Chapter 13

Endocytosis in Notch Signaling Activation

Elisa Sala, Luca Ruggiero, Giuseppina Di Giacomo and Ottavio Cremona

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/48767

1. Introduction

In mechanistic terms, endocytosis is the process by which plasma membrane (PM) components, together with extracellular solutes, macromolecules and particles, are internalized in the cell. Once the endocytic vesicle (or vacuole) is formed by fission of the PM, it is generally delivered to a specialized membrane compartment – the endosome – for recycling, degradation or re-routing.

In cell-physiological terms, endocytosis exerts multiple functions, which are only partially known and characterized. At a minimum, it maintains PM homeostasis by counterbalancing the apposition of new membrane (due to exocytosis) and by renewing PM components. More extensively, endocytosis constantly modulates PM composition and takes an active part in a variety of normal and pathological cell processes, including cell nutrition, cell motility, mitosis, neurotransmission, immune response, and microorganism entry (reviewed in [1-8]).

1.1. Endocytosis and signaling

In recent years, much of the effort to investigate this extensive endocytic activity has been focused upon unveiling the reciprocal interplay between endocytosis and cell signaling. In this introductory section, we provide a quick overview of the key concepts in the field to explain the endocytic function in Notch signaling. We refer those readers who wish to explore the relationship between endocytosis and cell signaling in details to other papers in this volume, and to recent reviews in the field [3, 9-11].

Originally, endocytosis was linked to the termination of PM-generated signals by reducing the availability of membrane receptors for ligand binding, and by degrading the ligand-receptor complex (reviewed in [10, 12-14]). Hence, blockage or dysregulation of endocytosis closely correlates with increased cell proliferation by the activation of receptor-tyrosine kinase pathways and cancer promotion (reviewed in [10, 15, 16]). More recently, new strategies for the endocytosis-mediated regulation of signaling have been uncovered: (i) endocytosis can

activate/modulate some PM-generated signals either directly (e.g. by controlling ligand availability, as in the case of the cell-to-cell Eph/Ephrin signaling pathway [17]), or indirectly (e.g. by regulating the composition of specific signaling platforms, as in the case of phospholipase C and PI3Kinase signaling activated via EGF receptor [18]); (ii) endocytosis can propagate signals to intracellular compartments, especially the endosomal compartment, where these signals are sustained, specified, spread over long distances or rerouted (reviewed in [9, 19-22]); (iii) endocytosis can ensure spatial restriction to signaling responses emanating from the PM and/or from the endosome (e.g. the endocytosis/recycling function in the spatial restriction of signaling controlling migratory programs (reviewed in [23], or in determining the timing, levels, and localization of guidance receptors, thus determining the outcome of guidance decisions [24]). On the other hand, signaling can modulate endocytosis: (i) activation of specific signaling pathways can upregulate or downregulate endocytosis, thus modulating other PM- and/or endosome-generated signals (e.g. EGF receptor activation increases SRC kinase-mediated phosphorylation of the clathrin heavy chain, which redistributes to the cell periphery, potentiating endocytosis [25]); (ii) actin dynamics/signaling takes an active part in the endocytic reaction by helping membrane invagination [26, 27], vesicle transportation [28, 29], and endosomal microdomain organization [30]. We will see that many of these endocytic strategies to control signaling are exploited in Notch signaling.

1.2. Types of endocytosis and their regulation

In order to promote its many functions, endocytosis relies on a variety of specialized mechanisms and accessory factors to guarantee selectivity, vectoriality and plasticity.

Regarding these mechanisms, there are multiple forms of endocytosis that act concomitantly in the cell (reviewed in [4, 31-33]). The best studied, and perhaps the most common, forms are clathrin-based. Their central paradigm is the recruitment and assembly of clathrin to the PM, triggered by a variety of adaptor proteins, which bind (and sometimes bend) the PM by means of lipid- and protein-interacting domains (reviewed in [34]). Invagination of the PM to form a bud depends on a concerted action of the clathrin lattice rearrangement (which shapes the high curvature profile of the bud [35]), polymerization of bending proteins (which shape the neck of the bud (reviewed in [36])), and actin polymerization (which helps the extension and constriction of the neck of the bud [37, 38]). Constriction of the bud and its fission to form free clathrin-coated vesicles requires the pinchase action of the GTPase dynamin(s) (reviewed in [39-41]). Free vesicles are then stripped of their coat by an uncoating complex, composed of the ATPase heat shock cognate 70 (HSC70) [42] and the J-domain-containing co-chaperone auxilin [43]. Structural requirements for the uncoating reaction are reviewed in [44, 45]. An essential function is also carried out by phosphoinositides, and more specifically by the PtdIns(4,5)P_2 present in the membrane of coated vesicles, which has to be hydrolyzed by synaptojanin for efficient release of endocytic adaptors, which precedes clathrin disassembly [46, 47].

Clathrin-mediated endocytosis (CME) is not the only type of endocytosis. It is now many years since evidence for clathrin-independent endocytosis (CIE) has been accumulated,

although the mechanism behind this process is as yet poorly characterized. The development of specialized techniques, reagents and markers to trace endocytosis has unveiled a whole new world of internalization routes that persist after inhibition of the clathrin function (reviewed in [32, 48-50]). A common finding is the exquisite sensitivity of CIE to cholesterol depletion, although CME is also somewhat sensitive to cholesterol levels, and some forms of CIE can still occur without membrane cholesterol [51]. On the PM, cholesterol is transiently enriched in microdomains, commonly known as lipid rafts [52]. This fact, together with the absence of rafts in clathrin intermediates and with the observation that most of the raft components are endocytosed by non-clathrin-dependent pathways, has led to the idea that most CIE occurs in these lipid microdomains. At rafts, signaling events are subcompartmentalized in specific nanoplatforms, whose composition and, therefore, activity is continuously changing [52]. The wealth of proteins that participate in raft signaling events also gives rise to a number of different CIE pathways which differ for (i) fission machinery (i.e. dynamin-dependence), (ii) coat composition and (iii) Rab effector specificity (reviewed in [4, 32, 50]). Both CME and CIE forms participate in Notch signaling activation and regulation.

Regarding accessory factors, tens of molecules, both proteins and lipids, interact with endocytic machinery at various stages. Several recognition modules have been identified, including protein-lipid (e.g. PH domain) and protein-protein interaction modules (e.g. BAR, SH3-, proline rich-, EH-, coiled-coil domains, ubiquitin interacting motifs (UIM)). It is conceivable that this array of interactions may help regulate both CME and CIE by: (i) assisting coat assembly/disassembly, (ii) regulating membrane shaping/sculpting and fission, and (iii) mediating interaction of the coat with signaling molecules and the cytoskeleton (reviewed in [4, 9, 34, 53-55].

In this review, we will focus on the molecular details at the basis of the endocytic control of Notch activation. Specific emphasis will be made on genetic data in mammals and invertebrates that support or validate *in vitro* interaction and/or models. Since dysregulation of Notch signaling contributes to the multi-step progression of a variety of cancers by inducing uncontrolled proliferation, the possible therapeutic value of this information can be clearly envisaged.

2. Overview of the Notch signaling pathway

The first Notch gene was identified in *Drosophila melanogaster* by J.S. Dexter, in T.H. Morgan's laboratory about a century ago, as a dominant mutant with a peculiar toothed/notched wing margin in heterozygosity [56]; this phenotype was later associated with additional defects, including thickened wing veins, and bristle abnormalities [57]. In hemizygosity, or in homozygous females in flies, Notch loss-of-function mutations are embryonic lethal with neuralization of important parts of the ectoderm, leading to hypertrophy of the central nervous system and corresponding hypotrophy of the epidermis of the fully developed embryo [58, 59]. As anticipated by these data, in higher metazoan the Notch pathway is one of a handful of signaling pathways (including Wnt/wingless,

BMP/TGF-beta, Sonic Hedgehog, receptor tyrosine kinases, nuclear receptors, JAK/STAT) that act reiteratively in cell fate decision and determination in tissues that derive from all three germ layers (reviewed in [60, 61]). After development, Notch signaling is required for the homeostasis of tissues and stem cells, as underscored by the high number of tumors associated with Notch signaling dysregulation, which was an early finding in Notch research in mammals.

The Notch signaling is a cell-to-cell communication pathway that is activated when Notch ligands (**D**elta/**S**errate/**L**ag2-DSL in invertebrates and Delta-like/Jagged, in mammals) on the sending cell bind to Notch receptor(s) on the receiving cell. This triggers a sequence of proteolytic cleavages, which starts with an ADAM-mediated cleavage at **s**ite **2** (S2) [62]. While ADAM17/TACE seems to be the main metalloprotease able to cleave Notch receptors *in vitro* [63], animal models point to ADAM10/Kuzbanian metalloprotease for this essential function in vivo [62, 64-68]. ADAM proteases leaves a short-lived fragment anchored to the PM, called NEXT (for **N**otch **ex**tracellular **t**runcation, see Fig.1), which becomes a substrate for the aspartyl-protease presenilin(s), a component of the γ-secretase complex [69, 70]. This protease complex (which includes four core proteins, i.e. presenilin 1 or 2, **a**nterior **ph**arynx defective 1 (APH1), nicastrin, and **p**resenilin **en**hancer 2 (PEN2) [71]) operates an intramembrane cleavage at site 3 (S3), which releases the **N**otch **i**ntra**c**ellular **d**omain (NICD). Then, NICD translocates to the nucleus and turns a transcription factor of the CSL family (**C**p-**b**inding **f**actor 1 (CBF-1)/**r**ecombination signal sequence-**b**inding **p**rotein Jk (RBP-Jk) in mammals, Su(H) (***Su****ppressor of* ***H****airless)* in *Drosophila*, and LAG-1 in nematodes) from a repressor [72-74] to a transcriptional activator. Although Notch signaling has such a broad impact in a variety of cellular functions, only a limited number of Notch primary targets have so far been identified, of which the best characterized are the helix-loop-helix transcription factors of the **H**airy/**e**nhancer of **s**plit (Hes) and **Hes**-**r**elated (Hesr, also known as Hey/HRT, CHF and gridlock) families (reviewed in [75-78]). CSL binding sites have also been identified in the promoter region of other genes, including c-myc, cyclinD1, p21/Waf1, NFk B2, glial fibrillary acidic protein *(GFAP),* Nodal, GATA3, bcl-2 and CD25 (alpha chain of the IL-2 receptor), although the role of these genes as direct Notch targets has still not been unambiguously shown (reviewed in [78, 79]).

2.1. Domain structure of Notch components

Let us now briefly analyze the architecture of Notch receptors and ligands to highlight those structural features that are key factors in endocytosis-mediated signaling activation (reviewed in [80, 81]).

While *Drosophila* has a single Notch receptor gene, C. Elegans has two (Glp-1 and Lin-12 [82], which are highly redundant [83]), and mammals have four paralogues (Notch1-4) with only partially superimposable functions. The Notch receptor is a type-I transmembrane protein which is cleaved in mammals by a furin-like convertase at an external site close to the PM (the site 1 (S1)). Proteolytic cleavage occurs in the trans-Golgi network to generate a heterodimer at the cell surface composed of two non-covalently associated fragments: the

Notch extracellular domain (NECD) and the Notch transmembrane domain (NTMD); NTMD contains a small portion of the extracellular region, the transmembrane region, and the intracellular domain [84, 85] (see Fig.1). The impact of cleavage on signaling activation is a matter of open discussion: while it was seen by some laboratories to be a prerequisite for delivery of receptors to the cell surface [84, 85], other groups have shown that Notch receptors which are defective for S1 cleavage are normally exposed to the cell surface, but fail in ligand-mediated activation of canonical Notch signaling [86, 87]; these latter data support the hypothesis that dissociation of NECD from NTMD (by endocytosis, see later) may be a prerequisite for S2 proteolysis. Notably, S1 cleavage-defective Notch receptors exhibit little change in their crystal and NMR structure in comparison with wild-type receptors [88]; this is in contrast to what happens with some viruses (including avian influenza virus, HIV-1, measles and papilloma virus [89-92]) in which furin cleavage induces major conformational changes leading to protein activation (reviewed in [93]).

2.1.1. Notch receptor architecture

Notch receptors are multidomain proteins, which have been conserved from invertebrates to man. Going from the N- to the C-terminus, mammalian Notch receptors contain five regions (Fig.1): (i) a variable number of **E**pidermal **g**rowth **f**actor (EGF)-like domains (spanning from 29 to 36 domains in mammals), many of which contain **c**alcium-**b**inding sites (cbEGF-like domains); (ii) three Lin-12-Notch repeats (LNR); (iii) a hydrophobic region for receptor **h**etero**d**imerization (HD domain), which is cleaved at the S1 site (at 70 amino acids from the transmembrane domain), and which contains the S2 site (at 12 amino acids from the transmembrane domain); (iv) the transmembrane domain, which bears the S3 cleavage site, a substrate for **r**egulated **i**ntramembrane **p**roteolysis (RIP) by the presenilin/γ-secretase complex to liberate the intracellular domain; (v) the NICD, which contains a RAM domain (for Notch binding to the transcription factor CSL/CBF1/Suppressor of Hairless/Lag-1)), seven **ank**yrin repeats (ANK), a **t**ranscription **a**ctivation **d**omain (TAD) and a PEST domain (which is implicated in NICD degradation by proteolysis and whose mutation leads to increased receptor stability , a condition that closely correlates with cancer, including some T-cell leukemias) (reviewed in [80]). LNRs, plus the heterodimerization domain, form the so-called **n**egative **r**egulatory **r**egion (NRR), which folds onto the S2 cleavage site by means of extensive interdomain interactions [94-98]. As analyzed in greater details in section 3.1.1, this conformation makes the S2 site inaccessible to ADAM metalloproteases, thus protecting the Notch receptor from ligand-independent activation; the key importance of this region is underlined by the fact that mutations of the NRR, which activates the Notch receptor, closely correlates with T-cell acute lymphoblastic leukemia lymphoma (T-ALL) [95]. The TAD region is found in Notch-1/-2, but it is not present in Notch-3/-4 in mammals.

2.1.2. Notch ligand architecture

As for Notch receptors, Notch ligands are genes that have been conserved throughout evolution. *Drosophila* has two such ligand genes, Delta and Serrate, while there are five mammalian ligands, three belonging to the **D**e**l**ta-**l**ike family (Dll-1, -3 and -4) and two to

the Jagged (Serrate homologous) family (Jagged-1 and -2). Starting from the N-terminus, the domain structure of the Notch ligands can be outlined as follows (Fig.1): (i) a **m**odule at the **N**-terminus of **N**otch **l**igands (also known as the MNNL domain) of unknown structure but functionally relevant since Jag-1 mutations in this region are present in a subset of patients with the Alagille syndrome [99, 100]; (ii) a DSL domain; (iii) a number of EGF-like repeats (ranging in number from 16 in the Jagged family to 5–9 in the Delta-like family); (iv) a **c**ysteine-**r**ich **d**omain (CRD) present in Jagged but not in Dll ligands; (v) a transmembrane domain and (vi) an intracellular domain, highly divergent among Notch ligands, but with a conserved PDZ-binding domain in the mammalian Jagged-1, Delta-like-1 and -4. The function of this latter domain is unknown, although there is some evidence that its interaction with PDZ-containing adherens-junction proteins inhibits cell motility and favors epithelial cell assembly [101-103]. Similar to Notch receptors, Notch ligands undergo ectodomain shedding by ADAM metalloproteases [104, 105] and RIP) by the presenilin/γ-secretase complex [104] with the release of a C-terminal intracellular fragment (CTIF). As for many other γ-secretase products, including Notch and **a**myloid **p**recursor **p**rotein (APP) intracellular domains, CTIF translocates to the nucleus where it may help transcriptional activities [104]. In particular, Jagged1 CTIF selectively stimulates the expression of reporter genes driven by the AP-1 response element, but not by other broad-spectrum enhancer elements [104]; these data point to a possible role of CTIF as a transcriptional co-activator.

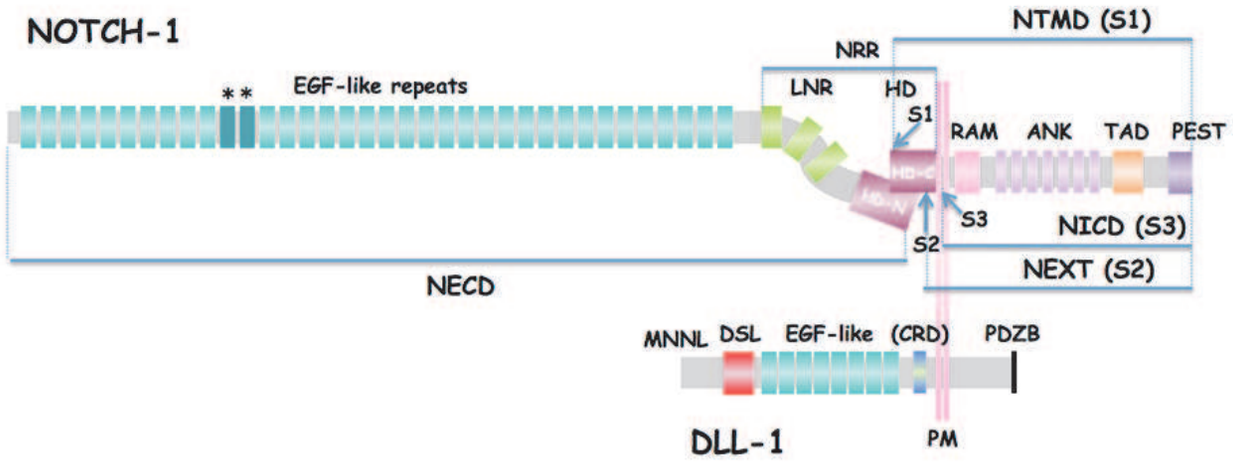

Figure 1. The domain architecture of mammalian Notch receptors (i.e. Notch-1) and Notch ligands/DSLs (i.e. Dll-1) is schematized in this drawing. Asterisks (*) indicate the EGF-like 11 and 12 of Notch receptors, which are keys for ligand binding. PM=Plasma Membrane; for other abbreviations, please refer to the text.

2.2. Notch ligand-receptor interaction

In the last few years, major advances have been made in clarifying the structural details of Notch-DSL interaction. This information is highly relevant to understand the effect of internalization and membrane trafficking on Notch signaling activation.

2.2.1. Structural requirements

By screening *Drosophila* Notch deletion mutants for their ability to promote aggregation of S2 cells [106], the EGF-like repeats 11-12 of Notch has been identified as the major interacting site for DSLs [107, 108]. Subsequent analyses have confirmed this initial observation, but have also shown that optimal binding between Notch and its ligands requires many of the 36 EGF-like repeats: a minimal Notch interacting fragment composed only of the EGF domains 10–13 has a 45-fold lower binding ability to Delta-expressing cells in comparison with full-length Notch receptors [109]. The relatively large size of EGF domains, their need for extensive disulphide bonding and, possibly, glycosylation for proper folding, have hampered the possibility to obtain structural data for Notch ligand-receptor interaction for many years. However, when an unglycosylated fragment of Notch-1 encompassing the EGF domains 11-13, was *in vitro* redox-refolded and demonstrated to be able to bind Notch ligands in a calcium-dependent manner, the way to get structural data for EGF repeats was discovered: the NMR structure of this fragment was readily solved, showing a well-defined, rod-like orientation of EGF-like 11-12, rigidified by calcium [110]. In the meantime, other studies have identified the DSL domain of Jagged-1 as the minimum binding site for Notch-2, both *in vivo* and *in vitro*; interestingly, the EGF-like repeats of Jagged-1 immediately downstream of the DSL domain, in particular the first and second EGFs, had been shown to considerably improve this interaction with their stabilizing action [111]. The convergence of previous information has made it possible to better define the structure of the Notch ligand-receptor interaction: the crystal structure of the minimal DSL binding site (i.e. the Jagged1 fragment comprising DSL plus EGF 1-3 ($Jagged1_{DSL\text{-}EGF3}$)), and that of its Notch receptor counterpart (i.e. the Notch-1 EGF 11-13 fragment ($Notch\text{-}1_{11\text{-}13}$)), were separately obtained [112]. Although it was not possible to make a co-crystal of the interaction, *in silico* docking of $Jagged1_{DSL\text{-}EGF3}$ and $Notch\text{-}1_{11\text{-}13}$ structures using restraints from parallel NMR binding data gave precious information: a single DSL surface is responsible for both *cis*-inhibiting and *trans*-activating complex of ligand and receptor [112]. A parallel study from the same group has also demonstrated that selective mutagenesis of the calcium binding site in the EGF-like repeat 12 abrogates ligand binding, thus strongly supporting the idea that this EGF repeat is actually the major DSL-binding site [113].

2.2.2. Glycosylation function

Post-translational modifications play a key role in modulating Notch activation. NECD is heavily glycosylated and many studies have tried to address the impact of these modifications on Notch signaling.

A **p**rotein **O**-**fu**cosyl**t**ransferase (Ofut1 in *Drosophila* and Pofut1 in mammals [114]) binds fucose to specific serine and threonine residues of the EGF-like repeats (reviewed in [115]); afterwards, acetylglucosaminyltransferases of the Fringe family (Fringe in *Drosophila*, Lunatic fringe (Lfng), Radical fringe (Rfng) and Manic fringe (Mfng) in mammals) can elongate the sugar chain by adding *N*-acetylglucosamine residues [116].

In *Drosophila*, Ofut1 downregulation by RNAi or gene inactivation results in classic Notch loss-of-function phenotypes, including neurogenic defects [115, 117]. The requirement of fucosylation for Notch activation is even more evident in mammals, where constitutive inactivation of the Pofut1 gene produces developmental defects that are undistinguishable from the most aggressive Notch mutants [118]; notably, defects due to Pofut1 absence can be fully rescued by expressing a constitutively active form of Notch1, at least in the hematopoietic compartment [119]. Taken together, these data show that fucosylation is required for proper Notch signaling activation in all species.

The precise role played by Pofut1 in this process was partially addressed by creating a mouse mutant bearing a Notch1 allele which was deficient for the fucosylation in a critical EGF-like repeat for DSL binding, i.e. EGF-12: trans-heterozygous mice carrying the Notch1^{12f} allele and a Notch1 null allele exhibit embryonic lethality, and defects similar to Notch1 knockouts [120]. However, homozygous Notch1^{12f} mice are viable, but with defects in T cell specification and functions and, notably, a sharply decreased binding capacity to Delta1-expressing cells [120], thus pointing to a key function of fucosylation in regulating the affinity of Notch receptors for Notch ligands.

Quite recently, experimental evidence has accumulated for other key roles of Ofut1 besides fucosylation. In *Drosophil*a, depletion of Ofut1 determines Notch accumulation in the endoplasmic reticulum, where the fucosyltransferase is resident; since transfection of the mouse Pofut1 rescues this accumulation defect, it was proposed that Ofut1 might have additional chaperone activity for the trafficking of Notch out of the endoplasmic reticulum [121].

Gene knockdown of the *Drosophila* **G**DP-4,6-**m**annose-**d**eshydratase (GMD, a cytosolic enzyme that converts GDP-mannose in GDP-4-keto-6-deoxymannose, an intermediate in the synthesis of GDP-fucose [122]) generates a loss-of-function Notch phenotype with increased Notch degradation but no accumulation in the ER; instead, co-silencing of Ofut1 and GMD restored ER accumulation, thus further supporting to the idea that an additional Ofut1 activity (independent from *O*-fucosylation) is required for proper exiting of Notch from the ER [123]. An Ofut1 chaperone function was suggested to be related to a quality control mechanism that scrutinizes Notch receptors for inappropriate inter- or intramolecular bonds between EGF-like repeats [124-127]; evidence of such a conserved function in mammals is lacking, at the moment.

Regarding the extension of *O*-fucosylated residues, Fringe deletion in flies results in a partial Notch phenotype with dorsal-ventral boundary defects during wing development [128, 129], thus formally proving the relevance of this additional glycosylation step at least for some aspects of Notch signaling activation (reviewed in [130, 131]). In mammals, evidence for a similar conserved function is accumulating. Although the three Fringe homologues have very similar enzymatic activity, substrate specificities and tissue distribution [132], only Lfng inactivation results in Notch-related defects, including (i) impairment of T cell maturation (since a developmental stage–specific expression of Lfng is required for the access of T cell progenitors to intrathymic niches that support Notch1-dependent T cell

development [133]), and (ii) subversion of somitogenesis in some body districts, with major alterations in vertebral and rib cage morphogenesis [134, 135]). Conversely, Rfng and Mfng knockouts display no obvious phenotypic defects. Furthermore, no synergistic defects were observed in mice lacking all fringe genes, thus questioning function redundancy in this gene family [136-138]. Notably, *in vitro* glycosylation and ligand binding studies and *in vivo* genetic data have established that the addition of N-acetylglucosamine onto O-fucose indeed modulates the affinity of Notch ligand-receptor interaction in an opposite manner, i.e. by enhancing Notch binding to Delta and inhibiting Notch binding to Serrate [139-141].

In addition to fucosylation, Notch receptors are also modified by *O*-glucosylation. Genetic studies in flies and mammals have shown that inactivation of the only enzyme responsible for the addition of O-glucose to EGF-like repeats, i.e. **p**rotein **O**-**glu**cosyl**t**ransferase (Poglut/Rumi), results in severe Notch phenotypes [142, 143]. Notably, Rumi activity is required in the signal-sending cell, where it has neither chaperone-like activity [142, 143] nor a function in ligand binding, since Notch in fly rumi knockdown cells binds Delta as efficiently as in control cells [142]. The concentration of NICD is dramatically reduced in several tissues of Rumi mutants [142], thus pointing to a function of *O*-glucosylation in Notch proteolysis, rather than in ligand binding. The structural basis of this function is unknown, but it is plausible that O-glucosylation may affect the structure of the NECD so that an initial constraint for S2 cleavage is removed [131, 144].

Therefore, genetic and in vitro data on Notch glycosylation indicate (i) that *O*-fucosylation and *O*-glucosylation play a general role in Notch signaling activation (albeit with different mechanisms), while *N*-acetylglucosamine addition is required for more specific aspects of this signaling activation, and (ii) that *O*-fucosylation and its acetylglucosamine extension mainly acts by regulating the affinity of DSL-Notch interaction.

3. Endocytosis in notch signaling activation

An absolute requirement for endocytosis was an early finding in Notch studies. Notably, the *shibire* mutant (i.e. a temperature-sensitive mutant of the endocytic fission protein dynamin in flies [145]), results in a developmental phenotype with an excess of neural cells when raised to restrictive temperature, i.e. with defects which closely phenocopy the Notch mutant [146, 147]. This phenotype is in sharp contrast to other signaling pathways, which are not severely disrupted in the *shibire* mutant (e.g. wingless [148], although even this signaling is affected by endocytic defects [149-151], as for most other signaling pathways (reviewed in [152])). The staminal observation of genetic interaction between dynamin and Notch prompted investigation of the requirement for dynamin function (i.e. endocytosis) for Notch signaling during the segregation of sensory bristles of the fly [153]. Overexpression of activated Notch isoforms (either membrane tethered or soluble) suppresses the *shibire* phenotype, thus indicating that endocytosis main action is upstream of the signal transduction promoted by Notch activation [153]. Notably, when wild-type Notch has to be activated by its ligand Delta, dynamin is required in both signaling and receiving cells, as shown by mosaic analysis in which bristles along the border can be either wild-type or mutant [153].

Localization studies of Notch and Delta during fly development showed that both Notch and Notch ligands were in a dynamic equilibrium between a PM pool and an intracellular vesicle pool, with a transition to internalized pool upon interaction of adjacent cells [154]. Delta is detected at both the PM and in vesicles only at some stages of specific developmental systems, while it is mostly internalized in others, including all stages and cell types of retinal development [154-158]. Morphological analyses of Delta subcellular localization in this latter development system have clarified that most, if not all, Delta-containing vesicles have an endocytic origin [159, 160]: Delta is re-localized to the PM in the full endocytic mutants, *hook* and *shibire*, thus supporting the idea that Delta is initially transported to the cell surface, but then it is taken up very quickly and efficiently by endocytosis to be delivered to the endocytic compartment [159, 160]. These and other observations [106, 161] suggested that an endocytic event could precede Notch activation. Direct evidence in support of this hypothesis came from (i) antibody uptake assays in living *Drosophila* tissue and in mammalian cells, which showed that DSLs are rapidly and efficiently internalized upon antibody binding and clustering [162-164], (ii) transfection assays with endocytosis-defective DSLs, which provided direct evidence that Notch ligand internalization is required to activate Notch signaling [164, 165], and (iii) uptake assays of recombinant forms of DSL (Delta1-Fc chimeric protein) [166] and of Notch-1 (N1Fc chimeric protein) [167], which showed that even soluble fragments of Notch ligands and Notch receptors, upon clustering, could potently promote the internalization of their cognate partners. Under these conditions, the Delta1-Fc chimera was also proved to be able to fully activate canonical Notch signaling [166].

Thus, the paradigm in the field is that Notch signaling critically depends on DSL endocytosis for its activation and modulation. We shall now analyze the molecular machinery involved in this process. At present, as the reader will see, only partial information is available, with (many) puzzling and (some) conflicting results.

3.1. Notch ligand endocytosis

The molecular characterization of DSL endocytosis began to attract great interest when it was published an in-depth morphological analysis of the effect of the *shibire* mutation on the localization of Delta and Notch in retinal development. This seminal work prompted a wealth of new studies on the relationship between endocytosis and Notch signaling [165]. In this paper, it was shown with stunning morphological data that NECD detaches from the Notch receptor on the signal-receiving cells (i.e. on the latticework cells) and is internalized or, more specifically, *trans*-endocytosed in the signal-sending cells (i.e. in the cone cells) in a complex with Delta. Notably, this process tightly correlates with the Notch signaling activation that underlies the cell fate specification of the retinal latticework, thus supporting the idea that NECD *trans*-endocytosis is requested for Notch activation. As expected, Notch receptor dissociation and its *trans*-endocytosis were severely hampered in the *shibire* mutant (i.e. in a condition in which endocytosis is blocked at the fission reaction of the clathrin-coated pit from the PM - see above), as well as when endocytosis-defective mutants of Delta were expressed in cultured cells [165]. Furthermore, this trans-endocytic mechanism was

also found to be active in another developmental system under strict Notch control, i.e. wing vein development, thus suggesting its universal use in Notch activation [165]. Besides further supporting a role of endocytosis in Notch activation, these data suggest a possible mechanism of how DSL endocytosis might control Notch signaling: the dissociation of NECD, which is triggered by DSL endocytosis, is the event that activates Notch by possibly giving access to its cleavable sites. In partial support of this hypothesis, previous studies indicated that Delta proteins lacking the intracellular domain (i.e. lacking the binding site for endocytic adaptors so that DSL endocytosis cannot occur) acted as dominant-negative proteins for Notch signaling in *Drosophila* and in vertebrates [168-170].

However, direct evidence that NECD shedding by endocytosis is required to trigger the Notch proteolytic cascade was lacking in the *Drosophila* analyses [165]. This issue was addressed by a follow-up study on mammalian cultured cells by another group [87]. In this latter paper, it was demonstrated that an NTMD construct (Fig.1) transfected in mammalian cells was constitutively active in a reporter assay for Notch activation, and that treatment with BB94 (a metalloprotease inhibitor) and/or DAPT (a γ-secretase inhibitor) reduced the level of this signaling. These data indicate that, when the NTMD fragment is generated, it will be constitutively processed by proteolytic cleavage to free up the NICD. These findings support a two-step model in which (1st step) ligand endocytosis non-enzymatically dissociates and internalizes NECD in ligand cells, and then (2nd step) the membrane-bound NTMD undergoes constitutive cleavage by ADAM metalloproteases to produce NEXT, followed by γ-secretase cleavage to produce NICD (see Fig.1 for fragments description).

3.1.1. Mechanistic models

Following the staminal observation by Parks et al. [165], many laboratories investigated the machinery and the types of endocytosis that are at the basis of DSL-mediated Notch activation. A large collection of data has been produced which points at two distinct, but not mutually exclusive, models (see Fig.2): (i) according to the "pulling force" model (that derives directly from the observation of NECD shedding [165]), DSL internalization can exert a mechanical stretching, or detaching, action on the NECD to unmask the cleavage sites (especially S2) of Notch receptors; (ii) alternatively ("Notch ligand/DSL trafficking model"), or in combination with the previous model, an inactive DSL is activated either by trafficking through a recycling compartment ("ligand maturation" or "recycling" model), or by transcytosis to a membrane domain where interaction with Notch receptor occurs with increased frequency ("highly polarized cells" model).

It is plausible that, if (and/or when) the two models combine, they will act sequentially: DSL activation by intracellular trafficking should precede the mechanical shedding of the Notch receptor by DSL endocytosis, making the two mechanistic models not just compatible, but even synergistic.

The initial hint for the existence of a trafficking event that could activate the Notch ligand in order to make it competent for Notch activation came from the analysis of the fly mutant *liquid facet* (*lqf*), whose gene encodes the *Drosophila* epsin ([171] and see later). In this work,

epsin was found to be implicated in a subset of DSL endocytic events which were able to activate Notch, while the bulk of DSL endocytosis (i.e. the constitutive endocytosis of Delta) was neither related to epsin function nor to Notch signaling [171]. Rescue of epsin absence was achieved by expressing a chimeric DSL, in which the intracellular tail was replaced by a short internalization signal of the LDL receptor, which was known to mediate the internalization and recycling of many proteins through the endosome [172]. Further studies in *Drosophila* and in mammalian cells have substantiated this initial observation of the existence of a possible trafficking step for the maturation of Notch ligands: (i) a defect in Delta trafficking through the recycling endosome was proposed to cause the aberrant cell fate transformation in sec15 mutant sensory lineages (see next paragraph for links of this developmental pathway to Notch signaling) [173]; (ii) expression of a dominant negative Rab11 (a small GTPase which regulates trafficking from the recycling endosome to the PM) was associated with DSL accumulation in endosomes and Notch signaling failure in a mammalian co-culture system [174]; (iii) an ubiquitylation-defective mutant of Dll1 can be efficiently endocytosed, but in contrast to the wild-type isoform is unable to recycle back to the cell surface and, possibly as a consequence of this trafficking defect, to efficiently bind Notch1 in a mammalian cell system [175].

An important question on the "ligand maturation" model regards the nature of the DSL activation process. In the *lqf* paper [171], a proteolytic step for *lqf* was identified, which was absent in epsin mutants but present in wild type-cells; it was speculated that this processing could indeed be Delta's activation step. Other Authors have looked for DSL processing in another system in which Notch ligand trafficking is essential, i.e. the **s**ensory **o**rgan **p**recursors (SOP) system (see next paragraph), but they failed to detect any evidence of DSL pre-cleavage [176], thus leaving unsolved the question of which molecular action eventually makes DSLs competent for Notch activation.

Intracellular trafficking can also activate DSLs with another mechanism, i.e. by re-localizing DSL from a membrane domain where it cannot interact with Notch to a membrane domain where this interaction can efficiently occur. This "highly-polarized cell" model is supported by at least two key sets of experiments undertaken in the *Drosophila* SOP system. This system is related to the development of the sensory organs (i.e. the mechanosensory bristles) located along the cuticle of the adult *Drosophila*, and is critically dependent on Notch: during a program of three rounds of asymmetric cell division [177, 178], each division generates one daughter cell that assumes the signal-sending role and uses DSLs to activate Notch in its sibling, which acts as a signal-receiving cell [179]. In the signal-sending cells of SOP, Delta localizes both at the apical and at the basolateral membrane, while Notch accumulates apically [180]. By using a pulse chase antibody uptake assay coupled to confocal microscopy sectioning, it was demonstrated that the basolateral pool of Delta is continuously endocytosed and delivered to the apical PM, where the interaction with Notch is most likely to occur [180]. This observation was extended by the same Authors to highly-polarized mammalian cells: by using a compartmentalized antibody uptake assay, they showed that Dll1 is similarly internalized from the basolateral membrane of Madin-Darby canine kidney cells and then transcytosed to the apical plasma membrane where Notch1 accumulates

[180]. In a second set of experiments on SOP the function of two primary regulators of actin dynamics was explored, i.e. the Arp2/3 complex and the **W**iskott-**A**ldrich **s**yndrome **p**rotein (Wasp) (reviewed in [181-183]). It was found that Arp2/3 and WASp were responsible for the nucleation of a filamentous **a**ctin-**r**ich **s**tructure (termed ARS) underneath the PM of pIIb (signal-sending) and pIIa (signal-receiving) cells at two different locations: (i) the apical domain, where it is responsible for microvilli elongation, and (ii) the lateral cell-cell contacts between pIIa and pIIb cells following SOP division [176]. Besides giving rise to this specialized actin cytomatrix, Arp2/3 and WASp also regulated the trafficking of Delta along the ARS in quite a unique manner: Delta was internalized from that part of the apical cell surface where microvilli were not clustered, and travelled basally, where it was then re-localized apically to the microvilli-rich portion, exactly where the contact with Notch usually occurs [176]. By inactivating Arp2/3 function, the ARS architecture was perturbed while Delta was still internalized. However, Delta failed to be delivered to the apical microvillar portion of the PM, being stopped in the basal portion of pIIb cells. Collectively, these data support a fundamental function of actin cytoskeleton in Delta trafficking, which is requested in the SOP system in order to localize DSL where interaction with Notch can occur. Conversely, a role of actin in DSL internalization is not requested in this developmental organ system [176, 184], while it is essential in other systems, cells and organisms (reviewed in [185-188].

A universal requirement for DSL trafficking does not seem exist for all tissues or developmental systems. Rab11 function, which was found to be essential for Delta trafficking and activation in the SOP system, is not required for *Drosophila* eye development [189], nor for germinal cell signaling [190]. In both these Notch-dependent events, endocytosis was required and a specific need for epsin, but not recycling, was evident at least in eye development [189].

More recently, the emerging structural findings described in section 2.2.1 have steered and re-focused the attention on the "pulling force" model. Direct evidence supports this model. As already discussed, using classical techniques to study membrane trafficking events it was possible to demonstrate that DSL-mediated receptor dissociation precedes and permits the proteolytic activation of Notch both in flies and in mammalian cells [87, 165]. A better structural appreciation of this event was acquired by using a material science technique to study surface morphology at the atomic level. Atomic force microscopy, applied to protein (or other molecules) interactions, can quite precisely measure the force applied to make contact between two interacting surfaces (the contact force) and the force applied to detach them after contact (the detachment force). This technique was then adapted to measure cell-cell adhesion [191] and was used to characterize Notch interaction [192]. A specific setup was engineered to mount a single S2-Delta-expressing *Drosophila* cell on the "tip-less" cantilevers, while immobilizing a S2-Notch-expressing cell in a plate well, and adhesion forces derived from this cell-cell interaction were measured [192]. The results of this elegant experiment showed that (i) expression of full-length Notch is required to produce maximal adhesion force (in the order of ~14 nN, comparable to a cell-cell adhesion contact) and signaling with S2-Delta cells; (ii) upon contact, this considerable adhesion force is lost within

minutes (~10min.), as a direct result of the proteolytic cleavage of Notch, then signaling starts to rise very quickly (this timing is compatible with the time-course of Notch signaling in lateral inhibition models where the signaling event is accomplished in less than 20 min [193, 194]); (iii) Ofut1 RNAi in S2-Notch cells abolish the detachment force from S2-Delta cells, consistent with a reduced binding capacity of unfucosylated Notch to Delta [120, 195] (iv) if pulling is applied to S2-Delta cells by cantilever retraction, the detachment force from the S2-Notch cell drops with an increased kinetics, suggesting that a stretching action accelerates proteolytic cleavage of Notch. Taken together, these observations support the notion that Notch proteolytic cleavage depends on the strength of Delta binding [192], thus providing key structural data to support the "pulling force model" of Notch activation.

Since Notch proteolysis proceeds constitutively after exposing the S2 cleavage site (either by shedding of the Notch ectodomain or through its stretching), this unmasking reaction has to be considered the true rate limiting step of Notch signaling activation [196]. What are the structural constraints that keep S2 in an inactive silent state, preventing unwanted activation before ligand interaction? How are these constraints lifted/eased/modified during Delta-mediated NECD pulling? As anticipated in section 2.1.1, experimental evidence points to the NRR region of NECD for this key inhibitory action of Notch cleavage. Receptors that lack EGF-like repeats cannot undergo constitutive proteolytic cleavage and are functionally inert [70, 94, 108, 197-199]; conversely, an NTMD construct undergoes constitutive cleavage to release NICD [87]. Taken together, these data indicate that the restraints on ligand-independent activation of Notch receptors reside in a region downstream of EGF-like repeats but upstream of NTMD. This region corresponds to the three LNR repeats plus the HD domain, i.e. the NRR (see Fig.1). Key evidence to support this idea came from the isolation of Notch gain-of-function or loss-of-function phenotypes directly related to the NRR. (i) Antibodies raised against the NRR region did not compete with ligand binding to the receptor, but strongly inhibited Notch activation [96, 200]. Notably, those inhibitory antibodies recognized a conformational epitope lying on a face where the first LNR repeat (LNR-A) approaches the β-sheets of the HD (HD2), supporting the idea that autoinhibition is due to the clamp of LNR1 and HD2 together (see later for structural considerations) [96]. Conversely, an anti-Notch-1 antibody that recognized a linear epitope in the LNR1 domain only was activating Notch signaling, possibly by inducing a conformational change of the LNR1 that opened the access to the S2 site [96]. (ii) Mutations in the NRR of Notch receptors produced gain-of-function phenotypes in various biological contexts, including invertebrate developments. An activating mutation of the *glp-1* Notch receptor in *C. Elegans* was located in the LNR1 of the molecule [201], while a bunch of activation mutations of the other Notch receptor in worms (i.e. *lin-12*) were found to be spread in both the LNR and HD region, with a preference for the latter domain [202]. (iii) A subset of patients, who develop T-cell acute lymphoblastic leukemia, have gain-of-function mutations of Notch-1, which clusters in two regions: the HD domain and the C-terminus (including the TAD and PEST domains). While the C-terminal mutations were found to increase NICD stability, the HD mutations increase Notch-1 proteolysis, as suggested by the blocking of their stimulatory activity by γ-secretase inhibitors [203].

Many structural data have been collected in recent years that have helped to clarify the mechanistic details of NRR function, in particular (i) the NRR role in protecting S2 from constitutive cleavage and (ii) the kinetics of S2 autoinhibition. At present, the crystal structure of the NRR of human Notch-1 [88], of human Notch-2 [98], and the co-crystal of inhibiting antibodies, together with their target NRR epitopes [204], have been solved at high resolution. The NRR of Notch-2, the first NRR analyzed, was seen to form a very compact structure with overall dimensions of 60Åx45Åx25Å [98]. The three irregularly folded LNRs wrap around the HD domain forming "a cauliflower-like shape, in which the LNRs 'florets' cover and protect the HD domain 'stem'" [98]. The two halves of the HD domain, (i.e. the HD-N before the S1 site and HD-C after this site, see Fig.1) form an intimately intertwined α/β sandwich containing three α-helices and five β-strands connected by several conserved loops [98]. The inner, concave face of the HD domain has hydrophobic residues pointing toward its center. The S2 site is on the β5-strand of the HD-C and it is actually buried in a small pocket that prevents protease accessibility; the pocket is formed by the hydrophobic residues of α-HD -C and of the LNR-AB linker. In particular, it is thought that a leucine residue (L1457) extends from the LNR-AB linker toward a critical valine residue (V1666) at the C-terminus of the S2, thus obliterating the access to the ADAM cleavage site [98]. The α3-helix above the S2 site is stabilized by hydrophobic interactions with residues in the LNR-B and in the LNR-AB linker plus a conserved hydrogen bond from LNR-A [98]. Consistent with previous structural data, deletion of LNR-A, the LNR-AB linker and LNR-B makes a constitutively activated Notch-2 [98]. The Notch-1 NRR structure is similar, although not identical, to that of the human Notch-2 NRR, with the classic conformation of the LNR-AB linker providing a key leucine residue that packs tightly against the C-terminal valine of the S2 site. As for NRR2, the folding of the HD domain has a rather stiff structure that is stabilized by extensive interaction between helices and strands. These data confirm a common autoinhibition strategy that is implemented among Notch family members [95].

Additional and fundamental structural data on Notch NRR function and dynamics came from the field of Notch immunotherapy and from the application of unconventional structural techniques. In an effort to overcome problems generated by the clinical use of presenilin inhibitors to silence the Notch pathway (in particular, the lack of selectivity for this pathway with a consequent broad toxicity), phage display technology was used to generate highly specific antibodies that could selectively antagonize a single Notch paralog (i.e. able to distinguish between Notch-1 and Notch-2) [204]. A co-crystal of this interaction shows that inhibitory, anti-NRR1 Fab-fragments bridge the LNR and HD domains, thus locking the NRR in a clamped conformation, which makes the S2 site unreachable for metalloproteases [204]. Further key data for the understanding of NRR-dependent S2 activation came from the application of hydrogen exchange mass spectrometry, a technique that monitors the exchange of deuterium between the solvent and the backbone amides during conformational changes [205, 206]. More specifically, when a surface of a protein is exposed it is rapidly deuterated, while when it is masked the exchange of hydrogen for deuterium is slow, or it does not happen at all. This technique was used to monitor the accessibility of the S2 cleavage site in a condition which should mimic ligand-dependent Notch activation, i.e. by chelation of Ca2+, a condition which causes the dissociation of the Notch receptor and triggers its signaling [207] (although widely used, Ca2+ chelation cannot

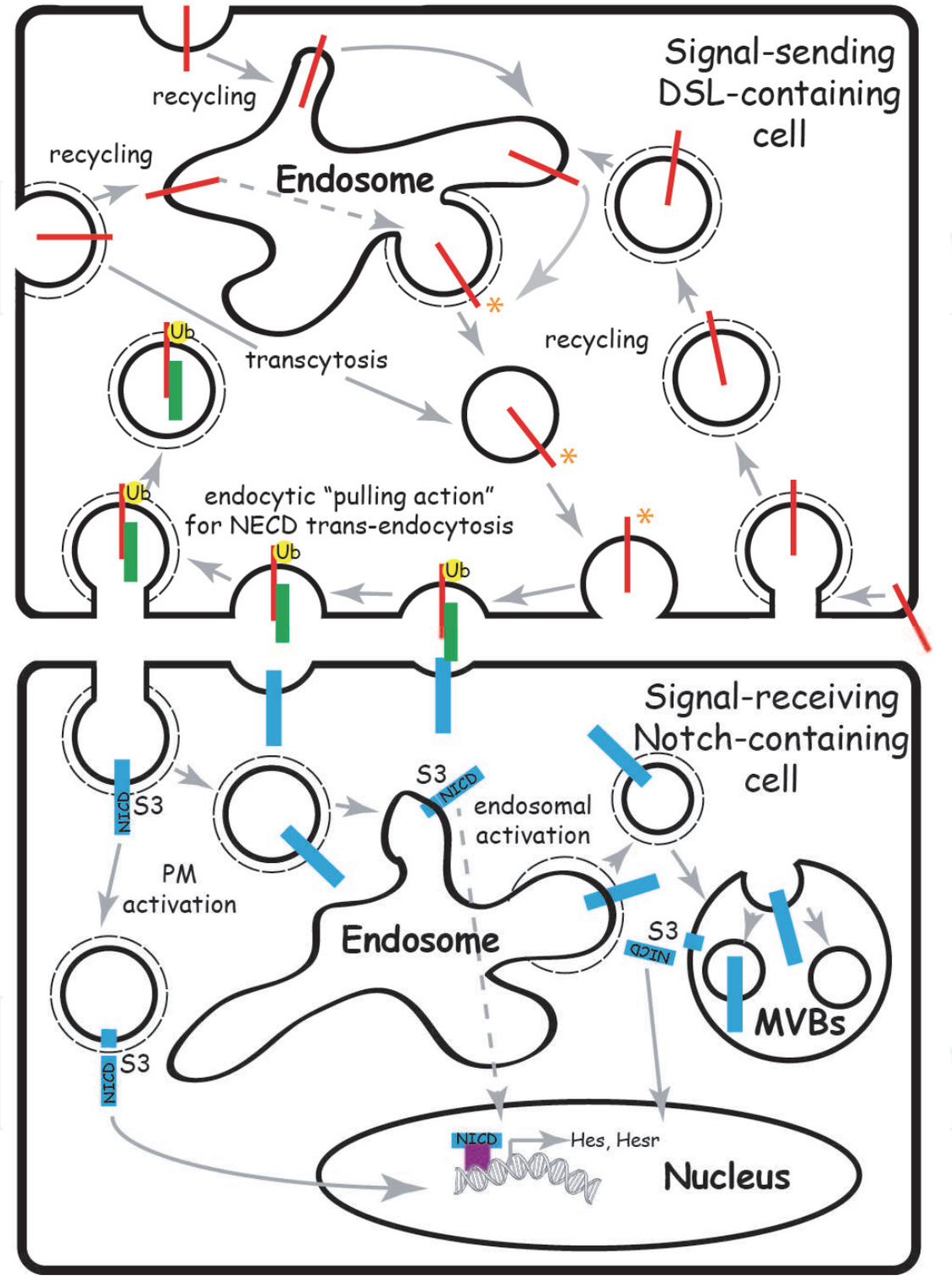

Figure 2. The different recycling and activation pathways of Notch ligands (DSLs) and of Notch receptors are outlined. In Red=Notch ligands/DSLs, in green=NECD, in blue=NTDM, in violet=the Notch transcriptional complex, PM=plasma membrane, S3=the Notch S3 site, which is cleaved by the γ-secretase complex to release NICD (where indicated, it represents the location of its presumable action); the orange (*) asterisk indicates a putative, activated state of DSL, after its recycling. For abbreviations, please refer to the text and to Fig.1.

be considered a surrogate of DSL action on the Notch receptor but obvious experimental constraints prevented the use of a more physiological condition). The results of these experiments showed that (i) upon Ca2+ chelation, LNR-A unfolding was the first event to occur, followed by the unfolding of LNR-B; (ii) after unfolding of the first two LNRs, the S2 site became accessible to the external environment, thus confirming previous results with deletion mutants in which removal of the LNR-A and LNR-B regions was sufficient to obtain a constitutively activated receptor [98]; (iii) Ca2+ is fundamental in stabilizing the secondary structure of LNR repeats [98]; (iv) HD-N and HD-C do not separate when S2 is exposed, and the HD domain maintains its folding for a very long time after Ca2+ chelation (i.e. well beyond the proteolytic cleavage of the receptor is terminated). This latter observation may indicate that ectodomain shedding is not an absolute prerequisite for the activation of the Notch proteolytic cascade [97]. To summarize, these structural data suggest that LNR-A and –B repeats are the fundamental gatekeepers of Notch activation as they control access to the Notch S2 cleavage site. Interestingly, in a recent paper, topology-based coarse-grained and physics-based atomistic molecular dynamics simulations were used to predict the conformational changes that occurred in the NRR by intrinsic and force-induced mechanisms [208]. These computer simulations showed that LNR unfolding is not sufficient to unmask the S2 site, but the continuous application of an external stretching/pulling force is needed to unfold the HD domain and, in particular, its β-5 strand [208]. Notably, the extension force required to unfold the β5 strand should be much lower than the force needed for heterodimer dissociation [208], suggesting that dissociation of Notch receptor is not needed for its activation, since an intermediate state with exposed S2 site might persist for a significant period of time before global unfolding and heterodimer disassociation occur. These predictions provide new and unforeseen roles for HD in Notch activation that definitely need experimental support.

3.1.2. Specialized endocytic machinery

Genetic evidence in invertebrates and mammals points to ubiquitylation (also referred to as ubiquitination) as the master regulatory mechanism controlling the endocytosis implicated in Notch signaling activation (reviewed in [209-211]).

Ubiquitylation, i.e. the conjugation of ubiquitin to proteins, is a rather common post-translational modification that regulates protein stability, localization, and activity (reviewed in [9, 11, 212-215]). Ubiquitin is a small conserved protein, whose C-terminal glycine (Gly76) can be engaged in a covalent isopeptide bond with the ε-amino group of lysine residues in substrate proteins. Ubiquitin can serve as an acceptor to form a polyubiquitin chain via one of its seven lysine residues (K6, K11, K27, K29, K33, K48 and K63). A hierarchical set of three enzymes acts in a sequential process to operate ubiquitin modification: (i) ubiquitin-activating (E1), (ii) -conjugating (E2), and (iii) -ligating (E3) enzymes. The large numbers of the latter enzymes (of which, the best studies are the **R**eally **I**nteresting **N**ew **G**enes (RING)-type and **H**omologous to the **E**6-AP **C**arboxyl **T**erminus (HECT)-type E3s) provide specificity to this post-translational modification in determining which substrate proteins will be modified. Ubiquitin can be attached in different amounts

which have an impact on protein stability, localization and activity: (i) as a single molecule (monoubiquitylation), (ii) as multiple monomers linked to different lysine residues of a protein (multiubiquitylation), and/or (iii) as chains of ubiquitin molecules of various lengths and linkages (polyubiquitylation). Based on the linkage type, polyubiquitylation can be homotypic or heterotypic: it is homotypic when the same lysine residue is used for the sequential conjugation of ubiquitin moieties, while it is heterotypic (or mixed-linkage) when different ubiquitin's lysines are used to add monomers to the growing chain [216]. Furthermore, polyubiquitin chains can be linear (usually when homotypically built) or ramified (when the heterotypical linkage is used). Ubiquitin moieties are recognized and non-covalently bound by specific modular elements, collectively called **u**biquitin-**b**inding **d**omains (UBDs), which are now classified in different families, according to their structural homology [217]. Ubiquitylation is requested for many cellular processes, including proteasomal targeting and degradation of proteins, cell division, apoptosis, immune response, cytoskeleton dynamics, DNA transcription and repair, signal transduction, quality control and, last but not least, membrane trafficking, of which endocytosis and endosomal sorting are the best characterized ubiquitin-regulated events (reviewed in [6, 11, 212, 214, 218-224]}. Ubiquitylation can be reversed by multiple deubiquitinating enzymes (DUBs), the study of which constitutes a fast growing field of research (reviewed in [225-230]).

The first hint that ubiquitylation might be a necessary step for Notch activation came from the correlation between two sets of data, obtained almost twenty years apart: (i) a mutation screening in *Drosophila* identified *neuralized* (*neur)* among various genes phenocopying Notch neurogenic defects [59], thus indicating genetic interaction between that protein and Notch signaling; (ii) *neur* was seen to encode for a RING-type ubiquitin E3 ligase [231, 232]}, whose mutations in the catalytic domain were not able to rescue neur mutant embryos, thus formally proving that ubiquitylation is essential for Notch signaling *in vivo* [232]. In the same year, Xenopus neuralized was seen to carry out the same functional and biochemical activities as for the fly homologue [233].

Since then, an impressive number of experiments has been carried out on Neuralized activity and action in invertebrates. Key advances can be summarized as follows. (i) The RING domain was found to be critically required for Delta endocytosis: as expected, when the mutant *neur* is expressed, Delta stays mainly on the PM but re-localizes to internal vesicles upon (over) expression of the wild-type gene [163, 231, 234, 235]. The activity of Neur was firmly localized in the Notch-sending/DSL-bearing cell following cell-transplantation experiments [234]. As a collateral observation, it was documented that fly cells overexpressing Neur had a reduced level of Delta due to increased proteasomal activity, secondary to massive polyubiquitylation [231, 234]. (ii) Two critical lysine residues for Neur-mediated ubiquitylation (K688, K742R) were identified in a screening of Delta mutants for aberrant subcellular trafficking (i.e. mutants with a stable PM localization) [236]. Similar results were seen in a study on Serrate to uncover motifs leading to its internalization: two highly conserved lysines (K1272, K1290) were identified which are conserved between *Drosophila* Serrate and mammalian Jagged, and whose mutation resulted in blockage of DSL endocytosis and Notch activation [237]. However, the sites and types of

ubiquitylation for endogenous DSLs are not yet known [237]. (iii) Neuralized-binding motifs, independent of ubiquitylated lysines, were identified on Delta and Serrate in the form of an NXXN sequence conserved among species, [238, 239]. (iv) Since a function of Neur in *cis*-inhibition was supposed due to its activity in Delta degradation [231, 233], it was demonstrated that overexpression of Neur indeed causes *cis*-inhibition, but Neur activity is not requested for this fundamental function during development [237].

In mammals, two **neur**alized-**l**ike genes, Neurl1 and Neurl2, are present. Quite surprisingly, inactivation of these genes does not result in major phenotypic defects [240-242]. Only subtle defects were scored in Neurl1$^{-/-}$ mice: (i) male mutants are sterile due to a defect in the axonemal organization of spermatozoa that leads to immotile sperm [242]; (ii) female KOs are defective in the final stages of mammary gland maturation during pregnancy [242]; (iii) Neurl1$^{-/-}$ mice are hypersensitive to ethanol effects on motor coordination and exhibit a defect in olfactory discrimination [241]. Only these latter defects can be putatively connected to an impairment of some subtle (yet to be defined) function of Notch in mammalian neurons, but no classical Notch signaling defects are identifiable in these mutants. Clearly, a compensation by the remaining Neurl2 gene was suspected, but, surprisingly and unexpectedly, inactivation of both Neurl1 and 2 did not result in any overt Notch defect in mice [240].

Neurls are not the only Notch-ligand specific E3 ligases present in vertebrate genomes. Another family, named after its first member *mind bomb* (*mib*), was identified in Zebrafish in a screening for neurogenic phenotypes (in which several Notch signaling components were also isolated [164]). Mib encodes for another RING-type E3 ligase, whose loss-of-function mutants cause major Notch developmental defects in the *Danio R.* [164]. Mib and Neuralized show complementary functions: (i) as for Neur, Mib(s) act(s) in the signal-sending cell [164] by promoting endocytosis of various DSLs, including Xenopus Delta [243] and Zebrafish Delta [164]; (ii) two mib genes are present in *Drosophila* with tissue distribution that complements that of Neur: inactivation of Mib indeed caused Notch defects in flies, but only in those tissues in which Neur was not expressed, while in tissues in which both Neur and Mib(s) were expressed, Notch phenotypes arose only upon co-inactivation of all E3 ligases [162, 243-245]. (iii) Mib1 cannot rescue *Drosophila neur* mutants [162], and, conversely, Neur and Mib1 cannot compensate for *mib2* defects in myoblast fusion and muscle homeostasis [246], thus showing that Mib(s) and Neur probably have other functions besides the ubiquitylation of DSL substrates.

Inactivation of Mib1 in mice finally results in a pure Notch phenotype, which recapitulates the most severe mammalian mutants of this signaling pathway [240]. Surprisingly, triple Neurl1/Neurl2/Mib2 knockout mice do not show major phenotypic defects, suggesting that Mib1 is the only essential E3 ligase for Notch activation. In support of these genetic data, knockdown of mib1 expression by siRNA dramatically reduces Notch activation in mammalian co-culture experiments [247, 248].

Activation of DSL internalization by ubiquitin moieties requires UBDs recognition and functional binding. Genetic experiments in mammals and invertebrates point to epsin family members as the principal actors in linking endocytosis, ubiquitylation and Notch

activation. Epsins are highly conserved genes with two homologues in yeast (Ent1, Ent2) [249], one in Drosophila (Lqf) [250], and three epsin genes (Epn1, 2 and 3) in mammals. While epsins 1 and 2 are expressed in all tissues [251, 252], epsin 3 is restricted to surface epithelia [253, 254]. Epsins have a characteristic, highly conserved, three domain structure: (i) a **E**psin **N**-**T**erminal **H**omology (ENTH)-domain for phosphoinositides binding, in particular PtdIns(4,5)P2 [255, 256]; (ii) a central region which interacts with clathrin and its adaptor AP2; (iii) a C-terminal domain with multiple NPF motifs for the recognition of **E**ps15-**h**omology (EH)-domain-containing proteins, including the endocytic adaptors Eps15(R) and intersectin1/2 [251, 257]; (iv) multiple **U**biquitin **I**nteracting **M**otifs (UIMs) between the ENTH domain and the central domain for mono/polyubiquitin binding and for epsin (mono)ubiquitylation [258]. Epsin was initially characterized to be at the center of a highly regulated network of ubiquitinating and deubiquitinating enzymes: (i) Drosophila epsin (*lqf*) is the substrate of *fat facets* (*faf*), a deubiquitinating enzyme whose mutation is embryonically lethal in the fly [250]; (ii) RPM1/Highwire/Hiw, an E3 ligase of the RING type, regulates synaptic morphology (in flies and nematodes [259]), where a *lqf* function was also demonstrated [260]; (iii) although Lqf is not a substrate for Highwire [259], Hiw and Fat facets interact genetically and act as mutually antagonistic regulators of presynaptic growth [261]; (iv) epsin in neurons undergoes cycles of multi(mono)ubiquitylation/deubiquitylation, that change epsin affinities for interactors [262]. Based on this interaction, epsins were classified as housekeeping **c**lathrin-**a**ssociated **s**orting **p**roteins (CLASPs) with specificity for ubiquitylated cargos (e.g. the EGF receptor upon ligand binding [251, 258, 263-267]), with the additional function of promoting membrane curvature [256, 268]. Genetic studies in yeast and *Dictyostelium* have also shown an additional role of epsin hortologues in the actin dynamics, which correlates with the endocytic function [269, 270]. However, at least in yeast, endocytosis has different requirements, being actin- but not clathrin-dependent, as in multicellular organisms (reviewed in [187, 188]).

Genetic studies in invertebrates have shown that the only epsin gene present in these species is required for the activation of Notch signaling [171, 245, 271, 272], and that this function is closely related to DSL ubiquitylation [245]. Genetic experiments in mammals have confirmed those studies and firmly established the essential role of epsin1 and 2 in Notch activation in vertebrates [273]: (i) the absence of epsin1/2 expression during mouse development correlates with embryonic lethality at midgestation, with multiorgan defects highly reminiscent of the most severe Notch mutants; (ii) accordingly, expression of Notch primary target genes is severely reduced in epsin1/2 double knockout embryos. Surprisingly, housekeeping forms of clathrin-mediated endocytosis were not impaired in cells deriving from those embryos [273].

A very recent study has provided evidence that epsins might have a previously unforeseen role in membrane fission [274]. In particular, predictions based on biophysical models support the idea that amphipathic helices (as those present in the epsin ENTH domain) could create a higher energy state due to their limited insertion into the polar head region, but not into the hydrocarbon region of the PM. This accumulated energy, when released, will crucially favor the fission reaction. This hypothesis was carefully tested *in vitro* by cell-

free vesiculation assays and some correlative morphological tests in cultured cells: results confirm the prediction, thus sustaining a role for epsins that could parallel to or substitute that of dynamin [274]. Furthermore, it was found that simultaneous depletion of epsin1/2/3 by knockdown experiments results in the impairment of all current paradigms of clathrin-dependent endocytosis, thus suggesting a general role of epsin in the core machinery of this endocytic pathway [274]. Interestingly, single epsin KDs, or any combination of two of them, have little effect on endocytosis [274].

Taken together, these experiments suggest that epsins are the best candidates to explain the molecular action of ubiquitylation in DSL endocytosis, although the machinery behind this function has still to be fully uncovered. Triple epsin knockout mice could be the key to shed light on this molecular network.

Regarding the types of endocytosis, most of the evidence cited in section 3.1 strongly supports a clathrin-dependent pathway for DSL uptake. However, in invertebrates and, more specifically, in their oogenesis, Delta endocytosis could occur in an AP-2- and clathrin-independent way, as assayed by Notch activation of surrounding follicular cells triggered by germline clones bearing mutations of clathrin and AP-2 adaptor subunits, but not dynamin [190]. In the same system, it was also analyzed the dependence of Notch activation on endosomal trafficking in signal-sending cells: germline clones mutant for small GTPases that critically regulate the endosomal compartment, including Rab5 and Rab11, normally activate Notch in follicular cells. Taken together, these data support the absolute requirement for dynamin in DSL uptake. Conversely, neither CME nor endosomal entry of DSLs are universally required for Notch activation [190] (and, see section 3.1.1).

3.2. Notch receptor endocytosis

As discussed at the beginning of section 3, a strict requirement for endocytosis in the signal-receiving cell is supported by *Drosophila* studies on the *shibire* mutation in the sensory bristle development [153]. However, the mechanistic and molecular information available for Notch receptor endocytosis is very poor (and, sometimes, contradictory) in comparison with the large amount of data available for DSL internalization and trafficking.

3.2.1. Notch receptor internalization and PM-emanating signals

Some recent results seem to question the requirement of Notch receptor internalization for the activation of its signaling. In mammalian HeLa cells, overexpression of a dominant negative form of dynamin (the K44A mutation) does not prevent the processing of a chimeric NEXT to generate the NICD, which then translocates to the nucleus and activates signaling [275]. Blockage of the internalization step increases γ-secretase-mediated Notch processing and downstream signaling, suggesting that Notch receptor endocytosis might tame the Notch signaling emanating from the PM, as observed for other signaling pathways (see section 1).

This puzzling result is supported by other observations both *in vivo* and *in vitro*. (i) Presenilin can cleave any single-pass transmembrane protein provided that its extracellular domain is sufficiently small (<300 amino acids) [276]. Such presenilin substrates can normally be processed in the initial rounds of neuroblast segregation in *shibire*^{TS} embryos, suggesting that presenilin-dependent cleavage is not inherently dependent on Notch receptor endocytosis (at least during the first few hours upon temperature shifting, while experiments in [153] were scored after more than 6 hours) [276]. (ii) Proteolysis at S3 does not occur at a unique site but at multiple sites of NEXT, both in HEK293T cells and in a cell-free system. NICD fragments showed different stability and, therefore, signaling intensity, according to the proteasome N-end rule, where N-terminal valine provided maximal stability and signaling. Notably, PM-derived NICDs contain preferentially N-val, i.e. the most stable NICD, while endosome-generated NICD showed the lowest stability [277].

In the same set of experiments on HeLa cells, the machinery responsible for Notch internalization was also partially characterized. It was found that Notch uptake is strictly dependent on clathrin, since it is suppressed by knockdown of this latter gene and of its adaptor AP-2, while it is attenuated in the absence of epsin1 [275]. Notably, epsin1 interaction with Notch was ubiquitin-dependent, and the HECT domain-containing E3 ligase Nedd4 was found to participate in that action [275]. In the *Drosophila* system, Nedd4 is a negative regulator of Notch signaling by targeting Notch and Deltex (see later) to endocytosis and degradation, possibly protecting unstimulated cells from sporadic activation of Notch signaling [278].

To summarize, these data suggest that, in specific cell systems, PM emanating signals (from Notch receptors) can be (down)regulated by endocytosis, which uses the same machinery of the Notch signal-sending cell, i.e. clathrin-mediated endocytosis triggered by ubiquitylation with a role of epsin in coat formation and membrane invagination (and perhaps fission). The suppressive action of endocytosis on Notch activation can have many functions, including the termination of Notch signaling and the cell-fate determination of the Notch signal-sending cell, as Numb function seems to suggest (see next section).

3.2.2. Notch receptor trafficking and endosomal-emanating signals

In elegant morphological experiments, Notch receptor localization, processing, and signaling output in subsequent steps of its endocytic route were monitored by analyzing imaginal discs in *Drosophila* bearing homozygous mutations for key endocytic factors [279]. In the *shibire* and *Rab5* mutations, Notch accumulated at or below the plasma membrane, respectively, with no signaling effect in either case as scored by activation of a transcriptional reporter of Notch signaling. These data confirm the role of dynamin in Notch activation (in sharp contrast with that the role of endocytosis reported in the previous section), but, more importantly, they identify a new membrane compartment that is required for Notch activation, i.e. the endosome, in which Rab5 regulates the entry of endocytic cargos (reviewed in [280]). As expected, wing discs that express a constitutively active Rab5 show strong up-regulation of signaling, but similar results were also obtained

with overexpression of Hrs, which regulates entry into multivescicular bodies (MVBs). Taken together, these experiments suggest that the transport of Notch receptor to endosomes and to MVBs potently stimulates Notch signaling possibly making the endosome the preferential station for the full activation of Notch receptor.

In a search for factors that regulate Notch activation in endosomes, it was found that mutations of the vacuolar proton pump (V-ATPase) produce defects in the processing of the internalized Notch receptor and its signaling [281, 282]. These results, together with the observation that presenilin works optimally in an acidic environment such as that present in the endosome/lysosome [283], support the idea that endosomal sorting of Notch is required for best activation of its S3 cleavage. However, unrestricted access of Notch receptors to the endosome should be prevented, since the acidic pH could dissociate the NECD, thus triggering ligand-independent Notch activation [210].

Another somewhat newer protagonist in Notch activation from endosomes is Deltex, whose mutation results in a lethal phenotype when associated to a gene dosage defect of one of the DSLs or Notch. Deltex encodes for a highly conserved gene endowed with three domains (reviewed in [284]): (i) a N-terminal WWE domain which binds the ANK repeats of Notch, (ii) a central proline-rich region for the binding to yet unknown SH3 domain-containing proteins, and (iii) a C-terminal RING-domain which has the signature of an E3 ligase, yet formal evidence of a Deltex direct ubiquitylation of Notch is lacking [284]. All domains are necessary for Deltex function, whose action has been studied intensively in recent years.

Data support a Deltex action both in Notch internalization and activation. Evidence for these functions can be summarized as follows: (i) in the *Deltex*-null *Drosophila* mutant, Notch accumulates on the cell surface and in some unknown endosomal compartment, but failes to be efficiently incorporated into internalized vesicles from the PM and in transport vesicles from early endosomes to lysosomes [285]; (ii) Deltex overexpression promotes Notch accumulation in late endosomes, where its signaling activity is potently stimulated [286]; (iii) Deltex makes a functional complex with critical regulators of late endosome formation or maturation [287], i.e. AP-3 (which selects cargos for late endosomes and lysosomes [288]) and HOPS (which participates in late endosome maturation in lysosomes [289]). To summarize, Deltex regulates Notch activation by stably localizing Notch in the late endosomal compartment, thus avoiding its delivery to MVBs where signaling is suppressed (since internalization of Notch in MVBs would prevent NICD release in the cytosol, see Fig.2).

However, the positive or negative outcome of endosomal sorting on Notch activation depends on other regulatory factors that control or antagonize the action of Deltex. (i) A member of the Nedd4 family of E3 ligases, *Suppressor of Deltex (Su(dx)),* permits the exit of Notch from the late endosomal compartment to incorporation into MVBs, thus terminating Notch signaling by avoiding the cytoplasmic release of NICD [287]. It was hypothesized that this negative regulatory function might be favored by a direct ubiquitylation of Notch by Su(dx), which, however, has not yet been detected [284]. In contrast, other members of Nedd4 family promote Notch ubiquitylation and degradation both in Drosophila and

mammals (see section 3.2.1). (ii) A binding partner of Deltex is Kurtz (Krz), the homologue of mammalian non-visual β-arrestins. This protein family is involved in the desensitization and endocytosis of G-coupled receptors [290], TGF β [291] and Frizzled 4 [292]. Deltex, Krz and Notch form a complex in endocytic vesicles [293]. *Krz* mutants show upregulation of Notch signaling without altering Deltex levels, thus suggesting that the trimeric complex is important in the ubiquitin/proteasome-mediated degradation of Notch and subsequent signaling termination [293]. (iii) A critical component of the ESCRT III complex, Shrub, also seems to be involved in Deltex-Krz dependent Notch degradation. Shrub affects Notch trafficking and induces Notch accumulation in MVBs by promoting its polyubiquitylation and antagonizing Deltex, that instead promotes monoubiquitylation in the absence of Shrub [294]. Those opposite ubiquitylation states modulate Notch ligand-independent activation, by regulating how the receptor is trafficked in the endocytic path: polyubiquitylation targets Notch to MVBs for degradation, whereas monoubiquitylation is associated to Notch activation by γ-secretase [294]. Taken together, these results strongly support a preferential activation of Notch receptor during its intracellular trafficking and, more specifically, after its delivery to the (late) endosomal compartment, a trafficking event that is critically controlled by Deltex, at least in invertebrates.

A key aspect of Notch signaling is the need to establish differential signaling between two cell populations, i.e. the signal-receiving cells in which Notch activation can be triggered and the signal-sending cells in which Notch activation is suppressed. In invertebrates, Notch expression at the cell surface of the signal-sending cell is dramatically downregulated in order to inactivate Notch signaling in this cell population. One way of obtaining this effect is to target the Notch receptor to endosomal degradation with a specialized machinery. During the first division of the SOP, a membrane-associated protein called Numb is asymmetrically partitioned in the pIIb cell, which is committed to become the Notch signal-sending cell [295]. Loss of *NUMB* function causes all SOP descendants to differentiate in outer support cells, i.e. in Notch signal-receiving cells. Conversely, ectopic Numb expression during SOP division results in overproduction of neuronal precursors, i.e. of cells with the Notch signal-sending phenotype [295]. The epistaticity between Numb and Notch is further supported by genetic data in which reduction of Notch function can partially suppress the phenotypes resulting from loss of Numb [296]. Experiments in mammalian cells have shown that Numb is an endocytic factor, which binds the α-adaptin subunit of the clathrin-adaptor AP-2 [297, 298] and, together with this adaptor, co-localizes with internalizing receptors in mammalian cells [297]. In the SOP system in flies, Numb asymmetrically segregates AP-2 in the pIIb cell, and mutant isoforms of α-adaptin that no longer bind Numb fail to asymmetrically partition and cause Numb-like defects in SOP division [299]. Direct evidence of Numb function in Notch internalization was recently seen in anti-Notch antibody uptake experiments in the SOP lacking Numb expression [300].

Since Numb can co-exist with Notch in some cell systems without antagonizing its function (as in the lateral inhibition of *Drosophila* neuroectoderm [300]), it is plausible that other factors come into play to force the functional interaction of Numb with Notch receptors. In particular, two proteins have been identified as critical Numb-Notch interactors: (i) the

HECT-domain E3 ubiquitin ligase Itch, which ubiquitylates Notch receptors [301, 302] and many other proteins involved in key signaling pathways in mammalian cells (for a review see [303]) and (ii) the four-pass transmembrane protein Sanpodo (Spdo) [304, 305]. Numb, cooperatively enhances Itch-dependent ubiquitylation of Notch-1 [302]. This action requires direct and simultaneous binding of Numb to the ANK repeats of the Notch receptor and to the WW1-2 domains of Itch [302]. Therefore, Numb acts as an adaptor, facilitating or stabilizing the interaction between Itch and its substrate and, therefore, its catalytic activity. Numb-dependent endosomal sorting of Notch-1 in C2C12 cells critically depends on Itch function, since Numb mutants that do not interact with Itch (or that cannot interact with endocytic proteins) fail to promote Notch-1 degradation. All together, these experiments support a scenario in which Itch-mediated ubiquitylation is used to re-route Notch receptors to the late endosome for degradation and signaling suppression [306].

Another key interactor of Numb is Spdo, which is expressed in flies in both the Notch ligand-bearing and Notch receptor-bearing cells, where it acts differentially: in neuroblast division, Spdo is required for the activation of the Notch receptor in the A cell (a cell with Notch-dependent fate) [305] while in the B (signal-sending) cells it stimulates the endocytic degradation of the Notch receptor, in concert with Numb [305]. Notably, Numb in pIIb (signal-sending) cells of SOP induces the endocytosis of Spdo in early and late (but not recycling) endosomal vesicles. As for Numb internalization, Spdo endocytosis requires α–adaptin both in SOP [307] and in the neuroblast divisions in the flies [298]. As a result of *SPDO* loss-of-function, SOP cell stem cells divide symmetrically into two pIIb (signal-sending) cells, confirming that Spdo is required for Notch activation [300, 307]. In the case of Spdo ectopic overexpression, pIIb cells are generated as a result of Numb/Spdo-induced downregulation of Notch from the PM [307, 308]. Hence, Spdo may either activate or inhibit Notch signaling, depending on the presence or absence of Numb, and both actions are related to endocytosis.

4. Conclusions

In 2013, it will be one hundred years since the first Notch gene was discovered. During this century, fundamental aspects of gene functioning have been uncovered, including the key molecular mechanisms involved in the normal and pathological activation of Notch signaling. What emerged is that endocytosis is the master regulator of Notch activation. This function is exerted by means of a specialized endocytic machinery, which acts differentially in the Notch signal-sending cell compared with the Notch signal-receiving cell.

In Notch signal-sending, genetic, cell biology, structural, and biophysical studies point to a mechanical action of the Notch ligand on its receptor, so that critical proteolytic sites are uncovered for constitutive activation. Although the molecular machinery has not been fully characterized, genetic evidence in vertebrates and invertebrates supports clathrin-mediated endocytosis of ubiquitylated DSLs, as being the key mechanism that exerts the pulling action on the Notch receptor. In some developmental and cell culture systems, trafficking of the Notch ligand by transcytosis is another crucial mechanism which exerts the fundamental

action of locating the Notch ligands in PM domains, where the interaction with the Notch receptor occurs with the highest efficiency. Evidence in specialized developmental systems in invertebrates supports a third function of endocytosis in the Notch signal-receiving cell, where DSL trafficking through the recycling endosome may serve the purpose of making Notch ligands competent for interaction with Notch receptors. However, the molecular event that pre-activates the Notch ligand is unknown, and no evidence has been provided yet to support a similar request in mammalian cells.

In comparison with the Notch signal-sending cell, where an endocytosis requirement is well established and many molecular details of its action are known, very little information is available, especially in vertebrates, to help us understand the need for endocytosis in the Notch signal-receiving cell. Genetic and cell-biology studies suggest that Notch signaling preferentially spreads from the endosomal compartment, where the acidic environment favors the γ-secretase release of the Notch active fragment (i.e. the NICD). As in the signal-sending cell, ubiquitylation is requested for this process, and its modulation by a variety of factors either firmly localizes Notch in a membrane trafficking compartment for signal activation, or quickly moves it to lysosomes for signal suppression.

Although we are beginning to see the "the big picture", crucial mechanisms are still missing. Although incomplete, some of the available endocytosis-related information has already entered medical experimentation [309]. A clear example is γ-secretase inhibitors (GIS), whose action is exploited in many current clinical trials for T-ALL, breast carcinoma, colon cancer, medulloblastoma, glioblastoma, osteosarcoma, pancreatic cancer, small-cell lung carcinoma, and melanoma, just to cite some of these studies. Analyses of GIS have also been extended to basically all cell lines and animal models in which a function of Notch for tumor promotion, progression and spreading was not only proved, but merely supposed. However, GIS use in current medical practice is far from established since the molecules that have so far been tested are plagued by significant human toxicity involving gastrointestinal bleeding and immunosuppression, which is attributable to widespread suppression of Notch signaling in many tissues. As discussed throughout this review, Notch actually plays a key role in the homeostasis of a variety of adult tissues, and its suppression thus hampers the functionality of many organs and systems. More unconventional approaches of Notch-related therapy are based on raising inhibiting or activating antibodies that regulate the level of Notch signaling by interfering with the Notch ligand-Notch receptor interaction, and, consequently, by directly or indirectly affecting the endocytic regulation of Notch signaling. Some of these antibodies are already in the initial phases of clinical trials, and they promise to offer better selectivity in targeting specific Notch components, thus minimizing side effects.

Notch-targeting therapies have a wide potential spectrum of application besides cancer, which includes developmental, vascular, cardiac, and other diseases associated with Notch pathway malfunction, or where Notch function could be exploited profitably for their treatment. It is not difficult to envisage a future interest for a highly-specific "endocytic-based" therapeutic approach to Notch dysregulation.

Author details

Elisa Sala, Luca Ruggiero and Giuseppina Di Giacomo
Università Vita-Salute San Raffaele, Milano, Italy

Ottavio Cremona
Università Vita-Salute San Raffaele, Milano, Italy
Istituto Nazionale di Neuroscienze, Italy

Acknowledgement

We thank Michael John for critically editing this review and Lorenzo Cremona for the artwork. Our work is supported by grants to OC from Ministero dell'Istruzione dell'Università e della Ricerca Scientifica (MIUR - PRIN2008 grant), Fondazione Cariplo, Fondazione Telethon (grant GGP10099), and Associazione Italiana per la Ricerca sul Cancro.

5. References

[1] Mercer J, Schelhaas M, Helenius A (2010) Virus entry by endocytosis. Annu. Rev. Biochem. 79: 803-833.
[2] Margadant C, Monsuur HN, Norman JC, Sonnenberg A (2011) Mechanisms of integrin activation and trafficking. Curr. Opin. Cell Biol. 23: 607-614.
[3] Shilo BZ, Schejter ED (2011) Regulation of developmental intercellular signalling by intracellular trafficking. EMBO J. 30: 3516-3526.
[4] McMahon HT, Boucrot E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol. 12: 517-533.
[5] Haucke V, Neher E, Sigrist SJ (2011) Protein scaffolds in the coupling of synaptic exocytosis and endocytosis. Nat. Rev. Neurosci. 12: 127-138.
[6] Jiang X, Chen ZJ (2012) The role of ubiquitylation in immune defence and pathogen evasion. Nat. Rev. Immunol. 12: 35-48.
[7] Fu W, Jiang Q, Zhang C (2011) Novel functions of endocytic player clathrin in mitosis. Cell Res. 21: 1655-1661.
[8] Dittman J, Ryan TA (2009) Molecular circuitry of endocytosis at nerve terminals. Annu. Rev. Cell Dev. Biol. 25: 133-160.
[9] Sigismund S, Confalonieri S, Ciliberto A, Polo S, Scita G, Di Fiore PP (2012) Endocytosis and signaling: cell logistics shape the eukaryotic cell plan. Physiol. Rev. 92: 273-366.
[10] Sorkin A, von Zastrow M (2009) Endocytosis and signalling: intertwining molecular networks. Nat. Rev. Mol. Cell Biol. 10: 609-622.
[11] Haglund K, Dikic I (2012) The role of ubiquitylation in receptor endocytosis and endosomal sorting. J. Cell Sci. 125: 265-275.
[12] Abella JV, Park M (2009) Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases. Am J Physiol Endocrinol Metab. 296: E973-984.
[13] Bache KG, Slagsvold T, Stenmark H (2004) Defective downregulation of receptor tyrosine kinases in cancer. EMBO J. 23: 2707-2712.

[14] Haglund K, Rusten TE, Stenmark H (2007) Aberrant receptor signaling and trafficking as mechanisms in oncogenesis. Crit. Rev. Oncog. 13: 39-74.
[15] Baass PC, Di Guglielmo GM, Authier F, Posner BI, Bergeron JJ (1995) Compartmentalized signal transduction by receptor tyrosine kinases. Trends Cell Biol. 5: 465-470.
[16] Mosesson Y, Mills GB, Yarden Y (2008) Derailed endocytosis: an emerging feature of cancer. Nat. Rev. Cancer. 8: 835-850.
[17] Pitulescu ME, Adams RH (2010) Eph/ephrin molecules--a hub for signaling and endocytosis. Genes Dev. 24: 2480-2492.
[18] Haugh JM, Meyer T (2002) Active EGF receptors have limited access to PtdIns(4,5)P(2) in endosomes: implications for phospholipase C and PI 3-kinase signaling. J. Cell Sci. 115: 303-310.
[19] Howe CL, Mobley WC (2004) Signaling endosome hypothesis: A cellular mechanism for long distance communication. J. Neurobiol. 58: 207-216.
[20] Hupalowska A, Miaczynska M (2012) The new faces of endocytosis in signaling. Traffic. 13: 9-18.
[21] Wegner CS, Rodahl LM, Stenmark H (2011) ESCRT proteins and cell signalling. Traffic. 12: 1291-1297.
[22] Gonnord P, Blouin CM, Lamaze C (2012) Membrane trafficking and signaling: Two sides of the same coin. Semin. Cell Dev. Biol. 23: 154-164.
[23] Disanza A, Frittoli E, Palamidessi A, Scita G (2009) Endocytosis and spatial restriction of cell signaling. Mol. Oncol. 3: 280-296.
[24] Winckler B, Mellman I (2010) Trafficking guidance receptors. Cold Spring Harb Perspect Biol. 2: a001826.
[25] Wilde A, Beattie EC, Lem L, Riethof DA, Liu SH, Mobley WC, Soriano P, Brodsky FM (1999) EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. Cell. 96: 677-687.
[26] Ferguson S, Raimondi A, Paradise S, Shen H, Mesaki K, Ferguson A, Destaing O, Ko G, Takasaki J, Cremona O, E OT, De Camilli P (2009) Coordinated actions of actin and BAR proteins upstream of dynamin at endocytic clathrin-coated pits. Dev. Cell. 17: 811-822.
[27] Romer W, Pontani LL, Sorre B, Rentero C, Berland L, Chambon V, Lamaze C, Bassereau P, Sykes C, Gaus K, Johannes L (2010) Actin dynamics drive membrane reorganization and scission in clathrin-independent endocytosis. Cell. 140: 540-553.
[28] Shupliakov O, Bloom O, Gustafsson JS, Kjaerulff O, Low P, Tomilin N, Pieribone VA, Greengard P, Brodin L (2002) Impaired recycling of synaptic vesicles after acute perturbation of the presynaptic actin cytoskeleton. Proc. Natl. Acad. Sci. U. S. A. 99: 14476-14481.
[29] Soldati T, Schliwa M (2006) Powering membrane traffic in endocytosis and recycling. Nat. Rev. Mol. Cell Biol. 7: 897-908.
[30] Puthenveedu MA, Lauffer B, Temkin P, Vistein R, Carlton P, Thorn K, Taunton J, Weiner OD, Parton RG, von Zastrow M (2010) Sequence-dependent sorting of recycling proteins by actin-stabilized endosomal microdomains. Cell. 143: 761-773.

[31] Doherty GJ, McMahon HT (2009) Mechanisms of endocytosis. Annu. Rev. Biochem. 78: 857-902.
[32] Sandvig K, Pust S, Skotland T, van Deurs B (2011) Clathrin-independent endocytosis: mechanisms and function. Curr. Opin. Cell Biol. 23: 413-420.
[33] Grant BD, Donaldson JG (2009) Pathways and mechanisms of endocytic recycling. Nat. Rev. Mol. Cell Biol. 10: 597-608.
[34] Traub LM (2009) Tickets to ride: selecting cargo for clathrin-regulated internalization. Nat. Rev. Mol. Cell Biol. 10: 583-596.
[35] Cheng Y, Boll W, Kirchhausen T, Harrison SC, Walz T (2007) Cryo-electron tomography of clathrin-coated vesicles: structural implications for coat assembly. J. Mol. Biol. 365: 892-899.
[36] Kozlov MM, McMahon HT, Chernomordik LV (2010) Protein-driven membrane stresses in fusion and fission. Trends Biochem. Sci. 35: 699-706.
[37] Ferguson SM, Raimondi A, Paradise S, Shen H, Mesaki K, Ferguson A, Destaing O, Ko G, Takasaki J, Cremona O, E OT, De Camilli P (2009) Coordinated actions of actin and BAR proteins upstream of dynamin at endocytic clathrin-coated pits. Dev. Cell. 17: 811-822.
[38] Boulant S, Kural C, Zeeh JC, Ubelmann F, Kirchhausen T (2011) Actin dynamics counteract membrane tension during clathrin-mediated endocytosis. Nat. Cell Biol. 13: 1124-1131.
[39] Ferguson SM, De Camilli P (2012) Dynamin, a membrane-remodelling GTPase. Nat. Rev. Mol. Cell Biol. 13: 75-88.
[40] Schmid SL, Frolov VA (2011) Dynamin: functional design of a membrane fission catalyst. Annu. Rev. Cell Dev. Biol. 27: 79-105.
[41] Campelo F, Malhotra V (2012) Membrane Fission: The Biogenesis of Transport Carriers. Annu. Rev. Biochem. DOI: 10.1146/annurev-biochem-051710-094912.
[42] Schlossman DM, Schmid SL, Braell WA, Rothman JE (1984) An enzyme that removes clathrin coats: purification of an uncoating ATPase. J. Cell Biol. 99: 723-733.
[43] Ungewickell E, Ungewickell H, Holstein SE, Lindner R, Prasad K, Barouch W, Martin B, Greene LE, Eisenberg E (1995) Role of auxilin in uncoating clathrin-coated vesicles. Nature. 378: 632-635.
[44] Harrison SC, Kirchhausen T (2010) Structural biology: Conservation in vesicle coats. Nature. 466: 1048-1049.
[45] Ungewickell EJ, Hinrichsen L (2007) Endocytosis: clathrin-mediated membrane budding. Curr. Opin. Cell Biol. 19: 417-425.
[46] Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, McCormick DA, De Camilli P (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell. 99: 179-188.
[47] Kim WT, Chang S, Daniell L, Cremona O, Di Paolo G, De Camilli P (2002) Delayed reentry of recycling vesicles into the fusion-competent synaptic vesicle pool in synaptojanin 1 knockout mice. Proc. Natl. Acad. Sci. U. S. A. 99: 17143-17148.
[48] Howes MT, Mayor S, Parton RG (2010) Molecules, mechanisms, and cellular roles of clathrin-independent endocytosis. Curr. Opin. Cell Biol. 22: 519-527.

[49] Hansen CG, Nichols BJ (2009) Molecular mechanisms of clathrin-independent endocytosis. J. Cell Sci. 122: 1713-1721.
[50] Mayor S, Pagano RE (2007) Pathways of clathrin-independent endocytosis. Nat. Rev. Mol. Cell Biol. 8: 603-612.
[51] Rodal SK, Skretting G, Garred O, Vilhardt F, van Deurs B, Sandvig K (1999) Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. Mol. Biol. Cell. 10: 961-974.
[52] Lingwood D, Simons K (2010) Lipid rafts as a membrane-organizing principle. Science. 327: 46-50.
[53] Husnjak K, Dikic I (2012) Ubiquitin-Binding Proteins: Decoders of Ubiquitin-Mediated cellular functions. Annu. Rev. Biochem. DOI: 10.1146/annurev-biochem-051810-094654.
[54] Rao Y, Haucke V (2011) Membrane shaping by the Bin/amphiphysin/Rvs (BAR) domain protein superfamily. Cell. Mol. Life Sci. 68: 3983-3993.
[55] Canton I, Battaglia G (2012) Endocytosis at the nanoscale. Chem. Soc. Rev. 41: 2718-2739.
[56] Dexter JS (1914) The analysis of a case of continuous variation in Drosophila by a study of its linkage relations. Am. Nat. 48: 712–758.
[57] Welshons WJ (1958) The analysis of a pseudoallelic recessive lethal system at the notch locus of Drosophila melanogaster. Cold Spring Harb. Symp. Quant. Biol. 23: 171-176.
[58] Poulson DF (1937) Chromosomal Deficiencies and the Embryonic Development of Drosophila Melanogaster. Proc. Natl. Acad. Sci. U. S. A. 23: 133-137.
[59] Lehmann R, Jimenez F, Dietrich U, Campos-Ortega JA (1983) On the phenotype and development of mutants of early neurogenesis in Drosophila melanogaster. Dev. Genes Evol. 192: 62-74.
[60] Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science. 284: 770-776.
[61] Artavanis-Tsakonas S, Muskavitch MA (2010) Notch: the past, the present, and the future. Curr. Top. Dev. Biol. 92: 1-29.
[62] van Tetering G, van Diest P, Verlaan I, van der Wall E, Kopan R, Vooijs M (2009) Metalloprotease ADAM10 is required for Notch1 site 2 cleavage. J. Biol. Chem. 284: 31018-31027.
[63] Brou C, Logeat F, Gupta N, Bessia C, LeBail O, Doedens JR, Cumano A, Roux P, Black RA, Israel A (2000) A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. Mol. Cell. 5: 207-216.
[64] Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, Russell WE, Castner BJ, Johnson RS, Fitzner JN, Boyce RW, Nelson N, Kozlosky CJ, Wolfson MF, Rauch CT, Cerretti DP, Paxton RJ, March CJ, Black RA (1998) An essential role for ectodomain shedding in mammalian development. Science. 282: 1281-1284.
[65] Hartmann D, de Strooper B, Serneels L, Craessaerts K, Herreman A, Annaert W, Umans L, Lubke T, Lena Illert A, von Figura K, Saftig P (2002) The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum. Mol. Genet. 11: 2615-2624.

[66] Lieber T, Kidd S, Young MW (2002) kuzbanian-mediated cleavage of Drosophila Notch. Genes Dev. 16: 209-221.
[67] Sotillos S, Roch F, Campuzano S (1997) The metalloprotease-disintegrin Kuzbanian participates in Notch activation during growth and patterning of Drosophila imaginal discs. Development. 124: 4769-4779.
[68] Zhang C, Tian L, Chi C, Wu X, Yang X, Han M, Xu T, Zhuang Y, Deng K (2010) Adam10 is essential for early embryonic cardiovascular development. Dev. Dyn. 239: 2594-2602.
[69] De Strooper B, Annaert W, Cupers P, Saftig P, Craessaerts K, Mumm JS, Schroeter EH, Schrijvers V, Wolfe MS, Ray WJ, Goate A, Kopan R (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature. 398: 518-522.
[70] Schroeter EH, Kisslinger JA, Kopan R (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature. 393: 382-386.
[71] Francis R, McGrath G, Zhang J, Ruddy DA, Sym M, Apfeld J, Nicoll M, Maxwell M, Hai B, Ellis MC, Parks AL, Xu W, Li J, Gurney M, Myers RL, Himes CS, Hiebsch R, Ruble C, Nye JS, Curtis D (2002) aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. Dev. Cell. 3: 85-97.
[72] Morel V, Lecourtois M, Massiani O, Maier D, Preiss A, Schweisguth F (2001) Transcriptional repression by suppressor of hairless involves the binding of a hairless-dCtBP complex in Drosophila. Curr. Biol. 11: 789-792.
[73] Ghai V, Gaudet J (2008) The CSL transcription factor LAG-1 directly represses hlh-6 expression in C. elegans. Dev. Biol. 322: 334-344.
[74] Dou S, Zeng X, Cortes P, Erdjument-Bromage H, Tempst P, Honjo T, Vales LD (1994) The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. Mol. Cell. Biol. 14: 3310-3319.
[75] Andersson ER, Sandberg R, Lendahl U (2011) Notch signaling: simplicity in design, versatility in function. Development. 138: 3593-3612.
[76] Tanigaki K, Honjo T (2010) Two opposing roles of RBP-J in Notch signaling. Curr. Top. Dev. Biol. 92: 231-252.
[77] Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell. 137: 216-233.
[78] Borggrefe T, Oswald F (2009) The Notch signaling pathway: transcriptional regulation at Notch target genes. Cell. Mol. Life Sci. 66: 1631-1646.
[79] Hansson EM, Lendahl U, Chapman G (2004) Notch signaling in development and disease. Semin. Cancer Biol. 14: 320-328.
[80] Chillakuri CR, Sheppard D, Lea SM, Handford PA (2012) Notch receptor-ligand binding and activation: Insights from molecular studies. Semin. Cell Dev. Biol DOI: 10.1016/j.semcdb.2012.01.009.
[81] van Tetering G, Vooijs M (2011) Proteolytic cleavage of Notch: "HIT and RUN". Curr. Mol. Med. 11: 255-269.
[82] Yochem J, Greenwald I (1989) glp-1 and lin-12, genes implicated in distinct cell-cell interactions in C. elegans, encode similar transmembrane proteins. Cell. 58: 553-563.

[83] Fitzgerald K, Wilkinson HA, Greenwald I (1993) glp-1 can substitute for lin-12 in specifying cell fate decisions in Caenorhabditis elegans. Development. 119: 1019-1027.
[84] Blaumueller CM, Qi H, Zagouras P, Artavanis-Tsakonas S (1997) Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell. 90: 281-291.
[85] Logeat F, Bessia C, Brou C, LeBail O, Jarriault S, Seidah NG, Israel A (1998) The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc. Natl. Acad. Sci. U. S. A. 95: 8108-8112.
[86] Bush G, diSibio G, Miyamoto A, Denault JB, Leduc R, Weinmaster G (2001) Ligand-induced signaling in the absence of furin processing of Notch1. Dev. Biol. 229: 494-502.
[87] Nichols JT, Miyamoto A, Olsen SL, D'Souza B, Yao C, Weinmaster G (2007) DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. J. Cell Biol. 176: 445-458.
[88] Gordon WR, Vardar-Ulu D, L'Heureux S, Ashworth T, Malecki MJ, Sanchez-Irizarry C, McArthur DG, Histen G, Mitchell JL, Aster JC, Blacklow SC (2009) Effects of S1 cleavage on the structure, surface export, and signaling activity of human Notch1 and Notch2. PLoS ONE. 4: e6613.
[89] Kido H, Okumura Y, Takahashi E, Pan HY, Wang S, Yao D, Yao M, Chida J, Yano M (2012) Role of host cellular proteases in the pathogenesis of influenza and influenza-induced multiple organ failure. Biochim. Biophys. Acta. 1824: 186-194.
[90] Day PM, Schiller JT (2009) The role of furin in papillomavirus infection. Future Microbiol. 4: 1255-1262.
[91] Moulard M, Hallenberger S, Garten W, Klenk HD (1999) Processing and routage of HIV glycoproteins by furin to the cell surface. Virus Res. 60: 55-65.
[92] Bolt G, Pedersen IR (1998) The role of subtilisin-like proprotein convertases for cleavage of the measles virus fusion glycoprotein in different cell types. Virology. 252: 387-398.
[93] Harrison SC (2005) Mechanism of membrane fusion by viral envelope proteins. Adv. Virus Res. 64: 231-261.
[94] Sanchez-Irizarry C, Carpenter AC, Weng AP, Pear WS, Aster JC, Blacklow SC (2004) Notch subunit heterodimerization and prevention of ligand-independent proteolytic activation depend, respectively, on a novel domain and the LNR repeats. Mol. Cell. Biol. 24: 9265-9273.
[95] Gordon WR, Roy M, Vardar-Ulu D, Garfinkel M, Mansour MR, Aster JC, Blacklow SC (2009) Structure of the Notch1-negative regulatory region: implications for normal activation and pathogenic signaling in T-ALL. Blood. 113: 4381-4390.
[96] Li K, Li Y, Wu W, Gordon WR, Chang DW, Lu M, Scoggin S, Fu T, Vien L, Histen G, Zheng J, Martin-Hollister R, Duensing T, Singh S, Blacklow SC, Yao Z, Aster JC, Zhou BB (2008) Modulation of Notch signaling by antibodies specific for the extracellular negative regulatory region of NOTCH3. J. Biol. Chem. 283: 8046-8054.
[97] Tiyanont K, Wales TE, Aste-Amezaga M, Aster JC, Engen JR, Blacklow SC (2011) Evidence for increased exposure of the Notch1 metalloprotease cleavage site upon conversion to an activated conformation. Structure. 19: 546-554.
[98] Gordon WR, Vardar-Ulu D, Histen G, Sanchez-Irizarry C, Aster JC, Blacklow SC (2007) Structural basis for autoinhibition of Notch. Nat. Struct. Mol. Biol. 14: 295-300.

[99] Spinner NB, Colliton RP, Crosnier C, Krantz ID, Hadchouel M, Meunier-Rotival M (2001) Jagged1 mutations in alagille syndrome. Hum. Mutat. 17: 18-33.

[100] Ropke A, Kujat A, Graber M, Giannakudis J, Hansmann I (2003) Identification of 36 novel Jagged1 (JAG1) mutations in patients with Alagille syndrome. Hum. Mutat. 21: 100.

[101] Mizuhara E, Nakatani T, Minaki Y, Sakamoto Y, Ono Y, Takai Y (2005) MAGI1 recruits Dll1 to cadherin-based adherens junctions and stabilizes it on the cell surface. J. Biol. Chem. 280: 26499-26507.

[102] Six EM, Ndiaye D, Sauer G, Laabi Y, Athman R, Cumano A, Brou C, Israel A, Logeat F (2004) The notch ligand Delta1 recruits Dlg1 at cell-cell contacts and regulates cell migration. J. Biol. Chem. 279: 55818-55826.

[103] Popovic M, Bella J, Zlatev V, Hodnik V, Anderluh G, Barlow PN, Pintar A, Pongor S (2011) The interaction of Jagged-1 cytoplasmic tail with afadin PDZ domain is local, folding-independent, and tuned by phosphorylation. J. Mol. Recognit. 24: 245-253.

[104] LaVoie MJ, Selkoe DJ (2003) The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. J. Biol. Chem. 278: 34427-34437.

[105] Parr-Sturgess CA, Rushton DJ, Parkin ET (2010) Ectodomain shedding of the Notch ligand Jagged1 is mediated by ADAM17, but is not a lipid-raft-associated event. Biochem. J. 432: 283-294.

[106] Fehon RG, Kooh PJ, Rebay I, Regan CL, Xu T, Muskavitch MA, Artavanis-Tsakonas S (1990) Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. Cell. 61: 523-534.

[107] Rebay I, Fleming RJ, Fehon RG, Cherbas L, Cherbas P, Artavanis-Tsakonas S (1991) Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell. 67: 687-699.

[108] Rebay I, Fehon RG, Artavanis-Tsakonas S (1993) Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell. 74: 319-329.

[109] Xu A, Lei L, Irvine KD (2005) Regions of Drosophila Notch that contribute to ligand binding and the modulatory influence of Fringe. J. Biol. Chem. 280: 30158-30165.

[110] Hambleton S, Valeyev NV, Muranyi A, Knott V, Werner JM, McMichael AJ, Handford PA, Downing AK (2004) Structural and functional properties of the human notch-1 ligand binding region. Structure. 12: 2173-2183.

[111] Shimizu K, Chiba S, Kumano K, Hosoya N, Takahashi T, Kanda Y, Hamada Y, Yazaki Y, Hirai H (1999) Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods. J. Biol. Chem. 274: 32961-32969.

[112] Cordle J, Johnson S, Tay JZ, Roversi P, Wilkin MB, de Madrid BH, Shimizu H, Jensen S, Whiteman P, Jin B, Redfield C, Baron M, Lea SM, Handford PA (2008) A conserved face of the Jagged/Serrate DSL domain is involved in Notch trans-activation and cis-inhibition. Nat. Struct. Mol. Biol. 15: 849-857.

[113] Cordle J, Redfieldz C, Stacey M, van der Merwe PA, Willis AC, Champion BR, Hambleton S, Handford PA (2008) Localization of the delta-like-1-binding site in human Notch-1 and its modulation by calcium affinity. J. Biol. Chem. 283: 11785-11793.

[114] Wang Y, Spellman MW (1998) Purification and characterization of a GDP-fucose:polypeptide fucosyltransferase from Chinese hamster ovary cells. J. Biol. Chem. 273: 8112-8118.

[115] Okajima T, Irvine KD (2002) Regulation of notch signaling by o-linked fucose. Cell. 111: 893-904.

[116] Moloney DJ, Panin VM, Johnston SH, Chen J, Shao L, Wilson R, Wang Y, Stanley P, Irvine KD, Haltiwanger RS, Vogt TF (2000) Fringe is a glycosyltransferase that modifies Notch. Nature. 406: 369-375.

[117] Sasamura T, Sasaki N, Miyashita F, Nakao S, Ishikawa HO, Ito M, Kitagawa M, Harigaya K, Spana E, Bilder D, Perrimon N, Matsuno K (2003) neurotic, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. Development. 130: 4785-4795.

[118] Shi S, Stanley P (2003) Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. Proc. Natl. Acad. Sci. U. S. A. 100: 5234-5239.

[119] Yao D, Huang Y, Huang X, Wang W, Yan Q, Wei L, Xin W, Gerson S, Stanley P, Lowe JB, Zhou L (2011) Protein O-fucosyltransferase 1 (Pofut1) regulates lymphoid and myeloid homeostasis through modulation of Notch receptor ligand interactions. Blood. 117: 5652-5662.

[120] Ge C, Stanley P (2008) The O-fucose glycan in the ligand-binding domain of Notch1 regulates embryogenesis and T cell development. Proc. Natl. Acad. Sci. U. S. A. 105: 1539-1544.

[121] Okajima T, Xu A, Lei L, Irvine KD (2005) Chaperone activity of protein O-fucosyltransferase 1 promotes notch receptor folding. Science. 307: 1599-1603.

[122] Becker DJ, Lowe JB (2003) Fucose: biosynthesis and biological function in mammals. Glycobiology. 13: 41R-53R.

[123] Glavic A, Lopez-Varea A, de Celis JF (2011) The balance between GMD and OFUT1 regulates Notch signaling pathway activity by modulating Notch stability. Biol. Res. 44: 25-34.

[124] Stahl M, Uemura K, Ge C, Shi S, Tashima Y, Stanley P (2008) Roles of Pofut1 and O-fucose in mammalian Notch signaling. J. Biol. Chem. 283: 13638-13651.

[125] Vodovar N, Schweisguth F (2008) Functions of O-fucosyltransferase in Notch trafficking and signaling: towards the end of a controversy? J. Biol. 7: 7.

[126] Okajima T, Reddy B, Matsuda T, Irvine KD (2008) Contributions of chaperone and glycosyltransferase activities of O-fucosyltransferase 1 to Notch signaling. BMC Biol. 6: 1.

[127] Lowe JB (2005) Cell biology. Does Notch take the sweet road to success? Science. 307: 1570-1572.

[128] Irvine KD, Wieschaus E (1994) fringe, a Boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during Drosophila wing development. Cell. 79: 595-606.

[129] Johnston SH, Rauskolb C, Wilson R, Prabhakaran B, Irvine KD, Vogt TF (1997) A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway. Development. 124: 2245-2254.
[130] Tien AC, Rajan A, Bellen HJ (2009) A Notch updated. J. Cell Biol. 184: 621-629.
[131] Rana NA, Haltiwanger RS (2011) Fringe benefits: functional and structural impacts of O-glycosylation on the extracellular domain of Notch receptors. Curr. Opin. Struct. Biol. 21: 583-589.
[132] Rampal R, Li AS, Moloney DJ, Georgiou SA, Luther KB, Nita-Lazar A, Haltiwanger RS (2005) Lunatic fringe, manic fringe, and radical fringe recognize similar specificity determinants in O-fucosylated epidermal growth factor-like repeats. J. Biol. Chem. 280: 42454-42463.
[133] Visan I, Tan JB, Yuan JS, Harper JA, Koch U, Guidos CJ (2006) Regulation of T lymphopoiesis by Notch1 and Lunatic fringe-mediated competition for intrathymic niches. Nat. Immunol. 7: 634-643.
[134] Evrard YA, Lun Y, Aulehla A, Gan L, Johnson RL (1998) lunatic fringe is an essential mediator of somite segmentation and patterning. Nature. 394: 377-381.
[135] Zhang N, Gridley T (1998) Defects in somite formation in lunatic fringe-deficient mice. Nature. 394: 374-377.
[136] Zhang N, Norton CR, Gridley T (2002) Segmentation defects of Notch pathway mutants and absence of a synergistic phenotype in lunatic fringe/radical fringe double mutant mice. Genesis. 33: 21-28.
[137] Moran JL, Shifley ET, Levorse JM, Mani S, Ostmann K, Perez-Balaguer A, Walker DM, Vogt TF, Cole SE (2009) Manic fringe is not required for embryonic development, and fringe family members do not exhibit redundant functions in the axial skeleton, limb, or hindbrain. Dev. Dyn. 238: 1803-1812.
[138] Svensson P, Bergqvist I, Norlin S, Edlund H (2009) MFng is dispensable for mouse pancreas development and function. Mol. Cell. Biol. 29: 2129-2138.
[139] Xu A, Haines N, Dlugosz M, Rana NA, Takeuchi H, Haltiwanger RS, Irvine KD (2007) In vitro reconstitution of the modulation of Drosophila Notch-ligand binding by Fringe. J. Biol. Chem. 282: 35153-35162.
[140] Panin VM, Papayannopoulos V, Wilson R, Irvine KD (1997) Fringe modulates Notch-ligand interactions. Nature. 387: 908-912.
[141] Fleming RJ, Gu Y, Hukriede NA (1997) Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc. Development. 124: 2973-2981.
[142] Acar M, Jafar-Nejad H, Takeuchi H, Rajan A, Ibrani D, Rana NA, Pan H, Haltiwanger RS, Bellen HJ (2008) Rumi is a CAP10 domain glycosyltransferase that modifies Notch and is required for Notch signaling. Cell. 132: 247-258.
[143] Fernandez-Valdivia R, Takeuchi H, Samarghandi A, Lopez M, Leonardi J, Haltiwanger RS, Jafar-Nejad H (2011) Regulation of mammalian Notch signaling and embryonic development by the protein O-glucosyltransferase Rumi. Development. 138: 1925-1934.
[144] Irvine KD (2008) A notch sweeter. Cell. 132: 177-179.

[145] Kosaka T, Ikeda K (1983) Possible temperature-dependent blockage of synaptic vesicle recycling induced by a single gene mutation in Drosophila. J. Neurobiol. 14: 207-225.
[146] Poodry CA (1990) shibire, a neurogenic mutant of Drosophila. Dev. Biol. 138: 464-472.
[147] Ramaswami M, Rao S, van der Bliek A, Kelly RB, Krishnan KS (1993) Genetic studies on dynamin function in Drosophila. J. Neurogenet. 9: 73-87.
[148] Bejsovec A, Wieschaus E (1995) Signaling activities of the Drosophila wingless gene are separately mutable and appear to be transduced at the cell surface. Genetics. 139: 309-320.
[149] Seto ES, Bellen HJ (2006) Internalization is required for proper Wingless signaling in Drosophila melanogaster. J. Cell Biol. 173: 95-106.
[150] Rives AF, Rochlin KM, Wehrli M, Schwartz SL, DiNardo S (2006) Endocytic trafficking of Wingless and its receptors, Arrow and DFrizzled-2, in the Drosophila wing. Dev. Biol. 293: 268-283.
[151] Marois E, Mahmoud A, Eaton S (2006) The endocytic pathway and formation of the Wingless morphogen gradient. Development. 133: 307-317.
[152] Fischer JA, Eun SH, Doolan BT (2006) Endocytosis, endosome trafficking, and the regulation of Drosophila development. Annu. Rev. Cell Dev. Biol. 22: 181-206.
[153] Seugnet L, Simpson P, Haenlin M (1997) Requirement for dynamin during Notch signaling in Drosophila neurogenesis. Dev. Biol. 192: 585-598.
[154] Kooh PJ, Fehon RG, Muskavitch MA (1993) Implications of dynamic patterns of Delta and Notch expression for cellular interactions during Drosophila development. Development. 117: 493-507.
[155] Huppert SS, Jacobsen TL, Muskavitch MA (1997) Feedback regulation is central to Delta-Notch signalling required for Drosophila wing vein morphogenesis. Development. 124: 3283-3291.
[156] Parks AL, Huppert SS, Muskavitch MA (1997) The dynamics of neurogenic signalling underlying bristle development in Drosophila melanogaster. Mech. Dev. 63: 61-74.
[157] Klueg KM, Muskavitch MA (1999) Ligand-receptor interactions and trans-endocytosis of Delta, Serrate and Notch: members of the Notch signalling pathway in Drosophila. J. Cell Sci. 112:3289-3297.
[158] Klueg KM, Parody TR, Muskavitch MA (1998) Complex proteolytic processing acts on Delta, a transmembrane ligand for Notch, during Drosophila development. Mol. Biol. Cell. 9: 1709-1723.
[159] Parks AL, Turner FR, Muskavitch MA (1995) Relationships between complex Delta expression and the specification of retinal cell fates during Drosophila eye development. Mech. Dev. 50: 201-216.
[160] Kramer H, Phistry M (1996) Mutations in the Drosophila hook gene inhibit endocytosis of the boss transmembrane ligand into multivesicular bodies. J. Cell Biol. 133: 1205-1215.
[161] Fehon RG, Johansen K, Rebay I, Artavanis-Tsakonas S (1991) Complex cellular and subcellular regulation of notch expression during embryonic and imaginal development of Drosophila: implications for notch function. J. Cell Biol. 113: 657-669.

[162] Le Borgne R, Remaud S, Hamel S, Schweisguth F (2005) Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. PLoS Biol. 3: e96.

[163] Le Borgne R, Schweisguth F (2003) Unequal segregation of Neuralized biases Notch activation during asymmetric cell division. Dev. Cell. 5: 139-148.

[164] Itoh M, Kim CH, Palardy G, Oda T, Jiang YJ, Maust D, Yeo SY, Lorick K, Wright GJ, Ariza-McNaughton L, Weissman AM, Lewis J, Chandrasekharappa SC, Chitnis AB (2003) Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev. Cell. 4: 67-82.

[165] Parks AL, Klueg KM, Stout JR, Muskavitch MA (2000) Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development. 127: 1373-1385.

[166] Hicks C, Ladi E, Lindsell C, Hsieh JJ, Hayward SD, Collazo A, Weinmaster G (2002) A secreted Delta1-Fc fusion protein functions both as an activator and inhibitor of Notch1 signaling. J. Neurosci. Res. 68: 655-667.

[167] Ladi E, Nichols JT, Ge W, Miyamoto A, Yao C, Yang LT, Boulter J, Sun YE, Kintner C, Weinmaster G (2005) The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. J. Cell Biol. 170: 983-992.

[168] Chitnis A, Henrique D, Lewis J, Ish-Horowicz D, Kintner C (1995) Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature. 375: 761-766.

[169] Jen WC, Wettstein D, Turner D, Chitnis A, Kintner C (1997) The Notch ligand, X-Delta-2, mediates segmentation of the paraxial mesoderm in Xenopus embryos. Development. 124: 1169-1178.

[170] Sun X, Artavanis-Tsakonas S (1996) The intracellular deletions of Delta and Serrate define dominant negative forms of the Drosophila Notch ligands. Development. 122: 2465-2474.

[171] Wang W, Struhl G (2004) Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development. 131: 5367-5380.

[172] Chen WJ, Goldstein JL, Brown MS (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J. Biol. Chem. 265: 3116-3123.

[173] Jafar-Nejad H, Andrews HK, Acar M, Bayat V, Wirtz-Peitz F, Mehta SQ, Knoblich JA, Bellen HJ (2005) Sec15, a component of the exocyst, promotes notch signaling during the asymmetric division of Drosophila sensory organ precursors. Dev. Cell. 9: 351-363.

[174] Emery G, Hutterer A, Berdnik D, Mayer B, Wirtz-Peitz F, Gaitan MG, Knoblich JA (2005) Asymmetric Rab 11 endosomes regulate delta recycling and specify cell fate in the Drosophila nervous system. Cell. 122: 763-773.

[175] Heuss SF, Ndiaye-Lobry D, Six EM, Israel A, Logeat F (2008) The intracellular region of Notch ligands Dll1 and Dll3 regulates their trafficking and signaling activity. Proc. Natl. Acad. Sci. U. S. A. 105: 11212-11217.

[176] Rajan A, Tien AC, Haueter CM, Schulze KL, Bellen HJ (2009) The Arp2/3 complex and WASp are required for apical trafficking of Delta into microvilli during cell fate specification of sensory organ precursors. Nat. Cell Biol. 11: 815-824.
[177] Gho M, Bellaiche Y, Schweisguth F (1999) Revisiting the Drosophila microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. Development. 126: 3573-3584.
[178] Reddy GV, Rodrigues V (1999) A glial cell arises from an additional division within the mechanosensory lineage during development of the microchaete on the Drosophila notum. Development. 126: 4617-4622.
[179] Zeng C, Younger-Shepherd S, Jan LY, Jan YN (1998) Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the Drosophila sensory organ lineage. Genes Dev. 12: 1086-1091.
[180] Benhra N, Vignaux F, Dussert A, Schweisguth F, Le Borgne R (2010) Neuralized promotes basal to apical transcytosis of delta in epithelial cells. Mol. Biol. Cell. 21: 2078-2086.
[181] Firat-Karalar EN, Welch MD (2011) New mechanisms and functions of actin nucleation. Curr. Opin. Cell Biol. 23: 4-13.
[182] Rottner K, Hanisch J, Campellone KG (2010) WASH, WHAMM and JMY: regulation of Arp2/3 complex and beyond. Trends Cell Biol. 20: 650-661.
[183] Kurisu S, Takenawa T (2010) WASP and WAVE family proteins: friends or foes in cancer invasion? Cancer Sci. 101: 2093-2104.
[184] Schejter ED (2009) Delta traffic takes a sh-Arp turn. Nat. Cell Biol. 11: 791-793.
[185] Kaksonen M, Toret CP, Drubin DG (2006) Harnessing actin dynamics for clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol. 7: 404-414.
[186] Anitei M, Hoflack B (2012) Bridging membrane and cytoskeleton dynamics in the secretory and endocytic pathways. Nat. Cell Biol. 14: 11-19.
[187] Conibear E (2010) Converging views of endocytosis in yeast and mammals. Curr. Opin. Cell Biol. 22: 513-518.
[188] Galletta BJ, Mooren OL, Cooper JA (2010) Actin dynamics and endocytosis in yeast and mammals. Curr. Opin. Biotechnol. 21: 604-610.
[189] Banks SM, Cho B, Eun SH, Lee JH, Windler SL, Xie X, Bilder D, Fischer JA (2011) The functions of auxilin and Rab11 in Drosophila suggest that the fundamental role of ligand endocytosis in notch signaling cells is not recycling. PLoS ONE. 6: e18259.
[190] Windler SL, Bilder D (2010) Endocytic internalization routes required for delta/notch signaling. Curr. Biol. 20: 538-543.
[191] Benoit M, Gabriel D, Gerisch G, Gaub HE (2000) Discrete interactions in cell adhesion measured by single-molecule force spectroscopy. Nat. Cell Biol. 2: 313-317.
[192] Ahimou F, Mok LP, Bardot B, Wesley C (2004) The adhesion force of Notch with Delta and the rate of Notch signaling. J. Cell Biol. 167: 1217-1229.
[193] Hoppe PE, Greenspan RJ (1990) The Notch locus of Drosophila is required in epidermal cells for epidermal development. Development. 109: 875-885.

[194] Baker NE, Yu SY (1998) The R8-photoreceptor equivalence group in Drosophila: fate choice precedes regulated Delta transcription and is independent of Notch gene dose. Mech. Dev. 74: 3-14.

[195] Okajima T, Xu A, Irvine KD (2003) Modulation of notch-ligand binding by protein O-fucosyltransferase 1 and fringe. J. Biol. Chem. 278: 42340-42345.

[196] Musse AA, Meloty-Kapella L, Weinmaster G (2012) Notch ligand endocytosis: Mechanistic basis of signaling activity. Semin. Cell Dev. Biol DOI: 10.1016/j.semcdb.2012.01.011.

[197] Struhl G, Adachi A (1998) Nuclear access and action of notch in vivo. Cell. 93: 649-660.

[198] Kopan R, Schroeter EH, Weintraub H, Nye JS (1996) Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. Proc. Natl. Acad. Sci. U. S. A. 93: 1683-1688.

[199] Lieber T, Kidd S, Alcamo E, Corbin V, Young MW (1993) Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. Genes Dev. 7: 1949-1965.

[200] Aste-Amezaga M, Zhang N, Lineberger JE, Arnold BA, Toner TJ, Gu M, Huang L, Vitelli S, Vo KT, Haytko P, Zhao JZ, Baleydier F, L'Heureux S, Wang H, Gordon WR, Thoryk E, Andrawes MB, Tiyanont K, Stegmaier K, Roti G, Ross KN, Franlin LL, Wang F, Chastain M, Bett AJ, Audoly LP, Aster JC, Blacklow SC, Huber HE (2010) Characterization of Notch1 antibodies that inhibit signaling of both normal and mutated Notch1 receptors. PLoS ONE. 5: e9094.

[201] Berry LW, Westlund B, Schedl T (1997) Germ-line tumor formation caused by activation of glp-1, a Caenorhabditis elegans member of the Notch family of receptors. Development. 124: 925-936.

[202] Greenwald I, Seydoux G (1990) Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans. Nature. 346: 197-199.

[203] Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 306: 269-271.

[204] Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, Finkle D, Venook R, Wu X, Ridgway J, Schahin-Reed D, Dow GJ, Shelton A, Stawicki S, Watts RJ, Zhang J, Choy R, Howard P, Kadyk L, Yan M, Zha J, Callahan CA, Hymowitz SG, Siebel CW (2010) Therapeutic antibody targeting of individual Notch receptors. Nature. 464: 1052-1057 .

[205] Wales TE, Engen JR (2006) Hydrogen exchange mass spectrometry for the analysis of protein dynamics. Mass Spectrom. Rev. 25: 158-170.

[206] Raphael K (2011) Deuterium reveals the dynamics of notch activation. Structure. 19: 443-444.

[207] Rand MD, Grimm LM, Artavanis-Tsakonas S, Patriub V, Blacklow SC, Sklar J, Aster JC (2000) Calcium depletion dissociates and activates heterodimeric notch receptors. Mol. Cell. Biol. 20: 1825-1835.

[208] Chen J, Zolkiewska A (2011) Force-induced unfolding simulations of the human Notch1 negative regulatory region: possible roles of the heterodimerization domain in mechanosensing. PLoS ONE. 6: e22837.
[209] Weinmaster G, Fischer JA (2011) Notch ligand ubiquitylation: what is it good for? Dev. Cell. 21: 134-144.
[210] Le Bras S, Loyer N, Le Borgne R (2011) The multiple facets of ubiquitination in the regulation of notch signaling pathway. Traffic. 12: 149-161.
[211] Baron M, Aslam H, Flasza M, Fostier M, Higgs JE, Mazaleyrat SL, Wilkin MB (2002) Multiple levels of Notch signal regulation (review). Mol. Membr. Biol. 19: 27-38.
[212] Macgurn JA, Hsu PC, Emr SD (2012) Ubiquitin and Membrane Protein Turnover: From Cradle to Grave. Annu. Rev. Biochem.
[213] Emmerich CH, Schmukle AC, Walczak H (2011) The emerging role of linear ubiquitination in cell signaling. Sci. Signal. 4: re5.
[214] Schaefer A, Nethe M, Hordijk PL (2012) Ubiquitin links to cytoskeletal dynamics, cell adhesion and migration. Biochem. J. 442: 13-25.
[215] Piper RC, Lehner PJ (2011) Endosomal transport via ubiquitination. Trends Cell Biol. 21: 647-655.
[216] Ikeda F, Dikic I (2008) Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO Rep. 9: 536-542.
[217] Dikic I, Wakatsuki S, Walters KJ (2009) Ubiquitin-binding domains - from structures to functions. Nat. Rev. Mol. Cell Biol. 10: 659-671.
[218] Maupin-Furlow J (2012) Proteasomes and protein conjugation across domains of life. Nat. Rev. Microbiol. 10: 100-111.
[219] Mocciaro A, Rape M (2012) Emerging regulatory mechanisms in ubiquitin-dependent cell cycle control. J. Cell Sci. 125: 255-263.
[220] Vucic D, Dixit VM, Wertz IE (2011) Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat. Rev. Mol. Cell Biol. 12: 439-452.
[221] Voutsadakis IA (2012) Ubiquitination and the Ubiquitin-Proteasome System as regulators of transcription and transcription factorsin epithelial mesenchymal transition of cancer. Tumour Biol DOI: 10.1007/s13277-012-0355-x.
[222] Wright DE, Wang CY, Kao CF (2012) Histone ubiquitylation and chromatin dynamics. Front. Biosci. 17: 1051-1078.
[223] Hammond-Martel I, Yu H, Affar el B (2012) Roles of ubiquitin signaling in transcription regulation. Cell. Signal. 24: 410-421.
[224] Benyair R, Ron E, Lederkremer GZ (2011) Protein quality control, retention, and degradation at the endoplasmic reticulum. Int Rev Cell Mol Biol. 292: 197-280.
[225] Burrows JF, Johnston JA (2012) Regulation of cellular responses by deubiquitinating enzymes: an update. Front. Biosci. 17: 1184-1200.
[226] Todi SV, Paulson HL (2011) Balancing act: deubiquitinating enzymes in the nervous system. Trends Neurosci DOI:10.1016/j.tins.2011.05.004.
[227] Ramakrishna S, Suresh B, Baek KH (2011) The role of deubiquitinating enzymes in apoptosis. Cell. Mol. Life Sci. 68: 15-26.

[228] Shabek N, Ciechanover A (2010) Degradation of ubiquitin: the fate of the cellular reaper. Cell Cycle. 9: 523-530.
[229] Komander D, Clague MJ, Urbe S (2009) Breaking the chains: structure and function of the deubiquitinases. Nat. Rev. Mol. Cell Biol. 10: 550-563.
[230] Reyes-Turcu FE, Ventii KH, Wilkinson KD (2009) Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu. Rev. Biochem. 78: 363-397.
[231] Lai EC, Deblandre GA, Kintner C, Rubin GM (2001) Drosophila neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. Dev. Cell. 1: 783-794.
[232] Yeh E, Dermer M, Commisso C, Zhou L, McGlade CJ, Boulianne GL (2001) Neuralized functions as an E3 ubiquitin ligase during Drosophila development. Curr. Biol. 11: 1675-1679.
[233] Deblandre GA, Lai EC, Kintner C (2001) Xenopus neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. Dev. Cell. 1: 795-806.
[234] Pavlopoulos E, Pitsouli C, Klueg KM, Muskavitch MA, Moschonas NK, Delidakis C (2001) neuralized Encodes a peripheral membrane protein involved in delta signaling and endocytosis. Dev. Cell. 1: 807-816.
[235] De Renzis S, Yu J, Zinzen R, Wieschaus E (2006) Dorsal-ventral pattern of Delta trafficking is established by a Snail-Tom-Neuralized pathway. Dev. Cell. 10: 257-264.
[236] Parks AL, Stout JR, Shepard SB, Klueg KM, Dos Santos AA, Parody TR, Vaskova M, Muskavitch MA (2006) Structure-function analysis of delta trafficking, receptor binding and signaling in Drosophila. Genetics. 174: 1947-1961.
[237] Glittenberg M, Pitsouli C, Garvey C, Delidakis C, Bray S (2006) Role of conserved intracellular motifs in Serrate signalling, cis-inhibition and endocytosis. EMBO J. 25: 4697-4706.
[238] Fontana JR, Posakony JW (2009) Both inhibition and activation of Notch signaling rely on a conserved Neuralized-binding motif in Bearded proteins and the Notch ligand Delta. Dev. Biol. 333: 373-385.
[239] Commisso C, Boulianne GL (2007) The NHR1 domain of Neuralized binds Delta and mediates Delta trafficking and Notch signaling. Mol. Biol. Cell. 18: 1-13.
[240] Koo BK, Yoon MJ, Yoon KJ, Im SK, Kim YY, Kim CH, Suh PG, Jan YN, Kong YY (2007) An obligatory role of mind bomb-1 in notch signaling of Mammalian development. PLoS ONE. 2: e1221.
[241] Ruan Y, Tecott L, Jiang MM, Jan LY, Jan YN (2001) Ethanol hypersensitivity and olfactory discrimination defect in mice lacking a homolog of Drosophila neuralized. Proc. Natl. Acad. Sci. U. S. A. 98: 9907-9912.
[242] Vollrath B, Pudney J, Asa S, Leder P, Fitzgerald K (2001) Isolation of a murine homologue of the Drosophila neuralized gene, a gene required for axonemal integrity in spermatozoa and terminal maturation of the mammary gland. Mol. Cell. Biol. 21: 7481-7494.
[243] Chen W, Casey Corliss D (2004) Three modules of zebrafish Mind bomb work cooperatively to promote Delta ubiquitination and endocytosis. Dev. Biol. 267: 361-373.

[244] Pitsouli C, Delidakis C (2005) The interplay between DSL proteins and ubiquitin ligases in Notch signaling. Development. 132: 4041-4050.
[245] Wang W, Struhl G (2005) Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in Drosophila. Development. 132: 2883-2894.
[246] Carrasco-Rando M, Ruiz-Gomez M (2008) Mind bomb 2, a founder myoblast-specific protein, regulates myoblast fusion and muscle stability. Development. 135: 849-857.
[247] Hansson EM, Lanner F, Das D, Mutvei A, Marklund U, Ericson J, Farnebo F, Stumm G, Stenmark H, Andersson ER, Lendahl U (2010) Control of Notch-ligand endocytosis by ligand-receptor interaction. J. Cell Sci. 123: 2931-2942.
[248] Yamamoto M, Morita R, Mizoguchi T, Matsuo H, Isoda M, Ishitani T, Chitnis AB, Matsumoto K, Crump JG, Hozumi K, Yonemura S, Kawakami K, Itoh M (2010) Mib-Jag1-Notch signalling regulates patterning and structural roles of the notochord by controlling cell-fate decisions. Development. 137: 2527-2537.
[249] Wendland B, Steece KE, Emr SD (1999) Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis. EMBO J. 18: 4383-4393.
[250] Cadavid AL, Ginzel A, Fischer JA (2000) The function of the Drosophila fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. Development. 127: 1727-1736.
[251] Chen H, Fre S, Slepnev VI, Capua MR, Takei K, Butler MH, Di Fiore PP, De Camilli P (1998) Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. Nature. 394: 793-797.
[252] Rosenthal JA, Chen H, Slepnev VI, Pellegrini L, Salcini AE, Di Fiore PP, De Camilli P (1999) The epsins define a family of proteins that interact with components of the clathrin coat and contain a new protein module. J. Biol. Chem. 274: 33959-33965.
[253] Spradling KD, McDaniel AE, Lohi J, Pilcher BK (2001) Epsin 3 is a novel extracellular matrix-induced transcript specific to wounded epithelia. J. Biol. Chem. 276: 29257-29267.
[254] Ko G, Paradise S, Chen H, Graham M, Vecchi M, Bianchi F, Cremona O, Di Fiore PP, De Camilli P (2010) Selective high-level expression of epsin 3 in gastric parietal cells, where it is localized at endocytic sites of apical canaliculi. Proc. Natl. Acad. Sci. U. S. A. 107: 21511-21516.
[255] Itoh T, Koshiba S, Kigawa T, Kikuchi A, Yokoyama S, Takenawa T (2001) Role of the ENTH Domain in Phosphatidylinositol-4,5-Bisphosphate Binding and Endocytosis. Science. 291: 1047-1051.
[256] Ford MG, Mills IG, Peter BJ, Vallis Y, Praefcke GJ, Evans PR, McMahon HT (2002) Curvature of clathrin-coated pits driven by epsin. Nature. 419: 361-366.
[257] Chen H, Slepnev VI, Di Fiore PP, De Camilli P (1999) The interaction of epsin and Eps15 with the clathrin adaptor AP-2 is inhibited by mitotic phosphorylation and enhanced by stimulation-dependent dephosphorylation in nerve terminals. J. Biol. Chem. 274: 3257-3260.
[258] Polo S, Sigismund S, Faretta M, Guidi M, Capua MR, Bossi G, Chen H, De Camilli P, Di Fiore PP (2002) A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature. 416: 451-455.

[259] Chang Q, Balice-Gordon RJ (2000) Highwire, rpm-1, and futsch: balancing synaptic growth and stability. Neuron. 26: 287-290.

[260] Bao H, Reist NE, Zhang B (2008) The Drosophila epsin 1 is required for ubiquitin-dependent synaptic growth and function but not for synaptic vesicle recycling. Traffic . 9: 2190-2205.

[261] DiAntonio A, Haghighi AP, Portman SL, Lee JD, Amaranto AM, Goodman CS (2001) Ubiquitination-dependent mechanisms regulate synaptic growth and function. Nature. 412: 449-452.

[262] Chen H, Polo S, Di Fiore PP, De Camilli PV (2003) Rapid Ca2+-dependent decrease of protein ubiquitination at synapses. Proc. Natl. Acad. Sci. U. S. A. 100: 14908-14913.

[263] Stang E, Blystad FD, Kazazic M, Bertelsen V, Brodahl T, Raiborg C, Stenmark H, Madshus IH (2004) Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits. Mol. Biol. Cell. 15: 3591-3604.

[264] Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, Di Fiore PP, Polo S (2005) Clathrin-independent endocytosis of ubiquitinated cargos. Proc. Natl. Acad. Sci. U. S. A. 102: 2760-2765.

[265] Hawryluk MJ, Keyel PA, Mishra SK, Watkins SC, Heuser JE, Traub LM (2006) Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. Traffic. 7: 262-281.

[266] Zoncu R, Perera RM, Sebastian R, Nakatsu F, Chen H, Balla T, Ayala G, Toomre D, De Camilli PV (2007) Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. Proc. Natl. Acad. Sci. U. S. A. 104: 3793-3798.

[267] Kazazic M, Bertelsen V, Pedersen KW, Vuong TT, Grandal MV, Rodland MS, Traub LM, Stang E, Madshus IH (2009) Epsin 1 is involved in recruitment of ubiquitinated EGF receptors into clathrin-coated pits. Traffic. 10: 235-245.

[268] Kweon DH, Shin YK, Shin JY, Lee JH, Lee JB, Seo JH, Kim YS (2006) Membrane topology of helix 0 of the Epsin N-terminal homology domain. Mol. Cells. 21: 428-435.

[269] Aguilar RC, Longhi SA, Shaw JD, Yeh LY, Kim S, Schon A, Freire E, Hsu A, McCormick WK, Watson HA, Wendland B (2006) Epsin N-terminal homology domains perform an essential function regulating Cdc42 through binding Cdc42 GTPase-activating proteins. Proc. Natl. Acad. Sci. U. S. A. 103: 4116-4121.

[270] Brady RJ, Damer CK, Heuser JE, O'Halloran TJ (2010) Regulation of Hip1r by epsin controls the temporal and spatial coupling of actin filaments to clathrin-coated pits. J. Cell Sci. 123: 3652-3661.

[271] Tian X, Hansen D, Schedl T, Skeath JB (2004) Epsin potentiates Notch pathway activity in Drosophila and C. elegans. Development. 131: 5807-5815.

[272] Overstreet E, Fitch E, Fischer JA (2004) Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. Development. 131: 5355-5366.

[273] Chen H, Ko G, Zatti A, Di Giacomo G, Liu L, Raiteri E, Perucco E, Collesi C, Min W, Zeiss C, De Camilli P, Cremona O (2009) Embryonic arrest at midgestation and disruption of Notch signaling produced by the absence of both epsin 1 and epsin 2 in mice. Proc. Natl. Acad. Sci. U. S. A. 106: 13838-13843.

[274] Boucrot E, Pick A, Camdere G, Liska N, Evergren E, McMahon HT, Kozlov MM (2012) Membrane Fission Is Promoted by Insertion of Amphipathic Helices and Is Restricted by Crescent BAR Domains. Cell. 149: 124-136.

[275] Sorensen EB, Conner SD (2010) gamma-secretase-dependent cleavage initiates notch signaling from the plasma membrane. Traffic. 11: 1234-1245.

[276] Struhl G, Adachi A (2000) Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. Mol. Cell. 6: 625-636.

[277] Tagami S, Okochi M, Yanagida K, Ikuta A, Fukumori A, Matsumoto N, Ishizuka-Katsura Y, Nakayama T, Itoh N, Jiang J, Nishitomi K, Kamino K, Morihara T, Hashimoto R, Tanaka T, Kudo T, Chiba S, Takeda M (2008) Regulation of Notch signaling by dynamic changes in the precision of S3 cleavage of Notch-1. Mol. Cell. Biol. 28: 165-176.

[278] Sakata T, Sakaguchi H, Tsuda L, Higashitani A, Aigaki T, Matsuno K, Hayashi S (2004) Drosophila Nedd4 regulates endocytosis of notch and suppresses its ligand-independent activation. Curr. Biol. 14: 2228-2236.

[279] Vaccari T, Lu H, Kanwar R, Fortini ME, Bilder D (2008) Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. J. Cell Biol. 180: 755-762.

[280] Spang A (2009) On the fate of early endosomes. Biol. Chem. 390: 753-759.

[281] Vaccari T, Duchi S, Cortese K, Tacchetti C, Bilder D (2010) The vacuolar ATPase is required for physiological as well as pathological activation of the Notch receptor. Development. 137: 1825-1832.

[282] Yan Y, Denef N, Schupbach T (2009) The vacuolar proton pump, V-ATPase, is required for notch signaling and endosomal trafficking in Drosophila. Dev. Cell. 17: 387-402.

[283] Pasternak SH, Bagshaw RD, Guiral M, Zhang S, Ackerley CA, Pak BJ, Callahan JW, Mahuran DJ (2003) Presenilin-1, nicastrin, amyloid precursor protein, and gamma-secretase activity are co-localized in the lysosomal membrane. J. Biol. Chem. 278: 26687-26694.

[284] Baron M (2012) Endocytic routes to Notch activation. Semin. Cell Dev. Biol. DOI: 10.1016/j.semcdb.2012.01.008.

[285] Yamada K, Fuwa TJ, Ayukawa T, Tanaka T, Nakamura A, Wilkin MB, Baron M, Matsuno K (2011) Roles of Drosophila deltex in Notch receptor endocytic trafficking and activation. Genes Cells. 16: 261-272.

[286] Hori K, Fostier M, Ito M, Fuwa TJ, Go MJ, Okano H, Baron M, Matsuno K (2004) Drosophila deltex mediates suppressor of Hairless-independent and late-endosomal activation of Notch signaling. Development. 131: 5527-5537.

[287] Wilkin M, Tongngok P, Gensch N, Clemence S, Motoki M, Yamada K, Hori K, Taniguchi-Kanai M, Franklin E, Matsuno K, Baron M (2008) Drosophila HOPS and AP-3 complex genes are required for a Deltex-regulated activation of notch in the endosomal trafficking pathway. Dev. Cell. 15: 762-772.

[288] Peden AA, Oorschot V, Hesser BA, Austin CD, Scheller RH, Klumperman J (2004) Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. J. Cell Biol. 164: 1065-1076.

[289] Rink J, Ghigo E, Kalaidzidis Y, Zerial M (2005) Rab conversion as a mechanism of progression from early to late endosomes. Cell. 122: 735-749.

[290] Luttrell LM, Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J. Cell Sci. 115: 455-465.

[291] Chen W, Kirkbride KC, How T, Nelson CD, Mo J, Frederick JP, Wang XF, Lefkowitz RJ, Blobe GC (2003) Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-regulation of its signaling. Science. 301: 1394-1397.

[292] Chen W, ten Berge D, Brown J, Ahn S, Hu LA, Miller WE, Caron MG, Barak LS, Nusse R, Lefkowitz RJ (2003) Dishevelled 2 recruits beta-arrestin 2 to mediate Wnt5A-stimulated endocytosis of Frizzled 4. Science. 301: 1391-1394.

[293] Mukherjee A, Veraksa A, Bauer A, Rosse C, Camonis J, Artavanis-Tsakonas S (2005) Regulation of Notch signalling by non-visual beta-arrestin. Nat. Cell Biol. 7: 1191-1201.

[294] Hori K, Sen A, Kirchhausen T, Artavanis-Tsakonas S (2011) Synergy between the ESCRT-III complex and Deltex defines a ligand-independent Notch signal. J. Cell Biol. 195: 1005-1015.

[295] Rhyu MS, Jan LY, Jan YN (1994) Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell. 76: 477-491.

[296] Guo M, Jan LY, Jan YN (1996) Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron. 17: 27-41.

[297] Santolini E, Puri C, Salcini AE, Gagliani MC, Pelicci PG, Tacchetti C, Di Fiore PP (2000) Numb is an endocytic protein. J. Cell Biol. 151: 1345-1352.

[298] Song Y, Lu B (2012) Notch Signaling Modulator Numb's Interaction with alpha-Adaptin Regulates Endocytosis of Notch Pathway Components and Cell Fate Determination of Neural Stem Cells. J. Biol. Chem DOI: 10.1074/jbc.M112.360719..

[299] Berdnik D, Torok T, Gonzalez-Gaitan M, Knoblich JA (2002) The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. Dev. Cell. 3: 221-231.

[300] Couturier L, Vodovar N, Schweisguth F (2012) Endocytosis by Numb breaks Notch symmetry at cytokinesis. Nat. Cell Biol. 14: 131-139 DOI: 10.1074/jbc.M112.360719..

[301] Qiu L, Joazeiro C, Fang N, Wang HY, Elly C, Altman Y, Fang D, Hunter T, Liu YC (2000) Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. J. Biol. Chem. 275: 35734-35737.

[302] McGill MA, McGlade CJ (2003) Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. J. Biol. Chem. 278: 23196-23203.

[303] Bernassola F, Karin M, Ciechanover A, Melino G (2008) The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell. 14: 10-21.

[304] Dye CA, Lee JK, Atkinson RC, Brewster R, Han PL, Bellen HJ (1998) The Drosophila sanpodo gene controls sibling cell fate and encodes a tropomodulin homolog, an actin/tropomyosin-associated protein. Development. 125: 1845-1856.

[305] O'Connor-Giles KM, Skeath JB (2003) Numb inhibits membrane localization of Sanpodo, a four-pass transmembrane protein, to promote asymmetric divisions in Drosophila. Dev. Cell. 5: 231-243.

[306] McGill MA, Dho SE, Weinmaster G, McGlade CJ (2009) Numb regulates post-endocytic trafficking and degradation of Notch1. J. Biol. Chem. 284: 26427-26438.

[307] Hutterer A, Knoblich JA (2005) Numb and alpha-Adaptin regulate Sanpodo endocytosis to specify cell fate in Drosophila external sensory organs. EMBO Rep. 6: 836-842.

[308] Babaoglan AB, O'Connor-Giles KM, Mistry H, Schickedanz A, Wilson BA, Skeath JB (2009) Sanpodo: a context-dependent activator and inhibitor of Notch signaling during asymmetric divisions. Development. 136: 4089-4098.

[309] Groth C, Fortini ME (2012) Therapeutic approaches to modulating Notch signaling: Current challenges and future prospects. Semin. Cell Dev. Biol. DOI: 10.1016/j.semcdb.2012.01.016.