Development 135, 1089-1095 (2008) doi:10.1242/dev.009530

Auxilin is essential for Delta signaling

Suk Ho Eun, Susan M. L. Banks and Janice A. Fischer*

Endocytosis regulates Notch signaling in both signaling and receiving cells. A puzzling observation is that endocytosis of transmembrane ligand by the signaling cells is required for Notch activation in adjacent receiving cells. A key to understanding why signaling depends on ligand endocytosis lies in identifying and understanding the functions of crucial endocytic proteins. One such protein is Epsin, an endocytic factor first identified in vertebrate cells. Here, we show in *Drosophila* that Auxilin, an endocytic factor that regulates Clathrin dynamics, is also essential for Notch signaling. Auxilin, a co-factor for the ATPase Hsc70, brings Hsc70 to Clathrin cages. Hsc70/Auxilin functions in vesicle scission and also in uncoating Clathrin-coated vesicles. We find that like Epsin, Auxilin is required in Notch signaling cells for ligand internalization and signaling. Results of several experiments suggest that the crucial role of Auxilin in signaling is, at least in part, the generation of free Clathrin. We discuss these observations in the light of current models for the role of Epsin in ligand endocytosis and the role of ligand endocytosis in Notch signaling.

KEY WORDS: Notch, Delta, Epsin, Clathrin, Auxilin, *liquid facets*, Signaling, Eye, *Drosophila*, Endocytosis

INTRODUCTION

Notch signaling is used universally in animals and in nearly every developmental context (Bray, 2006). In the signaling cells, transmembrane ligand endocytosis is necessary for signaling (Le Borgne et al., 2005; Le Borgne, 2006; Chitnis, 2006; Nichols et al., 2007a). In *Drosophila*, ligand endocytosis and signaling depend on Epsin (Overstreet et al., 2004; Wang and Struhl, 2004; Wang and Struhl, 2005), an endocytic factor first identified in vertebrates that functions in at the internalization step of endocytosis (Chen et al., 1998). *Drosophila* Epsin is encoded by the *liquid facets* (*lqf*) gene (Cadavid et al., 2000).

Recently, the endocytic protein Auxilin has been implicated in Notch signaling in *Drosophila* (Hagedorn et al., 2006; Eun et al., 2007). Auxilin is an adapter for the ATPase Hsc70 (Heat shock cognate 70); Auxilin binds Hsc70 with its J domain and also binds Clathrin cages, which are Hsc70 substrates. Auxilin catalyzes the binding of Hsc70 to Clathrin cages and stimulates its ATPase activity (Lemmon, 2001; Eisenberg and Greene, 2007). The evidence is most solid for two direct functions of Auxilin, one in internalization and a second in uncoating Clathrin-coated vesicles. At the internalization step, Hsc70/Auxilin exchanges Clathrin on cages forming at the plasma membrane, which might help to constrict vesicles prior to scission. After scission, Hsc70/Auxilin removes the Clathrin cages, allowing vesicle fusion with endosomes (Lemmon, 2001; Eisenberg and Greene, 2007).

Here, we show that *Drosophila* Auxilin is crucial in signaling cells for Delta internalization and signaling. In addition, we show that the requirement for Auxilin is, at least in part, to maintain the pool of free Clathrin. We infer that Clathrin has a key role in the signaling cells. We discuss how these observations impact current thinking on the roles of Epsin and endocytosis in the signaling cells.

Section of Molecular Cell and Developmental Biology, Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, USA.

*Author for correspondence (e-mail: jaf@mail.utexas.edu)

Accepted 10 January 2008

MATERIALS AND METHODS

Drosophila strains and chromosomes

From the Bloomington Stock Center: *FRT42D* (FBti0002072); *ubi-nGFP FRT40A/CyO* (FBti0015576, FBti0002071); *CyO, YFP* (FBti0058605); *w, eyFLP* (FBti0015982); *Act5C-gal4/CyO* (FBti0012293); *Dl-lacZ* (FBti0012268); and Chc^{1} (FBal0033511).

From our laboratory: w^{1118}; *w; gaux+/CyO* (Eun et al., 2007); aux^{727}, aux^{D136}, aux^{K47}, aux^{D128} (Eun et al., 2007); lqf^{FDD9} (FBal0104483); *UAS-aux+* (FBal0190739); *UAS-GFP-Clc* (FBtp0017428); and N^{ts1} (FBal0012887).

Generated in this work: *w; FRT42D, gaux+, ubi-GFP/CyO, YFP;* aux^{727}*/TM6B*; *w; Tub-aux+, ubi-GFP, FRT40A/CyO, YFP;* aux^{727}*/TM6B*; *w, eyFLP; FRT40A/CyO, YFP;* aux^{D128}*/TM6B*; *w, eyFLP; FRT42D/CyO, YFP;* aux^{D136}*/TM6B*; *w, eyFLP; FRT42D/CyO, YFP;* aux^{D136} *Dl-lacZ/TM6B*; *w; ro-aux+/CyO;* aux^{K47}*/TM6B*; *w; Act5C-gal4/CyO;* aux^{727}*/TM6B*; *w; Act5C-gal4/CyO;* aux^{K47}*/TM6B*; *w; UAS-*aux^{CBD+J}*/CyO;* aux^{D128}*/TM6B*; *w; UAS-*aux^{K+PTEN}*/CyO;* aux^{D128}*/TM6B*; *w; UAS-*$aux^{\Delta J}$*/CyO;* aux^{D128}*/TM6B*; *w;* $gaux^{CBD+J}$*/CyO;* aux^{D128}*/TM6B*; *w; gChc+/CyO;* aux^{K47}*/TM6B*; *w; gChc+/CyO;* aux^{D128}*/TM6B*; and *w; gChc+/CyO;* lqf^{FDD9}*/TM6B*.

P-element constructs and transformation

Molecular biology was performed using standard procedures. Cloning enzymes and standard oligonucleotides were from New England Biolabs and Roche, custom oligonucleotides were from IDT, and DNA purification kits were from Qiagen. The DNA sequences of all PCR products were verified.

ro-aux+

An *aux+* cDNA in *pOT2* (*GH26574* from the *Drosophila* Genomics Resource Center, Bloomington, IN) was purified as an *Xho*I-*Eco*RI fragment and ligated into those sites of *pBSKSII+* (Stratagene) to generate *pBS-aux+*. The *Eco*RI site in *pBS-aux+* was changed to *Asc*I by ligating annealed oligonucleotides of the sequence 5′-AATTGGCGCGCC-3′ into the *Eco*RI site. Subsequently, the *Xho*I site in the resulting plasmid was changed to *Asc*I by ligating annealed oligonucleotides of the sequence 5′-TCGAGG-CGCGCC-3′ into the *Xho*I site. The 4.4 kb *Asc*I fragment containing the *aux+* cDNA was ligated into the *pRo* vector (Huang and Fischer-Vize, 1996) and the correct orientation determined by *Sal*I digestion.

The *aux+* cDNA coding region is 3498 nt long, including start and stop codons, and it encodes a protein of 1165 amino acids (aa), including the Met initiator codon.

UAS_t-aux^{CBD+J}

This construct contains nt 2071-3498 of the *aux+* cDNA coding region, corresponding to aa N691-A1165. A 1.4 kb region of the *aux+* coding region was amplified by PCR (Platinum PCR Supermix,

Invitrogen) using *pBS-aux+* as the template and the primer pair 5′-GGAATTCATGAACCAGGACTTGGATGATCTG-3′ and 5′-GTCTAGATTACGCATTAAACATATTTTGCTG-3′. The PCR product was subcloned into pGEM-T Easy (Promega). A 1.4 kb *Eco*RI-*Xho*I fragment containing aux^{CBD+J} was purified and ligated into $pUAS_t$ (Brand and Perrimon, 1993) restricted with *Eco*RI and *Xho*I.

UAS_t-aux^{K+PTEN}

This construct contains nt 1-2103 of the *aux+* cDNA coding region, corresponding to aa M1-E701. A 2.1 kb *Asc*I fragment containing aux^{K+PTEN} was generated by PCR using *pBS-aux+* as a template, the primers 5′-AAAGGCGCGCCATGGGCGAGTTCTTTAAGTCGCTC-3′ and 5′-AAAGGCGCGCCTCACTCAGGGTCTGGCAGATCATCCA-3′, and Pfu Turbo Polymerase (Stratagene). The amplified product was gel-purified, restricted with *Asc*I, and ligated into *Asc*I-restricted $pUAS_t$.

UAS_t-$aux^{\Delta J}$

This construct contains nt 1-3291 of the *aux+* cDNA coding region, corresponding to aa M1-D1097. A 3.3 kb fragment containing $aux^{\Delta J}$ was generated by PCR using *pBS-aux+* as template, the primers 5′-AAAGGCGCGCCATGGGCGAGTTCTTTAAGTCGCTC-3′ and 5′-GGCTAGCTTAATGTCCCACAACACTGTGTGCATAG-3′, and Herculase DNA Polymerase (Stratagene). The product was ligated into pGEMT-Easy, re-isolated as an *Asc*I-*Xba*I fragment, and ligated into $pUAS_t$ restricted with those enzymes.

$pgaux^{CBD+J}$

This construct is the 21 kb genomic DNA fragment in *pgaux+* (Eun et al., 2007), subcloned as an *Nhe*I fragment into the *Xba*I site of *pCasper3* (Thummel and Pirrotta, 1992) to generate *pCasper3-gaux+*, and with a deletion of 4101 bp that includes the kinase and PTEN domains, and an addition of two aa (Leu-Glu), corresponding to an *Xho*I site (5′-CTCGAG-3′) just downstream of the start codon. This was achieved by replacing an internal 10.7 kb *Kpn*I fragment with a 6.6 kb *Kpn*I fragment, which was generated by joining three PCR products (Herculase), a 5′ product, a middle product, and a 3′ product that generates the deletion. The 984 bp 3′ product was amplified using *pCasper3-gaux+* as template and the primers 5′-ACTCGAGAACCAGGACTTGGATGATCTGCCAG-3′ and 5′-GGTACCGCCTGTGGCTGCG-3′, ligated into pGEM-T and re-isolated as an *Xho*I-*Kpn*I fragment and ligated into *pBSII* restricted with the same enzymes. The middle fragment (2852 bp) was generated the same way using the primers 5′-AGATATCTCCGGATGGGCAGACACGAA-3′ and 5′-ACTCGAGCATTTTGGTGGTGGCCAATGCTA-3′, isolated as an *Eco*RV-*Xho*I fragment and ligated into those two sites in *pBSII* containing the 3′ fragment. The 5′ fragment (2789 bp) was generated the same way using the primers 5′-AGCGGCCGCGGTACCGCCCGAGCCCG-3′ and 5′-TCCGGATGTTGCAAACTTTCCAA-3′, isolated as a *Not*I-*Bsp*EI fragment, and ligated into those two sites in *pBSII* containing the 3′ and middle fragments. Finally, a 6.6 kb *Kpn*I fragment was isolated from the resulting *pBSII* plasmid and ligated into *pCasper3-gaux+* restricted with *Kpn*I.

pgChc+

A 14.3 kb *Avr*II-*Sac*II fragment of BAC22H11 (BACPAC Resources, Oakland, CA) containing *Chc+* genomic DNA was ligated into *pCMC105* (a gift of C.-M. Chen and G. Struhl, Columbia University, New York, NY) restricted with *Avr*II and *Sac*II. The resulting plasmid was restricted with *Avr*II, and a 4.5 kb *Nhe*I-*Xba*I fragment of *pAT806* (a gift of K. Basler and G. Struhl) containing a *w+* marker gene was ligated in.

Tub-aux+

An *αTub84B* (*Tub*) promoter fragment was excised from *pKB700* (a gift of K. Basler and G. Struhl) as a 2.6 kb *Not*I-*Kpn*I fragment and ligated into *pBSKSII* restricted with the same enzymes. The *Not*I site in the resulting plasmid was changed to *Avr*II by inserting the linker 5′-GGCCCCTAGG-3′. The *Tub* promoter was excised as an *Avr*II-*Kpn*I (3′ overhang removed) fragment. A 4.4 kb *Eco*RI-*Xho*I fragment containing the *aux+* cDNA was excised from clone *GH26574* and ligated into *pBSKSII* restricted with the same enzymes. The *Xho*I site of the resulting plasmid was changed to *Nhe*I using the linker 5′-TCGAGCTAGC-3′, the *Eco*RI site was changed to *Eco*RV using the linker 5′-AATTCGATATCG-3′, and the *Not*I site was changed to *Avr*II using the linker 5′-GGCCCCTAGG-3′. The *Tub* promoter fragment was ligated into the resulting plasmid restricted with *Eco*RV and *Avr*II, and an *Avr*II-*Nhe*I fragment containing *Tub-aux+* was excised. A 1.2 kb *Spe*I-*Xba*I fragment containing transcription termination signals was excised from *pAT806* and ligated into *pCasper4* (Thummel and Pirrotta, 1992) restricted with *Spe*I. A plasmid with the termination signal in the appropriate orientation was isolated, restricted with *Spe*I, and the *Tub-aux+* ligated in.

Transformation

P-element transformation of w^{1118} flies was performed according to standard methods in our laboratory or by Genetic Services (Sudbury, MA).

Auxilin antibodies

Antibodies were generated to Auxilin amino acids Q702-A1165. The corresponding DNA sequence was amplified by PCR using primers 5′-GGATCCCAGGTGACACCTCGGTTCTGCG-3′ and 5′-GAATTCCGCATTAAACATATTTTGCTGCGTG-3′, with plasmid *GH26574* as a template, and subcloned into pGEMT. A *Bam*HI-*Eco*RI fragment containing the *aux* coding sequences was ligated into pRSET (Invitrogen) restricted with the same enzymes, which added six His codons at the beginning of the open reading frame. Protein expression from pRSET-aux was in BL21-CodonPlus-RIL cells (Invitrogen) induced with 0.1 mM IPTG. The 6xHis-Aux protein was purified from sonicated cells using HisBind (Novagen) and used to raise antibodies in rats (Pocono Rabbit Farm and Laboratory, Canadensis, PA). Prior to use for immunostaining, the antisera were preadsorbed with fixed w^{1118} *Drosophila* embryos.

Analysis of eyes and wings

Third instar larval eye discs were fixed in PEMS and antibody incubations and washes were in PBST (see Fischer-Vize et al., 1992). Primary antibodies used were: mAb323 (1:2) obtained from Sarah Bray (University of Cambridge, UK); mAb202 (1:10) from Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA; rat anti-Elav (9:1) from DSHB; and anti-Aux (1:500). Secondary antibodies (Molecular Probes) were AlexaFluor 568 anti-mouse and AlexaFluor 633 anti-rat (1:300). AlexaFluor 568-phalloidin (Molecular Probes) was dried and resuspended in PBST at 0.1 unit/l. Eye discs were mounted in Vectashield (Vector) and viewed with a Leica TCSSP2 confocal microscope. Plastic sectioning of adult eyes was as described (Tomlinson and Ready, 1987), and wings were dehydrated in 70% ethanol and mounted in DPX (Fluka). Wing and eye sections were photographed with a Zeiss Axioplan and Axiocam HRc. Images were processed with Adobe Photoshop.

RESULTS

auxilin (*aux*) mutants have the same eye development defects as *lqf* or *Delta* mutants

Throughout this work, we refer to aux^{727}/aux^{D128} or aux^{727}/aux^{D136} heterozygous cells or animals as *aux–*. [The *aux* alleles were analyzed previously (Eun et al., 2007).] The nonsense mutant aux^{D136} is likely to be null because the truncated protein it may produce would lack the J domain (Fig. 1A), which we show below is necessary for Auxilin function. aux^{727} and aux^{D128} are also likely null alleles because of their early nonsense mutations (Fig. 1A), and because their phenotypes in trans to each other or aux^{D136} are indistinguishable. Also, we show below that the mutant eye phenotype of either *aux–* genotype is identical to *lqf* or *Delta* null mutants. Finally, in eye discs immunolabeled with a polyclonal antibody to the C-terminal 464 amino acids of Auxilin, whereas adjacent *aux+* cells showed strong signals, clones of aux^{727}/aux^{D128} (Fig. 1B,B′) cells showed no signal above background. Nevertheless, there could be some truncated and functional Auxilin protein produced by aux^{727} or aux^{D128} that escaped detection. In aux^{727} or aux^{D128} homozygous larvae, mutant *aux* mRNA accumulates to levels similar to those seen in wild type (data not

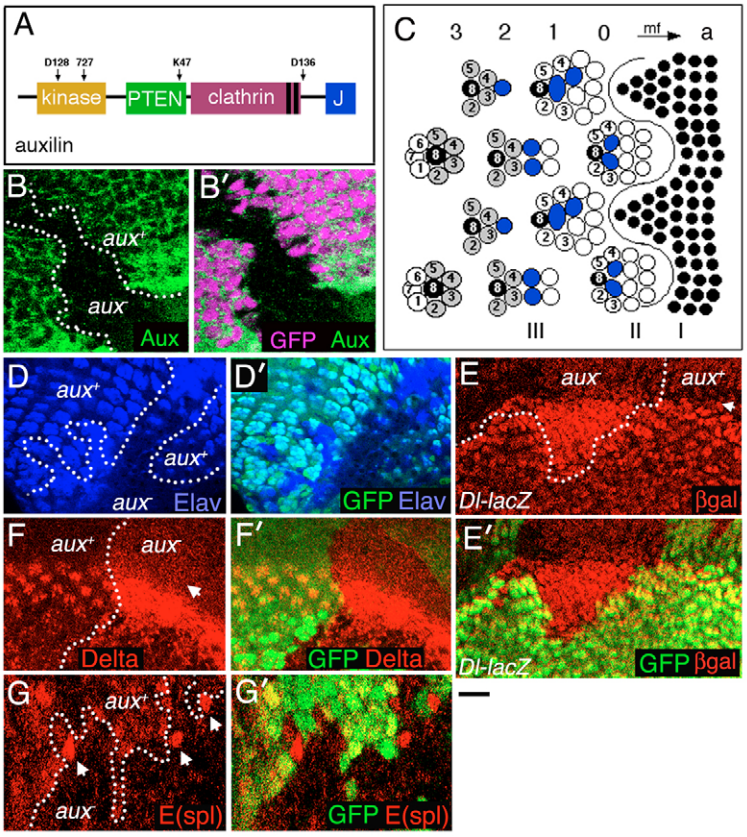

Fig. 1. Auxilin is required only in the Delta signaling cells. (**A**) Structure of *Drosophila* Auxilin. The vertical black bars within the Clathrin-binding domain are DPF motifs that in GAK bind AP2 (Umeda et al., 2000; Korolchuk and Banting, 2002). Approximate sites of the mutations in three *aux* nonsense mutant alleles and one missense allele (K47) are indicated (Eun et al., 2007). (**B**,**B′**) Confocal image of an aux^{727}/aux^{D128} clone (outlined) marked by the absence of GFP. Clones were generated in larvae of the genotype *w, eyFLP; FRT42D gaux+, Ubi-GFP/FRT42D;* aux^{727}/aux^{D128}. (**C**) Diagram of three Notch/Delta signaling events near the morphogenetic furrow. I, proneural enhancement; II, lateral inhibition; III, R-cell restriction. Numbers at top indicate rows of developing ommatidia. Numbers inside circles indicate R-cells. Blue cells are ectopic R-cells when R-cell restriction fails. mf, morphogenetic furrow; a, anterior. (**D-G′**) Confocal images of immunolabeled third instar larval eye discs. (D,D′) aux^{727}/aux^{D136} clone (outlined), marked by absence of GFP, generated in larvae of genotype *w, eyFLP; FRT42D gaux+, Ubi-GFP/FRT42D;* aux^{727}/aux^{D136}. R-cell nuclei are Elav+. Why Elav+ cells are observed that are not directly adjacent to the clone edge is not known. This was observed also in *lqf*– and *Dl*– clones (Baker and Yu, 1996; Overstreet et al., 2004). (E,E′) aux^{727}/aux^{D136} clone expressing β-galactosidase from a *Delta* enhancer trap (*Dl-lacZ*) generated in larvae of genotype *w, eyFLP; Tub-aux+, Ubi-GFP, FRT40A/FRT40A;* aux^{D136} *Dl-lacZ/*aux^{727}. The arrow indicates furrow. (F,F′) *aux*– clone generated as in B, outlined. The arrow indicates furrow. (G,G′) *aux*– clone generated as in D, outlined. Arrows indicate three *aux*– E(spl)+ nuclei. There are few *aux*– E(spl)+ nuclei observed because most of the cells at the clone border are R-cells (Elav+, see D), which do not express E(spl). Also, additional E(spl)+ nuclei are observed in other planes. Scale bar: 20 μm in B,B′,D-F′; 10 μm in G,G′.

shown), and thus through reinitiation of translation downstream of the nonsense mutation, aux^{727} or aux^{D128} potentially could produce truncated Auxilin protein. As we show that the C-terminal region of Auxilin containing the Clathrin-binding and J domains is sufficient for function, truncated Auxilin protein so produced could be functional. We could not test whether or not these alleles produce truncated protein undetectable by immunolabeling because the Auxilin antibody we generated does not detect Auxilin protein on blots of *Drosophila* wild-type protein extracts.

aux– clones in third instar larval eye discs displayed phenotypes consistent with the failure of three Delta signaling events near the morphogenetic furrow (Fig. 1C). Cells anterior to the furrow require Notch activation to achieve neural potential (proneural enhancement); when Notch activation fails at this point, no neurons develop (Baker, 2002). As cells exit the furrow, lateral inhibition results in evenly spaced ommatidial preclusters; failure of Notch activation at this point results in disordered photoreceptors (R-cells) (Baker, 2002). R-cell determination in *aux*– clones appeared identical to that in *Delta* or *lqf* null clones (Baker and Yu, 1996; Overstreet et al., 2004). No R-cells (Elav+) were present in the center of large *aux*– clones, and *aux*– R-cells present at clone borders were disorganized (Fig. 1D,D′). Another indicator of lateral inhibition failure is increased *Delta* transcription (Baker and Yu, 1998; Wang and Struhl, 2004). *aux*– clones showed increased expression of a *Delta-lacZ* enhancer trap (Fig. 1E,E′) and also increased Delta protein (Fig. 1F,F′). It was reported previously that *Delta-lacZ* is not upregulated in eye discs (Hagedorn et al., 2006). Perhaps the discrepancy is due to differences in the mutant *aux* alleles used. A later Notch signaling event is R-cell restriction, in which R2/5 and R3/4 signal precluster cells in the developing ommatidium to limit the number of outer R-cells to six (Fig. 1C) (Overstreet et al., 2004). Like *Delta* or *lqf* hypomorphs (Parks et al., 1995; Cadavid et al., 2000), *aux* hypomorphic eyes contain ommatidia with extra outer R-cells (Hagedorn et al., 2006; Eun et al., 2007).

As is the case for Epsin, the requirement for Auxilin in eye patterning appears limited to Notch signaling. Several different signaling pathways are needed for morphogenetic furrow movement, and Epidermal growth factor receptor signaling is essential for R-cell determination (Lee and Treisman, 2002; Kumar, 2002; Nagaraj et al., 2002). These pathways are operating in *aux*– clones because furrow movement inside *aux*– clones resembles that in wild type (Fig. 1F,F′), and cells expressed Elav, a marker of R-cell determination (Fig. 1D,D′). In addition, neither Epsin (Overstreet et al., 2004; Wang and Struhl, 2004) nor Auxilin is required for cell viability.

Auxilin is essential in signaling cells only

aux– cells become R-cells at clone borders, where they are adjacent to *aux*+ cells that signal and impart proneural potential (Fig. 1D,D′). This suggests that *aux*– cells can receive Notch activation signals. Indeed, *aux*– cells expressed Enhancer of split proteins [E(spl)], a direct response to Notch activation (Jennings et al., 1994), but only when adjacent to *aux*+ cells at clone borders (Fig. 1G,G′). In addition, in *aux* hypomorphic eyes, expression of *aux*+ in R2/5 and R3/4 by a transgene (*ro-aux+*) rescued the mutant phenotypes of ommatidia with ectopic R-cells: ~25% of facets in aux^{K47}/aux^{D128} were wild-type, and the addition of *ro-aux+* increased the fraction to ~95% (data not shown). The *ro* expression construct is remarkably specific for R2/5 and R3/4

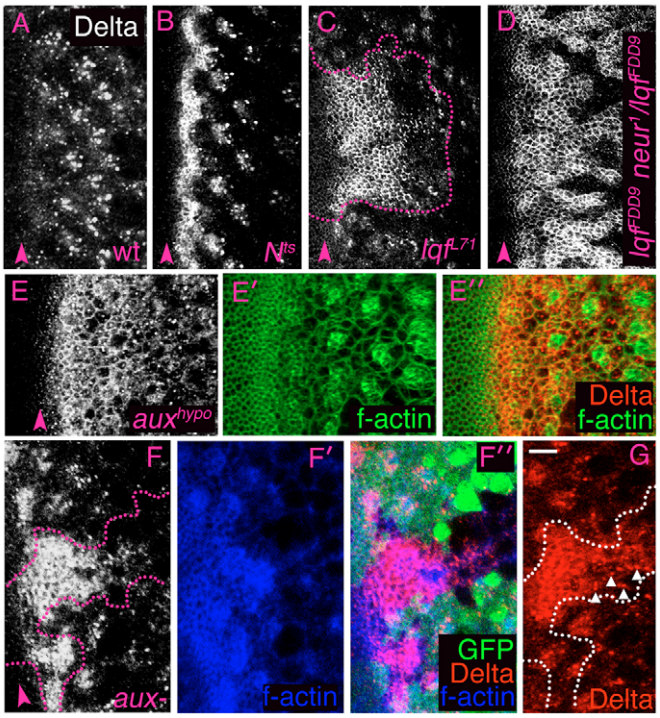

Fig. 2. Auxilin is required for some Delta internalization. Confocal images of immunolabeled third instar larval eye discs. Black-and-white images (A-D,E,F) show Delta protein. Arrowheads indicate furrow. (**A**) Wild type (wt). (**B**) N^{ts1} disc at 29°C for 6 hours. (**C**) *lqf*– clone (outlined). (**D**) *lqf* hypomorph heterozygous for strong *neur* mutation. (**E-E″**) aux^{K47}/aux^{D136} showing Delta and F-actin (plasma membrane). (**F-F″**) aux^{727}/aux^{D128} clone (outlined) marked by absence of GFP, generated in larvae of genotype *w, eyFLP; FRT42D gaux+, Ubi-GFP/FRT42D;* aux^{727}/aux^{D128}. (**G**) Same clone as in F, but more basal. Arrowheads indicate some of the numerous puncta. Scale bar: 10 μm in G for A-G.

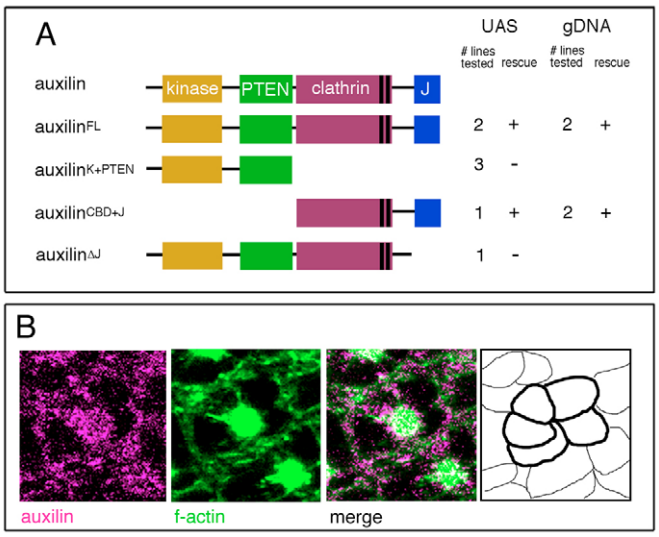

Fig. 3. The Clathrin-binding and J domains of Auxilin are sufficient and the J domain is necessary for Delta signaling. (**A**) *Drosophila* Auxilin (1165 amino acids) and four transgene constructs. To the right is a summary of the complementation test results. UAS, UAS-cDNA; gDNA, genomic DNA-based constructs; +, indicates complementation of all *aux*– mutant phenotypes including lethality (rescue); –, indicates no activity (no rescue). The *aux*– genotypes tested for complementation are aux^{727}/aux^{D128} (strong) and aux^{K47}/aux^{D128} (hypomorphic). Three independent *UAS-auxilin*$^{\Delta J}$ lines were tested, and in an aux^{727}/+ background, two lines showed *aux* mutant-like wing and eye phenotypes (data not shown) indicative of dominant-negative activity. No *auxilin*$^{K+PTEN}$ lines are dominant negative. The *auxilin*$^{\Delta J}$ line tested for complementation expresses protein; it has dominant-negative activity and protein is detected in immunolabeled eye discs (data not shown). (**B**) Confocal images of a facet (diagram of cell outlines at far right) in wild-type third instar larval eye disc immunolabeled with anti-Auxilin antibodies and phalloidin (F-actin).

(Overstreet et al., 2004). Thus, R-cell restriction fails when R2/5 and R3/4 have little *aux*+ activity because they cannot signal to extraneous precluster cells. We conclude that *aux*– cells in the eye can receive, but not send, Notch signals.

Auxilin is required for some, but not all, Delta internalization

Membrane-localized Delta is almost undetectable in immunolabeled wild-type eye discs (Fig. 2A) (Parks et al., 1995; Baker and Yu, 1998; Overstreet et al., 2003; Overstreet et al., 2004). Rather, nearly all Delta detected is in endocytic vesicles that are probably irrelevant to signaling, as they are observed in *lqf*– cells that do not signal (Fig. 2C) (Overstreet et al., 2004; Wang and Struhl, 2004). In *lqf*– clones, lateral inhibition failure results in Delta overexpression (Baker and Yu, 1998; Wang and Struhl, 2004), which allows detection of Delta on the plasma membrane (Fig. 2C). This is observed also in eye discs that are hypomorphic for both *lqf* and heterozygous for a *neuralized* (*neur*) mutation (Fig. 2D) (Overstreet et al., 2004). *neur* encodes a Ubiquitin ligase required for Delta ubiquitination and signaling (Le Borgne et al., 2005; Le Borgne, 2006). Delta eventually clears from the membrane and is observed again only in intracellular puncta several rows posterior to the furrow (Overstreet et al., 2003; Overstreet et al., 2004). A similar phenomenon is observed with *Notch* temperature sensitive (N^{ts}) mutant discs raised to the restrictive temperature for 6 hours (Fig. 2B) (Baker and Yu, 1998). However, in N^{ts} discs, where lateral inhibition fails owing to a failure in signal reception, Delta clears from the membrane quickly and only a tight band of membrane-associated Delta is apparent at the furrow (Fig. 2B) (Baker and Yu, 1998). By contrast, in *lqf*– cells, Delta remains on the membrane for a longer time (more rows of cells) posterior to the furrow (Fig. 2C,D) (Overstreet et al., 2003; Overstreet et al., 2004) and we interpret this as inefficient endocytosis. This observation is similar to that in the wing disc, where Delta was overexpressed using a transgene and clones of *lqf*– cells accumulated more Delta on the membrane than did adjacent wild-type cells (Wang and Struhl, 2004). Delta also persisted on the membrane in *aux* mutant cells (Fig. 2E-E″,F-F″). Also, as in *lqf*– clones (Overstreet et al., 2004; Wang and Struhl, 2004), Delta puncta were present in *aux*– cells (Fig. 2F,G). We conclude that like Epsin, Auxilin is required for some, but not all, Delta internalization. Similarly, in yeast and vertebrate cells, *aux* mutations result in decreased endocytosis (Pishvaee et al., 2000; Umeda et al., 2000).

An Auxilin fragment including the Clathrin-binding domain and J domain is sufficient for Notch signaling

Drosophila Auxilin, like vertebrate auxilin 2 or GAK (cyclin G-associated kinase), has N-terminal kinase and PTEN (phosphatase and tensin-related) domains (Fig. 3), the functions of which are unclear (Umeda et al., 2000; Lemmon, 2001; Korolchuk and Banting, 2002; Lee et al., 2006; Massol et al., 2006; Eisenberg and

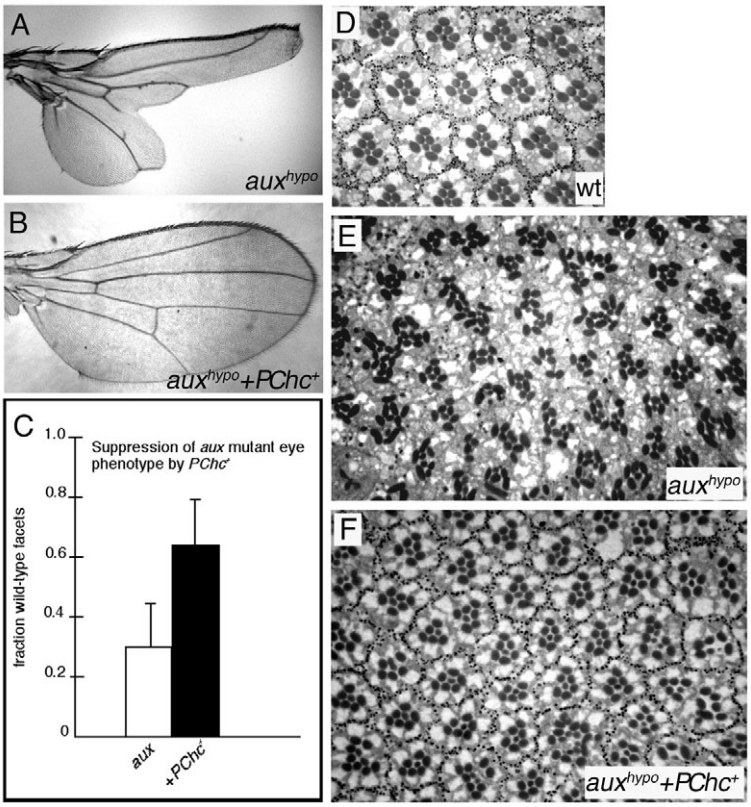

Fig. 4. A *Chc*+ transgene suppresses *aux* eye and wing mutant phenotypes. (**A**) aux^{K47}/aux^{D128} *Drosophila* wing. (**B**) aux^{K47}/aux^{D128} wing with one copy of *PChc*+. The wing is indistinguishable from that of wild type. (**C**) Bar chart showing the degree of suppression of aux^{K47}/aux^{D128} eyes by a single copy of *PChc*+. For each genotype, 51-124 facets in each of ten eyes were examined. Error bars represent one standard deviation from the mean calculated for each eye. The effect of *PChc*+ is significant (Student's *t*-test, $P<0.001$). (**D-F**) Tangential sections of adult eyes are shown as examples of data tabulated in C. *PChc*+ complements Chc^1 completely (Bazinet et al., 1993). The mutant phenotype of *aux* hypomorphs reflects some defects in lateral inhibition (see Fig. 2E), and mainly defects in R-cell restriction. The same phenomenon was observed for *lqf* hypomorphs (Overstreet et al., 2004). This observation might reflect a particular requirement for efficient Delta signaling in R-cell restriction, or mechanistic differences in signaling at different times during eye development.

Greene, 2007). The auxilins of yeast and *C. elegans*, which lack kinase and PTEN domains, uncoat clathrin-coated vesicles (CCVs) in vitro and in vivo (Gall et al., 2000; Pishvaee et al., 2000; Greener et al., 2001), and the Clathrin-binding and J domains of GAK are sufficient for uncoating in vivo (Holstein et al., 1996; Greener et al., 2000). The presence of the kinase and PTEN domains in *Drosophila* Auxilin suggests that the function of Auxilin in Notch signaling might not be limited to Clathrin uncoating or exchange.

To determine whether or not the kinase and PTEN domains or the J domain are required for Notch signaling, we generated transformants with the four different *UAS* transgenes shown in Fig. 3A. The transgenes were tested in single copy for their ability, when expressed ubiquitously with *Act5C-gal4*, to complement strong *aux*– mutants and hypomorphs. We found that $Auxilin^{FL}$ or $Auxilin^{CBD+J}$ complemented all *aux*– mutant phenotypes, and that $Auxilin^{K+PTEN}$ and $Auxilin^{\Delta J}$ had no activity (Fig. 3A). *Act5C>auxilin*$^{CBD+J}$ might overexpress $Auxilin^{CBD+J}$ relative to endogenous Auxilin. Because genomic transgenes are likely to be expressed at physiological levels, we also generated a transgene ($gaux^{CBD+J}$) that expresses $Auxilin^{CBD+J}$ in the context of a genomic DNA construct, *gaux*+ (Eun et al., 2007). A single copy of either *gaux*+ or $gaux^{CBD+J}$ complemented all *aux*– phenotypes (Fig. 3A). These results suggest that the ability of $Auxilin^{CBD+J}$ to complement *aux*– is unlikely to require its overexpression.

We conclude that the J domain is necessary and a C-terminal Auxilin fragment including the Clathrin-binding and J domains is sufficient for function in Notch signaling. Thus, the role of Auxilin is likely to depend on its function as a link between Hsc70 and Clathrin cages. Consistent with functions in endocytic vesicle internalization and uncoating, Auxilin was detected apically in cytoplasmic puncta near the plasma membrane (Fig. 3B).

The *Notch* pathway phenotypes of *aux* mutants are partially suppressed by an extra copy of the *Chc*+ gene

In *aux* mutants, it would be expected that the levels of free Clathrin are limiting owing to inefficient uncoating of CCVs. In yeast and *C. elegans*, free clathrin is depleted in *aux* mutants (Gall et al., 2000; Pishvaee et al., 2000). Consistent with Clathrin depletion in *Drosophila aux* mutants, we found that a loss-of-function mutation in the *Clathrin heavy chain* gene, Chc^1, is a strong dominant enhancer of *aux* hypomorphs: Chc^1/+ flies appeared normal, and aux^{K47}/aux^{D128} adult flies were viable with *Notch*-like phenotypes, but Chc^1/+; aux^{K47}/aux^{D128} adults could not be obtained. It was reported previously that expression of GFP-Clathrin light chain (Clc) enhances *aux* phenotypes (Hagedorn et al., 2006), and this result was repeatable in our hands. We do not think, however, that this genetic interaction contradicts the results with *Chc* mutants. Rather, as GFP-Clc does not colocalize with Chc in the eye disc (data not shown), we propose that GFP-Clc acts as a dominant negative. There are no *Clc* mutants available to test for complementation by *GFP-Clc*.

We also tested whether an additional copy of the *Chc*+ gene (*PChc*+) suppresses the *Notch*-like phenotypes of *aux*– hypomorphs. We found that *PChc*+ suppresses the wing phenotype completely (Fig. 4A,B) and the eye phenotype significantly (Fig. 4C-F). Similarly, in *C. elegans*, *aux(RNAi)* mutant phenotypes are suppressed by overexpression of clathrin heavy chain (Greener et al., 2001). Taken together, these results suggest that Notch pathway phenotypes of *aux* mutants are due at least in part to Clathrin depletion. Clathrin is likely to be needed in some manner for Delta internalization. Indeed, we have shown that Delta endocytosis is inefficient in *aux* mutants. Thus, the requirement for Auxilin does not necessarily infer a requirement

in the signaling cells for trafficking of Delta-containing endosomes downstream of internalization, as proposed previously (Hagedorn et al., 2006).

DISCUSSION

A role for Clathrin in Notch signaling cells was originally inferred from the observation that *Chc* mutants are strong dominant enhancers of *lqf* hypomorphs (Cadavid et al., 2000). As Epsin has both Ubiquitin- and Clathrin-binding motifs, and also binds the plasma membrane, the simplest scenario imaginable for Clathrin and Epsin function in Delta internalization is for Epsin to act as a Clathrin adapter that recognizes ubiquitinated Delta, and brings Clathrin to the membrane for CCV formation (Wendland, 2002). However, in light of evidence that Epsin-dependent endocytosis of ubiquitinated transmembrane proteins such as Delta may not occur through formation of CCVs (Chen and De Camilli, 2005; Sigismund et al., 2005; Aguilar and Wendland, 2005; Hawryluk et al., 2006; Barriere et al., 2006; Madshus, 2006), it became unclear how to interpret the *Chc*/*lqf* genetic interaction. The results presented here point to a crucial role for Clathrin in Notch signaling cells. One intriguing possibility is that Delta internalization depends on Clathrin not because Delta is endocytosed in CCVs, but because Clathrin is a positive regulator of Epsin function. More experiments are required to test this idea.

Why do tissues that lack Epsin or Auxilin display *Delta*-like phenotypes, rather than phenotypes indicating failure of many signaling pathways or even cell death? One possibility is that the apparent specificity of both Epsin and Auxilin might simply reflect the usual redundancy of endocytic protein functions, and an unusual dependence of Notch signaling on efficient endocytosis. Alternatively, a special function of Epsin might be crucial to Notch signaling cells. Two kinds of models have been proposed to explain why Notch signaling requires ligand endocytosis by the signaling cells (Le Borgne et al., 2005; Le Borgne, 2006; Chitnis, 2006; Nichols et al., 2007a). One idea (the 'pulling model') is that after receptor binding, ligand endocytosis generates mechanical forces that result in cleavage of the Notch intracellular domain (Notch activation), either by exposing the proteolytic cleavage site on the Notch extracellular domain, or by causing the heterodimeric Notch receptor to dissociate (Parks et al., 2000; Nichols et al., 2007b). Alternatively, ligand internalization prior to receptor binding might be required to process the ligand endosomally, and recycle it back to the plasma membrane in an activated form (the 'recycling model') (Le Borgne and Schweisguth, 2003; Wang and Struhl, 2004; Wang and Struhl, 2005; Emery et al., 2005; Jafar-Nejad et al., 2005). Epsin might generate an environment particularly conducive to either pulling or recycling, and Auxilin might be required specifically by Notch signaling cells because it activates Epsin, perhaps by providing free Clathrin. Alternatively, Auxilin might be needed to provide free Clathrin because Delta is internalized through CCVs. In this case, if Auxilin is required in Notch signaling solely to provide free Clathrin, the implication would be that efficient CCV uncoating is not important for generating uncoated Delta-containing vesicles per se, which are prerequisite for travel through an endosomal recycling pathway. Further understanding of the role of Auxilin in Notch signaling cells might be key to understanding the role of ligand endocytosis.

For flies, plasmids and antibodies we thank Nick Baker, Sarah Bray, Helmut Kramer, Ira Mellman, Marc Muskavitch, Gary Struhl and the Bloomington *Drosophila* Stock Center. We also thank Erin Overstreet for the N^{ts} image in Fig. 2, Xuanhua Xie for important discussions, and three anonymous reviewers who helped to improve the manuscript greatly. This work was supported by a grant to J.A.F. from the National Institutes of Health, Institute for Child Health and Human Development (RO1-HD30680).

References

Aguilar, R. C. and Wendland, B. (2005). Endocytosis of membrane receptors: two pathways are better than one. *Proc. Natl. Acad. Sci. USA* **102**, 2679-2680.

Baker, N. E. (2002). Notch and the patterning of ommatidial founder cells in the developing Drosophila eye. In *Drosophila Eye Development* (ed. K. Moses), pp. 35-58. Berlin: Springer-Verlag.

Baker, N. E. and Yu, S.-Y. (1996). Proneural function of neurogenic genes in the developing Drosophila eye. *Curr. Biol.* **7**, 122-132.

Baker, N. E. and Yu, S.-Y. (1998). The R8-photoreceptor equivalence group in Drosophila: fate choice precedes regulated *Delta* transcription and is independent of *Notch* gene dose. *Mech. Dev.* **74**, 3-14.

Barriere, H., Nemes, C., Lechardeue, D., Kahn-Mohammed, M., Fruh, K. and Lukacs, G. L. (2006). Molecular basis of oligoubiquitin-dependent internalization of membrane proteins in mammalian cells. *Traffic* **7**, 282-297.

Bazinet, C., Katzen, A. L., Morgan, M., Mahowald, A. P. and Lemmon, S. K. (1993). The Drosophila *clathrin heavy chain* gene: clathrin function is essential in a multicellular organism. *Genetics* **134**, 1119-1134.

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

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* **7**, 678-689.

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

Chen, H. and De Camilli, P. (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.

Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P. and De Camilli, P. (1998). epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* **394**, 793-797.

Chitnis, A. (2006). Why is Delta endocytosis required for effective activation of Notch? *Dev. Dyn.* **235**, 886-894.

Eisenberg, E. and Greene, L. E. (2007). Multiple roles of auxilin and Hsc70 in clathrin-mediated endocytosis. *Traffic* **8**, 640-646.

Emery, G., Hutterer, A., Berdnik, D., Mayer, B., Wirtz-Peitz, T., Gaitan, M. G. and Knoblich, J. A. (2005). Asymmetric Rab11 endosomes regulate Delta recycling and specify cell fate in the Drosophila nervous system. *Cell* **122**, 763-773.

Eun, S. H., Lea, K., Overstreet, E., Stevens, S., Lee, J.-H. and Fischer, J. A. (2007). Identification of genes that interact with Drosophila *liquid facets*. *Genetics* **175**, 1163-1174.

Fischer-Vize, J. A., Rubin, G. M. and Lehmann, R. (1992). The *fat facets* gene is required for Drosophila eye and embryo development. *Development* **116**, 985-1000.

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

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

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

Hagedorn, E. J., Bayraktar, J. L., Kandachar, V. R., Bai, T., Englert, D. M. and Chang, H. C. (2006). *Drosophila melanogaster auxilin* regulates the internalization of Delta to control activity of the Notch signaling pathway. *J. Cell Biol.* **173**, 443-452.

Hawryluk, M. J., Keyel, P. A., Mishra, S. K., Watkins, S. C., Heuser, J. E. and Traug, L. M. (2006). Epsin is a polyubiquitin-selective clathrin-associated sorting protein. *Traffic* **7**, 262-281.

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

Huang, Y. and Fischer-Vize, J. A. (1996). Undifferentiated cells in the developing Drosophila eye influence facet assembly and require the Fat facets ubiquitin-specific protease. *Development* **122**, 3207-3216.

Jafar-Nejad, H., Andrews, H. K., Acar, M., Bayat, B., Wirtz-Peitz, F., Mehta, S. Q., Knoblich, J. A. and Bellen, H. J. (2005). Sec15, a component of the exocyst, promotes Notch signaling during the asymmetric division of Drosophila sensory organ precursors. *Dev. Cell* **9**, 351-363.

Jennings, B., Preiss, A., Delidakis, C. and Bray, S. (1994). The Notch signaling pathway is required for Enhancer of split protein expression during neurogenesis in the Drosophila embryo. *Development* **120**, 3537-3548.

Korolchuk, V. I. and Banting, G. (2002). CK2 and GAK/auxilin2 are major protein kinases in clathrin-coated vesicles. *Traffic* **3**, 428-439.
Kumar, J. (2002). The epidermal growth factor receptor in Drosophila eye development. In *Drosophila Eye Development* (ed. K. Moses), pp. 59-71. Berlin: Springer-Verlag.
Le Borgne, R. (2006). Regulation of Notch signalling by endocytosis and endosomal sorting. *Curr. Opin. Cell Biol.* **18**, 213-222.
Le Borgne, R. and Schweisguth, F. (2003). Unequal segregation of Neuralized biases Notch activation during asymmetric cell division. *Dev. Cell* **5**, 139-148.
Le Borgne, R., Bardin, A. and Schweisguth, F. (2005). The roles of receptor and ligand endocytosis in regulating Notch signaling. *Development* **132**, 1751-1762.
Lee, D.-W., Wu, X., Eisenberg, E. and Greene, L. E. (2006). Recruitment dynamics of GAK and auxilin to clathrin-coated pits during endocytosis. *J. Cell Sci.* **119**, 3502-3512.
Lee, J. D. and Treisman, J. E. (2002). Regulators of the morphogenetic furrow. In *Drosophila Eye Development* (ed. K. Moses), pp. 21-58. Berlin: Springer-Verlag.
Lemmon, S. K. (2001). Clathrin uncoating: auxilin comes to life. *Curr. Biol.* **11**, R49-R52.
Madshus, I. H. (2006). Ubiquitin binding in endocytosis – how tight should it be and where does it happen? *Traffic* **7**, 258-261.
Massol, R. H., Boll, W., Griffin, A. M. and Kirchhausen, T. (2006). A burst of auxilin recruitment determines the onset of clathrin-coated vesicle uncoating. *Proc. Natl. Acad. Sci. USA* **103**, 10265-10270.
Nagaraj, R., Canon, J. and Banerjee, U. (2002). Cell fate specification in the Drosophila eye. In *Drosophila eye development* (ed. K. Moses), pp. 73-88. Berlin: Springer-Verlag.
Nichols, J. T., Miyamoto, A. and Weinmaster, G. (2007a). Notch signaling-constantly on the move. *Traffic* **8**, 959-969.
Nichols, J. T., Miyamoto, A., Olsen, S. L., D'Souza, B., Yao, C. and Weinmaster, G. (2007b). DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *J. Cell Biol.* **176**, 445-458.
Overstreet, E., Chen, X., Wendland, B. and Fischer, J. A. (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., Fitch, E. and Fischer, J. A. (2004). Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. *Development* **131**, 5355-5366.
Parks, A. L., Turner, F. R. and Muskavitch, M. A. T. (1995). Relationships between complex Delta expression and the specification of retinal cell fates during Drosophila eye development. *Mech. Dev.* **50**, 201-216.
Parks, A. L., Klueg, K. M., Stout, J. R. and Muskavitch, M. A. (2000). Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development* **127**, 1373-1385.
Pishvaee, B., Costaguta, G., Yeung, B. G., Ryazantsev, S., Greener, T., Greene, L. E., Eisenberg, E., McCaffery, J. M. and Payne, G. S. (2000). A yeast DNA J protein required for uncoating of clathrin-coated vesicles in vivo. *Nat. Cell Biol.* **2**, 958-963.
Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P. P. and Polo, S. (2005). Clathrin-independent endocytosis of ubiquitinated cargos. *Proc. Natl. Acad. Sci. USA* **102**, 2760-2765.
Thummel, C. S. and Pirrotta, V. (1992). Technical notes: new pCaSpeR P element vectors. *Drosoph. Inf. Serv.* **71**, 150.
Tomlinson, A. and Ready, D. F. (1987). Neuronal differentiation in the Drosophila ommatidium. *Dev. Biol.* **120**, 366-376.
Umeda, A., Meyerholz, A. and Ungewickell, E. (2000). Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation. *Eur. J. Cell Biol.* **79**, 336-342.
Wang, W. and Struhl, G. (2004). Drosophila epsin mediates a select endocytic pathway the DSL ligands must enter to activate Notch. *Development* **131**, 5367-5380.
Wang, W. and Struhl, G. (2005). Distinct roles for Mind bomb, Neuralized and epsin in mediating DSL endocytosis and signaling in Drosophila. *Development* **132**, 2883-2894.
Wendland, B. (2002). Epsins: adaptors in endocytosis? *Nat. Rev. Mol. Cell Biol.* **3**, 971-977.