The role of ligand endocytosis in notch signalling

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The Notch signalling receptor is a mechanoreceptor that is activated by force. This force elicits a conformational change in Notch that results in the release of its intracellular domain into the cytosol by two consecutive proteolytic cleavages. In most cases, the force is generated by pulling of the ligands on the receptor upon their endocytosis. In this review, we summarise recent work that shed a more detailed light on the role of endocytosis during ligand-dependent Notch activation and discuss the role of ubiquitylation of the ligands during this process.

Introduction

The Notch pathway is an ancient signalling system whose elements can be already found at the roots of the evolutionary tree (Gazave et al., 2009; Vlachakis et al., 2020). It mediates cell communication between cells that are in direct contact and is often employed in binary cell fate decisions (Artavanis-Tsakonas et al., 1999). The pathway is discovered and initially characterised in Drosophila melanogaster, but later found to mediate a plethora of cell decisions during development and homeostatic events in probably all metazoans, including humans. In addition, it is also associated with an increasing number of diseases, such as cancer and stroke (Siebel & Lendahl, 2017).

The pathway has three core elements, which are in Drosophila, the ligands Serrat (Ser) and Delta (Dl), the Notch receptor and a transcription factor of the CSL family, named Suppressor of Hairless (Su(H)) (Kovall et al., 2017). Mammals have several orthologs for most element in their genomes: three Dl orthologs (Dl-like1, 3 and 4), two Ser-orthologs (Jagged1, 2), four Notch receptors (Notch1-4), but only one CSL transcription factor. In addition to these core elements, other factors are required for the activation of the pathway, which perform more general functions and have substrates in addition to the Notch elements. Notch receptors are heterodimers which are connected via a Ca\(^{2+}\) Cysteine salt bridge. The two parts arise from one precursor that is cleaved by Furin convertases in the ER (S1 cleavage).

Notch receptors are large type I transmembrane proteins (TMPs) that contain 29–36 EGF-like repeats in their extracellular domain (ECD) followed by the Negative Regulatory Region (NRR) and the transmembrane domain. The canonical ligands of the pathway belong to the DSL (Dl-Ser-Lag2) family of type I TMPs who’s binding to Notch elicits two sequential proteolytic cleavages of Notch (Figure 1). The first cleavage is performed by Kuzbanian (Kuz, S2 cleavage), which is the Drosophila ortholog of mammalian ADAM10 (Lovendahl et al., 2018). The S2 cleavage is the crucial regulated step during Notch activation, which is elicited by the interaction with the ligands. It creates a truncated membrane anchored Notch, termed Notch EXtracellular Truncation (NEXT), that lacks most of its ECD. The second cleavage occurs through the \(\gamma\)-secretase...
Ligand Endocytosis Promotes the Activation of the Notch Pathway

The canonical activation of Notch signalling is initiated by the interaction between the Notch and a DSL ligand in trans. This interaction elicits two proteolytic cleavages that result in the release of NICD. NICD migrates into the nucleus where it activates the transcription of target genes as part of an activating transcription complex with CBF1/Su(H) and further co-factors. The first cleavage of Notch requires its conformational change that is induced by a pulling force generated by CME of the ligand. This force changes the conformation of the NRR and exposes the S2 site to ADAM10 for cleavage. The resulting truncated, but still membrane-anchored NEXT is subsequently cleaved by the γ-secretase complex at the S3 site to release the NICD into the cytosol. CME of the ligands is initiated by ubiquitylation of their ICD. The ubiquitylated ligand is connected to the core machinery of CME by the EA. The CCP further invaginates and is eventually pinched off as a Clathrin-coated vesicle by Dynamin. Finally, the Clathrin coat is disassembled by HSP70 that is recruited to the early endosomal vesicle by Aux. This uncoating is required for the fusion of the early endosomal vesicles with an early endosome.
complex (S3 cleavage), which appears to cleave many TMPs with an ECD smaller than 150 amino acids in *Drosophila* (Struhl & Adachi, 2000). The S3 cleavage results in the release of the intracellular domain of Notch (NICD) into the cytosol from which it enters the nucleus and associates with Su(H)/CSL to activate the transcription of the target genes.

While the interaction of the DSL ligands with Notch in *trans* (between cells) activates the pathway, their interaction in *cis* (in the same cell) suppresses the activation (Figure 1) (del Álamo et al., 2011; Doherty et al., 1996; Doherty et al., 1997; Klein et al., 1997). This suppressive effect is termed *cis*-inhibition (CI) and its mechanism is not completely understood, but it depends on the concentration ratio of the ligand and receptor (Yaron & Sprinzak, 2011).

In the prevailing model of ligand-dependent Notch activation, the critical regulatory step is the generation of a pulling force by the interaction between Notch and a ligand (Figure 1). This force results in a conformational change in the NRR of Notch, which exposes the S2 cleavage site to Kuz/ADAM10. The pulling force is thought to be generated by endocytosis of the ligand in the signal-sending and Notch in the signal-receiving cell (Figure 1) (Chastagner et al., 2017; Gordon et al., 2015; Henrique & Schweisguth, 2019; Langridge & Struhl, 2017; Meloty-Kapella et al., 2012b; Shergill et al., 2012).

Moreover, while it is clear that endocytosis is required to generate the pulling force, it might have additional functions for ligand-dependent activation of Notch. It might be also required to initiate a recycling pathway in which a modification occurs that is essential for the activity of the ligands (Wang & Struhl, 2004; Wang & Struhl, 2005; Weinmaster & Fischer, 2011).

The purpose of this review is to summarise recent work that shed new light on the role of endocytosis during ligand-dependent activation of the Notch pathway with the focus on the connection of the ligands to the endocytic machinery.

**A short introduction to endocytosis**

Endocytosis is the cellular process by which material, such as TMPs and nutrients, is internalised into the cell. It also counterbalances the continuous delivery of membrane to the cell surface by exocytosis, regulates the turnover of proteins in the plasma membrane, regulates the surface abundance of signalling receptors and mediates cellular uptake of nutrients. It is also exploited by pathogens to enter cells. In addition, it is essential for the activation of the evolutionary conserved Notch signalling pathway, as well as the regulation of signal duration of several other pathways, such as the BMP and EGFR pathways (Huotari & Helenius, 2011; Sorkin & von Zastrow, 2009).

Endocytosis is initiated by invagination of a small area of the plasma membrane into a pit, which progresses into deeper invaginations and results in the formation of intracellular membrane vesicles, termed early endosomal vesicles, from which the early endosomes (EEs) arise by homotypic fusion (Huotari & Helenius, 2011). Several types of endocytosis exist that can be classified into Clathrin-dependent and -independent processes. The best studied and prevailing type is Clathrin-mediated endocytosis (CME), which is relevant for Notch signalling and is described in more detail below.

Clathrin-mediated endocytosis is characterised by the prevailing coat formed by Clathrin (Kaksonen & Roux, 2018; Mettlen et al., 2018). Clathrin itself is a protein complex that consists of three light and three heavy chain subunits that assemble into a triskelion-shaped Clathrin monomer. Clathrin cannot bind to membranes itself. Therefore, a bi-layered coat forms during CME, consisting of an inner coat, formed by endocytic adapters (EAs) and cargo that also recruits the outer Clathrin coat. Clathrin molecules assemble into flat or curved lattices depending on whether the triskelia assemble into only hexamers (flat), or pentamers and hexamers (curved). The lattices can stabilise different phases of CME. Recent investigations using fluorescence recovery after photobleaching revealed that the Clathrin coat is highly dynamic (Mettlen et al., 2018). Thus, it can adapt quickly to the changing curvature during membrane invagination. The nascent Clathrin-coated vesicle is eventually pinched off from the plasma membrane by the activity of the GTPase Dynamin, which is recruited to the stalk of the deeply invaginated pit by the endocytic machinery (Haucke & Kozlov, 2018). Dynamin is recruited by BAR domain containing proteins that sense the curvature of the neck of the elongated Clathrin coated pits (CCPs) and assembles into helical oligomers around that neck. It undergoes a conformational change upon GTP consumption,
which constricts the neck of the CCP sufficiently to elicit fusion of the opposing membranes.

Once the Clathrin-coated vesicle is pinched off, it requires uncoating of the Clathrin coat in order to be able to fuse with its destination compartment, the EE. The disassembly of the coat is initiated by Auxilin (Aux), which acts as an adapter for the HSP70 chaperon (Figure 1). Aux can bind simultaneously to membrane phospholipids, Clathrin and, through its family defining J domain, to HSP70. After recruitment by Aux, HSP70 induces the dissociation of the Clathrin coat under ATP consumption (Roosen et al., 2019). Importantly, Aux also binds to Dynamin. This is most likely important for the coordination between uncoating and fission of the nascent early endosomal vesicle (Figure 1). The recently discovered dynamism of the Clathrin coat raise the question which factors drive the exchange of the Clathrin molecules. It is possible that Aux/HSP70 have a so far unappreciated role in this process. In addition to its established role in the late phase of endocytosis, HSP70 could transform the hexameric lattice into the pentameric containing one, required for a curved membrane state.

Transmembrane proteins, such as the Notch receptor and its ligands, are a major class of cargo incorporated into the cell by CME (Schnute et al., 2018). They are recruited into CCPs by several means. Some TMPs possess internalisation sequences in their intracellular domain (ICD) that are directly recognised by the endocytic core machinery (e.g., by the common EA AP-2) while others are actively labelled. One major reversible label for endocytosis that is important here is the polypeptide ubiquitin. It is added to the ICD of TMPs in the process of ubiquitylation (ubi). An ubiquitylated TMP is recognised by certain EAs as cargo and incorporated into the inner coat of CCPs.

After a TMP enters the endosomal pathway, its fate depends on several criteria, such as the type of modification (Mettlen et al., 2018). Several receptors for uptake of material, such as cholesterol or transferrin, are separated in the EE from their ligands and recycled back to the plasma membrane for further cycles ligand uptake. These recycled receptors often possess direct recognition sites for the endocytic core machinery and are not ubiquitylated. The separation of the ligands from the receptor occurs via acidification of the lumen of the EE, which lowers the affinity between the two partners.

TMPs labelled with ubiquitin are in most cases destined for degradation in the lysosome. They are transferred from the limiting membrane of the endosome into its lumen during maturation by pinching off membrane vesicles into the lumen of the endosome. The formation of these intraluminal vesicles (ILVs) is mediated by a large machinery, termed Endosomal sorting complex required for transport (ESCRT) (Hurley, 2015). ILV formation assures the complete degradation of TMPs after fusion of the maturing endosome with the lysosome, as it also incorporates their ICDs into the endosomal lumen. Besides by ligands, Notch can also be activated ligand independently in the limiting membrane of the maturing endosomes on its route to the lysosome. This mode of activation is not addressed here, as there are several recent reviews covering this topic (Palmer & Deng, 2015; Schnute et al., 2018).

**Endocytic adapters**

Clathrin-mediated endocytosis is initiated by a set of around 50 proteins (Haucke & Kozlov, 2018). An important class among these are the EAs. EAs select and concentrate cargo, recruit Clathrin and induce the formation of membrane curvature, which leads to formation of CCPs. An excellent overview on EAs is given in Tehran et al. (2019).

The most important EA in CME is AP-2, a complex of four subunits of different sizes, termed AP2α, AP2β, AP2μ and AP2σ. AP-2 can directly recognise cargo which contain tyrosine- or dileucine-based recognition sequences in their ICDs (Bonfaccino & Traub, 2003; Mettlen et al., 2018). In addition, AP-2 binds to Clathrin and to the plasma membrane through binding to the characteristic phospholipid PtdIns(4,5)P2 (PIP2), which is characteristic for the inner leaflet of the plasma membrane. The coincidence of the recognition motif in the cargo and PIP2 at the membrane causes conformational change that stabilises the binding of AP-2 and recruits Clathrin. AP-2 cannot induce membrane curvature required for the formations of CCPs. Moreover, although AP-2 is present in most CCPs, CME of TMPs can occur with a lower frequency in its absence (Motley et al., 2003; Pascolutti et al., 2019).

Besides AP-2, other adapters, generally monomeric, are present in the inner coat of nascent CCPs. They mediate the incorporation of proteins that do not directly bind to AP-2. Some of them recognise motifs in their cargo, while others...
recognise modifications, such as phosphorylation or ubiquitylation (Mettlen et al., 2018).

Many of the EAs have additional functions, such as inducing membrane curvature. CALM and AP180 are major monomeric EAs, as abundant as AP-2 and also arrive at the nascent CCP with the same kinetics as AP-2 (Takatori & Tomita, 2019). They bind PIP2 via their N-terminal ANTH domain and Clathrin and AP-2 via their unstructured C-terminus. However, they lack ubiquitin binding domains. Although cell culture experiments suggest that CALM is required for DSL signalling in mammals (Meloty-Kapella et al., 2012b), the genetic analysis fails to confirm their involvement. Loss of function of CALM in *Drosophila* and also mouse does not cause phenotypes that are typical for a defect in Notch signalling (Suzuki et al., 2012b; Zhang et al., 1998). However, it cannot be excluded that they have a redundant or tissue-specific function in signalling.

Another important EA, which is essential for ligand-induced Notch signalling is Epsin. It is encoded by liquid facets (*lqf*) in *Drosophila* (Messa et al., 2014b; Tian et al., 2004; Wang & Struhl, 2004). It consists of an N-terminal ENTH domain, followed by two Ubiquitin Interacting Motifs (UIMs) and an unstructured C-terminus. The C-terminus includes binding sites for Clathrin (Clathrin-binding motif, CBM), for AP-2 (DPW motif), for actin and for EH domain containing EAs (NPF sites), such as the EA Eps15. The ENTH domain binds to PIP2 and can induce membrane curvature (Horvath et al., 2007). Importantly, Epsin can initiate CCP formation in the absence of AP-2 (Legendre-Guillemin et al., 2004). Moreover, due to its ability to bind AP-2, it can also be incorporated in AP-2 containing CCPs together with its associated cargo.

Epsin recognises ubiquitylated cargo via its two UIMs (Hawryluk et al., 2006). Ubi is performed by E3 ligases that transfer ubiquitin polypeptides to the ε amino group of the side chain on lysines (Ks) of cargo, for example, the ICD of DSL-ligands. This transfer can be induced by activation of a signalling receptor, like in the case of Casistas B-lineage lymphoma that binds to phospho-tyrosines in the activated EGF-receptor, or can occur constitutively, like it might be the case for DSL ligands (Swatek & Komander, 2016). A recent study analysing mouse embryonic fibroblasts in which all three Epsin orthologs are inactivated found that CME is severely impaired, indicating that Epsin is an essential player in this process (Messa et al., 2014b). The Epsin-deficient CCPs lack a number of important endocytic factors such as HIPR and, important here, also Dynamin, which executes the abscission of the endocytic vesicle. Moreover, they are unable to coordinate the actin cytoskeleton for proper invagination. Hence, Epsin is essential for the maturation of the CCP, but although possessing curvature inducing abilities, it is not essential for pit formation. However, a recent in vitro reconstitution study shows that Epsin, but not AP-2, is able to support the formation of Clathrin-coated vesicles (Brod et al., 2020). A recent study indicates that Epsin is also involved in Clathrin-independent endocytosis of the EGF receptor (Sigismund et al., 2005). For yeast, it has been shown that its Epsin ortholog ENT1 interacts with Sla2, the ortholog of the ANTH domain containing Huntingtin interacting protein 1 (HIP1) (Takatori & Tomita, 2019). The ANTH domain is similar to and binds to the same phospholipid as the ENTH domain of Epsin. In addition, the ENTH and ANTH domains mediate the interaction between the two proteins. The co-assembly of ENT1 and Sla2 can transform flat membranes into tubules in in vitro experiments, revealing a potent ability for plasma membrane deformation. Whether a similar dimerisation occurs between Epsin and ANTH domain containing proteins, such as HIP1 and CALM, in metazoan cells is an outstanding question.

**Evidence for a role of CME-mediated endocytosis in ligand-dependent Notch signalling**

The first evidence for a requirement of endocytosis in ligand-dependent Notch signalling was provided by the analysis of the function of the gene encoding Dynamin in *Drosophila, shibire* (*shi*) (Poodry, 1990). Loss of *shi* function resulted in death during embryogenesis. Surprisingly, the mutant embryo displayed a hyperplasia of the nervous system at the expense of epidermal tissue, a phenotype termed neurogenic phenotype. This neurogenic phenotype is characteristic for the loss of function of genes encoding elements of the Notch pathway (Lehmann et al., 1983). It turned out that Shi is required on the signal-sending as well as signal-receiving side of the pathway (Seugnet et al., 1997b; Windler & Bilder, 2010a). Later several other components were identified.
Table 1 | Endocytic factors required for signalling of DSL ligands

| Drosophila | Wing imaginal discs | Germline | Mammals | References |
|------------|---------------------|----------|---------|------------|
| Lqf/Epsin  | +                   | +        | +       | Meloty-Kapella et al. (2012a); Messa et al. (2014a); Tian et al. (2004); Wang and Struhl (2004); Wang and Struhl (2005); Windler and Bilder (2010b) |
| Clathrin   | ?                   | −        | +       | (Windler & Bilder, 2010b), (Meloty-Kapella et al., 2012a) |
| Mind Bomb1 | +                   | ?        | +       | (Barsi et al., 2005; Kang et al., 2013; Koo et al., 2005; Koo et al., 2007b; Lai et al., 2005; Le Borgne et al., 2005; Li et al., 2018; Meloty-Kapella et al., 2012a; Wang & Struhl, 2005; Zhang et al., 2011a) |
| Neuralized | +                   | ?        | +       | (Lai et al., 2001; Le Borgne et al., 2005; Lee et al., 2020a; Pavlopoulos et al., 2001a; Song et al., 2006a; Yeh et al., 2001a) |
| Dynamin    | +                   | +        | +       | (Meloty-Kapella et al., 2012a; Okano et al., 2016; Seugnet et al., 1997a; Windler & Bilder, 2010b) |
| Actin      | ?                   | ?        | +       | (Meloty-Kapella et al., 2012a) |
| SNX18      | ?                   | ?        | +       | (Meloty-Kapella et al., 2012a) |
| Auxilin    | +                   | −        | ?       | (Banks et al., 2011; Eun et al., 2008a; Hagedorn et al., 2006a; Kandachar et al., 2008) |
| CALM       | ?                   | ?        | +       | (not required in vivo?) |

*‘+’ is required; ‘−’ not required; ‘?’ not investigated*

that suggested a role for endocytosis during Notch signalling (see Table 1). In various developmental processes of *Drosophila*, a requirement for two E3 ligases, Epsin and also Aux in ligand-dependent Notch signalling was found (Eun et al., 2008b; Hagedorn et al., 2006b; Lai et al., 2005; Le Borgne et al., 2005; Pavlopoulos et al., 2001b; Wang & Struhl, 2004; Yeh et al., 2001b). The fact that Aux was required and that Notch signalling in aux *Drosophila* mutants could be re-established by over-expression of Clathrin, indirectly revealed a requirement for Clathrin in imaginal discs (Eun et al., 2008b). Following studies in mammals revealed a similar requirement for endocytosis in ligand-dependent signalling (Meloty-Kapella et al., 2012b). While they confirm a requirement for Epsin, the E3 ligase Mib1 and Dynamin, they identified the EA CALM and actin as additional factors (Table 1). The requirement of CALM for signalling is not clear. While cell culture experiments suggest a role in Notch signalling, its loss of function in mouse does not cause Notch mutant phenotypes (Takatori & Tomita, 2019; Tehran et al., 2019). Accordingly, also the loss of function in *Drosophila* does not produce phenotypes characteristic for loss of Notch function (Zhang et al., 1998).

It is worth mentioning that AP-2 is not involved in Notch signalling, although present in most CCPs. In *Drosophila*, loss of function of the α-adaptin subunit of AP-2 does not cause a Notch mutant phenotype (González-Gaitán & Jäckle, 1997). Moreover, the analysis of Notch signalling in the female germline, a situation where the signalling cell can be unambiguously separated from the signal-receiving cell, showed that AP-2 is not required (Windler & Bilder, 2010a). Similarly, the loss of AP-2 function in mouse results in death during embryogenesis, but again the mutant phenotypes do not suggest a role in Notch signalling (Tehran et al., 2019). Recent work revealed that different types of CME exist (Pascolutti et al., 2019). In wild-type cells, a small fraction of CCPs is devoid of AP-2, but contains Epsin. In the absence of AP-2, CME is reduced, but occurs. In these remaining events of CME, Epsin plays a central role.

Epsin directly interacts with HIP1 in yeast via their ENTH and ANTH domains, respectively (Skrzynzy et al., 2015). Thus, it is possible that HIP1 plays a role in Notch signalling. In *Drosophila*, only one report has addressed this question so far. It reports that the over-expression of HIP1 affects the activity of the Notch pathway during neurogenesis.
Endocytosis and Notch signaling

Figure 2 | Model of Epsin-Dependent Endocytosis of DSL Ligands During Notch Signalling

The E3 ligases Mib1 or Neur ubiquitylate the ICD of the ligands on Ks. The ubiquitylated ligand is recognised by Epsin/Lqf via its UIMs. Epsin is a central organiser of CCP formation, since it also binds to the plasma membrane and induces membrane curvature alone and in concert with other endocytic factors. In addition, it recruits Clathrin and further endocytic factors, for example, actin, Eps15 and Dynamin. Epsin is also essential for the deeper invagination of the CCP by organising the actin cytoskeleton. The fully formed and invaginated CCP is finally abscised by Dynamin, which is recruited by Epsin and BAR proteins to the neck of the invaginated CCP. Besides Epsin-/Clathrin-mediated endocytosis, there seems to be a pathway that regulates the bulk endocytosis of the ligands. Whereas bulk endocytosis of Dl seems to occur ubi-independently, bulk endocytosis of Ser requires ubi. Which other components are required for bulk endocytosis is not known.

(Moores et al., 2008). However, this link has to be confirmed with loss of function studies. Nevertheless, although loss of function alleles of bip1 are available in Drosophila, no Notch-related phenotypes have been reported so far.

Based on the requirement of endocytosis for Notch signalling, the following model that is founded on results from diverse model systems was suggested (Figure 2). Ligand endocytosis is initiated by ubi of their ICDs by the E3 ligases Neuralized (Neur) and Mind bomb1 (Mib1). The ubiquitylated ligands are recognised by Epsin via its two UIMs. Epsin also recruits Clathrin to generate CCPs and induces membrane curvature in concert with other redundantly acting endocytic factors. Subsequently, it induces the deeper invagination of the pit by organising the actin cytoskeleton. The invaginated CCP is eventually abscised by Dynamin that is probably recruited by Epsin and BAR proteins at the neck of the CCP. Through the interaction of the ligand with Notch on neighbouring cells, the Epsin-mediated CME creates the necessary pulling force to expose the S2 cleavage site of Notch to Kuz/ADAM10 (Figure 1).

Although this model is plausible, closer scrutiny of the evidence suggests a more complicated picture of the role of endocytosis and the requirements of each of the described endocytic components. For example, it appears that the requirement of some components of the endocytic machinery is tissue specific: The analysis in the female germline of Drosophila, a situation where the signal-sending cells can be discriminated from the receiving cell, revealed that while Epsin and Dynamin are essential, Clathrin and Aux are not required for signalling (Banks et al., 2011; Windler & Bilder, 2010a). Hence, it appears that a Clathrin-independent type of endocytosis elicits DSL signalling in this tissue. Nevertheless, it has to be mentioned that, during oogenesis, Clathrin is required on the receptors side of the pathway during signalling (Windler & Bilder, 2010a).
The analysis of endocytosis and activity of Ser and Dl in mib1 and epsin/lqf mutants indicates a more detailed picture. Endocytosis of Dl is only very mildly affected upon loss of mib1 or lqf (Overstreet et al., 2004; Wang & Struhl, 2004; Wang & Struhl, 2005). In contrast, endocytosis of Ser is nearly abolished upon loss of function of mib1, but only mildly affected by loss of epsin/lqf function (Le Borgne et al., 2005; Wang & Struhl, 2005). Hence, there appears to be a pathway that regulates the bulk of ligand endocytosis (bulk endocytosis) and an Epsin-dependent one that elicits ligand-dependent signalling (Le Borgne et al., 2005; Wang & Struhl, 2005). The Epsin-dependent pathway constitutes only a fraction of endocytic events. In Drosophila, it was revealed by experiments where the endosomal pathway was challenged by over-expression of a ligand (Le Borgne et al., 2005; Wang & Struhl, 2005). In the light of this finding, it is interesting that a small percentage of CCPs in mouse embryonic fibroblasts does not contain AP-2 and depends on the activity of Epsin (Pascolutti et al., 2019). Perhaps, these relatively rare Epsin-dependent, but AP2-independent CME events mediate DSL signalling. This would explain the weak effect of loss of Epsin function on endocytosis of the ligands and why the Epsin-mediated endocytosis is limiting in Drosophila.

In the next sections, we will discuss new results that refine the current model of the role of endocytosis in ligand-dependent activation of Notch. We focus on two central questions: the role of ubi and how the pulling force is generated. A Drosophila centred view is presented, since most of the results are obtained in this system.

What is the function of the E3 ligases?
Several experiments indicate that the DSL ligands are ubiquitylated during Notch activation (e.g., see Daskalaki et al., 2011; Zhang et al., 2011b). Generally, Ubi is mediated by a cascade of 3 types of enzymes (E1-3) with the E3 ligases being the specific end point that transfers the activated ubiquitin to Ks of a substrate (Swatek & Komander, 2016). There are multiple ways of ubi: adding one ubiquitin to a single K is termed mono-, adding more than one ubiquitin to a single K is referred to as oligo-, adding a single ubiquitin to several Ks of a substrate is multi- and adding a chain to a K is poly-ubiquitylation. For Dl, it has been reported that either multi- or poly-ubiquitylation occurs (Daskalaki et al., 2011). Taking into account that the UIMs of Epsin1, which is essential for signalling, recognise poly-ubiquitin chains, Dl and Ser are probably poly-ubiquitinated by the E3 ligases (Hawryluk et al., 2006). Cell culture experiments identified ubi of a single K, K63, as essential for signalling of Dll1 (Zhang et al., 2011b). In the case of Dl, the loss of a single evolutionary conserved K in its ICD had no noticeable effect on signalling (Daskalaki et al., 2011). For Ser, two Ks have been identified that are essential for function, although it has not been tested whether these Ks are ubiquitylated (Glittenberg et al., 2006). These data suggest that ubi of several Ks in the ICDs of DSL ligands is needed, which act in a redundant fashion during signalling. However, this notion has to be proven.

Two conserved E3 ligases have been identified to be involved in ligand-dependent Notch signalling, termed Mib1 and Neur (Eun et al., 2008b; Hagedorn et al., 2006b; Lai et al., 2005; Le Borgne et al., 2005; Pavlopoulos et al., 2001b; Wang & Struhl, 2004; Yeh et al., 2001b). Both possess a Really Interesting New Gene (RING) Finger domain (RF) that binds a ubiquitin-conjugated E2 enzyme and transfers the ubiquitin moiety onto Ks of the ligand ICDs. Beyond the RFs, the two E3 ligases share no obvious sequence homologies. In Drosophila, both are required in predominantly complementary subsets of Notch-mediated processes in the signal-sending cell.

Mib1
Mib1 is a large cortical protein of around 1200 amino acids that contains an MZM and REP domain in its N-terminus, followed by 8 Ankyrin repeats and three RFs (Figure 3A). Only the last (third) RF is required for function (Lai et al., 2005; Le Borgne et al., 2005). The MZM and REP domains are responsible for the binding to the ICD of the ligands. Recently, the detailed study of the binding of Mib1 to Jagged1 (Jag1) has revealed a bipartite binding mode of Mib1 (McMillan et al., 2015). Two epitopes in the ICD of Jag1, termed N- and C-box, interact with the MZM and REP domains of MIB1, respectively. These interactions are essential for signalling. Corresponding sequences were also identified in the ICD of Dl, termed ICD2 (= N-Box) and ICD3 (= C-Box) (Daskalaki et al., 2011) (Figure 3B). They are conserved among the ICDs of insect Dl orthologs. However, in contrast
Endocytosis and Notch signaling

Figure 3 | Domain Organisation of Drosophila E3 ligases Mind bomb1 (Mib1) and Neur (Neur) and of the DSL ligands intracellular domains (ICDs)

(A) Both E3 ligases belong to the class of RING containing E3 ligases, meaning they contain at least one C-terminal catalytic RING domain that catalyses the ubiquitination of the ICD of the DSL ligands. Mib1 possesses two additional RING domains, eight Ankyrin repeats, as well as two N-terminally located ligand binding domains, termed MZM and REP, that bind to the N- and C-box in the ligand ICDs, respectively. Neur has two N-terminal Neuralized Homology Repeat (NHR) domains. NHR1 binds to a motif in the ICD of the ligands with a NxxN consensus sequence. The less well-understood NHR2 domain appears to be also essential for full E3 activity. Together with NHR1, the NHR2 domain contributes also to oligomerisation of Neur. (B) The ICD of Dl is composed of an N-terminal NxxN consensus motif (Neuralized-Binding Motif (NBM)), followed by the N- and C-boxes, that are responsible for the interaction with MZM and REP-domains of Mib1. In the case of Dl, only the N-box seems to be important for ligand function. Similar to the Dl-ICD, the ICD of Ser possesses predicted NBM, N- and C-Boxes. However, the functionality of the boxes has not been demonstrated. For comparison, the corresponding sequences of the predicted boxes in the mammalian ligands are shown. For Dll1 and Dll4, no C-box-like sequences have been found, but it is possible that non-related sequences can functionally replace the C-box in these ligands.

Neur

Neur is a peripheral membrane protein that possesses several domains important for its function apart from its RING Finger (RF) at the C-terminus (Figure 3A; Liu & Boulianne, 2017). It has two Neuralized Homology Repeats (NHRs) N-terminal to its RF. NHR1 is required for the binding of Neur to the ICD of both ligands and also for its homo-oligomerisation. It recognises a NxxN consensus motif (Neur-box, NEQN in Dl), which lies N-terminally to the N-box in the ICD of Dl and the predicted N-box of Ser (Bardin & Schweisguth, 2006; Comisson & Boulianne, 2007; Daskalaki et al., 2011; Fontana & Posakony, 2009; He et al., 2009; Liu & Boulianne, 2017) (Figure 3B). The function of the NHR2 is less well defined, but appears to contribute to the full ubiquitination activity and, together with NHR1, to oligomerisation of Neur (Liu et al., 2012). A basic stretch at the N-terminus, before NHR1, reaching from aa82-87 (KKIKKR) mediates the binding to specific phospholipids of the plasma membrane (Skwarek et al., 2007). During Drosophila wing development, Neur can compensate for the loss of mib1 function, suggesting that they have a similar function during DSL signalling (Berndt et al., 2017; Le Borgne et al., 2005; Wang & Struhl, 2005). In vitro experiments suggest that Neur and also its mammalian orthologs Neurl1 and Neurl2 undergo auto-ubiquitination (Koutelou et al., 2008; Yeh et al., 2001b). It has also been shown that Neurl2 cooperates with Mib1 to activate Dl in mammalian cell culture (Song et al., 2006b).

The function of Neur in Drosophila is largely restricted to neural processes, such as selection of neural precursors in the central and peripheral nervous system. During these processes, Neur is expressed in a restricted pattern or single cells (Yeh et al., 2001b). In contrast, Mib1 is ubiquitously expressed, indicating that most Notch signalling is mediated by Mib1. While Neuralized is clearly involved in Notch signalling in the vertebrate model Xenopus laevis (Deblandre et al., 2001), its role in mammalian Notch signalling is not clear. Even the concomitant loss of...
function of Neurl1 and Neurl2 does not produce the developmental defects seen in Notch mutants, suggesting that it is not involved in Notch signalling during development (Koo et al., 2007a; Weinmaster & Fischer, 2011). On the other hand, cell culture experiments revealed that Neurl2 can interact and ubiquitylate Dll1 and Neurl1 can activate Jag1 (Weinmaster & Fischer, 2011). Moreover, a putative consensus binding site for Neur is present in the ICD of most Dll1 and Jag1 orthologs at the expected site (Fontana & Posakony, 2009). It is possible that the requirement of Neurl1 and 2 is restricted to post-embryonic processes. Good candidates are processes underlying learning and memory where Neur and Notch are shown to be involved (see Lee et al., 2020b and citations therein).

In general, E3 ligases have several substrates. For example, Neur regulates epithelial morphology by interacting with Stardust to down-regulate Crumbs via endocytosis (Perez-Mockus et al., 2017). Moreover, Neurl1 also poly-ubiquitylates the cGMP-specific phosphodiesterase 9A to label it for degradation (Taal et al., 2019). Mib1 is shown to bind and to ubiquitylate EPB41L5, resulting in its degradation (Matsuda et al., 2016).

How important is ubi during ligand-induced Notch signalling?

Cell culture experiments indicated that Mib1 and Neur can ubiquitylate Dl, as well as Ser and that ubi of at least Dl depends on the presence of Ks in its ICD (Berndt et al., 2017; Daskalaki et al., 2011). Moreover, the two E3s are required for the activity of the ligands, suggesting that ubi is an important event during DSL signalling. Recent work conducted with Dl in Drosophila elucidated some unexpected twists in the importance of ubi. It showed that although ubi is required for its full activity, Dl can also signal independently of ubi in a biologically significant manner (Berndt et al., 2017). To demonstrate this, a variant of Dl, which cannot be ubiquitylated because all Ks of its ICD were replaced by the similar arginine was used (DIK2R-HA). This analysis revealed that DIK2R is weakly active, suggesting that ubi is not absolutely required for signalling. In agreement with this notion, it was also found that Dl can weakly signal in mib1 mutants. Two naturally occurring developmental processes that depend on Dl/Notch signalling were identified that are largely independent of ubi: the selection of the sensory organ precursor and the asymmetric division of the ganglion mother cell in the brain occurred also when Dl was replaced by DIK2R. Hence, Dl appears to be able to signal in a biologically significant manner without ubi of its ICD. Nevertheless, ubi of Ks in the ICD of Dl is clearly required for its full activity. While it is known that more than one K has to be ubiquitylated, it is not known how many and which Ks in Dl must be ubiquitylated.

It has to be noted that the consequences for endocytosis of Dl are weak if its ubi is abolished. DIK2R is only slightly less efficiently endocytosed compared to Dl and a Dl variant that lacks the Mib1 binding N-box is endocytosed (Berndt et al., 2017; Daskalaki et al., 2011). This is in line with the previous observation that loss of function of mib1 or epsin/lqf does not strongly affect the endocytosis of Dl, although it strongly reduces its ability to signal (Le Borgne et al., 2005; Wang & Struhl, 2004; Wang & Struhl, 2005). A similar behaviour was found for a K-free version of the mammalian Dl-ortholog Dll1, termed Dll1K17R, in cell culture experiments (Chastagner et al., 2017; Heuss et al., 2008). In summary, the work so far indicates that, in the case of Dl, the link between ubi and endocytosis is not absolute. Moreover, it confirms that one has to discriminate between bulk endocytosis that is irrelevant for Notch activation and a distinct activating pathway that requires only a fraction of the available Dl and depends on Epsin.

Another important conclusion is that, in the event of bulk endocytosis, Dl must be linked to the endocytic machinery in an ubi-independent manner. Either other unknown EAs connect Dl to the endocytic core machinery, or it can interact directly with elements such as AP-2, as it has been shown, for example, the transferrin receptor. However, a sequence analysis of the ICD of Dl did not identify any of the so far identified recognition motifs for AP2 or Clathrin. Recent work revealed that bulk endocytosis at synapses occurs independently of Clathrin and Dynamin (Wu et al., 2014). Thus, it is also possible that bulk endocytosis of Dl occurs via a Clathrin-independent pathway.

One caveat of the results obtained with DIK2R is that they were obtained with strong Gal4 overexpression and have to be confirmed with the used Dl variants expressed at endogenous level to exclude
Endocytosis and Notch signaling

Review

artefacts. Despite this caveat, the findings make sense in the light of evolution. The comparison of the presence of the Notch pathway components in metazoan phyla suggests that DI is the more ancient of the two DSL ligand subtypes. It was probably a crucial element of the Notch signalling system before Mib1 was recruited to the system (Gazave et al., 2009). Hence, for a certain time in evolution, DI had probably activated Notch without Mib1 and ubi. Interestingly, a clear Mib1 ortholog is missing in the clade Placozoa, although DI and Notch are present.

In contrast to DI, the activity and endocytosis of Ser, which apparently has been added to the Notch system after appearance of Mib1, completely depends on Mib1 function (Berndt et al., 2017; Le Borgne et al., 2005; Wang & Struhl, 2004). Thus, in the case of Ser, a clear positive correlation exists between ubi by Mib1, endocytosis and activity. Nevertheless, the loss of epsin/lqf function, which is also absolutely essential for Ser signalling, affects the endocytosis of Ser only very mildly (Wang & Struhl, 2005). Thus, also for Ser, a bulk- and signal-relevant (Epsin-dependent) endocytosis can be separated. In contrast to DI, also bulk endocytosis of Ser depends on ubi. This implies that another so far unidentified EA, which can bind to ubiquitin, is required for the bulk endocytosis of Ser. So far only a few EAs are known to fulfil these criteria during CME, e. g. Eps15 and Eps15R (Tehran et al., 2019).

A complication of this issue is that the ICD of Ser actually contains a conserved dileucine-based sequence that fits the consensus for binding to AP-2 (Glittenberg et al., 2006). Interestingly, the loss of this sequence nearly abolishes endocytosis, but has weak effects on Ser signalling. Thus, it is possible that AP-2 is involved in Ser bulk endocytosis, but only in context with an EA that can recognise the ubiquitilation status of Ser.

The analysis of the di-leucine motif of Ser indicates that a possible reason why the EAs for bulk endocytosis of Ser and DI have not yet been identified, is that their loss of function probably does not affect Notch signalling and, hence, does not cause a ‘Notch-specific’ phenotype.

The different phenotypes of mib1 and epsin/lqf mutants highlight that only a small fraction of ubiquitylated Ser enters the Epsin-dependent pathway that leads to ligand-dependent activation of the Notch pathway. It is unlikely that discrimination between bulk and Epsin-regulated endocytosis occurs via differential Mib1-mediated ubi, as loss of its function abolishes both endocytosis pathways. We therefore favour the possibility that Epsin is the limiting element in this process. It can only bind to a fraction of the available ubiquitylated Ser (and DI) to mediate a distinct endocytic event that is required for pulling force generation during DSL signalling. In this light, it is interesting that a small fraction of CCPs have been observed in mammalian cells that contain Epsin, but not AP-2 (Pascolutti et al., 2019). Perhaps only these rare CME events are relevant for signalling.

Which feature is responsible for the activation of Notch via the Epsin pathway? Experiments with the extra-cellular ligand binding domain of Notch1 fused to the immunoglobulin Fc fragment (N1Fc) revealed that while soluble N1Fc endocytosis by cells does not require Epsin, N1Fc linked to PrtA beads does (Meloty-Kapella et al., 2012b). This finding also revealed the existence of two endocytic pathways and suggests that Epsin-mediated pathway generates more force, that enables a cell to incorporate larger particles. This notion is strongly supported by elegant recent in vivo experiments in Drosophila (Langridge & Struhl, 2017). Thus, only the Epsin pathway is able to generate the necessary force for Notch activation.

The alternative model for function of endocytosis during Notch signalling

Although it is now well established that endocytosis is required for generation of a pulling force, an alternative, non-exclusive model for the role of endocytosis in DSL signalling has been proposed (Heuss et al., 2008; Parks et al., 2000; Wang & Struhl, 2005). It states that, via endocytosis, the ligands enter a recycling pathway in which they become modified. Only in this modified state, they can activate Notch after their return to the plasma membrane. Our recent work provided evidence that a recycling function of endocytosis might be required in addition and previous to pulling force generation (Berndt et al., 2017). We found that the loss of Ks in its ICD strongly increases the cis-inhibitory abilities of DI. Moreover, CI by the ligands is also increased in mib1 mutants. The increase of CI probably contributes to the observed corresponding loss of Notch activity in mib1 mutants. At the same time, the signalling abilities of
The ligands are strongly reduced in the mutant. These findings suggest that Mib1 induced endocytosis is required to suppress CI of the ligands. CI is caused by the interaction of the ligand with Notch on the same cell (Klein et al., 1997; Sprinzak et al., 2010). Assuming that newly synthesised Dl arrives in cis-association with Notch on the cell surface (cis-pair), we speculate that Mib1 suppresses CI by initiating the endocytosis of the cis-pair. During recycling, the pair is separated, possibly in the acidic environment of the lumen of the EE. Dl, released from the cis-pair, returns to the plasma membrane and can now activate Notch on the adjacent cell. Thus, in this scenario, Mib1 has two functions: to induce the recycling required for separation of the cis-pair and subsequently to induce the pulling force by initiating the endocytosis of the ligand bound to Notch.

An additional function of Neur
The analysis of DlK2R also revealed that Neur can activate Dl in a manner that is independent of ubi of the ICD of Dl (Berndt et al., 2017). The crucial finding is that co-expression of Neur and DlK2R in mib1 mutant wing imaginal discs resulted in a strong activation of the Notch pathway that is comparable to co-expression of Neur with Dl. Thus, it appears that the Ks in Dl and therefore ubi of Dl ICD is neglectable for Dl signalling induced by Neur. The ubi-independent activation requires the direct binding of Neur to the ICD of Dl. It further requires the RF domain of Neur, suggesting that ubi of a substrate other than Dl is required. So far there is no obvious candidate that is ubiquitylated by Neur other than Dl. However, it has been shown that Neur can undergo auto-ubi (Koutelou et al., 2008; Yeh et al., 2000). An attractive possibility is that Neur serves as a co-adapter that binds to Dl and, if ubiquitylated interacts with Epsin/Lqf via its UIMs. In this way, Neur would activate Dl in a manner independently of ubi of its ICD (Figure 4).

Force generating steps in endocytosis
Overwhelming evidence supports the notion that endocytosis generates the pulling force exerted by the
ligands to activate the Notch mechano-receptor in many processes (see, e.g., Henrique & Schweisguth, 2019). This pulling force is probably generated through invagination and subsequent scission of a spot of plasma membrane in which ligands are inserted in the process of endocytosis.

Recent studies indicate that membrane invagination during endocytosis can be subdivided into an early phase where the endocytic components assemble on a flat membrane to generate a shallow CCP and a later phase where the pit transforms into a deeper dome-shaped invagination, which is eventually abscised. The initial invagination is achieved by induction and stabilisation of membrane curvature by the interplay of the forming coat consisting of early endocytic factors and Clathrin (Haucke & Kozlov, 2018). In principal, there are two mechanisms to induce membrane curvature. One is the hydrophobic insertion or wedging mechanism. The insertion of a hydrophobic loop or helix into the inner leaflet of the plasma membrane results in a tendency to bulge the membrane in the direction of the insertion. Among the known endocytic factors, Epsin uses this mechanism. The amphiphatic helix0 in the membrane binding ENTH domain of Epsin inserts into the membrane to induce curvature (Legendre-Guillemin et al., 2004). Endocytic proteins that act like this contribute to the induction of the initial shallow pit. This ability of Epsin is in apparent contrast to its reported loss of function phenotype (Messa et al., 2014b). CME is impaired at an early pit stage, indicating that curvature is generated, but the CCP does not transform into deeper invaginations. These observations indicate that Epsin has an essential function for the progression of the invagination of the CCP, but not its initiation. In support of this notion is also a thorough structure/function analysis of the *Drosophila* Epsin, Lqf, which revealed that the curvature inducing ENTH domain is of minor importance for DSL/Notch signalling (Xie et al., 2012). However, it is clear that several curvature-inducing proteins act redundantly in the early phase of CCP formation. These proteins probably compensate for the loss of the Epsin function and obscure its contribution to curvature induction.

Some of these other early acting proteins use the other way to generate local membrane curvature, the scaffolding mechanism. This involves the formation of a rigid intrinsically bent scaffold that interacts with the membrane and enforces curvature on it (Haucke & Kozlov, 2018). Examples for such scaffolding proteins are the family of F-BAR domain containing proteins, such as the early acting endocytic components FCHo1 and FCHo2. These F-BAR-domain proteins induce curvature of the membrane by mere formation of a lattice on the membrane and probably induce the first shallow endocytic pits. They act in a redundant manner indicated by the fact that, at least in the investigated cases, their individual inactivation has no detectable effect on endocytosis (Tehran et al., 2019).

The concomitant recruitment of Clathrin by these early acting factors is likely to stabilise membrane curvature. Clathrin can either assemble into flat or curved lattices, depending on whether it assembles into lattices that contain only Clathrin hexagons (flat) or hexagons and pentagons (curved). This flexibility can stabilise several intermediates of the pit. Notably, Epsin can recruit Clathrin, even in the absence of any other EA (Ford et al., 2002).

Actin is required for the further membrane bending and progress to deeper invaginations. Actin filament formation is the likely mechanism to create the force to advance from a pit to a dome either alone or in concert with motor proteins. Polymerisation at the plasma membrane generates a flow of actin filaments from the base of the dome-shaped invagination towards the tip. To contribute to membrane curvature the polymerisation must be coupled to the membrane. This is probably achieved through binding to the APs. In yeast, polymerisation of a branched actin network is essential role for endocytosis (Kaksonen & Roux, 2018; Mund et al., 2018). The network at the site of endocytosis is initiated by a peripheral ring-like assembly of the actin nucleation factor Wiskott–Aldrich syndrome protein (WASP), which is recruited by endocytic factors such as SNX9. WASP in turn recruits the ARP2/3 complex for induction of the branched actin filament network at the site of invagination. The branched polymerisation of actin is thought to generate a pushing force at the edge that deepens the invagination. In this model, the binding of Epsin to elongating actin filaments at the clathrin-coated opposite located tip of the invagination creates an additional pulling force (Kaksonen & Roux, 2018; Trylinski & Schweisguth, 2019). Epsin is known to interact with actin via the DPW and NPF regions in its C-terminus (Kaksonen...
It is therefore likely that the loss of *Epsin* function leads to a failure to generate the pulling force and therefore to a failure of the CCP to advance into later deeper invaginated stages. Although, it is likely that this pulling force inducing function of Epsin is relevant for DSL signalling, this assumption has to be validated, for example, by demonstrating that a variant of Epsin that cannot interact with actin fails to mediate DSL signalling.

Note, however that in metazoans, the requirement of WASP and ARP2/3 and therefore probably the pushing force for endocytosis is not absolute. It has been proposed that a pushing force caused by branched actin polymerisation is required only in situations of high membrane tension (Kaksonen & Roux, 2018; Mund et al., 2018; Trylinski & Schweisguth, 2019). In yeast, high membrane tension is caused by the turgor and therefore the contribution of the WASP and ARP2/3-mediated pushing force is essential. In metazoans, the activity of WASP and ARP2/3 is probably only essential in situations where membrane tension is extraordinarily high, for example, during cytokinesis, to allow endocytosis to occur (Trylinski & Schweisguth, 2019). Hence, its requirement for Notch signalling is restricted to these situations. One example is the lineage of the sensory organ precursor of *Drosophila* (see, e.g., Trylinski & Schweisguth, 2019). In most situations, the tension appears to be low enough to allow Notch signalling to be induced solely by an Epsin-mediated pulling force.

Dynamin is involved in endocytosis and also in Notch signalling and has been shown to contribute to the pulling force (Meloty-Kapella et al., 2012b). Its well-established role is the fission of the invaginated CCPs. However, it is not clear how the abscission of an endocytic vesicle contributes to the generation of a pulling force. It is possible that the rapid constriction generates a short peak of force necessary for the change in the NRR. However, at the time of abscission, the pit is already maximally invaginated (Ferguson & De Camilli, 2012). Thus, a strong increase is not to be expected. Moreover, previous work showed that Dynamin actually prevents exaggerated invagination of the pit, as its loss of function results in super-invaginated pits (Wu et al., 2014). The Clathrin coat of these super invaginated pits is restricted to the tip of the invagination and its elongated neck is decorated with actin, as well as curvature sensing BAR proteins, for example, SNX9 and Endophilin2. Intuitively, it is difficult to understand that this exaggerated membrane invagination is not sufficient to create the necessary pulling force for activation of Notch, especially since the ligand in these invaginations is probably located in the Clathrin-coated tip. Thus, although clearly required, it is not really clear how Dynamin contributes to the generation of the ligand-mediated pulling force. A solution to this conundrum could be the so far less appreciated and not understood role of Dynamin during earlier phases of endocytosis (Mettlen et al., 2018). For example, in mammalian cells, overexpressing a version of Dynamin1 that is defective in self-assembly (and therefore abscission) accelerates CME. This finding suggests that non-assembled Dynamin is involved in the regulation of early steps of CME. It is possible that this early function is relevant in force generation during DSL signalling. Future work should investigate this avenue.

**Conclusions and outlook**

Endocytosis is an essential part of the mechanism of ligand-dependent Notch signalling. The recent work shed light on so far outstanding questions. It showed that the correlation between ubi and endocytosis is not absolute. It revealed how Mib1 binds to the ICDs of Dl and Jag1. Moreover, it revealed that there are differences in the activity and/or regulation of the two types of ligands, in *Drosophila*, Ser and Dl. So far, the similarities were more emphasised than the differences by showing that both ligands can activate the Notch pathway in a similar manner, for example, during *Drosophila* wing development (Le Borgne et al., 2005; Wang & Struhl, 2005). The recent work also revealed different requirements for ubi among Ser and Dl, which are the founders of the two sub-families of DSL ligands. While Ser probably requires ubi for signalling and also for bulk endocytosis, Dl signalling is partial and bulk endocytosis completely independent on it. It further revealed that the ICD, via ubi, adjusts the strength of the cis-inhibitory abilities of Dl. Whether this also holds true for Ser, is not known and should be investigated.

As always, writing a review raises more questions that should be answered in the future. A selection of these questions is listed in the following.
Endocytosis and Notch signaling

(1) The analysis of Dl and Jag1 has revealed that Mib1 binds to related N- and C-boxes in their ICDs. Although all ligands depend on the function of Mib1, it is not clear whether this bi-partite binding mode applies to the ICDs of the other mammalian ligands and also Ser. In total, there are five mammalian ligands whose ICDs, at least by direct sequence alignment, do not appear to have always corresponding N- and/or C-boxes. It is possible that they have functional correlates for at least one of the boxes that are not recognisable by sequence comparison. (2) For Dl, it has been shown that Ks in the ICD are important for its full function. It should be determined how many and also which of the 12 Ks are important. This should be then extended to other ligands to see whether there are general rules in ubi. (3) It should be determined whether the relevant Ks of the ligands are poly- or multi-ubiquitylated. (4) Another unexpected new avenue to explore is the recently discovered mechanism of Dl activation by Neur that is independent of the ubi of its ICD. Can Neur also activate other ligands in this manner? Is Neur acting as a co-adapter as suggested here and does it also act as a classical E3 ligase? (5) It is clear that Epsin-dependent endocytosis is crucial. This pathway should be investigated in more detail. One urgent question is, whether its binding to actin is important for signalling. (6) The function of Dynamin has to be specified in more detail: Is the so far not appreciated role in early phases of endocytosis or the established abscission function important for signalling? (7) It is not known how bulk endocytosis of the ligands occurs and whether the E3 ligases can – via ubi of different Ks – determine whether the ligands enter the pathway for signalling or bulk endocytosis. Addressing this question might also reveal whether the choice of the type of endocytosis is influenced by the interaction of the ligand and Notch in cis and trans. (8) So far, the model for the function of endocytosis and ubi depends on data obtained from different model systems, such as cell culture, mouse, the nematode C. elegans and Drosophila. It should be rigorously tested in one model system. Analysis in the female germline of Drosophila showed that some signalling events depend on an endocytic event that requires Epsin, but not Clathrin and Aux. In contrast Clathrin and Aux are required for signalling in the disc epithelium. Thus, different Clathrin-dependent and -independent endocytosis mechanisms can elicit Notch signalling events, even in one organism in different tissues. The different underlying mechanisms should be analysed in more detail.

Thus, although it is well established that endocytosis is important for ligand-dependent signalling through the Notch pathway, many important questions remain open and have to be answered in the future. Answering them will enable us to intervene more precisely in the activity of the Notch pathway. Given its importance in many pathological processes in humans, the efforts are necessary and will be rewarded.

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Review

**Endocytosis and Notch signaling**

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