Classification: BIOLOGICAL SCIENCES, Cell Biology

The intracellular region of Notch ligands Dll1 and Dll3 regulates their trafficking and signaling activity

Sara Farrah Heuss[*], Delphine Ndiaye-Lobry[*], Emmanuelle M. Six[†], Alain Israël[*] and Frédérique Logeat[*‡]

[*]Unité de Signalisation Moléculaire et Activation Cellulaire, URA CNRS 2582, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris cedex 15, France

Fax: 33 1 40 61 30 40

[†]Unité du Dévelopement Normal et Pathologique du Système Immunitaire, Unité INSERM 768, Groupe Hospitalier Necker-Enfants Malades, 149 rue de Sèvres, 75743 Paris Cedex 15, France

[‡]**Corresponding author:** Frédérique Logeat, email: flogeat@pasteur.fr

Number of text pages: 18

Number of figures: 6 + 3 supplementary figures

Abstract

Genetic studies have shown that ubiquitination and endocytosis of the *Drosophila* ligand Delta in signal-sending cells are required for activation of Notch signaling, but how these events promote Notch activation remains poorly understood. Here we show that an ubiquitination-defective mutant of the murine Delta-homologue Dll1 is endocytosed but, in contrast to the wild-type Dll1, is unable to subsequently recycle back to the cell surface nor to bind Notch1 efficiently. These results demonstrate that ubiquitination, although not required for endocytosis, is essential for Dll1 recycling, and that recycling is required to acquire affinity for the receptor. On the other hand a chimeric molecule encompassing the extracellular domain of Dll1 and the transmembrane/intracellular domain of Dll3 (which contains no lysine) is endocytosed, recycled and interacts with Notch1, but is unable to induce trans-endocytosis of the extracellular region of Notch1 nor to signal. These observations suggest that the chimera uses an ubiquitination-independent signal to recycle, allowing it to acquire affinity for Notch1. Our results support the idea that ligand recycling determines its competence to bind efficiently to the receptor, but that this is unsufficient to allow it to perform trans-endocytosis, an event required for activation of Notch signaling. Finally the present study indicates that Dll1 partially localizes to lipid microdomains, whereas both ubiquitination-defective Dll1 and the Dll1-3 chimera are excluded from these compartments, suggesting that these microdomains provide the environment necessary for Dll1 to activate Notch signaling.

Key words: Notch, Dll1, ubiquitination, recycling, membrane microdomains.

Introduction

Notch signaling is an evolutionary conserved pathway, which ensures correct specification of multiple cell types through local cell-cell interactions (1). Ligands of the DSL (Delta/Serrate/Lag2) family expressed at the cell surface interact with heterodimeric Notch molecules on adjacent cells and activate a signaling cascade, which relies on consecutive Notch cleavages. The linear nature of this signaling pathway apparently leaves little space for fine tuning and/or amplification, but a number of data suggest that specific events affecting receptors and ligands contribute to regulate Notch signaling (2). In particular endocytosis has been shown to play a key role in Notch signaling. In *Drosophila* Seugnet *et al* have shown that dynamin-dependent endocytosis is required for Notch activation in both signal-receiving and signal-sending cells (3). Two main models have been proposed to explain this requirement in signal-sending cells (for review see (4) (5) (6)). The first one postulates that endocytosis is required after ligand/Notch interaction to generate a sufficient "pulling" strength to dissociate the heterodimeric Notch receptor, or to modify its conformation, in order to allow Notch cleavages. The second one postulates that endocytosis followed by recycling back to the plasma membrane is necessary to generate an "active" ligand, although how the ligand would become active is currently unknown. Incidentally the two models are not mutually exclusive and therefore endocytosis might be required both to generate an active ligand, and to induce Notch cleavages. Genetic studies in *Drosophila* have identified two E3 ubiquitin ligases, Neuralized (Neur) and Mind bomb (Mib), as regulators of ligand activity. Neur and Mib have been shown to promote ligand ubiquitination and internalization (5).

To establish a link between ligand ubiquitination, trafficking and activation of the Notch signaling cascade, we examined trafficking and signaling activity of three Dll1 derivatives which differ essentially in their intracellular domain: wild-type murine Delta-like 1 (Dll1), a ubiquitination-defective Dll1 mutant that contains no acceptor lysine residue in its

intracellular domain and a chimeric molecule encompassing the extracellular region of Dll1 and the transmembrane and the intracellular region of murine Delta-like 3 (Dll3), a Delta homologue naturally devoid of lysine in its intracellular domain. The ability of the three molecules to interact with Notch1, induce trans-endocytosis of the extracellular region of Notch1 (NECD) and activate the subsequent signaling cascade were assayed. Our results demonstrate that Dll1 ubiquitination is not required for its internalization but is necessary for its recycling back to the plasma membrane and efficient interaction with Notch1. However the intracellular region of Dll3, although it allows recycling and interaction with Notch of the chimeric molecule, does not allow it to induce NECD trans-endocytosis, nor to activate signaling. Finally we investigated whether wild-type and mutant Dll1 reside in membrane microdomains. By providing an ordered membrane microenvironment, lipid rafts (7) or other domains may contribute to the clustering of Dll1 molecules, or to the interaction with specific cofactors (4). Interestingly a genetic screen performed in *C. elegans* identified BRE-5/Brainiac as a positive regulator of Notch signaling that acts before the ligand-induced cleavage of Lin-12 and may target the ligands (8). Brainiac is an enzyme that participates in the biosynthesis of glycosphingolipid, a component of lipid rafts. Indeed we observed that wild-type Dll1 preferentially localizes to lipid microdomains, whereas the ubiquitin-defective Dll1 mutant and the Dll1-3 chimera were almost excluded from these fractions.

Results

Ubiquitination and endocytosis of wild-type and mutant Dll1. To define the importance of ubiquitination in the regulation of wild-type (wt) Dll1 trafficking, we generated a Dll1 mutant, Dll1K17R, by replacing the 17 intracellular lysine residues with arginine. HEK 293T cells were transiently cotransfected with Dll1 and wild-type His-tagged ubiquitin (wt His-Ub), lysed and ubiquitinated proteins were analyzed. Figure 1A (lane 6) indicates that two major

species of Dll1 are detected and undergo ubiquitination, the full length (FL) and the metalloprotease-mediated cleavage product (TMIC for TransMembrane and IntraCellular domains, (9)). The apparent molecular weight difference between the TMIC or the FL forms present in the crude extract (Fig. 1A lanes 1-3) and those bound to Nickel beads (Fig. 1A lanes 5-6) suggest the latter represent poly- or multiubiquitinated species. To clarify this issue, we used a His-tagged ubiquitin construct in which all lysine residues were mutated to arginine (ko His-Ub). Nickel beads pull down experiments using the ko His-Ub confirmed that Dll1 was monoubiquitinated at several positions (Fig. 1A lane 5). A longer exposure revealed that Dll1-TMIC was also monoubiquitinated on one or two lysines (Fig. 1B lanes 5-6). We then analyzed Dll1K17R and Dll3, which do not contain any lysine in their intracellular domain. As expected no ubiquitinated species of either protein could be isolated on Nickel beads (Fig. 1 panels C and D respectively). To compare the role of the intracellular sequence of Dll1 and Dll3, we generated a Dll1-3 chimera encompassing the extracellular domain of Dll1 and the transmembrane and intracellular domain of Dll3. No ubiquitinated species of Dll1-3 was detected by Nickel beads pull down experiment (data not shown).

To investigate the physiological significance of Dll1 monoubiquitination, we compared the efficiency and kinetics of internalization of Dll1, Dll1K17R and Dll1-3 stably transfected into OP9 stromal cell lines, using a reversible biotinylation strategy involving surface biotinylation at 4°C followed by endocytosis at 37°C. Preliminary experiments showed that maximal internalization took place 20 min after temperature switch (data not shown). When cells were kept at 4°C, therefore preventing internalization, treatment with the membrane-impermeable reducing agent MesNa abrogated detection of the ligands, indicating that it successfully removed 90-100% of surface biotin (Fig. 1E lanes 2, 5 and 8). After 20 min at 37°C, biotinylated species of the three Dll1 derivatives could be detected, indicating that they had been internalized (Fig. 1E lanes 3, 6 and 9). The extent of Dll1-3 internalization was similar

to that of wt Dll1. Dll1K17R was also internalized, though not as efficiently as wt Dll1. These results indicate that Dll1 ubiquitination is not essential for its internalization.

Recycling of wt and mutant Dll1. As direct evidence of recycling of Dll1 or other Notch ligands has not been provided so far, we assayed this event through an extension of the endocytosis assay used above. After 20 min of endocytosis and a first MesNa treatment, cells were incubated at 37°C to allow transport through recycling endosomes for various periods of time (10, 20 or 30 min). At each time point, cells were re-exposed, or not, to MesNa to strip biotin from ligands that had reached the cell surface. Immunoblots were quantified as described in Supporting Information. At least 60-70% of Dll1 and Dll1-3 had recycled to the cell surface after 10 min (Fig. 2). This rate was constant during 30 min. The amount of Dll1K17R was identical whether the cells were treated with MesNa or not, indicating that the mutant Dll1K17R was not able to recycle. As an internal control we used cadherin, whose recycling has been largely documented (10). Supplementary figure (Fig S1) indicates that the half-life of surface expressed and endocytosed Dll1 and Dll1K17R is different. Dll1K17R is degraded 2.5 fold more rapidly than the wt ligand, most likely as a consequence of its inability to recycle, which implicated a feed-back into the biosynthetic and degradation rates. All together our data suggest that Dll1 recycling is dependent on its ubiquitination, but that the intracellular domain of Dll3 allows recycling in the absence of ubiquitination.

Binding of Notch to cells expressing wt or mutant Dll1. We next asked whether the three different Dll1 derivatives were able to interact with the extracellular domain of Notch1 fused to the Fc domain of human immunoglobulin (NECDFc). NECDFc preclustered with an anti IgG-Fc has previously been shown to interact with surface-expressed Dll1 (11). We transiently transfected HeLa cells with either wt Dll1, Dll1K17R or Dll1-3 molecules tagged with an extracellular VSV tag and incubated them with NECDFc preclustered with a FITC-conjugated anti IgG-Fc. Cells were then fixed and immunolabelled with a Cy3-conjugated

anti-VSV to detect the ligands. Figure 3A shows that under conditions of similar level of surface expression of the ligands, wt Dll1 (panels a-c and j-l) as well as Dll1-3 (panels d-f and m-o) are able to interact with NECDFc. Notch binding to Dll1-expressing cells was detected at low (panel k) and high (panel b) Notch concentration, whereas binding to Dll1K17R was only weakly detectable at high Notch concentration (panel h), indicating that Dll1K17R interacts with the receptor with a much lower affinity than the wt Dll1 or Dll1-3. To confirm our observations, these experiments were repeated using four different NECDFc concentrations. The quantification of this experiment (Fig 3B) shows that Dll1 and Dll1-3 exhibit a similar affinity for NECDFc whereas that of Dll1K17R is much lower. All together these results demonstrate that ligand recycling is necessary to acquire a strong affinity for the receptor.

Signaling activity of wt or mutant Dll1. To test the signaling activity of Dll1 derivatives, we took advantage of the OP9-Dll1 co-culture system that enables in vitro T cell differentiation of hematopoietic precursor cells through Notch signaling (12). Murine Sca1+ precursor cells were co-cultured with OP9 cell lines expressing (or not) one of the three Dll1 derivatives (at similar levels). After 21 days, flow cytometric analysis showed that OP9 cells expressing wt Dll1 induced differentiation of precursor cells to double positive CD4+ CD8+ T cells (Fig. 4). OP9 cells expressing Dll1K17R only activated Notch at a very low level – giving rise to 12 fold less double positive T cells than wt Dll1. Interestingly, despite its strong physical interaction with the receptor, Dll1-3 did not induce, in this cellular context, any T cell differentiation, indicating its inability to activate Notch signaling.

Trans-endocytosis of Notch1 extracellular domain in cells expressing wt or mutant Dll1. In 2000 Parks *et al* showed that endocytosis of *Drosophila* Delta is required to trans-endocytose the extracellular region of Notch (13), and more recently Nichols *et al* demonstrated that these events correlate with Notch activation in mammalian cells (14). To

characterize, in our cellular system, the endocytosis of Notch1 into Dll1-expressing cells, immunofluorescent staining was performed on OP9 cells stably expressing VSV-tagged Dll1 co-cultured with MEF cells expressing a human HA-tagged Notch1 receptor. After fixation, cells were permeabilized or not to distinguish between surface expressed and intracellular molecules. In permeabilized cells, positive vesicles costained with anti HA and anti VSV antibodies were detected (Fig. S2-a). In contrast, in non-permeabilized cells no such positive vesicles were detected, even when cells expressing Notch1 and Dll1 were clearly in contact (Fig. S2-b). To further specify the endocytic nature of the Notch1-Dll1 containing vesicles, cells were incubated with fluorescent dextran, a fluid phase tracer, for 4 hours before processing. Several of the Notch1 and Dll1 positive vesicles colocalized with the fluorescent dextran (Fig. S3) indicating they were of endocytic origin.

These data prompted us to investigate whether uptake of NECD could occur in cells expressing Dll1-3 or Dll1K17R compared to cells expressing wt Dll1. We co-cultured MEF cells stably expressing a HA-tagged Notch1 receptor with HeLa cells transiently transfected with VSV-tagged ligands. HA-positive intracellular vesicles appeared only in wt Dll1-expressing cells, where they colocalize with VSV staining (Fig. 5a) indicating that NECD was trans-endocytosed into the wt Dll1-expressing cells. On the contrary we could not detect any HA staining in cells expressing Dll1-3 (Fig. 5b) or Dll1K17R (Fig. 5c).

Distribution of wt and mutant Dll1 in membrane microdomains. Lipid rafts are examples of membrane microdomains that have been suggested to function as signaling platforms. To test this hypothesis in the context of Notch signaling, we isolated membrane lipid microdomains of ligand-expressing OP9 cells on the basis of their relative insolubility in non-ionic detergents and their ability to float in density gradients (7). As expected, caveolin-1 was detected in detergent insoluble membrane fractions (DIM) (Fig. 6, lane 3) and ß-tubulin was found in soluble fractions (Fig. 6, lanes 6-10). Interestingly, wt Dll1 was specifically enriched

in the buoyant fractions (DIM) where caveolin-1 segregates (Fig. 6 lane 3), suggesting an association with lipid microdomains. Dll1K17R could be found throughout the gradient but showed an enrichment in the fractions corresponding to soluble material (Fig. 6, lanes 6-10) while Dll1-3 was only found in the soluble fractions (Fig. 6, lanes 6-10), copurifying with ß-tubulin.

Discussion

Several authors have reported that ubiquitination and endocytosis of Notch ligands in signal-sending cells are required for Notch activation in signal-receiving cells (for review see (5)). To gain some insight into the role of these various events in the "activation" of Notch ligands, we assayed endocytosis, recycling, localization in membrane microdomains, NECD binding, trans-endocytosis and Notch signaling activation using three Dll1 derivatives which differ in their intracellular domain.

The first conclusion we reached was that ubiquitination is not necessary for Dll1 internalization. Indeed the ubiquitination-defective Dll1K17R and the lysineless Dll1-3 molecules are effectively internalized, although the Dll1K17R is reproducibly less efficiently endocytosed than the wt molecule. Many yeast surface proteins have been demonstrated to require ubiquitination for their internalization (15), but the situation is less clear in mammals. In the case of the EGF receptor, mutation of multiple intracellular lysines within the kinase domain of the protein that lead to an almost complete loss of EGF receptor ubiquitination did not result in the decrease of its internalization rate (16). Regarding Notch ligands it has been shown that the E3 ligase Mind Bomb 1 promotes ubiquitination and internalization of mammalian Dll1 (17). It has also been reported that in order to be active, *Drosophila* Delta has to traffic through a Neur/Mib and epsin-dependent pathway, although the majority of Delta molecules traffic in a non-productive manner through a different pathway, which is

ubiquitination-dependent but epsin-independent, and may lead to degradation (18). As we now demonstrate that Dll1K17R is endocytosed but is unable to signal, these results can be interpreted in two ways: i) bulk endocytosis of Dll1 is mildly affected by the lack of ubiquitination, while the small amount of ligand that follows the activating pathway can no longer be endocytosed in the absence of ubiquitination ii) ubiquitination is required for a subsequent step of Dll1 trafficking. Our results suggest that the latter explanation is correct, and that ubiquitination is necessary for Dll1 recycling. It has been suggested that trafficking through Rab11-positive recycling endosomes ensures proper Delta activity (19). Sec15, an effector of Rab11 (20), has also been shown to be required for Delta recycling and signaling activity in signal-sending cells. However direct evidence of ligand recycling has not been provided. Our experiments demonstrate that wt Dll1 is effectively endocytosed and recycled back to the cell surface, while Dll1K17R is endocytosed but not recycled. These results are in agreement with a model proposed by Wang and Struhl that suggests that epsin/lqf directs ubiquitinated ligands into a recycling pathway that is necessary to produce active ligands (21). In mammalian cells internalization of cell surface proteins occurs through both clathrin-dependent and clathrin-independent pathways, the latter generally depending on cholesterol-rich membrane domains, which have been postulated to play an important role in cell signaling (22). We demonstrate here that the Dll1K17R mutant is unable to enter the membrane microdomains as well as to recycle, two apparent requirements for activation of the signaling pathway. The epistatic relationship between localization in membrane microdomains and recycling remains however to be characterized.

In contrast to Dll1K17R, the chimeric ligand Dll1-3, although not ubiquitinated, is able to recycle to the cell surface, indicating that an unknown signal present in the intracellular domain of Dll3 can bypass the requirement for ubiquitination. However this molecule is unable to signal. Geffers *et al* have generated a similar Dll1-3 chimeric molecule and also

found that this molecule did not activate Notch signaling; however the details of its trafficking had not been analyzed (23).

When binding of clustered NECD to cells expressing either wt or mutant Dll1 molecules was tested, we observed that while Dll1 and the Dll1-3 chimera were able to bind NECD with a similar efficiency, binding by Dll1K17R was much less efficient, suggesting that recycling is somehow required for Dll1 to acquire a strong affinity for the receptor. However when we assayed NECD trans-endocytosis by the wt and mutant Dll1 molecules, we observed that wt Dll1 was the only one to be able to perform this task. Similarly, when using the hematopoietic co-culture system, a sensitive and physiological readout for Dll1-mediated activation of Notch1, we observed that only wt Dll1 was able to markedly induce T cell differentiation. Thus, if ligand recycling is necessary to generate a molecule able to interact with a soluble clustered form of the receptor, this recycling is not sufficient to activate Notch signaling in a T cell differentiation assay. A specific event may take place either during endocytosis or recycling of Dll1 and Dll1-3 (or during both) and cause their differential behavior. As membrane microdomains have been postulated to be involved in the assembly of signaling complexes, we tested the localization of the three Dll1 derivatives and observed that Dll1, but not Dll1K17R nor Dll1-3, was present in these microdomains. The inability of the Dll1-3 chimera to trans-endocytose NECD may be due to the fact that this molecule is excluded from these microdomains.

In conclusion, our results support a model whereby specific intracellular endocytosis/recycling events of the Notch ligands are necessary both before contact with Notch, to somehow "activate" the ligand, and after the interaction, to allow NECD trans-endocytosis and the subsequent cleavages necessary for Notch activation. Lipid microdomains may play an important role in endowing Dll1 with the ability to trans-endocytose the extracellular region of Notch. The challenge is now to identify the different

adaptor proteins implicated in these processes, and to identify more precisely the sequential events that allow intracellular "activation" of the ligands.

Materials and methods

Constructs. Dll1- and VSV-Dll1-coding constructs in pcDNA6 or in MSCV-IRES-GFP (MIG) vector have been described previously (9). To generate a Dll1K17R-expressing construct, the sequence coding for the extracellular and the transmembrane domain of Dll1 was amplified by PCR and was inserted into ICK17R-pcDNA3 (ATG:biosynthetics) encoding a Dll1 intracellular domain in which all intracellular lysines were replaced with arginines (mutations are at the following amino acid positions: 572, 575, 600, 613, 617, 618, 628, 629, 633, 648, 660, 664, 675, 689, 699, 702 and 713). To generate a Dll1-3-expressing construct, the sequence coding for the extracellular region of Dll1 and the sequence coding for the transmembrane and the intracellular domain of Dll3 were amplified by PCR, and inserted into pcDNA3. 6His-ubiquitin wt and ko plasmids were a gift from A. Treier and D. Bohmann.

Antibodies. The antibody directed against the C-terminal part of Dll1 (anti-Dll1CT) has been previously described (9). The antibody directed against Dll3 (anti-Dll3ic) was generated in rabbit against intracellular peptides aa 551-566 and 573-585 derived from the intracellular domain of the molecule (Eurogentec).

Cell culture, transfection and cell line establishment. HEK 293T, HeLa, Plat-E and stromal bone marrow-derived OP9 cell lines were cultured and transfected as described previously (12). MEF cells stably expressing human HA-Notch1 were generated by retroviral infection (C. Brou and P. Chastagner, unpublished data).

Ubiquitination assay. HEK 293T transfected with a ligand-expressing construct and with a plasmid expressing a His-Ub variant (24) were lysed with 8 M urea buffer. Cell extracts were centrifuged at 186,000*g*, ubiquitinated proteins were isolated on Nickel-agarose columns

according to the manufacturer's instructions (GE Healthcare) and analyzed by immunoblotting with anti-Dll1CT or anti-Dll3ic antibody.

Endocytosis and recycling assays. Reversible biotinylation assays were performed as described in Fournier *et al* with minor modifications ((25), see Supporting Information).

NECD binding assay. HeLa cells transiently transfected with a VSV-tagged ligand-expressing construct, were incubated for 40 min at 37°C with a recombinant protein containing the first 12 EGF-like repeats of rat Notch1 fused to the Fc domain of human immunoglobulin (NECDFc, R&D system), preclustered with a FITC-conjugated anti human IgG-Fc (Jackson ImmunoResearch). Cells were washed with PBS and analysed by immunofluorescence as described previously (12). Quantitation and colocalization were performed using AxioVision 4.6.3.

Notch1 extracellular domain trans-endocytosis assay. HeLa cells were transfected with VSV-tagged ligand-expressing plasmids (VSV is localized in the extracellular domain of the ligands). Eight hours after transfection, mouse embryonic fibroblasts (MEF) stably expressing human Notch1 were seeded on the HeLa cells (Notch1 is HA-tagged in its extracellular domain). After overnight co-culture, cells were analysed by immunofluorescence as described previously using a Cy3-conjugated anti-VSV and an Alexa488-conjugated anti-HA (Invitrogen).

Notch activation assay. Bone marrow was isolated from six weeks-old C57BL/6 mice. After red cell depletion using NH_4Cl, bone marrow-derived Sca1+ hematopoietic stem cells (HSC) were isolated using Sca1-PE, an anti-PE microbead kit and VarioMACS (Miltenyi Biotec), according to the manufacturer's protocol. T-lymphoid potential was tested by plating Sca1+ HSC on OP9 stromal cells stably expressing Dll1, Dll1K17R or Dll1-3. After 21 days of co-culture HSC were analyzed by flow cytometry using the following combination of antibodies: PE anti-CD8 and APC anti-CD4 (12). A 7AAD-GFP-cell gate was set in order to exclude

non-viable cells and Dll1-expressing 0P9 cells from the analysis; the data were analyzed using FlowJo software.

Lipid microdomain isolation. OP9 cells stably expressing Dll1 derivatives were lysed in buffer A (20 mM Tris pH 7,5; 150 mM NaCl) containing 1% Brij 98 for 30 min on ice. Cell lysates were homogenized by passing through a 23-G needle 10 times, mixed with an equal volume of buffer B (85% sucrose; 1% Brij 98 in buffer A), transferred in centrifuge tubes and successively 2 mL of 30% sucrose in buffer A and 1 mL of 5% sucrose in buffer A were layered on the samples. After a centrifugation at 230,000*g* for 18 h, fractions were collected from the top to bottom and analyzed by immunoblotting with anti-Dll1CT, anti-Dll3ic, anti caveolin-1 (BD transduction laboratories), or anti ß-tubulin (Sigma) antibody.

Acknowledgments

We thank C. Brou for critically reading the manuscript. We are grateful to J.C Aster (Harvard Medical School) for providing HA-tagged hNotch1 cDNA and M. Treier (EMBL, Heidelberg) for 6histidine-tagged ubiquitin constructs. We also thank A. Danckaert (PFID, Institut Pasteur) for her help with the quantification software. This work was supported in part by EC FP6 grant Rubicon. Sara Heuss is supported by a grant from Ligue Nationale Contre le Cancer.

References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. *Science* 284:770-776.
2. Bray SJ (2006) Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 7:678-689.

3. Seugnet L, Simpson P, Haenlin M (1997) Requirement for dynamin during Notch signaling in Drosophila neurogenesis. *Dev Biol* 192:585-598.

4. Chitnis A (2006) Why is delta endocytosis required for effective activation of notch? *Dev Dyn* 235:886-894.

5. Le Borgne R (2006) Regulation of Notch signalling by endocytosis and endosomal sorting. *Curr Opin Cell Biol* 18:213-222.

6. Nichols JT, Miyamoto A, Weinmaster G (2007) Notch signaling - constantly on the move. *Traffic* 8:959-969.

7. Simons K, Toomre D (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 1:31-39.

8. Katic I, Vallier LG, Greenwald I (2005) New positive regulators of lin-12 activity in Caenorhabditis elegans include the BRE-5/Brainiac glycosphingolipid biosynthesis enzyme. *Genetics* 171:1605-1615.

9. Six E*, et al.* (2003) The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. *Proc Natl Acad Sci U S A* 100:7638-7643.

10. Le TL, Yap AS, Stow JL (1999) Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. *J Cell Biol* 146:219-232.

11. Ladi E*, et al.* (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.

12. Six EM*, et al.* (2004) The notch ligand Delta1 recruits Dlg1 at cell-cell contacts and regulates cell migration. *J Biol Chem* 279:55818-55826.

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

14. Nichols JT, *et al.* (2007) DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *J Cell Biol* 176:445-458.

15. Hicke L, Dunn R (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* 19:141-172.

16. Huang F, Goh LK, Sorkin A (2007) EGF receptor ubiquitination is not necessary for its internalization. *Proc Natl Acad Sci U S A* 104:16904-16909.

17. Koo BK, *et al.* (2005) Mind bomb-2 is an E3 ligase for Notch ligand. *J Biol Chem* 280:22335-22342.

18. 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.

19. Emery G, *et al.* (2005) Asymmetric Rab 11 endosomes regulate delta recycling and specify cell fate in the Drosophila nervous system. *Cell* 122:763-773.

20. Jafar-Nejad H, *et al.* (2005) Sec15, a component of the exocyst, promotes notch signaling during the asymmetric division of Drosophila sensory organ precursors. *Dev Cell* 9:351-363.

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

22. Lajoie P, Nabi IR (2007) Regulation of raft-dependent endocytosis. *J Cell Mol Med* 11:644-653.

23. Geffers I, *et al.* (2007) Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo. *J Cell Biol* 178:465-476.

24. van der Horst A, *et al.* (2006) FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. *Nat Cell Biol* 8:1064-1073.

25. Fournier KM, Gonzalez MI, Robinson MB (2004) Rapid trafficking of the neuronal glutamate transporter, EAAC1: evidence for distinct trafficking pathways differentially regulated by protein kinase C and platelet-derived growth factor. *J Biol Chem* 279:34505-34513.

Figure legends

Fig. 1. Ubiquitination and endocytosis. Ubiquitination of wt Dll1 (A and B; B is a longer exposure of the area boxed in panel A), Dll1K17R (C) and Dll3 (D). HEK 293T cells were transfected with ligands, without (-) or with wild-type (wt) or lysineless (ko) His-Ub. Whole cell extracts (wce) and fractions retained on Nickel beads (Ni) were analyzed by immunoblotting with anti-Dll1CT (A, B and C) or anti-Dll3ic antibody (D). (E) Surface proteins of OP9 stromal cell lines stably expressing Dll1, Dll1K17R or Dll1-3 were labelled with NHS-SS-biotin, incubated for 20 min at 37°C (20') or left at 4°C (0'). Cell surface biotin was then stripped with MesNa. Whole cell extracts (wce) and fractions retained on streptavidin-agarose (b) were analyzed by immunoblotting with anti-Dll1CT or anti-Dll3ic antibody. Arrowheads in B indicate ubiquitinated forms of Dll1-TMIC. The star (D) shows a band arising from a non-specific reaction with the anti-Dll3ic antibody. FL: full length Dll1 species, TMIC: Dll1 TMIC species. NDB: not de-biotinylated cells.

Fig. 2. Dll1 and Dll1-3, but not Dll1K17R are recycled back to the plasma membrane. After internalization of biotinylated proteins for 20 min as described in Fig. 1E, cells were re-incubated at 37°C for 10', 20' and 30', then submitted (+) or not (-) to a second MesNa treatment (Mesna2). Cadherin was used as an internal positive recycling control. Whole cell extracts (wce) and fractions retained on streptavidin-agarose (b) were analysed by immunoblotting with anti-Dll1CT (αD1ct), anti-Dll3ic (αD3ic) or anti-pan cadherin (αcadh) antibody.

Fig. 3. Preclustered NECDFc binding to Dll1, Dll1-3 or Dll1K17R-expressing cells.

A. HeLa cells were transiently transfected with VSV-tagged Dll1 (a-c, j-l), Dll1-3 (d-f, m-o) or Dll1K17R (g-i, p-r) construct and incubated with 2 µg/mL or 0.05 µg/mL NECDFc preclustered with a FITC-conjugated anti-Fc antibody (panels b, e, h, k, n and q). Cells were stained with a Cy3-conjugated anti-VSV antibody (a, d, g, j, m and p). Hoechst staining is shown. Scale bar: 20 µm. **B.** Same experiment as in A but with four different concentrations of NECDFc. The amount of surface ligand colocalizing with NECDFc versus the total amount of surface ligand was quantified using AxioVision 4.6.3 (n=10) and plotted as a function of NECDFc concentration.

Fig. 4. Dll1K17R and Dll1-3 cannot efficiently induce Notch activation. Bone marrow derived hematopoietic stem cells were co-cultured with Dll1, Dll1K17R or Dll1-3-expressing OP9 cells. After 3 weeks, co-cultures were analyzed by flow cytometry using CD4 and CD8 T cell markers. The percentages of double positive T cell population are indicated.

Fig. 5. Notch1 is transendocytosed into Dll1-, but not Dll1K17R- nor Dll1-3-expressing cells. Hela cells transiently transfected with VSV-tagged Dll1 (a) or Dll1-3 (b) or Dll1K17R (c) construct were co-cultured with MEFs that stably express HA-tagged Notch1 receptor. Cells were stained with Cy3-conjugated anti-VSV and Alexa488-conjugated anti-HA antibodies; Hoechst staining is shown.

Fig. 6. Dll1 but neither Dll1K17R nor Dll1-3 localizes to lipid microdomains. OP9 stromal cell lines stably expressing Dll1, Dll1K17R or Dll1-3 were lysed in 1% Brij98 at 4°C and subjected to ultracentrifugation on sucrose gradients. Fractions were collected from top (fraction 1, low density) to bottom (fraction 10, high density) and analyzed by immunoblotting with anti-Dll1CT and anti-Dll3ic antibody. Caveolin-1 (24 kD) was used as a marker of lipid microdomains while ß-tubulin (55 kD) was used as a marker of detergent soluble subcellular fractions.