Monoubiquitination and endocytosis direct γ-secretase cleavage of activated Notch receptor

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Activation of mammalian Notch receptor by its ligands induces TNFα-converting enzyme–dependent ecto-domain shedding, followed by intramembrane proteolysis due to presenilin (PS)-dependent γ-secretase activity. Here, we demonstrate that a new modification, a mono-ubiquitination, as well as clathrin-dependent endocytosis, is required for γ-secretase processing of a constitutively active Notch derivative, ΔE, which mimics the TNFα-converting enzyme–processing product. PS interacts with this modified form of ΔE, ΔE^u. We identified the lysine residue targeted by the monoubiquitination event and confirmed its importance for activation of Notch receptor by its ligand, Delta-like 1. We propose a new model where monoubiquitination and endocytosis of Notch are a prerequisite for its PS-dependent cleavage, and discuss its relevance for other γ-secretase substrates.

Introduction

During the development of both invertebrates and vertebrates, the Notch pathway ensures correct specification of multiple cell types. The Notch gene encodes a large single-pass transmembrane (TM) receptor, matured in the secretory pathway by a convertase of the furin family, and presented at the cell surface as a heterodimeric molecule (Logeat et al., 1998). Binding of Notch ligands (Delta or Jagged in mammals) causes a proteolytic cleavage in the extracellular domain of Notch due to a protease of the ADAM family, TNFα-converting enzyme (TACE; Brou et al., 2000; Mumm et al., 2000). The remaining membrane-tethered Notch is then cleaved within its TM domain, leading to the release of the intracellular domain of Notch1 (ICv; (Kopan et al., 1994, 1996; Jarriault et al., 1995). The ICv domain translocates into the nucleus, where it participates in transcriptional activation of target genes together with CSL and Mastermind (Kitagawa et al., 2001). TM cleavage of Notch is due to a γ-secretase-like activity, analogous to the one that cleaves amyloid β precursor protein (APP; Fortini, 2002). Recent data suggest that γ-secretase cleavage is mediated by a large protein complex containing an aspartyl–protease activity. This high molecular weight complex may consist of one of the two presenilins (PSs) carrying the enzymatic activity, and of other regulatory

proteins, namely Nicastrin/APH-2, APH-1, and Pen-2 (Fortini, 2002). The majority of the PS pool is located within the ER and the Golgi apparatus (De Strooper et al., 1997; Zhang et al., 1998; Annaert et al., 1999), but more recent evidence suggests a distribution at the cell surface and in endocytic compartments (Georgakopoulos et al., 1999; Ray et al., 1999; Lah and Levey, 2000; Cupers et al., 2001; Kaether et al., 2002). Analyses of Nicastrin suggest that the mature, PS- and Nicastrin-containing γ-secretase complex is found in compartments distal to the ER (Yang et al., 2002) and in lysosomal membranes (Pasternak et al., 2003). Moreover, the γ-secretase activity itself has been suggested to be located in a compartment distal to the ER–Golgi (Cataldo et al., 2000; Cupers et al., 2001; Grbovic et al., 2003).

Endocytosis has been shown to play a role in Notch signaling in *Drosophila* and in *Caenorhabditis elegans*, although its direct role in γ-secretase cleavage of the receptor has not been proven yet. *Shibire*, the fly dynamin homologue, is necessary in ligand-expressing cells as well as in signal-receiving cells for proper Notch signaling (Seugnet et al., 1997). Dynamin is involved in endocytosis through its role in the formation and pinching off of clathrin-coated vesicles from the plasma membrane. Its involvement in the Notch pathway suggests the activity of both ligand and receptor is regulated by endocytosis. In the emitting cell, it has been shown

that Delta can trigger transendocytosis of the Notch extracellular domain after TACE cleavage (Parks et al., 2000). However, in the receiving cell, endocytosis of the receptor itself and its function in the signaling process has been poorly documented. In *C. elegans*, endocytosis-mediated down-regulation of the lin12/Notch pathway has been described (Shaye and Greenwald, 2002), but it could correspond to a constitutive internalization allowing recycling or degradation of the receptor. In *Drosophila*, it has been proposed that γ-secretase proteolysis of Notch occurs within the apical membrane or in an associated endosomal compartment (Lopez-Schier and St. Johnston, 2002). Based on these data, we hypothesized that after ligand binding, some trafficking of Notch and/or of the PS-containing complex is required for the γ-secretase cleavage. Here, we demonstrate that PSs interact with a monoubiquitinated form of activated Notch. This modification, as well as an endocytosis step, precedes and is necessary for γ-secretase cleavage of Notch.

Results

Endocytosis is required before γ-secretase cleavage of Notch

To elucidate the events occurring after TACE proteolysis, we took advantage of the Notch ΔE expression vector (Ko-

pan et al., 1996). ΔE encodes ΔE_{TM}, a truncated form of the Notch1 receptor (see Fig. 2 B) lacking most of its extracellular domain, but containing the signal peptide fused to the 21 amino acids NH_2-terminal to the TM domain, the TM domain, and the intracellular domain. ΔE_{TM} is very analogous to the natural TACE-processing product of Notch1 that is generated after ligand binding (Brou et al., 2000). It is constitutively cleaved by γ-secretase (De Strooper et al., 1999), thus releasing the transcriptionally active nuclear form of Notch, ICv (Fig. 1 A, A1). We mainly used COOH-terminally truncated forms of the constructs, where the last 355 amino acids of the Notch receptor have been replaced by 6-myc epitopes (Kopan et al., 1996). This truncation deletes the PEST domain, and the derived ICv is more stable than its full-length counterpart (Gupta-Rossi et al., 2001). To determine whether an endocytosis step was involved in ΔE_{TM} processing, we inhibited endocytosis by transfecting a GFP-conjugated Eps15 dominant-negative mutant (Eps15DN) deleted of its EH domains (Benmerah et al., 1999). As a control, we monitored endocytosis of the transferrin receptor by using a fluorescent dye–labeled transferrin (CY3-Tf). In the cells overexpressing Eps15DN (Fig. 1 A, B1), endocytosis of the transferrin receptor (Fig. 1 A, B2) was inhibited, as no intracellular fluorescence could be detected (Fig. 1

Figure 1. **Endocytosis is required for γ-secretase cleavage of Notch ΔE.** (A) Endocytosis is required for nuclear localization of ΔE derivatives. HeLa cells were transiently transfected with ΔE (A and C) and/or a dominant-negative form of GFP-fused Eps15 (B and C, EPS15DN). In A and B, living cells were first incubated with CY3-Tf for 1 h at 37°C. Eps15DN was visualized directly by the fluorescence emitted by GFP (B1, B3, C1, and C3), whereas ΔE was detected using an anti-myc antibody (Alexa 488® labeled in green: A1 and A3; CY3 labeled in red: C2 and C3). CY3-Tf reveals the internalization of transferrin receptor (in red: A2, A3, B2, and B3). Panels numbered 3 are merges of the corresponding panels 1 and 2. (B) γ-Secretase processing of ΔE is inhibited by overexpression of dominant-negative forms of dynamin2 (dynK44A) and Eps15 (Eps15DN). 293T cells were transfected with plasmids encoding ΔE (lanes 1–4), ΔE along with dynK44A (lanes 5 and 6) or Eps15DN (lanes 7 and 8), or ICv (lane 9). Cells were pulse labeled with [^{35}S]methionine and chased for 1 h. 50 µM MW167 (lanes 3 and 4) was added as indicated in the Materials and methods. Anti-myc immunoprecipitated cell extracts were analyzed by SDS-PAGE followed by autoradiography. ΔE_{TM} indicates the ΔE membrane-anchored form, asterisks mark 95- and 150-kD migrating products corresponding respectively to dynK44A (lanes 5 and 6) and Eps15DN (lanes 7 and 8). The ratio between ICv and ΔE_{TM} was quantified in this representative experiment using phosphorimager analysis to 38, 9, and 20%, corresponding respectively to lanes 2, 6, and 8. The open arrowhead shows a slower migrating band stabilized in the presence of MW167 (lanes 3 and 4).
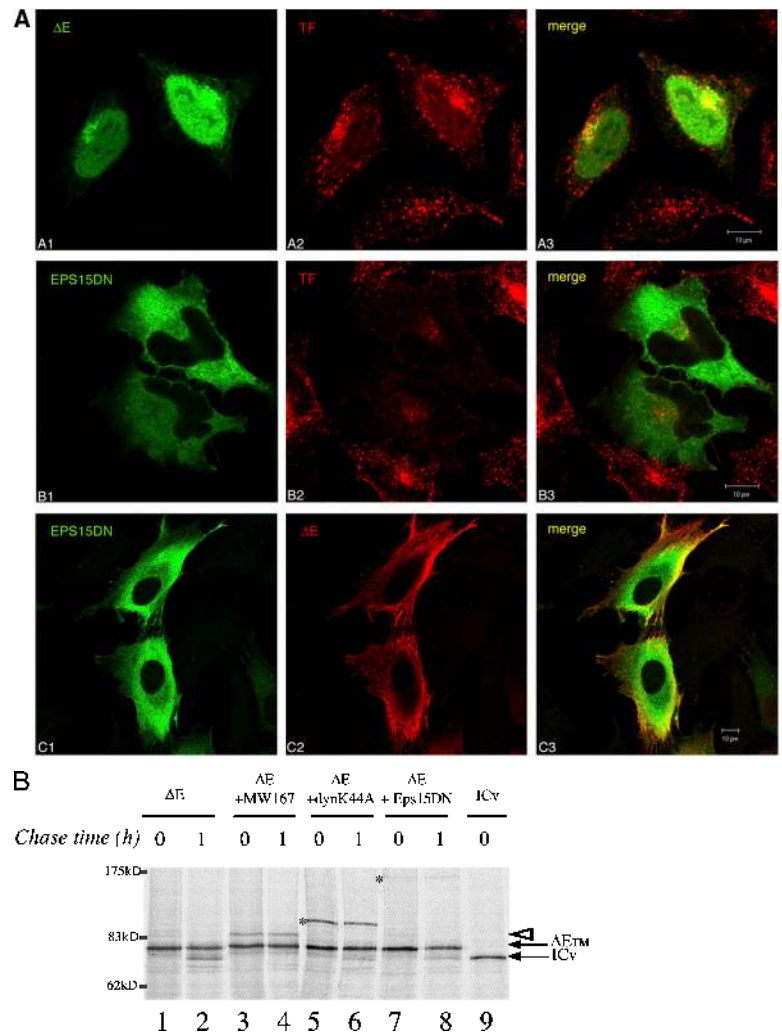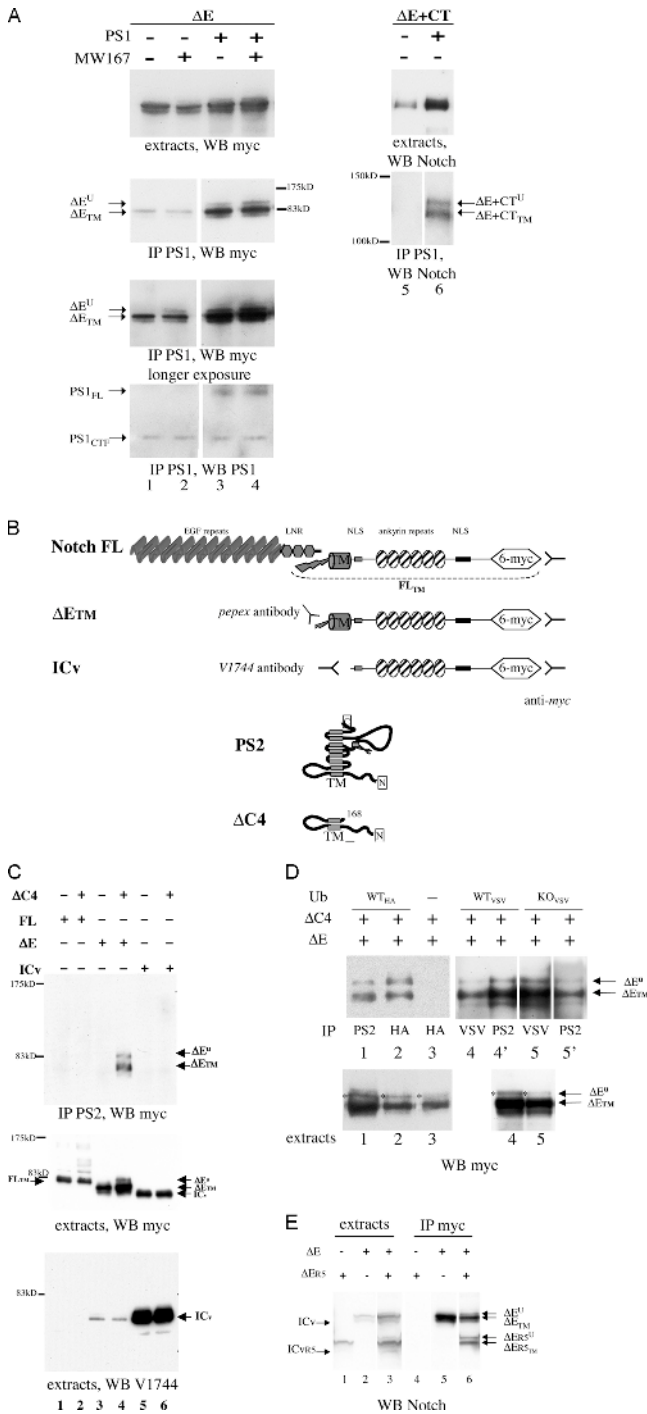
Figure 2. **PSs interact with a monoubiquitinated form of ΔE, ΔE^u.**
(A) Analysis of PS1–Notch interaction. 293T cells were transfected with ΔE (lanes 1–4), ΔE+CT (lanes 5 and 6), and PS1 (lanes 3, 4, and 6) expression vectors for 24 h, then treated with MW167 for 3 h when indicated. Extracts or PS1 immunoprecipitates (IP) were analyzed by SDS-PAGE (4–12%, lanes 1–4; 5%, lanes 5 and 6) and immunoblotted with antibodies against myc, Notch, or the loop domain of PS1 (recognizing PS1$_{FL}$ and PS1$_{CTF}$). ΔE$_{TM}$ and ΔE+CT$_{TM}$ represent the membrane-anchored forms encoded by the expression vectors; ΔE^u and ΔE+CT^U are the novel modified forms revealed by this assay. (B) Constructs used in this paper. Top, Notch derivatives: FL, ΔE, and ICv forms, and the epitopes recognized by the antibodies used are represented. Note that *pepex* and V1744 respectively recognize the membrane-anchored ΔE and the γ-secretase cleavage product ICv, whereas anti-myc and anti-Notch (not depicted) anti-

bodies detect all the derivatives. FL$_{TM}$ represents the TM subunit of Notch heterodimer resulting from furin cleavage. Bottom, PS2 and its derivative, ΔC4. ΔC4 encodes the NH$_2$-terminal 168 aa of PS2, containing its two first TM domains. (C) ΔC4 specifically interacts with ΔE-derived forms. 293T cells were transfected with either Notch FL (lanes 1 and 2), ΔE (lanes 3 and 4), or ICv (lanes 5 and 6), along with ΔC4 (even-numbered lanes). Total cell extracts (middle and bottom) and their immunoprecipitates obtained with anti-PS2 antibody (top) were analyzed by Western blotting using anti-myc (top and middle) or V1744 (bottom). (D) ΔE^u results from monoubiquitination of ΔE. 293T cells were transiently transfected with ΔE, along with ΔC4 and either WT ubiquitin (HA-tagged, lanes 1 and 2; VSV-tagged, lanes 4 and 4') or VSV-tagged UbKO (lanes 5 and 5'). In the top panel, cell extracts were immunoprecipitated with anti-PS2 (lanes 1, 4', and 5'), anti-HA (lanes 2 and 3), or anti-VSV (lanes 4 and 5) before SDS-PAGE analysis. In the bottom panel, 2% of total protein lysates used for IP were loaded. Extracts from WT$_{VSV}$ or KO$_{VSV}$ Ub-transfected cells (bottom, lanes 4 and 5, respectively) were divided into two halves for IP with either VSV (lanes 4 and 5) or PS2 (lanes 4' and 5'). Immunoblots were performed using anti-myc antibody. Asterisks indicate the ΔE^u form in whole-cell extracts. (E) Dimerization of ΔE molecules. 293T cells were transfected with myc-tagged ΔE and/or an untagged, COOH-terminally truncated ΔE, ΔER5, as indicated. The corresponding cell extracts were immunoblotted with anti-Notch antibody directly (lanes 1–3) or after anti-myc immunoprecipitation (lanes 4–6). The products derived from each construct are indicated: ΔE^u, ΔE$_{TM}$, and ICv; and ΔER5^u, ΔER5$_{TM}$, and ICvR5. White lines indicate that intervening lanes have been spliced out.

A, B3; cell surface receptor labeling expected here being washed out by Triton permeabilization). Overexpression of Eps15DN in ΔE-transfected cells led to nuclear exclusion of Notch products (Fig. 1 A, compare A1 with C2). When an expression vector encoding myc-tagged ICv was cotransfected with Eps15DN, the anti-myc labeling was exclusively nuclear, showing that ICv nuclear transport was not affected (unpublished data). We obtained the same results when replacing Eps15DN with a dominant-negative form of dynamin 2 (dynK44A; McNiven et al., 2000; unpublished data). These data suggest that inhibition of endocytosis prevents γ-secretase cleavage. To verify this hypothesis, we performed a pulse-chase analysis of ΔE-transfected cells in the presence or absence of dynK44A or Eps15DN (Fig. 1 B) in HEK 293T cells. ΔE$_{TM}$-derived ICv was visualized after a 1-h chase (Fig. 1 B, compare lane 2 with lane 9), but not after treatment with MW167 (Wolfe et al., 1998), a specific inhibitor of γ-secretase activity (Fig. 1 B, lanes 3 and 4). DynK44A coexpression inhibited ICv production; so did Eps15DN to a lesser extent (compare the ratio between ICv and ΔE$_{TM}$ in lanes 2, 6, and 8, quantified to 38, 9, and 20%, respectively). The 95- and 150-kD migrating products detected respectively in lanes 5 and 6 and in lanes 7 and 8 (Fig. 1 B, asterisks) probably correspond to dynK44A and Eps15DN, suggesting that they directly or indirectly associate with Notch. We conclude from these experiments that an endocytosis step is necessary before γ-secretase cleavage.

PSs interact with a modified form of ΔE

A slower migrating product (open arrowhead) that was stabilized in the presence of the γ-secretase inhibitor could be visualized in Fig. 1 B (lanes 3 and 4). We assumed that it might represent a specific modification that targets ΔE$_{TM}$

before γ-secretase cleavage. As γ-secretase is performed by a complex containing PS, PS (1 or 2) should coimmunoprecipitate with the substrate of the enzyme. PS1 indeed interacted with ΔE_{TM} when both proteins were overexpressed in HEK 293T cells (Fig. 2 A, lanes 3 and 4). The interaction between endogenous PS1 and ΔE_{TM} could also be detected on a longer exposure (Fig. 2 A, lanes 1 and 2). Furthermore, PS1 coimmunoprecipitation enabled us to reveal a novel form, ΔE^u, either when PS1 was overexpressed (Fig. 2 A, lanes 3 and 4) or when γ-secretase activity was blocked by MW167 treatment (Fig. 2 A, lane 2). Based on its apparent molecular weight, it could correspond to the labile form visualized in Fig. 1 B, lanes 3 and 4. To verify that COOH-terminal truncated forms of ΔE behave like their non-truncated counterparts, we coimmunoprecipitated PS1 with $\Delta E+CT$ and could detect $\Delta E+CT_{TM}$, as well as an upper-migrating band, $\Delta E+CT^U$ (Fig. 2 A, lanes 5 and 6). Then, we tested whether the NH_2-terminal part of PS1 or PS2 could account for Notch interaction, as previously described for APP–PS interaction (Pradier et al., 1999). Indeed, immunoprecipitating $\Delta C4$, which only contains the NH_2-terminal part of PS2 (Fig. 2 B), revealed ΔE_{TM} and resulted in enrichment of the ΔE^u form (Fig. 2 C, lane 4; compare top and middle panels). $\Delta C4$ did not coimmunoprecipitate with the ΔE_{TM}-derived (Fig. 2 C, lanes 3 and 4) or overexpressed ICv (Fig. 2 C, lanes 5 and 6). ICv is revealed by direct immunoblotting with the V1744 antibody (Fig. 2 C bottom, and see Fig. 2 B). Cotransfection of full-length Notch revealed that the furin-cleaved membrane-anchored fragment (Fig. 2 B, FL_{TM}) was not able to bind $\Delta C4$ either (Fig. 2 C, lanes 1 and 2). The same results were observed with a PS1-derived $\Delta C4$ construct (unpublished data). As both PS and $\Delta C4$ bind membrane-associated ΔE_{TM}-derived forms, we used $\Delta C4$ coimmunoprecipitation as a tool to select for γ-secretase substrates, this form being less toxic than the full-length PS.

Monoubiquitination accounts for the ΔE^u form

As the ΔE^u form probably derives from ΔE_{TM} and could represent an intermediate species necessary for either association with or cleavage by the γ-secretase, we decided to characterize the nature of this modification. Treatment with phosphatases or deglycosylases did not affect the ΔE^u band (unpublished data), and the apparent molecular weight difference of 8 kD between ΔE_{TM} and ΔE^u suggested that this modification could be due to a monoubiquitination event. Therefore, we cotransfected the cells with HA-tagged ubiquitin together with ΔE and $\Delta C4$. Immunoprecipitation with the anti-HA antibody, followed by anti-myc immunoblotting, specifically revealed the same migration products as those detected after anti-PS2 pull-down (Fig. 2 D, lanes 1–3), suggesting that ΔE^u contains an HA-ubiquitin moiety. We also used a ubiquitin molecule (UbKO) mutated on all its lysine residues and only able to form the first isopeptide bond between its last glycine (G76) and a lysine residue on the target protein (Wu et al., 2003). As shown in Fig. 2 D (lanes 4, 4′, 5, and 5′), immunoprecipitating ubiquitin or $\Delta C4$ followed by anti-myc immunoblotting revealed the same migrating products, whether ubiquitin wild type (WT) or KO were used. The fact that ΔE_{TM} was specifically coimmunoprecipitated with anti-ubiquitin as well as anti-PS2 antibodies in the presence of $\Delta C4$ can be explained by the formation of dimeric molecules. Dimerization of ΔE molecules was studied by cotransfecting ΔE along with a COOH-terminally truncated ΔE devoid of the myc epitopes (Fig. 2 E, $\Delta ER5$). Immunoprecipitation with anti-myc antibody followed by anti-Notch immunoblotting revealed ΔE_{TM} and ΔE^u, as well as $\Delta ER5_{TM}$ and $\Delta ER5^u$, showing that these proteins were associated (Fig. 2 E, lane 6). From these experiments, we propose that ΔE^u is derived from ΔE_{TM} by conjugation of a single ubiquitin moiety. This result was confirmed by anti-ubiquitin immunoblotting of $\Delta C4$ coimmunoprecipitates (Fig. 3 C).

Monoubiquitination takes place on a juxtamembrane lysine residue and is necessary for γ-secretase cleavage of ligand-activated Notch

Next, we tried to localize the lysine residue(s) targeted by this ubiquitination event. In one of our previous reports, we had used the ΔE-GAL-VP16 chimera ending at aa 1809 as a substrate for γ-secretase (Brou et al., 2000). It has also been shown that inserting a GAL-VP16 moiety 9 amino acids COOH-terminal to the γ-secretase cleavage site (at aa 1753) of mammalian Notch still allows γ-secretase cleavage (as measured by monitoring GAL-VP16 transcriptional activity; Taniguchi et al., 2002). Therefore, the Notch fragment immediately adjacent to the membrane probably contains any amino acid whose modification is required for γ-secretase cleavage. Comparison of Notch sequences derived from various species (Fig. 3 A) shows a good conservation of the juxtamembrane lysine residue. Therefore, we mutated lysine 1749 of murine Notch1 to arginine (K1749R). We also used the LLFF construct, a ΔE form mutated in the γ-secretase cleavage site (located after aa G1743; Schroeter et al., 1998). First, we analyzed the localization of these mutants when overexpressed in HeLa cells. In contrast to their WT counterpart, none of the mutants presented a nuclear staining (Fig. 3 B), suggesting that ICv production was impaired. The intracellular labeling may correspond to molecules trafficking to the membrane or, inversely, in the process of endocytosis. Immunofluorescence experiments showed that routing to the plasma membrane of these mutants was not affected (see Fig. 5). The substitution of lysine 1749 by arginine affected neither the localization nor the stability of the corresponding ICv, as confirmed by immunofluorescence and pulse-chase analysis (unpublished data). Therefore, these mutations near the TM domain do not interfere with correct addressing or stability of the proteins, but may prevent γ-secretase cleavage. Then, we determined whether monoubiquitination, as well as γ-secretase cleavage, was affected in these mutants. ΔE^u was detected by coimmunoprecipitation with $\Delta C4$ followed by immunoblotting with an antibody recognizing ubiquitin moieties (Fig. 3 C top, lanes 1–3). Interestingly, ΔE^u was only revealed when using WT or LLFF ΔE constructs, in contrast to K1749R, whereas ΔE_{TM} was coprecipitated with $\Delta C4$ in all cases (Fig. 3 C bottom, lanes 1–3; detected by reblotting with an anti-Notch antibody). On the other hand, we tested the cell ex-
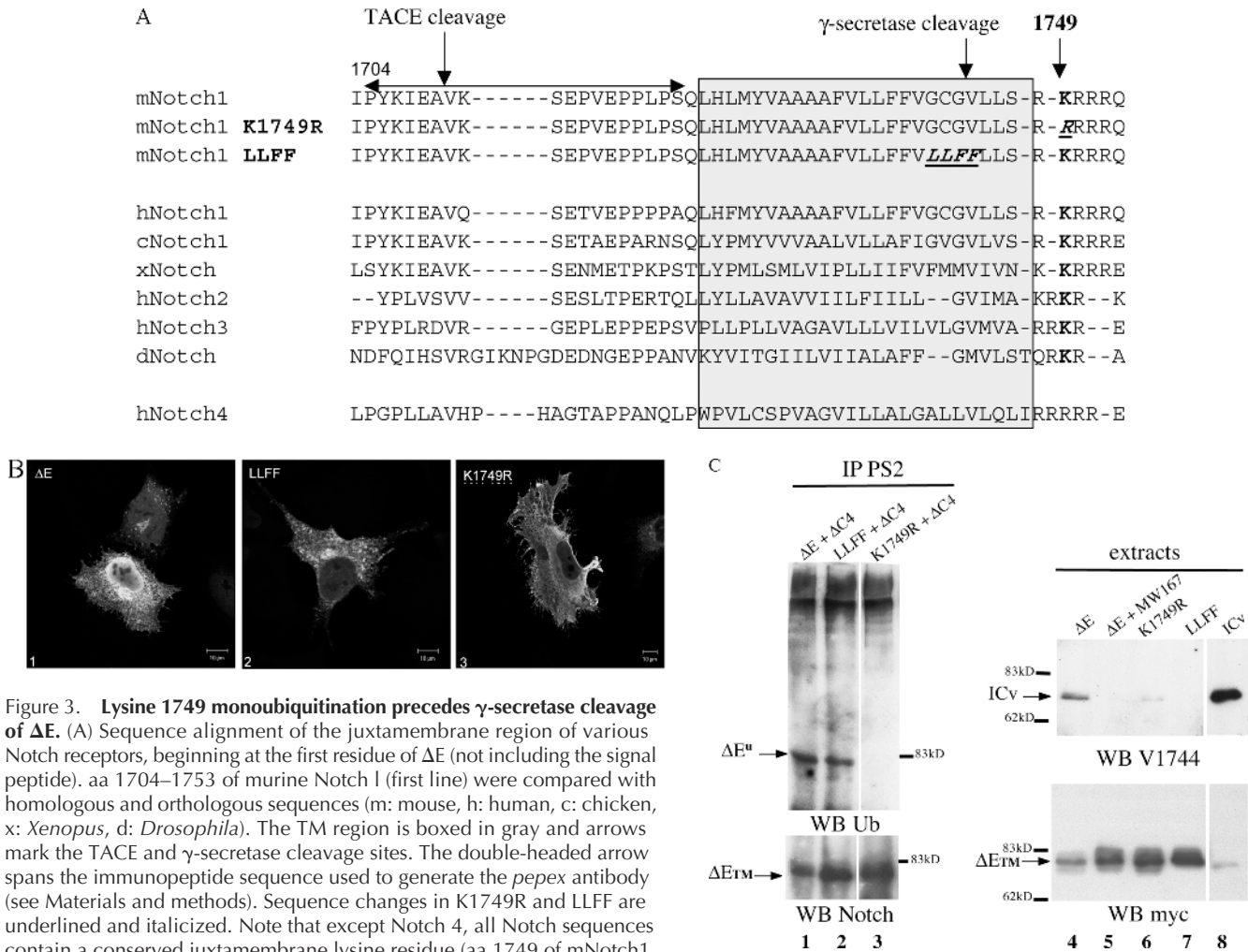
Figure 3. **Lysine 1749 monoubiquitination precedes γ-secretase cleavage of ΔE.** (A) Sequence alignment of the juxtamembrane region of various Notch receptors, beginning at the first residue of ΔE (not including the signal peptide). aa 1704–1753 of murine Notch l (first line) were compared with homologous and orthologous sequences (m: mouse, h: human, c: chicken, x: *Xenopus*, d: *Drosophila*). The TM region is boxed in gray and arrows mark the TACE and γ-secretase cleavage sites. The double-headed arrow spans the immunopeptide sequence used to generate the *pepex* antibody (see Materials and methods). Sequence changes in K1749R and LLFF are underlined and italicized. Note that except Notch 4, all Notch sequences contain a conserved juxtamembrane lysine residue (aa 1749 of mNotch1, arrow in bold). (B) Subcellular localization of mutant forms of ΔE. HeLa cells were transiently transfected by ΔE (panel 1), LLFF (panel 2), or K1749R (panel 3) expression vectors. Cells were fixed, Triton-permeabilized, and stained for fluorescence microscopy using the anti-myc antibody revealed by Alexa 488®–coupled secondary antibody. (C) Monoubiquitination of K1749 is required for γ-secretase cleavage. 293T cells were transfected with ΔC4 (lanes 1–3), various ΔE forms (WT: lanes 1, 4, and 5; LLFF: lanes 2 and 7; K1749R: lanes 3 and 6), or ICv (lane 8). In lanes 1–3, cell extracts were subjected to immunoprecipitation with anti-PS2 antibody, analyzed by immunoblotting with anti-ubiquitin antibody (top), and reprobed with anti-Notch antibody (bottom). In lanes 4–8, cell extracts were directly blotted with V1744 (top) or anti-myc (bottom) antibodies. White lines indicate that intervening lanes have been spliced out.

tracts in parallel for the presence of γ-secretase cleavage products with V1744 antibody (Fig. 3 C top, lanes 4–8) and for the total amount of Notch-derived products with anti-myc antibody (Fig. 3 C bottom, same lanes). ICv was indeed detected in extracts transfected with ΔE or ICv expression vectors (Fig. 3 C, lanes 4 and 8), but was not produced when cells were treated with MW167 (Fig. 3 C, lane 5) or when the LLFF construct was used (Fig. 3 C, lane 7). When using the K1749R mutant (Fig. 3 C, lane 6), ICv could still be detected, but to a much lower extent than its WT counterpart; this result being strengthened by the lower amount of total Notch products in Fig. 3 C, lane 4 vs. lane 6. Thus, the LLFF mutant can still be monoubiquitinated but not γ-secretase processed, whereas both events are strongly inhibited in the K1749R mutant.

To confirm our results in the context of the activation of full-length Notch receptor, we transiently transfected HeLa or HEK 293T cells with WT full-length Notch receptor (Notch FL) or its lysine 1749 mutant (FLK1749R) and incubated them with the preclustered extracellular domain of the Notch ligand, Delta-like 1 (Dl-Fc; Hicks et al., 2002). In comparison with Notch FL, FLK1749R-transfected cells exhibited decreased ICv production after ligand binding, as detected by immunoblotting (Fig. 4 A) and immunofluorescence (Fig. 4 B). FL or FLK1749R addressing was not affected by this mutation (Fig. 4 B). The residual ICv production detected in the K1749R mutants (FL and ΔE) may be due to an overexpression effect and/or to the use of secondary ubiquitination sites. These data suggest that ubiquitination occurs before and is required for γ-secretase cleavage and that lysine 1749 is crucial for this event.

Furthermore, as shown for ΔE, when cells were cotransfected with Notch FL along with the Eps15DN expression vectors, nuclear staining was no longer visible after activation in cells overexpressing Eps15DN (unpublished data). This result confirms that ligand-activated Notch is endocytosed before γ-secretase cleavage.
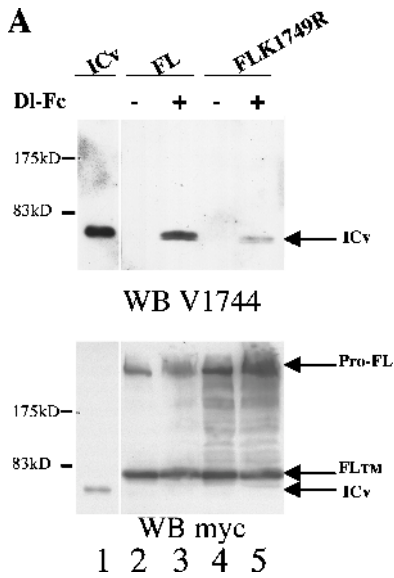
A

B

Figure 4. **Lysine 1749 is critical for Notch signaling.** (A) 293T cells were transfected with either ICv (lane 1), Notch FL (lanes 2 and 3), or FLK1749R (lanes 4 and 5). Notch FL activation was performed by overnight incubation with either control conditioned media (indicated as "−" in lanes 2 and 4) or clustered Dl-Fc–containing media (+, lanes 3 and 5). Total cell extracts were analyzed by V1744 Western blotting (top) and were reprobed with anti-myc antibody (bottom). Full-length Notch precursor is designated Pro-FL. (B) HeLa cells were transiently transfected with Notch FL (panels 1–4) or FLK1749R (panels 5–8). Ligand-binding assay was performed for 1 h with either control conditioned media (CM) or clustered Dl-Fc containing media (Dl-Fc). Notch was detected using an anti-myc antibody revealed by an Alexa 488®–coupled secondary antibody. Even-numbered panels show phase-contrast sections (differential interference contrast), odd-numbered panels an overlay of differential interference contrast and fluorescence. The bottom diagram represents the quantitative analysis of nuclear staining of Dl-Fc–activated cells (n = 28 and 23 for Notch FL and FLK1749R, respectively); 68% of WT Notch-expressing cells exhibited at least 40% nuclear staining when activated by the ligand, whereas none of the FLK1749R cells did.
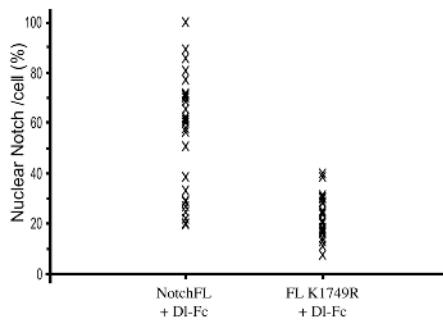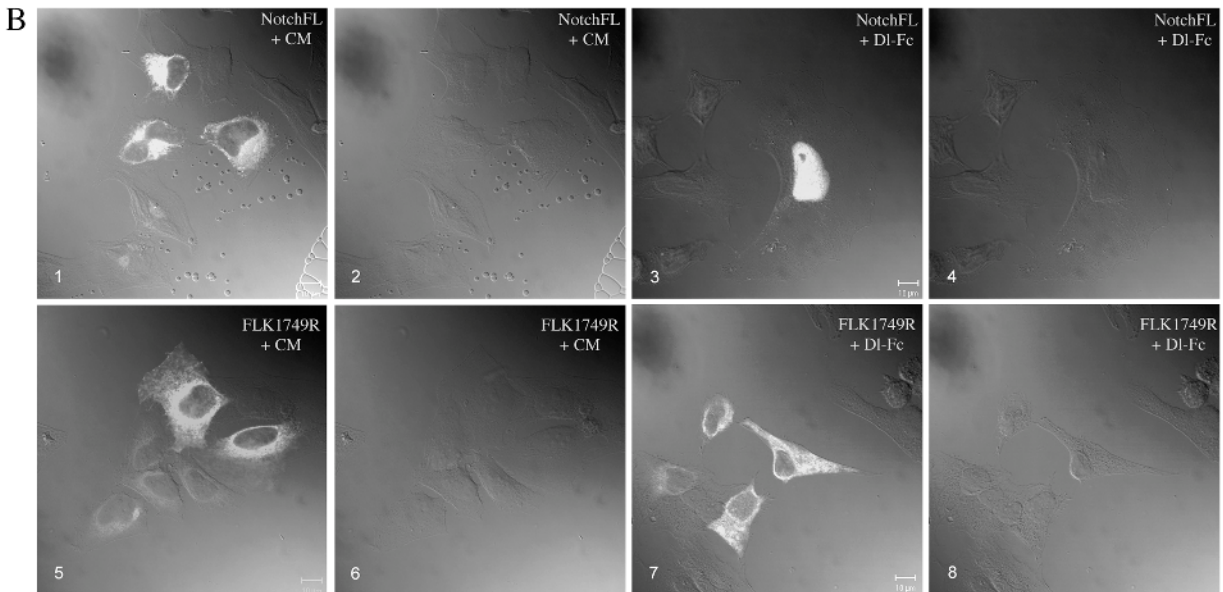
Monoubiquitination of Notch triggers its endocytosis-dependent γ-secretase cleavage

If monoubiquitination is required for Notch to reach the compartment where the γ-secretase is active, the LLFF mutant should be blocked in the subcellular location where γ-secretase cleavage takes place, whereas the K1749R mutant should remain more upstream in the endocytic pathway or at the plasma membrane, as the result of a lack of mono-ubiquitination. To confirm this hypothesis, we performed immunostaining on living cells using an antibody (*pepex*) directed against aa 1705–1722 of Notch1, i.e., the extracellular, NH_2-terminal part of ΔE (Fig. 2 B and Fig. 3 A). Thus, we could visualize only the ΔE molecules that have reached the plasma membrane and could thus bind the antibody without interference with the intracellular molecules accumulating in the ER or Golgi. We monitored, in parallel, en-
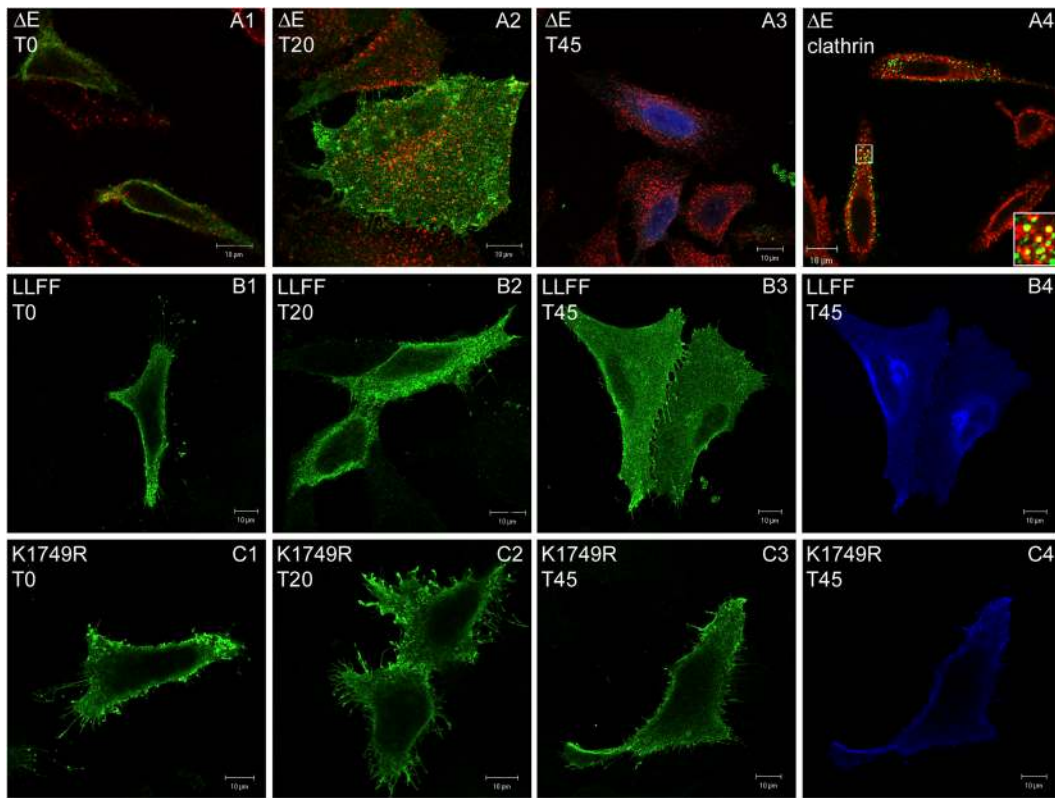
Figure 5. **Kinetics of ΔE endocytosis compared with LLFF and K1749R.** HeLa cells were transiently transfected with ΔE (A panels), LLFF (B panels), or K1749R (C panels). Living cells were incubated with an antibody directed against the extracellular part of Notch ΔE (*pepex*, in green: A1–A4, B1–B3, and C1–C3) and CY3-Tf (in red: A1–A3) at 4°C (T0), or were incubated at 37°C for either 20 min (T20; A2, A4, B2, and C2) or 45 min (T45). Cells were fixed, saponin permeabilized, and incubated with anti-myc (in blue: A3, B4, and C4) or anti-clathrin antibodies (in red: A4) and the appropriate dye-labeled secondary antibodies. Same fields are presented in B3 and B4, and in C3 and C4. The inset in A4 shows a higher magnification of the boxed region and highlights ΔE and clathrin colocalization (in yellow). Preparations were analyzed by confocal microscopy (0.3-μm sections).

docytosis of the transferrin receptor (Fig. 5, A1–A3; punctate red staining). At 4°C (T0), *pepex* antibody revealed membrane labeling of all three ΔE derivatives (Fig. 5, A1, B1, and C1), thus showing their correct addressing to the plasma membrane. After 20 min at 37°C, *pepex*-ΔE and *pepex*-LLFF complexes were internalized, whereas the K1749R mutant remained mainly membrane localized (Fig. 5, A2, B2, and C2). After 45 min, a red perinuclear staining showed the transferrin receptors in recycling endosomes (Fig. 5, A3), but none of the Notch derivatives were localized in this compartment (Fig. 5, A3; unpublished data). Moreover, at this time point, the ΔE-transfected cells no longer exhibited *pepex* staining, although they were positive for intranuclear Notch staining, as revealed after cell permeabilization, by the anti-myc antibody (Fig. 5, A3; in blue). Thus, it seems that all *pepex*-bound ΔE molecules were γ-secretase processed, eliminating the extracellular epitope. On the other hand, in accordance with the fact that γ-secretase processing could not occur at 45 min, LLFF and K1749R mutants were still *pepex* labeled (Fig. 5, B3 and C3), showing colocalization with the anti-myc antibody (Fig. 5, B4 and C4). The K1749R mutant presented less endocytosis than the LLFF mutant, suggesting that monoubiquitination may precede late endocytosis. Furthermore, partial colocalization of internalized ΔE molecules (20-min pepex incuba-

tion; Fig. 5, A4, green) with clathrin light chain (Fig. 5, CON.1 antibody, red) confirmed that a clathrin-dependent mechanism was involved. From these results and the biochemical data, we propose that the monoubiquitination step takes place at the plasma membrane or during early endocytosis, both events being required for the substrate to reach the compartment where the γ-secretase is active.

Discussion
A monoubiquitination event is involved in Notch signaling
The ubiquitination pathway involves a multiprotein cascade in which the substrate specificity is determined by the E3 component (Jackson et al., 2000). Multi-ubiquitin chains at least four subunits long are required for efficient recognition and degradation of ubiquitinated proteins by the proteasome, but ubiquitin has more recently been shown to endorse new functions that do not always involve the proteasome (Schnell and Hicke, 2003).

Our results show, for the first time, that a monoubiquitination event takes place on the ΔE molecule, a constitutively active form of the Notch receptor that mimics the intermediate TACE-processing product generated after ligand binding. This modification is a prerequisite for γ-secretase cleav-

age and targets one of the subunits of a dimeric membrane-anchored form of Notch ΔE. We localized the major site of monoubiquitination to a juxtamembrane, conserved lysine residue K1749 in mNotch1. We got access to the monoubiquitinated form ΔE^u by coimmunoprecipitation with endogenous PS1 when γ-secretase activity was inhibited by a specific drug. Thus, ΔE^u is a labile intermediate appearing before γ-secretase cleavage. This form could also be detected after coexpression of PS1 or ΔC4, a PS2-derived construct. These molecules are probably not included into PS-containing high molecular weight complexes, neither are γ-secretase components when transiently overexpressed (Kimberly et al., 2003). Thus, they highlight the existence of ΔE^u by extracting and stabilizing it out of the active complexes. We propose that this ubiquitination step is required in the context of the full-length receptor activated by ligand binding. Although we could not directly access the modified intermediate species derived from full-length Notch, mutating the crucial lysine residue impaired Dll1-mediated Notch signaling, in accordance with the ΔE results. It remains to be determined which E3 ubiquitin ligase is involved in this modification. Various proteins carrying such an activity have been associated with the Notch cascade and are candidates to be tested (Lai, 2002; Itoh et al., 2003), e.g., Deltex (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994), Suppressor of Deltex (Qiu et al., 2000), and Cbl (Jehn et al., 2002). Experiments are in progress to answer this question.

Monoubiquitination and endocytosis trigger γ-secretase cleavage of activated Notch

Our results show that endocytosis of Notch ΔE and of ligand-activated full-length Notch are necessary for γ-secretase cleavage. The involvement of a clathrin-dependent endocytosis event for Notch activation complies with the mosaic analysis performed in *Drosophila*, which revealed that *shibire* function is required in Notch-expressing cells receiving a lateral inhibition signal (Seugnet et al., 1997). We propose that monoubiquitination on a juxtamembrane lysine (K1749) and endocytosis occur after ligand-induced cleavage of the Notch extracellular domain by TACE. Our data are in apparent contradiction with the model proposed by Lopez-Schier and St. Johnston (2002), according to which γ-secretase cleavage occurs at the plasma membrane. However, Lopez-Schier's data can be reinterpreted in light of our model. These authors argue that the TACE-processing product of Notch (similar to the ΔE construct we use) remains associated with the apical membrane in Nicastrin or PS mutant cells, and in WT cells only a small amount of this molecule can be found in endocytic vesicles. This result can be explained by the fact that ubiquitination is one of the limiting steps in Notch signaling, or that active PS is needed to direct the final steps of endocytosis of the ubiquitinated forms. Probably for the same reason, ΔE is very poorly cleaved by γ-secretase when overexpressed, and the ubiquitination event can hardly be detected. Our results are also in apparent contradiction with those of Struhl and Adachi (2000), who postulate that in *Drosophila*, PS-mediated proteolysis does not appear to require a particular sequence nor the presence of active dynamin. However, the assay used appears unusually sensitive, as it even detects the cleavage of a Notch molecule carrying a G1743V mutation of the γ-secretase cleavage site, a mutation which prevents activation in most other assays (Huppert et al., 2000) and, when introduced into mice, gives rise to an almost perfect Notch1 null phenotype. Therefore, a leakage due to overexpression might in some cases be responsible for the activity detected. Moreover, these authors use constructs where the intracellular juxtamembrane domain (containing the conserved lysine residue) is always present, therefore precluding any conclusion about the importance of this lysine. Finally, species-specific differences might also be responsible for these discrepancies. Experiments are in progress to test the effect of mutating the juxtamembrane lysine of *Drosophila* Notch.

Various papers have described monoubiquitination as a signal for internalization of receptors such as EGFR or glycine receptor (Buttner et al., 2001; Haglund et al., 2003; Mosesson et al., 2003). Our results do not allow one to discriminate between ubiquitination triggering endocytosis or being concomitant with the first steps of endocytosis. However, our observations show a more internal localization of LLFF compared with the K1749R mutant, and endocytosis of the K1749R ΔE mutant seems to be blocked at an earlier stage when compared with the WT or LLFF mutant. These data suggest that ubiquitination is necessary for late events driving Notch to compartments where γ-secretase cleavage can occur.

Are ubiquitination and endocytosis general features of γ-secretase cleavage?

In other regulated intramembrane proteolysis events, transport factors are needed to deliver the substrate to the compartment where the enzyme is active (Urban and Freeman, 2002). For example, SCAP is necessary for delivering SREBP to S2P, and Star for delivering Spitz to rhomboid (Tsruya et al., 2002; Urban et al., 2002). It is possible, as suggested by others (Ray et al., 1999), that ΔE–PS interaction takes place at the plasma membrane. Indeed, the non-monoubiquitinated ΔE molecules (mutated on lysine 1749) are mainly localized at the plasma membrane, but still interact with PS when overexpressed. So PSs could recruit ΔE-like activated Notch molecules at the membrane, the complex being then ubiquitinated and internalized to reach the acidic environment where the γ-secretase is active. Alternatively, PS could recruit monoubiquitinated forms of Notch before endocytosis. It is also of note that a ΔE^u-like slower migrating band seems to be stabilized in PS$^{-/-}$ cells (for example see Fig. 5 in Soriano et al., 2001), or in cells treated with a γ-secretase inhibitor (this paper). Furthermore, ΔE location varies from plasma membrane to intracytoplasmic vesicles depending on the mutation affecting PS (Moehlmann et al., 2002). These data suggest that ubiquitination could occur in the absence of active PS, but that Notch–PS interaction would be required for proper internalization. Thus, it is tempting to speculate that PSs are bifunctional proteins, acting first as ubiquitin-dependent chaperones that target or escort their substrates to the appropriate compartment, and second, as γ-secretase enzymes.

Evidence for such a hypothesis does also exist for other γ-secretase substrates. First, APP cleavage was shown to de-

pend on trafficking and membrane fluidity (Fassbender et al., 2001; Kojro et al., 2001; Wolozin, 2001). At least a small amount of Aβ results from cleavage of APP within the endosomal/lysosomal pathway, particularly in nonneuronal cells (Haass et al., 1992; Cataldo et al., 2000; Grbovic et al., 2003). Another relevant observation concerns E-cadherin, recently described as another substrate for γ-secretase. Under conditions of cell–cell dissociation or apoptosis, this TM molecule is first cleaved in its ectodomain by matrix metalloprotease, then by a γ-secretase activity (Marambaud et al., 2002). These events promote the disassembly of adherens junctions in response to apoptotic signals and the subsequent release of the intracellular region of E-cadherin in the cytosol. Hakai, a RING-H2 finger E3 ubiquitin ligase, mediates E-cadherin ubiquitination in a tyrosine phosphorylation–dependent manner, this event leading to E-cadherin internalization and consequently to the perturbation of cell–cell adhesions (Fujita et al., 2002). One can combine these data and imagine that ubiquitination and endocytosis are also required before γ-secretase cleavage in the case of E-cadherin, and maybe of other γ-secretase substrates.

Remarkably, a lysine residue is present in the juxtamembrane region of various γ-secretase substrates, such as Notch, Delta1, Jagged 1 and 2 (Bland et al., 2003; Ikeuchi et al., 2003; LaVoie and Selkoe, 2003; Six et al., 2003), APP, ErbB-4, CD44, N and E-cadherins, and a few amino acids COOH-terminal to their γ-secretase cleavage site (Ni et al., 2001; Lammich et al., 2002; Marambaud et al., 2003). Experiments are in progress to confirm the validity of this general model.

Materials and methods

Materials
All Notch1 constructs reported here were cloned into the pCS2+ vector. Notch1 constructs, ΔE, ΔE+CT, ICv, and Notch FL plasmids have been described previously (Jarriault et al., 1995; Kopan et al., 1996; Schroeter et al., 1998); LLFF was a gift from R. Kopan (Washington University, St. Louis, MO). PS1 and ΔC4 constructs were supplied by L. Pradier (Aventis, Vitry, France). HA-tagged ubiquitin was provided by M. Treier (EMBL, Heidelberg, Germany). Eps15DN, a GFP-tagged, EH-domain–deleted form of Eps15, the CY3-labeled transferrin, and the anti-clathrin CON.1 antibody were provided by A. Dautry-Varsat (Pasteur Institute, Paris, France). Antibodies were supplied by Cell Signaling (V1744 antibody, recognizing γ-secretase–cleaved Notch1, and P4D1, an mAb against ubiquitin), Molecular Probes, Inc. (Alexa Fluor® 488 conjugates), Amersham Biosciences (CY3-coupled anti–mouse), Biogenesis (anti-Fc), and Jackson ImmunoResearch Laboratories (CY5-labeled donkey anti–mouse). Anti-PS1 pAb, directed against either the first 19 amino acids of the molecule (for IP) or the loop domain (for WB), and anti-PS2 pAb (95041) against the NH₂ terminus of PS2 (Pradier et al., 1999) were gifts from L. Buée (INSERM U422, Lille, France), C. Haas (Ludwig-Maximillians University, Munich, Germany), and L. Pradier, respectively. Anti-Notch pAbs have been described previously (Logeat et al., 1998; Brou et al., 2000). A pAb, named *pepex,* was generated against a peptide corresponding to aa 1705–1722 of mNotch1 (Fig. 3 A), coupled to keyhole limpet hemocyanin (Sigma-Aldrich) via an NH₂-terminal cysteine. Anti-HA (12CA5), anti-VSV (P5D4), and anti-myc (9E10) mAbs were used for immunoprecipitations and isolated on protein G–sepharose (Amersham Biosciences). N-ethylmaleimide was purchased from Sigma-Aldrich and Mowiol was purchased from Calbiochem. HEK 293T, HEK 293T stably transfected with a Dl-Fc expression vector (a gift of G. Weinmaster, Jonsson Cancer Center, Los Angeles, CA; Hicks et al., 2002), and HeLa cells were cultured in DME supplemented with 10% FBS and 1% penicillin/streptomycin (GIBCO BRL).

Constructs
The K1749R mutant was generated from ΔE by site-directed mutagenesis using the oligonucleotide 5′-GGGTGCTGCTGTCCCGTCGACGCCG-

GCGGCAGC-3′ and its complementary DNA. The FLK1749R mutant was obtained by inserting a NotI–BglII fragment from ΔE K1749R into the Notch FL construct. The ΔER5 construct was obtained by inserting an oligonucleotide containing a stop codon at the EcoRV site of ΔE. Ubiquitins WT_VSV and KO_VSV were generated from Ub-WT and UbKO (Wu et al., 2003) by amplification and insertion into EcoRI–XbaI sites of GVSV-pcDNA3 (Brou et al., 2000). Each construct was checked by sequencing.

Cell extracts, immunoprecipitations, and immunoblots
293T cells grown on 100-mm dishes were transiently transfected with a total DNA amount of 10 μg equalized with pcDNA3 vector. 40 h after transfection, cells were harvested, washed in PBS buffer, and lysed in 100 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, and 1% NP-40) supplemented with 1× protease inhibitor cocktail (Boehringer), 5 mM N-ethylmaleimide, and phosphatase inhibitors (Sigma-Aldrich). After 20 min on ice, cell lysates were cleared at 14,000 rpm for 20 min at 4°C. Immunoprecipitations were performed with the appropriate antibody after dilution of the extracts to 50 mM Tris-HCl, 200 mM NaCl, and 0.5% NP-40. Samples were resuspended in Laemmli buffer for SDS-PAGE resolution on a 6.5% polyacrylamide gel, and immunoblots were performed as described previously (Brou et al., 2000). 2–20% (when using V1744 antibody) of total protein lysates were loaded on the same gels for direct immunoblotting. Immunoblots with anti-ubiquitin antibodies were performed according to manufacturer's instructions (Cell Signaling).

Metabolic labeling
Pulse-chase experiments were performed as described previously (Brou et al., 2000). When mentioned, 50 μM MW167 (Partners Neurology) was added 1 h before the pulse and was maintained during the chase. Extracts were submitted to anti-myc antibody immunoprecipitation and analyzed by SDS-PAGE followed by autoradiography.

Endocytosis and ligand-binding assays
HeLa cells were grown on glass coverslips in a 24-well plastic dish, transfected with FuGENE™ 6 as indicated by the supplier (Roche), and treated 24 h after transfection. Endocytosis of transferrin receptor was performed using the CY3-Tf as described previously (Benmerah et al., 1999), and the *pepex* antibody was added (to a 200-fold dilution) when mentioned (Fig. 5 A). Endocytosis was stopped by washing with ice-cold PBS and PFA fixing. For ligand activation, conditioned media were prepared from control conditioned media or Dl-Fc–expressing (Dl-Fc) 293T cells as described previously (Hicks et al., 2002). Anti-Fc antibody was added for 1 h at a 200-fold dilution, then the medium was diluted twice in serum containing DME and added 1 d after transfection to the cells, for 1 h or overnight at 37°C.

Immunofluorescence and confocal microscopy
Cells were fixed with 4% PFA-PBS for 20 min, quenched for 10 min in 50 mM NH₄Cl, permeabilized for 5 min in 0.2% Triton X-100, and incubated for 1 h at RT with the anti-myc antibody. *Pepex*-incubated coverslips were permeabilized with 0.02% saponin. Cells were incubated for 45 min with the appropriate dye-labeled secondary antibodies (anti–mouse/anti–rabbit Alexa® 488, anti–mouse CY3, or anti–mouse CY5). Cell preparations were mounted in Mowiol and images were acquired using a confocal laser imaging system (LSM 510; Carl Zeiss MicroImaging, Inc.). Quantification was performed using LSM software (LSM SCS combi, 2.8 sp1; Carl Zeiss MicroImaging, Inc.) on projections of optical sections to one plane. Both nucleus and total cell were bordered in phase contrast (differential interference contrast), and the ratio of nuclear staining was determined as: (nuclear surface × mean nuclear fluorescence)/(total cell surface × mean cell fluorescence).

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