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Report

Uninflatable and Notch Control the Targeting of Sara Endosomes during Asymmetric Division

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Summary

Cell fate decision during asymmetric division is mediated by the biased partition of cell fate determinants during mitosis [1–6]. In the case of the asymmetric division of the fly sensory organ precursor cells, directed Notch signaling from pIIb to the pIIa daughter endows pIIa with its distinct fate [1–6]. We have previously shown that Notch/Delta molecules internalized in the mother cell traffic through Sara endosomes and are directed to the pIIa daughter [6]. Here we show that the receptor Notch itself is required during the asymmetric targeting of the Sara endosomes to pIIa. Notch binds Uninflatable, and both traffic together through Sara endosomes, which is essential to direct asymmetric endosomes motility and Notch-dependent cell fate assignation. Our data uncover a part of the core machinery required for the asymmetric motility of a vesicular structure that is essential for the directed dispatch of Notch signaling molecules during asymmetric mitosis.

Results and Discussion

The Notch signaling pathway plays multiple roles in organisms ranging from flies and worms to mammals [7]. A powerful model system to elucidate the cell biology of Notch signaling is the *Drosophila* sensory organs. Each sensory organ precursor (SOP) cell divides asymmetrically to produce a pIIa cell and a pIIb daughter cell, which perform directed Notch signaling: pIIb signals to pIIa. Four independent endocytic mechanisms control asymmetric signaling in the SOP. These include asymmetric endocytic events mediated by the E3 ubiquitin ligase Neuralized [1], recycling endosomes [2], and the endocytic adaptors alpha- and gamma-adaptin together with Numb [3–5].

During SOP cytokinesis, a fourth mechanism involves a population of endosomes marked by the adaptor protein Sara. Sara endosomes contain as cargo a pool of endocytosed Notch and Delta molecules. We showed that Notch and Delta reach the Sara endosome 20 min after their endocytosis in the SOP and that this pool is dispatched into pIIa during cytokinesis [6]. In contrast, the pools of Notch in endosomal populations upstream (Rab5 early endosomes) or downstream (Rab7 late endosomes) of Sara endosomes are segregated symmetrically [6]. The specific pool of Notch in Sara endosomes is relevant for signaling: it is cleaved in a ligand- and gamma-secretase-dependent manner to release the transcriptionally active Notch intracellular domain (NICD) in pIIa [6].

A key question is what machineries control the asymmetric targeting of these endosomes. Is the cargo (the ligand Delta or its receptor Notch) playing a role on the specific targeting of these endosomes? To unravel the machinery regulating the behavior of Sara endosomes during SOP mitosis, we tested candidate factors from previously reported proteomics approaches or genetic screens for Notch signaling [8–10]. Thus, we identified Uninflatable as a factor involved in the asymmetric dynamics of Sara endosomes.

Uif Mediates the Asymmetric Targeting of Sara Endosomes

We generated MARCM homozygous mutant clones for a null allele of Uninflatable (*Uif*^{2B7}) and monitored the trafficking of Delta, Notch, and the Notch effector Sanpodo through Sara endosomes. To look at the motility of the endogenous population of Sara endosomes, we followed the cohort of internalized Delta molecules 20 min after its endocytosis in the SOP by means of a pulse-chase antibody uptake assay (iDl$^{20'}$; [6]). Delta, Notch, and Sanpodo traffic normally through Sara endosomes in the absence of Uif (Figures 1A and 1B), and these endosomes are targeted to the cleavage plane (the central spindle) in cytokinesis (Figures 1B, 2A, and 2D).

In Uif mutants or RNAi knockdown conditions, iDl$^{20'}$/Sara endosomes fail to be asymmetrically dispatched to pIIa after their targeting to the central spindle (Figures 2B–2C; Figures S1A–S1D and Movie S2 available online). These results indicate that Uif is not required to bring Notch to the Sara endosomes (Figure 1A) or to target the endosomes to the central spindle (Figures 1B, 2A, 2D, and S1A and Movie S2). However, once in the spindle, Uif is essential for the specific dispatch of Sara endosomes from the spindle into the pIIa cell.

This function of Uninflatable is specific to the asymmetric segregation of Sara endosomes (see the Supplemental Results and Discussion). To gain mechanistic insights into the mechanism of action of Uif, we have analyzed the density of microtubules in the central spindle and have shown that Uninflatable does not regulate the organization of the microtubular cytoskeleton (Figures S1I and S1J). In contrast, we have found that Uif controls the residence time of Sara endosomes on the central spindle: in control SOPs, Sara endosomes depart from the central spindle with a decay time of 103 ± 21 s, whereas upon Uif downregulation this decay time goes up to 175 ± 42 s (Figure 2E). These data indicate that Uif is not involved in the organization of the spindle, but rather in the motility properties of the endosomes, particularly their last step of departing from the central spindle and end up in pIIa.

Uif Contributes to Cell Fate Assignation in the SOP Lineage

Consistent with the role of Uif in the asymmetric targeting of Sara endosomes, Uif contributes to Notch-dependent cell fate assignation in the SOP lineage. To address this, we looked

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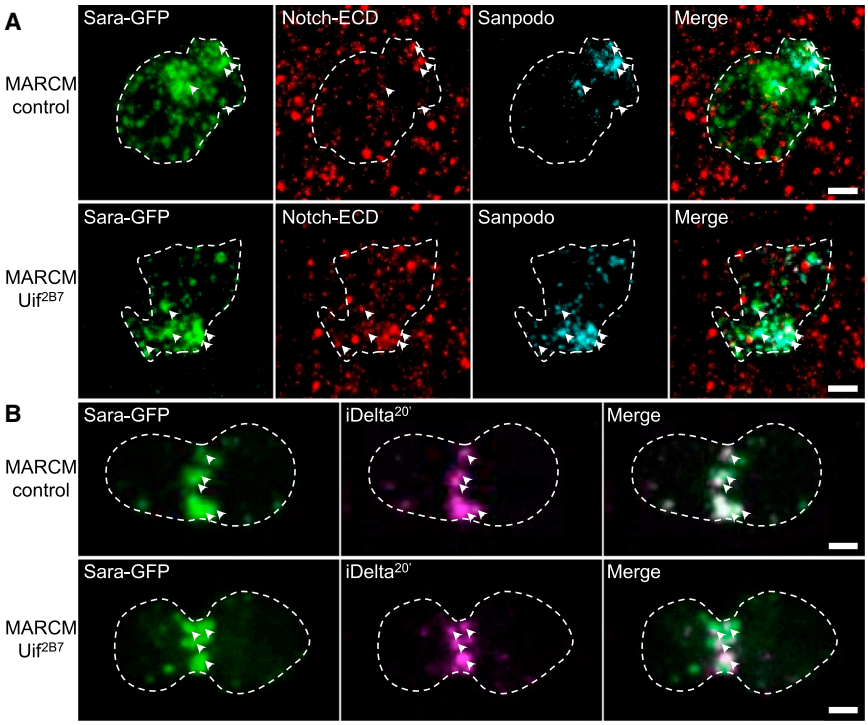

Figure 1. Targeting of Notch, Delta, and Sanpodo to Sara Endosomes and to the Cleavage Plane in *Uif*^{2B7}/*Uif*^{2B7} Cells

(A) Immunostainings (z projections of confocal slices) of Notch-ECD and Sanpodo in interphase SOPs in control (top) and *Uif*^{2B7}/*Uif*^{2B7} (bottom) MARCM clones. Arrowheads point to examples of Sara endosomes in which Notch-ECD and Sanpodo are colocalized. There is no statistically significant difference between the extent of the colocalization of both Notch and Sanpodo with Sara endosomes in control and mutant SOPs: in control SOPs, 63% ± 4.6% of Sanpodo is localized in Sara endosomes (n = 13 cells), whereas in *Uif*^{2B7}/*Uif*^{2B7} SOPs, 63% ± 7.1% of Sanpodo is localized in Sara endosomes (n = 6 cells); and in control SOPs, 47% ± 2.8% of Notch-ECD is localized in Sara endosomes (n = 13 cells), whereas in *Uif*^{2B7}/*Uif*^{2B7} SOPs, 51% ± 5.6% of Notch-ECD is localized in Sara endosomes (n = 6 cells). Scale bars, 2.5 μm.

(B) z projections of confocal slices of control (top) and *Uif*^{2B7}/*Uif*^{2B7} (bottom) SOPs in cytokinesis expressing Sara-GFP (green) and showing iDelta$^{20'}$ (magenta). Arrowheads point to examples of Sara endosomes containing iDelta$^{20'}$ that are targeted to the cleavage plane in cytokinesis. iDelta$^{20'}$ is present in Sara endosomes in both conditions: in control SOPs, 82% ± 3.3% of iDelta$^{20'}$ is localized in Sara endosomes (n = 16 cells), whereas in *Uif*^{2B7}/*Uif*^{2B7} SOPs, 80% ± 4.8% of iDelta$^{20'}$ is localized in Sara endosomes (n = 5 cells). Scale bars, 3 μm.

White dashed lines represent the SOP outline. The colocalization values are the Mander's coefficients given by the ImageJ JACoP plugin.

at the composition of SOP lineages in homozygous *Uif*^{2B7} MARCM clones or upon Uif RNAi. In wild-type animals, the SOP lineage consists of four different cells: two external cells (the shaft and the socket) originating from pIIa and two internal cells (the sheath and the neuron) from pIIb, which can be identified by immunostaining (Figure 2F; arrowheads, wild-type organs). In Uif mutant clones, instead of a sheath and a neuron per SOP lineage, two sheath cells can be frequently observed in the notum (Figures 2F and 2G; asterisk, two green cells), indicating a symmetric division in the pIIb lineage. Similarly, upon Uif downregulation in the postorbital SOPs, we observed duplications of sockets, which is diagnostic of symmetric divisions in the pIIa lineage (Figure 2H). These data uncover a role for Uninflatable in Notch-dependent asymmetric cell fate assignation that is mediated by the asymmetric dispatch of the Sara endosomes.

Uninflatable Colocalizes with Notch in Sara Endosomes

The Uif phenotype during asymmetric endosomal targeting and cell fate assignation prompted us to look whether Uif is a cargo of Sara endosomes. To detect the endogenous protein, we generated anti-Uif antibodies (see the Supplemental Experimental Procedures and Figure S2A). To look at Uif trafficking in vivo, we also generated transgenic flies expressing a Uif-GFP protein, which can provide activity to rescue the lethality of a Uif lethal mutation at least partly (Figures S2B–S2H).

We found that Uif-GFP is strongly colocalized with both Sara-GFP and iDelta$^{20'}$ (Figures 3A and S2L and the Supplemental Results and Discussion). Since a cargo of Sara endosomes is Notch itself (73% ± 2.7% of the vesicular population of Notch molecules is in Sara endosomes; see below and [6]), we looked at the presence of Notch cargo in Uif vesicles: 44% ± 4.7% of Uif-positive vesicular structures contain Notch (Figure 3C; n = 662 vesicles in 106 cells). Therefore, a population of Uninflatable and Notch traffics through Sara endosomes during SOP asymmetric mitosis.

Notch Binds Uninflatable

The fact that Uninflatable controls the asymmetric dispatch of the Sara endosomes, which contain internalized Notch and Uninflatable, prompted us to look at a possible molecular interaction between Uninflatable and Notch. We cotransfected Uif- and Notch-expressing plasmids in S2 cells and performed immunoprecipitation experiments by using anti-Uif-coupled beads, followed by immunoblotting with a clean anti-Notch antibody that we purified from a hybridoma cell line (DSHB #C17.9C6; see the Supplemental Experimental Procedures). Figure 3D shows that Uif can immunoprecipitate Notch. As shown in Figure 3E, this coimmunoprecipitation can be reproduced from lysates of S2 cells expressing Notch and Uif tagged with the PC peptide tag and anti-PC-coupled beads; as a control, other transmembrane proteins such as Tkv-GFP are not coimmunoprecipitated with Uif-PC (Figure 3F). Together, these results indicate a specific molecular interaction between Notch and Uif.

Uninflatable is a transmembrane protein that, like Notch, contains an array of epidermal growth factor (EGF) repeats [11]. It has been shown that Notch is engaged in protein-protein interactions through its EGF repeats with other factors containing EGF repeats. These include its ligand Delta [12, 13], but also a number of noncanonical Notch ligands, secreted or membrane proteins lacking the DSL domain characteristic of canonical Notch ligands (Dlk-1, Dlk-2, DNER, Trombospondin, LRP1, EGFL7, and Weary; reviewed in [9]). Consistently, it has recently been reported that a synergistic genetic interaction between Uif and Notch depends on Notch EGF repeats [14]. We therefore studied which EGF repeats of Uif could be involved in the molecular interaction with Notch. We performed a coimmunoprecipitation experiment in S2 cells

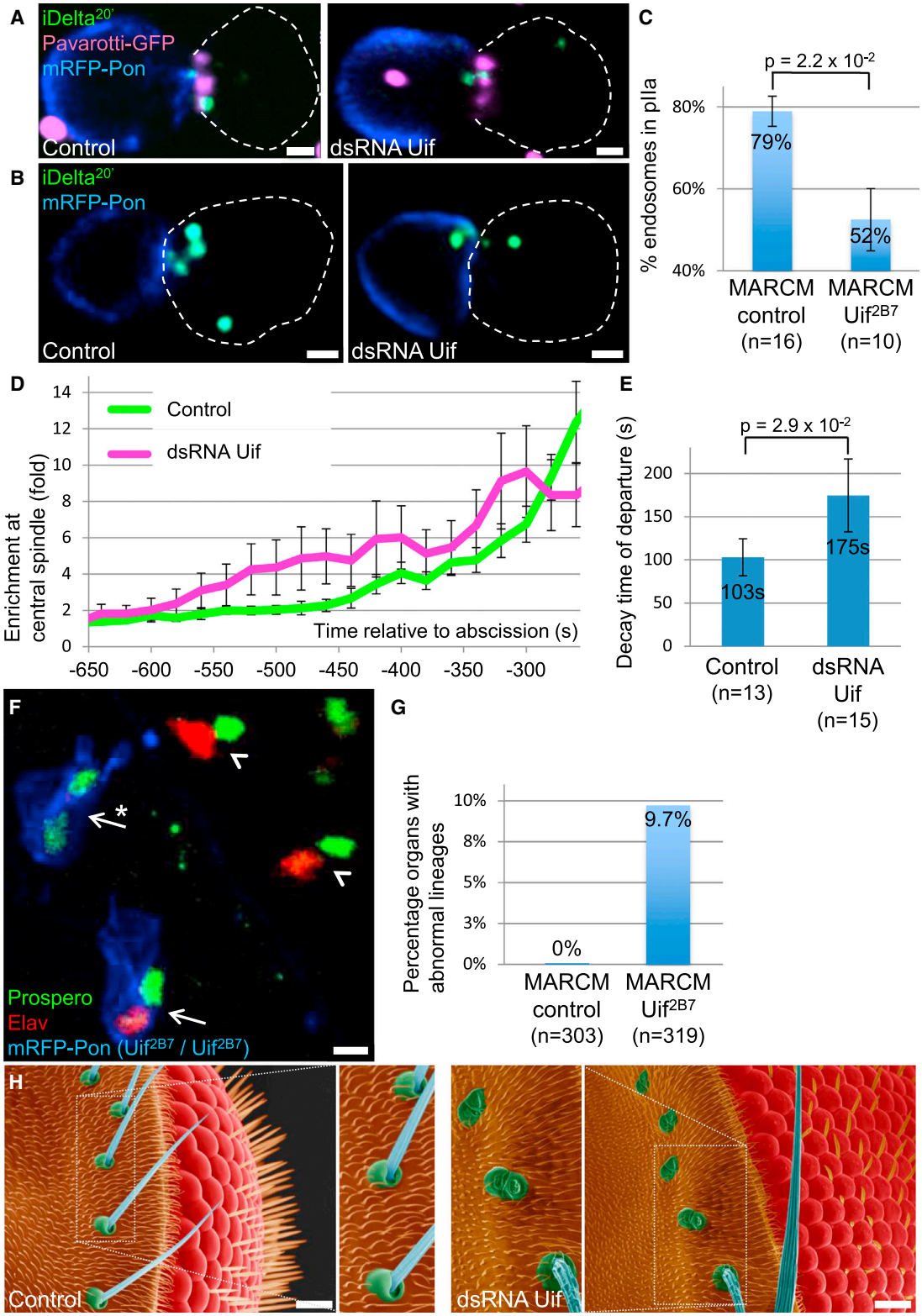

Figure 2. Uninflatable Mediates the Asymmetric Targeting of Sara Endosomes and Contributes to Cell Fate Assignation in the SOP Lineage

(A and B) Stills (z projections) of wild-type SOPs and SOPs expressing a double-stranded RNA (dsRNA) against Uif in cytokinesis (A) and at abscission (B). Pavarotti-GFP (in A) marks the central spindle midzone. In this figure and in the next ones, white dashed lines represent the pIIa cell membrane (unless otherwise specified). Scale bars, 1.9 μm.

(C) Percentage of iDelta$^{20'}$ endosomes segregating to the pIIa daughter cell upon division of SOPs from control MARCM clones and from *Uif*^{2B7} MARCM clones (n = 16 cells in four animals for control MARCM; n = 10 cells in two animals for *Uif*^{2B7} MARCM). This percentage was calculated by integrating the total endosomal signal in each cell after background subtraction and thresholding.

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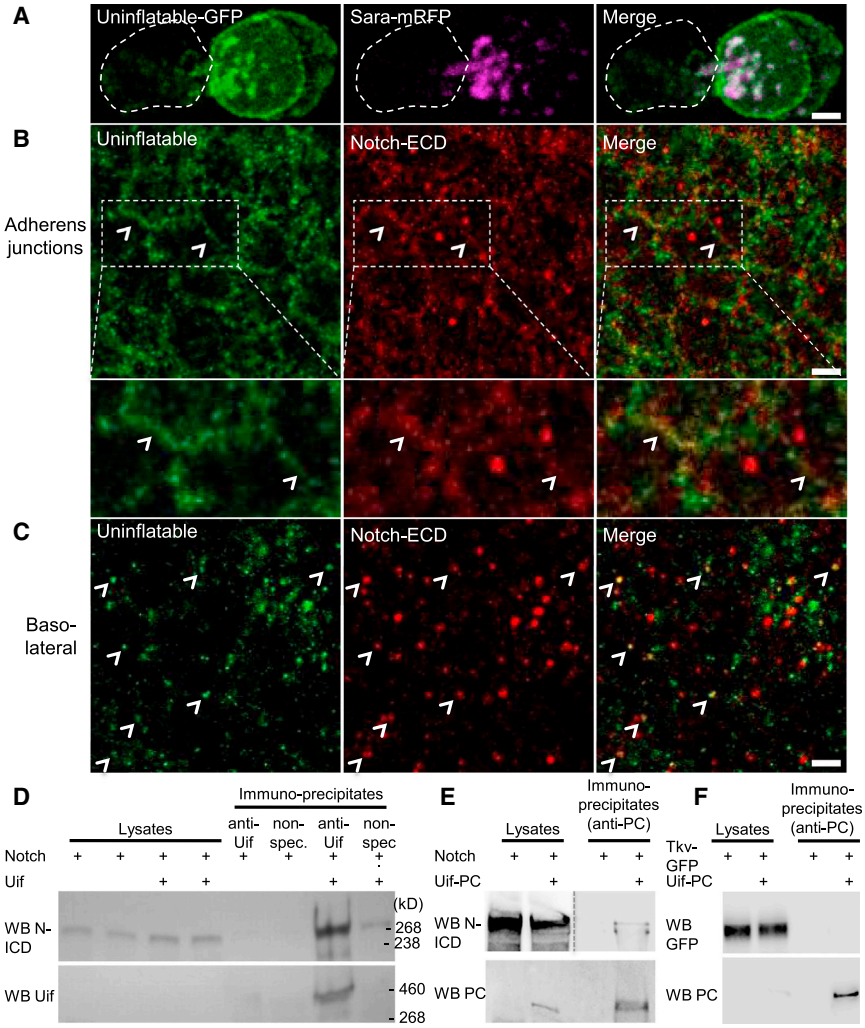

Figure 3. Notch/Uninflatable Binding and Colocalization

(A) Still (z projection) of a dividing SOP during abscission illustrating the colocalization of Uif-GFP and Sara-mRFP on Sara endosomes. Note that the outline of the pIIa daughter cell is visible in the Uif-GFP channel, due to the apical pool of Uif-GFP. The white dashed line indicates the pIIb outline in this case. Scale bar, 2 μm.

(B and C) Confocal slices of a pupal notum immunostained with anti-Uif and anti-Notch-ECD antibodies at the adherens-junctions level (B) and the basolateral level (C). Arrowheads indicate junctions or vesicles where Uif and Notch-ECD are in close proximity (blowups in B) or colocalized (C). Scale bars, 3 μm.

(D) Coimmunoprecipitation of Uif and Notch. Lysates from S2 cells overexpressing Notch or Notch and Uif were immunoprecipitated with anti-Uif antibodies or with nonspecific rabbit IgGs from preimmune sera and were immunoblotted with anti-Notch-ICD and anti-Uif antibodies.

(E) Coimmunoprecipitation of Uif-PC and Notch. Lysates from S2 cells overexpressing Notch or Notch and Uif-PC were immunoprecipitated with anti-PC antibodies and immunoblotted with anti-Notch-ICD and anti-PC antibodies.

(F) Control transmembrane proteins are not coimmunoprecipitated with Uif-PC. Lysates from S2 cells overexpressing Tkv-GFP or Tkv-GFP and Uif-PC were immunoprecipitated with anti-PC antibodies and immunoblotted with anti-GFP and anti-PC antibodies.

coexpressing Notch and an N-terminal, truncated form of Uif tagged with PC (Uif$^{\Delta Cter}$-PC) that lacks the four EGF domains flanking the transmembrane domain but still contains the other 17 EGF repeats and other extracellular domains. While full-length Uif-PC coimmunoprecipitates Notch, Uif$^{\Delta Cter}$-PC does not (Figure S2M). This indicates that the interaction between Uif and Notch may be mediated by the four EGF domains of Uif flanking its transmembrane domain.

Although Uif binds and colocalizes with Notch, it does not play a role in core Notch signaling: embryos deprived of maternal and zygotic Uif in germline clones do not show a Notch signaling phenotype, whereas they display loss of inflation of the trachea as previously reported (Figure S1E) [11]. Consistently, loss of Uif in wing mosaics does not cause a defect of Notch-dependent expression of Wingless at the wing margin (Figure S1F). This indicates that Uninflatable interaction with Notch is not essential during core Notch signaling, but rather during the asymmetric dispatch of Notch-containing Sara endosomes during asymmetric cell division. This prompted the possibility that Notch itself is required for the asymmetric motility of the endosomes.

Trafficking of a Notch-GFP Fusion at Endogenous Expression Levels

To study whether Notch plays a role during the asymmetric dispatch of Sara endosomes, we first wanted to study the

(D) Enrichment of Sara endosomes in the central spindle area in control SOPs (green) and upon expression of a dsRNA against Uif (magenta) (n = 8–26 cells for control; n = 6–10 cells for dsRNA Uif). The central spindle area was defined as a 3-μm-wide region centered on the cytokinetic furrow. Time is counted relative to abscission.

(E) Decay time of the departure of Sara endosomes from the central spindle in control SOPs and upon downregulation of Uif (n = 13 cells for control; n = 15 for dsRNA Uif). The decay time was measured for each cell by fitting of an exponential to the decay of the curve representing the enrichment of Sara endosomes in the central spindle area.

(F) Immunostainings in pupal nota (25 hr after puparium formation) for sheath cells (Prospero, green) and neurons (Elav, red) reveal sensory organs with abnormal lineages (asterisk) in *Uif^{2B7}/Uif^{2B7}* MARCM clones (arrows; marked by Neur:mRFP-Pon, blue). Arrowheads indicate SOP lineages in the control territory. Scale bar, 8 μm.

(G) Percentage of sensory organs composed of two sheath cells, zero neurons, and two external cells (of the pIIa lineage) in control and *Uif^{2B7}/Uif^{2B7}* MARCM clones (303 organs in ten pupae for MARCM control; 319 organs in 25 pupae for MARCM *Uif^{2B7}*).

(H) Scanning electron microscopy pictures of postorbital bristles of a wild-type fly (control) and of a fly in which SOPs express a dsRNA against Uif (dsRNA Uif). Colors indicate the socket cells (green), shafts (cyan), omatidia (red), and cuticle (light brown).

The graphs in (C)–(E) represent mean values ± SEM. See also Figure S1 and Movies S1 and S2.

trafficking of a Notch-GFP fusion expressed at endogenous levels. The idea was to confirm our previous observations using a Notch antibody uptake assay [6] to follow Notch expressed at endogenous levels. To achieve this, we set up to use a reported transgenic fly strain in which Notch-GFP fusion is driven by the Notch endogenous promoter and is expressed at endogenous levels [15]. In this fusion, GFP is inserted in the middle of the Notch-intra domain [15]. Since in protein fusions GFP is frequently cleaved out, we studied whether the fusion protein is intact (Figure S3; see also Supplemental Results and Discussion). This would be particularly important in this case, since a cleavage event would lead to a truncated Notch-intra peptide.

We indeed found that in these transgenic Notch-GFP flies, GFP is very efficiently cleaved out (74% of total GFP is cleaved; Figure S3B), leading to truncated Notch-intra peptides (Figures S3A and S3B) that can only partially support Notch function and thereby cause a highly penetrant mutant phenotype (Figures S3E and S3F). This precludes the usage of this reagent as a bona fide marker for Notch. In particular, the cytosolic GFP signal cannot be used as a readout of signaling as previously reported: a nuclear accumulation of the GFP signal in these flies [15] does not solely reflect the accumulation of Notch-intra-GFP, but rather the overall accumulation of different GFP-containing fragments.

We still studied whether, in these conditions, the pool of membrane associated GFP-Notch traffics through Sara endosomes and is asymmetrically dispatched to the pIIa cell. Only 11% ± 1.3% of the total GFP signal in these flies is membrane associated (plasma membrane and intracellular vesicular structures; Figure S4). The rest, representing the vast majority (89%), corresponds to cytosolic and nuclear cleaved GFP.

In Notch-GFP flies, 3.1% of the total GFP signal is associated with intracellular vesicular structures (Figure S4B; for a description of the measurement method, see the Supplemental Experimental Procedures and Figures S4E–S4G). These correspond to various intracellular vesicular compartments, including Notch in the secretory pathway, as well as in early endosomes, Sara endosomes, recycling endosomes, and late endosomes [6, 16, 17]. To measure the size of the specific pool of Notch in Sara endosomes, we performed a Notch antibody internalization assay and chased internalized Notch 20 min after its endocytosis (iNotch$^{20'}$), as previously established (Figure S4C) [6]: 73% ± 2.7% of Notch-GFP vesicles are positive for iNotch$^{20'}$. Of this iNotch$^{20'}$-positive pool, 79% would be targeted to pIIa (Figure 2C). This is consistent with only 65% ± 3.1% (n = 26 cells in seven animals) of the total pool of Notch-GFP being dispatched to pIIa (Figure S4D; see also "Asymmetric dispatch of internalized endogenous Notch in Sara endosomes" in the Supplemental Results and Discussion).

Notch-Dependent Targeting of Sara Endosomes to pIIa

We then addressed whether Notch itself plays a role on the asymmetric targeting of Sara endosomes. We depleted Notch in the SOP by expressing a previously validated Notch dsRNA [18] and looked at the behavior of Sara endosomes. Figures 4A–4D show that upon Notch knockdown in the SOP, iDl$^{20'}$/Sara endosomes are still targeted to the central spindle (see the arrowheads in cytokinesis in Figure 4A), but the subsequent directed dispatch to pIIa is defective (see also Movie S3). This indicates that Notch itself contributes to the endosomal recruitment of the machinery that endows the Sara endosomes with their asymmetric behavior.

We have previously shown that the targeting of Notch to Sara endosomes does not depend on Uninflatable (Figure 1A); we then sought to determine whether the recruitment of Uninflatable on Sara endosomes depended on Notch. Interestingly, we found that, conversely, the targeting of Uif to Sara endosomes is not controlled by Notch (Figure S2N). This implies that these two molecules use different machineries to get to the endosome, where they can interact and are both required for the asymmetric motility of the endosome.

Since the Notch receptor itself is required for the asymmetric targeting of Sara endosomes, we wondered whether Notch signaling plays a role in the process. We blocked Notch signaling by inactivating the ligand Delta through overexpression of Tom in the SOP cell [6, 19]; Tom overexpression leads to inactivation of the Ubiquitin ligase Neuralized and thereby blocks endocytosis-dependent activation of Delta [19]. Figure 4E shows that in the absence of Notch signaling, targeting of Sara endosomes to the central spindle and their asymmetric dispatch to the pIIa cell remains intact. This indicates that although the Notch receptor is essential for the asymmetric targeting of Sara endosomes, Notch signaling is not.

The Machinery for Asymmetric Dispatch of Sara Endosomes

In this report, we have started to unravel the machinery that mediates asymmetric endosome motility during asymmetric cell division. We showed that both Notch and Uninflatable play a key role in the last step of the asymmetric motility of endosomes: the final, specific stride of the Sara endosomes from the central spindle into the anterior pIIa cell. This is based on the following four key sets of observations.

First, we confirmed that a functional Notch-GFP fusion expressed at endogenous level does traffic through Sara endosomes, which are indeed dispatched asymmetrically during SOP mitosis (Figures S4A–S4C). Second, Notch binds Uninflatable (Figures 3D and 3E), and both colocalize in Sara endosomes (Figures 1A, 3A, 3C, and S2L). Third, neither Notch nor Uninflatable is essential for the targeting of Notch/Delta/Uif to the Sara endosomes (Figures 1 and S2N) or the targeting of those endosomes to the central spindle (Figures 1B, 2A, 2D, 4A, and S1A), but they are essential for the final dispatch from the central spindle into the pIIa cell (Figures 2C, 2E, 4B–4D, and S1B–S1D and Movies S2 and S3). Although Notch is necessary for this process, Notch signaling is not (Figure 4E). Fourth, Uninflatable is not an integral component of the Notch signaling pathway (Figures S1E and S1F), but it plays a role during asymmetric Notch signaling in the SOP, and therefore mutant *Uif* conditions lead to a lineage identity phenotype (Figures 2F–2H). It remains to be elucidated what machineries downstream of Notch/Uninflatable implement the control of the final step toward pIIa and what is asymmetrical in the cytoskeleton so that this final step occurs toward pIIa and not pIIb.

Supplemental Information

Supplemental Information includes Supplemental Results and Discussion, Supplemental Experimental Procedures, four figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.07.054.

Author Contributions

S.L., C.S., and M.G.-G. wrote the manuscript. S.L., C.S., A.M., A.D., M.F., and M.G.-G. designed the experiments. S.L., C.S., A.M., A.D., and M.F. performed the experiments. S.L., C.S., A.M., A.D., M.F., and M.G.-G. analyzed the data.

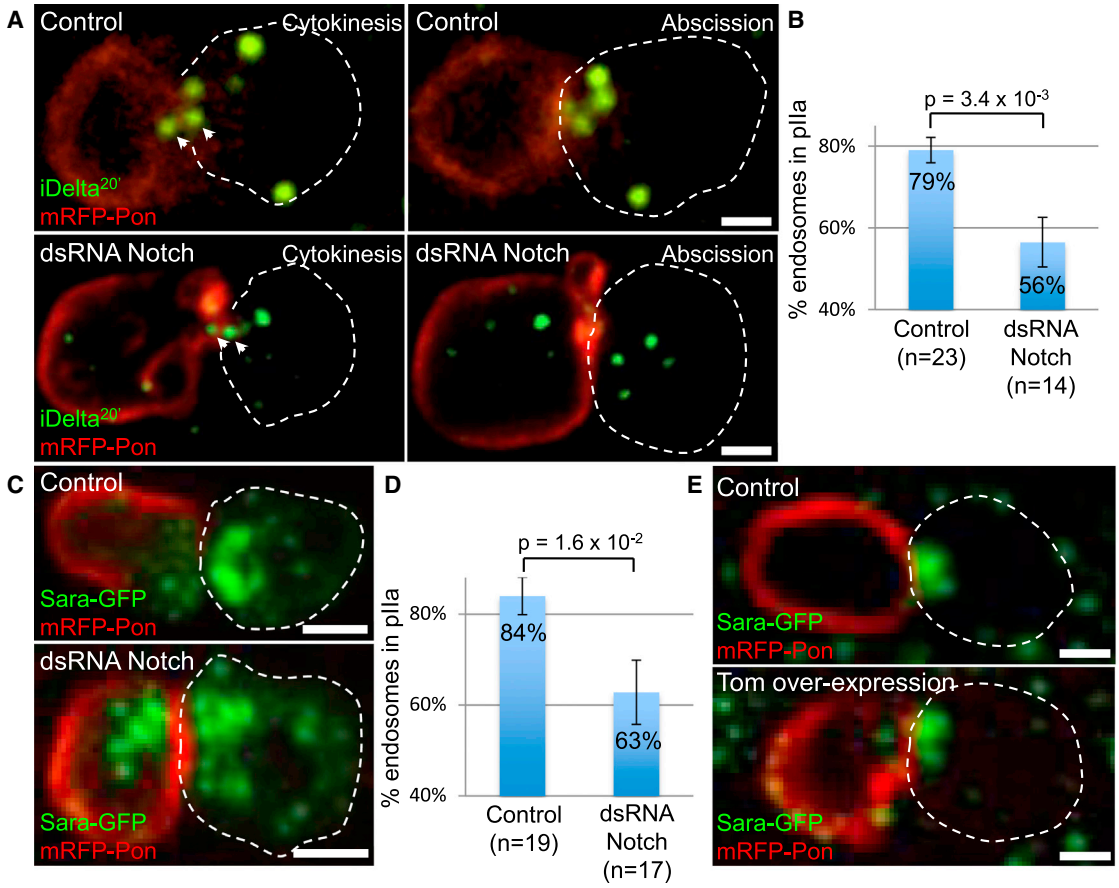

Figure 4. Notch-Dependent and Notch Signaling-Independent Targeting of Sara Endosomes to pIIa

(A) Stills (z projections) of a wild-type dividing SOP (top) and an SOP expressing a dsRNA against Notch (bottom) in cytokinesis (left) and at abscission (right). Arrowheads indicate endosomes targeted to the central spindle. Scale bar, 2.5 μm.

(B) Percentage of iDelta$^{20'}$ endosomes segregating to the pIIa daughter cell upon division of wild-type SOPs and SOPs expressing a dsRNA against Notch (n = 23 cells in six animals for control; n = 14 cells in three animals for dsRNA Notch).

(C) Stills (z projections) of a wild-type dividing SOP (top) and an SOP expressing a dsRNA against Notch (bottom) at abscission. Scale bars, 4 μm.

(D) Percentage of Sara-GFP endosomes segregating to the pIIa daughter cell upon division of wild-type SOPs and SOPs expressing a dsRNA against Notch (n = 19 cells in two animals for control; n = 17 cells in four animals for dsRNA Notch).

(E) Stills (z projections) of a wild-type dividing SOP (top) and an SOP overexpressing Tom (bottom) at abscission. There is no statistically significant difference between the proportion of Sara endosomes targeted to the pIIa daughter cell in either condition: 72% ± 2.8% in control SOPs (n = 38 cells in two animals) and 67% ± 2.1% upon overexpression of Tom (n = 80 cells in four animals) (p = 0.13). Scale bar, 2.5 μm. Endosomal localizations were quantified by integration of the total intensity of endosomes in each cell after background subtraction and thresholding.

The graphs in (B) and (D) represent mean values ± SEM. See also Movies S1 and S3.

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