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Neuralized Promotes Basal to Apical Transcytosis of Delta in Epithelial Cells

Najate Benhra,*† Françoise Vignaux,*† Aurore Dussert,*† François Schweisguth,‡§ and Roland Le Borgne*†

*Centre National de la Recherche Scientifique Unité Mixte de Recherche 6061, Institut de Génétique et Développement de Rennes, †Université de Rennes 1, 35000 Rennes, France; and ‡Drosophila Developmental Genetics Unit, Department of Developmental Biology, Institut Pasteur, F-75015 Paris, France; §Centre National de la Recherche Scientifique Unité de Recherche Associée 2578, 75015 Paris, France

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Notch receptors mediate short-range signaling controlling many developmental decisions in metazoans. Activation of Notch requires the ubiquitin-dependent endocytosis of its ligand Delta. How ligand endocytosis in signal-sending cells regulates receptor activation in juxtaposed signal-receiving cells remains largely unknown. We show here that a pool of Delta localizes at the basolateral membrane of signal-sending sensory organ precursor cells in the dorsal thorax neuroepithelium of *Drosophila* and that Delta is endocytosed in a Neuralized-dependent manner from this basolateral membrane. This basolateral pool of Delta is segregated from Notch that accumulates apically. Using a compartimentalized antibody uptake assay, we show that murine Delta-like 1 is similarly internalized by mNeuralized2 from the basolateral membrane of polarized Madin-Darby canine kidney cells and that internalized ligands are transcytosed to the apical plasma membrane where mNotch1 accumulates. Thus, endocytosis of Delta by Neuralized relocalizes Delta from the basolateral to the apical membrane domain. We speculate that this Neuralized-dependent transcytosis regulates the signaling activity of Delta by relocalizing Delta from a membrane domain where it cannot interact with Notch to another membrane domain where it can bind and activate Notch.

INTRODUCTION

Notch is a transmembrane receptor of an evolutionarily conserved cell–cell communication pathway used by metazoan to regulate numerous developmental decisions. Notch family receptors and most Delta, Serrate, Lag-2 family ligands are type I single pass-transmembrane proteins. Ligand-induced activation of Notch triggers the cleavage of the intracellular domain of Notch, which subsequently translocates to the nucleus and functions as a transcriptional regulator (Lai, 2004; Schweisguth, 2004; Kopan and Ilagan, 2009).

The dorsal thorax of *Drosophila* pupae, or notum, consists in a single-layered neuroepithelium that produces only two types of cells, epidermal cells and sensory organ cells. Notch regulates two successive cell fate decisions during sensory organ development (Hartenstein and Posakony, 1989, 1990). Notch controls first the emergence of regularly spaced sensory organ precursor cells (SOPs or pI cells) within the pupal notum via lateral inhibition. It also regulates binary cell fate decisions in the sensory organ lineage. Each SOP divides asymmetrically to generate two distinct cells: Notch signaling is activated in one of the two SOP daughter cells that becomes pIIa and is inhibited in the other cell that becomes pIIb. The ligand Delta (Dl) and the E3 ubiquitin ligase Neuralized (Neur) are required for both Notch signaling events (Deblandre *et al*., 2001; Lai and Rubin, 2001a,b; Pavlopoulos *et al*., 2001; Le Borgne and Schweisguth, 2003; Chanet *et al*., 2009). Previous studies have shown that Neur regulates the ubiquitin-dependent and Epsin-dependent endocytosis of Dl (Lai and Rubin, 2001a; Le Borgne and Schweisguth, 2003; Overstreet *et al*., 2004; Wang and Struhl, 2005). Neur is specifically expressed in signal-sending SOPs, localizes at one pole of dividing SOPs and is specifically inherited by the signal-sending pIIb cell. Despite intensive studies, the mechanism whereby Neur regulates Dl activity is not known (Deblandre *et al*., 2001; Lai and Rubin, 2001a,b; Pavlopoulos *et al*., 2001; Le Borgne and Schweisguth, 2003; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005; Commisso and Boulianne, 2007; Skwarek *et al*., 2007; Koutelou *et al*., 2008; for reviews, see Le Borgne *et al*., 2005; Fischer *et al*., 2006; Coumailleau and Gonzalez-Gaitan, 2008). Recent studies have suggested that ligand endocytosis in pIIb cells promotes ligand targeting to an endocytic recycling compartment and that Rab11- and Sec15-dependent recycling is required for Delta signaling (Emery *et al*., 2005; Jafar-Nejad *et al*., 2005). This notion is further supported by recent studies showing that the activities of Arp2/3 complex and Wiskott-Aldrich syndrome protein are required in the signal-sending cell for the recycling of internalized Delta into apical microvilli for proper Notch activation (Rajan *et al*., 2009). These data are consistent with a model whereby inactive ligands are internalized in an ubiquitin-dependent manner and are "activated" as they traffic through the endocytic and recycling compartments (Wang and Struhl, 2005). However, the molecular nature of the activation of Dl as well as the membrane domain where recycled active Dl interacts with Notch in sensory cells remain unclear (D'Souza *et al*., 2008).

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Address correspondence to: François Schweisguth (francois.schweisguth@pasteur.fr) or Roland Le Borgne (roland.leborgne@univ-rennes1.fr).

Here, we have examined the subcellular localization of Dl and Notch at the plasma membrane of epithelial cells of the pupal notum. We find that Dl localizes at the basolateral and apical membranes whereas Notch resides primarily at the apical plasma membrane. Using a pulse chase antibody uptake assay, we find that Neur promotes the internalization of Delta from the basolateral membrane. Both segregation of Notch and Dl to distinct apical–basal membrane domains and internalization of Dl by Neur from the basolateral membrane also were observed in polarized MDCK cells. Using a compartimentalized antibody uptake assay we further show that Neur promotes the basal to apical transcytosis of Dl. We propose that endocytosis of Dl by Neur triggers the relocalization of basolateral Dl to an apical membrane domain where it can interact with Notch.

MATERIALS AND METHODS

Fly Strains

Mitotic clones for *neur*^{IF65} and for *lqf* ^{L71} were induced using the FLP-FRT technique by heat shocking first instar larvae (30 min at 37°C). The following genotypes were used: 1) *y w hsFLP/w;* FRT82B, *neur*^{IF65}/FRT82B, *Ubi-GFP(S65T)nls*. *Neur*^{IF65} is a strong hypomorphic allele (Lai and Rubin, 2001a; Le Borgne and Schweisguth, 2003). 2) *y w hsFLP/w;* FRT80B, *lqf* ^{L71}/FRT80B, *Ubi-GFP(S65T)nls*, *lqf* ^{L71} carries a nonsense W73* mutation in the ENTH domain (Overstreet *et al.*, 2003). The apGAL4 driver was used to drive the expression of UAS-*Tom* (Bardin and Schweisguth, 2006). *shi*^{ts} flies were obtained from T. Lecuit (IBDML, Marseille, France). *shi*^{ts} pupae were incubated at 33°C for 5 min and then dissected and fixed on ice.

neur$^{P72\text{-}GAL4}$ was used to drive the expression of UAS-*GFP::Sec15* (a kind gift from H. Bellen, Baylor College of Medicine, Houston, TX; Emery *et al.*, 2005; Jafar-Nejad *et al.*, 2005), and UAS-*Rab5::GFP* was expressed under the control of *Tub-GAL4* (a kind gift from S. Eaton, Max Planck Institute, Dresden, Germany).

Immunocytochemistry

Madin-Darby canine kidney (MDCK) cells (NBL-2; ATCC CCL-34) were grown in DMEM (Invitrogen, Carlsbad, CA) with 8% fetal calf serum. Cells (1.5×10^6) were transfected using Lipofectamine 2000 (Invitrogen) and placed in transwell Costar filers (0.4 μm, clear polyester membrane 3460; Corning Life Sciences, Lowell, MA). Twenty-six hours after transfection, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) with $Ca^{2+}Mg^{2+}$ (Biowhitaker, Lonza, Verviers, Belgium) for 30 min at room temperature and then permeabilized with 0.1% Triton X-100. Pupal nota were dissected from staged pupae, fixed, and stained as described in Le Borgne and Schweisguth (2003). Primary antibodies (against *Drosophila* antigens unless specified) were mouse anti-Notch Extra Cellular Domain (NECD) (1:250, C458.2H; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), mouse anti-Notch IntraCellular Domain (NICD) (1:250, C17.9C6; Developmental Studies Hybridoma Bank), rabbit anti-murine Notch1 (1:1000; a gift from C. Brou, Pasteur Institute, Paris, France), mouse anti-Dl Extra Cellular Domain (1:250, C594-9B; Developmental Studies Hybridoma Bank), Guinea Pig anti-Dl Extra Cellular Domain (1:3000; a gift from M. Muskavitch, Boston College, Chestnut Hill, MA), rabbit anti-murine Delta-like1 (Dll-1) (1:1000; a gift from F. Logeat, Pasteur Institute, Paris, France), mouse anti-Cut (1:100, 2B10; Developmental Studies Hybridoma Bank), rabbit anti-Par-6 (1:1000; a gift from J. Knoblich, Institute of Molecular Biotechnology, Vienna, Austria), rat or rabbit anti-Sanpodo (1:2000; a gift from J. Skeath, Washington University School of Medicine, St. Louis, MO), rat anti-DE-Cadherin (E-Cad) (1:100, DCAD2; Developmental Studies Hybridoma Bank), and mouse anti-vesicular stomatitis virus-glycoprotein (VSV-G) (1:100, P5D4; Sigma-Aldrich, St. Louis, MO). Cy-2, Cy3-, and Cy5-coupled secondary antibodies (1:500) were from Jackson ImmunoResearch Laboratories (West Grove, PA), and Alexa-488-coupled secondary antibodies (1:500) were from Invitrogen. Images were acquired on SP2 or SPE confocal microscopes (Leica, Wetzlar, Germany). All images were processed and assembled using ImageJ (National Institutes of Health, Bethesda, MD) and Photoshop (Adobe Systems, Mountain View, CA). Rabbit anti-*Drosophila* Rab11 (1:1000; a kind gift from Don Ready, Purdue University, West Lafayette, IN).

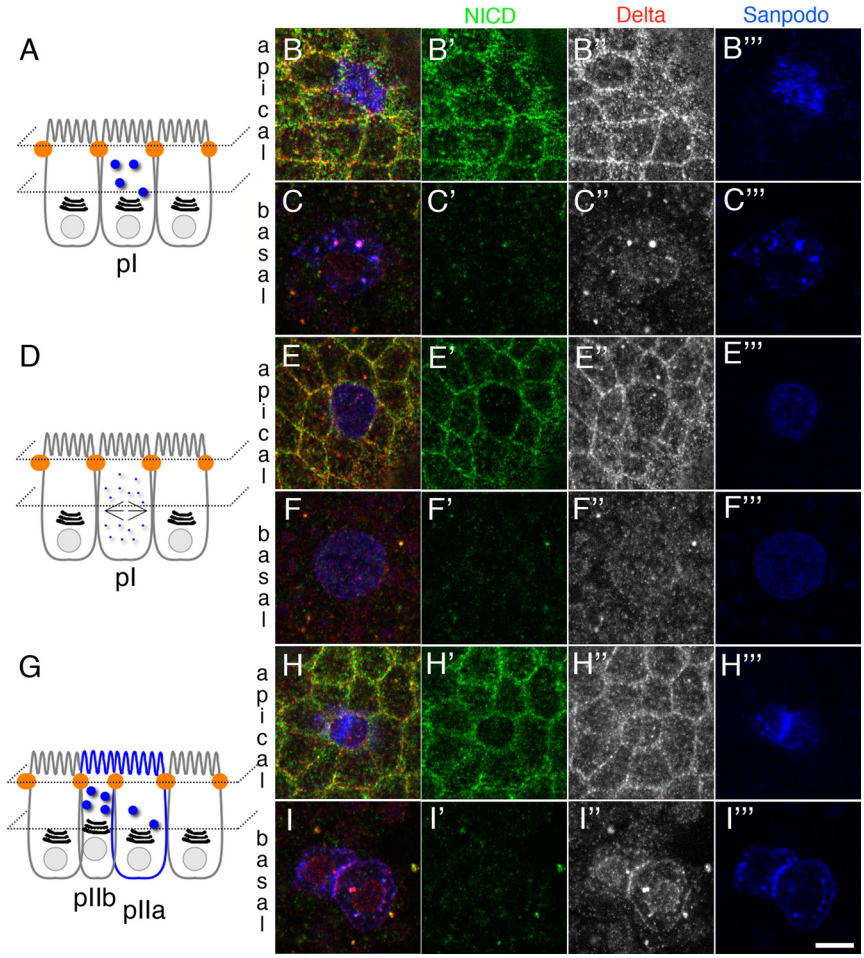

Figure 1. Apical-basal localization of Notch and Dl in the pupal notum. (A, D, and G) Schematic representation of apical to basal sections of epidermal, pI (SOP), pIIb, and pIIa cells from the pupal notum. Adherens junctions are depicted in orange and Sanpodo (Spdo) localization is schematized in blue. (B–I‴) Localization of NICD (green in B–I′), Dl (red in B–I, gray in B″–I″) and Spdo (blue in B–I, B‴–I‴) in epidermal and interphasic pI cells (B–C‴), pI at prometaphase (E–F‴), and pIIa/pIIb cells (H–I‴). B–B‴, E–E‴ and H–H‴ show apical confocal sections. C–C‴, F–F‴, and I–I‴ show basal confocal sections taken at the level of the nuclei. Sensory organ cells (pI, pIIb, and pIIa) were identified using Spdo (blue). Anterior is left. Bar, 5 μm (B–I‴).

Cell Surface Labeling and Antibody Uptake Experiments

For pulse-chase internalization experiment, pupal nota were dissected in Schneider's *Drosophila* medium (Invitrogen) containing 10% fetal calf serum (Invitrogen). After dissection, pupal nota were incubated in the presence of mouse monoclonal anti-Dl antibody (1:100) for 10 min on ice (cell surface staining). After three medium changes, nota were either directly fixed on ice (t = 0 min) or incubated with prewarmed medium at 25°C for 5 or 15 min before fixation. Localization of Dl was then revealed using secondary antibodies.

MDCK cells transfected as mentioned above with N-terminal-VSV-G–tagged version of Dll-1 (a gift from F. Logeat; Six *et al*., 2003) or VSV-G-Dll-1

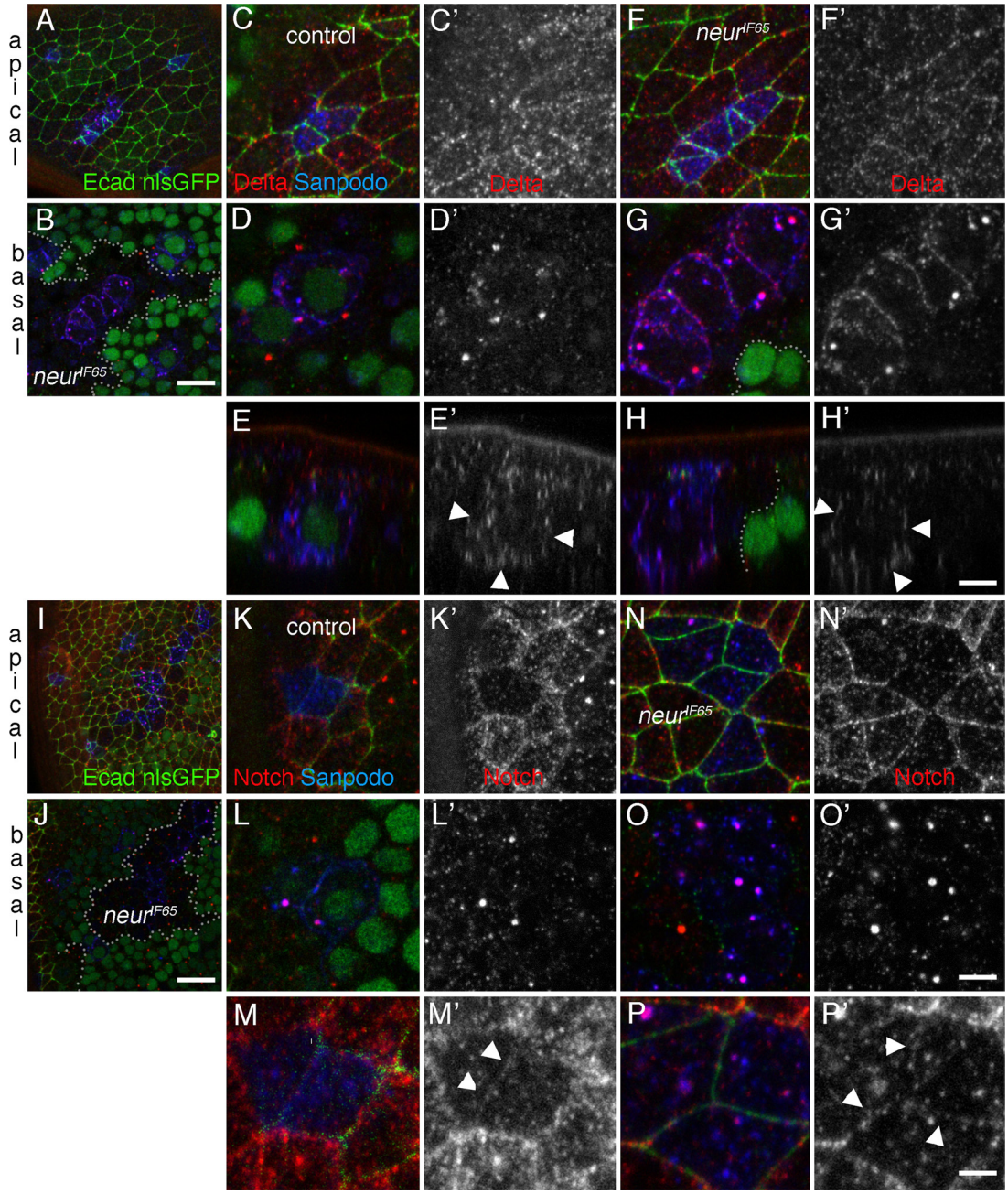

Figure 2. Dl accumulates at the basolateral plasma membrane in *neur* mutant cells. (A–H′) Localization of Dl (A–H′, red in A–E and F–H, gray in C′–E′ and F′–H′) and Notch (I–P′, red in I–M and I–P and gray in K′–M′ and N′–P′) in *neur*^{IF65} mutant (F–H′ and N–P′) versus control sensory organ cells (C–E′ and K–M′). A, C, C′, F, F′, I, K, K′, N, and N′ show confocal sections at the apical cortex taken at the level of the adherens junctions (E-Cad; green), and B, D, D′, G, G′, J, L, L′, O, and O′ show confocal sections taken at the level of the nuclei (basolateral cortex). (E, E′, H, H′) Panels are orthogonal sections of the cells shown in D, D′, G, G′, respectively. (M, M′, P, P′) Panels are higher magnifications of the apical sections of the cells shown in K, K′, N, N′, respectively. In *neur*^{IF65} mutant cells, identified by the loss of nuclear GFP (A B, and F–H; green in B, G, and H), Dl localized at the level of E-Cad and at the basolateral cortex below E-Cad. By contrast, Notch was found at the apical cortex (I, N, N′, P, and P′, red in I and N–P) and in intracellular dotted structures (red in O and O′) but not at the basolateral cortex of *neur*^{IF65} mutant cells. A, B and I, J are lower magnifications of wild-type and mutant organs illustrated in C–H′ and K–P′, respectively. Arrowheads in E′ and H′ highlight Dl localizing at the basolateral membrane. Arrowheads in M′ and P′ are pointing to Notch localizing apically at the interface between sensory organ daughter cells. Sensory organ cells were identified using Spdo (blue in A–E, F–H, I–M, and N–P). Anterior is left. Bar, 25 μm (I and J), 15 μm (A, B), 5 μm (C–H′, K–O′), and 2.5 μm (M–P′).

K17R chimera (a gift from F. Logeat; Heuss *et al.*, 2008) alone or together with a murine orthologue of Neur, Neuralized-like 2 (mNeur2) constructs (a gift from Y. Y. Kong; Song *et al.*, 2006) and seeded at high density on transwell Costar filters (Corning Life Sciences). Filter-grown MDCK cells were incubated with anti-VSV-G (1:100) diluted in tissue culture medium applied to either the bottom or the top compartment to gain access to the basolateral or apical membrane respectively for the indicated period at 4°C. After three washes, epithelial cells were fixed and stained with goat anti-mouse secondary antibodies to monitor cell surface staining. Alternatively, for transcytosis experiment, MDCK cells prepared as described above were incubated with anti-VSV-G antibody (1:100) in the basal compartment and anti-mouse coupled to Cy3 (1:500) in the apical compartment for 120 min in tissue culture medium at 37°C. After three washes, cells were fixed and stained for Dll-1 and actin using Atto 647N-phalloidin (Sigma-Aldrich).

RESULTS

Basolateral Internalization of Dl in Drosophila Sensory Organ Cells

To gain insight into how Neur regulates the signaling activity of Dl, we first examined the subcellular distribution of Delta in SOPs and their progeny cells. As described previously, Dl was detected into intracellular endocytic structures in SOPs, pIIa, and pIIb (Le Borgne and Schweisguth, 2003). Dl also was detected, albeit at a lower level, into intracellular dots in surrounding epidermal cells. We find here that Dl can also be detected at the apical plasma membrane where it colocalized with Notch, E-Cad, and Par-6 (Figure 1, Supplemental Figure S1, and Supplemental Movies 1–3). Our analysis of the subcellular localization of Dl further revealed that a low level of Dl can be detected along the basolateral membrane of pI (Figure 1, F and F″) and pIIa cell as well as at the pIIa/pIIb interface (Figure 1, I and I″; also see Parks *et al.*, 1997). Basolateral localization of Dl in these cells was confirmed by cell surface anti-Dl staining (Supplemental Figure S1, E–E″). In contrast, Dl was not consistently detected along the basolateral membrane of epidermal cells (Figure 1, C″, F″, and I″), possibly due to the low level of Dl expression in these cells (see below). These data indicate that a low amount of Dl is present at the basolateral membrane of sensory organ cells.

To further examine the distribution of Dl at the cell surface, we genetically blocked the endocytosis of Dl by using a thermosensitive allele of the fly orthologue of Dynamin, *shibire* (*shi*^{ts}) (Seugnet *et al.*, 1997); a mutant allele of the fly orthologue of Epsin, *liquid facets* (*lqf*) (Overstreet *et al.*, 2004; Wang and Struhl, 2004); and a strong hypomorphic allele of *neur* (Lai and Rubin, 2001a; Le Borgne and Schweisguth, 2003). In all three

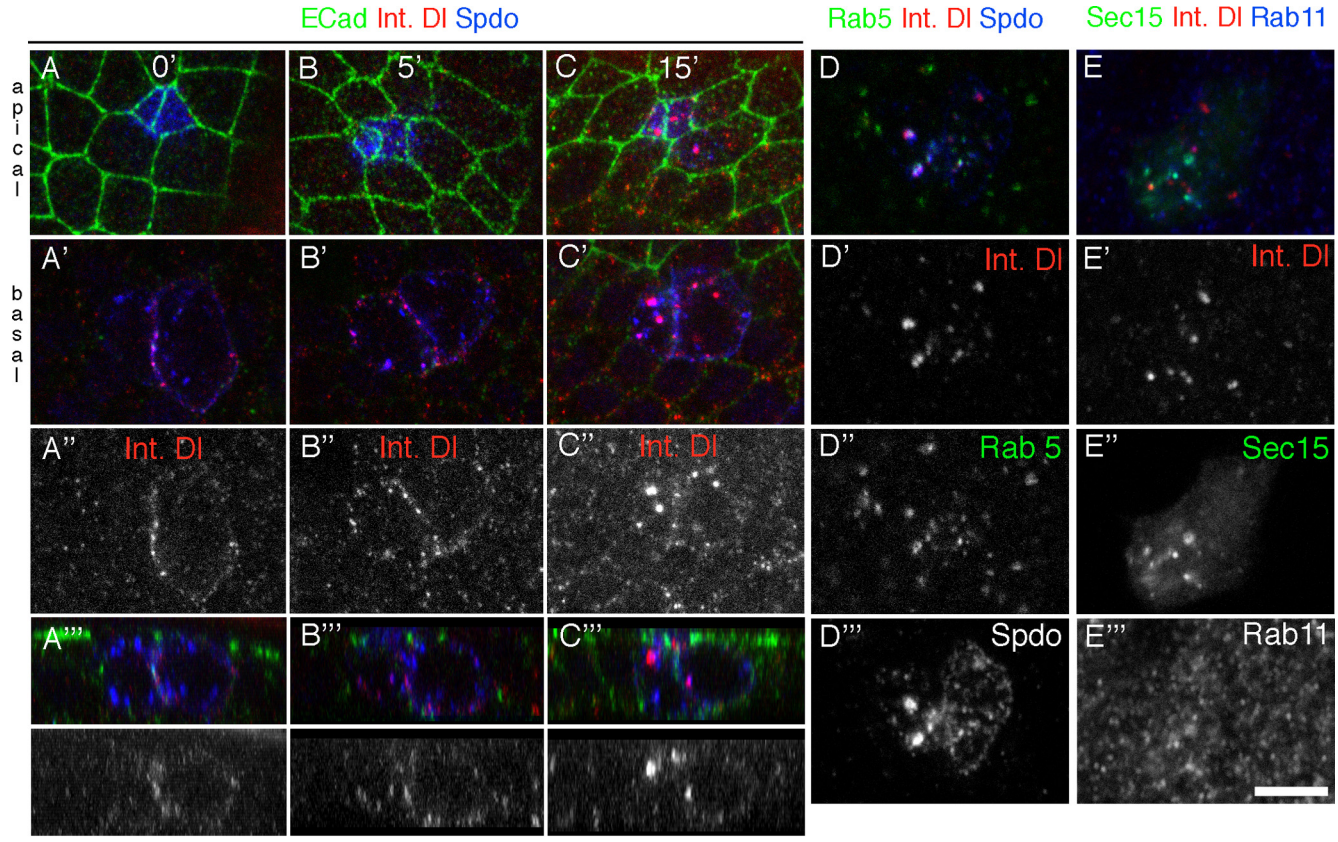

Figure 3. Dl is internalized from the basolateral membrane in the pupal notum. (A–C‴) Pulse-chase labeling experiments to monitor Dl internalization in living pupae. Dl internalization (red in A–C‴ and separate channel in A″–C″) at the pIIa/pIIb cell stage was examined at three time points (0, 5, and 15 min). A–C show confocal sections at the apical cortex taken at the level of the adherens junctions labeled with E-Cad (green), whereas panels A′–C′ show confocal sections taken at the level of the nuclei (basolateral cortex). Sensory organ cells were identified using Spdo (blue in A–C, A‴–C‴, and D). At 0 min, Dl was detected at the basolateral plasma membrane of the pIIa cell as well as at the pIIa/pIIb interface, below E-Cad (green). After 5 min of internalization, clustered Dl still present at the plasma membrane and/or small endocytic vesicles containing Dl were detected in both pIIa and pIIb cells. At 15 min, Dl was predominantly detected in vesicular compartments within the pIIa and pIIb cells. Most of these vesicles were located in vicinity to E-Cad-positive junctions of the pIIb cell. A‴–C‴ show confocal sections along the *z*-axis of images A–C′, respectively. D–E‴, distribution of Dl 15 min after internalization (D, D′, E, E′; red in D, E) relative to Rab5-GFP (D, D′; green in D), Sec15-GFP (E, E″; green in E) and Rab11 (E, E‴; blue in E). Sensory organ cells were identified using Spdo (blue in A–C′, A‴, C‴–D and E). Anterior is left. Bars, 10 μm.

mutant contexts, Dl accumulates along the basolateral membrane of sensory cells (Figure 2, G–H′ and Supplemental Figure S2). In particular, low amounts of Dl are detected at the level or above the adherens junctions marked by E-Cad in *neur* mutant cells (Figure 2, A, B, F, F′, H, and H′), suggesting that the activity of *neur* is required for the apical accumulation of Dl. In addition, Dl could also be detected along the basolateral membrane of epidermal cells in *laf* mutant cells (Supplemental Figure S2, C–C″). The observation that Dl accumulates in this membrane domain upon inhibition of its endocytosis suggests that a low level of Dl is present along the basolateral membrane of epidermal cells. Together, our data suggest that a small basolateral pool of Dl exists in both epidermal and sensory cells in this epithelium and that Dl is internalized from this basolateral domain in a Dynamin- and Epsin-dependent manner (Wang and Struhl, 2004, 2005).

To further investigate Dl trafficking, we performed pulse-chase labeling experiments to monitor Dl internalization in living pupae. Dl internalization was examined at three time points (Figure 3). At 0 min, Dl was detected at the basal surface of the pIIa cell and at the pIIa/IIb interface, below the adherens junctions. No staining was detected in the apical part of the pIIa and pIIb cells. At 5 min after internalization, basal dots were detected in both pIIa and pIIb cells. These dots may correspond to clustered Dl at the cell surface and/or to internalized Dl. At 15 min, Dl was mostly found in vesicles within the pIIa and pIIb cells, which colocalized with Sanpodo (Spdo), a membrane protein required for Notch signaling in asymmetrically dividing cells (O'Connor-Giles and Skeath, 2003). Most of these vesicles were located apically within the pIIb cell. The bulk of internalized Dl reaches early endosomes marked with Rab5 within 15 min (Figure 3, D–D‴; 8.7 ± 3.2 Dl-positive structures colocalized with Rab5 per pIIa/pIIb cell pair, n = 12; these doubly stained structures represent 76% of all Dl-positive structures). A smaller fraction of the Dl-positive structures colocalized with Rab11, a recycling endosome marker, and Sec15 that marks secretory vesicles, respectively, and is required for Notch activation in pIIa (Jafar-Nejad *et al*., 2005): 1.6 ± 0.6 Dl-positive structures colocalized with both Rab11 and Sec15 per pIIa/pIIb cell pair (n = 15; these triply stained structures represent 11% of all Dl-positive structures; Figure 3, E–E‴). These data suggest that Dl is internalized from the basolateral membrane targeted to Rab5-positive endosomes and that a fraction of internalized Dl is recycled back to the cell surface via Rab11- and Sec15-positive vesicles.

Segregation of Dl and Notch in Drosophila Sensory Organ Cells

In contrast with Dl, Notch was not detected along the basolateral membrane at steady state (Figure 1, C, C′, F, F′, I, and I′) nor upon cell surface staining using the anti-NECD antibody (data not shown). Instead, Notch colocalized with E-Cad and Par-6 at the apical cortex (Supplemental Figure S3 and Supplemental Movies 1–3) (also see Sasaki *et al*., 2007). A low level of Notch also was detected at the interface between pIIa/pII, colocalizing with E-Cad (Figure 2, K, K′, M, and M′; also see Supplemental Figure S3C′; Notch levels in SOPs and progeny cells seemed to be low compared with neighboring epidermal cells). These data indicate that the main pools of Dl and Notch localize to distinct membrane domains at steady state.

Loss of *neur* activity had no effect on the localization of Notch that remained localized at the apical cortex (Figure 2, N, N′, P, and P′) and that, in contrast with Dl, did not accumulate along the basolateral membrane (Figure 2, O and O′). Similar results were obtained in cells overexpressing Twin of m4 (Tom), an inhibitor of Neur-mediated endocytosis of Dl (Bardin and Schweisguth, 2006; De Renzis *et al*., 2006; Supplemental Figure S4). These observations indicate that Notch and Dl localized in two distinct membrane domains in the absence of *neur* activity. Thus, physical segregation correlated with defective Notch activation. These data therefore raised the hypothesis that Neur promotes the internalization of Dl from the basolateral membrane to promote its accumulation to the apical membrane where it can interact with Notch.

Basal-to-Apical Transcytosis of Delta in MDCK Cells

To test the hypothesis that Neur regulates the transcytosis of Dl from the basolateral to the apical membrane, we developed a transcytosis cell-based assay in polarized MDCK cells in which both apical and basal membranes domains are independently accessible for experimentation. The localization of Notch and Dl was first analyzed on filter-grown polarized MDCK cells. Murine Notch1 or a VSV-G tagged version of Dll-1 (Six *et al*., 2003) were transiently expressed in MDCK cells that were allowed to polarize on filters.

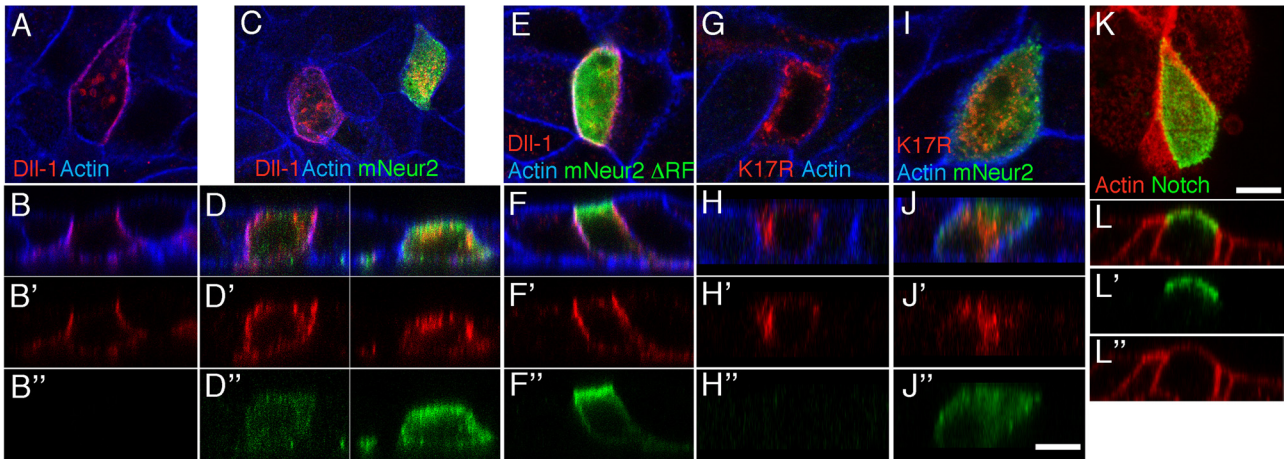

Figure 4. Apical-basal localization of Notch and Dl in polarized MDCK cells. Distribution of a VSV-G–tagged version of wild-type murine Dll-1 (A–F″, red) or K17R Dll11 a mutant version of Dll1 (G–J″) in polarized MDCK cells (phalloidin in blue) in the absence of mNeur2 (A–B″) or the presence of mNeur2 (C–D″, I–J″) or mNeur2-ΔRF (E–F″) (in C–J″; mNeur2 was detected using the HA tag in green). B–B″, D–D″, F–F″, H–H″, and J–J″ show confocal Z-sections of images shown in A, C, E, G, and I, respectively. (K–L″) Distribution of murine Notch1 (green) in polarized MDCK cells (phalloidin in red). Apical is up. Bar, 10 μm (A–H″, J–J″).

Notch1 and Dll1 are expressed in separate populations of cells such as they do not interact in *cis* in this experimental setup. Dll-1 localized at the basolateral membrane and in intracellular dotted structures (Figure 4, A–B″). In contrast, Notch1 accumulated at the apical plasma membrane (Figure 4, K–L″). This indicated that ectopically expressed Notch1 and Dll-1 segregate in two distinct membrane domains along the apical–basal axis in MDCK cells. This segregation is similar to the one observed in sensory organ cells upon inhibition of Dl internalization (Figure 2).

To recapitulate the endocytosis of Dll-1 in MDCK cells, we expressed mNeur2 in these cells. Expression of a hemagglutinin (HA)-tagged version of mNeur 2 (Song *et al*., 2006) resulted in a loss of Dll-1 from the basolateral membrane. This loss correlated with the accumulation of Dll-1 into dotted intracellular structures located in the apical portion of polarized MDCK cells (Figure 4, C–D″; compare Figure 4, A–B″ with C–D″; and Figure 6A). A similar effect was seen with Neuralized-like1 (mNeur1): Dll-1 was similarly redistributed upon expression of a GFP-tagged version of mNeur1 (data not shown). Anti-VSV-G uptake assays indicated that this effect of mNeur1 and mNeur2 resulted from the internalization of Dll-1 from the basolateral membrane (data not shown). In addition, this change in Dl localization required the catalytic RING domain activity of mNeur2 (Figures 4, E–F″, and 6A). Finally, the localization of K17R, a version of Dll1 that cannot be ubiquitinated, i.e., with all 17 lysine residues of its intracellular tail mutated into arginine residues (Heuss *et al*., 2008), is insensitive to mNeur2 expression (Figures 4, G–J″ [note that the bulk of K17R is intracellular], and 6A). Together, these data indicate that mNeur1 and mNeur2 promote the endocytosis of Dll-1 from the basolateral domain of MDCK cells.

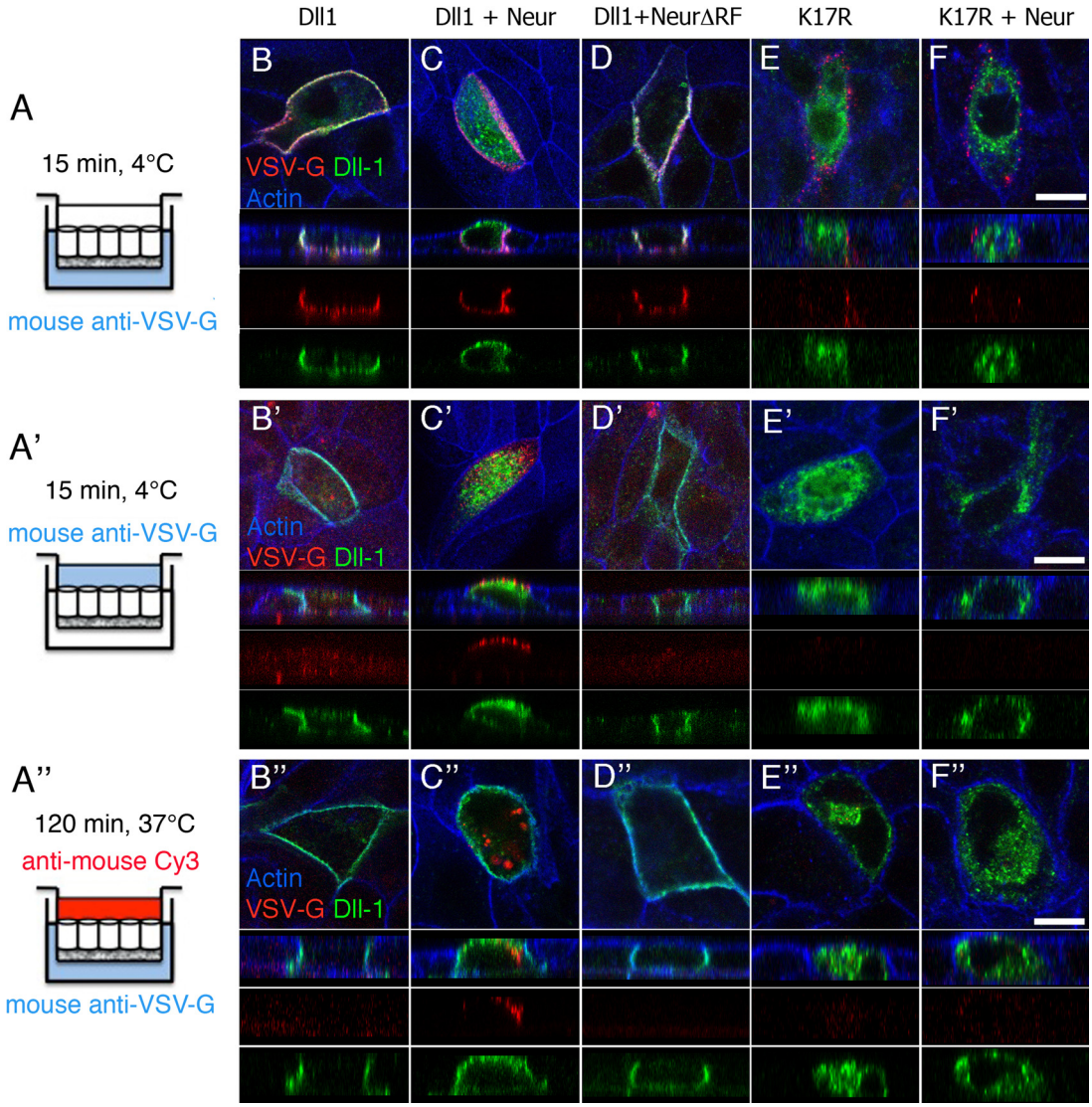

Figure 5. Neur promotes basal to apical transcytosis of Dl in MDCK cells. (A–A″) Schematic representation of the cell surface accessibility assay (A and A′) and transcytosis assay (A″). (B–F″) Polarized MDCK cells were transfected with VSV-G–tagged mouse Dll-1 (B–D″) or K17R Dll1 (E–F″) alone or together with wild type mNeur2 (C–C″, F–F″), or mNeur2-ΔRF (D–D″). They were then incubated with anti-VSV-G antibodies in the basal (A, B–F) or apical compartment (A′, B′–F′) at 4°C for 15 min. Alternatively, polarized MDCK cells were incubated with anti-VSG-G and anti-mouse Cy3 in the basal and apical compartment respectively for 120 min at 37°C (A″, B″–F″). Dll-1 was detected using rabbit anti-Dll-1 (green; phalloidin in blue). Orthogonal confocal sections taken along the *z*-axis are shown below images B–F″. The faint diffuse red staining seen in B″, D″, E″, and F″ corresponds to background (laser intensity and detector sensitivity of the confocal were increased compared with C″). Bar, 10 μm (B–F′) and 5 μm (B″–F″).

To then test whether Dll-1 was transcytosed from the basolateral domain to the apical domain of polarized MDCK cells upon mNeur2 expression, we made use of the extracellular VSV-G tag of Dll-1 (Figure 5, A–A″). Polarized MDCK cells that had been transfected with Dll-1, with or without mNeur2, were incubated for 15 min at 4°C with anti-VSV-G added in the basolateral (Figure 5A) or the apical (Figure 5A′) side of the cell monolayer. In the absence of mNeur2, anti-VSV-G cell surface staining detected Dll-1 only in the basolateral compartment (Figure 5B). Indeed, Dll-1 was not detected at the apical surface using anti-VSV-G (Figure 5B′). In contrast, cell surface labeling with anti-VSV-G showed that Dll-1 localized at the apical plasma membrane in the presence of mNeur2 (Figure 5, C–C′). These data indicate that mNeur2 regulated the distribution of Dll-1. This activity of mNeur2 required the catalytic domain of mNeur2 (Figure 5, D and D′) and the presence of lysine residues in the intracellular tail of Dll-1 (Figures 5, E–F′, and 6B). This redistribution of Dll-1 by mNeur2 could result from two distinct mechanisms. First, newly synthesized Dll-1 may be directly targeted to the apical plasma membrane in the presence of mNeur2. Alternatively, Dll-1 may be first transported to the basolateral membrane and then rerouted toward the apical plasma membrane by transcytosis.

To test this second possibility, anti-VSV-G was added on the basolateral side and fluorescent anti-mouse immunoglobulin (Ig)G was added on the apical side (Figure 5A″). We observed that anti-mouse IgG Cy3 was efficiently internalized from the apical plasma upon cotransfection of mNeur2 and Dll-1 (Figure 5C″). This indicates that anti-VSV-G antibody bound at the basolateral plasma membrane has reached the apical plasma membrane to interact with anti-mouse Cy3. Apical anti-mouse Cy3 uptake began after a 60- to 90-min latency (data not shown), a kinetic comparable with the basal-to-apical transcytosis of pIgA receptor (Apodaca *et al*., 1994). Together, these data indicate that mNeur2 promotes the transcytosis of Dll-1 from the basolateral membrane to the apical plasma membrane in polarized MDCK cells. This transcytosis of Dll-1 required the E3 ubiquitin activity of mNeur2 because deletion of the RING finger abolished this activity (Figure 5D″). Consistent with this, the K17R mutant form of Dll-1 is unable to transcytose even in the presence of mNeur2, indicating that transcytosis required the lysine residues from the intracellular tail of Dll1 (Figures 5, E″ and F″, 6C). We therefore conclude that ubiquitination and internalization of basolateral Dll-1 by mNeur 2 are required for its relocalization to the apical membrane domain. We therefore suggest that Neur-dependent transcytosis of Dl1-1 may overcome the segregation of Notch1 and Dll-1 into distinct membrane domains in polarized epithelial cells.

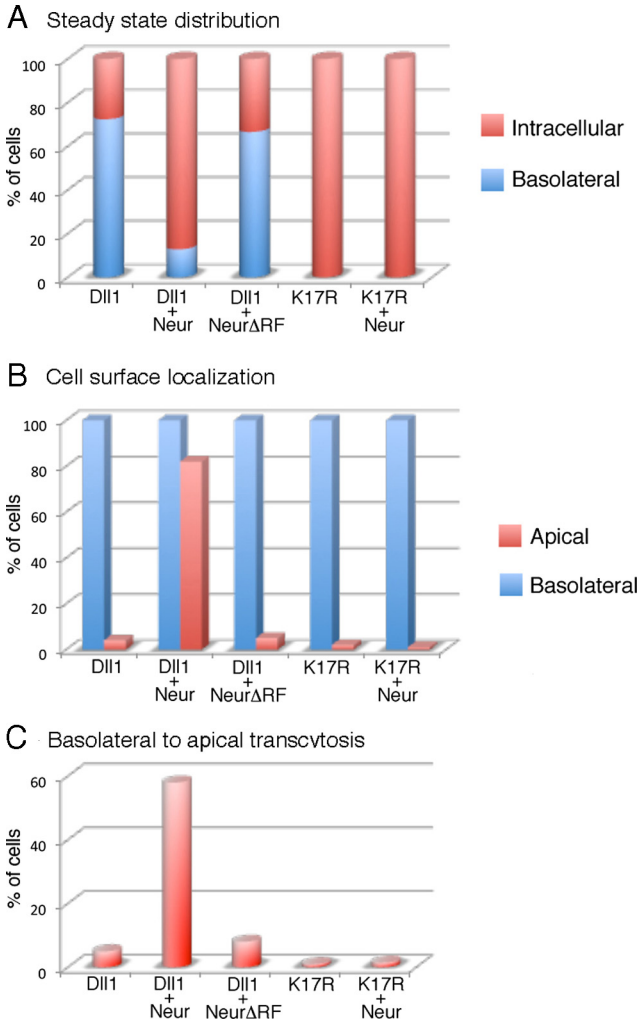

Figure 6. Quantitation of Dll-1 localization and transcytosis in polarized MDCK cells. (A) Steady-state distributions of Dll-1 (n = 48), Dll-1 + mNeur2 (n = 47), Dll-1+mNeur2-ΔRF (n = 47), K17R Dll-1 (K17R; n = 45), and K17R Dll1 + mNeur2 (n = 50). In this semiquantitative analysis of confocal images, basolateral staining refers to cells in which Dll1 was predominantly observed at the basolateral membrane (see Figure 4, A, B and E, F). In cells where Dll1 predominantly localized in intracellular compartment (see Figure 4, C, D and G–J′), staining was classified as intracellular. (B) Quantitation of apical and basolateral cell surface immunostaining of Dll-1 (n = 49 and n = 46, respectively), Dll-1 + mNeur2 (n = 68 and n = 60, respectively), Dll-1 + mNeur2-ΔRF (n = 54 and n = 48, respectively), K17R Dll1(n = 49 and n = 56, respectively), and K17R Dll1 + mNeur2 (n = 45 and n = 58, respectively) illustrated in Figure 5, B–F and B–F′. Numbers into brackets refer to the number of cells analyzed for apical and basolateral cell surface staining, respectively. Apical versus basolateral cell surface staining was done on separate transwell filters. In this quantitation the occurrence of the immunostaining (binary: yes or no) at the apical or basolateral membrane was assessed. All transfected cells exhibited a basolateral staining, including cells transfected with K17R Dll1 mutant that showed low plasma membrane staining. This apparent discrepancy is likely due to the fact that the anti-VSVG antibody is more sensitive than anti-Dll1. By contrast, apical staining was almost exclusively detected upon coexpression of mNeur2 and Dll-1 (80% of the cells expressing Dll1 showed a VSG-G staining at the apical surface). (C) Quantitation of basal to apical transcytosis of Dll-1 (n = 49), Dll-1 + mNeur2 (n = 90), Dll-1 + mNeur2-ΔRF (n = 54), K17R Dll1 (n = 56), and K17R Dll1 + mNeur2 (n = 58) as shown in Figure 5, A″–F″. In all cases, quantitation was performed on results obtained from at least three independent experiments. Experiments performed in parallel indicated that mNeur2 and Dll-1 constructs are typically coexpressed in 90% of transfected cells.

DISCUSSION

A conserved function of Neur is to mediate the internalization of Dl. Our analysis of Dl internalization in two types of epithelial cells, the precursor cells of adult sensory organs in *Drosophila* and polarized MDCK cells, indicated that Dl is, at least in part, internalized from the basolateral membrane. In addition, this Neur-dependent internalization is followed by the transcytosis of Dl from the basolateral membrane to the apical membrane in MDCK cells. We discuss below the potential implications of these observations.

Segregation of Notch and Delta as a Control Mechanism

We have shown that Neur promotes the internalization of Delta from the basolateral membrane domain that is largely

devoid of Notch. After internalization, Dl can be retargeted to the apical membrane where Notch accumulates. In agreement with our findings, the Arp2/3 complex and WASp have recently been shown to be required for apical trafficking of internalized/recycled Delta into apical microvilli for proper Notch activation (Rajan *et al*., 2009). Thus, Neur may counteract the segregation of Dl and Notch into distinct membrane domains in polarized epithelial cells. Segregation of receptors and ligands to distinct membrane domains has been proposed previously as a control mechanism, in particular for the regulation of the ErbB2/ErbB3 receptor tyrosine kinase by its ligand ErbB1: ErbB3 is normally sequestered within the lateral domain, away from its ErbB3 coreceptor that localizes at the apical domain and from the luminal ligand ErbB1. On loss of tight junction barrier, the heterodimeric ErbB2/ErbB3 receptor can form apically and respond to ErbB1, thereby acting as a sensor for epithelial damage (Carraway and Carraway, 2007). Thus, in analogy with the regulation of the ErbB2/ErbB3 receptor tyrosine kinase by its ligand ErbB1, we propose that transcytosis of Dl may regulate Notch receptor activation.

Transcytosis as an Activation Mechanism

Two models have been proposed to explain the role of ubiquitin-dependent endocytosis of Dl in Notch receptor activation (D'Souza *et al*., 2008). First, the "pulling" model proposes that internalization of Dl bound to its receptor exerts pulling forces on N (Klueg and Muskavitch, 1999; Nichols *et al*., 2007) and induces a conformational change exposing the S2 cleavage site to metalloproteases (Gordon *et al*., 2007; Nichols *et al*., 2007). In this model, endocytosis is temporally and spatially linked to Notch receptor activation. Alternatively, the activation/recycling model proposes that internalization is required to promote the formation of *active* ligands that are recycled back to the cell surface to activate Notch (Wang and Struhl, 2004, 2005). This model suggests that endocytosis is only indirectly linked to Notch activation and that endocytosis of Dl can be temporally and/or spatially uncoupled from activation of Notch. These two models are not mutually exclusive and endocytosis/recycling may precede pulling.

Our data indicating that Neur promotes the transcytosis of Dl from the basolateral to the apical membrane are consistent with the activation/recycling model. Accordingly, basolateral Dl would be *inactive*, presumably because this pool of Dl would not be able to interact with Notch, whereas apical Dl would be *active* because it could now interact with Notch present at the surface of neighboring cells. Thus, transcytosis would be part of the *activation* mechanism. Importantly, our data do not, however, contradict nor argue against the pulling model. Indeed, we observed that Dl is internalized from the apical plasma membrane in the presence of Neur. Although we cannot test for its Neur dependence, the apical internalization of Dl could contribute to generate the proposed pulling forces. Such a scenario is consistent with the proposed existence of two rounds of ligand endocytosis: a first round would serve to activate the ligand, whereas a second round would exert pulling forces on the receptor (Heuss *et al*., 2008). Future studies will investigate whether and how transcytosis of Dl is causally linked to Notch receptor activation.

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