Drosophila melanogaster auxilin regulates the internalization of Delta to control activity of the Notch signaling pathway

Elliott J. Hagedorn, Jennifer L. Bayraktar, Vasundhara R. Kandachar, Ting Bai, Dane M. Englert, and Henry C. Chang

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907

We have isolated mutations in the *Drosophila melanogaster* homologue of auxilin, a J-domain–containing protein known to cooperate with Hsc70 in the disassembly of clathrin coats from clathrin-coated vesicles in vitro. Consistent with this biochemical role, animals with reduced auxilin function exhibit genetic interactions with Hsc70 and clathrin. Interestingly, the *auxilin* mutations interact specifically with *Notch* and disrupt several Notch-mediated processes. Genetic evidence places auxilin function in the signal-sending cells, upstream of Notch receptor activation, suggesting that the relevant cargo for this auxilin-mediated endocytosis is the Notch ligand Delta. Indeed, the localization of Delta protein is disrupted in auxilin mutant tissues. Thus, our data suggest that auxilin is an integral component of the Notch signaling pathway, participating in the ubiquitin-dependent endocytosis of Delta. Furthermore, the fact that auxilin is required for Notch signaling suggests that ligand endocytosis in the signal-sending cells needs to proceed past coat disassembly to activate Notch.

Introduction

Endocytosis, a process characterized by the internalization of extracellular materials and membrane proteins via vesicular intermediates, plays many roles in regulating cell–cell signaling pathways. In addition to the well-established role of attenuating signaling activity by clearing active receptor molecules from the cell surface, endocytosis has been proposed to facilitate signaling by transporting active receptor molecules to sites where downstream effectors are localized (Entchev et al., 2000; Dubois et al., 2001; Sorkin and Von Zastrow, 2002). A novel role of endocytosis has recently been proposed for the Notch signaling cascade, in which the internalization of the ligand facilitates activation of the receptor (Lai, 2004; Le Borgne et al., 2005a), although the exact mechanism of this critical event remains elusive.

The Notch pathway is a signaling module that is highly conserved in all metazoans and has been implicated in a variety of developmental processes (Artavanis-Tsakonas et al., 1999). How Notch transduces signals from the plasma membrane and affects gene regulation has been extensively analyzed in *Drosophila melanogaster*, as well as several other model systems. It is now apparent that proteolytic processing of the Notch receptor is tightly associated with its ability to transduce signals (Chan and Jan, 1998; Artavanis-Tsakonas et al., 1999). Notch is first cleaved during its transit through the biosynthetic pathway, thereby reaching the cell surface as a heterodimer of Notch extracellular domain (NECD) and a membrane-tethered intracellular domain (Blaumueller et al., 1997; Logeat et al., 1998). The binding of Notch to its ligand induces two additional cleavage events, releasing a signaling-competent Notch intracellular domain fragment from the plasma membrane (Kopan et al., 1996; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998). Notch intracellular domain then translocates into the nucleus and regulates gene expression by acting as a transcriptional coactivator (Jarriault et al., 1995; Struhl and Adachi, 1998).

Endocytosis appears to play a key role in regulating the activity of the Notch pathway. The importance of vesicular trafficking in Notch signaling was first noticed when mutations in *D. melanogaster* dynamin, a GTPase required for the detachment of vesicles from plasma membrane (Kosaka and Ikeda, 1983; van der Bliek and Meyerowitz, 1991), was found to produce a Notch-like phenotype (Poodry, 1990). Clonal analysis suggested that in Notch signaling, dynamin function is required in both signal-sending and signal-receiving cells (Seugnet et al., 1997),

E.J. Hagedorn and J.L. Bayraktar contributed equally to this work.

Correspondence to Henry C. Chang: hcchang@purdue.edu

Abbreviations used in this paper: CCV, clathrin-coated vesicle; Clc, clathrin light chain; Dl, Delta; DSL, Dl, Ser, and *Caenorhabditis elegans* Lag-2 protein family; EGFR, EGF receptor; NECD, Notch extracellular domain; Ser, Serrate; UAS, upstream activating sequence.

The online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 173, No. 3, May 8, 2006 443–452
http://www.jcb.org/cgi/doi/10.1083/jcb.200602054

suggesting that endocytosis impinges on the pathway at two independent steps. Although the role of endocytosis in signal-receiving cells is less clear, the internalization of ligand for the Notch receptor in the signal-sending cells appears to be a key event in activating the Notch cascade (Parks et al., 2000).

In *D. melanogaster*, there are two known Notch ligands, Delta (Dl) and Serrate (Ser), members of the Dl, Ser, and *Caenorhabditis elegans* Lag-2 protein family (DSL). Both Dl and Ser appear to use an ubiquitin-mediated endocytic pathway to activate Notch receptors (Lai et al., 2005; Le Borgne et al., 2005b; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). The covalent addition of ubiquitin to polypeptides, besides being a tag for proteasome-mediated protein degradation, can serve as a sorting signal for membrane protein internalization (Hicke and Riezman, 1996; Terrell et al., 1998). The ubiquitination of Dl and Ser for subsequent internalization is mediated by *neuralized* (*neur*) and *mind bomb* (*mib1*), which encode two structurally unrelated E3 ubiquitin ligases (Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001; Itoh et al., 2003; Le Borgne and Schweisguth, 2003; Koo et al., 2005). Although Neur and dMib regulate distinct Notch-dependent processes, they appear to be interchangeable in mediating the ubiquitination and internalization of the DSL ligand (Lai et al., 2005; Le Borgne et al., 2005b; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). Another critical component of this process is *liquid facets* (*lqf*), the *D. melanogaster* homologue of epsin (Cadavid et al., 2000). *Lqf* contains an ubiquitin-interacting motif (Polo et al., 2002; Shih et al., 2002), as well as motifs that bind to clathrin and other classes of adaptors (Bonifacino and Traub, 2003). Thus, it is thought that lqf functions as a cargo-specific clathrin adaptor, capable of recognizing and sequestering monoubiquitinated DSL ligand into clathrin-coated vesicles (CCVs; Overstreet et al., 2003, 2004; Wang and Struhl, 2004), although an alternative function for epsin in nonclathrin endocytosis has been proposed (Chen and De Camilli, 2005; Sigismund et al., 2005).

Although a requirement of ligand endocytosis for Notch activation seems clear, the mechanism of how the internalization of the DSL ligand in the signal-sending cells promotes the proteolytic processing of Notch in the neighboring signal-receiving cells remains poorly understood. One set of models proposed that the internalization of Notch bound DSL ligand could either clear NECD from the extracellular space or generate physical force to dissociate NECD from the membrane-tethered intracellular domain, allowing the subsequent cleavage processing to occur (Parks et al., 2000). Alternatively, it has been suggested that endocytosis is required to transport DSL ligand to subcellular compartments, where the ligand is rendered signaling competent before being recycled back to the cell surface (Wang and Struhl, 2004; Emery et al., 2005). Because, at present, the analysis of the roles of DSL endocytosis in Notch signaling relies on those mutations disrupting the assembly of cargo-containing CCVs, it is difficult to distinguish whether it is the internalization by itself or the transit of Dl through specific endocytic compartments that is critical for Notch activation. To better understand the mechanism of this critical process, the effects of additional endocytic mutations in Notch signaling need to be assessed.

The clathrin coats of newly formed CCVs need to be dissociated so the vesicles can fuse with target organelles and the released clathrin triskelions can be reutilized for subsequent rounds of endocytosis. *D. melanogaster* Hsc70, a constitutively expressed member of the Hsp70 chaperone family, has been implicated in promoting the release of clathrin triskelions and other coat proteins from CCVs in vitro (Schlossman et al., 1984; Chappell et al., 1986; Ungewickell et al., 1995).

In addition to Hsc70, another important factor in the clathrin uncoating reaction is thought to be auxilin, which contains clathrin binding domains, as well as a J-domain (Ungewickell et al., 1995; Umeda et al., 2000). The J-domain, a conserved motif shared by members of the DnaJ protein family, can bind to Hsp70 family proteins and stimulate their low intrinsic ATPase activity (Ungewickell et al., 1995). Thus, auxilin is thought to function as a cofactor in the uncoating reaction by recruiting ATP bound Hsc70 proteins to CCVs (Ungewickell et al., 1995; Holstein et al., 1996). In support of this, inhibition of auxilin function in vivo using yeast mutants, RNAi, or injection of interfering peptides can disrupt clathrin function (Gall et al., 2000; Pishvaee et al., 2000; Greener et al., 2001). Recent biochemical analysis suggests that auxilin participates in other steps of the CCV cycle, in addition to clathrin coat disassembly (Newmyer et al., 2003). Still, it is unclear what the relevant endocytic cargo of auxilin may be under physiological conditions or whether auxilin has any role in regulating cell–cell signaling in metazoan systems.

To further understand the roles of endocytosis in cell signaling during animal development, we sought to generate loss-of-function mutations in auxilin from an F_2 complementation screen in *D. melanogaster*. From this screen, we isolated six loss-of-function mutations in *auxilin*. In support of previous biochemical data, we find that auxilin interacts genetically with Hsc70 and clathrin. In addition, the location of the genetic lesion in one of our alleles suggests that the putative lipid binding tensin domain plays a role in regulating clathrin function. The *auxilin* mutations also interact specifically with *Notch* and disrupt several Notch-mediated processes, suggesting that auxilin participates in an endocytic event critical for regulating the Notch cascade. Indeed, our analysis suggests that *D. melanogaster* auxilin is required for internalization of the Dl proteins that are critical for activating the Notch receptor.

Results

Isolation of loss-of-function mutations in *D. melanogaster auxilin* gene

The *D. melanogaster* genome contains a single *auxilin* homologue (*CG1107*; hereafter referred to as *dAux*) located at the base of the third chromosome right arm (82A1). Conceptual translation of the *dAux* ORF reveals a polypeptide of 1,165 amino acids, with an NH_2-terminal kinase domain, followed by a tensin-related domain, a clathrin binding domain, and a COOH-terminal DnaJ domain (Fig. 1 A). The presence of this NH_2-terminal kinase domain suggests that dAux is structurally more similar to the ubiquitously expressed cyclin G–associated kinase (Greener et al., 2000; Umeda et al., 2000) than the

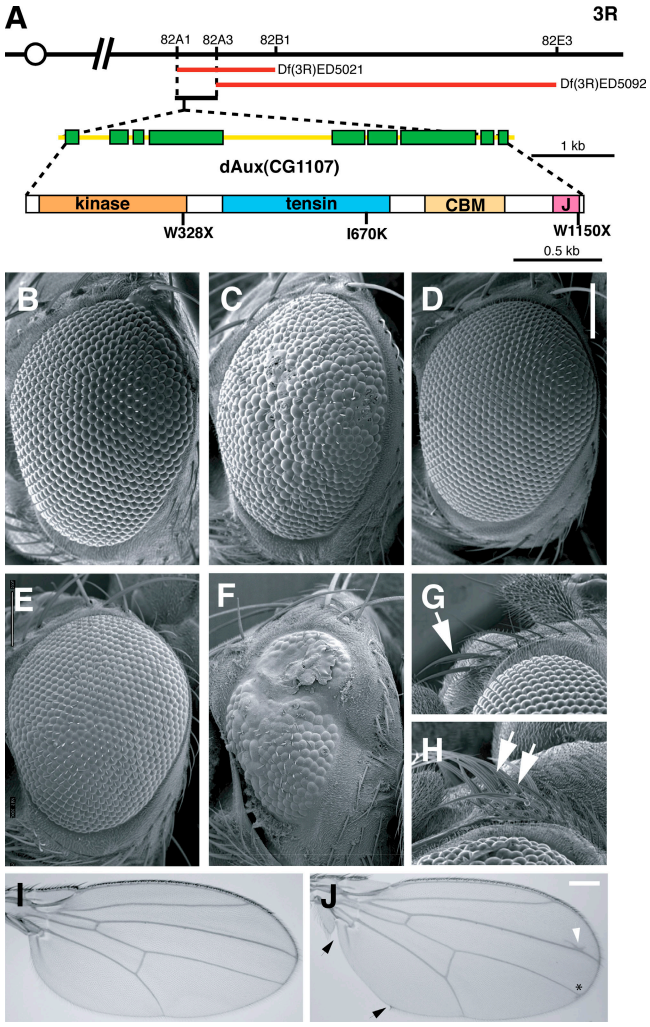

Figure 1. **Hypomorphic *dAux* mutations cause a rough eye.** (A) A schematic diagram of the *dAux* locus. The regions removed by the two deletions are indicated in red. The exons of *dAux* transcript are indicated by green boxes. (B–F) Scanning electron micrographs of the adult eyes of wild-type (B), $dAux^{I670K}/dAux^{I670K}$ (C), *UAS-dAux; Act5C-GAL4/+;* $dAux^{I670K}/dAux^{I670K}$ (D), $dAux^{I670K}/dAux^{I670K}$ grown at 18°C (E), and $dAux^{I670K}/dAux^{W328X}$ grown at 18°C (F). Bar, 100 μm. (G–H) Scanning electron micrographs of the adult heads of wild type (G) and $dAux^{I670K}/dAux^{I670K}$ (H). The vibrissae bristles are indicated by arrows. (I and J) Whole mount wings of wild type (I) and $dAux^{I670K}/dAux^{W328X}$ grown at 18°C (J). The missing wing margins and the extra vein material are indicated by black and white arrowheads, respectively. The DI-like vein junction is indicated by an asterisk. Bar, 200 μm.

neuronal cell-specific bovine auxilin (Ungewickell et al., 1995). Indeed, as with cyclin G–associated kinase, dAux appears to be ubiquitously expressed throughout embryonic development, although higher levels of dAux expression are detected in embryonic Garland cell primordium and larval Garland cells (Tomancak et al., 2002).

To understand the role of auxilin under physiological conditions, we set out to isolate loss-of-function mutations in *dAux* using an F_2 noncomplementation screen with two deletions, *Df(3R)ED5021* (81F6-82A5) and *Df(3R)ED5092* (82A1-E7; FlyBase). Because of the cytological location of *dAux*, we reasoned that loss-of-function mutations in *dAux* should fail to complement *Df(3R)ED5021* by lethality but complement *Df(3R)ED5092* (Fig. 1 A). Using these criteria, we isolated one mutation in *dAux* from ~1,600 chemically mutagenized third chromosomes. Sequencing analysis of this mutant revealed a single nucleotide change, which alters the Ile at position 670 to a Lys in the tensin-related domain (Fig. 1 A). Although isolated by lethality exhibited when placed over a deletion, homozygous $dAux^{I670K}$ animals could survive until adulthood, suggesting that $dAux^{I670K}$ is a partial loss-of-function allele. Furthermore, the emergence of homozygous mutant adults suggests that there are no other recessive lethal mutations on that chromosome.

Five additional *dAux* alleles were isolated by screening ~4,000 ethyl methyl sulfonate–mutagenized chromosomes for noncomplementation with $dAux^{I1670K}$. Two of them, $dAux^{W328X}$ and $dAux^{W1150X}$, have been characterized molecularly and contain nonsense mutations at amino acids Trp328 and -1150, respectively (Fig. 1 A). However, animals homozygous for these stronger mutations die before the larval stages, precluding the phenotypic analysis of the larval tissues.

Mutant adults homozygous for $dAux^{I670K}$ exhibited several morphological defects, including rough eyes, extra bristles, missing wing veins, and male and female sterility. Although wild-type compound eyes displayed regular arrays of ~800 ommatidia (Fig. 1 B), the eyes of $dAux^{I670K}$ mutants were grossly disorganized, with patches of brown necrotic tissues on the surface (Fig. 1 C). This rough eye phenotype exhibited by homozygous $dAux^{I670K}$ mutants showed temperature dependence, as the eye roughness was significantly milder for mutants raised at 18°C (Fig. 1 E) than those grown at 25°C (Fig. 1 C). At a higher temperature, such as 29°C, the homozygous mutant state was completely lethal.

Although $dAux^{W328X}$ and $dAux^{W1150X}$ are lethal over $dAux^{I670K}$ at 25°C, mutant animals of heteroallelic combinations for $dAux^{W328X}/dAux^{I670K}$ and $dAux^{W1150X}/dAux^{I670K}$ could occasionally survive until adulthood when raised at 18°C. The eye phenotypes of these animals, although raised at 18°C, were more drastic than the eye roughness exhibited by $dAux^{I670K}$ at 25°C (Fig. 1 F), suggesting that the increase in the severity of eye defects correlates with the decrease in dAux activity.

To ensure that the mutation in the *dAux* gene was responsible for this fully penetrant rough eye phenotype, we introduced a wild-type copy of the *dAux* gene in $dAux^{I670K}$ mutants using the upstream activating sequence (UAS)–GAL4 expression system (Brand and Perrimon, 1993). Expression of *dAux* using the *Act5C-GAL4* driver completely rescued the rough eye phenotype of $dAux^{I670K}$ (Fig. 1 D).

In addition to rough eyes, homozygous $dAux^{I670K}$ mutants contained supernumerary vibrissae (Fig. 1, compare G and H) and, frequently, extra anterior sternopleural bristles. Occasionally, extra bristles were also detected on the notum or scutellum of mutant animals. Furthermore, at a low frequency, some mutant animals had wings with incompletely formed posterior crossveins, and absent wing vein material at the posterior wing vein margin (unpublished data). Similar to the rough eye phenotype, the penetrance and the severity of these phenotypes are more pronounced in mutant animals heteroallelic for $dAux^{W328X}/dAux^{I670K}$ and $dAux^{W1150X}/dAux^{I670K}$ raised at 18°C (Fig. 1, compare I and J). Interestingly, each of the adult phenotypes

observed in *dAux*^{I670K} mutants resembled *Notch* phenotypes, suggesting a link between the role of dAux in endocytosis and Notch signaling.

dAux interacts with *clathrin* and *Hsc70* in vivo

Because auxilin has been implicated in Hsc70-mediated disassembly of the CCVs in vitro, we asked whether this *dAux*^{I670K} interacts with *clathrin* and *Hsc70* in vivo. We expressed a dominant-negative form of *Hsc70-4* (Elefant and Palter, 1999) in the eye using the *GMR-GAL4* driver (Hay et al., 1994). Expression of this ATP hydrolysis-defective Hsc70-4 caused a rough eye (Fig. 2 A), presumably the result of defective endocytosis on developmental signaling pathways. Mutating one copy of the *dAux* gene with *dAux*^{I670K} intensified the rough eye phenotype, indicating genetic interaction of *dAux* and *Hsc70* in vivo (Fig. 2 B).

To test whether dAux interacts with clathrin, we expressed the clathrin light chain (Clc) fused to GFP (*Clc-GFP*; Chang et al., 2002) using *Act5C-GAL4* in a *dAux* homozygous background. Although expression of *Clc* under the control of *Act5C-GAL4* had no detectable effect on eye development or viability in wild-type animals, the expression of *Clc-GFP* greatly reduced the viability of dAux mutants. In rare escapers, the eyes of *Act5C-GAL4, UAS-Clc-GFP/+; dAux*^{I670K}*/dAux*^{I670K} were rougher, and there was dramatic enhancement of the wing phenotypes, including severe notching, wing vein thickening, and ectopic vein formation (Fig. 2, compare C and D). Together, these data indicated that *dAux*^{I670K} interacts genetically with *Hsc70* and *clathrin* in vivo.

To understand the role of dAux in vesicular trafficking, we examined the subcellular localization of dAux proteins, using a fluorescently tagged dAux fusion (*UAS-dAux-mRFP*). Both dAux-mRFP and Clc-GFP were expressed in larval Garland cells using Act5c-GAL4. Although intense vesicular Clc staining was seen around the cell periphery, the vesicular dAux-mRFP appeared more centrally localized and showed little overlap with *Clc* (Fig. 2, E–G). Interestingly, although *dAux*^{I670K} interacts with the Clc in vivo, no apparent difference in Clc-GFP pattern was detected between wild type and homozygous mutant Garland cells by confocal microscopy (unpublished data).

dAux is required for the proper specification of photoreceptor cell fate

To further understand the roles of *dAux* during development, we investigated the cause of the rough eye phenotype by tangential sectioning of the adult retina. In wild-type eyes, rhabdomeres of the eight photoreceptors are organized in a stereotypical manner in lattices of ommatidia (Fig. 3 A). In contrast, sections of *dAux*^{I670K} mutant retina showed that regular arrays of ommatidia were disrupted. Furthermore, supernumerary photoreceptor cells were detected in 39.67% (n = 510) of *dAux*^{I670K} mutant clusters (Fig. 3 B). The identities of these extra photoreceptors could be either outer (cells with large rhabdomeres; 21.42%) or inner (cells with small rhabdomeres; 18.25%), suggesting that *dAux*^{I670K} is not affecting the determination of a particular photoreceptor cell fate.

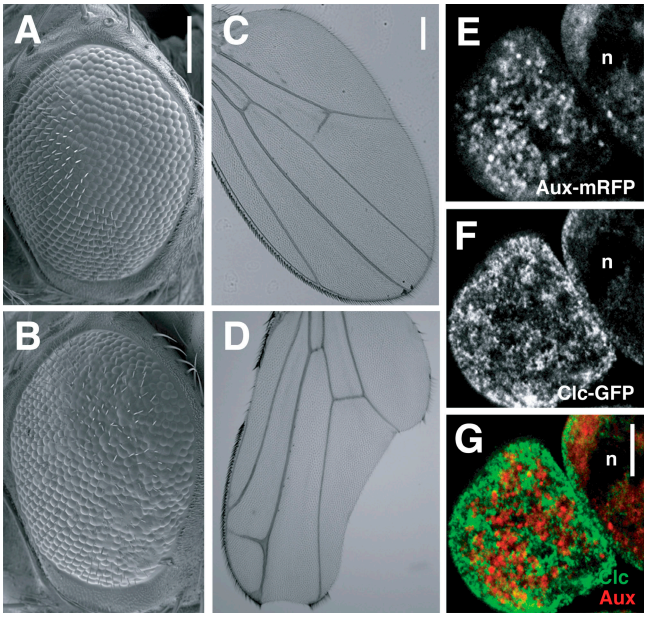

Figure 2. ***dAux*^{I670K} interacts genetically with *Hsc70* and *clathrin*.** (A and B) Scanning electron micrographs of the adult eyes of *UAS-Hsc70-4*^{K71S}*/+; GMR-GAL4/+* (A) and *UAS-Hsc70-4*^{K71S}*/+; GMR-GAL4/dAux*^{I670K} (B). Bar, 100 μm. (C and D) Adult wings of *dAux*^{I670K}*/dAux*^{I670K}(C) and *Act5C-GAL4, UAS-Clc-GFP/+; dAux*^{I670K}*/dAux*^{I670K} (D). Bar, 200 μm. (E–G) Confocal micrographs of *Act5C-GAL4, UAS-Clc-GFP/UAS-dAux-mRFP* larval Garland cells. The optical section of the adjacent cells shows a tangential view of the cell on the left and a cross-section of the cell on the right. (G) The subcellular localizations of dAux (E) and Clc (F) are indicated in red and green, respectively. n, nucleus. Bar, 5 μm.

To understand the origin of these extra photoreceptors, we stained eye imaginal discs, the monolayer epithelia that give rise to adult eyes, with αElav antibody, which labels the nuclei of neuronal photoreceptor cells (Robinow and White, 1988). Organized clusters of eight normal Elav-positive cells were seen in wild-type eye discs (Fig. 3 C, inset). In contrast, Elav-positive cell clusters were clearly disorganized in *dAux*^{I670K} tissues, and there were supernumerary Elav-positive cells in some of the clusters (Fig. 3 D, inset). These data suggest that the extra photoreceptor cells in adult retina were the result of a disruption in photoreceptor recruitment caused by *dAux*^{I670K}.

To be sure that this defect in photoreceptor recruitment results from a decrease in dAux function, we examined the number of Elav-positive cells in eye discs isolated from mutants raised at 25°C but heteroallelic for *dAux*^{W328X}*/dAux*^{I670K}. The number of Elav-positive cells appeared to increase in eye discs mutant for stronger *dAux* alleles (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200602054/DC1), suggesting that dAux activity is critical in controlling formation of the proper number of photoreceptor cells.

To analyze this disruption in the organization of ommatidia arrays, we stained the eye discs with αBoss antibody, which specifically labels the apical surfaces of the R8 cells, the first photoreceptor specified in each cluster. Although the Boss staining is evenly spaced in wild-type eye discs (Fig. 3 E), the spacing between Boss-positive cells in *dAux*^{I670K} mutant discs varied greatly, and clusters with multiple Boss-positive cells were occasionally detected (Fig. 3 F, inset). This periodic

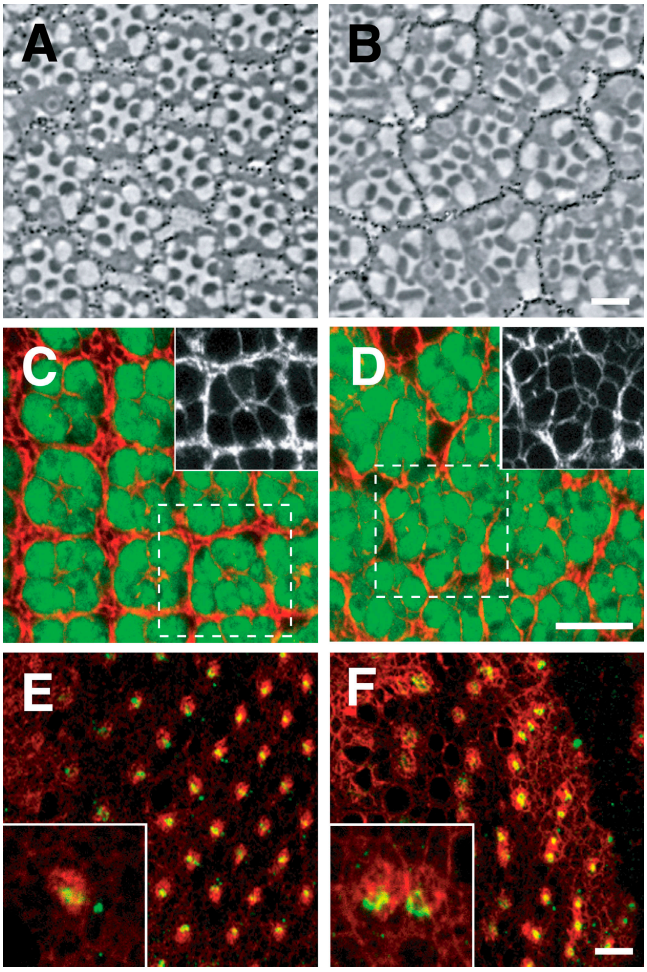

Figure 3. ***dAux*^{I670K} causes the formation of supernumerary photoreceptor cells.** (A and B) Tangential section of adult retina of wild type (A) and *dAux*^{I670K}/*dAux*^{I670K} (B). Bar, 10 μm. (C and D) Eye imaginal discs of wild type (C) and *dAux*^{I670K}/*dAux*^{I670K} (D) stained with α-Elav antibodies (green) and phalloidin (red). The boxed regions are shown in the insets without Elav staining to highlight the number of cells in a cluster. Bar, 10 μm. (E and F) Eye imaginal discs of wild type (E) and *dAux*^{I670K}/*dAux*^{I670K} (F) stained with α-Boss antibodies (green) and phalloidin (red). A single cluster is shown in the inset at a higher magnification. Bar, 15 μm.

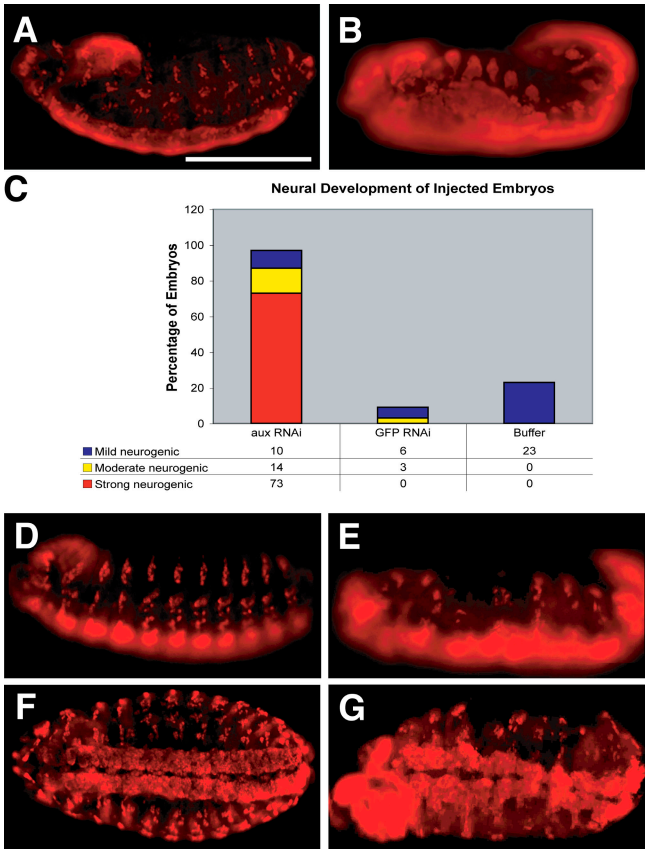

Figure 4. ***dAux* is required for neural development during embryogenesis.** (A and B) Elav staining of stage 15 embryos injected with buffer (A) or *dAux* dsRNA (B). Anterior is oriented toward the left, and dorsal is upward. Bar, 200 μm. (C) Classification of neural development in injected embryos. Wild-type embryos injected with buffer, *GFP* dsRNA, or *dAux* dsRNA were aged to stage 15 and immunostained with α-Elav antibody to reveal their neural development. Embryos were designated as members of one of four classes: normal, mild, moderate, and strong. Normal class embryos exhibited wild-type Elav staining, with highly organized nervous tissues. The latter three classes refer to the degree of neural hypertrophy (neurogenic phenotype) observed. Mild embryos exhibited slight excess in numbers of neurons in the peripheral or central nervous system but overall were similar to wild type. The moderate class is composed of embryos with more significant excess in neuropositive cells but with distinction maintained between chordotonal and sensory neurons, and the amount of epidermal tissue was reduced moderately. In the strong class, embryos exhibited a nearly complete transformation of epidermis into neuropositive cells, a neurogenic phenotype characteristic of Notch signaling mutants. In some embryos, morphological defects were observed as well. However, only the degree of Elav staining is reported in this table. (D–G) Lateral (D and E) and ventral (F and G) views of stage 13 embryos immunostained with α-Elav antibody. Wild-type (D and F) and an embryo maternally deficient but zygotically heterozygous for *dAux*^{I670K} (E and G).

spacing of R8s is thought to require Notch-mediated lateral inhibition (Baker, 2002), and the phenotype exhibited by *dAux*^{I670K} suggests a disruption in this process.

dAux is required for the proper patterning of neural tissues during embryonic development

The Notch signaling cascade participates in the formation of neuronal tissues during embryonic development. To determine whether dAux has a role in the specification of neuronal cell fate during embryogenesis, we inhibited dAux function using dsRNA injection and stained the injected embryos with αElav antibodies. 73% of embryos injected with *dAux* dsRNA exhibited a strong neurogenic phenotype, with transformation of nearly all epidermis to neural tissues (Fig. 4 B). In contrast, injection of buffer (Fig. 4 A) or *GFP* dsRNA (not depicted) did not affect neural patterning, suggesting that the phenotype of embryos injected with *dAux* dsRNA was specific. A quantitative summary of the phenotypes exhibited by injected embryos is tabulated in Fig. 4 C. This RNAi data, along with other dAux phenotypes, suggests that dAux acts in the general regulation of neuronal development.

To determine whether the formation of embryonic neural tissues depends on the zygotic or the maternal *dAux* transcripts, embryos derived from the mating of *dAux*^{I670K} homozygous mutant females with wild-type males were stained with αElav antibodies. Elav staining of embryos derived from wild-type

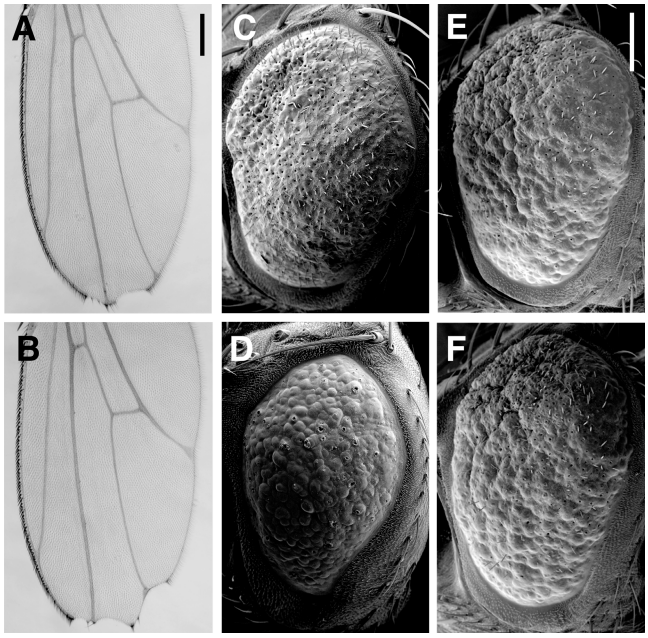

Figure 5. ***dAux^{J670K}* interacts specifically with *Notch*.** Adult wings of $N^{264\text{-}39}$/+ (A) and $N^{264\text{-}39}$/+; $dAux^{J670K}$/+ (B). Bar, 200 μm. Scanning electron micrographs of the adult eyes of *UAS-N/+; GMR-GAL4/+* (C), *UAS-N/+; GMR-GAL4, dAux^{J670K}/+* (D), *UAS-egfr/+; GMR-GAL4/+* (E), and *UAS-egfr/+; GMR-GAL4, dAux^{J670K}/+* (F). Bar, 100 μm.

parents revealed highly organized central and peripheral nervous systems with characteristic numbers of neuropositive cells (Fig. 4, D and F). In contrast, embryos maternally deficient but zygotically heterozygous for *dAux* showed mild hypertrophy in the ventral nerve cord, disorganization and slight reduction in the peripheral nervous system, and abnormal body morphology (Fig. 4, E and G). This suggests that the maternal dAux contribution plays a critical role in embryonic patterning of neural tissues and may explain the apparent female sterility associated with $dAux^{J670K}$ mutant adults.

dAux exhibits specific genetic interactions with *Notch*

The $dAux^{J670K}$ phenotypes of supernumerary photoreceptors, bristles, and embryonic neuronal cells are all reminiscent of those exhibited by mutants deficient in Notch signaling. To test for dAux participation in Notch pathway regulation, we asked whether the *dAux* mutations interact with mutant alleles of *Notch*. 36.36% ($n = 44$) of heterozygous $N^{264\text{-}39}$, a null allele of *Notch*, exhibits a haploinsufficient phenotype of wing "notching" at the posterior margins (Fig. 5 A). Mutating one copy of *dAux*, by $dAux^{J670K}$, increased the severity of both the penetrance (100%; $n = 36$) and the phenotype (Fig. 5 B), indicating that *dAux* interacts genetically with *Notch*. In support of the notion that $dAux^{J670K}$ represents a loss-of-function allele, a similar increase in the penetrance and the severity of the phenotype was seen with other alleles of *dAux*, as well as the deletion that removes the entire *dAux* locus.

In addition to wing development, Notch is involved in many developmental decisions during retina formation, and overexpression of a full-length *Notch* (Go et al., 1998), using the *GMR-GAL4* driver, causes a rough eye (Fig. 5 C), presumably disrupting some of these Notch-dependent processes. Although animals heterozygous for $dAux^{J670K}$ are normal, disruption of one copy of the *dAux* gene strongly reduced the eye size and worsened the rough eye of *UAS-Notch; GMR-GAL4* (Fig. 5 D), suggesting that dAux has a role in regulating the Notch-mediated signaling pathway.

To test the specificity of this interaction, we overexpressed a full-length *EGF receptor* (*EGFR*) under the control of *GMR-GAL4*. EGF signaling is involved in the differentiation of all cell types during retina development (Freeman, 1996), and as for *Notch*, overexpression of *EGFR* with *GMR-GAL4* causes a rough eye (Fig. 5 E). However, mutating one copy of the dAux gene by $dAux^{J670K}$ had no effect on the rough eye phenotype caused by *UAS-egfr; GMR-GAL4* (Fig. 5 F). This suggests that dAux does not participate in all signaling pathways required for eye development but that it may specifically regulate signaling activity of the Notch cascade.

dAux acts upstream of a constitutively activated Notch in the signal-sending cells

To ask which cells require functional dAux for activation of the Notch pathway, we performed mosaic analysis in the adult retina. We reasoned that if dAux is required in the signal-sending cells, mutant clusters near the border of the clone would be less affected than those near the center of the clone because they can receive signals from the nearby wild-type cells. On the other hand, if dAux is required in the signal-receiving cells, mutant clusters should exhibit defects regardless of their location in the clone. Because of the proximity of *dAux* to the centromere, mitotic clones of $dAux^{J670K}$, marked by the absence of the *white* gene, were generated by γ-ray irradiation. Tangential sections through these clones showed that, consistent with the homozygous mutant eyes, some mutant clusters contained supernumerary R-cells. Mutant clusters near the border of the clone were mostly wild type, whereas the mutant clusters in the center of the clones exhibited a stronger mutant phenotype (Fig. 6 A). Thus, it appears that dAux acts noncell autonomously in Notch activation.

To position the function of dAux in the Notch pathway, we performed epistasis tests using $dAux^{J670K}$ and a truncated form of Notch, which mimics the signal-competent Notch fragment after proteolytic cleavage (Go et al., 1998; Matsuno et al., 2002). Consistent with its role in lateral inhibition, expression of this activated form of *Notch* in the eye discs using the *GMR-GAL4* driver greatly reduced the number of Elav-positive cells, indicating a strong inhibition of photoreceptor recruitment (Fig. 6 C). In contrast, there were excessive Elav-positive cells in homozygous $dAux^{J670K}$ eye discs (Fig. 6 D). However, in mutant eye discs that also expressed the activated form of Notch, the number of Elav-positive cells was reduced (Fig. 6 E), indicating that activated *Notch* is epistatic to *dAux*. This suggests that dAux acts upstream of the generation of this signal-competent fragment of Notch.

dAux^{1670k} causes an increase of Dl proteins near cell periphery

Recent evidence indicates that internalization of the DSL ligand may play a critical role in regulating Notch activity. Because our

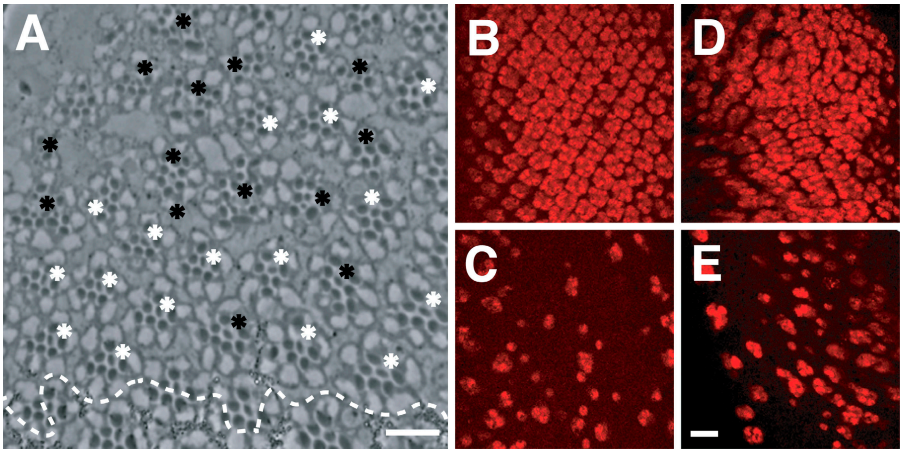

Figure 6. ***dAux* functions upstream of *Notch*.** (A) A tangential section of a *dAux* mutant clone in adult retina. The boundary of the clone is delineated by a dashed line. Genotypically mutant but phenotypically wild-type clusters are indicated by white asterisks, and genotypically and phenotypically mutant clusters are indicated by black asterisks. Bar, 10 μm. (B–E) Confocal micrographs of eye discs stained with α-Elav antibodies (red). (B) *GMR-GAL4/+*; (C) *UAS-N^{Act}/+; GMR-GAL4/+*; (D) *GMR-GAL4/+; dAux^{I670K}/dAux^{I670K}*; and (E) *UAS-N^{Act}/+; GMR-GAL4, dAux^{I670K}/dAux^{I670K}*. Bar, 15 μm.

data suggest that dAux acts noncell autonomously and upstream of activated Notch in Notch signaling, we suspected that Dl endocytosis could be regulated by dAux. To investigate whether the trafficking of Dl proteins is disrupted, eye discs from wild-type, homozygous *dAux^{I670K}*, and *dAux^{W328X}/dAux^{I670K}* mutant animals were dissected and stained with antibody raised against the extracellular domain of Dl.

In wild-type eye discs, evenly spaced Dl staining was first seen in cells that are recruited to form photoreceptor clusters behind the morphogenetic furrow (Fig. 7, A and D). The Dl staining in these cells appeared to overlap with cortical phalloidin (not depicted), suggesting that most Dl protein initially localized at or proximal to the plasma membrane. In more mature clusters located in the posterior region of the eye disc, the Dl staining became more vesicular. These Dl-positive structures showed little or no colocalization with the Clc, Rab11, or Grasp65, which are markers for clathrin-coated structures, recycling endosomes, and Golgi, respectively, but overlapped moderately with Rab5 and extensively with Rab7, suggesting that most of the internalized Dl proteins are in the early and late endosomes (unpublished data). To ensure that our determination of Dl subcellular localization was not influenced by fixatives or histological techniques, we generated *UAS-Dl-mRFP*, a functional Dl chimera with an mRFP (Campbell et al., 2002) fused to the intracellular COOH terminus of the Notch ligand, placed under the control of UAS regulatory element (Brand and Perrimon, 1993). As with the antibody staining of endogenous Dl, Dl-mRFP in live eye discs also appeared vesicular and exhibited overlap with Rab5 and -7 endosomal markers (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200602054/DC1).

In *dAux^{I670K}* homozygous mutant eye discs, Dl staining appeared more excessive and disorganized in cells behind the furrow, reflecting the defects in photoreceptor specification (Fig. 7, B and E). Although Dl- and Rab7-positive vesicular structures could still be detected in mature clusters in the more posterior region of the mutant eye discs (not depicted), there appeared to be an increase in the peripheral staining of Dl in the first few rows of cells immediately posterior to the furrow (Fig. 7, B and E). This suggests that Dl internalization in these cells, where Notch signaling is thought to participate in the proper spacing of photoreceptor cell clusters, was disrupted. The *dAux^{W328X}/dAux^{I670K}* eye discs showed even more severe disruption of Dl localization, with excessive, peripheral Dl staining extended from the region behind the furrow to the posterior edge of the disc, consistent with a greater decrease in dAux activity in heteroallelic animals (Fig. 7, C and F).

The apparent increase in Dl staining in the *dAux^{I670K}* mutants could result from either a block in Dl internalization and degradation or an increase in Dl gene expression. Because an increase in Dl expression in larval eye discs mutant for *lqf* was previously reported (Wang and Struhl, 2004), it seemed likely that the level of Dl expression would be affected in *dAux* mutant cells. To determine if this was the case, we monitored the transcriptional activities of the endogenous Dl promoter by measuring the β-gal activities from a Dl enhancer trap line (Fanto and Mlodzik, 1999). Unlike *lqf*, we did not observe a significant increase in β-gal activity in *dAux^{I670K}* eye discs (unpublished data), suggesting that the apparent increase in Dl staining was not due to elevated Dl transcription.

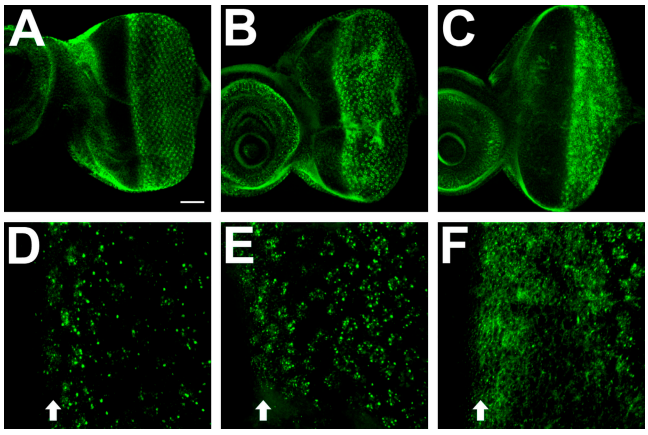

Figure 7. **Dl endocytosis is disrupted in *dAux* mutant eye discs.** Confocal micrographs of third instar larval eye discs immunostained with Dl antibody at low (A–C) and high magnification (E–F). (A and D) Wild-type *dAux^{I670K}/dAux^{I670K}* (B and E) and *dAux^{W328X}/dAux^{I670K}* (C and F). The cells immediately posterior to the morphogenetic furrows are shown in the higher magnification images. The location of the morphogenetic furrow is indicated by the arrows. Images are the projections of 4 (low magnification) or 10 optical sections (high magnification). Bar, 100 μm.

Discussion

To understand the physiological roles of J-domain–containing proteins during metazoan development, we isolated and characterized mutants in *D. melanogaster auxilin*. In support of its well-known biochemical role in Hsc70-mediated disassembly of CCVs, we showed that this $dAux^{I670K}$ mutation interacts genetically with *Hsc70-4* and the *Clc*. The in vivo link between auxilin and Hsc70 is further strengthened by the observation that a nonsense mutation ($dAux^{W1150X}$) near the very COOH terminus, where the J-domain is located, can strongly disrupt dAux function. These genetic observations are in agreement with in vivo analyses of auxilin function from other systems, which showed that clathrin function was disrupted in auxilin-deficient cells (Gall et al., 2000; Greener et al., 2001; Morgan et al., 2001). In addition, our genetic data of $dAux^{I670K}$ suggest a relevance of the tensin-related domain, a putative lipid binding domain, in clathrin-mediated endocytosis, despite the fact that it does not appear to be required for catalyzing the dissociation of clathrin triskelions from CCVs in vitro (Holstein et al., 1996; Newmyer et al., 2003).

It has been suggested that, in addition to disassembling clathrin coats, auxilin participates in the dynamin-mediated constriction during CCV formation (Newmyer et al., 2003). However, our subcellular localization analysis did not reveal dAux proteins colocalizing with clathrin at the cell periphery. Instead, most auxilin proteins appear to be associated with intracellular structures, in regions devoid of clathrin staining. This lack of overlap between dAux and Clc seems more consistent with the notion that auxilin is required for the dissociation of clathrin coats from CCVs under physiological conditions.

Our analysis of *dAux* clearly suggests that auxilin plays an important role in the Notch cascade in multiple Notch-dependent processes. Supportive evidence comes from the strong genetic interactions between dAux and Notch and the phenotypic similarities ranging from eye and wing development to neural development during embryogenesis. Moreover, the in vivo function of auxilin in the Notch signaling cascade seems specific, as $dAux^{I670K}$ has no dominant effect on the phenotype caused by the overexpression of EGFR. Together, these observations argue that dAux acts specifically as a general component in the Notch cascade.

Analysis from several groups has suggested that ligand internalization is a key event for Notch activation. The neurogenic phenotypes exhibited by $dAux^{I670K}$ tissues and other genetic data further support this notion. The distribution of phenotypically mutant clusters in a genotypically mutant clone suggests that dAux acts noncell autonomously. In addition, the epistasis analysis places dAux function upstream of an activated form of Notch. Based on the phenotypic resemblance of $dAux^{I670K}$ to those reported for *neur* (Lai et al., 2001; Pavlopoulos et al., 2001) and *lqf* (Overstreet et al., 2003), we suspect that dAux functions along with neur and lqf in the ubiquitin-dependent endocytic pathway in the signal-sending cells.

The identification of dAux as a critical factor in Notch ligand endocytosis has strong implications on the mechanism of Notch activation. Unlike neur and lqf, which are postulated to tag and sequester cargos into vesicles, auxilin is thought be involved in disassembly of clathrin coats. Thus, the revelation of dAux as another component in this pathway suggests that Dl-containing endocytic vesicles need to proceed past the clathrin uncoating step to activate Notch. One possible mechanism is that recycling of Dl is a prerequisite to form signaling-competent Dl-containing exosomes (Mishra-Gorur et al., 2002), although the presence of these structures under physiological conditions remains to be demonstrated. Alternatively, it may be that, as previously proposed, the DSL ligand is not signaling competent before endocytosis but is "activated" during transit through recycling compartments. Indeed, the transit through Rab11-positive recycling endosomes has been suggested as a critical step for Dl activity (Emery et al., 2005). However, although Dl appears to colocalize extensively with coalesced perinuclear Rab11-positive structures in the sensory organ precursor cells (Emery et al., 2005), our analysis found little spatial overlap between Rab11 and Dl in cells near the furrow. One possible explanation for this apparent difference is that the transit of Dl through Rab11-positive structures in the eye disc cells occurs more transiently, therefore evading detection by immunostaining at a steady state.

Another explanation for the relevance of ligand endocytosis hypothesizes that Dl internalization causes a mechanical stress on the Notch receptors, which then induces subsequent cleavages. A variation of this model proposes that the objective of Dl internalization is to remove the NECD fragment from the intercellular space so proteolytic processing can occur. If auxilin is solely involved in clathrin-coat disassembly, it will be difficult to reconcile our data with these two models because the internalization of Dl into CCVs, the presumed force-generating event, should have already been completed in *dAux* mutants.

Materials and methods

Fly genetics

All fly crosses were performed at 25°C in standard laboratory conditions unless otherwise specified. To screen for loss-of-function *dAux* alleles, *w; iso 2; 3* males were mutagenized with 25 mM ethyl methane sulfonate (Sigma-Aldrich) and mass mated with *w/w; TM3, Sb/TM6B, Hu* virgins. Progeny were then individually mated with *Df(3R) ED5021/TM3, Sb* flies, and those that failed to complement the deletion were recovered and maintained over *TM6B* or *TM3* balancers. Using these criteria, 11 lines were isolated from ~1,600 crosses. They were then mated with *Df(3R)ED5092/TM3, Sb* flies, and those that complemented were characterized further. Of the 11 lines, 3 complemented *Df(3R)ED5092*.

For the genetic interaction and epistasis analysis, $UAS\text{-}Hsc70\text{-}4^{K71S}$ (Elefant and Palter, 1999), *UAS-Clc-EGFP* (Chang et al., 2002), UAS-Notch, $UAS\text{-}N^{Act}$ (Go et al., 1998), *UAS-egfr* (Freeman, 1996), $N^{264\text{-}39}$, *GMR-GAL4*, and *Act5C-GAL4* were used. To label subcellular structures, *UAS-GFP-Rab5* (Wucherpfennig et al., 2003), *UAS-GFP-Rab7* (Entchev et al., 2000), *UAS-GFP-Rab11* (this study), *UAS-dGrasp65-GFP* (this study), and *UAS-Clc-EGFP* (Chang et al., 2002) were used.

For mosaic analysis, *w; P[SUPor-P]KG08740/TM3* flies were mated with $w/w;\ dAux^{I670K}/TM6B$. First instar larvae were then irradiated with a 1,000 rad γ-ray (Gammacell 220), and flies containing mosaic clones were identified by the presence of w^- patches on the adult retina.

Histology and immunohistochemistry

Immunostaining of eye discs and tangential sections of adult retina were performed according to Wolff (2000). Embryos were aged to stage 15 after injection and fixed as described previously (Kennerdell and Carthew, 1998). Rat α-Elav 7E8A10 (Developmental Studies Hybridoma Bank), mouse α-Boss, and mouse α-Dl C594.9B (Developmental Studies Hybridoma Bank)

were used at 1:100, 1:3,000, and 1:100 dilutions, respectively. Alexa Fluor 568 phalloidin (Invitrogen) and fluorescently conjugated secondary antibodies were used according to the manufacturer's instructions. Fluorescently labeled samples were mounted in VECTASHIELD Mounting Medium (Vector Laboratories). Light micrographs and fluorescent images were acquired at 25°C with 20× (0.5) and 40× (0.75) lenses on a microscope (BX61; Olympus) equipped with a camera (DP70; Olympus) and DP Manager software. All confocal microscopy images were acquired at 25°C with 20× (0.5) and 60× (1.25) lenses using a confocal microscope (OPTIPHOT-2 [Nikon]; MRC1024 system [Bio-Rad Laboratories]) and LaserSharp 3.0 software (Bio-Rad Laboratories). Images were 3D reconstructed in Volocity (Improvision) and then processed in Photoshop (Adobe) to adjust γ levels and image size.

Adult wings were dissected and mounted in Gary's magic mountant. Scanning electron microscopy was performed as previously described with a scanning electron microscope (JSM-840 [JEOL]; Wolff, 2000).

Molecular biology

To construct UAS-dAux, an EcoRI–XhoI fragment containing the entire auxilin (CG1107) ORF was excised from GH26574 (Research Genetics) and subcloned into pUAST. To construct UAS-dAux-mRFP, a 0.7-kb COOH-terminal portion (containing the internal SpeI site) of *dAux* was PCR amplified and cloned into pHFK-mRFP-KB as an EcoRI–XhoI fragment. After sequencing verification, the NH_2-terminal half was inserted as a 2.9-kb EcoRI–SpeI fragment, and the entire *dAux* fusion was then subcloned as an EcoRI–NotI fragment into pUAST. To construct UAS-Dl-mRFP, PCR-amplified mRFP was fused the to the COOH terminus of Dl (pBS-Dl, DGRC) as an NdeI–HindIII fragment, and the resulting Dl-mRFP was cloned into pUAST an EcoRI–XhoI fragment. To construct UAS-GFP-Rab11, Rab11 coding sequence was amplified by PCR and subcloned as a ClaI–BamHI fragment into the COOH terminus of GFP in pHFK-GFP-RC. To construct UAS-dGrasp65-GFP, dGrasp65 coding sequence was amplified by PCR and subcloned as an EcoRI–KpnI fragment into the NH_2 terminus of GFP in pHFK-GFP-KB. The resulting GFP-Rab11 and dGrasp65-GFP fusions were verified by sequencing and subcloned as NotI fragments into pUAST, respectively. Transgenic flies carrying these constructs were generated by P-element–mediated transformation as previously described (Rubin and Spradling, 1982).

dsRNA synthesis and RNAi injections

Synthesis of dsRNA was done according to Kennerdell and Carthew (1998). PCR primers bearing T7 promoter sequence at the 5′ ends were used to amplify 610- and 599-bp fragments of *auxilin* (5′-TCGAGTCGACGTACAAGACG-3′ and 5′-GCCTGATACAACCGCATTTT-3′) and *GFP* (5′-GTCAGTGGAGAGGGTGAAGG-3′ and 5′-CCCAGCAGCTGTTACAAACTC-3′) coding sequence, respectively. In vitro transcription of the PCR fragments produced dsRNAs, which were then prepared as 5-μM aliquots for injection as described previously (Kennerdell and Carthew, 1998). RNAi injections into *w1118* embryos were done as previously described (Kennerdell and Carthew, 1998).

Online supplemental material

Fig. S1 shows that the severity of photoreceptor recruitment defects correlates with the decrease in dAux function. Fig. S2 shows that Dl proteins are localized in Rab5- or Rab7-positive vesicular structures in wild-type eye discs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200602054/DC1.

We would like to thank Spyros Artavanis-Tsakonas, Marc Muskavitch, Annette Parks, Marcos A. González-Gaitán, the Bloomington Stock Center, and the Developmental Studies Hybridoma Bank for providing fly strains and antibodies. We thank Donna Fekete, Don Ready, and Lin Reynolds for helpful comments regarding the manuscript. We thank Debby Sherman and Chia-Ping Huang for technical assistance with scanning electron microscopy. We thank Ira Mellman for providing generous support.

Submitted: 10 February 2006
Accepted: 5 April 2006

References

Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. *Science.* 284:770–776.

Baker, N.E. 2002. NOTCH and the patterning of ommatidial founder cells in the developing *Drosophila* eye. *In Drosophila* Eye Development. K. Moses, editor. Springer-Verlag Berlin Heidelberg, New York. 35–58.

Blaumueller, C.M., H. Qi, P. Zagouras, and S. Artavanis-Tsakonas. 1997. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell.* 90:281–291.

Bonifacino, J.S., and L.M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu. Rev. Biochem.* 72:395–447.

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 118:401–415.

Cadavid, A.L., A. Ginzel, and J.A. Fischer. 2000. The function of the *Drosophila* fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. *Development.* 127:1727–1736.

Campbell, R.E., O. Tour, A.E. Palmer, P.A. Steinbach, G.S. Baird, D.A. Zacharias, and R.Y. Tsien. 2002. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA.* 99:7877–7882.

Chan, Y.M., and Y.N. Jan. 1998. Roles for proteolysis and trafficking in Notch maturation and signal transduction. *Cell.* 94:423–426.

Chang, H.C., S.L. Newmyer, M.J. Hull, M. Ebersold, S.L. Schmid, and I. Mellman. 2002. Hsc70 is required for endocytosis and clathrin function in *Drosophila*. *J. Cell Biol.* 159:477–487.

Chappell, T.G., W.J. Welch, D.M. Schlossman, K.B. Palter, M.J. Schlesinger, and J.E. Rothman. 1986. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell.* 45:3–13.

Chen, H., and P. De Camilli. 2005. The association of epsin with ubiquitinated cargo along the endocytic pathway is negatively regulated by its interaction with clathrin. *Proc. Natl. Acad. Sci. USA.* 102:2766–2771.

Dubois, L., M. Lecourtois, C. Alexandre, E. Hirst, and J.P. Vincent. 2001. Regulated endocytic routing modulates Wingless signaling in *Drosophila* embryos. *Cell.* 105:613–624.

Elefant, F., and K.B. Palter. 1999. Tissue-specific expression of dominant negative mutant *Drosophila HSC70* causes developmental defects and lethality. *Mol. Biol. Cell.* 10:2101–2117.

Emery, G., A. Hutterer, D. Berdnik, B. Mayer, F. Wirtz-Peitz, M.G. Gaitan, and J.A. Knoblich. 2005. Asymmetric rab11 endosomes regulate delta recycling and specify cell fate in the *Drosophila* nervous system. *Cell.* 122:763–773.

Entchev, E.V., A. Schwabedissen, and M. Gonzalez-Gaitan. 2000. Gradient formation of the TGF-beta homolog Dpp. *Cell.* 103:981–991.

Fanto, M., and M. Mlodzik. 1999. Asymmetric Notch activation specifies photoreceptors R3 and R4 and planar polarity in the *Drosophila* eye. *Nature.* 397:523–526.

Freeman, M. 1996. Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell.* 87:651–660.

Gall, W.E., M.A. Higginbotham, C. Chen, M.F. Ingram, D.M. Cyr, and T.R. Graham. 2000. The auxilin-like phosphoprotein Swa2p is required for clathrin function in yeast. *Curr. Biol.* 10:1349–1358.

Go, M.J., D.S. Eastman, and S. Artavanis-Tsakonas. 1998. Cell proliferation control by Notch signaling in *Drosophila* development. *Development.* 125:2031–2040.

Greener, T., X. Zhao, H. Nojima, E. Eisenberg, and L.E. Greene. 2000. Role of cyclin G-associated kinase in uncoating clathrin-coated vesicles from non-neuronal cells. *J. Biol. Chem.* 275:1365–1370.

Greener, T., B. Grant, Y. Zhang, X. Wu, L.E. Greene, D. Hirsh, and E. Eisenberg. 2001. *Caenorhabditis elegans* auxilin: a J-domain protein essential for clathrin-mediated endocytosis in vivo. *Nat. Cell Biol.* 3:215–219.

Hay, B.A., T. Wolff, and G.M. Rubin. 1994. Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development.* 120:2121–2129.

Hicke, L., and H. Riezman. 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell.* 84:277–287.

Holstein, S.E., H. Ungewickell, and E. Ungewickell. 1996. Mechanism of clathrin basket dissociation: separate functions of protein domains of the DnaJ homologue auxilin. *J. Cell Biol.* 135:925–937.

Itoh, M., C.H. Kim, G. Palardy, T. Oda, Y.J. Jiang, D. Maust, S.Y. Yeo, K. Lorick, G.J. Wright, L. Ariza-McNaughton, et al. 2003. Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell.* 4:67–82.

Jarriault, S., C. Brou, F. Logeat, E.H. Schroeter, R. Kopan, and A. Israel. 1995. Signalling downstream of activated mammalian Notch. *Nature.* 377:355–358.

Kennerdell, J.R., and R.W. Carthew. 1998. Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the Wingless pathway. *Cell.* 95:1017–1026.

Koo, B.K., H.S. Lim, R. Song, M.J. Yoon, K.J. Yoon, J.S. Moon, Y.W. Kim, M.C. Kwon, K.W. Yoo, M.P. Kong, et al. 2005. Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development.* 132:3459–3470.

Kopan, R., E.H. Schroeter, H. Weintraub, and J.S. Nye. 1996. Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc. Natl. Acad. Sci. USA.* 93:1683–1688.

Kosaka, T., and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster*, *shibire*^{ts1}. *J. Cell Biol.* 97:499–507.

Lai, E.C. 2004. Notch signaling: control of cell communication and cell fate. *Development.* 131:965–973.

Lai, E.C., G.A. Deblandre, C. Kintner, and G.M. Rubin. 2001. *Drosophila* neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. *Dev. Cell.* 1:783–794.

Lai, E.C., F. Roegiers, X. Qin, Y.N. Jan, and G.M. Rubin. 2005. The ubiquitin ligase *Drosophila* Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. *Development.* 132:2319–2332.

Le Borgne, R., and F. Schweisguth. 2003. Unequal segregation of Neuralized biases Notch activation during asymmetric cell division. *Dev. Cell.* 5:139–148.

Le Borgne, R., A. Bardin, and F. Schweisguth. 2005a. The roles of receptor and ligand endocytosis in regulating Notch signaling. *Development.* 132:1751–1762.

Le Borgne, R., S. Remaud, S. Hamel, and F. Schweisguth. 2005b. Two distinct E3 ubiquitin ligases have complementary functions in the regulation of Delta and Serrate signaling in *Drosophila*. *PLoS Biol.* 3:e96.

Lecourtois, M., and F. Schweisguth. 1998. Indirect evidence for *Delta*-dependent intracellular processing of notch in *Drosophila* embryos. *Curr. Biol.* 8:771–774.

Logeat, F., C. Bessia, C. Brou, O. LeBail, S. Jarriault, N.G. Seidah, and A. Israel. 1998. The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc. Natl. Acad. Sci. USA.* 95:8108–8112.

Matsuno, K., M. Ito, K. Hori, F. Miyashita, S. Suzuki, N. Kishi, S. Artavanis-Tsakonas, and H. Okano. 2002. Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling. *Development.* 129:1049–1059.

Mishra-Gorur, K., M.D. Rand, B. Perez-Villamil, and S. Artavanis-Tsakonas. 2002. Down-regulation of Delta by proteolytic processing. *J. Cell Biol.* 159:313–324.

Morgan, J.R., K. Prasad, S. Jin, G.J. Augustine, and E.M. Lafer. 2001. Uncoating of clathrin-coated vesicles in presynaptic terminals: roles for Hsc70 and auxilin. *Neuron.* 32:289–300.

Newmyer, S.L., A. Christensen, and S. Sever. 2003. Auxilin-dynamin interactions link the uncoating ATPase chaperone machinery with vesicle formation. *Dev. Cell.* 4:929–940.

Overstreet, E., X. Chen, B. Wendland, and J.A. Fischer. 2003. Either part of a *Drosophila* epsin protein, divided after the ENTH domain, functions in endocytosis of delta in the developing eye. *Curr. Biol.* 13:854–860.

Overstreet, E., E. Fitch, and J.A. Fischer. 2004. Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. *Development.* 131:5355–5366.

Parks, A.L., K.M. Klueg, J.R. Stout, and M.A. Muskavitch. 2000. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development.* 127:1373–1385.

Pavlopoulos, E., C. Pitsouli, K.M. Klueg, M.A. Muskavitch, N.K. Moschonas, and C. Delidakis. 2001. *neuralized* encodes a peripheral membrane protein involved in Delta signaling and endocytosis. *Dev. Cell.* 1:807–816.

Pishvaee, B., G. Costaguta, B.G. Yeung, S. Ryazantsev, T. Greener, L.E. Greene, E. Eisenberg, J.M. McCaffery, and G.S. Payne. 2000. A yeast DNA J protein required for uncoating of clathrin-coated vesicles in vivo. *Nat. Cell Biol.* 2:958–963.

Pitsouli, C., and C. Delidakis. 2005. The interplay between DSL proteins and ubiquitin ligases in Notch signaling. *Development.* 132:4041–4050.

Polo, S., S. Sigismund, M. Faretta, M. Guidi, M.R. Capua, G. Bossi, H. Chen, P. De Camilli, and P.P. Di Fiore. 2002. A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature.* 416:451–455.

Poodry, C.A. 1990. *shibire*, a neurogenic mutant of *Drosophila*. *Dev. Biol.* 138:464–472.

Robinow, S., and K. White. 1988. The locus *elav* of *Drosophila melanogaster* is expressed in neurons at all developmental stages. *Dev. Biol.* 126: 294–303.

Rubin, G.M., and A.C. Spradling. 1982. Genetic transformation of *Drosophila* with transposable element vectors. *Science.* 218:348–353.

Schlossman, D.M., S.L. Schmid, W.A. Braell, and J.E. Rothman. 1984. An enzyme that removes clathrin coats: purification of an uncoating ATPase. *J. Cell Biol.* 99:723–733.

Schroeter, E.H., J.A. Kisslinger, and R. Kopan. 1998. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature.* 393:382–386.

Seugnet, L., P. Simpson, and M. Haenlin. 1997. Requirement for dynamin during Notch signaling in *Drosophila* neurogenesis. *Dev. Biol.* 192:585–598.

Shih, S.C., D.J. Katzmann, J.D. Schnell, M. Sutanto, S.D. Emr, and L. Hicke. 2002. Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nat. Cell Biol.* 4:389–393.

Sigismund, S., T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P.P. Di Fiore, and S. Polo. 2005. Clathrin-independent endocytosis of ubiquitinated cargos. *Proc. Natl. Acad. Sci. USA.* 102:2760–2765.

Sorkin, A., and M. Von Zastrow. 2002. Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell Biol.* 3:600–614.

Struhl, G., and A. Adachi. 1998. Nuclear access and action of Notch in vivo. *Cell.* 93:649–660.

Terrell, J., S. Shih, R. Dunn, and L. Hicke. 1998. A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol. Cell.* 1:193–202.

Tomancak, P., A. Beaton, R. Weiszmann, E. Kwan, S. Shu, S.E. Lewis, S. Richards, M. Ashburner, V. Hartenstein, S.E. Celniker, and G.M. Rubin. 2002. Systematic determination of patterns of gene expression during *Drosophila* embryogenesis. Genome Biol. 3:RESEARCH0088.1–0088.14.

Umeda, A., A. Meyerholz, and E. Ungewickell. 2000. Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation. *Eur. J. Cell Biol.* 79:336–342.

Ungewickell, E., H. Ungewickell, S.E. Holstein, R. Lindner, K. Prasad, W. Barouch, B. Martin, L.E. Greene, and E. Eisenberg. 1995. Role of auxilin in uncoating clathrin-coated vesicles. *Nature.* 378:632–635.

van der Bliek, A.M., and E.M. Meyerowitz. 1991. Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature.* 351:411–414.

Wang, W., and G. Struhl. 2004. *Drosophila* Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. *Development.* 131:5367–5380.

Wang, W., and G. Struhl. 2005. Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in *Drosophila*. *Development.* 132:2883–2894.

Wolff, T. 2000. Histological Techniques for the *Drosophila* eye. Parts I and II. *In Drosophila* Protocols. W. Sullivan, M. Ashburner, and R.S. Hawley, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 201–244.

Wucherpfennig, T., M. Wilsch-Brauninger, and M. Gonzalez-Gaitan. 2003. Role of *Drosophila* Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *J. Cell Biol.* 161:609–624.

Yeh, E., M. Dermer, C. Commisso, L. Zhou, C.J. McGlade, and G.L. Boulianne. 2001. Neuralized functions as an E3 ubiquitin ligase during *Drosophila* development. *Curr. Biol.* 11:1675–1679.