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The Functions of Auxilin and Rab11 in Drosophila Suggest That the Fundamental Role of Ligand Endocytosis in Notch Signaling Cells Is Not Recycling

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Abstract

Notch signaling requires ligand internalization by the signal sending cells. Two endocytic proteins, epsin and auxilin, are essential for ligand internalization and signaling. Epsin promotes clathrin-coated vesicle formation, and auxilin uncoats clathrin from newly internalized vesicles. Two hypotheses have been advanced to explain the requirement for ligand endocytosis. One idea is that after ligand/receptor binding, ligand endocytosis leads to receptor activation by pulling on the receptor, which either exposes a cleavage site on the extracellular domain, or dissociates two receptor subunits. Alternatively, ligand internalization prior to receptor binding, followed by trafficking through an endosomal pathway and recycling to the plasma membrane may enable ligand activation. Activation could mean ligand modification or ligand transcytosis to a membrane environment conducive to signaling. A key piece of evidence supporting the recycling model is the requirement in signaling cells for Rab11, which encodes a GTPase critical for endosomal recycling. Here, we use Drosophila Rab11 and auxilin mutants to test the ligand recycling hypothesis. First, we find that Rab11 is dispensable for several Notch signaling events in the eye disc. Second, we find that Drosophila female germ line cells, the one cell type known to signal without clathrin, also do not require auxilin to signal. Third, we find that much of the requirement for auxilin in Notch signaling was bypassed by overexpression of both clathrin heavy chain and epsin. Thus, the main role of auxilin in Notch signaling is not to produce uncoated ligand-containing vesicles, but to maintain the pool of free clathrin. Taken together, these results argue strongly that at least in some cell types, the primary function of Notch ligand endocytosis is not for ligand recycling.

Introduction

Virtually all signaling pathways have an endosomal component [1]. Notch signaling, however, is remarkable in its absolute dependence on endocytosis [2–7]. The Notch receptor and its ligands (Delta and Serrate in Drosophila) are transmembrane proteins [8]. Although the roles of ligand and receptor internalization are unclear, endocytosis is essential to both Notch signaling and signal reception. Most counterintuitive is the requirement for ligand endocytosis into the signaling cells. Two classes of models have been proposed to explain why ligand needs to be internalized in order to signal [2–7]. The “pulling” model proposes that endocytosis of ligand bound to the Notch receptor exerts a mechanical force that activates the receptor either by exposing a proteolytic cleavage site on the receptor extracellular domain, or by dissociating the subunits of the receptor heterodimer. In contrast, ligand is endocytosed prior to receptor binding in the “recycling” model, and via an endosomal pathway, it is returned to the plasma membrane either in an activated form that can bind ligand, or to a new membrane environment favorable to receptor interaction.

Several results support the pulling model. First, when separated from its transmembrane domain and secreted, the extracellular domain of Delta blocks Notch activation [9]. Second, the extracellular domains of Notch and Delta are sometimes found together in endosomes inside signaling cells [10,11]. Third, structural studies suggest that the ADAM protease site on the Notch extracellular domain, which must be cleaved to activate the receptor, is exposed by ligand binding [12]. Finally, there is evidence that ligand internalization into signaling cells depends on the presence of Notch in adjacent cells [13]. There is also evidence in favor of the recycling model. For example, in some epithelial cells, the GTPase Rab11, which is required for endosomal recycling [14], is needed in signaling cells for signaling and for...
Delta recycling [15–17]. In addition, the ligand intracellular domain, which is normally ubiquitinated by specific ubiquitin ligases that are necessary for signaling and ligand endocytosis [10–26], may be replaced by the internalization and recycling signals from the vertebrate LDL receptor [27]. Finally, Delta transcytosis has been observed, and it is thought to relocate ligand to a site on the plasma membrane near Notch in the adjacent cell [15–17,28].

The pulling and recycling models are not necessarily mutually exclusive. It has been proposed that two ligand internalization events are required, the first to activate ligand through recycling, and the second to activate the receptor on an adjacent cell through pulling [28,29].

Epsin and auxilin are two endocytic proteins required in signaling cells for ligand endocytosis and signaling [27,30–36]. Epsin, which has been shown to be an essential component of the Notch pathway in C. elegans [36] and vertebrates [37], as well as in Drosophila [27,30], has binding sites for the plasma membrane, ubiquitin, clathrin, and other proteins present in clathrin-coated vesicles [38]. Although the mechanism of epsin function in Notch signaling is not well understood, studies of epsin in other contexts suggest that epsin probably links ubiquitinated ligand with endocytic vesicles [38]. Another endocytic protein, auxilin, is also required in Notch signaling cells in all Drosophila tissues tested [31–34]. Auxilin brings the ATPase Hsc70 to clathrin cages, and stimulates Hsc70 to uncoat clathrin coated vesicles [39]. At first glance, it would appear that the requirement for auxilin supports the recycling model; uncoating of newly internalized clathrin-coated vesicles containing ligand is prerequisite for trafficking of ligand through an endosomal pathway for recycling. However, it is also possible that auxilin is required only to maintain the pool of free clathrin, and not for production of uncoated vesicles [33].

In addition, it was shown recently that to send Delta signals, Drosophila female germline cells require epsin-mediated endocytosis, but not clathrin [40]. Vertebrate epsin is known to function in both clathrin-dependent and clathrin-independent endocytosis [41–43]. However, this result suggests the possibility that epsin function in Notch signaling is generally clathrin-independent, and thus the function of auxilin in signaling cells might be other than its characterized role in clathrin dynamics.

Here, we performed genetic experiments in Drosophila to test the roles of Rab11 and auxilin in Notch signaling, and ultimately to test the recycling model. First, we found that Rab11 is not required for Notch signaling events in the eye disc that require both epsin and signaling cells. Rab11 mediates fusion of early endosomes with the sorting endosome, an event required for trafficking through any endosomal pathway, and Rab11 is required for subsequent routing of an endosome through the recycling pathway [14]. First, we asked about one characterized event early in eye development, called R-cell restriction [30]. Photoreceptors R2/R5 and R3/R4 in early ommatidial preclusters signal via Delta to other precluster cells, preventing them from becoming ectopic photoreceptors (R-cells). When this signaling event fails (for example in hypomorphic lqf or aux mutants), ommatidia have one or several extra photoreceptors [30,32,33,44]. When dominant negative shibire (encodes Drosophila dynamin) or Delta genes are expressed specifically in R2/R5 and R3/R4 using arough (ro) gene expression vector, ommatidia in adult eyes have extra R-cells due to failure of R-cell restriction [30]. Using the samero expression vector, we generated transgenes expressing dominant negative forms of Rab5 or Rab11 (ro-Rab5N122I and ro-Rab11N224I). Rab11N224I has been shown to act as a dominant negative late in eye development, where it blocks transport of rhodopsin to rhabdomeres and formation of multivesicular bodies in late endosomes [45]. Neither transgene had an effect on eye development, even when present in as many as four copies (data not shown). These results suggest that neither Rab5 nor Rab11 is required for this Notch signaling event, but there are other plausible explanations for the failure of these transgenes to interfere with Notch signaling. For example, expression levels that are too low for effective competition with wild-type proteins.

To overcome the problem in interpreting results obtained with dominant negative transgenes, we wanted to generate Rab5- or Rab11- (null) clones in the eye disc. Rab5 null clones have an overgrowth phenotype that would obscure a Notch signaling defect [46]. Rab11 null clones in the eye have not been reported, but we were able to generate them, and they were not hypertrophic (see below). The Rab11 null allele we used, Rab11FRT, has a deletion of the promoter and first two exons, and produces no protein [47]. We used Rab11 null clones to ask whether or not well-characterized signaling events in the eye disc required Rab11. The adult Drosophila eye develops from the larval eye imaginal disc, a monolayer epithelium [48]. Rows of ommatidia assemble stepwise posterior to the morphogenetic furrow, as it moves from the posterior to the anterior of the disc. The first cells to join the facets are the eight photoreceptors (R1-R8), and they do so in an invariant order in every ommatidium. Nearly every step of ommatidial assembly involves Notch signaling [49,50], and so elimination of the Notch pathway in clones of mutant cells is catastrophic to eye development. In Notch- clones, no cells are specified as photoreceptors because Notch signaling is required anterior to the furrow to give cells neural potential, a process called proneural enhancement [51]. In Delta- clones, there are no photoreceptors in the middle of the clone. At the clone border, however, Delta- cells do become photoreceptors because they receive Notch signals from adjacent wild-type cells. Discrete ommatidia do not form within the clone because subsequent lateral inhibitory signaling cannot occur between adjacent Delta- cells, and the result is that too many cells adopt neural fate [51]. Clones of either lqf- (liquid facets) or aux- (auxiliary) cells in the eye disc appear identical to Delta- clones, consistent with the idea that epsin and auxilin are required in the signaling side of the Notch pathway [30,33]. In accord with the developmental mutant phenotype, reporters for Notch activation are not expressed at all in N- cell clones, and are expressed in Dl-, lqf-, or aux- clones only in cells at the clone border, adjacent to wild-type cells that can signal [30,33,34,31, and see below].

We tested whether or not Rab11- (null) clones in eye discs would suffer severe defects in early ommatidial assembly, and whether or not Rab11- cells, especially those in the middle of the clone, would activate Notch. First, we observed Rab11- clones in eye discs

Results

Rab11 was dispensable for Notch signaling events in the eye disc

We wanted to determine whether or not ligand recycling is required for Notch signaling during eye development. If so, it would be expected that the two GTPases Rab5 and Rab11 would both be required in signaling cells. Rab5 mediates fusion of early endosomes with the sorting endosome, an event required for trafficking through any endosomal pathway, and Rab11 is required for subsequent routing of an endosome through the recycling pathway [14]. First, we asked about one characterized
immunostained with anti-Elav, which labels photoreceptor nuclei [52]. We found that compared with the calamitous effect on development in N-, Dl-, lqf-, or aux- clones [30,33,34,51], ommatidial assembly was not obviously disrupted within the Rab11- clones; discrete ommatidia were present in the middle of the clone and at the clone borders (Fig. 1E1, E1'). This is consistent with results of similar experiments performed with Rab11 homopmorphs, where eye morphogenesis defects observed were due mainly to late events: cell death and the failure to form light-gathering rhodobemes [45,53]. These eye discs also contain a reporter transgene called m\textsuperscript{6}-lac\textsubscript{Z} which is transcribed in R4 when Notch is activated in response to Delta signaling by R5 [54,55]. This Notch signaling event distinguishes R3 and R4 [54–56]. No cells in Notch- (null) clones expressed m\textsuperscript{6}-lac\textsubscript{Z} (Fig. 1A, A'), while Delta- (null) cells did express m\textsuperscript{6}-lac\textsubscript{Z} but only when they were adjacent to wild-type cells at the clone edges (Fig. 1B, B'). We found that like Delta- cells, lqf- (null) or aux- (null) cells at the clone edge activated m\textsuperscript{6}-lac\textsubscript{Z} (Fig. 1C–D'). This result is consistent with other evidence that lqf+ and aux+ function in the signaling cells [27,31,33,34,40], and that in imaginal discs and embryos, auxilin and possibly epsin are needed, but clathrin is dispensable [40]. One possibility suggested by this observation is that epsin-like proteins promote clathrin-dependent endocytosis of ligand in imaginal discs, and that in imaginal discs and embryos, auxilin and possibly also clathrin perform functions other than clathrin-mediated endocytosis. Alternatively, as epsin is known to facilitate both kinds of endocytic pathways [41–43], epsin may promote ligand endocytosis through a clathrin-independent pathway in female germline cells, and through a clathrin-dependent pathway in imaginal discs. In this scenario, auxilin would perform its known function in clathrin dynamics, which is uncoating clathrin-coated vesicles after internalization [39].

One way to distinguish between these two alternatives is to determine if the function of auxilin in Notch signaling is separable from the function of clathrin, and so we tested whether or not aux+ was required in the female germline. In the ovary, the sixteen germ-line cells in the nurse cell/oocyte complex signal to surrounding somatic follicle cells at stage 6 of oogenesis, and Notch receptor activation may be monitored by expression of the target gene Hindsight (Hnt) (Fig. 2A) [40,46]. In wild-type ovaries, Hnt is present in the nuclei of all surrounding follicle cells following stage 6 (Fig. 2B.B'). In mosaic ovaries in which the follicle cells are aux+ and the germline cells are aux- (null), the follicle cells nevertheless express Hnt (Fig. 2C–D'). Identical results were observed previously in ovaries mosaic for Chev- and Che- cells [46] (see legend to Fig. 2). The same results were obtained using two different aux- backgrounds: aux\textsuperscript{136}/aux\textsuperscript{277} or aux\textsuperscript{9356}/ homozygotes, aux\textsuperscript{136} [32,33] and aux\textsuperscript{9356} [34] have nonsense mutations positioned between the codons for the clathrin binding domain and the J domains, which binds Hsc70. Thus, C-terminally truncated auxilin proteins that could in theory be produced would lack the J domain, which is essential for auxilin function in Notch signaling [33,34]. aux\textsuperscript{277} has a nonsense mutation early in the open reading frame, and an N-terminally truncated protein containing both the clathrin binding and J domains, produced by translation reinitiation, could function in Notch signaling [33,34]. No auxilin protein from aux\textsuperscript{277} was detectable with immunofluorescence using an auxilin antibody, and the genetic behavior of aux\textsuperscript{277} was indistinguishable from that of aux\textsuperscript{136} [34]. Thus, we conclude that the germline cells, which do not require clathrin for signaling, also do not require auxilin. This result indicates that germline and eye and wing disc cells simply internalize ligand through different endocytic pathways. Thus, the requirement for clathrin and auxilin in eye and wing discs most likely means that auxilin regulates clathrin dynamics in Notch signaling cells in the eye disc.

Overexpression of clathrin heavy chain and liquid facets suppressed the semi-lethality and severe eye defects caused by strong auxilin mutations

The requirement for auxilin by the signaling cells provides a tool for testing the recycling model. Auxilin uncoats clathrin-coated vesicles, an expected prerequisite for fusing of newly endocytosed vesicles with the sorting endosome and subsequent transit through an endocytic pathway [39]. Auxilin activity, however, in addition to producing uncoated ligand-containing vesicles, also produces free clathrin. Indeed, free clathrin is depleted in the absence of auxilin [57,58], and Delta endocytosis is inefficient in aux mutants [33]. Thus, it is possible that auxilin is required by signaling cells not to provide uncoated ligand-containing vesicles, but to provide free clathrin for use in the internalization step. If so, then providing free clathrin through different means should obviate the need for auxilin in signaling cells. Indeed, it was observed that Che+ overexpression partially suppressed the Notch signaling defects in eyes (and wings) associated with strong aux mutants [33]. Here, we tested the extent to which the lethality associated with aux mutations is also be suppressed by Che+ overexpression. In addition, we tested whether or not auxilin overexpression also suppresses the aux mutant phenotype, and if the extent of suppression would be increased by co-overexpressing clathrin heavy chain and auxin.

First, we wondered how well the lethality of aux mutants, presumably caused by the failure of Notch signaling in early development [31], was suppressed by Che+ overexpression. Heterozygotes for one weak missense mutation and one strong

auxilin was not required for clathrin-independent Notch signaling in the ovary

Auxilin is known to be required for Notch signaling in the eye, wing, and embryo [31–34]. Strong genetic interactions between clathrin heavy chain (che) and lqf [44], and the requirement for aux in signaling cells [31–34] suggested that epsin promotes clathrin-mediated endocytosis of ligand in signaling cells. Therefore, we were puzzled by the observation that for signaling by female germ-line cells, epsin is needed, but clathrin is dispensable [40]. One possibility suggested by this observation is that epsin-like proteins promote clathrin-independent endocytosis of ligand in imaginal discs, and that in imaginal discs and embryos, auxilin and possibly also clathrin perform functions other than clathrin-mediated endocytosis. Alternatively, as epsin is known to facilitate both kinds of endocytic pathways [41–43], epsin may promote ligand endocytosis through a clathrin-independent pathway in female germline cells, and through a clathrin-dependent pathway in imaginal discs. In this scenario, auxilin would perform its known function in clathrin dynamics, which is uncoating clathrin-coated vesicles after internalization [39].
nonsense mutation in aux (auxK47/auxD128) rarely reach adulthood when grown at 25°C (Table 1). In addition, adult escapers have severely malformed imaginal disc-derived structures, including their eyes (Fig. 3A,B,F–H). Addition to the auxK47/auxD128 flies of a transgene containing a genomic DNA copy of the Chc+ gene (PgChc+) that can substitute for the endogenous Chc+ gene increases the eclosion frequency of adults markedly (Table 1). Also, as reported previously, the mutant eye phenotype of those rescued adults was suppressed somewhat (from 8% to 28% wild-type ommatidia) (Fig. 3D, J).

Next, we wondered whether epsin overexpression, either alone or in combination with Chc+ overexpression, would suppress the auxK47/auxD128 mutant phenotype. We reasoned that if epsin links ligand to clathrin, it may be freed along with clathrin when auxilin uncoats clathrin from newly endocytosed vesicles. Alternatively, increased epsin levels in aux mutants may result in more efficient plasma membrane localization of the remaining free clathrin. We found that a transgene with a genomic DNA copy of the lqf+ gene (Pglqf+) that complements lqf null mutants (similar to the transgene in ref. 44; X. X. and J.A.F., manuscript in preparation) rescued the

Figure 1. Rab11 is not required for Notch signaling in eye discs. Confocal microscope images of third instar larval eye discs with clones of mutant cells are shown. The discs are immunolabeled to reveal Notch activation (anti-ßgal), photoreceptor cell nuclei (anti-Elav), and F-actin (phalloidin). Homozygous mutant cell clones are marked by the absence of nuclear GFP expression. Clones are outlined in white. Arrow heads point to some of the mutant cells within the clones that express ß-gal, indicating that Notch is activated. (A,A’) A Notch null (N-) clone was generated in larvae of the genotype N5kek FRT19A/ubi-npg FRT19A; ey-gal4, UAS-flp/++; mδ-lacZ+. (B,B’) A Delta null (Δl) clone was generated in larvae of the genotype ey-flp;mδ-lacZ/+; FRT82B Df104/FRT82B ubi-npg (C,C’) lqf- clones generated in larvae of the genotype ey-flp; mδ-lacZ/+; lqf+ FRT80B/ubi-npg FRT80B. (D,D’) aux- clones were generated in larvae of the genotype ey-flp; mδ-lacZ/+; auxF956*/FRT5-5Z3515 tub-npg. (E-E‘) The same Rab11-clone is shown in all panels, generated in larvae of the genotype ey-flp; mδ-lacZ/+; Rab11D FRT/FRT5377 Hrb98DE::GFP. Scale bar 20 μm.
lethality of auxK47+/auxD128 mutants (Table 1) and suppressed their mutant eye phenotype even better than PgChc+ did (62% wild-type ommatidia) (Fig. 1E,K). Moreover, auxK47+/auxD128 flies carrying both Pglqf+ and PgChc+ had remarkably normal-appearing eyes (97% wild-type ommatidia) (Fig. 3C,I). However, no increase in viability was detected in these flies above the level observed with Pglqf+ alone (Table 1; see also legend).

Thus, a single extra copy of either the Chc+ gene or the lqf+ gene suppressed the aux mutant phenotype, including lethality, significantly. Remarkably, a single extra copy of both the Chc+ and lqf+ genes suppressed nearly completely the severe morphological abnormalities due to Notch signaling defects in aux mutants. This indicates that supplying free clathrin heavy chain and additional epsin to the cells bypasses the large part of the need for auxilin in Notch signaling. We conclude that the primary role of auxilin in Notch signaling cells is to maintain the pool of free clathrin, and possibly also epsin.

Discussion

There are three major results of this work. First, we found that Rab11 is not required for several Notch signaling events in the developing Drosophila eye that require epsin and auxilin. Thus, as in the female germline cells, ligand recycling, at least via a Rab11-dependent pathway, is not necessary for Notch signaling in the eye disc. Second, we found that the one Notch signaling event presently known to be clathrin-independent is also auxilin-independent. This result reinforces the idea that rather than performing some obscure function, the role of auxilin in Notch signaling cells is to regulate clathrin dynamics. Finally, we showed that overexpression of both clathrin heavy chain and epsin rescues to nearly normal the severely malformed eyes and semi-lethality of aux mutants. Presumably, vesicles uncoated of clathrin fuse with the sorting endosome, and so it seems reasonable to assume that uncoating clathrin-coated vesicles containing ligand is prerequisite for trafficking ligand through endosomal pathways. Thus, if ligand endocytosis is prerequisite to recycling, efficient production of uncoated vesicles would be required. In aux mutants with severe Notch-like mutant phenotypes, clathrin vesicle uncoating is inefficient. We presume that this remains so even when clathrin and epsin are overexpressed, yet the eye defects and lethality are nearly absent. Thus, we reason that auxilin is required not for efficient production of uncoated vesicles per se, but for the other product of auxilin activity – free clathrin (and possibly also

Figure 2. Female germline cells do not require auxilin to send Delta signals to follicle cells. (A) A diagram of an oocyte/nurse cell complex (stage 6–7) is shown. The fifteen nurse cells are diploid, and the cytoplasmic bridges of the nurse cells and the oocyte are interconnected by cytoplasmic bridges. (B–D') Confocal microscope images of oocyte/nurse cell complexes are shown. The complexes were immunolabeled to reveal Notch activation in the follicle cells (anti-Hnt) and F-actin (phalloidin). Homozygous mutant cell nuclei are marked by the absence of GFP. (B,B') Wild-type (WT) complexes are shown. Notch was activated in the follicle cells. (C,C') A mosaic complex with aux- germ-line cells and aux+ follicle cells is shown. Notch was activated in the follicle cells. The clone was generated in females of the genotype hs-flp/+; ubi-gfp tub-aux FRT40A/FRT40A; aux136/+aux727. (D,D') As in (C,C), except the genotype was hs-flp/+; FRT5-5Z3515, auxF956*/FRT5-5Z3515, ubi-ngfp. Reduced levels of Hnt were seen at the poles of the aux+/aux- mosaic oocyte/nurse cell complexes, as was also observed in Chc+/Chc- mosaics [SLW and DB, unpublished observation]. This is quite distinct, however, from the absence of Hnt throughout the follicle epithelium observed with lqf- or Dl- germ line clones [40]. Scale bar 20 µm.

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Table 1. Rescue of lethality of aux mutants by overexpression of epsin and/or clathrin heavy chain.

| genotype* | # flies# | # expectedd |
|-----------|----------|-------------|
| w;+/*CyO; aux47/auxD128 | 2 | 0 |
| w; glf/+; aux47/auxD128 | 84 | 61 |
| w; PgChc+/+; aux47/auxD128 | 44 | 61 |
| w; PgChc+/PgChc+; aux47/auxD128 | 52 | 61 |
| w; +/*CyO; aux7/TM6B | 69 | 122 |
| w; PgChc+/+; aux7/TM6B | 114 | 122 |
| w; PgChc+/CyO; aux7/TM6B | 193 | 122 |
| w; PgChc+/Pglqf+; aux7/TM6B | 114 | 122 |
| total | 672 | 671 |

*The flies of the genotypic classes listed were obtained from crosses of three w; gChc/+; aux47/auxD128 males with eight w; glf+/+; aux47/auxD128/TM6B virgin females, kept at 25°C, and transferred to new food vials every 2–3 days for 5 days. Flies with glf+ only were differentiated from gChc+; glf+ flies by the latter having darker eye color.

# aux means either aux47 or auxD128.

The important comparison is between the first row and the three rows beneath. Addition of either or both PgChc+ or PgChc+ transgenes increases drastically the viability of aux47/auxD128 adults. It is not clear why the effect of both transgenes is not greater than the effect of a single transgene. One possibility, suggested by the expected frequency of adults (see d below) is that each transgene rescues viability completely. In this case, the differences from expectation would be due to the effects of other aspects of the genotype, such as the presence or absence of CyO, and transgene insertion sites.

The expected numbers were calculated making three simplifying assumptions: (1) aux47/auxD128 is completely lethal; (2) one copy of either transgene rescues viability fully; (3) no aspect of the genotype other than aux47/auxD128 affects viability.

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Figure 3. Overexpression of clathrin heavy chain and/or epsin suppresses the adult eye defects in aux loss-of-function mutants. (A–E) Light micrographs of adult external eyes of the genotypes indicated beneath are shown. (F) A diagram of an apical tangential section of a single ommatidium is shown. The numbers are photoreceptor cells R1 – R7. The black circular projections from each cells are the light-gathering organelles called rhabdomeres. The hexagonal shape is formed by pigment cells. (G–K) Small fields of apical tangential sections of adult eyes are shown. (H) A diagram of an apical tangential section of a single ommatidium is shown. The numbers are photoreceptor cells R1 – R7. The black circular projections from each cells are the light-gathering organelles called rhabdomeres. The hexagonal shape is formed by pigment cells. (I–K) Addition of genomic DNA transgenes that express Chc+ or lqf+ suppresses the eye morphology defects of aux hypmorphs. The fraction of phenotypically wild-type (wt) ommatidia was determined by observing 300–500 ommatidia in 4–5 eyes of each genotype. The error is one standard deviation. Scale bar 10 μm (G–K) and 60 μm (A–E).

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Materials and Methods

Drosophila mutants and transgenes

The alleles and transgenes used are listed below. Flybase id numbers (http://flybase.org/) are provided when available. Chromosomes and genotypes used in particular experiments are indicated in Figure Legends. Mutant alleles: auxF956* (FBal0240439), auxK47 (FBal0197315), auxD128 (FBal0197310), auxF956* (FBal0197311), aux727 (FBal0197300), Rab11FRT18A [47], DmRFT82B (FBal0029366), A52511 (FBal012701). Transgenes: P[Chc+ [33], tub-aux [33], cy-flp (FB0015982), mdr-lacZ (on 2 and 3; FBrp0010977), hs-flp122 (on X), ubi-ngfp (on X,2L,3R), Hh99D::GFP [47], FRT82B (FBal0002074), FRT78A (FBal0002070), FRT41A (FBal0002071), FRT5377 [47], FR75-25115 [34], eye-flp (on 2), UAS-flp (on 2), UAS-Rab11FRT82B (FBal0190955). Transgenes generated in this work: P[glqf+ (on 2), ro-Rab11FRT82B (multiple lines), ro-Rab11FRT82B (multiple lines).
Transgene construction

Pglqf+. This construct is an ∼16,240 bp Not I – Xho I fragment of Drosophila genomic DNA containing the lgl gene obtained from a subclone called 19G [44], with the C-terminal codons fused to Ala6-GFP, ligated into pCaSpeR4 restricted with Not I and Xho I. The GFP tag was inserted using a two-step PCR method (X.X. and J.A.F., manuscript in preparation).

ro-Rab5N122I. Total RNA from 5 w1118 females was isolated using TRI reagent (Molecular Research Center), and 5 μg was used for reverse transcription with SuperScriptII (Invitrogen). The primers used were Rab5F (5’-AAAGGGCCGCCATGCACCACTTCACGGC-3’) and Rab5R (5’-AAGGGCCGCCCTCACTGGACAGTGTGTGGC-3’). The cDNA was diluted to 200 μl and 2 μl was used as the template for the following PCR reactions. The mutant Rab5 cDNA was generated in two steps. First, two PCR reactions were performed with mutagenic primers, Rab5CF (5’-GGCGCGCATCAAGGCAG-3’) and Rab5NR (5’-CTGCTCGTAGTGCGGC3’). One reaction used the primer pairs F and NR, and the other used R and CF. Next, the amplification products from each reaction were mixed, and used together as a template for PCR with primers F and R. The resulting amplification product was ligated as an Asc I fragment into BluescriptIIKS+ [59].

ro-Rab11N122I. The mutant Rab11 cDNA was obtained by PCR using as template genomic DNA from flies containing UAS-Rab11N122I [45], and the primers Rab11F (5’-AAGGGCCGCCATGGGTGCAAGAGAAGACGA-3’) and Rab11R (5’-CTGCTCGTAGTGCGGC3’). The resulting ~660 bp amplification product was ligated as an Asc I fragment into pUAS–Xu [57] restricted with Asc I.

Analysis of eyes

Plastic sectioning of adult eyes was performed as described [60], and sections were viewed and photographed with a Zeiss Axioplan 10. Parks AL, Klueg KM, Stout JR, Muskavitch MAT (1995) Ligand endocytosis not mainly for recycling. Development 132: 1751–1762.

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Author Contributions

Conceived and designed the experiments: SMLB BC SHE JHL SLW XX DB JAF. Performed the experiments: SMLB BC SHE JHL SL XX. Analyzed the data: SMLB BC SHE JHL SLW XX DB JAF. Contributed reagents/materials/analysis tools: SMLB BC SHE JHL SLW XX DB JAF. Wrote the paper: JAF SLW.

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