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Asymmetric Rab11 Endosomes Regulate Delta Recycling and Specify Cell Fate in the *Drosophila* Nervous System

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Summary

***Drosophila* sensory organ precursor (SOP) cells are a well-studied model system for asymmetric cell division. During SOP division, the determinants Numb and Neuralized segregate into the pIIb daughter cell and establish a distinct cell fate by regulating Notch/Delta signaling. Here, we describe a Numb- and Neuralized-independent mechanism that acts redundantly in cell-fate specification. We show that trafficking of the Notch ligand Delta is different in the two daughter cells. In pIIb, Delta passes through the recycling endosome which is marked by Rab11. In pIIa, however, the recycling endosome does not form because the centrosome fails to recruit Nuclear fallout, a Rab11 binding partner that is essential for recycling endosome formation. Using a mammalian cell culture system, we demonstrate that recycling endosomes are essential for Delta activity. Our results suggest that cells can regulate signaling pathways and influence their developmental fate by inhibiting the formation of individual endocytic compartments.**

Introduction

Drosophila external sensory (ES) organs are a well-established model for studying asymmetric cell division (Bellaiche and Schweisguth, 2001). ES organs consist of four cells, which are generated from a single sensory organ precursor (SOP) cell in a stereotyped lineage (Gho et al., 1999; Hartenstein and Posakony, 1989). The SOP first divides into a posterior pIIa and an anterior pIIb cell. While the pIIa cell gives rise to the two outer cells (hair and socket), the pIIb cell gives rise to the two inner cells (neuron and sheath). During SOP division, the proteins Numb and Neuralized localize to the anterior cell cortex (Le Borgne and Schweisguth, 2003; Rhyu et al., 1994). They segregate into the anterior pIIb cell and are required for the establishment of distinct fates in the two daughter cells. Asymmetric localization of Numb and Neuralized in SOP cells requires the conserved Par protein complex (Henrique and Schweisguth, 2003). Already in interphase, this complex localizes to the posterior cell cortex of the SOP cell (Bellaiche et al., 2001b). During mitosis, it is required for the asymmetric localization of Numb and Neuralized to the opposite, anterior, cortex. The Par complex contains a protein kinase called aPKC, which phosphorylates and inactivates a cytoskeletal protein called Lethal (2) giant larvae (Lgl). Through an unknown mechanism, Numb and Neuralized are recruited to the cortical area that is not occupied by the Par proteins and presumably contains the active, unphosphorylated form of Lgl (Betschinger et al., 2003). In mutants affecting the Par proteins (like *bazooka*) or their asymmetric localization (like *discs large*), Numb and Neuralized are no longer asymmetric (Bellaiche et al., 2001b; Le Borgne and Schweisguth, 2003; Roegiers et al., 2001). However, many SOP cells can still divide asymmetrically, indicating that a second, Par protein-independent mechanism might help to establish distinct cell fates in pIIa and pIIb.

Numb and Neuralized act by regulating the Notch pathway. Notch is a large transmembrane receptor that is activated by binding to its ligand Delta (Artavanis-Tsakonas et al., 1995). Upon ligand binding, a conformational change allows proteolytic cleavage of the receptor just outside the transmembrane domain. A second proteolytic event releases the intracellular domain, which subsequently translocates into the nucleus and acts as a transcriptional coactivator. Notch and Delta are expressed both in pIIa and pIIb, but Numb and Neuralized ensure that Notch signaling is only activated in the pIIa cell (Schweisguth, 2004). Both proteins influence the endocytic trafficking of components of the Notch pathway. Numb polarizes the distribution of α-Adaptin, an endocytic protein that recruits transmembrane proteins into Clathrin-coated vesicles and removes them from the plasma membrane (Berdnik et al., 2002). Numb can bind the Notch receptor (Guo et al., 1996) but also a four transmembrane protein called Sanpodo that is required for Notch signaling in the SOP lineage (O'Connor-Giles and Skeath, 2003). Whether Sanpodo or Notch is the critical target for Numb-induced endocytosis is not clear. Neuralized is an E3 ubiquitin ligase that ubiquitinates the Notch ligand Delta (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001). Ubiquitination stimulates the endocytosis of Delta in the pIIb cell and increases its ability to activate Notch on the neighboring pIIa cell (Le Borgne and Schweisguth, 2003). Upon activation, Notch is cleaved and its extracellular domain is trans-endocytosed together with Delta into the signal-sending pIIb cell (Klueg and Muskavitch, 1999; Parks et al.,

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2000). It is unclear what happens to Delta after endocytosis and how Delta endocytosis increases Notch signaling.

To gain further insight into the regulation of Notch/Delta signaling by endocytic trafficking and to identify a potential second Numb- and Neuralized-independent pathway, we have analyzed the subcellular distribution of Rab5-, Rab7-, and Rab11-positive endosomes during asymmetric cell division. While Rab5 and Rab7 are symmetric, Rab11 endosomes accumulate around the centrosome shortly after division in pIIb but not pIIa. Unlike Numb and Neuralized localization, Rab11 asymmetry is maintained in *discs large* mutants and upon overexpression of nonphosphorylatable Lgl, suggesting that it is established through a distinct pathway. We identify the protein Nuclear fallout as an important component of this pathway and show that it might influence cell fate by regulating recycling of the Notch ligand Delta. Our results identify a mechanism that can ensure the asymmetric outcome of cell division and contributes to cell-fate specification in the *Drosophila* peripheral nervous system.

Results

Asymmetric Distribution of the Rab11 Protein

Numb and Neuralized differentially regulate endocytosis in pIIa and pIIb. To analyze the distribution of endocytic compartments in the pIIa and pIIb daughter cells, we expressed GFP fusions to Rab GTPases (Zerial and McBride, 2001; Figure 1). Rab5-GFP (Wucherpfennig et al., 2003) was used to label early endosomes, Rab7-GFP (Entchev et al., 2000) for late endosomes, and Rab11-GFP for recycling endosomes (Figure 1A). GFP fusions were specifically expressed in SOP cells and visualized by time-lapse confocal microscopy of *Drosophila* pupae. Rab5-GFP labels small vesicles, which are symmetrically distributed in mitosis and behave similarly in both daughter cells (Figure 1A; see Movie S1 in the Supplemental Data available with this article online). Rab7-GFP labels fewer but larger endosomes that also do not show any asymmetric distribution (Figure 1A; Movie S2). Rab11-GFP, in contrast, shows a striking asymmetry (Figure 1A; Movie S3). During mitosis, Rab11-GFP-positive vesicles are symmetrically distributed and segregate equally into both daughter cells. Immediately after mitosis, Rab11-GFP distribution is similar in the two daughter cells. Around 3.5 min after the completion of cytokinesis, however, Rab11-GFP accumulates in a perinuclear region distal to the cleavage plane in only one of the two daughter cells (arrowhead in Figure 1A). In the other daughter cell, this accumulation is very weak or does not occur at all. Rab11-GFP accumulation is highly dynamic, and Rab11-positive vesicles rapidly move in and out of the perinuclear region (Figure 1B). A similar asymmetric accumulation can be seen using a Rab11 antibody indicating that the GFP fusion correctly reveals the localization of endogenous Rab11 (Figure 1E). Quantification of fluorescence intensity from 3D movies (Figure 1G; Movie S4, see Experimental Procedures for details) shows that total Rab11-GFP concentrations are similar in the two daughter cells, even when Rab11 accumulates asymmetrically (Figure 1H). Thus, the subcellular distribution but not the total amounts of Rab11 are different in the two daughter cells. When cytokinesis is inhibited by expression of a dominant-negative mutant of *rho* (*rhoN19*; Strutt et al., 1997), Rab11-GFP accumulates around both centrosomes (Figure 1D; Movie S8). Thus, the asymmetric localization of Rab11 is not due to an intrinsic difference between the two centrosomes but might be generated by a cytoplasmic factor present in only one of the two daughter cells.

During cellularization of *Drosophila* embryos, Rab11 colocalizes with the Golgi and with recycling endosomes (Pelissier et al., 2003). In SOP cells, Rab11-GFP does not colocalize with a Golgi marker (Figure 1F) but double staining with γ-Tubulin shows that Rab11 accumulates around the centrosome in one of the two daughter cells (Figure 1E). Recycling endosomes are located around the centrosome, suggesting that the Rab11 asymmetry reflects a different distribution of the Rab11 recycling endosomal compartment in the two daughter cells. In epithelial cells that are located right next to the SOP but do not undergo asymmetric cell division, Rab11-GFP accumulates around the centrosomes of both daughter cells (Figure 1C; Movie S5). This suggests that Rab11 is asymmetric because its pericentrosomal accumulation is suppressed in one of the two daughter cells.

Rab11 Asymmetry Is Established through a Distinct Mechanism

To determine whether Rab11 accumulation is suppressed in the pIIa or pIIb cell, we expressed Rab11-GFP in SOP cells together with an RFP fusion to the localization domain of Pon (partner of Numb; Figure 2). Pon is a Numb binding protein that colocalizes with Numb throughout mitosis (Lu et al., 1998), and a GFP fusion to its localization domain has been successfully used to follow Numb localization in real time (Lu et al., 1999). Like Numb, Pon-RFP localizes to the anterior SOP cell cortex in metaphase and segregates into the anterior pIIb cell (Figure 2A; Movie S6). Immediately after mitosis, the distribution of Rab11-GFP is similar in both daughter cells. Around 3.5 min after the completion of cytokinesis, however, perinuclear accumulation of Rab11-GFP is seen in the daughter cell that also inherits Pon-RFP (96% of n = 50 cells). Thus, Rab11 accumulation is inhibited in the pIIa cell, which does not inherit any of the known cytoplasmic determinants. Consistent with this, Rab11-GFP localization is unaffected in *numb* (Figure 2B) or *neuralized* (Figure 2C) mutants.

A molecular pathway for Numb and Neuralized localization has been identified (Bellaiche et al., 2001b; Betschinger and Knoblich, 2004). In SOP cells, the proteins Discs-large (Dlg) and Partner of Inscuteable (Pins) localize to the anterior cell cortex. They direct the Par complex to the opposite, posterior cortex. The Par-complex in turn phosphorylates Lgl and is required for localization of Numb, Neuralized, and Pon to the anterior cell cortex. To test whether Rab11 localization uses the same pathway, we analyzed Rab11-GFP in *dlg* mutants (Figure 2D) and upon expression of nonphosphorylatable Lgl (lgl3A, Figure 2E). In both situations,

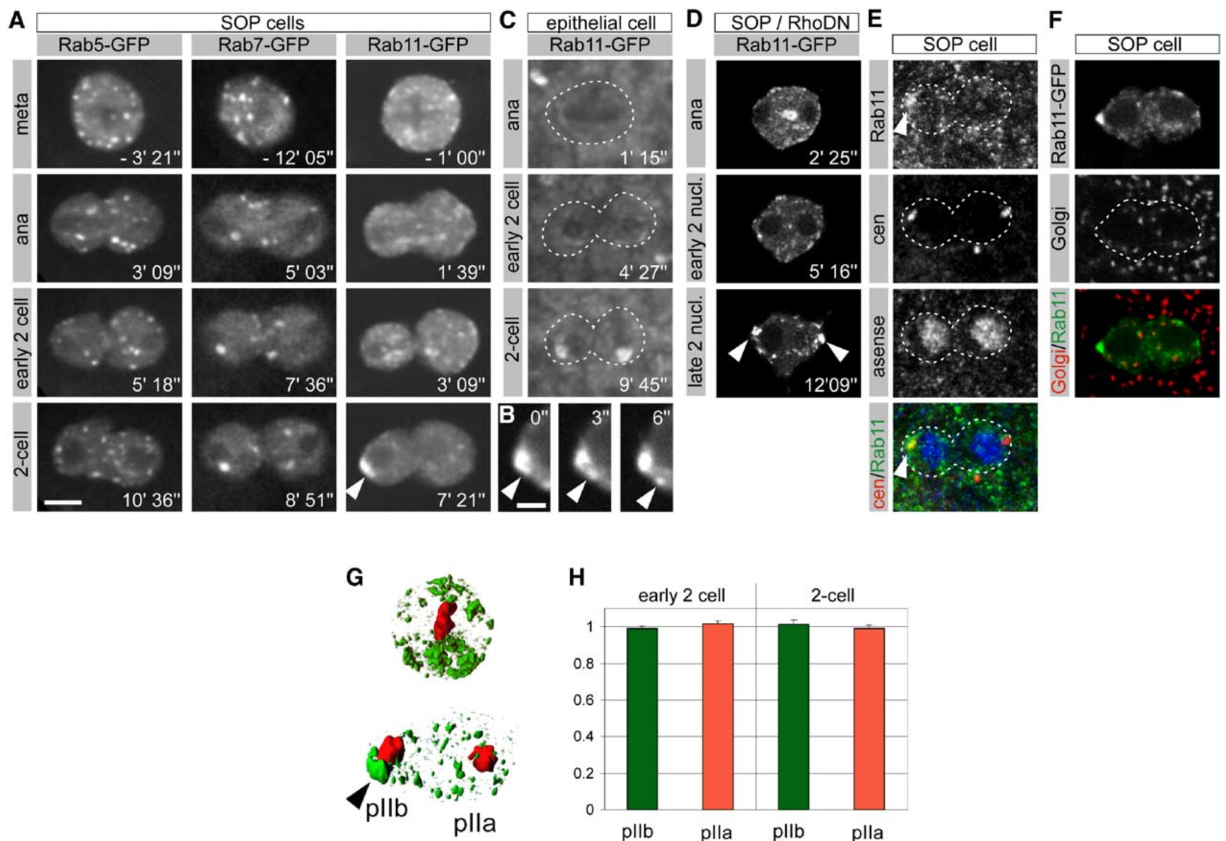

Figure 1. Distribution of Endocytic Compartments during Asymmetric Cell Division

(A) Rab5-GFP (early endosomes), Rab7-GFP (late endosomes), and Rab11-GFP (recycling endosomes) fusion proteins are expressed in SOP cells using *neuralized-Gal4* (Bellaiche et al., 2001a) and visualized through a spinning disk confocal microscope. Anterior is left; indicated times are relative to sister chromatid separation (t = 0). Asymmetric accumulation of Rab11-GFP is indicated by arrowhead. Scale bar is 5 μm.

(B) Three consecutive frames at 3 s intervals showing Rab11-GFP-positive vesicles (arrowhead) emanating from the recycling endosome. Scale bar is 2 μm.

(C) Rab11-GFP expressed in epithelial cells using *daughterless-Gal4*. Dashed line marks cell outlines; arrowhead marks Rab11-GFP accumulation in both daughter cells.

(D) In an SOP where cytokinesis has been impaired by the expression of *rhoN19* (RhoDN), Rab11-GFP accumulates around both centrosomes (arrowheads).

(E) SOP cell after division stained for endogenous Rab11 (green), γ-Tubulin (red), and the SOP marker Asense (blue).

(F) Rab11-GFP-expressing SOP cell (GFP in green) stained with the Golgi marker anti-gp120 (red). Dashed line marks cell outline.

(G) 3D reconstruction of Rab11-GFP and Histone-RFP in metaphase (top) and after mitosis (bottom), arrowhead: asymmetric Rab11 accumulation.

(H) Rab11-GFP intensity (reflecting Rab11-GFP concentration) in pIIa and pIIb when Rab11 is symmetric (early two cell) or asymmetric (two cell). Mean and SEM of four experiments are shown as a fraction of average Rab11-GFP intensity in both cells.

Pon-RFP fails to localize asymmetrically and is found in both daughter cells. Rab11-GFP, however, still accumulates in only one of the two daughter cells (Figures 2D and 2E) suggesting that it is established by a distinct mechanism. The polarity of SOP cells can be completely inverted by expression of the adaptor protein Inscuteable (Bellaiche et al., 2001b). Upon *inscuteable* expression, Par-proteins localize anteriorly and both Numb and Neuralized (data not shown) segregate into the posterior daughter cell. We analyzed Pon-RFP and Rab11-GFP in SOP cells expressing *inscuteable*. In 42% of 46 *inscuteable* overexpressing SOP cells, Pon-RFP and Rab11-GFP are still seen in the same daughter cell (Figure 2F). In 34%, however, the Rab11 asymmetry is lost (Figure 2F′), and in 24%, Rab11-GFP accumulates in the cell that does not inherit Pon-RFP (Figure 2F′′). Thus, Pon-RFP and Rab11-GFP respond differently to an inversion of Par protein polarity. While Pon-RFP follows the reversed polarity and segregates into the posterior daughter cell, Rab11 asymmetry is essentially randomized between the two daughter cells. These experiments demonstrate that asymmetric localization of Par proteins and Lgl phosphorylation are not essential for Rab11 asymmetry and suggest that a distinct mechanism is used to suppress Rab11 endosome formation in the pIIa cell. This mechanism might act redundantly with the Par proteins to establish Rab11 asymmetry. Alternatively, it can be manipulated by the presence of Inscuteable in a way that is independent of Par protein asymmetry.

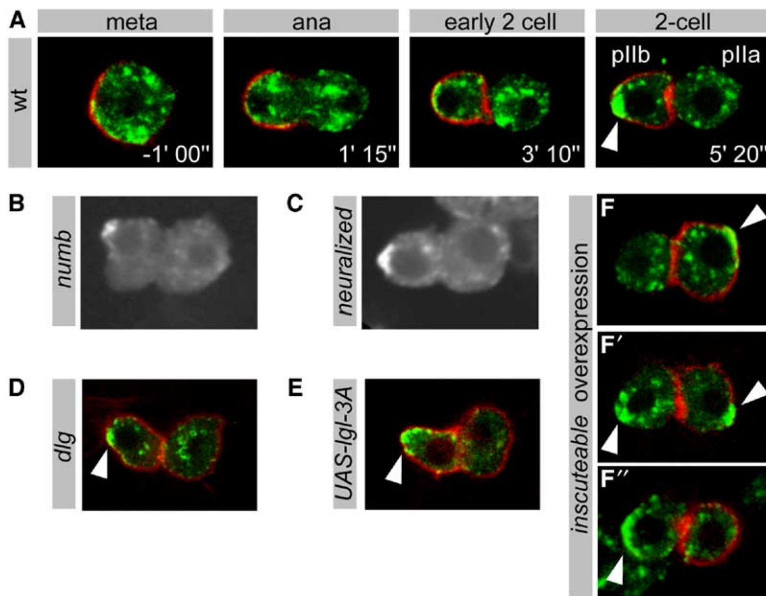

Figure 2. Rab11 Asymmetry Can Be Uncoupled from Known Pathways of Asymmetric Cell Division

(A) Rab11-GFP (green) and Pon-RFP (red) are coexpressed in SOP cells using *neuralized-Gal4*. Both proteins accumulate in the pIIb cell. Times are as in Figure 1.

(B and C) Rab11-GFP is still asymmetric in *numb* or *neuralized* mutant clones generated using the MARCM system.

(D–F) Rab11-GFP and Pon-RFP expressing SOP cells from *dlg*^{1P20} mutants (D) or from pupae expressing nonphosphorylatable *lgl* (lgl3A, [E]) or *inscuteable* (F) from *neuralized-Gal4*. Anterior is left. In *dlg* mutant or *lgl3A* overexpressing cells, Pon-RFP is symmetric but Rab11-GFP is asymmetric. Upon *inscuteable* overexpression, Pon-RFP enters the posterior daughter cell but Rab11-GFP is randomized. In 42% (n = 46 cells), it is posterior (F), in 34%, equal (F′), and in 24%, Rab11-GFP accumulates in the anterior cell (F″).

Rab11 Asymmetry Involves Nuf Accumulation on One of the Two Daughter Cell Centrosomes

Our results indicate that an important regulator of Rab11 endosomes is missing or inhibited in the pIIa daughter cell. Expression of this factor in both daughter cells might rescue Rab11 accumulation in the pIIa cell. To identify this factor, we overexpressed genes in SOP cells using a collection of EP lines (Rorth et al., 1998) and screened for changes in Rab11 localization. From a collection of lines previously shown to cause defects in ES organ formation (Abdelilah-Seyfried et al., 2000), two lines affect Rab11-GFP localization when crossed to neuralized-Gal4 (Figure 3A; Movie S7). In the lines EP(3)3324 and EP(3)3339, Rab11-GFP accumulates around the centrosome in metaphase but is lost from centrosomes in anaphase. After mitosis, Rab11-GFP localization resumes, but in contrast to wild-type SOP cells, this occurs in both daughter cells. EP(3)3339 and EP(3)3324 are inserted respectively 482 bp upstream of the gene *nuclear fallout* and 54 bp upstream of an alternative start of *nuclear fallout* (*nuf*; Abdelilah-Seyfried et al., 2000), the *Drosophila* homolog of Arfophilin 1 (also called Eferin or Rab11-FIP3) and Arfophilin 2. Like its vertebrate homologs (Hales et al., 2001; Hickson et al., 2003; Meyers and Prekeris, 2002; Prekeris et al., 2001), Nuf concentrates at centrosomes (Riggs et al., 2003) and binds to Rab11. In early embryos, Nuf is required for localizing Rab11 to the recycling endosome and for recruiting proteins to the plasma membrane during cellularization (Rothwell et al., 1999). To test whether Nuf localization is compatible with a role in generating Rab11 asymmetry, we used a Nuf antibody (Rothwell et al., 1998) to follow the protein through SOP division. During mitosis, Nuf is located in the cytoplasm and on vesicles that show no obvious asymmetric distribution (data not shown). After mitosis, Nuf concentrates at the centrosome of only one of the two daughter cells at the time when Rab11 becomes asymmetric (Figure 3B). In symmetrically dividing epithelial cells (Figure 3C) the protein is seen at the centrosomes of both daughter cells. Upon overexpression, Nuf distribution is similar in pIIa and pIIb (Figures 3D and 3E), suggesting that the mechanism inhibiting Nuf localization in pIIa can be saturated. These experiments suggest that the recruitment of Nuf to the centrosome is inhibited in the pIIa cell and this is responsible for the asymmetric distribution of Rab11. Overexpression of *nuf* in SOP cells provides a useful tool to induce Rab11 accumulation in the pIIa cell and study the functional relevance of Rab11 asymmetry. This is particularly valuable since loss-of-function studies are complicated by the fact that the available *nuf* alleles are specific for early embryonic development and *nuf* null alleles are expected to be cell lethal—as are null alleles of *rab11*.

Endocytosed Delta Is Trafficking through Recycling Endosomes in pIIb

Rab11 plays a well-established role in regulating protein recycling back to the plasma membrane (Ullrich et al., 1996). Both Rab11 and Nuf are required for protein recycling in early *Drosophila* embryos (Dollar et al., 2002; Pelissier et al., 2003; Riggs et al., 2003). To test whether Notch or Delta recycling is different in pIIa and pIIb, we analyzed dividing SOP cells in which Rab11-GFP is asymmetric (Figure 4). While Notch-positive vesicles did not show any polarized distribution (data not shown), Delta-positive vesicles are often found around the Rab11-positive centrosome (Figure 4A). Image quantifications revealed a significant enhancement of anti-Delta staining around the centrosome of pIIb (Figure S1). Furthermore, a higher fraction of Delta-positive vesicles is positive for Rab11 in pIIb (Figure 4C) while in pIIa, more Delta vesicles stain for Hrs, a marker for the degradative pathway (Figures 4B and 4C).

To directly monitor Delta trafficking in pIIa and pIIb, we adapted an endocytosis assay in which endocytosed Delta protein is followed by a bound antibody (Le Borgne and Schweisguth, 2003). We modified the assay

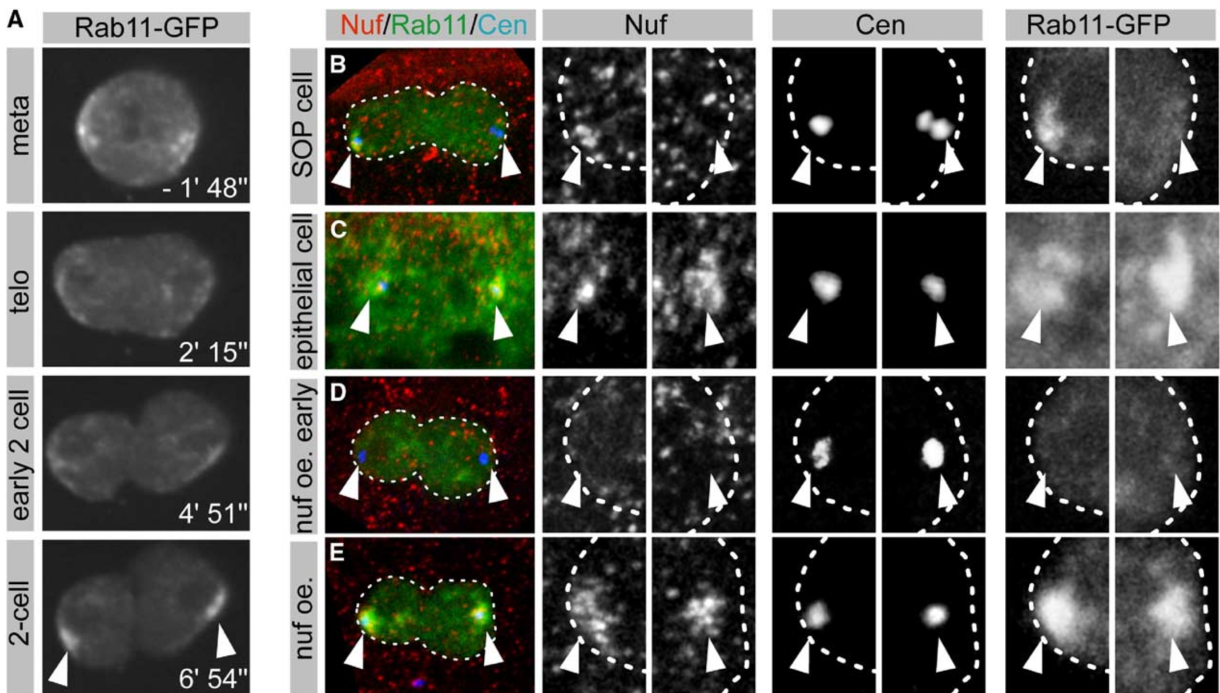

Figure 3. Nuf Is Involved in Generating Rab11 Asymmetry

(A) Rab11-GFP in an SOP cell overexpressing *nuf* from EP(3)3339. Rab11-GFP accumulation is indicated in both cells by arrowheads; time is as in Figure 1.

(B–F) Immunostainings of dividing cells expressing Rab11-GFP. Rab11-GFP (green), Nuf (red), and γ-Tubulin (blue) are shown. Right side shows Nuf, γ-Tubulin (cen), and Rab11-GFP at higher magnification. Dashed lines indicate cell outlines. Arrowheads indicate position of the centrosome.

(B) In asymmetrically dividing SOP cells, Nuf and Rab11-GFP accumulate around the centrosome in pIIb but not pIIa.

(C) In epithelial cells, Nuf and Rab11-GFP concentrate around both centrosomes.

(D and E) In SOP cells overexpressing *nuf*, Nuf and Rab11-GFP are not at the centrosome immediately after division (D). Later after mitosis, the two proteins accumulate at both centrosomes (E).

to allow a time course of Delta trafficking (see Experimental Procedures) and analyzed only those cells in which Rab11-GFP is asymmetric. Five minutes after endocytosis, Delta is found in many small vesicles (Figure 4D) that are equally distributed in the two daughter cells. These vesicles do not colocalize with Rab11 (Figure 4E) and presumably are early endosomes. Fifteen minutes (Figure 4D) or thirty minutes (data not shown) after endocytosis, Delta is found in larger endosomes and in smaller vesicles that often accumulate around the Rab11-positive centrosome (arrowhead in Figure 4D). In pIIa, only very few of the Delta vesicles colocalize with Rab11 (Figure 4E) and are located around the centrosome (Figure S1). In pIIb, however, around 30% of Delta-positive vesicles are Rab11 positive (Figure 4E), indicating that they are recycling endosomes. When Rab11 accumulation is induced in pIIa by expression of *nuf*, however, Delta enters Rab11-positive vesicles in both daughter cells (Figure 4F). Thus, Delta passes through a Rab11-positive—presumably recycling endosomal—compartment in pIIb but not pIIa.

To directly test an involvement of Rab11 in Delta trafficking, a dominant-negative form of *rab11* (*rab11SN*) was expressed in SOP cells. To avoid cell death and cytokinesis defects that arise from unconditional expression of *rab11SN* (data not shown), we used transgenic flies in which tissue-specific expression of *rab11SN* can be induced by Flp-induced recombination (see Experimental Procedures). Upon expression of *rab11SN*, Delta accumulates in abnormally enlarged, Hrs-positive vesicles (arrowheads in Figure 4B, Rab11SN panels). We conclude from this experiment that Delta normally traffics through the recycling pathway and this trafficking is inhibited by dominant-negative Rab11.

Recycling Endosomes Regulate Delta Activity

Trafficking through a recycling compartment was recently postulated to be important for Delta activation (Wang and Struhl, 2004). Rab11 loss-of-function analysis is complicated by its well-demonstrated role in cytokinesis (Wilson et al., 2005). Hypomorphic *rab11* alleles are available but do not show cell fate transformations in ES organs (Jankovics et al., 2001). However, an accompanying manuscript in *Developmental Cell* demonstrates that the Rab11 effector Sec15 is required for the activation of Notch signaling in the pIIb cell (Jafar-Nejad et al., 2005), possibly due to a defect in Delta trafficking.

To directly characterize the role of recycling endosomes in Delta activation, we used a mammalian cell culture assay in which lymphoid progenitors are cocultured with the bone marrow stromal cell line OP9 trans-

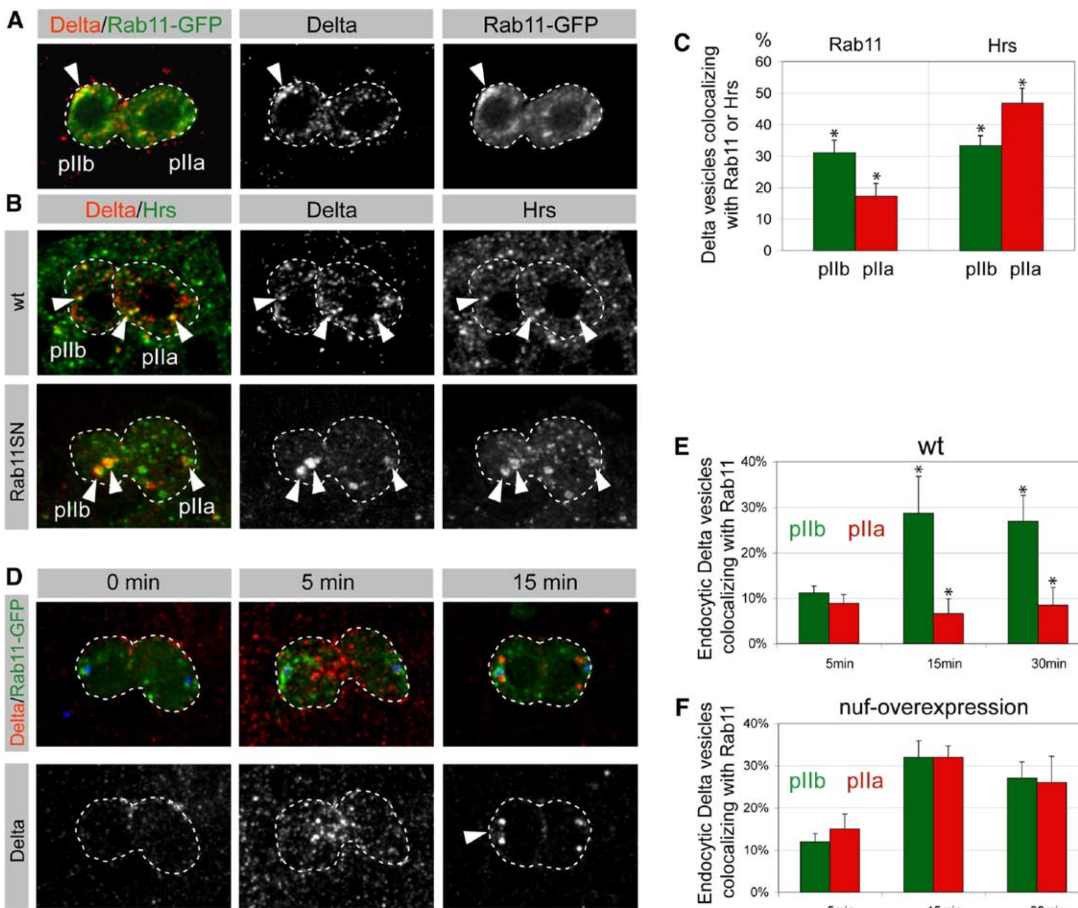

Figure 4. Delta Trafficking Is Different in pIIa and pIIb

(A) SOP cells expressing Rab11-GFP stained for GFP (green) and Delta (red). Dashed line outlines the cell. Delta vesicles are enhanced in pIIb where Rab11-GFP accumulates (arrowhead).

(B) wt (lower panels) or *rab11SN*-expressing (upper panels) SOP cells stained for Hrs (green) and Delta (red). In wt cells, more Delta vesicles colocalize with Hrs in pIIa than in pIIb (arrowheads). In cells expressing *rab11SN*, Delta accumulates in enlarged vesicles that colocalize with Hrs (arrowheads). Note that Delta asymmetry is not visible because the focus is not on accumulating Rab11-GFP.

(C) Quantification of the results in (A) and (B) (average and SEM of n = 15 cells). All Delta vesicles were analyzed for colocalization with Hrs or Rab11-GFP in wt.

(D) Time course of Delta endocytosis in pIIa and pIIb (see Experimental Procedures). Only the small fraction of pIIa and pIIb cells with clearly asymmetric Rab11-GFP was analyzed. Rab11-GFP (green), endocytosed anti-Delta (red), and anti-centrosomin (blue) are shown 0′, 5′ and 15′ after temperature shift to 25°C.

(E) Quantification of results in (D) (average and SEM). Six to nine Z stacks were analyzed for each time point; total number of Delta vesicles at 5′, 15′, and 30′ is 25 ± 4, 6.4 ± 1, and 8.5 ± 1.7 in pIIa and 27 ± 5, 6.0 ± 1, and 7.2 ± 1.6 in pIIb.

(F) Same experiment in pupae overexpressing *nuf* (EP(3)3339). Asterisks indicate a statistically significant difference ($p < 0.05$) between pIIa and pIIb (Student's t test, two-tailed equal variance).

fected with the Delta homolog Delta-like-1 (Dll1; Hoflinger et al., 2004; Schmitt and Zuniga-Pflucker, 2002). Pro-B cells from Pax5$^{-/-}$ mice lack the ability to differentiate into B cells (Nutt et al., 1999). When Notch is activated in these cells by Dll1 expressed in OP9 cells, however, Pax5$^{-/-}$ pro-B cells differentiate into T cells (Hoflinger et al., 2004). The first sign of Notch activation is the upregulation of the c-Kit receptor (Hoflinger et al., 2004). In OP9 cells, Dll1 is mostly found in vesicles (Figures 5A and 5B). These vesicles do not colocalize with internalized Transferrin (Figure 5C), a marker for recycling endosomes. Upon expression of dominant-negative Rab11, trafficking from the recycling endosome to the plasma membrane is strongly reduced (Ullrich et al., 1996). Under these conditions, Transferrin accumulates in recycling endosomes that also contain Dll1 and Rab11 (Figures 5A and 5D) and are located around the centrosome (Figure 5B, arrowhead). We used the number of c-Kit-positive pro-B cells attached to OP9 cells as a readout for the ability of Dll1 to activate Notch (Figures 5E and 5F). On average, 1.2 such cells are detected per Dll1-expressing OP9 cell (Figure 5F, green bar representing untransfected control cells in Dll1-Rab11SN or Dll1-Rab7NI). In OP9 cells cotransfected with dominant-negative Rab11 (Figure 5F, Dll1-Rab11SN, red bar), however, this number drops to 0.4—the background level observed for OP9 cells not expressing Dll1 at all (Figure 5F, con). Expression of a

dominant-negative form of Rab7, however, has no effect on pro-B cell attachment (Figure 5F, Rab7NI, red bar). Since Rab7 regulates trafficking to late endosomes, we conclude that the degradative pathway is not involved in regulating Dll1 activity in OP9 cells. Thus, trafficking through recycling endosomes is essential for the signaling capacity of the mouse Delta homolog Dll1.

Rab11 Asymmetry Is Involved in Cell-Fate Specification

To test whether Rab11 asymmetry is important for cell-fate specification, we induced Rab11 accumulation in the pIIa cell by *nuf* expression (Figures 5G, 5G′, and 5H). We used postorbital ES organs, which can easily be scored in fairly high numbers. Cell-fate transformations upon *nuf* overexpression were described before (Abdelilah-Seyfried et al., 2000), but surprisingly, they do not occur at high frequency. Such transformations can, however, be observed upon coexpression of constitutively active Rab11. Upon expression of nonphosphorylatable *lgl*, Numb and Neuralized asymmetry are disrupted, but most ES organs still develop normally. When both pathways are disrupted by coexpression of *lgl3A* and *nuf*, however, a large fraction of ES organs shows cell-fate transformations that are consistent with a higher level of Delta activity in pIIa. Lineage analysis shows cell-fate transformations in 44% of postorbital ES organs, and in 18% of these, pIIb cells are transformed into pIIa cells (6% in ES organs expressing *lgl3A* alone). Twenty-five percent of the cell fate transformations affect the first (SOP) while 75% affect the second (pIIa) division, indicating that Rab11 asymmetry also plays a role in other divisions of the SOP lineage. Taken together, our results suggest that two partially redundant pathways exist to generate asymmetry in the SOP lineage: the Par proteins phosphorylate Lgl to direct Numb and Neuralized into the pIIb cell where they repress Notch or activate Delta, respectively. In the pIIa cell, inhibition of Nuf and Rab11 inhibits Delta by preventing its trafficking through the recycling endosome.

Discussion

Signal Transduction Can Be Regulated by Inhibiting Protein Recycling

In many signal transduction pathways, vesicle trafficking of ligands or receptors are key regulatory events (Gonzalez-Gaitan, 2003; Piddini and Vincent, 2003; Sorkin and Von Zastrow, 2002). In most cases, ubiquitination or binding to adaptor proteins recruits ligands or

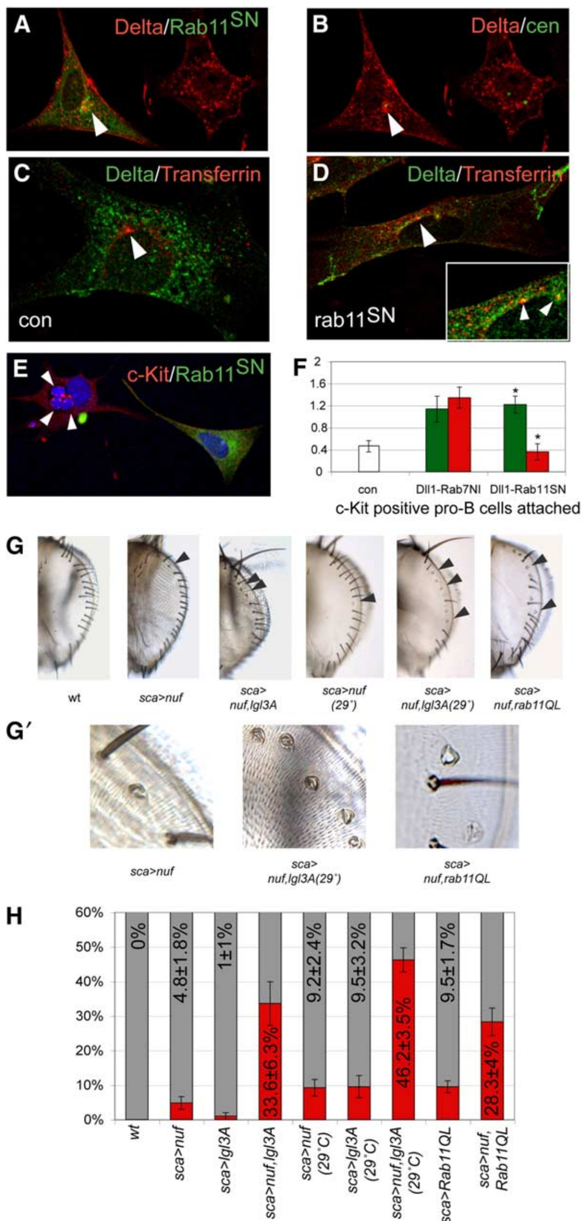

Figure 5. Functional Analysis of Delta Recycling

(A and B) Delta homolog Dll1 (red) expressed in mouse OP9 cells is in vesicles (right cell) but accumulates around the centrosome (green in [B]) 24 hr after transfection with dominant-negative Rab11SN-GFP (left cell, GFP green in [A]).

(C and D) Dll1 (green) accumulates in the recycling endosome (marked by internalized Transferrin, red, arrowheads) 24 hr after transfection with Rab11SN-GFP (D) but not in untransfected cells (C). Inset in (D) is high magnification of the same cell.

(E) c-Kit-positive pro-B cells are found on Dll1 expressing control (left, arrowheads) but not on Rab11SN-GFP (green)-transfected OP9 cells.

(F) Average number of c-Kit-positive pro-B cells attached to each OP9 stromal cell. Con shows untransfected OP9 cells (white bar), while all other bars are OP9 cells stably expressing Dll1. Dll1-expressing cells were transiently transfected with GFP-fused dominant-negative Rab7 (Dll1-Rab7NI) or Rab11 (Dll1-Rab11SN). Red bars are untransfected and green bars are transfected cells. Between 24 and 79 cells were analyzed for each bar. Statistically significant differences ($p < 0.01$ in Student's t test, two-tailed, equal variance) are marked by asterisks.

(G) Postorbital bristles of wt flies or *scabrous-Gal4* flies overexpressing *nuf* (EP(3)3324) with and without nonphosphorylatable *lgl* (raised at 25°C or at 29°C). Arrowheads indicate ES organs containing multiple sockets but no hair indicative of cell-fate transformations due to higher Notch activity. (G′), high magnification views of postorbital bristles as shown in (G).

(H) Quantification of the experiment in (G). Red bars and numbers indicate average percentage of transformed ES organs. Error bars are SEM.

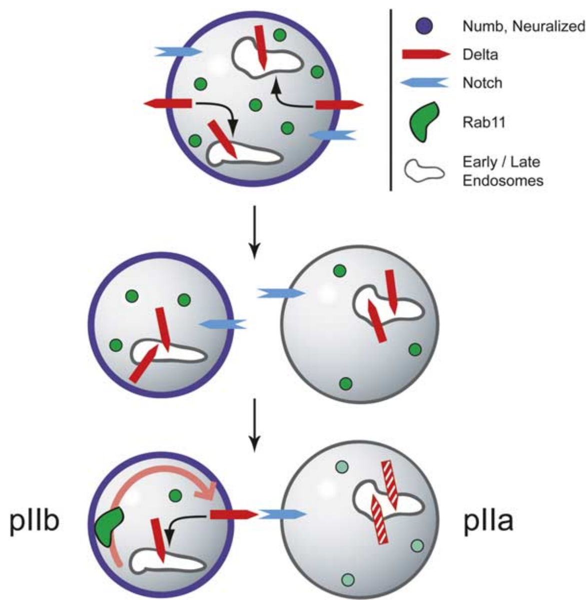

Figure 6. Model for Delta Regulation by Rab11-Dependent Protein Recycling

Delta protein endocytosed before mitosis is found in Rab11-positive endosomes after mitosis in pIIb but not in pIIa.

receptors into particular endocytic compartments to enhance or suppress their signaling capacity. Our results suggest that cells can also regulate signal transduction pathways by controlling the formation or distribution of whole endocytic compartments. After SOP division, Rab11-positive vesicles accumulate around the centrosome in this cell but not in pIIa. Rab11 plays a well-documented role in controlling vesicular protein transport through recycling endosomes to the plasma membrane (Zerial and McBride, 2001). Dominant-negative forms of Rab11 inhibit the recycling of endocytosed Transferrin receptors (Ren et al., 1998; Ullrich et al., 1996) or recruitment of H^+-K^+-ATPase to the plasma membrane (Duman et al., 1999) suggesting that Rab11 regulates trafficking of vesicular cargo through the recycling endosomal compartment. In SOP cells, the asymmetric localization of Rab11 reflects a different ability of pIIa and pIIb cells to recycle the Notch ligand Delta. Rab11 asymmetry is observed 3.5 min after cytokinesis but Delta is in recycling endosomes only 15 min after endocytosis. Thus, the protein is endocytosed before mitosis and recycles back to the plasma membrane in pIIb but not in pIIa (Figure 6). In pIIa, we observe more Delta/Hrs double-positive vesicles, indicating that the protein enters a late-endosomal pathway.

Several observations indicate that passage through recycling endosomes is essential for Delta to signal. In OP9 cells, inhibition of recycling endosomes dramatically reduces Delta signaling capacity. Similarly, blocking the recycling pathway by overexpression of a dominant-negative form of Rab11 in SOP cells causes relocalization of Delta into enlarged late endosomes. In *Drosophila* wing discs, Delta has been postulated to pass through a specific endocytic recycling pathway to acquire signaling capacity (Wang and Struhl, 2004). Finally, an accompanying manuscript (Jafar-Nejad et al., 2005) demonstrates that the Rab11 binding partner Sec15 is required both for Delta trafficking and Notch activation in the SOP lineage. Sec15 is a component of the exocyst and was shown to be a Rab11 effector (Zhang et al., 2004). Although Sec15 is not asymmetric itself, it is conceivable that the higher amounts of GTP bound Rab11 in pIIb increase its activity in delivering Delta to the plasma membrane. A difference between Delta trafficking in pIIa and pIIb has been observed before (Le Borgne and Schweisguth, 2003), but both Delta/Hrs vesicles and total number of Delta vesicles were actually higher in pIIb in these previous experiments. While these experiments analyzed the whole two cell stage, we focus on the short time interval right after mitosis where Rab11 is asymmetric. This explains the different outcome and might in fact indicate that pIIb cells switch from an initial phase where Delta is recycled to a later phase where trafficking is regulated by *neuralized*-dependent endocytosis.

An Alternative Pathway for Asymmetric Cell Division

Although many cell types in different organisms undergo asymmetric cell division, only one mechanism has been identified so far that directs this important biological process in animals. This mechanism involves the Par proteins, which phosphorylate Lgl on one side and direct cell fate determinants to the opposite side of the cell cortex. Several results indicate that other pathways might exist: in dividing progenitor cells of the mammalian brain, Numb segregates into one of the two daughter cells (Zhong et al., 1996) and is required for lineage specification (Petersen et al., 2002). However, some of these divisions are asymmetric, although their orientation predicts that Numb would be inherited by both daughter cells (Kosodo et al., 2004; Wodarz and Huttner, 2003). In *Drosophila* SOP cells, *lgl3A* overexpression affects both Numb and Neuralized localization (data not shown) but has only a minor influence on the asymmetric outcome of the division (Figure 5). Our results indicate that the asymmetric distribution of Rab11 is established through a distinct pathway. First, Rab11 asymmetry is unaffected in SOP cells overexpressing *lgl3A*. Second, Rab11 is still asymmetric in *dlg* mutants where Par proteins do not localize and Numb and Neuralized segregate into both daughter cells. Third, Rab11 asymmetry can be uncoupled from Numb and Neuralized localization by the expression of *inscuteable*. Finally, the events responsible for Rab11 asymmetry seem to occur in the pIIa cell, but none of the known determinants is inherited by this daughter cell. Although our observations could also be explained if Numb or Neuralized would relieve a general suppression of recycling endosome formation in the SOP lineage, this is unlikely since Rab11 asymmetry is unaffected in *numb* or *neuralized* mutants. More likely, an unknown factor could act on Nuf or the centrosome in the pIIa cell to prevent Rab11 accumulation. Nuf localization is cell cycle regulated, and a key regulatory component could be missing in pIIa. For example, Nuf is highly phosphorylated (Rothwell et al., 1998) and dif-

ferential activity of a kinase or phosphatase could prevent its pericentriolar localization in the pIIa cell. Homologs of Nuf exist and bind to Rab11 in vertebrates. Their expression pattern has not yet been described but it will be interesting to determine whether these homologs regulate Notch signaling in vertebrates and are responsible for asymmetric cell division in the mammalian brain.

Experimental Procedures

Constructs, Flystrains, and Antibodies

For Rab11-GFP, the *rab11* coding region was cloned from the EST LD14551 into the Xho1 site of *pEGFP-C3* (Clontech). The resulting fusion was cloned into the Xba1 site of *pUAST*. To generate constitutively active *rab11*, Gln70 was changed to Leu (*rab11QL*) in LD14551 using the quickchange mutagenesis kit (Stratagene) and mutant forms were cloned into *pUAST*. Flp inducible dominant-negative *rab11*, where the Ser25 was changed to Asn (*rab11SN*), will be described elsewhere (E. Marois and S. Eaton, unpublished data). Briefly, *rab11SN* is expressed under UAS control with a Flp-removable Stop cassette between the coding region and the UAS sites. For *rab11SN* expression (Figure 4B), the flip-out cassette was removed using *hsFlp* (37°C for 90 min at 0–1 hr apf) and expression was driven by *neuralized*-Gal4. To generate *Pon-RFP* and *Histone-RFP*, the Pon localization domain (Lu et al., 1999) or the entire coding region of Histone 2A were PCR-cloned into *pDONR* (Invitrogen) by BxP recombination. LxR recombination with the destination vectors *pUAST-DEST14* (Pon) or *pUAST-DEST15* (Histone) containing monomeric red fluorescent protein (RFP; Campbell et al., 2002) generated *pUAST-mRFP1::Pon.LD* or *pUAST-His::mRFP1*, which were used for transgenic flies. Pon-RFP colocalizes with Pon-GFP throughout mitosis. Ubx-Flp was generated by inserting two copies of the Ubx enhancer fragment PBX-41 (Christen and Bienz, 1994; gift from M. Bienz) into *pCaSpeR-hsFlp*, which carries the Flp recombinase under control of a complete *hsp70* promoter. *Ubx-Flp* induces recombination in all imaginal discs. Other fly lines were as follows: Rab5-GFP (Wucherpfennig et al., 2003), Rab7-GFP (Entchev et al., 2000), UAS-insc (Kraut et al., 1996), UAS-lgl3A (Betschinger et al., 2003), UAS-RhoN19 (Strutt et al., 1997), *neuralized*-Gal4 (Bellaiche et al., 2001a), *scabrous*-Gal4 (Brand and Perrimon, 1993), *daughterless*-Gal4 (Brand and Perrimon, 1993), and *dlg*^{1P20} (Bellaiche et al., 2001b). EP(3)3324 and EP(3)3339 were obtained from the Szeged *Drosophila* stock center. *Numb*^{2} (Figure 2B) and *neuralized*^{P72} (Figure 2C) mutant clones were created using MARCM system (Lee et al., 2000) and *Ubx-Flp*. Rabbit anti-Rab11 was generated against the peptide H2N-CQKQIRDPPEGDVIRPS-CONH2 coupled to KLH and affinity purified. The following other antibodies were used: guinea pig anti-Delta (Huppert et al., 1997), rabbit anti-Nuf (Bill Sullivan), guinea pig anti-Hrs (Hugo Bellen), rabbit anti-centrosomin (Tom Kaufmann), guinea pig anti-Asense (Y.N. Jan), mouse anti-γ-Tubulin (Sigma), mouse anti-gp120 (Calbiochem), mouse anti-Notch-extra (DSHB) rabbit anti-GFP (Abcam).

Time-Lapse Microscopy and Image Quantification

For time-lapse microscopy, *Drosophila* pupae were dissected and imaged essentially as described (Bellaiche et al., 2001a). Monochrome movies were generated on a spinning disk confocal microscope using 3 s time intervals. Two color movies were generated on a Zeiss LSM510 confocal microscope using 5 s time intervals. Images were processed using Metamorph (Universal Imaging) and ImageJ software. For 3D reconstructions and quantifications of Rab11-GFP, image stacks were taken every 30 s at 1.3 μm intervals. Gauss-filtered and thresholded images were used for surface rendering (Figure 1F; Movie S4) using Imaris software (Bitplane AG). Rab11-GFP intensity was calculated for all Z-planes using ImageJ. Delta fluorescence intensity (Figure S1) was measured after background subtraction in fixed pupae expressing Rab11-GFP and stained for Delta and centrosomin. Average Delta fluorescence intensity within a 1 μm sphere around the centrosome was measured using Metamorph software and compared to the average fluorescence intensity in the same focal planes.

Delta Endocytosis Assay

The assay was carried out essentially as described (Le Borgne and Schweisguth, 2003) but modified to allow a time course of Delta trafficking. *neurGal4*>>*Rab11-GFP* Pupae were preincubated for 15 min at 4°C with monoclonal anti-Delta (1:10, DSHB) and switched to 25°C for the times indicated. After fixation, endocytosed Delta antibodies were detected by a secondary antibody. Only cells in which Rab11-GFP is clearly asymmetric were analyzed. Around 100 pupae were dissected for each time point.

Mammalian Cell Culture Experiments

OP9 cells stably expressing GFP (note that soluble GFP is lost during fixation) or Dll1 and GFP (described in Schmitt and Zuniga-Pflucker [2002]) were plated 36 hr and transfected with Rab7NI-GFP or Rab11SN-GFP (provided respectively by Jean Gruenberg and Bruno Goud) using Lipofectamine (Invitrogen) 20–24 hr prior to the experiment. For Dll localization, cells were stained using rabbit anti-Delta (H-265, Santa Cruz Biotechnologies). Transfected cells (red bars in Figure 5F) were identified by GFP fluorescence or by HA staining using monoclonal rat anti-HA (3F10). Labeling of recycling endosomes by endocytosis of Transferrin-Cy3 (Molecular Probes) was as described (Gagescu et al., 2000). For pro-B cell experiments (Hoflinger et al., 2004), 10^5 Pax5$^{-/-}$ pro-B cells were incubated with 10^4 OP9 cells for 16 hr. Cells were washed in PBS, fixed in −20°C MeOH for 4 min and stained using biotinylated anti-c-Kit mAb (CD117 (2B8)) and Cy3-streptavidin (Molecular Probes) using standard procedures. Culture media were essentially as described (Hoflinger et al., 2004).

Statistical Analysis

Pooled data were analyzed for equal variance using an f test. Statistical significance was determined using a Student's t test (two-tailed, equal variance).

Supplemental Data

Supplemental Data include one figure and eight movies and can be found with this article online at http://www.cell.com/cgi/content/full/122/5/763/DC1/.

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