Endosomal entry regulates Notch receptor activation in *Drosophila melanogaster*

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Signaling through the transmembrane receptor Notch is widely used throughout animal development and is a major regulator of cell proliferation and differentiation. During canonical Notch signaling, internalization and recycling of Notch ligands controls signaling activity, but the involvement of endocytosis in activation of Notch itself is not well understood. To address this question, we systematically assessed Notch localization, processing, and signaling in a comprehensive set of *Drosophila melanogaster* mutants that block access of cargo to different endocytic compartments. We find that γ-secretase cleavage and signaling of endogenous Notch is reduced in mutants that impair entry into the early endosome but is enhanced in mutants that increase endosomal retention. In mutants that block endosomal entry, we also uncover an alternative, low-efficiency Notch trafficking route that can contribute to signaling. Our data show that endosomal access of the Notch receptor is critical to achieve physiological levels of signaling and further suggest that altered residence in distinct endocytic compartments could underlie pathologies involving aberrant Notch pathway activation.

Introduction

Notch signaling controls a myriad of proliferation and differentiation decisions across animal species, and aberrant Notch signaling is associated with cancers, stroke, and a host of human developmental disorders (Artavanis-Tsakonas et al., 1999). The potency of Notch signaling requires that its activity be under tight cellular regulation, which involves several posttranslational modifications including glycosylation, ubiquitination, and proteolysis. These modifications contribute to the sensitive control of spatiotemporal domains and the degree of activation seen in vivo. In canonical Notch signaling, the signaling-competent Notch receptor exists at the plasma membrane as a heterodimer consisting of the Notch extracellular domain (NECD) and the transmembrane and intracellular domains (Notch extracellular truncation [NEXT]). In signal-sending cells, activated ligands of the Delta, Serrate, Lag-2 (DSL) family bind to Notch. Upon ligand binding, the NECD is separated from NEXT, which then becomes an efficient substrate for an intramembrane cleavage mediated by the γ-secretase enzyme. This cleavage releases the cytoplasmic Notch intracellular domain (NICD) from its transmembrane anchor. Free NICD translocates to the nucleus, where it interacts with transcription factors to relieve transcriptional repression of Notch targets (for review see Schweisguth, 2004).

Because Notch and its ligands are transmembrane proteins, vesicular trafficking could influence signaling activity. Indeed, endocytic internalization and recycling of DSL ligands in the signal-sending cell are needed for productive signaling (for reviews see Le Borgne et al., 2005; Nichols et al., 2007). In contrast, the role of Notch receptor endocytosis in the signal-receiving cell is poorly understood and controversial. In vertebrates, it is unclear whether or not γ-secretase cleavage requires receptor internalization, with evidence supporting either scenario (Gupta-Rossi et al., 2004; Chyung et al., 2005; Kaether et al., 2006). In *Drosophila melanogaster*, genetic analysis has suggested that Notch signaling requires internalization in the receiving cell; however, cleavage and activation of NEXT may not (Seugnet et al., 1997; Struhl and Adachi, 2000; Lopez-Schier and St Johnston, 2002). Recently, several *D. melanogaster* mutations that affect endocytosis after internalization have been described that appear to have disparate consequences for Notch activation, either decreasing, not affecting, or increasing signaling (Giebel and Wodarz, 2006).

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Abbreviations used in this paper: Avl, Avalanche; DAPT, *N*-[*N*-[3,5-difluorophenacetyl-L-alanyl]]-*S*-phenylglycine t-butyl ester; DSL, Delta, Serrate, Lag-2; EE, early endosomes; ESCRT, endosomal sorting complex required for transport; FC, follicle cell; Hnt, Hindsight; Lqf, Liquid facets; MVB, multivesicular body; NECD, Notch extracellular domain; NEXT, Notch extracellular truncation; NICD, Notch intracellular domain; Shi, Shibire; Wg, Wingless; WT, wild type.

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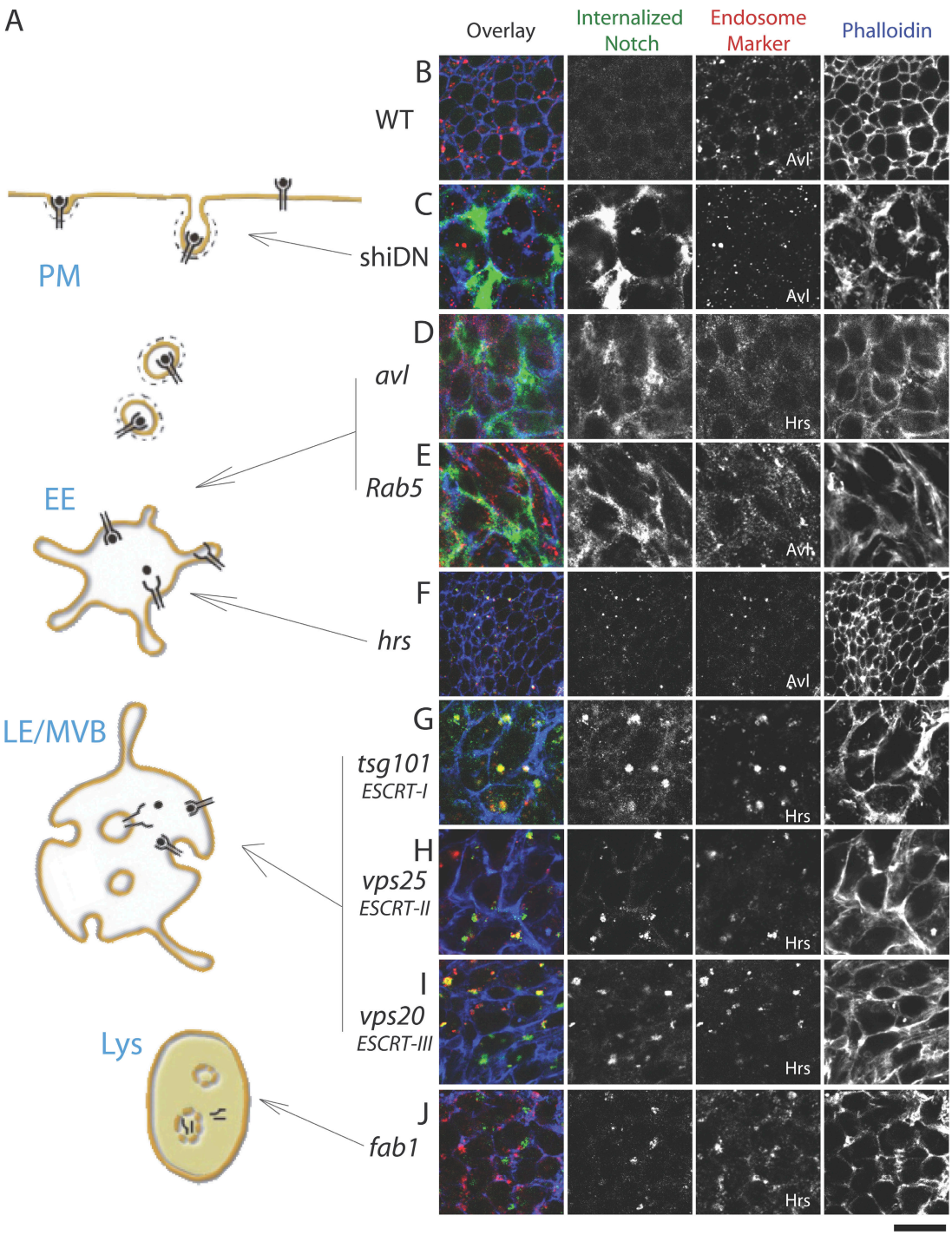

Figure 1. **Trafficking defects in endocytic mutant tissue lead to Notch accumulation in different compartments.** (A) Schematic of endocytic trafficking. Arrows indicate the approximate site of action of each gene product. See text for a description of gene product function. (B–J) Live trafficking assay for Notch in cultured eye or wing (C) imaginal discs. Avl and Hrs mark endosomal compartments and phalloidin reveals cell outlines. In WT tissue, surface-bound Notch is internalized and then degraded after 5 h (B). In mutant tissue, Notch is internalized but is not degraded, persisting 5 h after labeling (C–J). In *shiDN*, *avl*, and *Rab5* mutants, Notch is localized to the cell surface (C–E); in *hrs* mutants, Notch is bound to Avl-positive organelles (F); in *ESCRT* mutants, Notch is bound to Hrs-positive organelles (G and I); and in *fab1* mutants, Notch is bound in organelles that are neither Avl- nor Hrs-positive. Bar, 10 μm.

However, because of the varied approaches used in these studies, it is difficult to correlate the effects of Notch trafficking and signaling in a comparable manner. Hence, the significance of endocytic trafficking of the Notch receptor through various endosomal compartments during signaling remains an open question.

Here, we have used a set of *D. melanogaster* null mutations that disrupt representative and well-characterized core components of the endocytic machinery to systematically address this question by altering subsequent steps of endocytic trafficking and evaluating Notch localization, processing, and signaling output in vivo.

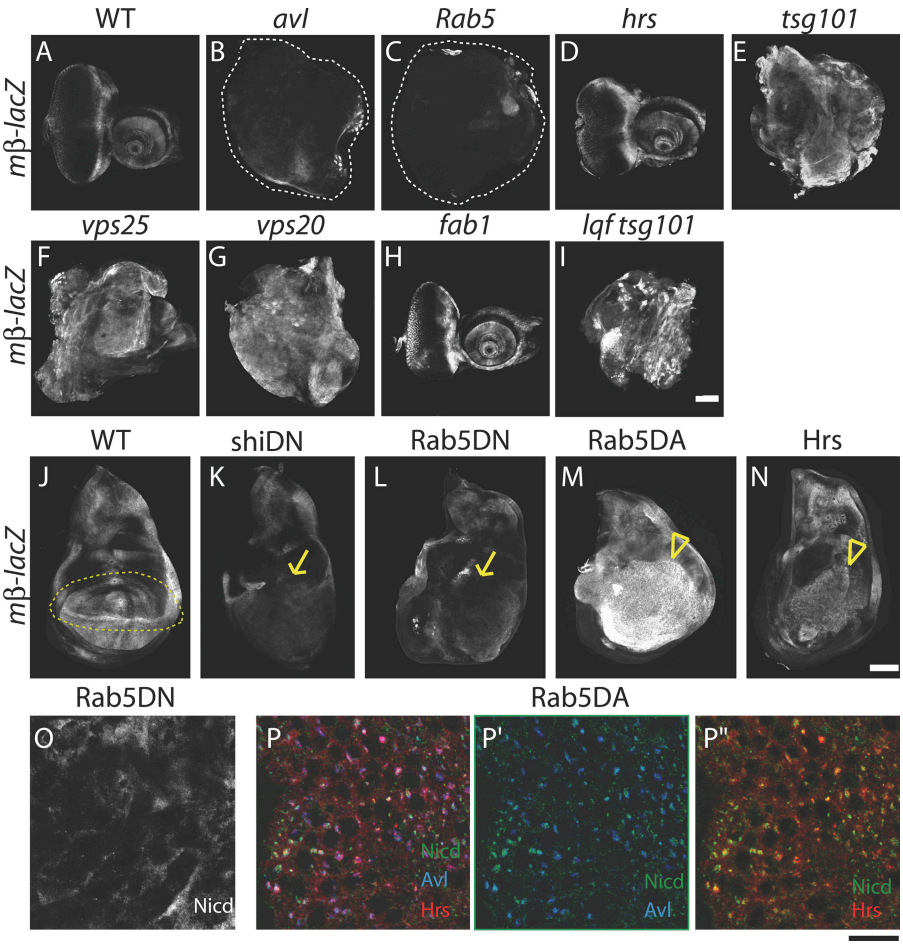

Figure 2. **Notch signaling displays a bimodal profile in endocytic mutant tissue.** (A–N) Eye (A–I) and wing (J–N) discs stained to detect the Notch reporter *mß-lacZ*. Compared with the WT (A and J), *mß-lacZ* expression is reduced in *avl* and *Rab5* mutant discs (outlined in B and C), unchanged in *hrs* and *fab1* mutant discs (D and H), and increased in *ESCRT* mutant discs (E–G and I). Expression of ShiDN or Rab5DN in the dorsal wing disc pouch (broken line in J) correlates with reduction of *mß-lacZ* (arrow in K and L). In contrast, expression of Rab5DA or Hrs correlates with enhancement of *mß-lacZ* (arrowhead in M and N). (O and P) Notch is at the cell periphery in Rab5DN-expressing discs (O) and in enlarged endosomes in Rab5DA-expressing discs (P). Bars: (A–N) 100 μm; (O and P) 10 μm.

Results and discussion

Notch endosomal access and multivesicular body (MVB) sorting correlate with signaling activation

We first determined the effects of endocytic mutants on in vivo trafficking of the endogenous Notch receptor (see Fig. 1 A and the text below for a description of the mutants used). We generated homozygous mutant imaginal discs and performed a live trafficking assay to follow Notch endocytosis (see Materials and methods). In wild-type (WT) tissue, most surface-labeled Notch is internalized from the apical plasma membrane and degraded within 5 h (Fig. 1 B; Lu and Bilder, 2005; Vaccari and Bilder, 2005). In contrast, in each endocytic mutant analyzed, Notch is not degraded and instead accumulates in distinct subcellular compartments (Fig. 1, C–J), often resembling its steady-state localization seen using standard immunohistochemistry. We monitored Notch accumulation with respect to phalloidin, which marks the cortical actin cytoskeleton, and either Avalanche (Avl) or Hrs, which mark distinct domains of early endosomes (EEs). As expected, in mutants for proteins that regulate cargo entry into EE, such as a dominant-negative form of the dynamin orthologue Shibire (Shi), ShiDN (Moline et al., 1999), the GTPase Rab5, and the endocytic syntaxin Avl (Wucherpfennig et al., 2003; Lu and Bilder, 2005), Notch accumulates at or below the plasma membrane (Fig. 1, C–E). In mutants for Hrs, which is thought to mediate recruitment of ubiquitinated cargo in the EE (Lloyd et al., 2002), surface-labeled Notch reached and accumulated in an Avl-positive EE compartment (Fig. 1 F). In mutants for the endosomal sorting complex required for transport (ESCRT) genes *tsg101*, *vps25*, and *vps20*, which control MVB sorting (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Herz et al., 2006), almost all Notch was present in Hrs- but not Avl-positive puncta, apparently corresponding to a more mature EE compartment (Fig. 1, G–I; and not depicted). Finally, in mutants for proteins that act in a post-MVB, prelysosomal compartment, such as the PIKFYVE orthologue Fab1 (Rusten et al., 2006), Notch accumulated in a structure that is distinct from EEs, as it did not colocalize with either Avl or Hrs (Fig. 1 J and not depicted). The site of Notch trapping in each mutant is consistent with the predicted function of the disrupted endocytic regulator and therefore provides an opportunity to correlate the endocytic localization of Notch with its associated signaling activity.

To investigate the effects of Notch accumulation on receptor activation, we examined expression of *mβ–lacZ*, a transcriptional reporter of Notch signaling (Fig. 2). *mβ–lacZ* can be reliably assayed in mutant imaginal discs, even in *shi*, *avl*, *Rab5*, and *ESCRT* mutants that behave as neoplastic tumor suppressor genes. In the latter mutants, epithelial organization is lost and tissue overgrows, but these cell-autonomous phenotypes are independent of Notch signaling (Lu and Bilder, 2005; Vaccari and

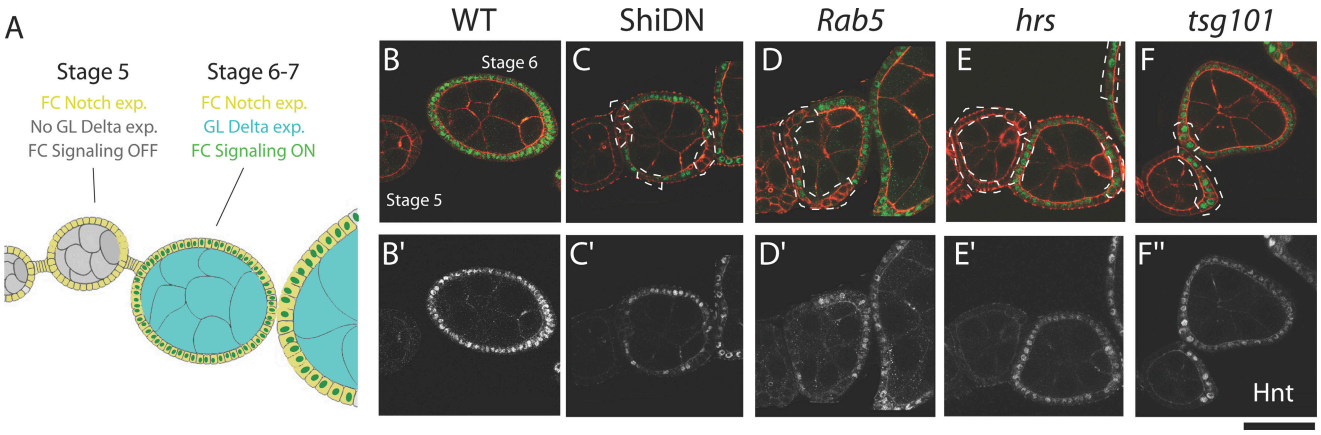

Figure 3. **Notch signaling activation in FCs is reduced in mutants that impair entry into EEs.** (A) Schematic of Notch signaling at stages 5–7 of oogenesis. Notch is predominantly expressed in the FC layer throughout oogenesis. After stage 5, Delta expression in the germline triggers activation of signaling and target gene expression, including Hnt, in FCs. (B–F) Egg chambers at stages 5 and 6 of oogenesis stained for Hnt (green) and actin (red). In WT cells, Hnt is absent at stage 5 but expressed from stage 6 onwards (B). In ShiDN-expressing and in *Rab5* FCs (outlined in C and D), Hnt is reduced, whereas in *hrs* FCs (outlined in E), Hnt is not affected. In *tsg101* FCs (outlined in F), Hnt is not altered at stage 6 but is precociously activated at stage 5. For clone boundaries, see Fig. S3 (available at http://www.jcb.org/cgi/content/full/jcb.200708127/DC1). Bar, 50 μm.

Bilder, 2005). As compared with the stereotyped levels and patterns seen in WT discs (Fig. 2, A and J; Nellesen et al., 1999), expression is uniformly reduced in *shiDN*-expressing disc cells and also in *avl* and *Rab5* mutant discs (Fig. 2, K, B, and C; Lu and Bilder, 2005). In contrast, the level of *mβ–lacZ* in *hrs* and *fab1* mutant discs was unchanged compared with the WT (Fig. 2, D and H). Finally, expression of *mβ–lacZ* is elevated in *ESCRT-I*, *-II*, and *-III* mutants (Fig. 2, E–G). In light of our Notch localization data, these results suggest a correlation between Notch endosomal access and its degree of signaling activation.

Transport to endosomes and to MVBs controls and promotes Notch signaling

Because loss-of-function mutants across the endocytic panel indicate that access of Notch to endosomes and MVBs correlates with signaling activation, we tested this idea by using a gain-of-function approach. We overexpressed dominant active Rab5 (Rab5DA), which enhances endosomal fusion (Entchev et al., 2000), as well as Hrs, which enhances MVB transport (Seto and Bellen, 2006), in the dorsal compartment of the wing disc and monitored Notch signaling.

As expected from our analysis of *Rab5* mutant eye discs, wing discs that express a dominant-negative form of Rab5 (Rab5DN) that inhibits endosomal fusion (Entchev et al., 2000) show reduced *mβ–lacZ*, mimicking the disruption of Shi function (Fig. 2, K and L). In striking contrast, wing discs that express Rab5DA show strong up-regulation of *mβ–lacZ* (Fig. 2 M). We obtained similar results, albeit with less potent enhancement of *mβ–lacZ* upon Hrs overexpression (Fig. 2 N). Consistent with enhanced endosomal fusion in Rab5DA- and Hrs-expressing discs, Notch localization is mostly endosomal (Fig. 2 P and not depicted), whereas Notch is largely cortical in Rab5DN-expressing discs (Fig. 2 O). Collectively, with our endosomal mutant studies, these ectopic expression studies provide evidence for a sharp transition in signal potentiation between the "active" and "inactive" states that depends critically upon Notch entry into endosomes.

Efficient signaling activation requires endocytosis of endogenous Notch receptor

A potential explanation for the signaling profile observed in endocytic mutant tissue would assign the differences to trafficking of DSL ligands for Notch rather than the receptor itself. To specifically assess the role of Notch receptor trafficking in signaling activation, we used *D. melanogaster* ovaries. In these organs, the Notch ligand Delta is up-regulated in the germ cells during stages 6–7 of oogenesis to activate Notch signaling in the adjacent somatic follicle cells (FCs; Fig. 3 A; Deng et al., 2001; Lopez-Schier and St Johnston, 2001). This signaling up-regulates the transcription factor Hindsight (Hnt) and simultaneously down-regulates Cut (Fig. 3 B; Sun and Deng, 2005, 2007) We made genetic mosaic ovaries in which some FCs were deficient for the function of *shi*, *Rab5*, *hrs*, or the ESCRT component *tsg101* (Fig. 3, C–F). In these ovaries, internalization of Notch in certain signal-receiving cells is altered but internalization of Delta in the signal-sending cell remains normal. We found a consistent reduction of Hnt expression at stage 6 in FCs expressing *shiDN* or mutant for *Rab5* (Fig. 3, C and D). In contrast, we found no reduction in *hrs* and *tsg101* mutant FCs (Fig. 3, E and F). Reciprocal results were obtained analyzing Cut, and the differences were not caused by disrupted cell polarity because Notch signaling was unaffected in comparable FCs mutant for the nonendocytic neoplastic tumor suppressor gene *lgl* (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200708127/DC1; Bilder et al., 2000).

Interestingly, we observed precocious Hnt expression in *tsg101* mutant FCs at stage 5 (Fig. 3 F), which suggests that signaling activation in ESCRT mutant FCs is ligand independent. Ligand-independent Notch activation has been demonstrated in cultured cells depleted of ESCRT components by RNAi (Thompson et al., 2005). To test ligand involvement directly in vivo, we assayed *mβ–lacZ* in imaginal disc cells lacking both the ESCRT component Tsg101 and the Epsin homologue Liquid facets (Lqf), which is required for ligand-dependent Notch signaling (Overstreet et al., 2003; Wang and Struhl, 2004).

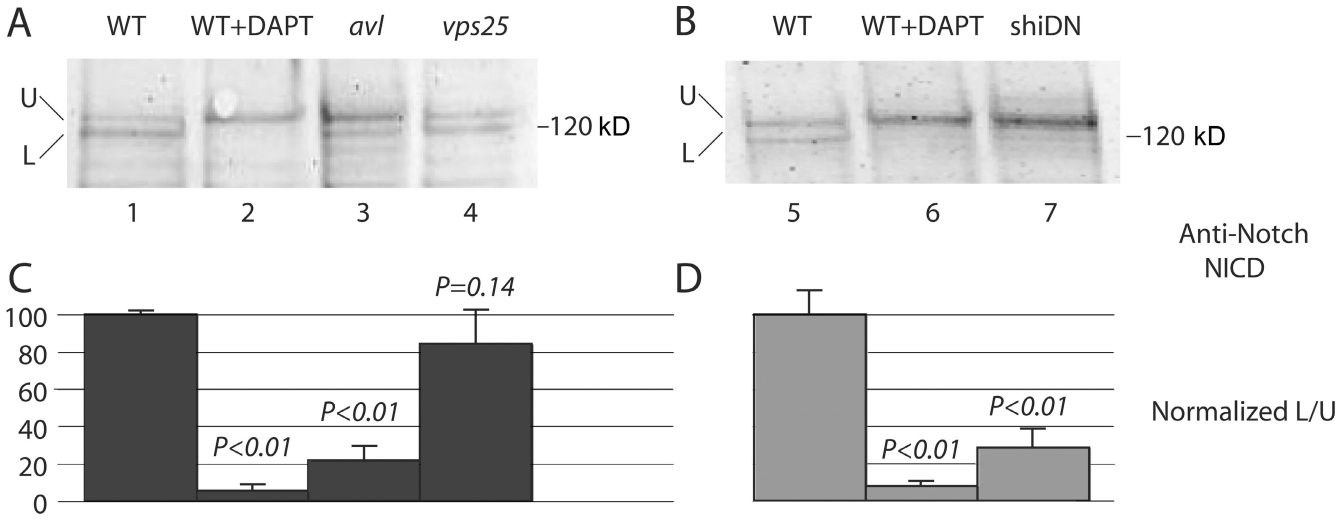

Figure 4. **The amount of Notch cleaved by γ-secretase is reduced in *avl* and shiDN extracts.** (A and B) Hypotonic extracts from eye (A) and wing (B) imaginal discs immunoblotted to detect the amount of NICD generated by γ-secretase cleavage, visualized as the lower migrating band (L) of the 120-kD doublet. Compared with WT and *vps25* extracts (lanes 1 and 4), WT extracts treated with DAPT or *avl* extracts extract generate only small amounts of free NICD (lanes 2 and 3). Similarly, generation of free NICD is strongly reduced in ShiDN-expressing extracts (B; lane 7). (C and D) Quantification of cleavage assay (normalized free NICD [L]/uncleaved NEXT [U] ratio), with standard deviation and statistical significance shown.

In *lqf tsg101* double mutant discs, *mβ–lacZ* expression was unchanged compared with that observed in *tsg101* mutant discs (Fig. 2 I). Collectively, these data demonstrate that the alterations in Notch signaling in endocytic mutant FCs parallel those seen in imaginal discs and indicate that endosomal entry of Notch itself is required for efficient signaling in epithelia.

The amount of cleaved Notch correlates with signaling activation

How could the entry of the Notch receptor into endosomes promote efficient signaling? Because signaling depends on intramembrane cleavage of Notch by γ-secretase, we asked whether altered Notch endosomal transport might affect its cleavage. We directly measured the amount of Notch in endocytic mutant tissue that can be cleaved by γ-secretase by using an assay that induces ligand-independent Notch cleavage in tissue extracts (see Materials and methods). Notch cleavage efficiency ex vivo is measured in Western blots by quantifying the amount of the lower band (corresponding to the γ-secretase cleavage product NICD) relative to the upper band (corresponding to its immediate precursor, the membrane-anchored γ-secretase substrate NEXT) of the ∼120-kD doublet recognized by an antibody against an NICD epitope. As expected, generation of free NICD in this assay is completely blocked by treatment with the γ-secretase inhibitor *N*-(*N*-[3,5-difluorophenacetyl-L-alanyl])-*S*-phenylglycine t-butyl ester (DAPT; Fig. 4 A, lanes 2 and 6). Strikingly, NICD generation is strongly reduced in *avl* mutant extracts compared with WT extracts, although it is not completely abolished (Fig. 4 A; lane 3), paralleling the reduced activation profile observed in situ with the *mβ–lacZ* reporter. To assess whether this reduction in γ-secretase cleavage is specific to *avl* or common to mutants that block endosomal entry, we tested *shiDN* extracts and found that they are also severely impaired (Fig. 4 B, lane 7). In contrast, no significant difference in cleavage was observed in extracts from *vps25* tissue in which, as in WT tissue, Notch can traffic to endosomes (Fig. 4 A, lane 4). This result is in apparent contrast with the elevation of Notch signaling observed in *vps25* mutants in vivo. However, elevated signaling is likely induced by persistence of trapped Notch in cleavage-proficient endosomes, and assessment of this increased cleavage over time is precluded by the ex vivo nature of the assay. These data indicate that access of Notch to endosomes correlates with increased ability to be cleaved by γ-secretase.

Notch activation in MVB sorting mutants can occur despite an early endocytic block

Although both Notch cleavage and signaling are substantially reduced in mutants that block endosomal entry, they are not completely abolished. We hypothesized that alternative low-efficiency Notch trafficking routes could account for such residual signaling. To test this hypothesis, we reasoned that any population of Notch that was internalized in endosomal entry mutants would subsequently accumulate and fail to be degraded in the absence of *ESCRT* activity. We therefore generated *avl tsg101* double mutant discs and found that, in contrast to *avl* discs, *avl tsg101* double mutant discs display some Notch immunoreactivity in Hrs-positive intracellular puncta that resemble those found in *tsg101* discs (Fig. 5, A, D, and G). This indicates that a small but significant amount of Notch can enter endosomes independent of *avl* function. We further found that *avl tsg101* discs display a level of *mβ–lacZ* intermediate between *avl* and *tsg101* alone (Fig. 5 H). Finally, to confirm this increase in Notch activation levels, we examined adult eyes resulting from genetically mosaic, rather than entirely mutant, eye discs. *ESCRT* cells in mosaic discs promote Notch-dependent overgrowth and give rise to bulging adult eyes (Fig. 5 F; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Herz et al., 2006), whereas adult eyes developing from *avl* mosaic discs are morphologically normal (Fig. 5 C). In contrast, adult eyes developing from *avl tsg101* mosaic discs display mild bulging, which is consistent

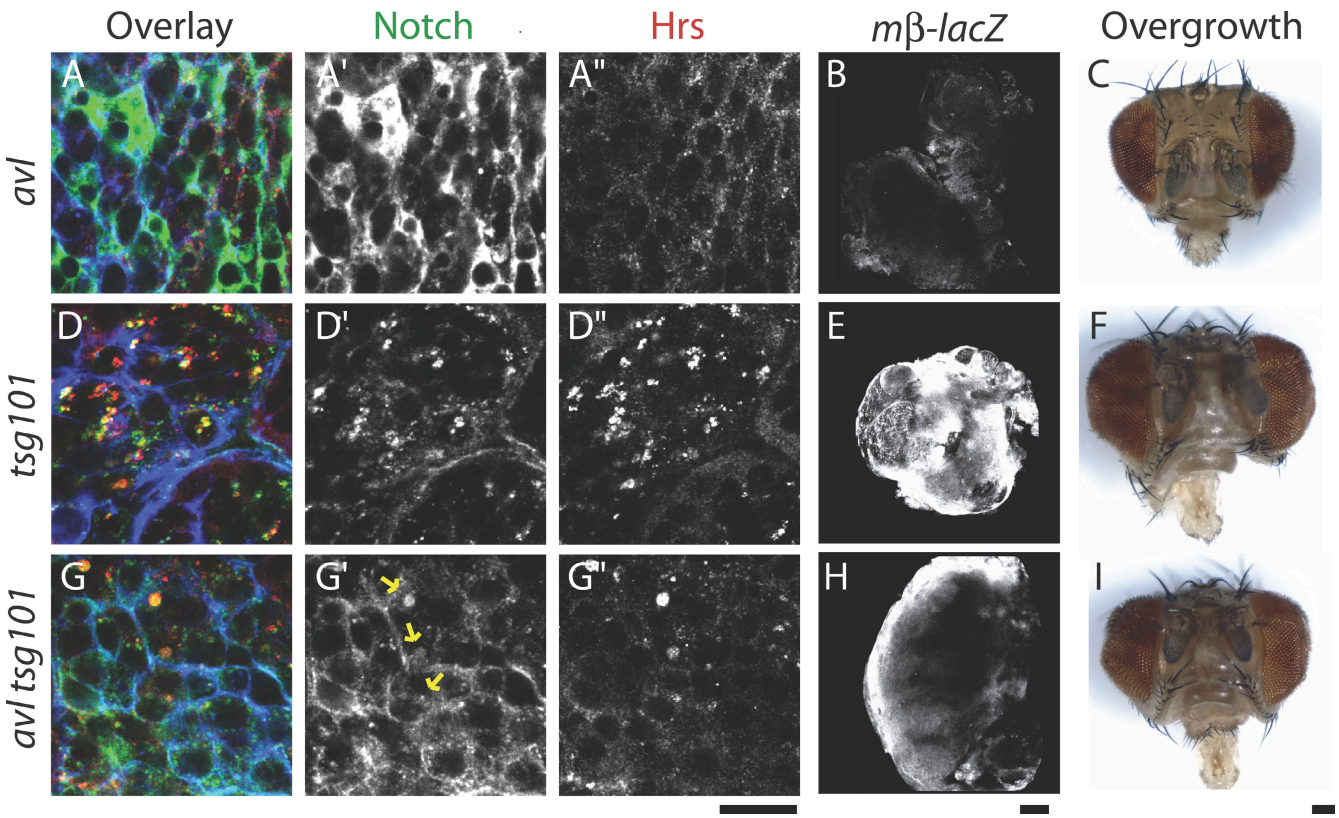

Figure 5. **Notch can be internalized and activated in an *avl*-independent fashion.** (A, D, and G) Notch staining of *avl* (A), *tsg101* (D), and *avl tsg101* double mutant (G) eye discs. Hrs marks endosomal compartments and phalloidin reveals cell outlines (blue). Intracellular Notch puncta that colocalize with Hrs (arrows in G′) are present in *avl tsg101* cells, albeit smaller than those seen in *tsg101* cells. (B, E, and H) *mß-lacZ* expression in eye discs. *avl tsg101* double mutants (H) express levels of β-gal intermediate between those seen in *avl* (B) and *tsg101* (E) discs. (C, F, and I) Adult eyes originating from mosaic *avl tsg101* and *avl tsg101* eye discs. Compared with eyes from *avl* (C) and *tsg101* (F) mosaics, *avl tsg101* mosaics (I) display an intermediate level of overgrowth. Bars: (A, D, and G) 10 μm; (B, E, and H) 100 μm; (C, F, and I) 100 μm.

with intermediate levels of Notch signaling hyperactivation (Fig. 5 I). These data suggest that more than one Notch internalization route exists and may account for residual Notch activation seen in mutants that impair cargo recruitment into endosomes.

Our findings place Notch among the growing number of signaling pathways in which activity is stimulated, rather than attenuated, by receptor endocytosis. Examples of such pathways that have been studied in *D. melanogaster* include Dpp and Wingless (Wg) signaling (Fischer et al., 2006). Although the effects of endocytosis on Wg and Dpp signaling appear complex, transport to endosomes of Wg- and Dpp-bound receptors can, in some circumstances, enhance activation of the respective pathways (Entchev et al., 2000; Piddini et al., 2005; Marois et al., 2006; Rives et al., 2006; Seto and Bellen, 2006). Interestingly, at least in the case of Wg receptors, signaling appears to be reduced by stimulation of MVB transport (Seto and Bellen, 2006), an effect opposite to the enhancement observed for Notch signaling under similar conditions. This example highlights how each step of endocytic traffic can exert distinct regulatory effects on different activated receptors and emphasizes the importance of defining the influence of endocytic trafficking on all receptor-mediated cell signaling pathways.

Previous studies have concluded that endocytosis of the activated NEXT fragment of Notch is not a requirement for signaling. For example, Struhl and Adachi (2000) showed that an overexpressed NEXT-like construct can be cleaved in *shi* mutant *D. melanogaster* embryos. We have performed analogous experiments in imaginal discs and also find that NEXT overexpression induces activation when *shi*-dependent internalization is disrupted (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200708127/DC1). We favor the hypothesis that the difference between these experiments and the data presented above involves the use of an overexpressed Notch mimic instead of endogenous Notch. Our data, which point to the existence of more than one route to deliver Notch to endosomes, can reconcile and clarify the above findings: alternative routes may permit sufficient internalization of overexpressed protein to account for the activation observed. A second, not mutually exclusive possibility is that a low and normally subthreshold degree of γ-secretase cleavage of NEXT at the plasma membrane is amplified by overexpression. Consistent with either scenario, in a study of bristle patterning, activation of Notch via overexpression did not require *shi* but activation of Notch via a missense mutation in the endogenous protein was *shi* dependent (Seugnet et al., 1997).

How might endosomal entry promote Notch signaling? The strong correlation between γ-secretase cleavage efficiency and the degree of Notch signaling observed in vivo, seen in both loss- and gain-of-function manipulations that alter endosomal traffic, leads us to propose that in WT *D. melanogaster cells*, γ-secretase cleavage occurs most efficiently in endosomes. This proposition

is consistent with the fact that γ-secretase can act in endosomes and has optimal activity at low pH, typical of endocytic organelles (Lah and Levey, 2000; Pasternak et al., 2003; Gupta-Rossi et al., 2004; Urra et al., 2007). Unliganded Notch, which is continually internalized, is normally rapidly transported through such endosomes; thus, the ligand-independent activation seen in *ESCRT* mutants could result from amplification of a subthreshold propensity of heterodimeric Notch to be cleaved because of altered endocytic kinetics and endosomal accumulation. Alternatively, considering that the ionic environment can affect shedding of the inhibitory extracellular portion of Notch (Rand et al., 2000), *ESCRT* mutant endosomes might possess a lumenal environment that weakens Notch heterodimeric interactions, leading to ligand-independent cleavage. Future investigation will test the relevance of ligand-independent activation in *ESCRT* mutants to physiological Notch signaling.

Our finding of an endosomal trafficking route that promotes ligand-independent Notch activation may be relevant to human neoplasias. A subset of leukemia-associated mutations display γ-secretase–dependent Notch hyperactivation and render Notch sensitive to ligand-independent cleavage by weakening the heterodimerization of Notch N- and C-terminal fragments (Malecki et al., 2006). Several γ-secretase inhibitors are currently under evaluation as potential therapeutic agents for Notch-regulated cancers (Kogoshi et al., 2007). The fact that endosomal trafficking of Notch appears closely tied to the γ-secretase–mediated cleavage step raises the possibility that endosomal routing pathways might offer additional prospective targets for clinical intervention.

Materials and methods

D. melanogaster genetics

The *avl*[1] (Lu and Bilder, 2005), *lqf*[f71] (provided from J. Fisher, University of Texas, Austin, TX), and *tsg101*[2] (provided by K. Moberg, Emory University School of Medicine, Atlanta, GA) alleles were used to generate recombinant chromosomes. Other chromosomes used include *Rab5*[2] *UAS-Rab5DA* and *UAS-Rab5DN* (provided by M. González-Gaitán, Département de Biochimie, Sciences II, Genève, Switzerland), *hrs*[D28] *and UAS-Hrs* (provided from H. Bellen, Baylor College of Medicine, Houston, TX), *vps25*[A3] (Vaccari and Bilder, 2005), *fab-1*[2-1] (provided by T.E. Rusten, University of Oslo, Oslo, Norway) and *vps20*[l3] (Vaccari and Bilder, 2005), *lgl*[4] (Bilder et al., 2000), *E(spl)mß-LacZ* (provided by J. Posakony, University of California, San Diego, La Jolla, CA), *UAS-shiK44A* (Bloomington Stock Center), *UAS-NotchFL*, *UAS-NICD* (provided from G. Struhl, Columbia University, New York, NY), and *MS1096 GAL4* (Bloomington Stock Center). *UAS-NEXT* expresses a protein consisting of the first 25 amino acids of Notch (including signal sequence) followed by an HA epitope tag, followed by Notch sequences from AAKHQ located ∼35 amino acids N terminal to the transmembrane domain to QQLGG in the intracellular domain, and ending with C-terminal V5 and His tags. Follicle cell clones were generated as described by Bilder et al. (2000). Mosaic eyes were generated as described by Tapon et al., (2001), whereas completely mutant eye discs (referred to in the text as mutant discs) were generated as described by Newsome et al., (2000).

Trafficking assay, immunostainings, and microscopy

Ovaries and imaginal disc tissues were fixed and stained under standard conditions with rhodamine-phalloidin and primary antibodies against the following antigens: Avl (Lu and Bilder, 2005), Hrs (Lloyd et al., 2002), β-gal (Capell) and NECD, NICD, Wg, Hnt (Developmental Studies Hybridoma Bank). Notch endocytosis assays were conducted as described by Vaccari and Bilder (2005). Secondary antibodies were obtained from Invitrogen. In Fig. 1, subapical cross-sections of epithelium anterior to the morphogenetic furrow (Fig. 1, B, F, and J) or in the wing pouch (C) are shown; other panels show equivalent sections, although these mutant cells with altered polarity do not display a clear apical side. All images are single confocal sections taken at room temperature with a microscope (TCS; Leica) using 16× NA 0.5 or 63× NA 1.4 oil lenses (Leica). Images were edited using Photoshop 7.0 (Adobe) and assembled with Illustrator 10 (Adobe).

Notch cleavage assay

For Notch cleavage assays, postnuclear extracts from WT and mutant discs were prepared using a hypotonic lysis buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, and Complete protease inhibitor (Roche; Hu et al., 2002). The low salt concentration and absence of detergents promotes preservation of in vivo compartmental localization of transmembrane proteins, whereas EDTA chelates Ca^{2+} and thus promotes ligand-independent Notch processing (Rand et al., 2000). To test γ-secretase cleavage of Notch after harvesting of discs, extracts were incubated ex vivo for 20 min in hypotonic buffer alone or supplemented with 100 µM DAPT to inhibit the γ-secretase enzyme. Western blots were probed with both anti-NICD and anti–β-tubulin (Sigma-Aldrich). Quantification in Fig. 4 C and Fig. 4 D, based on three and four independent experiments, respectively, was obtained by calculating of the intensity ratio of free NICD (L) band to uncleaved NEXT (U) band normalized to WT (normalized L/U). The distinct L/U ratios seen between WT eye and wing disc extracts are consistent and may reflect intrinsic tissue-specific differences in Notch processing. Statistical analysis was performed by applying a Student's *t* test on the quantified dataset. Image acquisition and quantification was performed with an imaging system (Odyssey; Li-Cor Biosciences).

Online supplemental material

Fig. S1 shows that the loss of Notch signaling in endocytic mutant FCs is not an indirect effect of cell polarity alterations. Fig. S2 shows that Notch ligand control can be bypassed by overexpression of a truncated Notch mimic in ShiDN-expressing cells. Fig. S3 shows GFP expression controls for the endocytic mutant FC clones shown in Fig. 3. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708127/DC1.

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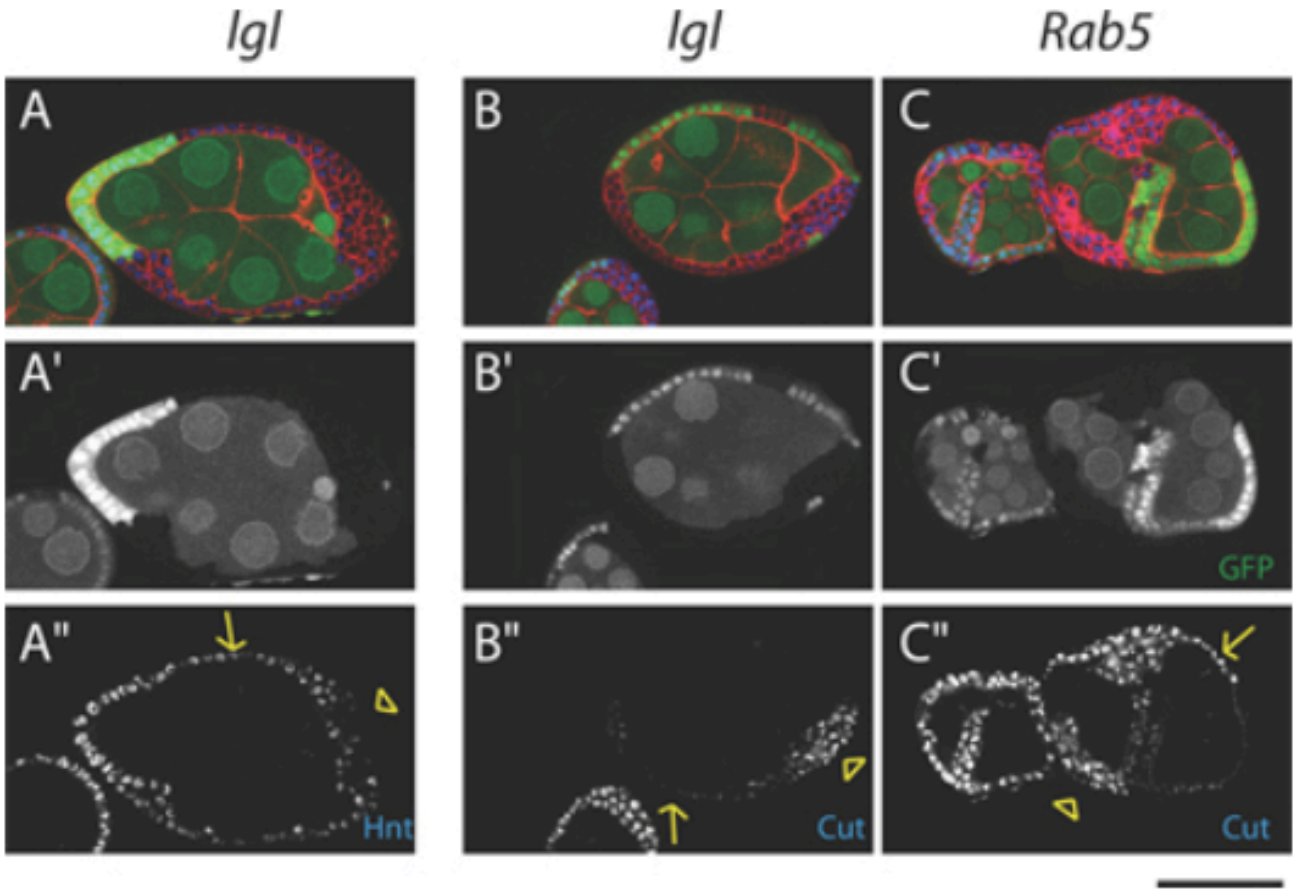

Figure S1. **Loss of Notch signaling in endocytic mutant FCs is not an indirect effect of cell polarity alterations.** (A–C) Egg chambers at stages 5–6 of oogenesis stained for Hnt (A), Cut (B and C), and actin (red). In *lgl* mutant FC clones (lacking GFP) that retain monolayered organization, activation of the positively regulated Notch target Hnt is not lost, a phenotype opposite to that observed in monolayered *Rab5* FC clones (compare arrow in A″ with Fig. 3 D). In multilayered *lgl* mutant FCs that do not contact the germline and are thus unable to receive the Delta signal, Hnt expression is lost as expected (A″, arrowhead). Reciprocal effects are observed looking at the negatively regulated Notch target Cut (B and C; arrows indicate examples of monolayered FC clones; arrowheads indicate examples of multilayered FC clones). Bar, 50 μm.

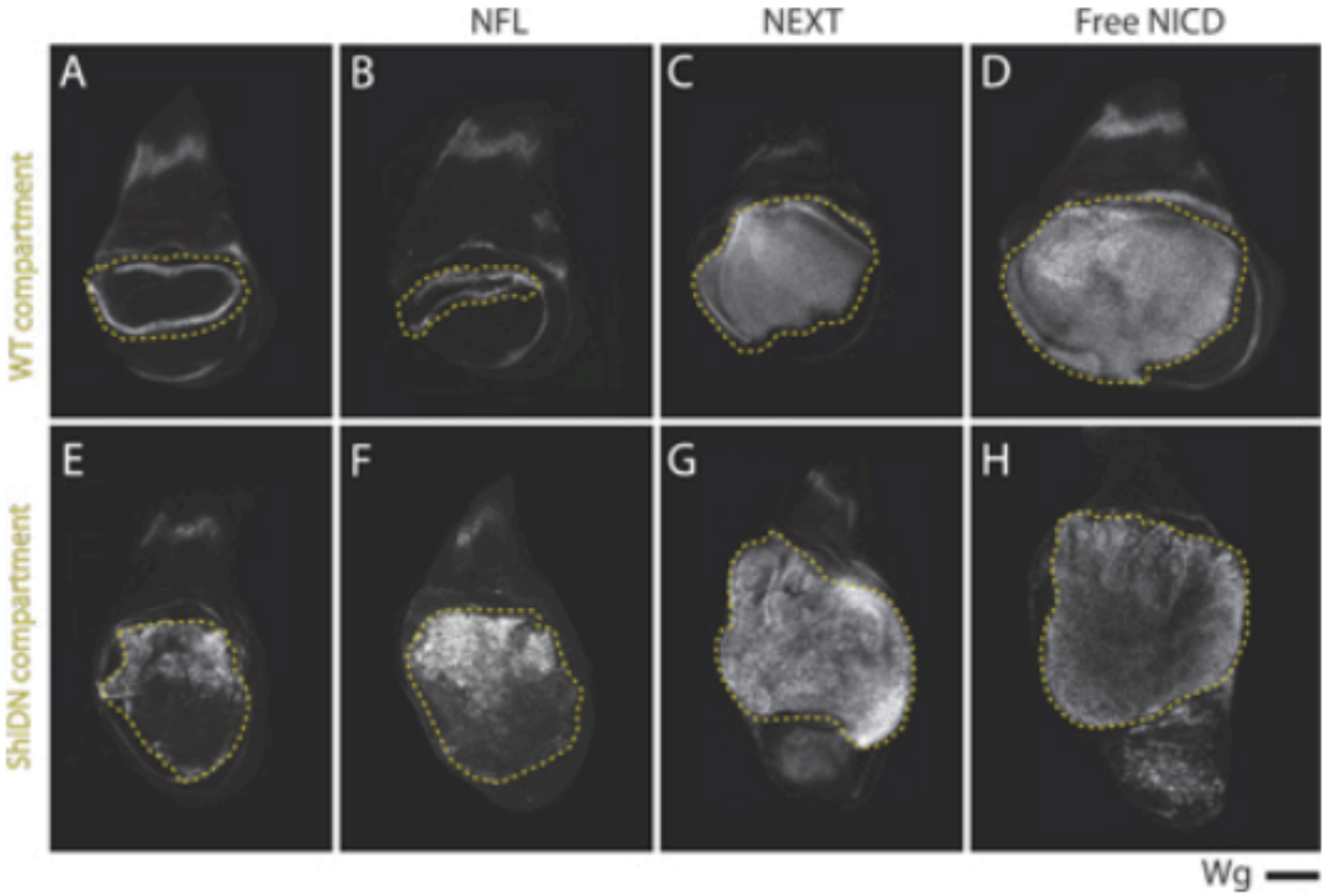

Figure S2. **Ligand control can be bypassed by overexpression of a truncated Notch mimic in ShiDN-expressing cells.** (A–D) WT wing discs (A) and discs expressing full-length Notch (NFL), NEXT, and free NICD (B–D) stained to detect expression of the Notch target Wg. Expression of Notch constructs in the dorsal compartment (outlined with a broken line) is driven by *MS1096-GAL4*. In contrast to discs expressing NFL, which retain ligand control (B), discs expressing NEXT (C) and free NICD (D) promote ectopic Wg expression throughout the wing pouch. (E–H) In discs expressing *shiDN* (E) and in discs expressing *shiDN* and NFL (F), the domain of Wg expression is expanded because of a lack of Wg endocytosis (Seto, E.S., and H.J. Bellen. 2006. *J. Cell Biol*. 173:95–106), but most *shiDN*-expressing tissue does not express Wg. In discs expressing *shiDN* and *NEXT* (G) and discs expressing *shiDN* and free *NICD* (H), all of the *shiDN*-expressing tissue expresses Wg. Bar, 100 µm.

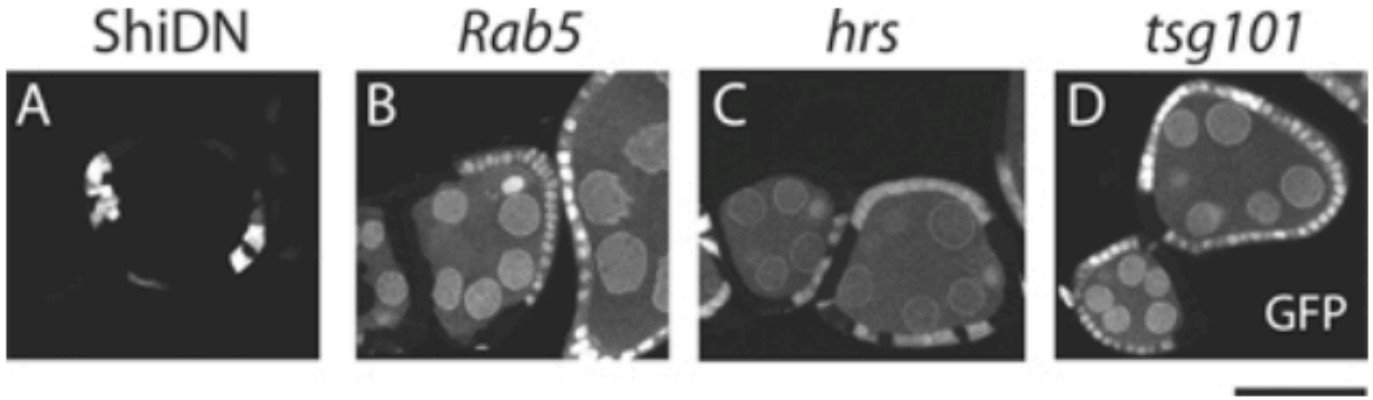

Figure S3. **Endocytic mutant FC clones marked by GFP expression.** GFP channel of the egg chambers presented in Fig. 2. (A) FC clones expressing *ShiDN* (corresponding to the clones outlined in Fig. 2 C) are marked by GFP expression. (B–D) FC clones mutant for *Rab5*, *hrs*, and *tsg101* (corresponding to the clones outlined in Fig. 2, D–F) lack GFP expression. Bar, 50 µm.