Numb regulates the balance between Notch recycling and late-endosome targeting in Drosophila neural progenitor cells

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INTRODUCTION

The Notch signaling pathway plays essential roles in both animal development and human disease. Regulation of Notch receptor levels in membrane compartments has been shown to affect signaling in a variety of contexts. Here we used steady-state and pulse-labeling techniques to follow Notch receptors in sensory organ precursor cells in Drosophila. We find that the endosomal adaptor protein Numb regulates levels of Notch receptor trafficking to Rab7-labeled late endosomes but not early endosomes. Using an assay we developed that labels different pools of Notch receptors as they move through the endocytic system, we show that Numb specifically suppresses a recycled Notch receptor subpopulation and that excess Notch signaling in numb mutants requires the recycling endosome GTPase Rab11 activity. Our data therefore suggest that Numb controls the balance between Notch receptor recycling and receptor targeting to late endosomes to regulate signaling output after asymmetric cell division in Drosophila neural progenitors.

ABSTRACT The Notch signaling pathway plays essential roles in both animal development and human disease. Regulation of Notch receptor levels in membrane compartments has been shown to affect signaling in a variety of contexts. Here we used steady-state and pulse-labeling techniques to follow Notch receptors in sensory organ precursor cells in Drosophila. We find that the endosomal adaptor protein Numb regulates levels of Notch receptor trafficking to Rab7-labeled late endosomes but not early endosomes. Using an assay we developed that labels different pools of Notch receptors as they move through the endocytic system, we show that Numb specifically suppresses a recycled Notch receptor subpopulation and that excess Notch signaling in numb mutants requires the recycling endosome GTPase Rab11 activity. Our data therefore suggest that Numb controls the balance between Notch receptor recycling and receptor targeting to late endosomes to regulate signaling output after asymmetric cell division in Drosophila neural progenitors.

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Abbreviations used: L(2)gl, Lethal (2) giant larvae; SOP, sensory organ precursor.

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The Delta ligand is ubiquitinylated by the conserved ubiquitin ligase Neuralized, endocytosed, and recycled through the Rab11 endosome and the Sec15–exocyst complex back to the apical region of the pIIb cell to activate Notch signaling in the pIIa cell (Lai et al., 2005; Benhra et al., 1994). After division of the SOP, Numb is exclusively inherited by one of the two daughter cells (the anterior pIIb cell) and excluded from the other cell (the posterior pIIa). Numb acts as a cell-autonomous inhibitor of Notch signaling in the pIIb cell, whereas in the pIIa cell, Notch signaling is required for proper cell fate (Uemura et al., 1989; Rhyu et al., 1994; Frise et al., 1996).

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microvillar membrane projections required for robust Notch activation in the pilla cell (Ben-Yaacov et al., 2001; Rajan et al., 2009).

Control of membrane trafficking is not limited to the Delta ligand in the pilla cell. Sanpodo, a four-pass transmembrane protein that interacts with Notch, promotes Notch receptor endocytosis (O’Connor-Giles and Skeath, 2003; Couturier et al., 2012; Upadhyay et al., 2013), whereas Numb inhibits membrane targeting of Notch and Sanpodo in the pilla cell (Couturier et al., 2012, 2013; Cotton et al., 2013). Notch–Sanpodo oligomers appear to be recycled in SOP cells (Le Bras et al., 2012; Cotton et al., 2013; Couturier et al., 2013, 2014; Upadhyay et al., 2013), but it remains unclear how Numb regulates membrane levels of Notch to modulate signaling in this system.

In this study, we sought to understand how vesicle trafficking controls targeting of Notch receptor pools in SOP cells during Notch-dependent cell fate decisions. We developed a technique to distinguish different populations of receptors as they trafficked from the plasma membrane to internal compartments or were recycled. Our observations confirm that Numb plays an important role in restricting recycling of a Notch receptor population, as opposed to promoting Notch endocytosis from the plasma membrane. Of importance, we find that Numb functions to reroute the receptor preferentially to Rab7-positive late endosomes in pilla cells. Our analysis further shows that Notch recycling is unaffected in Sec15-exocyst and WASp mutants but is regulated by conserved tumor suppressor and WD repeat–containing protein Lethal (2) giant larva (L(2)gl).

RESULTS
Numb regulates Notch trafficking to late endosomes
Notch receptors colocalize with markers of early and late endocytic compartments in pilla/pllb cells (Hutterer and Knoblich, 2005; Couturier et al., 2014). Previous studies proposed a role for Numb in regulating, either directly or indirectly, Notch endosomal targeting to limit Notch signaling (McGill et al., 2009; Couturier et al., 2013). We therefore quantified the number of Notch puncta and the colocalization of the Notch extracellular domain (NECD) with the markers of early (Rab5) and late endosomes (Rab7) in wild-type Numb-negative pilla and Numb-positive pllb cells in pulse-chase and steady-state experiments. In pulse-chase experiments in pllb and pilla cells, colocalization between Rab5 and Notch peaked between 20 and 30 min, whereas Notch colocalization with Rab7 peaked at 30 min.
We observed no difference in Notch colocalization in either Rab5- or Rab7-positive endosomes between the pIIa and pIIb cells in these flux assays, suggesting that the presence of Numb may not influence the rate of receptor trafficking. In steady-state labeling experiments, we saw no difference in pIIa and pIIb cell NECD colocalization with Rab5–green fluorescent protein (GFP)–labeled early endosomes in wild-type or numb mutants (Figure 1, B–D), confirming that Numb is unlikely to influence Notch trafficking through early endosomes (Couturier et al., 2013). However, we did note a significantly higher level of NECD puncta in pIIb cells than in pIIa cells in wild-type but not numb mutant cells (Figure 1D). In contrast to the results with Rab5, we observed a significantly higher level of NECD colocalization with Rab7 puncta in Numb-positive pIIb than in Numb-negative pIIa cells (Figure 2, A, C, E, and F). Notch-Rab7 colocalization in pIIa/pIIb cells decreased in numb mutants (Figure 2, B and G) and increased in cells overexpressing Numb (Figure 2, D and H); overexpression of Numb-myc results in loss of hair and socket cells in adult flies, resulting in a virtually bald thorax; unpublished data). The asymmetry in NECD levels and colocalization of NECD and Rab7 in wild-type pIIa and pIIb cells is numb dependent: in numb mutant and Numb-overexpression samples, the asymmetry is abolished (Figures 1D and 2, E–H). Furthermore, in numb mutants, both pIIa and pIIb cells had total NECD and NECD-Rab7 colocalization levels comparable to those of wild-type pIIa cells (Figures 1D and 2, E–G). In contrast, the Notch–Rab7 colocalization in both cells was comparable to the wild-type pIIb cell in Numb overexpression (Figure 2H). Our findings demonstrate that Notch levels in late endosomes at steady state are Numb dependent and higher in wild-type pIIb than in pIIa.

An assay to distinguish recycled from static membrane Notch receptors

In Drosophila, recent studies in SOP cells showed that Numb inhibits Notch/Sanpodo oligomer membrane targeting (Benhra et al., 2011; Couturier et al., 2012, 2013; Cotton et al., 2013; Upadhyay et al., 2013). From these studies, a model has emerged in which Numb has a conserved function in blocking endocytic recycling of Notch, thereby acting as a Notch signaling inhibitor (Couturier et al., 2013). However, testing this model has been challenging, as dynamic methods of following different populations of receptors have only recently been applied to understanding how Notch receptors are regulated (Courmaileau et al., 2009; Couturier et al., 2014). In this study, we developed an assay, adapted from a technique used previously for Sanpodo (Cotton et al., 2013), to specifically visualize the population of Notch receptors endocytosed and recycled back to the plasma membrane. We followed a multistep pulse-chase procedure (described in detail in Materials and Methods) in live tissue using an antibody that binds to the NECD, followed by a first and second secondary antibody, each coupled to a different fluorophore (FSA and SSA, respectively; Figure 3A). This approach has the potential to identify three distinct populations of Notch receptors: 1) a static pool of receptors (labeled by both FSA and SSA), which remains at the cell surface throughout the double pulse-labeling assay, 2) the recycled

(D) Overexpression of Numb abolished this bias, causing both cells to possess pIIb levels of colocalization (19 cell pairs, three flies). Wild-type (E, F) and numb2 (G, H) clonal pIIa/pIIb cells were quantified as average numbers of single (NECD) or colocalized (NECD + Rab7) puncta per cell. Genotypes: Ubx-flp; ftr40Gal80; neur-Gal4, Rab5-GFP/TM6y’ ckrfr40/Cyo (A), ckrfr40br’/Cyo (B), neur-Gal4 UAS-Rab7GFP (C), and UAS-numb-myc (D).
FIGURE 3: Notch recycling assay confirms Numb’s inhibition of Notch recycling. (A) Schematic of Notch recycling assay. Living explants are incubated with NECD primary antibody (black circles) for 10 min, allowing for internalization of bound receptors. After NECD antibody is removed, the green-labeled FSA is added for 10 min at 4°C to allow antibody binding, but receptor trafficking is inhibited. FSA is removed, and the sample is raised to room temperature to allow for Notch receptor trafficking to resume. Samples are then fixed and stained with the red-labeled SSA. Samples that contain high levels of recycled Notch are those with prominent SSA signal that is not also represented by a similar FSA signal. Samples with high levels of static Notch are those with colocalized SSA and FSA signals. (B–D) Recycling assay image series through multiple z-planes. (B) Wild-type clones marked with actin-GFP showed low accumulation of FSA and SSA antibodies (29 cell pairs, 10 flies). (C) Numb clones showed higher accumulation of SSA and low accumulation of FSA (37 cell pairs, 13 flies). (D, E) Quantifications of SSA and FSA intensity at the pilia/pllb cell interface region for wild-type and numb mutant clones (normalized intensity on the y-axis, samples on the y-axis). Horizontal lines indicate averages (*p < 0.001). (F) Wild-type and (G) numb mutant quantifications of FSA, SSA, and actin-GFP (blue, red, and green, respectively) show intensity levels in a representative sample. Borders of pilia and pllb cells are shown with yellow and blue rectangles, respectively. Genotypes: UAS-ActinGFP, neur-Gal4 (A–C), Ubx-flp; ckfrt40A; neur-Gal4 (B), and Ubx-flp; numb2frt40A; neur-Gal4 (C).

population of the receptor (labeled by SSA alone), which is internalized in the first step of the assay and subsequently returns to the plasma membrane, and 3) an internalized pool of receptors (labeled by FSA alone), which is endocytosed during the assay but remains in intracellular compartments (Figure 3A). In contrast, receptors sequestered in internal membrane compartments during the primary
Rab5 activity is required to reduce membrane Notch levels to control Notch signaling

To further investigate the regulation of membrane Notch levels using our trafficking assay, we expressed a dominant-negative form of Rab5 (Rab5DN) in pIIa/pIIb cells. Rab5DN is a mutant form that locks the Rab5 GTPase in the inactive state, preventing the fusion of endocytic vesicles (Stenmark et al., 1994; Marois, 2005). Expression of Rab5DN blocks formation of early endosomes labeled with Rab5-GFP (Figure 4A) and inhibits formation of large, colocalized Notch–Sanpodo punctae seen in wild-type cells (Figure 4B). We hypothesized that blocking early endosome formation would inhibit Notch signaling in pIIa cells due to inhibition of Delta trafficking. Surprisingly, we found that although Rab5DN overexpression in SOP cells resulted in bristle loss and loss of Su(H) expression in differentiated cells, a proportion of the remaining organs (18%) exhibited extra Su(H)-positive socket cells, an indication of excess Notch signaling activity (Figure 4, C–F). These results suggest that Rab5 activity can both promote and restrict Notch activation in SOP-lineage cells. We hypothesize that Rab5-dependent endocytosis of the Notch receptor is required to reduce overall plasma membrane levels of Notch and thereby reduce

antibody incubation step (newly synthesized receptors that have not yet reached the membrane or receptors endocytosed before primary antibody addition) are not labeled because only plasma membrane–exposed receptors are bound with primary antibody.

We conducted this assay on both wild-type and numb mutant pIIa/pIIb cells. We analyzed Notch membrane levels by measuring the signal intensity of both FSA and SSA at the interface between the pIIa and pIIb cells to exclude FSA and SSA signals from Notch receptors in neighboring epithelial cells. At the membrane interface of pIIa/pIIb cells, we found that FSA levels were low in both wild-type and numb mutant cells (Figure 3, B–E). In contrast, in numb mutant cells, we detect higher SSA signals at the subapical sections of the interface than with controls that exhibit low levels SSA in pIIa and pIIb cells (Figure 3, B–E). These findings show, consistent with previous observations, that Notch membrane levels are higher in numb mutant pIIa/pIIb than in wild-type cells (Couturier et al., 2012). Furthermore, the data from our assay suggest that the population of Notch receptors at the pIIa/pIIb cell interface in numb mutants represents a recycled pool of receptors rather than a static pool of receptors that remains at the membrane surface throughout the assay.

FIGURE 4: Rab5 dominant negative causes cell fate switch and accumulation of static Notch.

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levels of Notch signaling in SOP cells. Indeed, using our trafficking assay, we found that Rab5DN expression increased colocalization of FSA and SSA signals (which in our assay represent static Notch receptors at the membrane) at the pllb/plla cell interface and increased FSA signal levels compared with wild-type cells (Figures 3D and 4, G and I). These findings suggest that Rab5 inhibition increases the population of static Notch receptors at the membrane surface. Furthermore, Rab5DN expression in numb mutant cells increased the FSA signal at the plla/pllb cell interface (Figure 4, H, J, and K) compared with numb mutant cells (Figures 3E and 4K). These findings suggest that the pool of endocytically recycled Notch receptors (labeled by SSA) in numb mutant cells is dependent on Rab5 function.

**Rab11 activity is required for excess Notch signaling in numb mutant SOP cells**

The foregoing data confirm that Numb plays a role in suppressing Notch receptor recycling in pllb/plla cells. We hypothesized that excess Notch recycling in numb mutants drives increased Notch signaling in numb mutant cells, resulting in loss of neuronal cell fates. Therefore we tested whether disruption of Rab11-dependent Notch recycling by expression of a dominant-negative Rab11 (Rab11DN) would restore neuronal cell fates to numb mutant clones. Expression of Rab11DN in wild-type pllb/plla cells showed low levels of SSA and FSA signals, similar to wild-type cells alone (Supplemental Figure S3). To see whether expression of Rab11DN could alter cell fate phenotypes in SOP-linage cells, we used the neuronal marker ELAV to label and quantify neuronal fates in numb mutant sensory organs. External sensory organs in numb mosaic clones on the adult thorax showed the expected multiple socket phenotype that we and others reported previously (Uemura et al., 1989; Rhyu et al., 1994). In wild-type cells, every organ contained a single ELAV-labeled neuron (Figure 5, A and B). In contrast, 65% (76 cell clusters) of numb mutant organs had no detectable ELAV expression, indicating pllb-to-plla transformation (Figure 5, A and B). We found that Rab11DN expression in either wild-type or numb mutant sensory organ cells significantly increased the number of sensory organs containing neurons (Figure 5B). Surprisingly, ∼10% of all organs in numb/Rab11DN sensory organs exhibited multiple ELAV-expressing neuronal cells, a
phenotype that was not observed in either wild-type or numb mutant external sensory organs but consistent with plia-to-pilb cell fate transformations observed in Notch mutants (Guo et al., 1996). We conclude from these data that Rab11 activity contributes to excess Notch signaling activity in numb mutant pilb cells.

L(2)gl, but not WASp or exocyst component Sec15, regulates Notch recycling in SOP cells

In the pilb cell, Delta trafficking through the Rab11-dependent recycling endosome promotes Notch activation in the neighboring pila cell (Emery et al., 2005). Delta furthermore requires exocyst complex and WASp activity for recycling and membrane targeting in plia/pilb cells (Rajan et al., 2009). Because we found that Notch recycling in numb mutant cells also requires Rab11 in SOP cells, we wondered whether Notch, like Delta, requires exocyst complex and WASp activity for Notch recycling in plia/pilb cells. Using our recycling assay, we determined that FSA and SSA signal levels in wild-type, l(2)gl234 (A), and l(2)gl234, nb2 (B) clones. White dashed area indicates region of uncolocalized SSA. (F, G) Quantifications of l(2)gl234 (F) and l(2)gl234, nb2 (G). Intensity values represent the ratio of FSA or SSA relative to background nuclear staining. Averages for FSA and SSA are represented by gold and blue bar, respectively. Genotypes: Ubx-flp; Gal80frt40; NeurGal4, Rab5-GFP, l(2)gl234frt40A (A, D, F), l(2)gl234, nb2 frt40A (B, E, G), and y+ frt40A (C).

FIGURE 6: Notch recycling is suppressed by L(2)gl. (A, B) Sensory organ precursor cells were staged to 20 h after puparium formation (two-cell stage) and analyzed using the Notch recycling assay for l(2)gl234 (A; 36 cell pairs, 12 flies) and l(2)gl234, nb2 (B; 27 cell pairs, 10 flies). Clones showed accumulation of SSA but not FSA, indicating recycled Notch, at the plia/pilb interface (dashed region). (C–E) Z-stack representation of recycling assay for wild-type, l(2)gl234 (A), and l(2)gl234, nb2 (B) clones. White dashed area indicates region of uncolocalized SSA. (F, G) Quantifications of l(2)gl234 (F) and l(2)gl234, nb2 (G). Intensity values represent the ratio of FSA or SSA relative to background nuclear staining. Averages for FSA and SSA are represented by gold and blue bar, respectively. Genotypes: Ubx-flp; Gal80frt40; NeurGal4, Rab5-GFP, l(2)gl234frt40A (A, D, F), l(2)gl234, nb2 frt40A (B, E, G), and y+ frt40A (C).

To elucidate how Notch signaling is controlled during development and how Notch signaling can be dysregulated in disease, an understanding of the mechanisms underlying control of membrane levels of Notch pathway components is essential (Vaccari et al., 2008; Fortini and Bilder, 2009). From recent studies, it is clear that cellular context plays an important role in regulation of Notch receptor levels and in signaling output. In SOP cells, Numb, a known endocytic regulator, is asymmetrically localized during progenitor mitosis and acts to block Notch pathway activation cell-autonomously in the pilb daughter cell that inherits it. Recent evidence in flies and Caenorhabditis elegans points to a model in which Numb inhibits Notch receptor recycling, thereby decreasing Notch plasma membrane levels (Nilsson et al., 2008; Cotton et al., 2013; Couturier et al., 2013), whereas in mammalian cells, evidence points to Numb promoting Notch targeting to late endosome compartments through the ubiquitin ligase Itch (McGill and McGlade, 2003; McGill et al., 2009). These two functions may not be mutually exclusive. In this study, we analyzed the Notch levels in endocytic compartments and developed an assay that allows us to identify pools of Notch (recycled, static, internalized) in SOP cells in vivo. Our findings reveal that Numb is responsible for regulating Notch accumulation in Rab7-positive late endosomes and that Numb restricts a population of recycled Notch receptors in SOP daughter cells.

DISCUSSION

To elucidate how Notch signaling is controlled during development and how Notch signaling can be dysregulated in disease, an understanding of the mechanisms underlying control of membrane levels of Notch pathway components is essential (Vaccari et al., 2008; Fortini and Bilder, 2009). From recent studies, it is clear that cellular context plays an important role in regulation of Notch receptor levels and in signaling output. In SOP cells, Numb, a known endocytic regulator, is asymmetrically localized during progenitor mitosis and acts to block Notch pathway activation cell-autonomously in the pilb daughter cell that inherits it. Recent evidence in flies and Caenorhabditis elegans points to a model in which Numb inhibits Notch receptor recycling, thereby decreasing Notch plasma membrane levels (Nilsson et al., 2008; Cotton et al., 2013; Couturier et al., 2013), whereas in mammalian cells, evidence points to Numb promoting Notch targeting to late endosome compartments through the ubiquitin ligase Itch (McGill and McGlade, 2003; McGill et al., 2009). These two functions may not be mutually exclusive. In this study, we analyzed the Notch levels in endocytic compartments and developed an assay that allows us to identify pools of Notch (recycled, static, internalized) in SOP cells in vivo. Our findings reveal that Numb is responsible for regulating Notch accumulation in Rab7-positive late endosomes and that Numb restricts a population of recycled Notch receptors in SOP daughter cells.
In mammalian cells, Numb promotes Notch targeting to late endosomes through the ubiquitin ligase Itch (McGill and McGlade, 2003; McGill et al., 2009). Here we find that NECD punctate levels are higher in the Numb-positive pIIb cell than in the Numb-negative pIIa at steady state, and yet our study confirms previous observations that Numb does not influence Notch colocalization with the early endosomes in pIIa/pIIb cells (Couturier et al., 2013). However, our observation of a Numb-dependent Notch late endosome asymmetry, although consistent with findings in mammalian cells, are at odds with results obtained using Notch-GFP and Notch-Cherry fusion proteins in pIIa/pIIb cells, for which no asymmetry was detected at cytokinesis (Couturier et al., 2014). This may be due to our use of different approaches: our study followed Notch by antibody labeling of the extracellular domain of the receptor at time points after cytokinesis, whereas Couturier et al. (2014) used receptors fluorescently tagged within the intracellular domain. Furthermore, our marker for late endosomes, Rab7, may define a different population of endosomes from those defined by Couturier et al. (2014) based on differences in Notch-GFP and Notch-Cherry fusion protein signals. Nonetheless, our data indicate that, in our assay conditions, Numb has a conserved role in influencing Notch trafficking, perhaps specifically to late endosomes. This result is consistent with the Numb-dependent asymmetry of Sanpodo targeting to late endosomes in pIIb cells reported by Couturier et al. (2014). However, whether Numb regulates Notch trafficking through a ubiquitin-dependent mechanism, indirectly through Sanpodo, by direct interaction with the Notch receptor, or through some combination of these mechanisms remains unclear.

We chose to assess the role of L(2)gl in regulating Notch trafficking because L(2)gl plays an important role in restricting Notch activation and therefore promoting pIIb cell fate in the sensory lineage (Justice et al., 2003). In its role as polarity regulator, L(2)gl functions to regulate asymmetric targeting of Numb in both neuroblasts and SOP cells during metaphase of mitosis (Ohshiro et al., 2000; Peng et al., 2000; Langevin et al., 2005). However, L(2)gl is not required for Numb asymmetry to the pIIb cell at telophase, resulting in a delay, but not failure, to segregate Numb to pIIb (Justice et al., 2003; Langevin et al., 2005). Studies from yeast, flies, and vertebrate neurons have implicated L(2)gl in membrane fusion events and vesicle trafficking, including trafficking of Sanpodo (in SOP cells) and regulating Notch signaling by controlling endosome acidification in the Drosophila eye (Lehman et al., 1999; Langevin et al., 2005; Roegiers et al., 2005; Zhang, 2005; Grosshans, 2006; Wang et al., 2011; Parsons et al., 2014). The evidence we provide suggests that L(2)gl may play a role in suppressing Notch recycling and therefore may be part of a mechanism by which L(2)gl regulates pIIb cell fate. It is interesting to note that we see an increased level of recycled Notch in l(2)gl, numb mutant cells compared with numb mutants alone. Although L(2)gl may regulate Numb asymmetry at mitosis in SOPs, our findings suggest that the two genes may be working in parallel to control Notch recycling in SOP daughter cells after mitosis.

On the basis of our results, we propose that control of Notch signaling in pIIb/pIIa cells depends on the balance between the membrane and endosomal pools of Notch receptors. Rab5 and Sanpodo function to shunt Notch to the endosomal pool in the SOP. After asymmetric cell division, the presence of Numb in the pIIb cell increases levels of internalized Notch receptors in late endosomes, either directly or by decreased trafficking through the Rab11-dependent recycling endosome. Delta, on the other hand, is recycled in a Neuralized/Rab11/Sec15-dependent manner. The case in pIIa is different. There Sanpodo promotes Notch internalization to early endosomes at the same rate as that observed in pIIb. However, the absence of Numb in pIIa cells prevents sequestration of Notch in late endosomes, resulting instead in Rab11-dependent basolateral membrane Notch recycling and activation of Notch signaling. Of interest, Notch recycling is not strictly required for Notch signaling activity in the pIIa cell, as disrupting early endosomes blocks Notch recycling but does not affect pIIa cell fate determination. However, blocking early endosome function does result in both accumulation of static Notch at the plasma membrane and cell fate changes in the pIIb. In conclusion, this study provides direct evidence that Numb is responsible for regulating the endosomal sorting of Notch, answering the long-standing question of the function of Numb.

**MATERIALS AND METHODS**

**Immunostaining**

*Drosophila* stocks and crosses were maintained under standard laboratory conditions. To stage pupa for immunostaining experiments, we selected early-stage (white) pupae and aged them for 16–18 h at 25°C for staining of pIIa/pIIb cells. Samples were immediately fixed under standard conditions (4% paraformaldehyde [PFA] for 20 min at room temperature). After fixation, we used the antibodies mouse anti-Notch (NECD, C458.2H, 355 μg/ml; at 1:100; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and goat anti-mouse Alexa Fluor 488, 568, 633 (1:100; Thermo Fisher, Waltham, MA). Samples were imaged on a Nikon C1 confocal microscope using a 60×/numerical aperture 1.49 objective.

**Internalization and recycling assays**

The internalization assay was performed on dissected notum tissue from properly staged pupae and confirmed through live-cell imaging. Tissue was dissected in S2 cell medium at room temperature and then transferred to fresh medium with Notch antibody (NECD, C458.2H, 355 μg/ml used at 1:50) and allowed to incubate for 10 min. Samples were then quickly washed and transferred to fresh S2 medium and allowed to chase for the indicated time intervals. At the end of the indicated time, samples were fixed as described and stained with secondary antibody according to standard methods.

As in the internalization assay, the recycling assay was performed on live, staged notal tissue by labeling membrane Notch receptors (NECD). The tissue was incubated in S2 medium containing the NECD antibody (NECD, C458.2H, 355 μg/ml used at 1:50), which labels exposed receptors at the plasma membrane during a short time window (10 min). After an S2 medium wash, the tissue containing the cells was labeled with the fluorophore-coupled FSA at 4°C to block receptor endocytosis/recycling during this step and then allowed to internalize at 25°C for 10 min and washed. The tissue was fixed (4% PFA, 15 min), permeabilized (0.1% Triton X), and labeled with the fluorophore-coupled SSA (Figure 1A). The FSA and SSA were labeled with different fluorophores (Alexa 633 and 568, respectively; 2 mg/ml, used at 1:50), allowing us to follow the dynamics of different populations of Notch receptors. Only Notch present at the plasma membrane in the first step of the assay was labeled with the primary antibody. Subsequent movement of the receptor into early endosomes and back to the plasma membrane through recycling was labeled with the FSA, SSA, or both (Figure 1A). Three populations of receptors were therefore identified: a static pool of receptors (labeled by both FSA and SSA), a recycled pool (labeled by FSA only), and an internalized pool of receptors (labeled by SSA only).

**Quantification**

To quantitate the number of endosomes that contained Notch, we defined an endosome as being a roughly spherical object 0.5-1 μm in diameter. Colocalization was defined as when a collection of
Notch pixels of intensity at least 33% that of the maximum intensity overlapped in the general shape of the underlying endosome labeled with either Rab5-GFP or Rab7-GFP.

The recycling assay quantifications were performed using ImageJ software (National Institutes of Health, Bethesda, MD). To quantify levels of recycled Notch, antibody staining in the SSA channel was evaluated for its intensity at the interface between pIIa and pIIb cells. The SSA interface signal level was normalized to the level of background SSA found in the nucleus (we assumed that the nucleus contained no specific signal of the NECD, which is excluded from the nucleus). Similarly, to quantify internalized Notch, we also measured the FSA signal at the interface and normalized it to the background nuclear levels.

To represent static Notch, colocalization of FSA and SSA was assessed. To determine colocalization, signal intensity from FSA and SSA was set to a threshold equal to ~30% of maximum intensity. Masks of thresholds were then taken, and colocalized pixels were counted and are represented in yellow in the figures. The p values were obtained using a Wilcoxon rank sum test for use with paired samples of unequal variance (Table 1).

**Drosophila stocks**

We used neur-Gal4/TM6B, UAS-Rab5GFP, y,w;Ubx-flp, y,w; Gal80, frt40, y,w; y+; ck, frt40/Cyo, y,w; NB2; ck, frt40/Cyo, UAS-Rab7GFP, UAS-numb-myc, UAS-ActinGFP, UAS-Sanpodo-GFP (Tong et al., 2010), UAS-Rab11SN-YFP (Zhang et al., 2007), Gal80FRT82B, sec15; frt82b/TM6B, wsp3; frt82b, y,w; lgl334; frt40a, y,w; lgl334; nb2; frt40A, y,w; sca-Gal4.

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