

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

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**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.

## Monitoring Editor

Carl-Henrik Heldin  
Ludwig Institute for Cancer  
Research

Received: Nov 2, 2015

Revised: Jul 20, 2016

Accepted: Jul 21, 2016

## INTRODUCTION

The Notch signaling pathway is conserved throughout metazoan evolution and is used to control tissue patterning and cell fate determination in a diverse array of developmental contexts. Inappropriate activation of the pathway has been implicated in a variety of cancer types, as well as in human disease syndromes such as cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (Louvi and Artavanis-Tsakonas, 2012). Notch signaling occurs when Notch, a transmembrane receptor protein in the signal-receiving cell, binds to ligands of the Delta/Serrate/Lag-2 family in the signal-sending cell, resulting in a conformational change in the receptor. The ligand-dependent conformational change in the receptor triggers proteolytic cleavage of the receptor by the  $\gamma$ -secretase complex, releasing the intracellular domain of Notch to travel to the nucleus and act as a transcriptional activator in the receiving cell (Kopan and Ilagan, 2009). Activation of the path-

way therefore relies on mechanisms that control both the localization and the abundance of the ligands and receptor in membrane compartments (Kandachar and Roegiers, 2012).

A long-standing model in the study of regulation of Notch signaling in development is the *Drosophila* sensory organ precursor (SOP) cell (Singhania and Grueber, 2014). The SOP cell divides four times to give rise to four terminally differentiated cells (hair, socket, neuron, sheath) that make up the external sensory organ (Figure 1A). The SOP cell undergoes an asymmetric cell division along the anterior-posterior axis, characterized by targeting of a membrane-associated protein, Numb, to one side of the precursor cell during mitosis (Rhyu *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).

The Delta ligand is ubiquitinated 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.*, 2001; Pavlopoulos *et al.*, 2001; Le Borgne and Schweisguth, 2003; Emery *et al.*, 2005; Benhra *et al.*, 2010; Giagtoglou *et al.*, 2012). The apical cell interface between pIIa and pIIb is enriched with Arp2/3 complex and the Wiskott-Aldrich syndrome protein-dependent

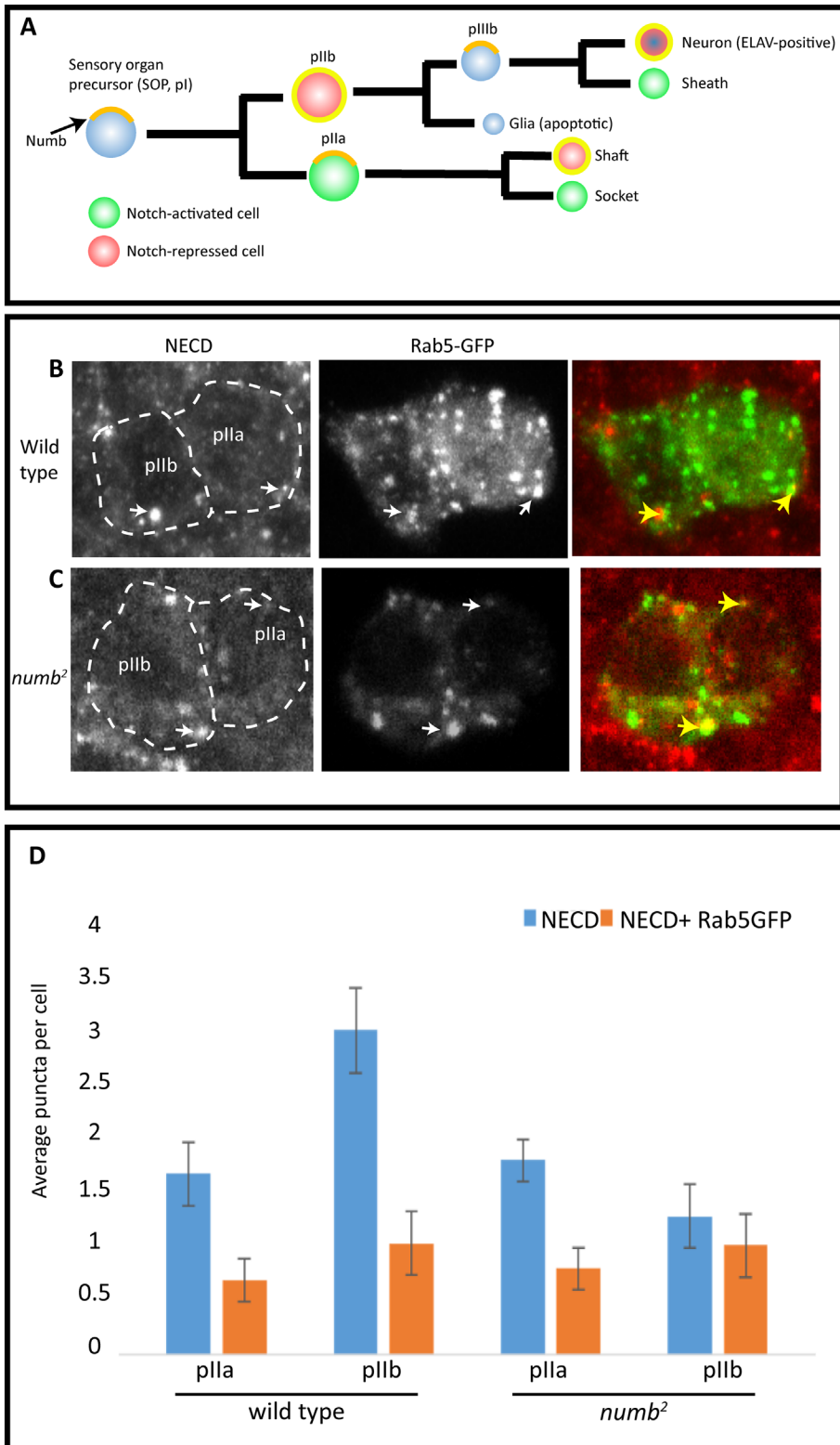
This article was published online ahead of print in MBoC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E15-11-0751>) on July 27, 2016.

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

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**FIGURE 1:** Notch recruitment to early endosomes is Numb independent. (A) SOPs divide asymmetrically, segregating Numb to one daughter cell to create Numb-negative p11a and Numb-positive p11b cells, which divide again to produce the four terminal cell fates (the neuronal cell expresses ELAV). (B, C) Sections of wild type (B; 23 cell pairs, five flies) and *numb<sup>2</sup>* (C; 21 cell pairs, four flies) clonal tissue displaying p11a/p11b cells that express Rab5-GFP and were stained with NECD. Rab5GFP endosomes that colocalized with NECD puncta (yellow arrows) were quantified and compared between wild-type and *numb* mutant p11a and p11b cells (D). Genotypes: *neur-Gal4, UAS-Rab5GFP/TM6y<sup>+</sup>* (B–D) and *Ubx-flp; numb<sup>2</sup>frt40A/frt40Gal80; neur-Gal4, Rab5-GFP/TM6y<sup>+</sup>* (C).

microvillar membrane projections required for robust Notch activation in the p11a cell (Ben-Yaacov *et al.*, 2001; Rajan *et al.*, 2009).

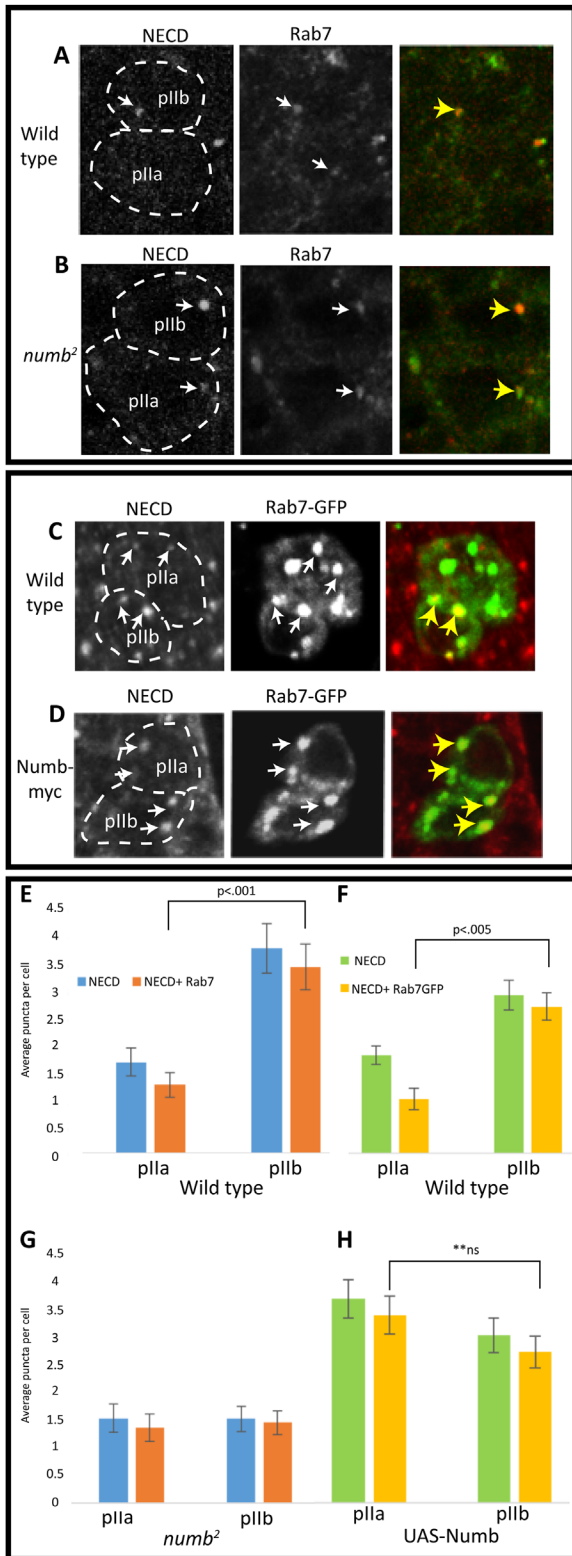
Control of membrane trafficking is not limited to the Delta ligand in the p11b cell. Sanpodo, a four-pass transmembrane protein that interacts with Notch, promotes Notch receptor endocytosis (O'Connor-Giles and Skeath, 2003; Couturier *et al.*, 2012; Upadhyay *et al.*, 2013), whereas Numb inhibits membrane targeting of Notch and Sanpodo in the p11b 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 p11b 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 p11a/p11b 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 punctae and the colocalization of the Notch extracellular domain (NECD) with the markers of early (Rab5) and late endosomes (Rab7) in wild-type Numb-negative p11a and Numb-positive p11b cells in pulse-chase and steady-state experiments. In pulse-chase experiments in p11b and p11a cells, colocalization between Rab5 and Notch peaked between 20 and 30 min, whereas Notch colocalization with Rab7 peaked at 30 min



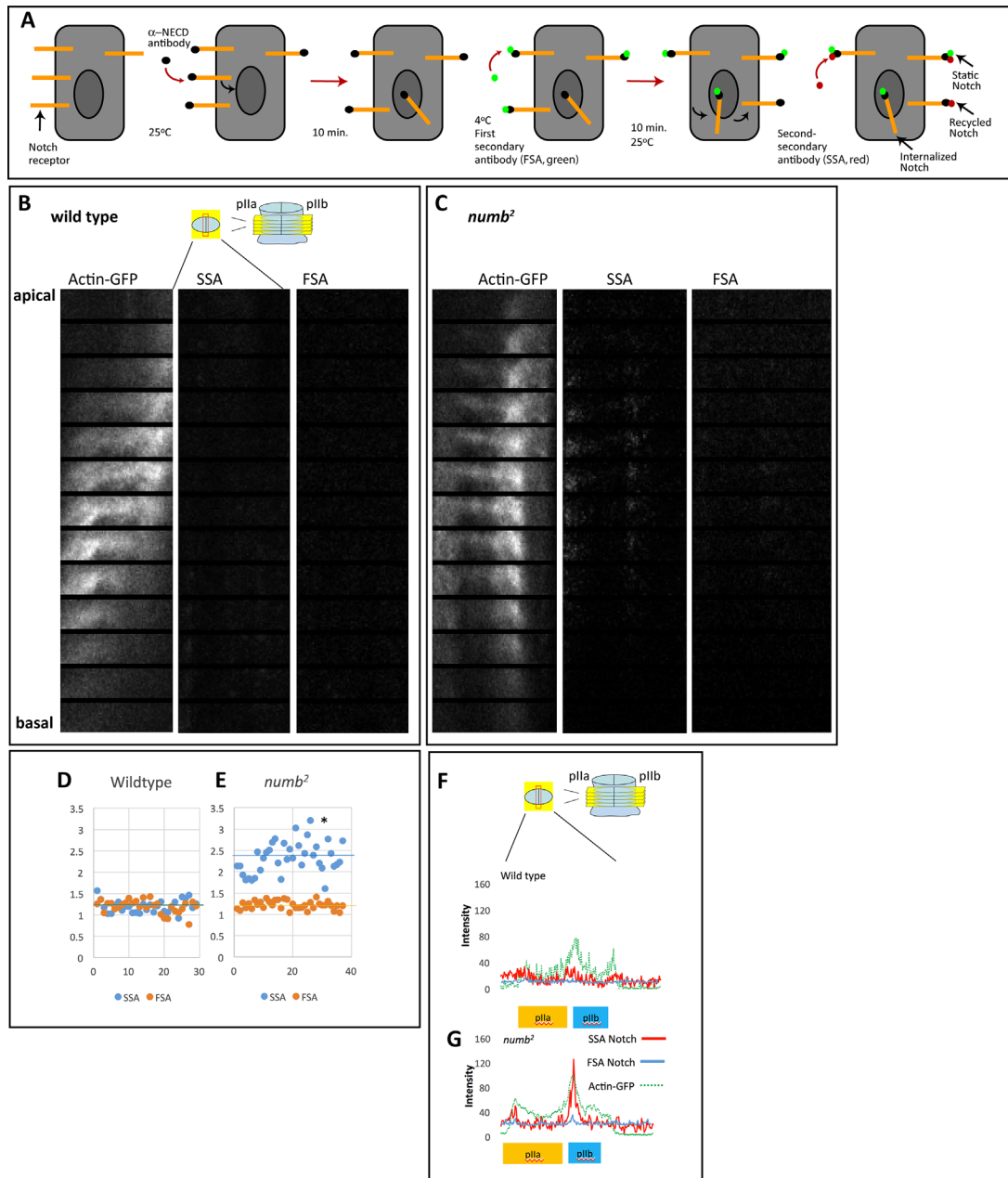
**FIGURE 2:** Numb is required for asymmetric Notch trafficking to late endosomes. (A) Wild-type clones marked with Rab5GFP were stained with antibody for NECD (red) and Rab7 (green; 21 cell pairs, five flies). NECD and Rab7 puncta (white arrows) were most often colocalized (yellow arrow) in pIIb cells. This asymmetry was abolished in *numb*<sup>2</sup> mutant clones (B) also stained for NECD (20 cell pairs, five flies). (C) Wild-type clones marked with Rab7GFP were stained with NECD (25 cell pairs, five flies). NECD and Rab7GFP punctae (white arrows) were also most often colocalized in pIIb cells (yellow arrows).

(Supplemental Figure S1, A and B). 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 punctae 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 punctae 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 (Coumilleau *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

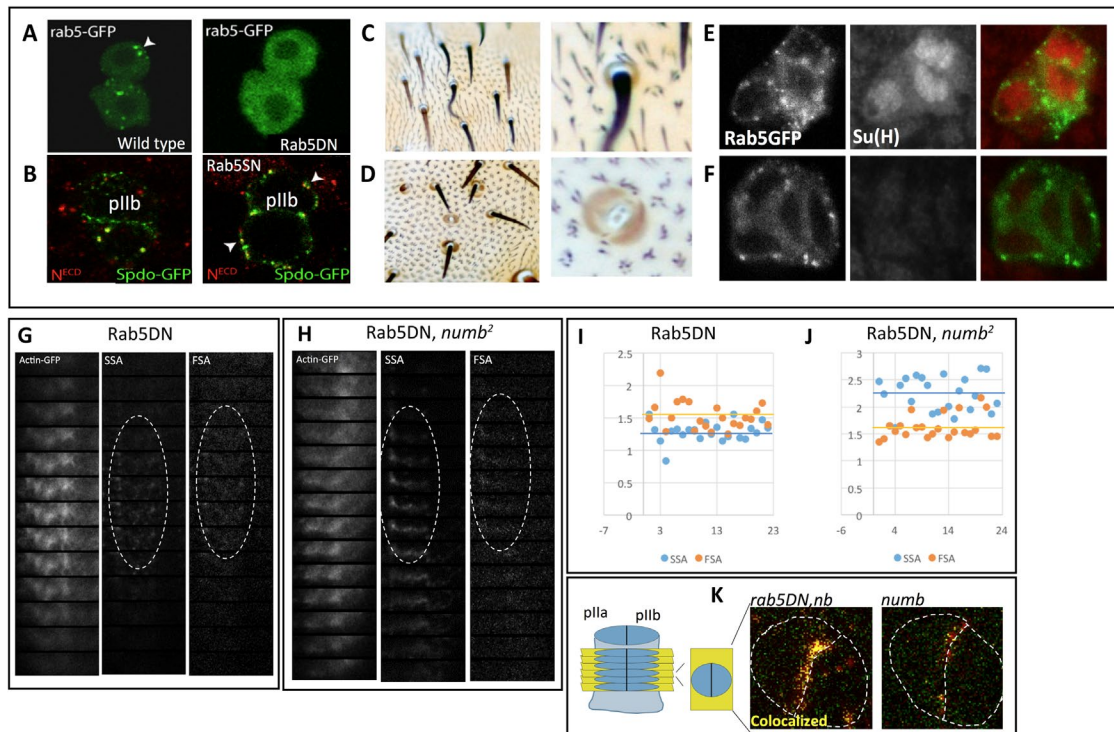
(D) Overexpression of Numb abolished this bias, causing both cells to possess pIIb levels of colocalization (19 cell pairs, three flies). Wild-type (E, F) and *numb*<sup>2</sup> (G, H) clonal pIIa/pIIb cells were quantified as average numbers of single (NECD) or colocalized (NECD + Rab7) puncta per cell. Genotypes: *Ubx-flp; frt40Gal80; neur-Gal4, Rab5-GFP/TM6y<sup>c</sup> ckfrt40/Cyo* (A), *ckfrt40nb<sup>2</sup>/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 *p11a/p11b* 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 *p11a* and *p11b* 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; numb<sup>2</sup>frt40A; 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



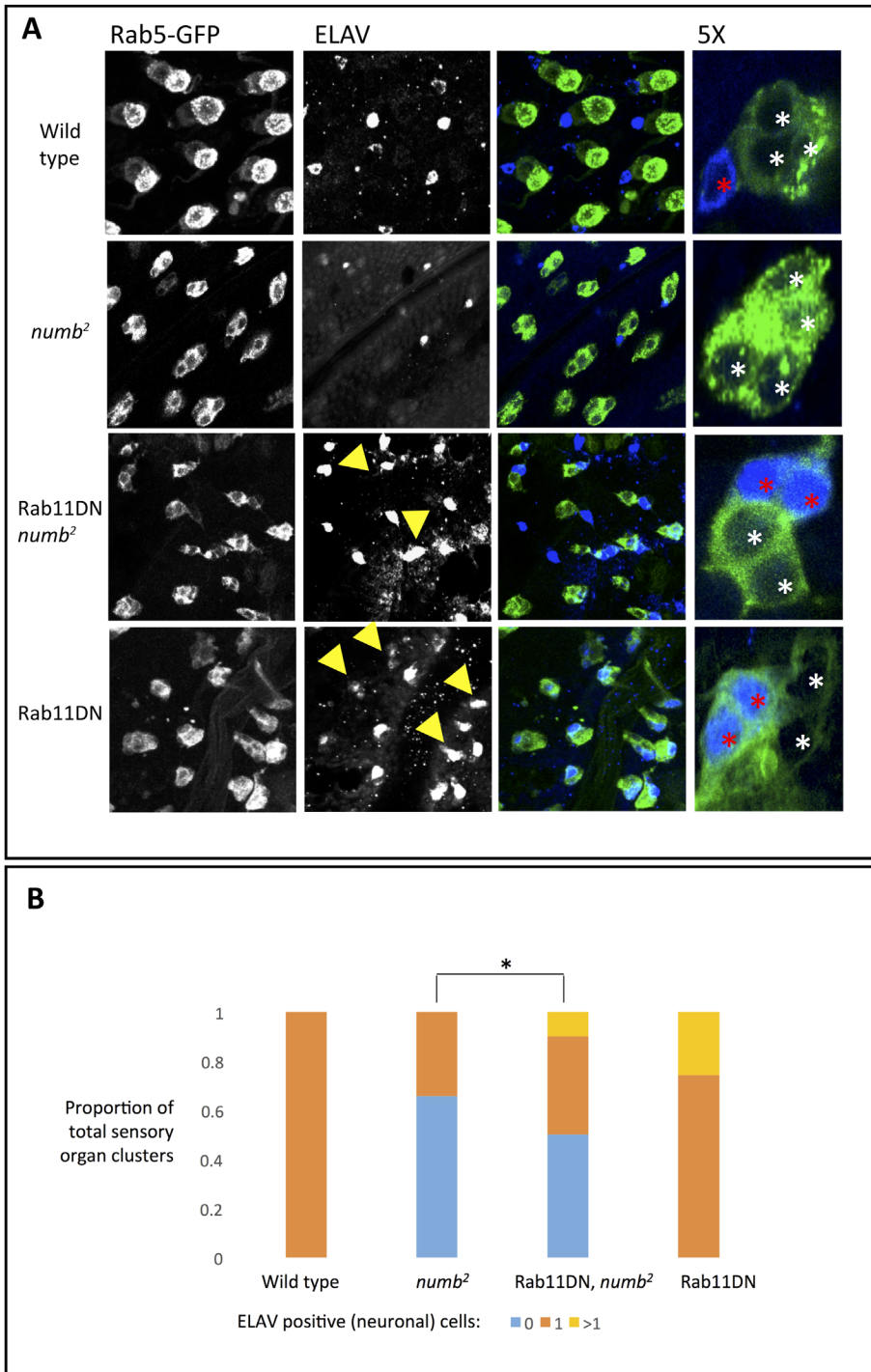
**FIGURE 4:** Rab5 dominant negative causes cell fate switch and accumulation of static Notch. (A) Expression of Rab5GFP in p11a and p11b labels discrete puncta of Rab5-GFP-labeled early endosomes (white arrowhead). Expression of Rab5 dominant negative (Rab5DN) prevents formation of Rab5 early endosomes. (B) NECD immunostaining in cells expressing Sanpodo-GFP (to mark early endosomes) in wild type shows NECD in intracellular early endosomes. Rab5 dominant negative-expressing cells (Rab5DN) show accumulation of NECD in subapical vesicles (white arrowhead). (C, D) Bristle phenotypes for wild-type (C) and Rab5DN (D) showed multiple sockets and areas of balding with expression of Rab5DN (six fly thoraces). Adult cell phenotypes were confirmed with Su(H) staining in sensory organ clusters at 24 h after puparium formation. Overexpression of Rab5SN resulted in clusters containing multiple (E) or zero (F) Su(H)-stained cells. (G, H) Recycling assays of Rab5DN-expressing (G) and Rab5DN, *numb*<sup>2</sup>-expressing (H) cells displayed as z-plane stacks. Dashed circles indicate areas of overlap between FSA and SSA. (I, J) Quantifications of FSA and SSA from recycling assays in G and H. Intensity values represent ratio of FSA or SSA relative to background nuclear staining. For I; 22 cell pairs and five flies; for J, 23 cell pairs and six flies. (K) Colocalization analysis of *nb*<sup>2</sup> and Rab5DN, *nb*<sup>2</sup>. Colocalized pixels above separate channel intensity threshold are represented in yellow and are increased in Rab5DN, *nb*<sup>2</sup> double mutants. Genotypes: *UAS-Sanpodo-GFP neur-Gal4* (A, C), *UAS-Rab5DN, neur-gal4* (C–E), and *UAS-Actin-GFP* (D, E).

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 p11a/p11b cells. We analyzed Notch membrane levels by measuring the signal intensity of both FSA and SSA at the interface between the p11a and p11b cells to exclude FSA and SSA signals from Notch receptors in neighboring epithelial cells. At the membrane interface of p11a/p11b 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 p11a and p11b cells (Figure 3, B–E). These findings show, consistent with previous observations, that Notch membrane levels are higher in *numb* mutant p11a/p11b than in wild-type cells (Couturier *et al.*, 2012). Furthermore, the data from our assay suggest that the population of Notch receptors at the p11a/p11b 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.

### 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 p11a/p11b 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 p11a 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

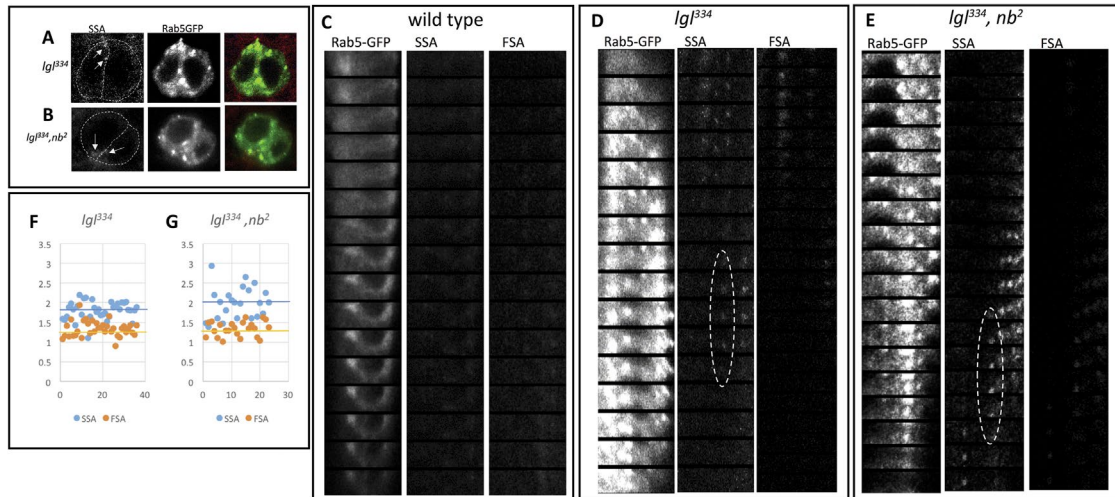


**FIGURE 5:** Notch signaling requires Rab11 recycling endosome. Notal tissue was staged to 22 h after puparium formation to capture the four-cell stage and immunostained with neuronal marker ELAV. (A) Wild-type GFP-expressing differentiated sensory organs (55 organs, three flies) show a single ELAV-positive (blue) cell (red asterisks) with three nonneuronal cells (white asterisks; 55 organs, three flies). Right, magnified cells. *numb* MARCM clones showed clusters with zero and one ELAV-positive cell cluster (76 organs, four flies). Rab11SN, *numb* double clones showed zero, one, and two ELAV-positive cell clusters (89 organs, two flies). Dominant-negative Rab11 (Rab11SN) showed one and two ELAV-positive cell clusters (two ELAV cells; yellow arrows; 31 organs, five flies). (B) Quantification of number of ELAV-positive cell clusters in each background. Genotypes: *UAS-Rab5GFP, neur-Gal4 UAS-Rab11SN-YFP Ubx-flp; numb²frt40, Gal80frt40; neur-Gal4, Rab5-GFP/TM6* (A). \**p* < 0.05.

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 p11b/p11a 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 p11a/p11b 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 p11a/p11b 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 p11a/p11b 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-lineage cells, we used the neuronal marker ELAV to label and quantify neuronal fates in *numb* mutant external 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 p11b-to-p11a 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



**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)gl<sup>B334</sup>* (A; 36 cell pairs, 12 flies) and *l(2)gl<sup>B334</sup>,nb<sup>2</sup>* (B; 27 cell pairs, 10 flies). Clones showed accumulation of SSA but not FSA, indicating recycled Notch, at the pIIa/pIIb interface (dashed region). (C–E) Z-stack representation of recycling assay for wild-type, *l(2)gl<sup>B334</sup>* (A), and *l(2)gl<sup>B334</sup>,nb<sup>2</sup>* (B) clones. White dashed area indicates region of uncolocalized SSA. (F, G) Quantifications of *l(2)gl<sup>B334</sup>* (F) and *l(2)gl<sup>B334</sup>,nb<sup>2</sup>* (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)gl<sup>B334</sup>frt40a* (A, D, F), *l(2)gl<sup>B334</sup>,nb<sup>2</sup> frt40a* (B, E, G), and *y+ frt40a* (C).

phenotype that was not observed in either wild-type or *numb* mutant external sensory organs but consistent with pIIa-to-pIIb 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 pIIb cells.

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

In the pIIb cell, Delta trafficking through the Rab11-dependent recycling endosome promotes Notch activation in the neighboring pIIa cell (Emery *et al.*, 2005). Delta furthermore requires exocyst complex and WASp activity for recycling and membrane targeting in pIIa/pIIb 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 pIIa/pIIb cells. Using our recycling assay, we determined that FSA and SSA signal levels in *sec15* and *wasp* mutants (Supplemental Figure S2) were indistinguishable from wild-type cells (Figure 1B). We next expanded our analysis to explore the role of *lethal (2) giant larvae (l(2)gl)* in regulating Notch trafficking in SOP lineage cells. L(2)gl is an evolutionarily conserved tumor suppressor that plays important roles in apical-basal cell polarity, asymmetric targeting of cell fate determinants (including Numb), and membrane trafficking (Vasioukhin, 2006; Wirtz-Peitz and Knoblich, 2006; Parsons *et al.*, 2014). In SOP cells, L(2)gl regulates cell fate: in *l(2)gl* mutants, sensory organ differentiation is disrupted, resulting in increased hair and socket cells at the expense of neurons, which is a phenotype reminiscent of *numb* mutants (Ohshiro *et al.*, 2000; Justice *et al.*, 2003; Langevin *et al.*, 2005). We hypothesized that L(2)gl may play a role in regulating Notch trafficking in SOP cells. We therefore performed the recycling assay cells in tissue containing *l(2)gl* mutant MARCM clones. We found increased SSA signal (but no change in FSA levels) at the membrane interface in *l(2)gl* mutant pIIa/pIIb cells compared with wild-type cells (Figure 6, A, C, D, and F), similar to that observed in *numb* mutant

cells (Figure 3C). However, in *l(2)gl* mutant cells, in contrast to *numb* cells, the recycled Notch signal was shift basolaterally (Figure 6D). In cells mutant for both *l(2)gl* and *numb*, we found a similar intensity in the SSA signal to that for either mutant alone (Figures 3E and 6, F and G); however, we observed the same basolateral shift in the SSA signal in *l(2)gl, numb* as in the *l(2)gl* mutant alone (Figure 6, D and E). These finding suggests that L(2)gl and Numb may function together to suppress the pool of recycled Notch receptors at the pIIa/pIIb cell interface and that L(2)gl may regulate the apical-basal polarity of the recycled pool receptors.

### 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 pIIb 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 endosome compartments through the ubiquitin ligase Itch (McGill and McGlade, 2003; McGill *et al.*, 2009). Here we find that NECD punctae levels are higher in the Numb-positive p11b cell than in the Numb-negative p11a at steady state, and yet our study confirms previous observations that Numb does not influence Notch colocalization with the early endosomes in p11a/p11b 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 p11a/p11b 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 have defined 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 p11b 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 p11b 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 p11b cell at telophase, resulting in a delay, but not failure, to segregate Numb to p11b (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 p11b 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 p11b/p11a 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 p11b 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 Neutralized/Rab11/Sec15-dependent manner. The case in p11a is different. There Sanpodo promotes Notch internalization to early endosomes at the same rate as that observed in p11b. However, the

absence of Numb in p11a 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 p11a cell, as disrupting early endosomes blocks Notch recycling but does not affect p11a 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 p11b. 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 p11a/p11b 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 quantify 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



	Wild-type FSA	wild-type SSA	numb FSA	numb SSA	IgI FSA	IgI SSA	Rab5DN, nb FSA	Rab5DN, nb SSA	Rab5DN FSA	Rab5DN SSA
numb FSA	ns			<0.001			<0.001			
numb SSA		<0.001					ns			
IgI FSA	ns		ns			<0.001				
IgI SSA		<0.001		<0.001						
IgI,numb FSA	ns		0.041		ns					
IgI,numb SSA		<0.001		0.032		ns				
Rab5DN FSA	<0.001						ns			<0.001
Rab5DN SSA		0.01							<0.001	
Wild-type FSA		ns								
Wild-type SSA										
Rab55DN,nb FSA										<0.001
Rab55DN,nb SSA										

The p values in all cases were obtained using a Wilcoxon rank sum test for use with paired samples of unequal variance. The p values are given for corresponding FSA or SSA data sets of compared genotypes. ns, not significant.

TABLE 1: Recycling-assay p values.

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, yw: Gal80, frt40, y,w: y+,ck, frt40/Cyo, y,w: nb<sup>2</sup>,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, sec-15<sup>2</sup>frt82b/TM6B, wsp<sup>3</sup>frt82b, y,w: Igl<sup>334</sup>frt40a, y,w:Igl<sup>334</sup>,nb<sup>2</sup> frt40A, y,w:sca-Gal4.

### ACKNOWLEDGMENTS

We thank Deborah Silverman, Esteban Martinez, and Danielle Mathis for technical assistance. We are especially grateful to Alana O'Reilly and Greg Bashaw for critical reading of the manuscript. In addition, we thank Steve Dinardo, Warren Pear, Meera Sundaram, and the anonymous reviewers for providing valuable insights and suggestions. We thank Marcos Gonzalez-Gaitan and Juergen Knoblich for sharing fly stocks. Stocks obtained from the Bloomington *Drosophila* Stock Center (National Institutes of Health P40OD018537) were used in this study. The NECD antibody, C458.2H, developed by S. Artavanis-Tsakonis, was obtained from the Developmental Studies Hybridoma Bank, created by the National Institute of Child Health and Human Development of the National Institutes of Health and maintained at the Department of Biology, University of Iowa (Iowa City, IA). Work from this study was funded by National Institutes of Health Grant R01-R56 NS059971 to F.R. and supported by a Fox Chase Cancer Center Core Grant and the University of Pennsylvania CAMB Graduate Program.

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