCF/Cullin); and the U-box-containing E3 ubiquitin ligases. Some members of the Ring finger family are part of multi-protein complexes that contain F-boxes. Members of three of these families (Ring finger, HECT, SCF/Cullin) have been involved in N signaling in *Drosophila* (Lai, 2002). The E3 ubiquitin ligases of the Ring finger and HECT families involved in the regulation of receptor and ligand endocytosis are shown in this figure. Not shown is SEL-10, a F-Box protein of a SCF (Skp1-Cul1-F-box-Rbx1)-type ubiquitin ligase, which was first identified as a negative regulator of *lin-12* and which was recently shown to bind the nuclear form of activated N in a phosphorylation-dependent manner to promote its proteasome-mediated degradation (reviewed by Lai, 2002). HERC2, Hect (homologous to the E6-AP carboxyl terminus domain and RCC1-like domain 2); WWE, WWE domain (named after three of its conserved residues); ZnF ZZ, ZZ-type Zinc Finger; UBA, ubiquitin-associated domain.

Replacing its ICD with a 21 amino acid peptide from the Low Density Lipoprotein receptor (LDLR), which is known to promote LDLR internalization and recycling, restored signaling (although not to control levels) (Wang and Struhl, 2004). Replacing the DI ICD with a monoubiquitin can, likewise, promote both DI endocytosis and N signaling (Wang and Struhl, 2004). Interestingly, the expression of the DI-LDLR chimera protein suppressed the *lqf* phenotype, whereas the DI-ubiquitin chimera did not. The LDLR peptide differs in its ability to promote recycling to the cell surface, perhaps highlighting an essential role for Lqf in recycling (Wang and Struhl, 2004). The use of these and similar chimeric proteins will hopefully tease apart each step in the trafficking of the DSL ligand from the cell surface, through the endosomes and back to the cell surface, and also shed light on when and where DSL activation occurs and the genes involved in this activation.

### Is ligand endocytosis always required for N activation?

The range of combined *neur* and *Dmib* mutant phenotypes strongly suggest that the endocytosis of DSL ligands is required for all, or most, N-mediated fate decisions in *Drosophila* (Le Borgne et al., 2005). The neurogenic phenotype observed in *lqf* mutant clones further strengthens the notion that the endocytosis of DSL ligands is required for N activation. However, this strict *in vivo* requirement for ligand endocytosis is not observed in transfected *Drosophila* S2 cells. Indeed, the activation of N receptor signaling that occurs upon

the aggregation of N- and DI-expressing S2 cells does not appear to require the endocytosis of DI. This is because the formalin fixation of DI-expressing S2 cells prior to cell aggregation does not block the activation of the *E(spl)-m3* N target gene in N-expressing S2 cells. Thus, DI molecules immobilized at the cell surface are still able to activate N, implying that endocytosis is not essential for N activation in this assay (Mishra-Gorur et al., 2002).

Studies in cultured mammalian cells may help solve this apparent paradox. Soluble, non-membrane bound ligands have been shown to retain signaling activity in some, but not all, cultured cell assays (Hicks et al., 2002; Li et al., 1998; Ohishi et al., 2002; Shimizu et al., 2000; Shimizu et al., 2002; Vas et al., 2004; Wang et al., 1998). The antibody-induced oligomerization of soluble ligands, which were produced as fusions with human IgG Fc, has been reported to increase the signaling activity of these ligands, suggesting that ligand clustering promotes signaling (Hicks et al., 2002; Shimizu et al., 2002). The immobilization of soluble ligands on beads or on a plastic surface also appeared to increase their activity (Maekawa et al., 2003; Ohishi et al., 2002; Varnum-Finney et al., 2000; Vas et al., 2004). Importantly, free soluble ligands can antagonize the activity of immobilized, soluble ligands, as well as that of membrane-bound ligands in cells assays (Hicks et al., 2002; Shimizu et al., 2002; Small et al., 2001; Trifonova et al., 2004; Vas et al., 2004). These observations are similar to those made in transgenic flies expressing secreted versions of DI and Ser, which indicate that soluble ligands act as N antagonists *in vivo* (Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997). These results can be interpreted as follows: secreted ligands can compete with membrane-bound ligands for N binding, but are very poor activators of N. Thus, soluble ligands may only be able to activate N in specific cultured cell assays in which their competition with endogenous ligands is reduced.

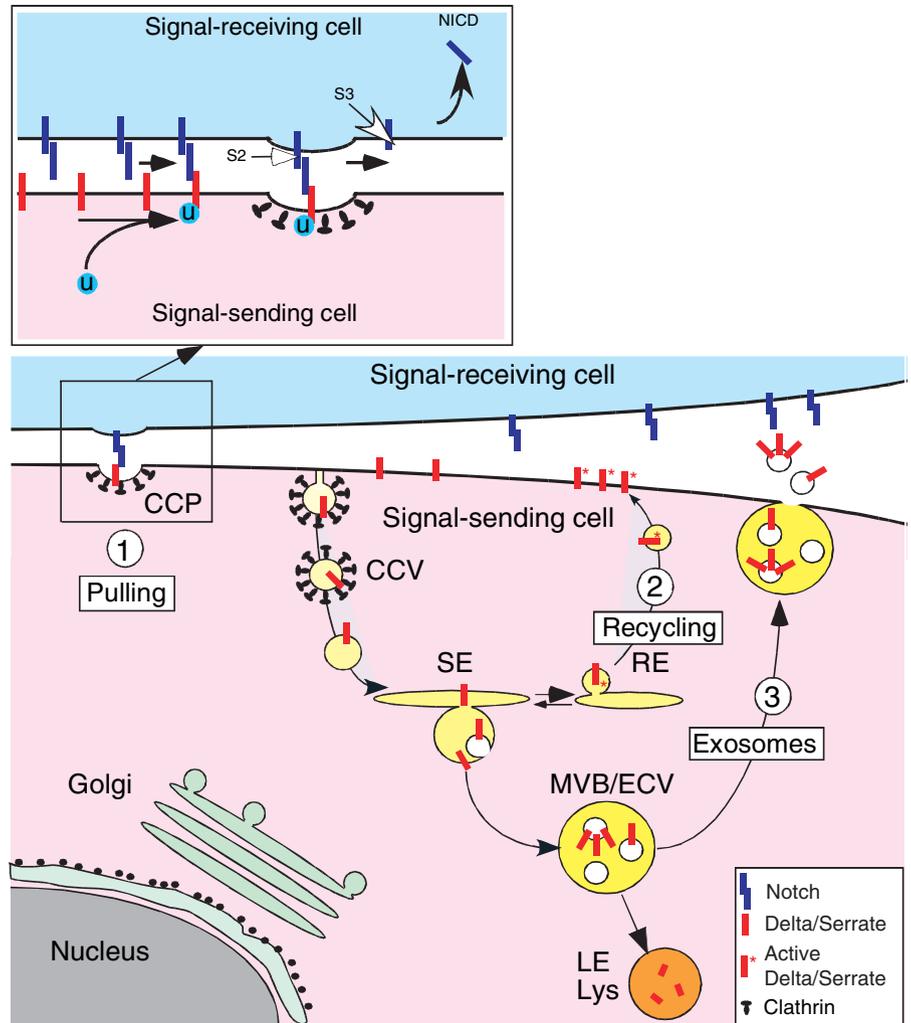
By contrast, ligand endocytosis may be largely dispensable for the activation of the *C. elegans* N family receptors GLP-1 and LIN-12. First, the ICD of LAG-2 can be replaced with a  $\beta$ -galactosidase fusion protein with no discernable consequences on GLP-1 and LIN-12 signaling (Fitzgerald and Greenwald, 1995; Henderson et al., 1994). Second, the *C. elegans* genome contains five genes that encode putative ligands for GLP-1 and LIN-12 that are predicted to be secreted. At least one of these predicted secreted ligands, DSL-1, acts as a bona fide ligand for LIN-12 (Chen and Greenwald, 2004). [It will be interesting to examine the evolutionary conservation of genes encoding secreted DSL ligands, and it is noteworthy that a secreted version of human Jagged1 can be generated by alternative splicing (Aho, 2004).] Finally, there is no clear *mib* homolog in the *C. elegans* genome, and RNAi-mediated inactivation of the putative *neur* homolog (F10D7.5) does not reveal that it is specifically required for LIN-12 and/or GLP-1 activation (<http://www.wormbase.org/db/gene/gene?name=neuralized>).

Together, these results indicate that ligand endocytosis is not strictly required for the activation of the GLP-1 and LIN-12 receptors in *C. elegans*, which contrasts with the strict requirement for endocytosis in *Drosophila*. However, a lack of requirement does not necessarily imply an absence of function, and it is conceivable that endocytosis modulates ligand activity in *C. elegans* too. Consistent with this speculation is that epsin activity is required for GLP-1 signaling in the *C. elegans* gonad (Tian et al., 2004). Whether *C. elegans* epsin regulates the

#### Box 2. Ubiquitin as an endocytosis signal

Ubiquitin is a highly conserved 76-amino acid polypeptide that is covalently linked to its protein substrates via an isopeptide bond between its carboxy-terminal glycine and the  $\epsilon$ -amino group of a lysine residue in the substrate protein, or, less frequently, the amino group at the N terminus of the substrate. Ubiquitination is a three-step reaction involving Ubiquitin-activating (E1), Ubiquitin-conjugating (E2) and Ubiquitin-ligase (E3) enzymes. Ubiquitin ligases (E3s) confer substrate specificity and catalyze the transfer of ubiquitin to target proteins. Three modes of ubiquitination with distinct biological functions have been described (Haglund et al., 2003). (1) Monoubiquitination, which is defined by the addition of a single ubiquitin to a substrate and which functions in endocytosis, membrane trafficking, and sorting proteins in MVBs. (2) Multiubiquitination (or multiple monoubiquitination), which consists of the monoubiquitination of several lysine residues in a protein substrate and which functions in endocytosis and membrane trafficking. (3) Polyubiquitination, in which a ubiquitin chain forms that is attached to a single lysine of the target protein. The chain forms through an iterative process in which seven lysine residues in ubiquitin are targeted by another ubiquitin. This results in target proteins being degraded via the 26S proteasome. How the ubiquitin machinery decides between mono versus polyubiquitination is not known. One possible mechanism involves an equilibrium being reached between ubiquitination and de-ubiquitination. Lqf, the *Drosophila* homolog of epsin, is an ubiquitinated protein that is a substrate for Fat facets (Faf), a de-ubiquitinating enzyme (Chen et al., 2002). These two enzymes function together to activate DI internalization and DI signaling (Overstreet et al., 2004). Faf might inhibit the accumulation of polyubiquitinated Lqf and thereby favor the accumulation of active Lqf.

**Fig. 3.** Models of how ligand endocytosis promotes N signaling. Three models have been proposed to resolve the paradox that N activation in the signal receiving cell requires the endocytosis of *Dl* and *Ser/Jagged* in the signal-sending cell, which removes the ligands from the cell surface where they have to reside to interact with N. (1) The endocytosis of Notch-bound DSL ligands might create pulling forces on N that induce conformational changes associated with the unmasking of the S2 cleavage site. (2,3) Newly synthesized inactive DSL ligands become active upon being trafficked through endosomal compartments. In model 2, internalized DSL ligands transit via the recycling endosomes (RE, see Box 1) where they would be activated by an as yet unknown post-translational modification (Wang and Struhl, 2004). In model 3, endocytosed DSL ligands are targeted inside the lumen of MVBs (see Box 2), leading either to their degradation upon maturation of the MVBs into lysosomes, or to the extracellular release of secreted vesicles, called exosomes, on the fusion of the MVBs with the plasma membrane. These models are not mutually exclusive. The pH gradient of vesicles is color-coded from neutral (pale yellow) to pH 5 (orange). CCP, clathrin-coated pit; CCV, clathrin-coated vesicle; ECV, endosomal carrier vesicles; LE, late endosome; Lys, lysosome; MVBs, multivesicular bodies; NICD, N intracellular domain; RE, recycling endosome; SE, sorting endosomes; U, ubiquitin.



signaling activity of a transmembrane ligand for GLP-1 or another process remains to be determined.

Finally, the existence of secreted ligands in *C. elegans* but not in *Drosophila* may reflect differences in developmental strategies. Indeed, anchoring a ligand to the cell surface restricts receptor activation to the few cells that are in direct contact with the signal-sending cell. This might ensure the tight spatial control of N activation. By contrast, secreted ligands may activate N in distant cells and have long-range effects. Thus, one may predict that the ability of a cell to respond to diffusible signals may be tightly regulated in organisms expressing secreted ligands. Indeed, both transcriptional and post-transcriptional mechanisms ensure that GLP-1 and LIN-12 are tightly developmentally regulated in *C. elegans*. Conversely, the ability of a cell to respond to membrane-bound signals may not need to be tightly regulated in organisms with membrane-anchored ligands. This developmental strategy is observed in *Drosophila*: N is broadly expressed in the embryo and the imaginal tissues, whereas the transcription of the *Dl*, *Ser* and *neur* genes is tightly regulated.

### Is N endocytosis required for S2 and/or S3 cleavage?

The strong correlation of endocytosis with DSL activity, as

discussed above, does not alone account for the neurogenic *shi<sup>ts</sup>* phenotype. Indeed, clonal analysis of the conditional *shi<sup>ts</sup>* mutation has indicated that dynamin-dependent endocytosis is also required in signal-receiving cells for N signal transduction (Seugnet et al., 1997).

Two observations have recently indicated that a regulatory step exists between the S2 and S3 cleavages of N. First, soluble Delta1 can bind to Notch2 at the surface of mammalian cultured cells and promote its S2 cleavage, but it cannot promote intracellular S3 cleavage nor the release of the Notch2 ICD and the subsequent activation of Notch2 reporter constructs (Shimizu et al., 2002). Second, although two distinct extracellular proteases, TACE and Kuzbanian (Kuz), can cleave an engineered form of N at the extracellular S2 site in *Drosophila* cells, only the S2-cleaved forms of N generated by Kuz are efficiently cleaved by the  $\gamma$ -secretase complex (Lieber et al., 2002).

A recent study has indicated that regulating the cleavage of N may involve its endocytosis. First, a truncated form of Notch1 (N1), N1 $\Delta$ E, that is similar to the S2-cleaved form of N1, could be endocytosed in 3T3 cells in an antibody-uptake assay (Gupta-Rossi et al., 2004). Second, inhibiting endocytosis using dominant-negative forms of either Dynamin2 or Eps15 blocked the  $\gamma$ -secretase processing of

N1ΔE (Gupta-Rossi et al., 2004). N1ΔE was mono-ubiquitinated at a conserved lysine residue, the mutation of which reduced both N internalization and S3 cleavage (Gupta-Rossi et al., 2004). These data suggest that S2-cleaved N is endocytosed prior to S3 cleavage and raise the possibility that endocytosis is required following S2 cleavage for N signal transduction. Although this possibility remains to be tested in a ligand-mediated signaling event, results from a sensitive assay for S3 cleavage of *Drosophila* N do not support this hypothesis (Struhl and Adachi, 2000) [see discussion in Gupta-Rossi et al. (Gupta-Rossi et al., 2004)]. It will thus be important to test the functional importance of this mono-ubiquitination for N signaling and, of course, to identify the E3 ubiquitin ligase(s) involved in this modification of N. Finally, we note that this model raises the possibility that endocytosis may be similarly required for the intracellular cleavage of Delta/Jagged by the  $\gamma$ -secretase complex (Bland et al., 2003; Ikeuchi and Sisodia, 2003; Kiyota and Kinoshita, 2004; LaVoie and Selkoe, 2003; Six et al., 2003).

Why would endocytosis of N be required for S3-cleavage? One model is that the  $\gamma$ -secretase is prevented from contacting its substrate at the plasma membrane, such that endocytosis is required to bring S2-cleaved N from the plasma membrane, where it is produced, to an intracellular compartment containing biologically active  $\gamma$ -secretase. Whether the S3 cleavage of N takes place at the plasma membrane or in an intracellular membrane compartment is an unsolved issue. Although a large pool of active  $\gamma$ -secretase complexes is known to reside in lipid rafts within the endosomal pathway (Pasternak et al., 2004; Vetrivel et al., 2004), it is difficult to exclude the presence of a minor pool at the cell surface that would be specifically involved in N S3 cleavage (Tarassishin et al., 2004). Further analysis of the compartment in which S3 cleavage of N occurs *in vivo* is required.

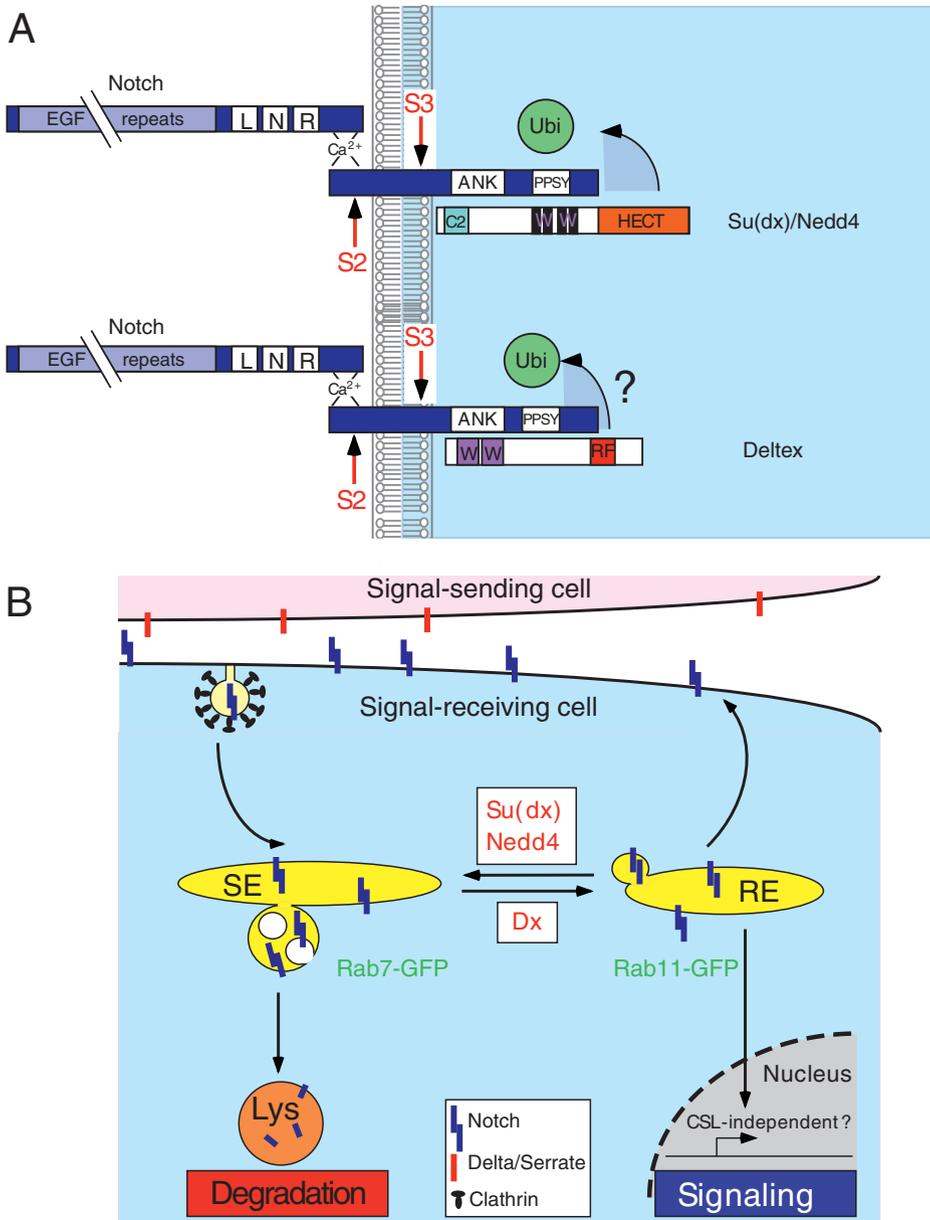
### How is N targeted for lysosomal down-regulation?

The studies reviewed above have indicated that endocytosis positively regulates N signaling. Endocytosis also appears to regulate the steady-state level of N at the cell surface by targeting N for lysosomal degradation. Results from several recent studies indicate that more than one mechanism may contribute to the downregulation of N.

Biochemical studies have indicated that murine N1 is targeted to the lysosomal compartment for degradation by Cbl (Jehn et al., 2002). N1 contains a YxxxP binding site for Cbl, which is a RING finger E3 ubiquitin ligase that regulates the internalization of various transmembrane receptors. Cbl co-immunoprecipitates with N1 in C2C12 cells, and this association increases upon treatment of C2C12 cells with the lysosomal inhibitor chloroquine. Immunoprecipitation experiments using an anti-ubiquitin antibody also revealed that full-length N1 is either mono-ubiquitinated or is associated with a ubiquitinated complex in C2C12 cells. Consistent with this observation, N intracellular and extracellular epitopes colocalize with Hrs in *Drosophila*, suggesting that N is endocytosed into Hrs-positive endosomes prior to S2 cleavage (Fehon et al., 1990; Wilkin et al., 2004). Moreover, N accumulates together with many other non-degraded ubiquitinated proteins into large vesicles in *hrs* mutant cells (Jekely and Rorth, 2003). Together, these results suggest that Cbl may be involved in the lysosomal degradation of N1 in mammals (Jehn et al., 2002).

Interestingly, Cbl-C, which is one of the three human Cbl family members, binds to AIP4/Itch, an E3 ubiquitin ligase of the Nedd4 family. These two interacting E3 ubiquitin ligases cooperate to regulate the internalization of the EGF receptor (Courbard et al., 2002; Waterman and Yarden, 2001). AIP4/Itch binds to and promotes the ubiquitination of N1 in cultured cells (Qiu et al., 2000). Moreover, mammalian Numb, an inhibitor of N signaling that has been implicated in the endocytosis of N (Berdnik et al., 2002; Santolini et al., 2000), has been shown to interact with AIP4/Itch and to promote the AIP4/Itch-dependent ubiquitination and degradation of N1 (McGill and McGlade, 2003) (Fig. 4A). Although there is no genetic evidence that Cbl regulates N signaling in *Drosophila* (Pai et al., 2000), two Nedd4 family members, Nedd4 and Suppressor of deltex [Su(dx); the putative *Drosophila* homolog of AIP4/Itch], appear to target N for degradation (Cornell et al., 1999; Fostier et al., 1998; Sakata et al., 2004; Wilkin et al., 2004). Nedd4 associates with full-length N in transfected *Drosophila* cells, and *in vitro* binding studies have indicated that Nedd4 binds the PPSY endocytic motif of N via its WW domain. Likewise, Su(dx) also interacts with full-length N via its WW domain (Qiu et al., 2000; Wilkin et al., 2004) (Fig. 2A). Additionally, ubiquitination of full-length N is abolished upon mutation of the PPSY motif or upon the RNAi-mediated downregulation of Nedd4 activity in S2 cells (Sakata et al., 2004). While these data suggest that Su(dx) and Nedd4 may target N for degradation, loss of Nedd4 and/or Su(dx) activity has surprisingly little effect on the accumulation and/or localization of endogenous N, or on the level of N signaling (Cornell et al., 1999; Fostier et al., 1998; Sakata et al., 2004; Wilkin et al., 2004). This might be due to functional redundancy between Nedd4, Su(dx) and Dsmurf, the three Nedd4 family members in *Drosophila*. Nevertheless, overexpression of Su(dx) or Nedd4 mimic a partial loss of N activity (Mazaleyrat et al., 2003; Sakata et al., 2004; Wilkin et al., 2004). Furthermore, the overexpression of both Su(dx) and N leads to the accumulation of N in intracellular vesicles that also contain Rab7-GFP, a late endosomal marker (Wilkin et al., 2004). By contrast, the overexpression of a truncated version of Su(dx), in which the HECT (Homologous to E6-AP C Terminus) catalytic domain has been removed, leads to the accumulation of overexpressed N in a distinct intracellular compartment that contains Rab11-GFP, a marker for the recycling endosome (Wilkin et al., 2004). Inhibition of Su(dx) activity, therefore, appears to promote the sorting of endocytosed N to the recycling endosome. Thus, Su(dx) and Nedd4 may act to direct N for degradation by regulating the endosomal sorting of N following its endocytosis from the plasma membrane.

Ubiquitination may not be the sole determinant of the rapid turnover of N. Downregulation of the *C. elegans* LIN-12 receptor during vulval development is mediated by a 'downregulation targeting signal' (DTS) that contains a 'Dileucine motif' (Shaye and Greenwald, 2002). This motif has been characterized in various transmembrane receptors and has been involved in both constitutive and regulated endocytosis (Box 1). However, this signal does not appear to be conserved in *Drosophila* and vertebrate N receptors. Consistent with a role of this motif in the internalization of LIN-12, the internalization and degradation of a LIN-12::GFP fusion protein was shown to depend on the DTS. Another potential determinant of N turnover has recently been identified in



**Fig. 4.** Models of how Dx and Su(dx) regulate N signaling. (A) Su(dx) and Nedd4 bind and ubiquitinate (Ubi) N. *Drosophila* Nedd4 and Su(Dx) interact with the PPSY endocytic motif of N via their WW domains, and promote the ubiquitination of N. Ubiquitination of N by Nedd4/Su(dx) leads to the endosomal degradation of N. Deltex (Dx) interacts with the Ankyrin (ANK) repeats of N and exhibits E3 ligase activity in vitro. However, it is not yet known whether Dx promotes the ubiquitination of N. It has been proposed that Su(Dx) and Nedd4 interact with the full-length N, as depicted here. Whether Dx interacts with full-length N or with the S2-cleaved form of N is not known. These interactions may occur at the plasma membrane or in endosomes. (B) Dx and Su(dx)/Nedd4 appear to act antagonistically to regulate N endosomal sorting. Upon arrival in sorting endosomes (SE), N may be targeted for degradation to a late endosomal, Rab7-GFP positive compartment. This sorting event appears to be regulated by Su(dx) and Nedd4. Dx may act antagonistically to Su(dx) and Nedd4 by sorting N towards a Rab11-GFP-positive compartment that may correspond to the recycling endosome (RE). This sorting event may promote a CSL- and DSL-independent activity of N. Localization studies have suggested that Su(dx)/Nedd4 and Dx are present in endosomes, but it is possible that they also act at the cell surface to mediate N endocytosis.

*Drosophila*. RNAi inactivation of the heparan sulfate 3-O sulfotransferase gene results in a loss of N signaling activity that correlates with an enlargement of lysosomal compartments, and in a strong reduction on N, but not of DL protein levels (Kamimura et al., 2004). One possible interpretation of these data is that loss of 3-O sulfation leads to a higher rate of degradation of various membrane proteins, including N, in lysosomes. Thus 3-O sulfation may protect N from being targeted to the lysosome. Whether N is a direct target of the heparan sulfate 3-O sulfotransferase is not yet known. Together, these studies suggest that the steady-state level of N at the cell surface is tightly regulated.

#### Does endosomal sorting regulate CSL-independent N signaling?

Thus far, we have only considered the role of endocytosis in ligand- and CSL-dependent N signaling. However, there is

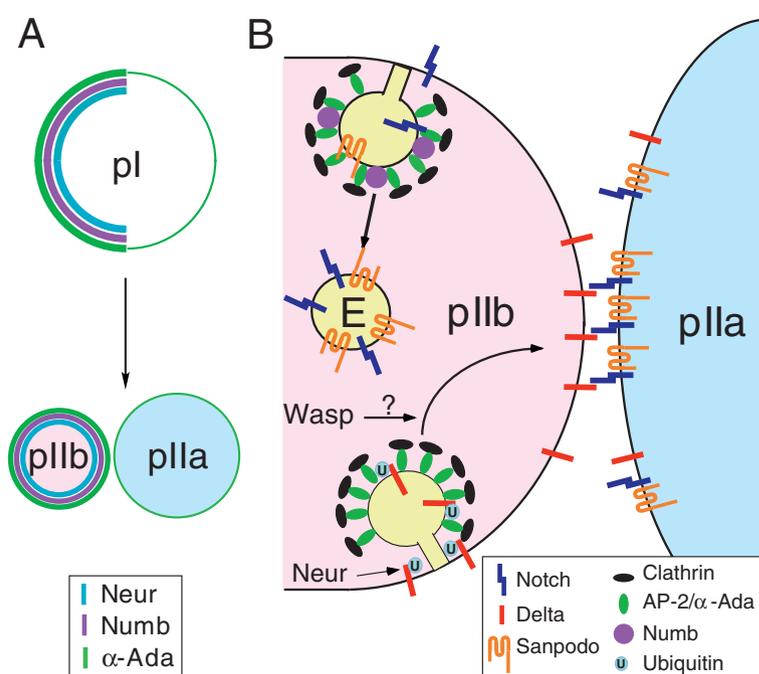
evidence, both in *Drosophila* and vertebrates, that N also promotes distinct cellular responses in a CSL-independent manner. Although this CSL-independent activity of N is not well characterized, several studies have suggested that it may involve the activity of the RING finger type E3 ubiquitin ligase Deltex (Dx).

Dx was first characterized as a positive regulator of N in *Drosophila* (Busseau et al., 1994; Matsuno et al., 1995; Xu and Artavanis-Tsakonas, 1990), and was later found to also regulate N signaling in mammals (Izon et al., 2002; Kishi et al., 2001; Matsuno et al., 1998). Dx binds N (Diederich et al., 1994; Matsuno et al., 1995) and has E3 ubiquitin ligase activity in vitro (Takeyama et al., 2003). Loss of *dx* activity in *Drosophila* leads to a slight reduction in the expression of N target genes during wing development. Conversely, overexpression of Dx results in the cell-autonomous, N-dependent, activation of N target genes that has been reported

to be independent of CSL (Hori et al., 2004). Genetic analysis of truncated N alleles in *Drosophila* has also suggested that N signals in a CSL-independent manner via Dx (Romain et al., 2001). Moreover, the activity of a N-regulated enhancer, the *vestigial* boundary enhancer, is not significantly affected in cells that are mutant for both *Ser* and *Dl* and that overexpress Dx. This indicates that Dx potentiates a signaling activity of N that is ligand-independent in *Drosophila* (Hori et al., 2004). This conclusion is further supported by results from transfection studies in S2 cells. These studies show that the expression of a mutant version of Nedd4, Nedd4<sup>C974FS</sup>, in which the catalytic cysteine used for ubiquitin transfer is mutated, promotes the ligand-independent activation of a N target gene, and that this effect is potentiated by the concomitant expression of Dx (Sakata et al., 2004). These results were interpreted as showing that Nedd4<sup>C974FS</sup> inhibits the targeting of N for degradation, and that Dx enhances the ligand-independent signaling activity of this pool of stabilized N, at least in S2 cells (Sakata et al., 2004). Consistent with this model, the overexpression of Dx leads to the stabilization of N in intracellular vesicles in vivo (Hori et al., 2004), indicating that Dx antagonizes the degradation of N that is thought to be promoted by Su(dx) and Nedd4. These studies suggest that Dx acts antagonistically to Nedd4 family members to protect N from being sorted to an endocytic degradation pathway. Thus, Su(dx)/AIP4/Itch, Nedd4 and, possibly, Dsmurf would regulate the endosomal sorting of N towards lysosomal degradation, whereas Dx would target N towards an undefined intracellular compartment, possibly the Rab11-positive recycling endosome, from which N signals in a ligand- and/or a CSL-independent manner (Fig. 4B). Confirmation of this model will require the molecular characterization of the ligand- and CSL-independent signaling activity of N, the biochemical identification of the forms of N that are endocytosed and sorted by the E3 ubiquitin ligases involved in these sorting events, and a more precise description of the compartment in which these sorting events takes place. Although these studies have been extremely useful at identifying a novel level of N signaling regulation, we note that the analysis of N endosomal sorting relies partly on experiments in which N, the E3 ubiquitin ligases that regulate N trafficking, and the small GTPases used as endosomal markers are overexpressed (Wilkin et al., 2004). Thus, an important challenge in the field is to develop tools that give access to the dynamics of N sorting in more physiologically relevant situations.

### What is the role of Numb and Wasp in N signaling?

One important issue in the field is whether all N signaling events similarly require endocytosis, or whether the regulation of N signaling by endocytosis is context dependent. Results from the study of N-mediated binary fate choices following asymmetric cell division clearly favor the second possibility. Two regulators of N signaling, Neur and Numb, act as cell-fate determinants in *Drosophila* (Le Borgne and Schweisguth,



**Fig. 5.** Regulation of N signaling during asymmetric cell division. (A) The cell fate determinants Neur (blue) and Numb (pink) are unequally segregated during the asymmetric division of the sensory organ precursor cell (SOP or pl) in *Drosophila* and are inherited by the pIIb daughter cell.  $\alpha$ -Adaptin (green) binds to Numb and is enriched at the anterior cortex of the dividing pl cell. (B) In the pIIb cell, the interaction of Numb with  $\alpha$ -Adaptin has been proposed to promote clathrin-mediated endocytosis of either N and/or Sanpodo to endosomes (E). Neur binds Delta (DI), ubiquitinates it and promotes its endocytosis in the pIIb cell. It is not known whether the endocytosis of DI is AP2-dependent. The endocytosis of DI in pIIb leads to the activation of N in the pIIa cell. A possible role of Wasp in endocytosis may be to promote DI internalization.

2003b; Rhyu et al., 1994). Numb is a conserved membrane-associated protein that acts upstream of the S3 cleavage to antagonize N signaling (Guo et al., 1996). Numb binds both NICD and the ear domain of  $\alpha$ -adaplin (Berdnik et al., 2002; Guo et al., 1996; Santolini et al., 2000). The latter is one of the subunits of the AP2 complex that, either directly or indirectly, links cargos recruited for endocytosis to the clathrin coat of the transport vesicles. Numb-mediated inhibition of N appears to require  $\alpha$ -adaplin function, suggesting that Numb may be directly involved in targeting N for endocytosis (Berdnik et al., 2002). Alternatively, or perhaps additionally, Numb may act by preventing the plasma membrane accumulation of Sanpodo (Spdo), a four-pass transmembrane protein that physically associates with both Numb and N, and that is strictly required for N signaling in many, if not all, Numb-mediated cell fate decisions (Dye et al., 1998; O'Connor-Giles et al., 2003; Skeath and Doe, 1998). However, whether Numb directs N and/or Spdo towards endocytosis remains to be demonstrated (Fig. 5).

Interestingly, Wasp, a conserved regulator of Arp2/3-dependent actin polymerization (Vartiainen and Machesky, 2004), is also required for N signaling in the specific context of Numb-mediated decisions in *Drosophila*, but it is dispensable for the unequal segregation of Numb (Ben-Yaacov

et al., 2001). Several studies have implicated Wasp in endocytosis (Chang et al., 2003; Engqvist-Goldstein and Drubin, 2003). In mammals, Wasp interacts with Syndapin, a dynamin-associated protein involved in endocytosis. Wasp also appears to promote local actin polymerization and to facilitate the detachment from the plasma membrane and the intracellular movements of clathrin-coated vesicles (Kessels and Qualmann, 2004). Because loss of *Wasp* activity results in a phenotype opposite to that caused by loss of *numb* activity, it is unlikely that Wasp regulates Numb-mediated endocytosis, or that this function could be masked by a requirement for Wasp activity in DI-N signaling. Whether Wasp regulates the endocytosis of DI and/or of activated N remains to be investigated.

### Conclusions and perspectives

Recent studies have begun to unravel the key role of endocytosis and endosomal sorting in the regulation of N receptor signaling. Many important questions, however, remain. Is the internalization of N (or of its ligands) clathrin-dependent? Or do alternative endocytic routes exist? What are the different compartments through which N and its DSL ligands traffic? What are the forms of N targeted for endocytosis and endosomal sorting? What are the membrane domains in which the S2 and S3 cleavages occur? Are different forms of N targeted to distinct endocytic compartments? What are the signals used for constitutive and regulated endocytosis, and what are the ones used to regulate the endocytosis of the different forms of N? In the case of ubiquitination signals, are N and its DSL ligands mono- and/or multi-ubiquitinated? When and where in the cell do ubiquitination and processing of N take place relative to each other? How is endosomal sorting regulated? Is the activity of the various E3 ubiquitin ligases known to regulate N developmentally regulated? Does activation of N, in turn, regulate the activity of the endocytic and sorting machineries? Some of the answers to these questions will certainly come from a combination of biochemical, genetic and in vivo imaging approaches, as illustrated by the recent elegant studies on endocytosis in yeast (Kaksonen et al., 2003).

We thank E. C. Lai for communicating unpublished data. We thank T. Klein, A. Martinez-Arias and the anonymous referees for their help in improving this review.

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