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

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**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 (Poody, 1990). Clonal analysis suggested that in Notch signaling, dynamin function is required in both signal-sending and signal-receiving cells (Seugnet et al., 1997),
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 Schweiguth, 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 (laf), the D. melanogaster homologue of epsin (Cadavid et al., 2000). Laf 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 (Bonifacio 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 DI 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 F2 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 DI 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 (CG 1107: 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 NH2-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 NH2-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
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 F2 noncomplementation screen with two deletions, Df(3R)ED5021 (81F6-82A5) and Df(3R)ED5092 (82A1-82A5; 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 dAuxI670K animals could survive until adulthood, suggesting that dAuxI670K 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 dAuxI670K . Two of them, dAuxW328X and dAuxW1150X, 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 dAuxI670K 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 dAuxI670K mutants were grossly disorganized, with patches of brown necrotic tissues on the surface (Fig. 1 C). This rough eye phenotype exhibited by homozygous dAuxI670K 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 dAuxW328X and dAuxW1150X are lethal over dAuxI670K at 25°C, mutant animals of heteroallelic combinations for dAuxW328X/dAuxI670K and dAuxW1150X/dAuxI670K 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 dAuxI670K 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 dAuxI670K 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 dAuxI670K (Fig. 1 D).

In addition to rough eyes, homozygous dAuxI670K 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 dAuxI670K/dAuxI670K and dAuxI670K/dAuxI670K raised at 18°C (Fig. 1, compare I and J). Interestingly, each of the adult phenotypes

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 (I), dAuxI670K/dAuxI670K (C), UAS-dAux; Act5C-GAL4/+; dAuxI670K/dAuxI670K (D), dAuxI670K/dAuxW328X grown at 18°C (E), and dAuxI670K/dAuxW328X grown at 18°C (F). Bar, 100 μm. (G–H) Scanning electron micrographs of the adult heads of wild type (G) and dAuxI670K (H). The vibrissae bristles are indicated by arrows. (I and J) Whole mount wings of wild type (I) and dAuxW328X/dAuxW328X grown at 18°C (J). The missing wing margins and the extra vein material are indicated by black and white arrowheads, respectively. The Df-like vein junction is indicated by an asterisk. Bar, 200 μm.
observed in dAuxI670K 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 dAuxI670K 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 dAuxI670K 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/+; dAuxI670K/dAuxI670K 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 dAuxI670K 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 dAuxI670K 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 the mutant retina showed that regular arrays of ommatidia were disrupted. Furthermore, supernumerary photoreceptor cells were detected in 39.67% (n = 510) of dAuxI670K 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 dAuxI670K is not affecting the determination of a particular photoreceptor cell fate.

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 (Robinson 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 dAuxI670K 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 dAuxI670K.

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 dAuxW328X/dAuxI670K. The number of Elav-positive cells appeared to increase in dAuxI670K mutant discs (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 dAuxI670K mutant discs varied greatly, and clusters with multiple Boss-positive cells were occasionally detected (Fig. 3 F, inset). This periodic
spacing of R8s is thought to require Notch-mediated lateral inhibition (Baker, 2002), and the phenotype exhibited by dAuxI670K 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 dAuxI670K homozygous mutant females with wild-type males were stained with αElav antibodies. Elav staining of embryos derived from wild-type
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 dAuxI670K mutant adults.

dAux exhibits specific genetic interactions with Notch

The dAuxI670K 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 N264-39/+; dAuxI670K/+ (Fig. 5 A) and N264-39/+; dAuxI670K/+(B). Scanning electron micrographs of the adult eyes of UAS-N/+; GMR-GAL4/+ (C), UAS-N/+; GMR-GAL4, dAuxI670K/+ (D), UAS-egfr/+; GMR-GAL4/+ (E), and UAS-egfr/+; GMR-GAL4, dAuxI670K/+ (F). Bar, 100 μm.

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-receiving 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 dAuxI670K, 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.

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

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To position the function of dAux in the Notch pathway, we performed epistasis tests using dAuxI670K 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 dAuxI670K 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.

dAuxI670K causes an increase of DI proteins near cell periphery

Recent evidence indicates that internalization of the DSL ligand may play a critical role in regulating Notch activity. Because our
data suggest that dAux acts noncell autonomously and upstream of activated Notch in Notch signaling, we suspected that DI endocytosis could be regulated by dAux. To investigate whether the trafficking of DI proteins is disrupted, eye discs from wild-type, homozygous dAux\textsuperscript{I670K}, and dAux\textsuperscript{W328X}/dAux\textsuperscript{I670K} mutant animals were dissected and stained with antibody raised against the extracellular domain of DI.

In wild-type eye discs, evenly spaced DI staining was first seen in cells that are recruited to form photoreceptor clusters behind the morphogenetic furrow (Fig. 7, A and D). The DI staining in these cells appeared to overlap with cortical phalloidin (not depicted), suggesting that most DI protein initially localized at or proximal to the plasma membrane. In more mature clusters located in the posterior region of the eye disc, the DI staining became more vesicular. These DI-positive structures showed little or no colocalization with the Clic, 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 DI proteins are in the early and late endosomes (unpublished data). To ensure that our determination of DI subcellular localization was not influenced by fixatives or histological techniques, we generated UAS-DI-mRFP, a functional DI 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 DI, DI-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\textsuperscript{I670K} homozygous mutant eye discs, DI staining appeared more excessive and disorganized in cells behind the furrow, reflecting the defects in photoreceptor specification (Fig. 7, B and E). Although DI- 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 DI in the first few rows of cells immediately posterior to the furrow (Fig. 7, B and E). This suggests that DI internalization in these cells, where Notch signaling is thought to participate in the proper spacing of photoreceptor cell clusters, was disrupted. The dAux\textsuperscript{W328X}/dAux\textsuperscript{I670K} eye discs showed even more severe disruption of DI localization, with excessive, peripheral DI 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 DI staining in the dAux\textsuperscript{I670K} mutants could result from either a block in DI internalization and degradation or an increase in DI gene expression. Because an increase in DI expression in larval eye discs mutant for lqf was previously reported (Wang and Struhl, 2004), it seemed likely that the level of DI expression would be affected in dAux mutant cells. To determine if this was the case, we monitored the transcriptional activities of the endogenous DI promoter by measuring the β-gal activity from a DI enhancer trap line (Fanto and Mlodzik, 1999). Unlike lqf, we did not observe a significant increase in β-gal activity in dAux\textsuperscript{I670K} eye discs (unpublished data), suggesting that the apparent increase in DI staining was not due to elevated DI transcription.

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 black asterisks, and genotypically and phenotypically mutant clusters are indicated by white asterisks. Bar, 10 μm. (B–E) Confocal micrographs of eye discs stained with α-Elav antibodies (red). (B) GMR-GAL4/+; (C) UAS-N\textsuperscript{Dm}/+; GMR-GAL4/+; (D) GMR-GAL4/+; dAux\textsuperscript{I670K}/dAux\textsuperscript{W328X}; and (E) UAS-N\textsuperscript{Dm}/+; GMR-GAL4, dAux\textsuperscript{I670K}/dAux\textsuperscript{W328X}. Bar, 15 μm.

Figure 7. DI endocytosis is disrupted in dAux mutant eye discs. Confocal micrographs of third instar larval eye discs immunostained with DI antibody at low (A–C) and high magnification (E–F). (A and D) Wild-type dAux\textsuperscript{I670K}/dAux\textsuperscript{W328X} (B and E) and dAux\textsuperscript{W328X}/dAux\textsuperscript{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 dAuxI670K 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 (dAuxW1150X) 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 dAuxI670K 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 dAuxI670K 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 dAuxI670K 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 dAuxI670K 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 DI-containing endocytic vesicles need to proceed past the clathrin uncoating step to activate Notch. One possible mechanism is that recycling of DI is a prerequisite to form signaling-competent DI-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 DI activity (Emery et al., 2005). However, although DI 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 DI in cells near the furrow. One possible explanation for this apparent difference is that the transit of DI 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 DI internalization causes a mechanical stress on the Notch receptors, which then induces subsequent cleavages. A variation of this model proposes that the objective of DI 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 DI into CCVs, the presumed force-generating event, should have already been completed in *dAux* mutants.

Materials and methods

### Fly genetics

All 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-Hsc70-dAuxI670K* (Elefant and Palter, 1999), *UAS-Clec-EGFP* (Chang et al., 2002), *UAS-Notch*, *UAS-lqf* (Overstreet et al., 2003), *UAS-Grasp65-GFP* (Chang et al., 2002), *UAS-dGrasp65-GFP* (this study), and *UAS-Clec-EGFP* (Chang et al., 2002) were used.

For mosaic analysis, *w; P[Sup+ Pki]KGO8740/TM3* flies were mated with *w/w; dAuxI670K/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 Wolf (2000). Embryos were aged to stage 15 after injection and fixed as described previously (Kemeder et al. and Carthew, 1998). Rat-α-Elev 7E8A10 (Developmental Studies Hybridoma Bank), mouse α-Boss, and mouse α-D C594.9B (Developmental Studies Hybridoma Bank)
Molecular biology

To construct UAS-dAux, an EcoRI–XhoI fragment containing the entire dAux 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–Xhol fragment. After sequencing verification, the NH2-terminal half was inserted as a 2.9-kb EcoRI–SpeI fragment, and the entire dAux fusion was then subcloned as an EcoRI–Not fragment into pUAST. To construct UAS-Dl-mRFP, PCR-amplified mRFP was fused to the COOH terminus of Dl (pBS-Dl, DGRC) as an NdeI–HindIII fragment. The resulting Dl-mRFP was cloned into pUAST an EcoRI–Xhol 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 PCR amplified and cloned into phFK-mRFP-KB. The resulting GFP-Rab11 and dGrasp65-GFP fusions were verified by sequencing and subcloned as Notl fragments into pUAST, respectively. Transgenic flies carrying these constructs were generated by P-element–mediated germline 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′ and 3′ ends were used to amplify 610- and 599-bp fragments of auxilin (5′-TCAAGTCC-AGCGTACAAGACG-3′ and 5′-GGCTATATAACCCGATTIT-3′) and GFP (5′-GTGATGGGAGAGGGAAGG-3′ and 5′-CCCCAAGAGCTGTTACA-AACTC-3′) coding sequence, respectively. In vitro transcription of the PCR fragments produced dsRNAs, which were then prepared as 5′-monomethylxanthosine oligo (N) duplexes 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 DI 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.

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