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Function and Regulation of *Drosophila* Epsin in Notch Signaling

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Function and Regulation of *Drosophila* Epsin in Notch Signaling

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Dedication

To my family and friends, especially Jing Li

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Thank you Janice, for your time, guidance, and encouragement. I learned everything from you to continue my career in academia. I will never forget the moments you helped me to decide what to do, and what not to do. I am also grateful to all my committee members for their constructive criticism and insightful suggestions. Thanks to everyone who wrote great recommendation letters for my applications for fellowships and postdoctoral positions. I deeply appreciate the support and friendship I have received from all the members in Fischer lab and our fly community. Finally, I want to give my special thanks to my wife, Jing Li, my family, and the rest of my friends for sharing all the happiness and sadness during my stay at the University of Texas at Austin.

Function and Regulation of *Drosophila* Epsin in Notch Signaling

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The University of Texas at Austin, 2011

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Epsin is an endocytic protein that binds Clathrin, the plasma membrane, Ubiquitin, and also a variety of other endocytic proteins through well-characterized motifs. Although Epsin is a general endocytic factor, genetic analysis in *Drosophila* and mice revealed that Epsin is essential specifically for internalization of ubiquitinated transmembrane ligands of the Notch receptor, a process required for Notch activation. How Epsin promotes ligand endocytosis and thus Notch signaling is unclear. Here, by generating *Drosophila* lines containing transgenes that express a variety of different Epsin deletion and substitution variants, I tested each of the five protein or lipid interaction modules of Epsin for a role in Notch activation by each of the two *Drosophila* ligands, Serrate and Delta. here are five main results of this work that impact present thinking about endocytic machinery/Epsin, Epsin/ligand, or ligand/receptor interactions at the plasma membrane. First, I discovered that deletion or mutation of both UIMs destroys Epsin's function in Notch signaling and has a greater negative effect on Epsin's ability to function than removal of any other module type. Second, only one of the two UIMs of Epsin is essential. Third, the lipid-binding function of the ENTH domain is required for maximal Epsin activity. Fourth, although the C-terminal Epsin modules that interact with Clathrin, the adapter protein complex AP-2, or endocytic accessory proteins are necessary collectively for Epsin activity, their functions are highly redundant. Finally,

I detected no ligand-specific requirements for Epsin modules. Most unexpected was the finding that Epsin's Clathrin binding motifs were dispensable. All of these observations are consistent with a model where Epsin's essential function in ligand cells is to link ubiquitinated Notch ligands to Clathrin-coated vesicles through other Clathrin adapter proteins.

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Chapter 1. Introduction

1.1. Notch signaling pathway

As life forms evolve from a single cell organism to a multi-cellular organism, cell-cell communication becomes indispensable to control cell growth, proliferation, differentiation, survival, and death. The Notch signaling pathway defines one of the most commonly used pathways to regulate cell-cell interactions in tissue development and morphogenesis in metazoans (Artavanis-Tsakonas et al., 1999). Both the Notch (N) receptors and the ligands are type I transmembrane proteins, which makes the signaling restricted to neighboring cells (Bray 2006). Notch activation requires four proteolytic cleavages (S1 – S4) of the receptor itself (Bray, 2006). The S2 cleavage is triggered by the direct interaction between the ligand and the receptor and processed by ADAM/TACE protease (Schweisguth 2004). The subsequent S3 and S4 cleavages are mediated by γ-secretase, and the Notch intracellular domain (NICD) is released into the cytosol and further trafficked into the nucleus (Schweisguth 2004). The activated NICD binds to CSL (human CBF1, *Drosophila* Suppressor of Hairless (Su(H)), and *C.elegans* Lag-1) proteins to activate the target genes (Ilagan and Kopan, 2007). One unique feature of Notch signaling is that both the receptor and the ligand need to be internalized in order to signal (Le Borgne et al., 2005; Nichols et al., 2007a, 2007b; Parks et al., 2000). The activities of the ligand and the receptor are also regulated by glycosylation and ubiquitination (Acar et al., 2008; Weinmaster and Fischer, 2011).

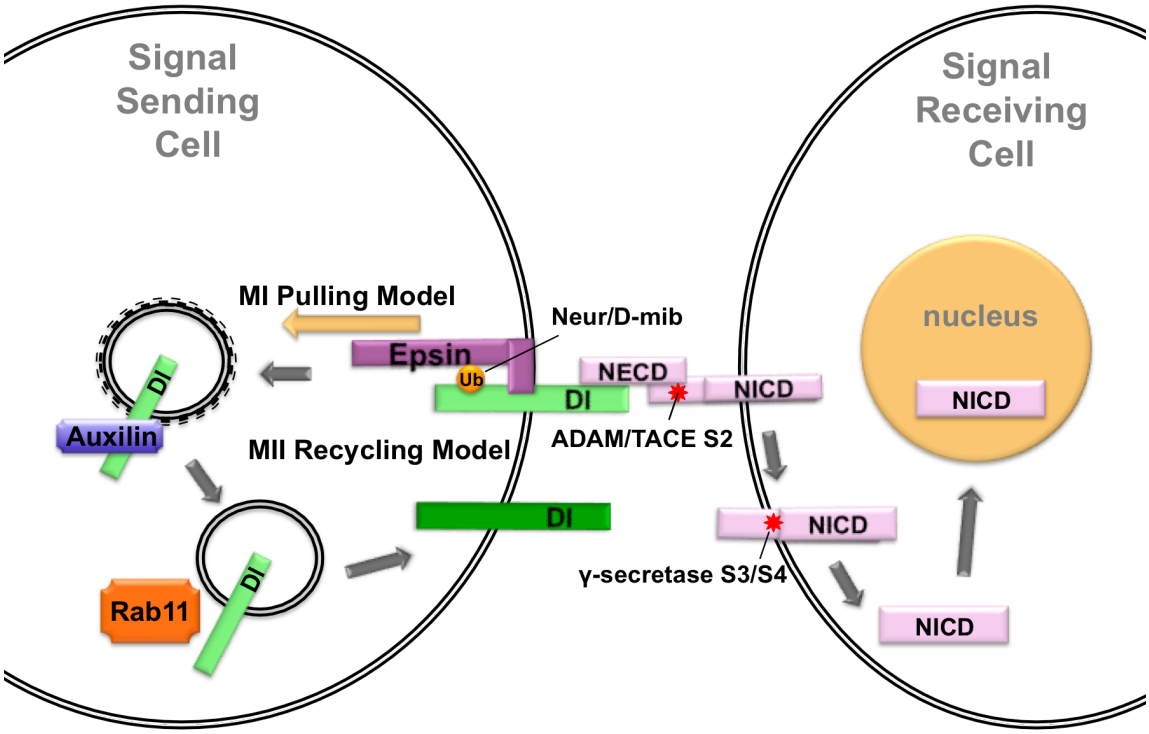


Figure 1.1. Notch signaling pathway. Notch protein undergos four cleavages to be activated. It is first cleaved by Furin (S1 cleavage) to form a heterodimer connected with non-covalent linkages. Upon the the binding of the ligand, S2 cleavage is performed by ADAM/TACE enzyme. After that, the NEXT fragment is processed by γ-secretase (S3/S4 cleavages) to form an active NICD fragment. Finally, the NICD fragment is released into the cytosol and trafficked into the nucleus to function as a transcription factor. There are mainly two models to explain why ligand endocytosis is necessary for Notch activation. In the "pulling" model, endocytosis of the ligand provides a mechanical force to promote the S2 cleavage of the Notch receptor. In the "recycling" model, the ligands need to be processed in the endosomes and recycled back to the plasma membrane as "active" ligands.

1.1.1. Notch ligands

Notch ligands are defined by an N-terminal DSL (Delta and Serrate from *Drosophila*, and Lag-2 from *C.elegans*) domain (Artavanis-Tsakonas et al., 1999). In *Drosophila*, there are two Notch ligands, Delta (Dl) and Serrate (Ser) (Schweisguth 2004). They are usually utilized in different developmental contexts to trigger Notch

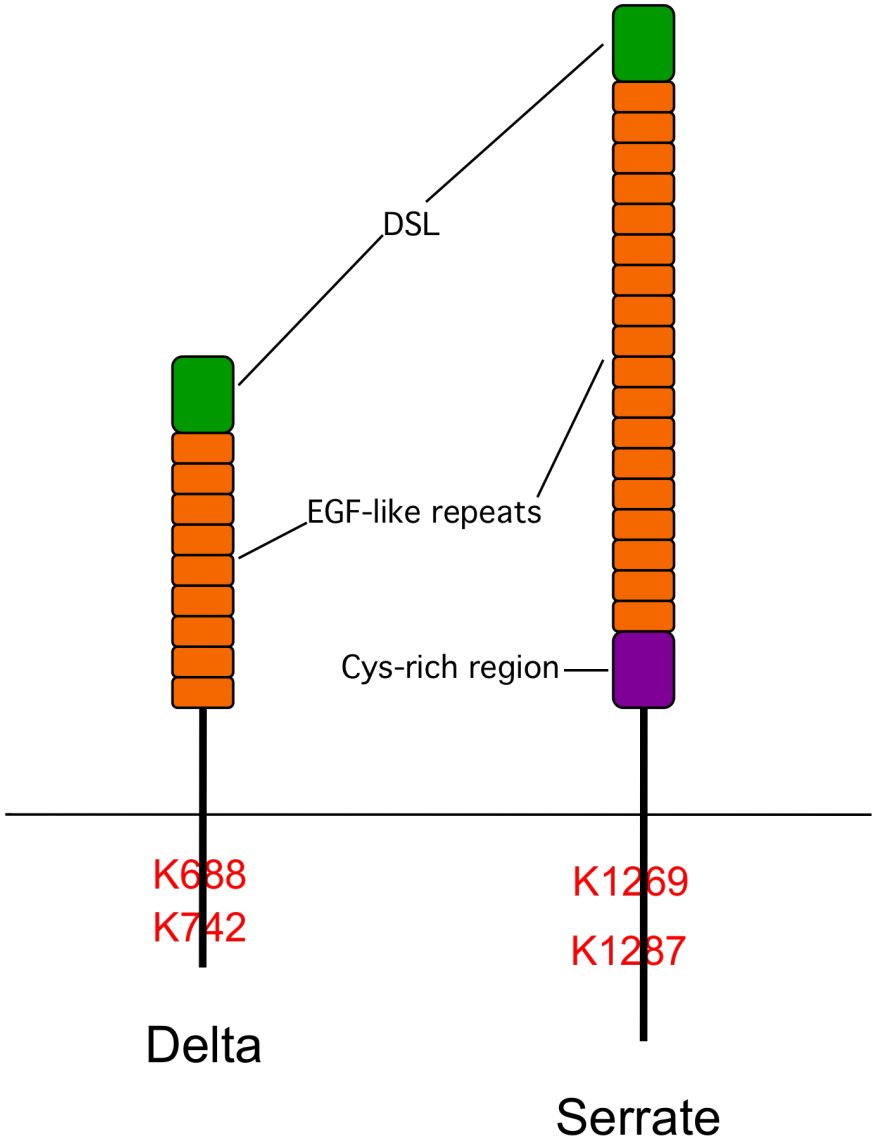


Figure 1.2. Sturcture of *Drosophila* Notch ligands. Notch ligands share an N-terminal DSL domain, followed by various numbers of ELR repeats (depending on different ligands). *Drosophila* Serrate also contains a Cystein-rich region in the extracellular domain. The intracellular domains of the ligands contain several lysines for ubiquitinaion. *Drosophila* Delta might be ubiquitinated on lysine 688, 742; while *Drosophila* Serrate could be ubiquitinated on lysine 1269, 1287.

signaling (Le Borgne et al., 2005). Occasionally both of them are involved in the same context, for example, during sensory organ precursor (SOP) development (Zeng et al., 1998). Other times only one of them is required, like Dl's role in lateral inhibition of

neural cells during embryogenesis (Alton et al., 1988). They also play overlapping roles for tissue development in the eye, wing, leg, and other areas (Micchelli et al., 1997; Li and Baker, 2004; Wang and Struhl, 2004; Lai et al., 2005).

Sequence analysis of the two ligands showed that they are very different in size, composition, and specific domains. However, both of them contain a DSL domain to interact with Notch receptor and several Epidermal growth factor-like repeats (ELRs) in the extracellular domains (Fig.1.2). While the DSL motifs mediate ligand-receptor interaction, other regions might be equally important for signal-sending. A single mutation in ELR3 of the Dl extracellular domain (C301Y) disrupts Dl trafficking and signaling (Parks et al., 2000). This indicates a structural requirement of the ligand for receptor binding. The intracellular domains of the ligands contain several lysines for ubiquitination, which seems to be involved in the ligand endocytosis (Lai et al., 2005; Wang and Struhl, 2005). Mutagenesis analysis showed that K688M and K742R affected the trafficking of the Dl proteins, suggesting these two lysines may be the ubiquitination sites of Dl (Parks et al., 2006). K1269A - K1287A double mutant Serrate failed to activate N signaling, which indicates that one or both of these two lysines could be the ubiquitination sites of Serrate (Glittenberg et al., 2006).

1.1.2. Notch ligands are ubiquitinated to signal

Notch ligands need to be activated through ubiquitination (Weinmaster and Fischer, 2011). Mutagenesis analysis with lysines indicated that Dl intracellular domain (Dl-ICD) is very likely to be multi-mono-ubiquitinated (Heuss et al., 2008). There are two ubiquitin E3 ligases, Neuralized (Neur) and *Drosophila* mindbomb (D-mib), to ubiquitinate the ligands in *Drosophila* (Weinmaster and Fischer, 2011). Neur binds to Dl through its NHR (Neuralized Homologue Region) domain (Commisso and Boulianne,

2007). D-mib binds to both Dl and Ser through its N-terminus (Le Borgne et al., 2005). Both of them contain RING (Really interesting new genes) domains in their C-terminus. Further analysis revealed an important interaction between phosphoinositides and Neur, which is required for the membrane localization of Neuralized. This Dl-independent targeting event is required to internalize Delta after its ubiquitination (Skwarek et al., 2007).

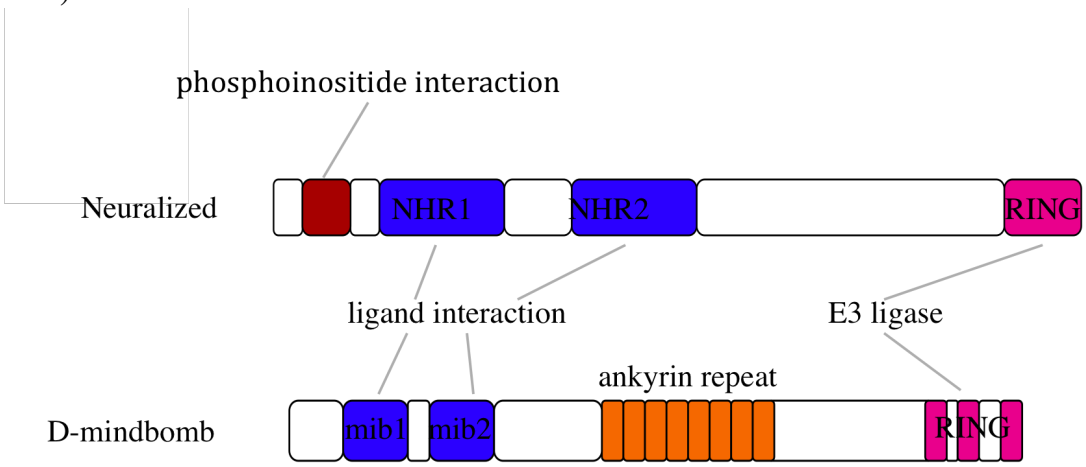


Figure 1.3. Neuralized and *Drosophila* Mindbomb. Both Neur and D-mib have RING domains in their C-terminus to function as E3 ligase. Neur binds to Dl through its NHR domain. D-mib binds to both Dl and Ser through its mib domains. Neur also interacts with phosphoinositides to internalize Delta after its ubiquitination.

Both E3 ligases have been shown to directly ubiquitinate and function with both Dl and Ser (Le Borgne et al., 2005; Lai et al., 2005). However, phenotypic analysis indicated that Neur and D-mib are used in different developmental contexts. While both of them are employed during SOP development, only Neur is used in lateral inhibition during embryogenesis (Le Borgne et al., 2005; Lai et al., 2005). And D-mib (but not Neur) is widely used in imaginal disc development, like eye growth, wing margin specification, and leg development (Lai et al., 2005). In some cases, Neur can replace the

function of D-mib. *Actin-Gal4* or *Ser-Gal4* driven Neur expression has been shown to complement D-mib's function in wing development. However, *neur-Gal4* driven D-mib expression failed to replace Neur's function during embryogenesis (Le Borgne et al., 2005). It seems Neur and D-mib have similar, but not identical activity in N ligand endocytosis and signaling.

1.1.3. Ligand endocytosis is required for Notch activation

One unique feature of Notch signaling is that ligand endocytosis in the signaling cells is required for the Notch activation in the adjacent signal-receiving cells (Parks et al., 2000; Le Borgne et al., 2005; Fischer et al., 2006; Le Borgne, 2006; Vaccari et al., 2008; Furthauer and Bonzalez-Gaitan, 2009; Windler and Bilder, 2010). There are two major models (which are not mutually exclusive) to explain why ligand endocytosis is necessary for Notch activation. The primary difference argues whether endocytosis is important before or after the ligand binds to the Notch receptor (Le Borgne et al., 2005). In the "pulling" model, endocytosis of the ligand provides a mechanical force that results in the S2 cleavage of the Notch receptor (Fig.1.1). In the "recycling" model, the ligands need to be processed in the endosomes and recycled back to the plasma membrane as "active" ligands (Le Borgne and Schweisguth, 2003; Wang and Struhl, 2004, 2005; Emery et al., 2005; Jafar-Hejad et al., 2005; Rajan et al., 2009).

Studies in different developmental contexts revealed requirements for different endocytic factors in Notch signal-sending cells. During *Drosophila* sensory organ precursor (SOP) development, Rab11, Sec15, Neuralized, Actin-related protein-3 (Arp3), Wiskott-Aldrich syndrome protein (WASp), and Epsin are required (Emery et al., 2005; Jafar-Hejad et al., 2005; Rajan et al., 2009). The activities of Arp2/3 complex and WASPs are required to recycle Dl into apical microvilli for its activation (Rajan and

Bellen, 2009). Further analysis indicates that Dl needs to be internalized from the basolateral plasma membrane and relocalized to the apical plasma membrane, where it meets Notch to trigger signaling events (Benhra et al., 2010). This Dl transcytosis requires Neur, Epsin, and Dynamin. Rab5 and Rab11 might also be involved in this context. These results support the "recycling model" (Fig.1.1). However, studies using *Drosophila* ovary tell a different story. Windler and Bilder demonstrated that Chc (Clathrin heavy chain), Rab5, Rab11, Sec15, Drip, MyoV, and AP-2 are not required, but Epsin and Dynamin are, for Dl signaling during oogenesis (Windler and Bilder, 2010). During *Drosophila* eye development, Epsin, Auxilin, Clathrin, and Neuralized are essential for signaling cells (Cadavid et al., 2000; Overstreet et al., 2003; Eun et al., 2008; Kandachar et al., 2008). It seems that ligand endocytosis can happen with various endocytic pathways to trigger Notch signaling.

1.2. Endocytic pathways

Intercellular signaling is highly regulated by intracellular trafficking during development (Le Roy and Wrana, 2005; Shilo and Schejter, 2011). Endocytosis serves a critical role for the cells to both send and receive signals. It acts in several distinct steps: recognizing the signaling molecules and initiating the plasma membrane curvature; budding vesicles from the plasma membrane; pinching off the vesicles; sorting the vesicles into different endocytic pathways; and sending the signal or degrading the signal molecules (Sorkin 2000). Among them, the first step is a pivotal one, since it provides the specificity for the signaling pathway and also determines the fate of the signaling molecules. Different endocytic events can be divided into three categories: Clathrin-dependent endocytosis (CDE), Clathrin-independent but Caveolin-dependent

endocytosis, and Clathrin-independent and Caveolin-independent endocytosis (McMahon and Boucrot, 2011).

1.2.1. Clathrin-dependent endocytosis

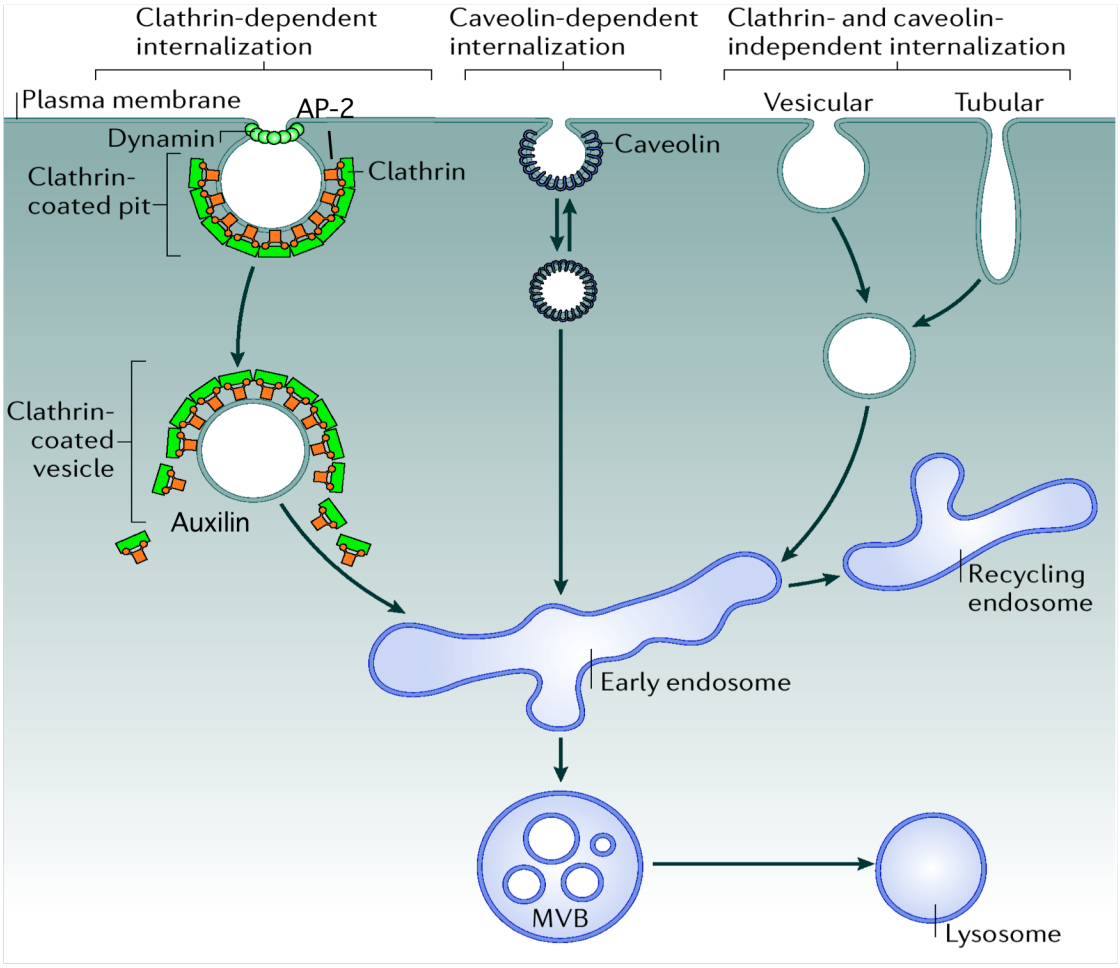


Figure 1.4. Endocytic pathways (modified from McMahon and Boucrot, 2011). Depending on whether Clathrin or Caveolin is required or not, endocytosis can be divided into three categories: Clathrin-dependent endocytosis (CDE), Clathrin-independent but Caveolin-dependent endocytosis, or Clathrin-independent Caveolin-independent endocytosis.

Vesicles can be formed from almost all types of cellular membranes: the plasma membrane, the endoplasmic reticulum, the Golgi complex, the endosomes, and the

lysosomes (Sorkin 2000). Most vesicles are covered with a protein coat and fall into one of three categories: Clathrin-coated vesicles (CCVs), COP1-coated vesicles, and COPII-coated vesicles (McPherson 2010). Electron micrographs of Clathrin coats showed that they consist of two different layers (Reider and Wendland, 2011). The inner layer is formed with adapter proteins that contact cargos and the vesicle membrane, while the outer layer is composed of Clathrin lattices. Clathrin forms trimers (triskelia) with 192kDa heavy chains and 22-25kDa light chains. The C-terminal regions are connected to form trimers and the N-terminus is free to interact with other triskelia or different endocytic proteins (McPherson 2010).

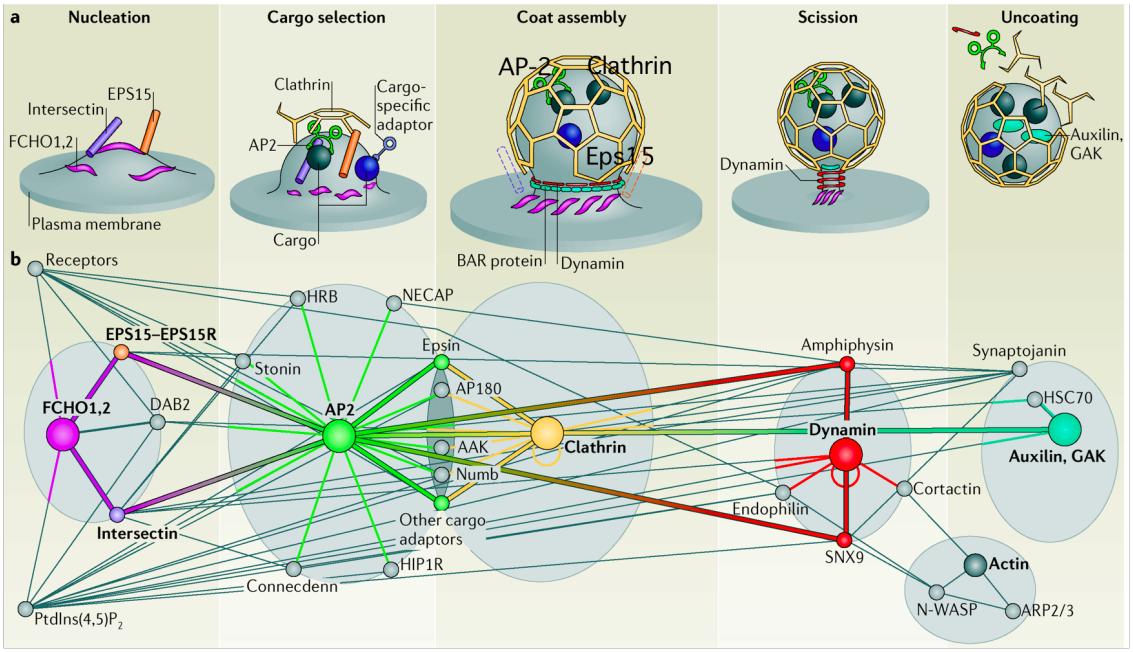


Figure 1.5. Clathrin-coated vesicle formation (modified from McMahon and Boucrot, 2011). Clathrin-coated vesicle (CCV) formation requires five steps: Nucleation, Cargo selection, Coat assembly, Scission, and Uncoating. Various regulators form a network to assembly the CCVs at various locations and uncoat the CCVs constantly. Major hubs include FCHO proteins, AP-2, Clathrin, Dynamin, and Auxilin. Clathrin, AP-2, and Eps15 present on mature CCPs.

The Clathrin protein inside the cell is dynamic. Clathrin-coated vesicle formation requires five steps (McMahon and Boucrot, 2011). Nucleation works through the

interaction between FCH domain only (FCHO) proteins, Eps15 (epidermal growth factor receptor pathway substrate 15)–Eps15R, intersectins, AP2, and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2)-rich zones of the plasma membrane. Cargo selection is mediated by AP-2, or other cargo-specific adapters (for example, stonin, HRB, and Numb). Coat assembly is initiated by Clathrin recruitment through AP-2 and progressed by Clathrin self-assembly. Scission requires the function of the GTPase Dynamin (Shibire in *Drosophila*). Actin polymerization further promotes vesicle formation. Uncoating requires cooperation between Auxilin (or cyclin G-associated kinase, GAK) and heat shock cognate 70 (HSC70). All the components of the Clathrin machinery can be recycled for another round of Clathrin-coated vesicle formation. The following vesicle sorting is controlled by two groups of proteins: Rabs (small GTPases) and SNAREs. Various regulators form a network to assemble the CCVs at various locations and uncoat the CCVs constantly. Major hubs include FCHO proteins, AP-2, Clathrin, Dynamin, and Auxilin (McMahon and Boucrot, 2011). They are essential to regulate Clathrin-mediated endocytosis.

1.2.2. Clathrin adapters and accessory factors

To form a Clathrin-coated vesicle (CCV), different proteins need to cooperate with each other to generate an internalized membrane structure (McMahon and Boucrot, 2011). Around 40 proteins have been shown to be involved in forming an endocytic CCV (Traub 2011). The interaction between cargos and Clathrin lattices is mediated by adapter proteins (APs), which form the inner shell of Clathrin coats (Bonifacino and Traub, 2003). To function as a Clathrin adapter, the protein has to bind to Clathrin, cargos, and/or phospholipids simultaneously (Oven et al., 2004). Adapter proteins are heterotetrameric complexes (AP-1, AP-2, AP-3, AP-4) that function at different sub-

cellular compartments (Bonifacino and Traub, 2003). AP-2 is the general Clathrin adapter for Clathrin-dependent endocytosis (CDE). It consists of four subunits: two large subunits (α2 and β2), one medium unit (μ2), and one small unit (σ2) (Bonifacino and

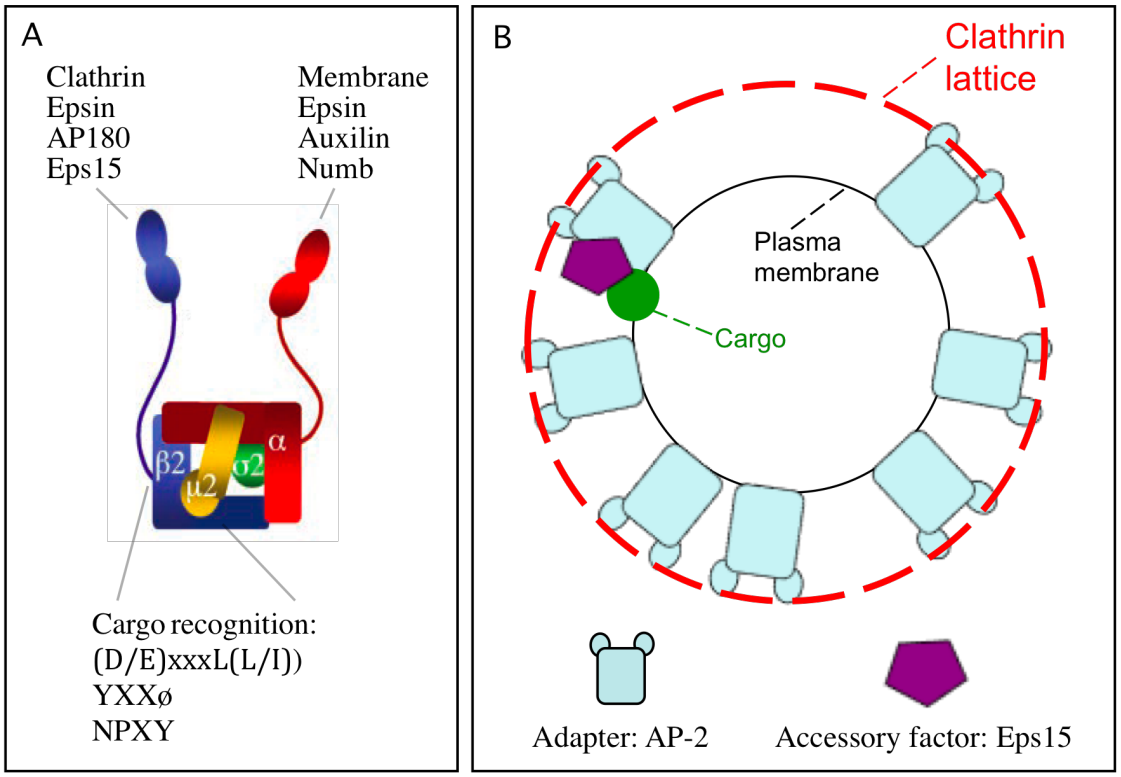


Figure 1.6. Adaptor proteins and accessory factors (modified from Bonifacino and Traub 2003). A. Clathrin adapters bind to Clathrin, cargos, and/or phospholipids simultaneously. Adapter AP-2 consists of α, β2, μ2, σ2 subunits. The cargo recognition is mediated through β2 subunit and μ2 subunit. The Clathrin interaction functions through the ear domains. The α subunit binds to phospholipids. B. Adapter proteins form the inner shell of a Clathrin-coated vesicle. Accessory factors facilitate the cargo selection, membrane curvature, or CCV formation.

Traub, 2003). The cargo recognition is mediated through the β2 subunit (recognizing di-leucine motifs, (D/E)xxxL(L/I)) and μ2 subunit (binding to tyrosine based motifs, YXXø). The Clathrin interaction requires the β2 subunit. The α2 subunit binds to the plasma membrane and other endocytic proteins like Epsin, Eps15, etc. When AP-2

function is disrupted, there are still some CCVs formed (Motley et al., 2003). This indicates that there are alternative Clathrin adapters to promote CDE (Reider and Wendland, 2011).

AP-2 can recognize proteins presenting di-leucine motifs [(D/E)xxxL(L/M/I)] or tyrosine-based motifs (Yxxϕ, ϕ-bulky hydrophobic residue) (Bonifacino and Traub, 2003). Proteins without these two motifs are internalized through the function of other adapters. One kind of signal for these proteins to use could be post-translational modifications, such as phosphorylation or ubiquitination. Recently mono-ubiquitination has been shown to be an important signal for endocytosis and endosomal trafficking (Sigismund et al., 2004). Alternative adapters include ubiquitin-interaction-motif (UIM)-containing proteins (Epsin, Eps15), Phosphotyrosine binding domain (PTB)-containing proteins (Dab-2, Numb, ARH, etc.), and β-Arrestin (Reider and Wendland, 2011). The main difference between these proteins and AP-2 is that they are all monomeric. Some of these adapters are connected to the core CDE through AP-2 interaction. Although most of them are Clathrin adapters, some have been shown to be involved in Clathrin-independent endocytosis (Reider and Wendland, 2011).

Except for the Clathrin adapters, there are other proteins present in the CCVs known as accessory factors (McMahon and Boucrot, 2011). They share modules to interact with membrane, cargo, and coat components. These proteins facilitate the cargo selection, membrane curvature, or CCV formation. The role of adapter proteins and accessory factors in the regulation of endocytosis is highly variable. It is critical to reveal the spatial and temporal relationships between cargo, adapters, and coat components to understand the purpose of vesicle trafficking.

1.2.3. Clathrin-independent endocytosis

Although most of the vesicles are covered with a coat, there are vesicles formed without a coat (Le Roy and Wrana, 2005). Ubiquitinated epidermal growth factor receptor (EGFR) is internalized through a Clathrin-independent, lipid raft-dependent pathway (Sigismund et al., 2004). It has also been shown that the actin cytoskeleton is involved in the caveolae-dependent internalization of GF—anchored alkaline phosphatase (Le Roy and Wrana, 2005). The low motility of caveolae is dependent on actin filaments, while the rapid movement of caveolin-vesicles depends on microtubules. The trafficking of the caveolin-vesicles also requires the function of PKCα. However, it is still largely unknown how the Clathrin-independent vesicles are formed, and how the trafficking is controlled.

1.3. Epsin function is required in the signaling cells for Notch activation

Epsin has been shown to be involved in different biological events. Most of the functions seem to be associated with endocytosis (Chen et al., 1998; Wendland et al., 1999). It is related to Influenza and HIV-I virus infection, and synaptic vesicle endocytosis (Bao et al., 2008; Chen and Zhuang, 2008; Huang et al., 2008; Jakobsson et al., 2008). Functional analysis of Epsins showed that the ENTH domain is necessary and sufficient in single-cell organisms like yeast and *Dictyostelium* (Aguilar et al., 2003, 2006; Brady et al., 2008). Epsin also plays roles in mitosis and autophagy (Csikos et al., 2009; Liu and Zheng, 2009). Genetic studies revealed that Epsin plays a conserved role in the Notch signaling pathway in worms (*C.elegans*), insects (*Drosophila melanogaster*), and Mammals (mouse, and humans) during development (Fig.1.7, Overstreet et al., 2004; Wang and Struhl, 2004; Tian et al., 2004; Chen et al., 2009). This role seems to be specific to Notch signaling, since Decapentaplegic, Wingless, and Hedgehog signaling pathways are not affected without Epsin (Overstreet et al., 2004; Wang and Struhl, 2004).

Further analysis has shown that Epsin is required for sensory organ precursor (SOP), wing, eye, and egg development (Overstreet et al., 2004; Wang and Struhl, 2004; Windler and Bilder, 2010), and probably for all Notch signaling events in *Drosophila*.

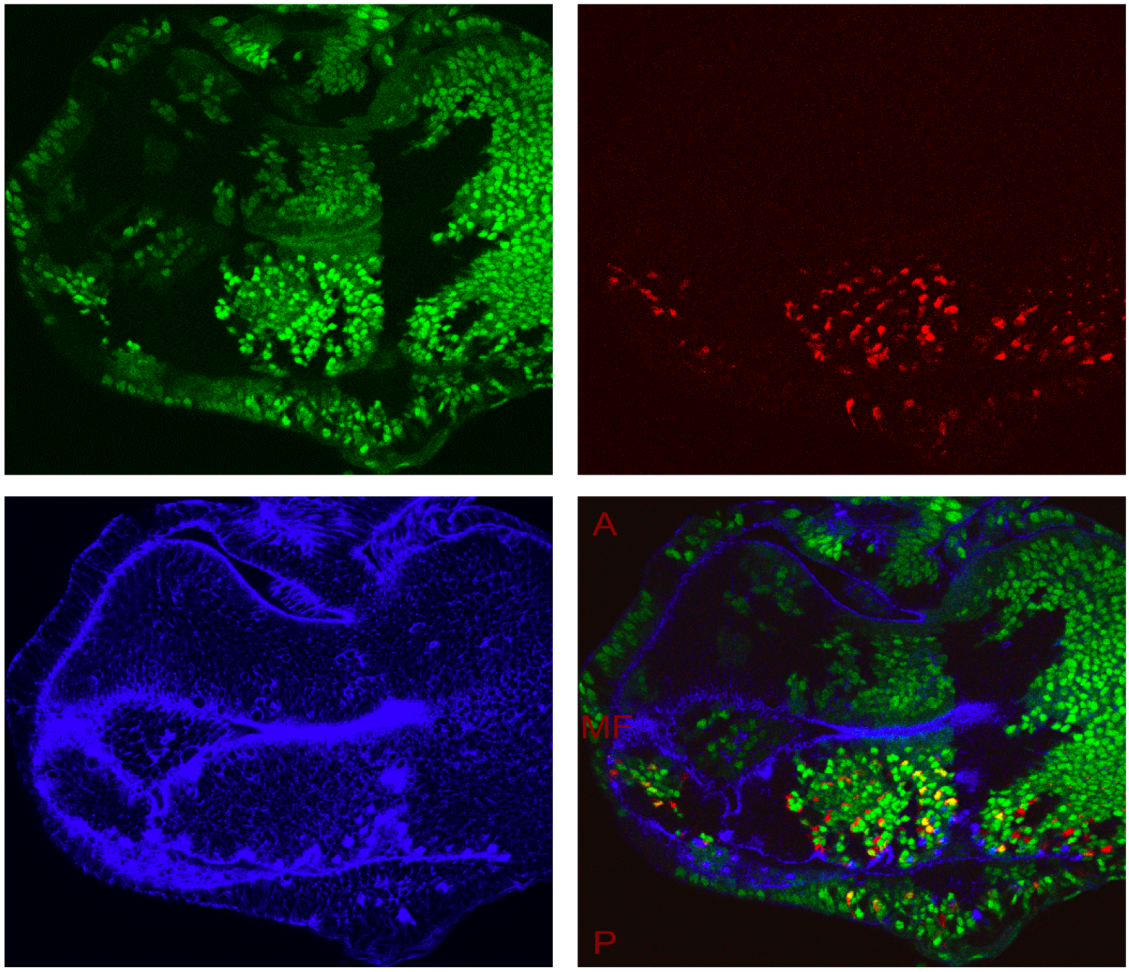


Figure 1.7. Epsin is required in the Notch signaling pathway. *Epsin* null clones were generated in the developing eye disc using mitotic recombination and labelled by the absence of the green GFP signal. No Notch signaling activity was detected in the middle of the clones, as indicated by the absence of mδ0.5-lacZ signal. Green: GFP; Red: mδ0.5-lacZ (Notch reporter); Blue: Phalloidin (Actin). A: anterior region of the eye disc; MF: morphogenetic forrow; P: posterior region of the eye disc.

Clonal analysis in the wing and eye discs demonstrated that Epsin is specifically required in the signaling cells during Notch signaling (Fig.1.8, Overstreet et al., 2004;

Wang and Struhl, 2004). A chimeric Dl with an LDL intracellular domain (targeting for the recycling endosome) to replace its own intracellular domain obviates the requirement for Epsin in Notch signaling (Wang and Struhl, 2004). This suggests that Epsin functions

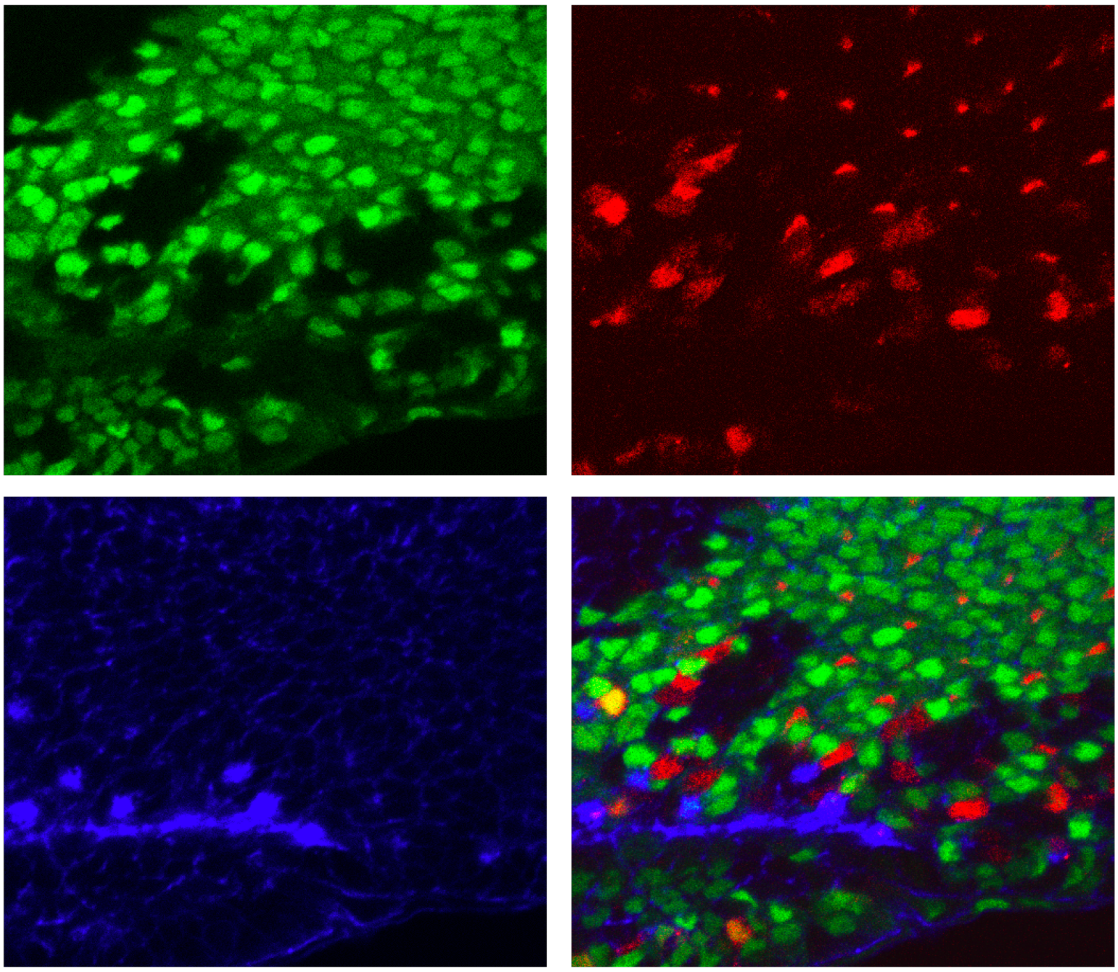


Figure 1.8. Epsin is required in Notch signaling cells. *epsin* null clones were generated in the developing eye disc. No Notch signaling activity was detected in the middle of the clones. *epsin* null cells on the boundary of the clones can still receive signals from adjacent wild-type cells. Green: GFP; Red: mδ0.5-lacZ (Notch reporter); Blue: Phalloidin (Actin).

to form a specialized endosome (presumably a recycling endosome) for Dl to be activated. This result seems to support the recycling model. But it is also consistent with the pulling model since endocytosis is the first step for recycling. It is critical to

understand the functional mechanism of Epsin in the Notch signaling pathway to elucidate why ligand endocytosis is required for Notch activation.

1.4. Epsin is a multi-modular protein

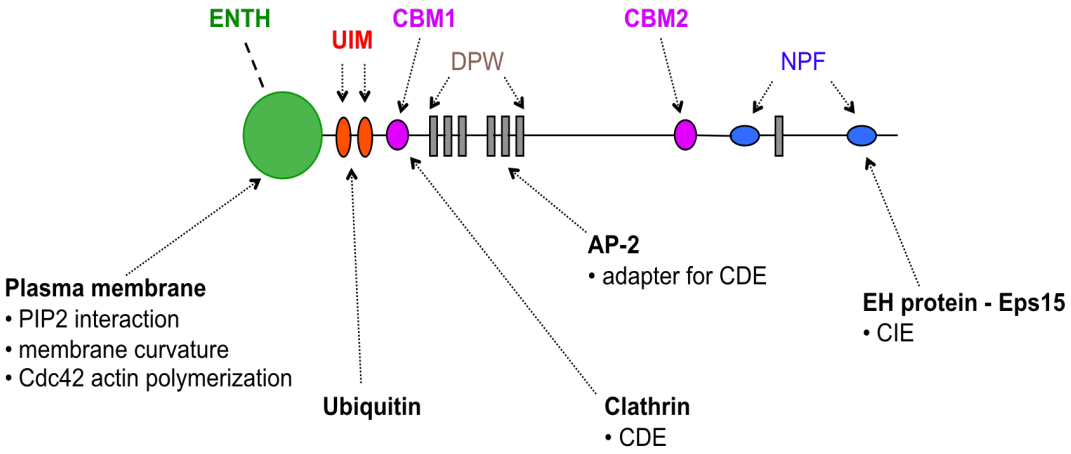


Figure 1.9. Structure of Epsin protein. Epsin has five modules to interact with other proteins or phospholipids. The N-terminal ENTH domain binds PIP2 and induce membrane curvature. There are two UIMs, two CBMs, seven DPW motifs, and two NPF motifs in the C-terminus.

Epsin was discovered as a binding partner of Eps15, and thus got its name as Eps15 interacting protein (Chen et al., 1998). Chen and colleagues pulled down rat Epsin by using the EH-domain of Eps15 as bait. They showed that Epsin protein is ubiquitously expressed in various tissues and interacts with AP-2. One year before rat Epsin was discovered, *Drosophila liquid-facets* (*lqf*) was isolated from a genetic screen as an enhancer of *faf* (*fat-facets*) and Lqf turned out to be endocytic Epsin (Fischer et al., 1997). Since then, many Epsins have been isolated and analyzed in different species. They all share a highly conserved globular module in the N-terminal region, called the Epsin N-terminal Homology (the ENTH domain). ENTH domain-containing proteins can be further divided into two groups: Epsin (with UIMs), and Epsin-R (Epsin-Related,

without UIMs). Phylogenic analysis indicated that the UIMs exist only in single cell organisms and animals (Gabernet-Castello et al., 2009).

There are two Epsin isoforms in *Drosophila*, produced by alternate splicing of the *lqf* mRNA (Cadavid et al., 2000). Both of them contain multiple modules, including a structured N-terminal ENTH domain (Kay et al., 1998; Rosenthal et al., 1999; De Camilli et al., 2001). The unstructured C-terminal region contains four protein-protein interaction motifs with varying numbers in different animal species (Kay et al., 1998; De Camilli et al., 2001). Each Epsin isoform (Fig.1.9) has two ubiquitin-interaction motifs (UIMs) (Hofmann and Falquet, 2001; Polo et al., 2002; Shih et al., 2002; Oldham et al., 2002; Miller et al., 2003; Klapsiz et al., 2002), two Clathrin-binding motifs (CBMs) (Aguilar et al., 2003; Drake et al., 2000), seven DPW motifs that bind the AP-2 endocytic adapter complex (Owen et al., 1999), and two NPF motifs that bind EH-domain-containing endocytic factors (Aguilar et al., 2003; Salcini et al., 1997; Paoluzi et al., 1998).

1.4.1. NPF motif

NPF-EH interaction is considered to be involved in trafficking and sorting of proteins inside the cell (Salcini et al., 1997). Epsins from different species contain various numbers of NPF motifs (three amino acids) in the C-terminus. EH proteins share a conserved EH (Eps15 homology) domain. In *Drosophila*, Eps15 is predominantly required in the nervous system to maintain high levels of multiple endocytic proteins like dynamin (Koh et al., 2007). Over-expression of peptides containing NPFs inhibited Clathrin coat assembly, which is critical for neurotransmitter release during synaptic vesicle recycling (Morgan et al., 2003). Yeast Epsins (Ent1/2) play redundant roles with Yap1801/2 (yeast AP180/CALM proteins) in scaffold protein dynamics through NPF-EH domain interaction, which is essential for Clathrin-mediated endocytosis (Maldonado-

Baez et al., 2008). Single amino acid change from NPFs to NPMs abolished the interaction between yeast Epsin and Eps15 (Aguilar et al., 2003). Eps15 has also been shown to be involved in Clathrin-independent endocytosis (Sigismund et al., 2004).

1.4.2. Epsin N-terminal Homology (ENTH) domain

All Epsin family members share an N-terminal ENTH domain, which is the only region that forms a tertiary structure (Kalthoff et al., 2002). The remaining Epsin protein hangs into the cytosol with a random structure (Kalthoff et al., 2002). In yeast and *Dictyostelium*, the ENTH domain is both necessary and sufficient for the function of Epsin (Aguilar et al., 2003, 2006; Brady et al., 2008).

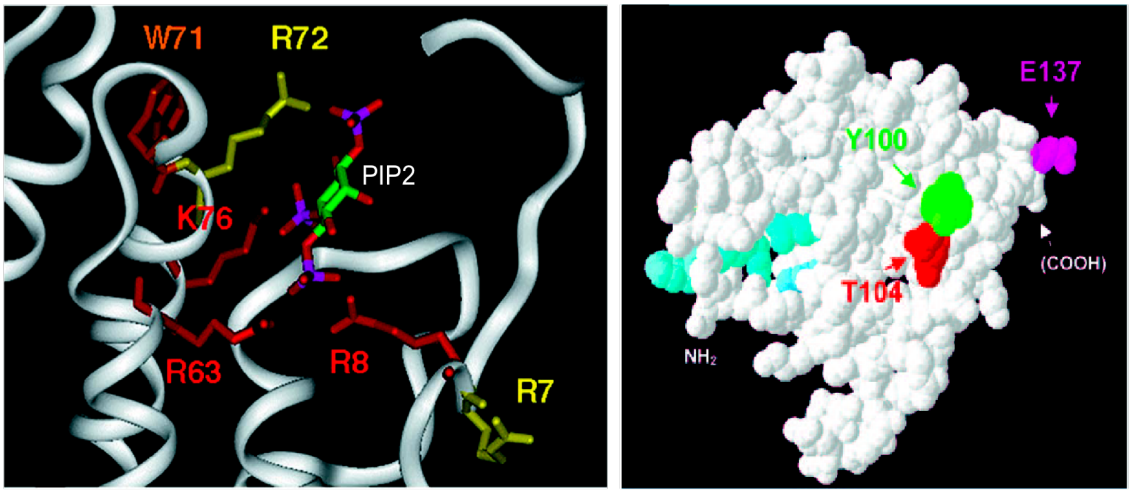


Figure 1.10. Epsin-ENTH domain binds to PIP2 or Cdc42-GAP with two isolated regions (modified from Aguilar et al., 2006). Nuclear magnetic resonance (NMR) analysis indicates that Arg63 - Lys76 patch binds to PIP2. Tyr100 and Thr104 are required for Cdc42-GAP interaction.

The whole ENTH domain consists of about 140 amino acids, which define two different functions with isolated regions. An *in vitro* liposome binding assay showed that the yeast ENTH domain preferably binds to phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2, or PIP2] and PtdIns(3,4,5)P3 (Itoh et al., 2001). Nuclear magnetic

resonance (NMR) analysis indicated that Arg63 and Lys76 are critical for the ENTH domain to bind to PIP2 (Fig.1.10). Another study demonstrated that the ENTH domain could initiate the membrane curvature *in vitro* (Itoh and Takenawa, 2009). Interestingly, the essential function of the ENTH domain in yeast is independent of lipid-binding. Aguilar and colleagues showed that the yeast ENTH domain binds Cdc42 GTPase-activating proteins (Cdc42 GAP) with both a yeast two-hybrid assay and an *in vitro* binding assay. Tyr100 and Thr104 of the ENTH domain are required for this interaction to regulate actin polymerization and cell polarity (Aguilar et al., 2006). It appears that the ENTH domain plays both an important role to interact with PIP2 during endocytosis and another essential role to regulate Cdc42 in yeast (Aguilar et al., 2003, 2006).

1.4.3. Ubiquitin interaction motif (UIM)

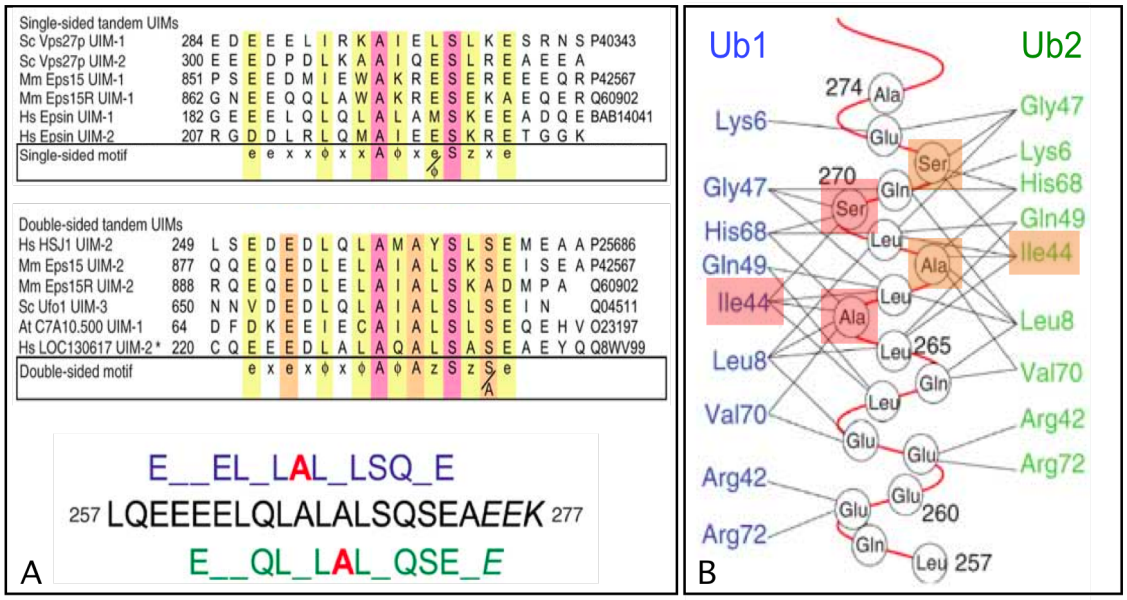


Figure 1.11. Ubiquitin interaction motifs (modified from Hirano et al., 2006). A. Alignment of single-sided and double-sided UIMs from different proteins. They all share a 15 amino acid consensus sequence. B. Double-sided UIMs can bind to two ubiquitin molecules at the same time to increase the ubiquitin binding affinity.

The UIM motif was discovered in the 26S protease subunit 5a (Young et al., 1998). It belongs to one of several ubiquitin-interacting domains (Hurley et al., 2006). UIMs can be divided into two groups: single-sided UIMs and double-sided UIMs (Hirano et al., 2006). Single-sided UIMs contain one consensus sequence to interact with one ubiquitin at a time. Whereas double-sided UIMs can bind to two ubiquitin molecules at the same time (Fig.1.11). The UIM motif forms an α-helix to bind to ubiquitin through a Ile44-centered patch (Hirano et al., 2006). It provides a post-translational way to regulate endocytosis and endosomal trafficking through ubiquitination (Madshus 2006; Traub and Lukacs, 2007). Efficient ubiquitin-binding requires more than one single-sided UIM. The two UIMs of yeast Epsin function in a cooperative manner to interact with ubiquitin (Aguilar et al., 2003). Both UIMs in Vps27p are required for ubiquitin binding (Shih et al., 2002).

Except for its role in transporting and sorting ubiquitinated cargos, UIMs are also involved in self-ubiquitination of Epsin (Oldham et al., 2002). Studies in *Xenopus*-Epsin showed that more than one UIM are required for efficient ubiquitination, and both UIMs are necessary and sufficient for its ubiquitination (Oldham et al., 2002).

1.4.4. Clathrin binding motif (CBM)

Epsin co-localizes with Clathrin in cultured cells and *in vivo* extensively (Chen et al., 2005). *In vitro* binding assays demonstrated that Epsin binds to Clathrin by directly associating with the N-terminal domain of Clathrin heavy chain (Chc-TD) (Drake et al., 2000; Drake and Traub, 2001). There are two kinds of Clathrin interaction modules: CBMs (Clathrin binding motifs) and CBDs (Clathrin binding domains) (Kang et al., 2009). CBMs are usually smaller than CBDs. The consensus sequence of CBMs is: L(L/I)(D/E/N)(L/F)(D/E) (Wendland, 2002). Epsin contains two separate CBMs (Chen et

al., 1998). Studies in rat Epsin1 indicated that the two CBMs cooperate in Clathrin recruitment (Drake et al., 2000).

Epsin has been shown to promote endocytosis in Clathrin-mediated pathways (Chen et al., 1998; Wendland, 2002). It is conceivable that Epsin recruits ubiquitinated proteins with the UIMs and interacts with Clathrin via the CBMs to trigger Clathrin-dependent endocytosis. If this is the case, both the UIMs and the CBMs should function positively to internalize ubiquitinated cargos. However, the De Camilli group suggested that Clathrin binding could prevent the interaction between Epsin and ubiquitinated cargos (Chen et al., 2005). This indicates that the Clathrin-CBM interaction may regulate Epsin's activity to bind to ubiquitinated cargos, instead of bringing free Clathrin to form CCVs on the plasma membrane.

1.4.5. DPW motif

Epsin contains two clusters of DPW motifs that interact with AP-2 (Chen et al., 1998). Sequential deletions of the DPWs in rat Epsin1 showed that more than one DPW is required to mediate efficient association between Epsin and AP-2 (Drake et al., 2000). Over-expression of DPW motifs blocked transferrin uptake and Clathrin-dependent EGFR endocytosis (Chen et al., 1998), which indicates that the DPW – AP-2 interaction is tightly regulated for Clathrin-dependent endocytosis. As the general Clathrin adapter, AP-2 can recognize and internalize transmembrane proteins that contain dileucine motifs or Yxxϕ motifs (Bonifacino and Traub, 2003). Some accessory factors function to facilitate cargo selection and internalization through AP-2 interaction (McMahon and Boucrot, 2011). In this case, the accessory factors bridge the cargos and the Clathrin-dependent endocytic machinery with the AP-2 interaction.

1.5. Possible ways that Epsin may be involved in Notch ligand signaling

Equipped with five modules to interact with different proteins and lipids, Epsin has the ability to function as a Clathrin adapter or accessory factor during Clathrin-dependent endocytosis, or work through Clathrin-independent endocytosis, or interact with Cdc42-GAP to regulate actin polymerization and cell polarity (independent of endocytosis). To understand the essential role of Epsin during development, it is important to elucidate which function(s) of Epsin is involved in Notch ligand signaling.

1.5.1. Epsin functions as a Clathrin adapter

Clathrin adapters mediate the interactions between Clathrin, cargo, and/or phospholipids simultaneously (Oven et al., 2004). They form a shell to bridge cargos, Clathrin cage and the plasma membrane (Bonifacino and Traub, 2003). Thus, the CBM-Clathrin interaction is essential if Epsin functions as a Clathrin adapter. Epsin has been indicated to define a specialized endosome to modify the Notch ligands (Wang and Struhl, 2004). If this is the case, Epsin is very likely to function as a Clathrin adapter to form unique CCVs for the ligands to enter. Given this, the UIMs, CBMs, and/or the ENTH domain should be indispensable for Epsin's role in Notch signaling.

1.5.2. Epsin functions as an accessory factor

Accessory factors function in a variety of steps to facilitate cargo selection, membrane curvature, or CCV formation (McMahon and Boucrot, 2011). Depending on how Epsin is involved in Notch ligand endocytosis, different modules might be essential. If Epsin functions to recruit ubiquitinated ligands to CCVs, then the UIMs and the CCV-interacting modules (like CBMs, DPWs, or NPFs) will be indispensable. If Epsin is required to facilitate membrane curvature, then the ENTH domain should be essential. If Epsin is involved in CCV formation, then the CBMs and the DPWs could be important.

1.5.3. Epsin functions in Clathrin-independent endocytosis

Epsin has been shown to be involved in Clathrin-independent pathways to internalize ubiquitinated cargos (Chen and De Camilli, 2005). Eps15 and/or Eps15R play redundant roles with Epsin to promote endocytosis of ubiquitinated EGFR through Clathrin-independent pathways (Sigismund et al., 2004). It is possible that Epsin functions independently in a Clathrin-independent pathway to internalize Notch ligands. If this is the case, the UIMs, but not the CBMs, should be necessary. Alternatively, Epsin might work through Eps15 to facilitate endocytosis of Notch ligands. In this case, the NPF motifs should be essential.

1.5.4. Epsin functions independent of endocytosis to regulate actin polymerization and cell polarity

Although Epsin has been extensively shown to be involved in endocytosis, it does define an endocytosis-independent function. The Cdc42-GAP interaction provides the essential role of Epsin in yeast (Aguilar et al., 2003, 2006), which is related to actin regulation and cell polarity. If *Drosophila* Epsin plays a similar role during Notch signaling, then only the ENTH domain will be required for its essential role during development.

1.6. Significance of my doctoral work

Previous studies using a *ro-hs* promoter showed that both the ENTH domain and the ΔENTH fragment function significantly in Notch signaling during eye development (Overstreet et al., 2003). The result from the ENTH domain is surprising because the UIMs seem to be dispensable. Since ligand ubiquitination is required for Notch activation (see 1.1.2), there should be a UIM-containing protein (for example, Epsin) to interact with ubiquitinated ligands. One of the problems of those experiments is that the Epsin

variants were over-expressed under the *ro-hs* promoter, which might cause unexpected gain-of-function effect. I decided to perform a systematic function/structure analysis of *Drosophila* Epsin using a 16kb genomic fragment containing the *lqf* gene. The results determined that the UIMs are the most important modules. The three C-terminal motifs play an essential redundant role. And the ENTH domain is required to interact with PIP2 to provide full activity of Epsin in Notch signaling. These results support a model that Epsin functions to link the ubiquitinated ligands to Clathrin-coated vesicles to promote endocytosis.

Chapter 2. *Drosophila* Epsin functions as an accessory factor in Notch signaling cells

2.1. Introduction

Epsin is a multi-modular endocytic protein present in metazoans and yeast that functions at the internalization step of endocytosis (Chen et al., 1998; Wendland et al., 1999). Genetic studies in *Drosophila,* nematodes, and mice revealed that Epsin is required specifically for Notch signaling, and probably for all Notch signaling events (Overstreet et al., 2004; Wang and Struhl, 2004; Tian et al., 2004; Chen et al., 2009).

Epsin has a structured N-terminal ENTH domain (Kay et al., 1998; Rosenthal et al., 1999; De Camilli et al., 2001) (Fig.1.9). The unstructured C-terminal region of Epsin contains four different protein-protein interaction motifs in varying numbers in different animal species (Kay et al., 1998; De Camilli et al., 2001) (Fig.1.9). There are two Epsin isoforms in *Drosophila*, produced by alternate splicing of the *liquid facets* (*lqf*) gene mRNA (Fig.2.1) (Cadavid et al., 2000). Each Lqf isoform has two Ubiquitin interaction motifs (UIMs) (Hofmann and Falquet, 2001; Polo et al., 2002; Shih et al., 2002; Oldham et al., 2002; Miller et al., 2003; Klapsiz et al., 2002), two Clathrin binding motifs (CBMs) (Aguilar et al., 2003; Drake et al., 2000), seven DPW motifs that bind the AP-2 endocytic adapter complex (Owen et al., 1999), and two NPF motifs that bind EH-domain-containing endocytic factors (Aguilar et al., 2003; Salcini et al., 1997; Paoluzi et al., 1998).

The mechanism of Epsin function appears to be complex and context-dependent. For example, the results of some studies suggest that Epsin functions in Clathrin-dependent endocytosis (Chen et al., 1998; Hawryluk et al., 2006), while other results

suggest that Epsin functions also in Clathrin-independent membrane internalization (Sigismund et al., 2005). In addition, Epsin may be an endocytic accessory factor that facilitates protein interactions at the Clathrin-coated vesicle or induces membrane curvature, or Epsin may itself be an autonomous adapter protein that links transmembrane cargo to Clathrin directly (Aguilar and Wendland, 2006b).

Different roles for yeast and *Drosophila* Epsin ENTH domains have also been reported. The yeast Ent1 ENTH domain alone is sufficient for the essential function of the protein. This function is not endocytosis, but the regulation of actin cytoskeleton dynamics (Aguilar et al., 2006a). Remarkably, when overexpressed, either the ENTH domain of *Drosophila* Epsin, or the remaining C-terminal endocytic modules provides significant Epsin activity when overexpressed (Overstreet et al., 2003). Thus, it is unclear which function of Epsin – the actin regulatory function or the endocytosis function or both – are important for Notch signaling under physiological conditions.

Both positive and negative roles for Epsin's UIMs and CBMs have been proposed also. The UIMs are required for Epsin ubiquitination (Hofmann and Falquet, 2001; Polo et al., 2002; Shih et al., 2002; Oldham et al., 2002; Miller et al., 203; Klapsiz et al., 2002), which inhibits Epsin function (Chen et al., 2002). However, as ubiquitination of Notch ligand intracellular domains promotes ligand endocytosis and is necessary for signaling (reviewed in Weinmaster and Fischer, 2011), Epsin may also recognize Notch ligands through its UIMs. Genetic evidence in *Drosophila* indicates a positive role for Clathrin in ligand endocytosis (Cadavid et al., 2000; Eun et al., 2007; Eun et al., 2008; Hagedorn et al., 2006), suggesting that Epsin's CBMs may serve simply to link ligand at the plasma membrane with Clathrin. However, there is also evidence in vertebrate cell culture that Clathrin binding through the CBMs may inhibit UIM binding to Ubiquitin

(Chen and De Camilli, 2005), and thus the CBMs may actually inhibit Epsin function in endocytosis of ubiquitinated transmembrane proteins.

The Notch receptor and its ligands (Delta and Serrate, in *Drosophila*) are transmembrane proteins (reviewed in Bray, 2006). Receptor activation leads ultimately to cleavage of an intracellular receptor fragment that enters the nucleus and acts a transcription factor (Bray, 2006). Epsin is required for Notch ligand endocytosis by the signaling cells, which is necessary for receptor activation by the signal receiving cells (Fig.1.7, 1.8, Overstreet et al., 2004; Wang and Struhl, 2004; Tian et al., 2004). Epsin-dependent ligand internalization by the signaling cells is most likely required to exert a pulling force on Notch that activates the receptor (Le Borgne et al., 2005; Nichols et al., 2007a, 2007b; Weinmaster and Fischer, 2011). The specific requirement for Epsin in Notch ligand cells may mean that Epsin is somehow intrinsic to the mechanism by which the ligand cell, through ligand endocytosis, activates the Notch receptor in an adjacent cell.

An important step forward in understanding the role of Epsin in Notch ligand cells is to determine which Epsin modules are required for this process in particular. Here, by generating a variety of transgenes that express amino acid deletion or substitution variants of Epsin in transformed flies, I tested each Epsin module type to determine whether or not it was needed in Notch signaling. The results suggest that for its role in Notch ligand cells, Epsin is divided into three necessary functional regions: the lipid binding function of the ENTH domain, a single UIM, and the C-terminus. The results, interpreted in the context of a large body of data regarding the functions of Epsin in other organisms and the roles of several other proteins in Notch ligand cells, are most consistent with a model where Epsin recognizes ubiquitinated ligands at the plasma

membrane, and links ligands to Clathrin-coated vesicles indirectly through other Clathrin adapter proteins.

2.2. Results

2.2.1. An assay for Epsin function in Notch signaling *in vivo*

My aim was to generate *Drosophila* lines that express mutant Epsin proteins with different modules deleted, at levels as close as possible to those of the endogenous gene. To this end, I generated a series of deletion mutant transgenes in the context of a 16 kilobase *Drosophila* genomic DNA fragment containing the *lqf* gene. The *lqf* genomic DNA fragment (Fig.2.1) complemented completely the lqf^{L71}/lqf^{ARI} mutant phenotype (Fig2.2). lqf^{L71} and lqf^{ARI} are null alleles (Fig.2.1) (Wang and Struhl, 2004; Cadavid et al., 2000; Overstreet et al., 2003). To facilitate protein quantification, most of the deletion transgenes were generated in a modified *lqf* genomic DNA that produced an Epsin-GFP fusion protein. The full-length *lqf-gfp* fusion transgene also complemented *lqf* null mutants (Fig.2.2). Moreover, endogenous Epsin and Epsin-GFP each colocalized extensively with clathrin in eye discs, and were present mainly in puncta at the apical plasma membrane (Fig.). The puncta may be clathrin-coated vesicles or clathrin plaques (Saffrian et al., 2009). Deleted versions of Epsin or Epsin-GFP proteins expressed by the transgenes in several different transformant lines were quantified using Western blots (Apendix), and one or two lines that matched normal endogenous Epsin levels most closely were used for functional tests.

I used the ability of a transgene to complement *lqf* loss-of-function mutant phenotypes as an assay for Epsin function in Notch signaling. *lqf* null mutants die as embryos, and *lqf* hypomorphs are semi-viable with eye, wing, and leg defects typical of Notch pathway mutants (Cadavid et al., 2000). Analysis of *lqf* null clones in developing

eyes and wings (imaginal discs) indicates a specific failure of Notch signaling (Overstreet et al., 2004; Wand and Struhl, 2004, 2005). Likewise, I think that *lqf* null flies die due to

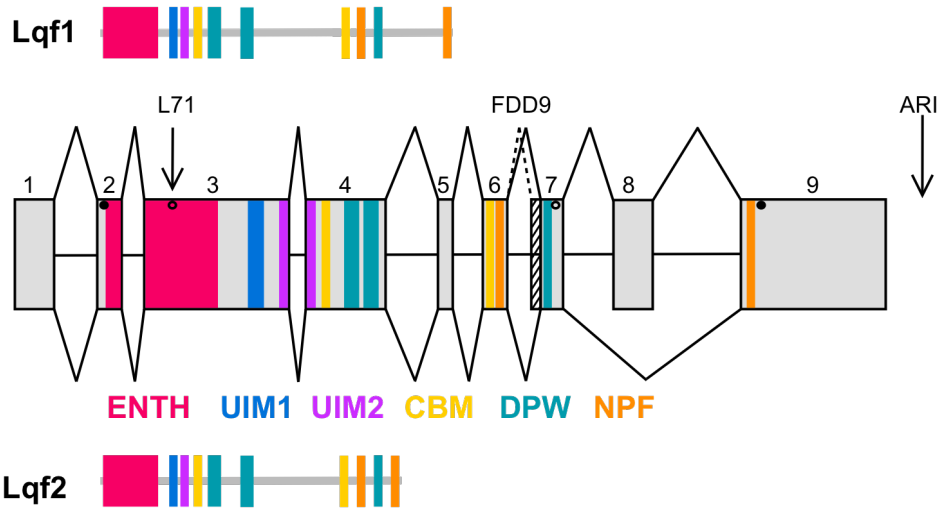


Figure 2.1. *Drosophila lqf* gene structure. A diagram of *lqf* genomic DNA is in the center. The nine exons are numbered and the two alternate splice forms of the mRNA are indicated by the bent lines connecting the exons above (*lqf1*) and below (*lqf2)* (Cadavid et al., 2000; Overstreet et al., 2003). The two protein isoforms (Lqf1 and Lqf2) are diagrammed. ENTH=Epsin N-terminal homology domain, UIM=Ubiquitin interaction motif, CBM=Clathrin binding motif, DPW=DPW motifs that bind AP-2, NPF=NPF motifs that bind EH-domain proteins. The two solid black ovals are the start and stop codons in the wild-type genomic DNA. The unfilled ovals are the stop codons used in the *lqfL71* and *lqfFDD9* alleles (Overstreet et al., 2003; see Materials and Methods). *lqfL71* is a nonsense mutation (Wang and Struhl, 2004; Overstreet et al., 2003). *lqfFDD9* is a temperature sensitive allele with a point mutation (G-to-A) that generates a cryptic splice acceptor site in intron 6 resulting in production of a single Epsin protein that is C-terminally truncated (the final 11 nucleotides of intron 6 become the 5'-end of exon 7, resulting in a 10 aa insertion and an early stop codon) and present at levels much lower than wild-type Epsin (Overstreet et al., 2003 and Materials and Methods). The dotted line indicates the 3'-splice acceptor site used by the *lqfFDD9* allele, and the lined box shows the addition to exon 7 present in *lqfFDD9* mRNA. *lqfARI* is a small deletion 3' to the transcribed region (Cadavid et al., 2000). *lqfARI* produces no detectable protein (Overstreet et al., 2003).

Notch signaling failure. Notch signaling is essential during embryonic development, and consequently homozygous null mutants in core Notch pathway genes die during embryogenesis (Poulson, 1937; Lehmann et al.,1983; Nusslein-Volhard et al.,1984). In

addition, different alleles of *lqf* affect viability and Notch-like morphology similarly (Cadavid et al., 2000; Overstreet et al., 2003, and see below). Also, mice with knock-out mutations in both Epsin genes (*epsin 1* and *epsin 2*) show typical Notch signaling defects throughout development, including embryogenesis (Chen et al., 2009).

I tested how well each transgene substituted for the endogenous *lqf* gene by generating lqf^{L71}/lqf^{ARI} flies containing one copy of each transgene. To detect low levels of Epsin activity, I also tested each transgene in a hypomorphic (lqf^{FDD9}) background (Cadavid et al., 2000; Overstreet et al., 2003). lqf^{FDD9} is a temperature sensitive allele with a point mutation that generates a cryptic splice acceptor site in intron 6 (Fig.2.1), resulting in production of a single Epsin protein that is C-terminally truncated and present at levels much lower than wild-type (Overstreet et al., 2003 and Apendix). lqf^{FDD9} homozygotes are viable at 25ºC, and have a variety of morphological defects, including missing leg segments, notched wings, and rough eyes (Cadavid et al., 2000).

In Table 1, the transgene structures, the amount of protein each produces, and the results of both complementation assays are summarized. The transgenes fell into four groups, based on their activity. Group I transgenes had complete Epsin activity; they complemented the lqf^{L71}/lqf^{ARI} (and lqf^{FDD9}) mutant phenotype fully, meaning that the flies are viable and have no apparent morphological defects. Group II transgenes retain significant Epsin activity; they complement lqf^{L71}/lqf^{ARI} partially (the flies are at least semi-viable and have morphological defects typical of *Notch* pathway mutants), and complement lqf^{FDD9} completely. Group III transgenes have only residual activity; they enable lqf^{L71}/lqf^{ARI} to survive only until they are pupae, and they complement the morphological mutant phenotype of lqf^{FDD9} partially. Group IV transgenes have no apparent Epsin activity; they fail to complement lqf^{L71}/lqf^{ARI} or lqf^{FDD9} detectably.

Quantitative analysis of complementation of the mutant eye phenotype and examples of mutant eyes observed in complementation tests are shown in Fig.2.2.

The *lqf* null phenotype is probably due entirely to the failure of Notch signaling, and thus the transgene complementation test is a reasonable assay for Epsin activity in the Notch pathway. This assumption is based on several observations. First, analysis of *lqf* null clones in developing eye and wing imaginal discs indicates a specific failure of Notch signaling (Overstreet et al., 2004; Wang and Struhl, 2004, 2005). Second, Notch signaling is essential during *Drosophila* embryonic development, and consequently homozygous null mutants in core Notch pathway genes die during embryogenesis (Poulson, 1937; Lehmann et al.,1983; Nusslein-Volhard et al.,1984). Likewise, mice with knock-out mutations in both Epsin genes (*Epsin 1* and *Epsin 2*) show typical Notch signaling defects throughout development, including embryogenesis (Chen et al., 2009). Finally, different mutant alleles of *lqf* affect viability and Notch-like morphology to a similar degree (Cadavid et al., 2000; Overstreet et al., 2003). Likewise, I always observed a linear correspondence between the ability of a transgene to rescue the lethality of *lqf* null mutants and Notch signaling defects later in development in either *lqf* null mutants or *lqf* hypomorphs (Fig.2.2).

2.2.2. Deletion of individual Epsin module types: the UIMs are the single module type most important to Epsin's ability to promote ligand signaling

My first aim was to generate *Drosophila* lines that express one of five different variants of Epsin in which all (or most in the case of the DPWs) copies of a particular module type were deleted (Fig.2.2). The Epsin-GFP proteins expressed by each transgene in several different transformant lines were quantified using Western blots (Apendix) and lines that matched normal endogenous Epsin levels most closely (Fig. 1) were used for

further analysis. First, I tested whether or not a single copy of each transgene could substitute for the endogenous gene as well as FL did. I found that three of the transgenes-

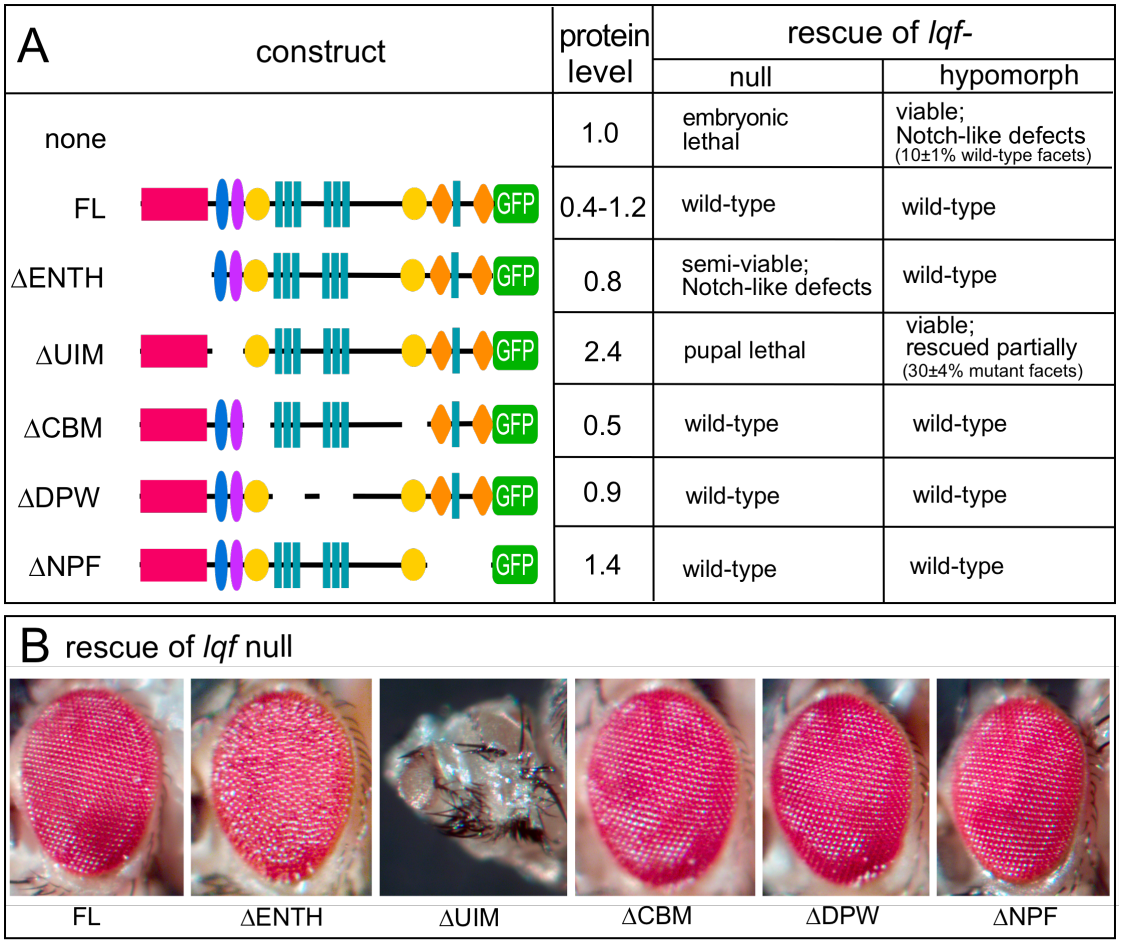


Figure 2.2. Epsin's ENTH domain and UIMs are each required for Notch signaling. (A) At left are diagrams of full-length (FL) Epsin-GFP protein and five deletion derivatives (described in text) expressed by P element transgenes. Protein levels from a single transgene copy were measured in eye discs and normalized to the amount of protein produced by one endogenous *lqf* gene copy as described in A.2.1. How well a single copy of each transgene rescues the mutant phenotypes of *lqf* nulls (*lqfL71/lqfARI*) and *lqf* hypomorphs (*lqfFDD9*) is indicated. (The *lqf* mutant alleles are described in Fig.2.1) The fraction of wild-type (wt) facets was obtained by counting ~100 facets in each of at least 3 adult eyes. Wild-type eyes have 100% wild-type facets. (B) External eyes of flies (or pupae for ΔUIM) that are *lqfL71/lqfARI* and have a single copy of the transgene indicated. All of the eyes are wild-type except for ΔENTH (the external eyes are slightly roughened due to defects in retinal development) and ΔUIM (dying pupae dissected from the pupal case have no eyes.)

ΔCBM, ΔDPW, and ΔNPF – complemented *lqf* null mutants completely (Fig.2.2). Although ΔENTH did not rescue the *lqf* nulls completely, it did retain significant gene activity as some viable adults eclosed with morphological defects typical of Notch signaling mutants (including malformed eyes, wings, and legs) (Fig.2.2). ΔUIM had considerably less *lqf*+ activity than ΔENTH; the developing animals died as pupae and no escapers ever eclosed (Fig.2.2). To better detect and resolve the low levels of *lqf*+ gene activity in the ΔENTH and ΔUIM transgenes, I also tested how well each of the transgenes rescued the morphological defects of *lqf* hypomorphs. Consistent with the idea that ΔENTH has much more *lqf*+ activity than ΔUIM, ΔENTH rescued lqf^{FDD9} to wild-type while ΔUIM rescued the lqf^{FDD9} defects only slightly (Fig.2.2).

2.2.3. Either UIM is sufficient for Epsin function in ligand signaling

Epsin has two UIMs (Fig.2.3A) and I was curious to know whether or not both of Epsin's UIMs are required for ligand cell siganling. I generated a *lqf-gfp* transgene in the context of the 16 kb genomic DNA as used above with a deletion of UIM2 *(ΔUIM2*). In transformed flies, *ΔUIM2* rescued the lethality of *lqf-* null mutants and the viable flies were almost completely wild-type morphologically (Fig. 5B,C). I conclude from this that only a single UIM is essential. To test if the single UIM could be UIM2 instead of UIM1, I first generated a transgene identical to *ΔUIM2*, except that three UIM1 consensus Glutamic acid codons were changed to Alanine codons ($UIM1^{EEE/AAA}$*ΔUIM2*; Fig. 5B). $UIM1^{EEE/AAA}$*ΔUIM2* had only a very small amount of Epsin activity in the *lqf-* phenotypic rescue assays (Fig. 5B). Thus, the UIM1 mutations rendered $UIM1^{EEE/AAA}$*ΔUIM2* essentially inactive. Next, I added back to $UIM1^{EEE/AAA}$*ΔUIM2* the UIM2 sequences to generate $UIM1^{EEE/AAA}$ (Fig. 5B). I found that $UIM1^{EEE/AAA}$ had significant activity, just below that of *ΔUIM2*. $UIM1^{EEE/AAA}$ rescued *lqf* null mutants to nearly wild-type (Fig.

5B,C). I conclude that only one UIM is necessary for very nearly full Epsin activity in ligand cells, and that either UIM1 or UIM2 is sufficient.

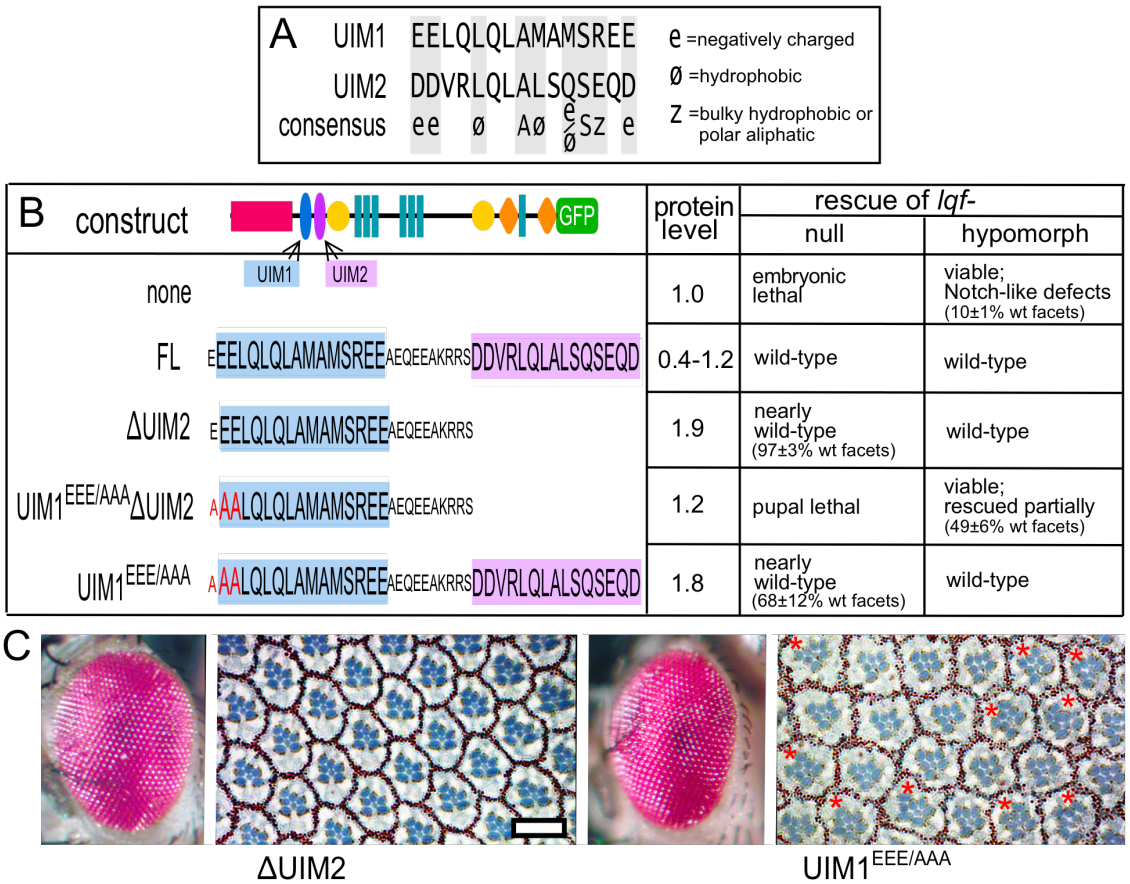


Figure 2.3. Either UIM is sufficient for Epsin function in ligand signaling. (A) UIM1 and UIM2 of *Drosophila* Epsin are shown and each conforms to the consensus for single-sided UIMs (Hirano et al., 2006). (B) At left, alterations in the UIM region of the three different Epsin variants indicated are shown. The transgenes in this experiment were all introduced into the same genomic location using a PhiC31 integrase vector (A.1.1). The red amino acids are altered. See Fig.2.2 legend for explanation of the remainder of the table. (C) Eyes of *lqf* null (*lqfL71/lqfARI*) adults expressing the Epsin variant indicated from a single copy of a transgene (external eyes at left and tangential sections at right) are shown. The red asterisks indicate mutant facets with extra photoreceptor cells. size bar: sections, ~20μm sections; external eyes, ~150 μm.

2.2.4. The CBMs, DPWs and NPFs are redundant with each other

Above I discovered that Epsin's function in Notch ligand cells is independent of its CBMs, DPW motifs, or NPF motifs in an otherwise intact protein. To determine whether the C-terminal modules are required at all, I generated a *lqf-gfp* transgene

A construct	protein level	rescue of *lqf-*	
		null	hypomorph
none	/	embryonic lethal	viable; Notch-like defects (10±1% wild-type facets)
FL GFP	1.2	wild-type	wild-type
ENTH-UIM GFP	4.8	embryonic lethal	viable; Notch-like defects (15±3% wt facets)
ΔCBM-ΔDPW1 GFP	1.0	wild-type	wild-type
ΔCBM-ΔDPW GFP	0.7	nearly wild-type (92±2% wt facets)	wild-type
4XNPF GFP	0.7	semi-viable; Notch-like defects	wild-type

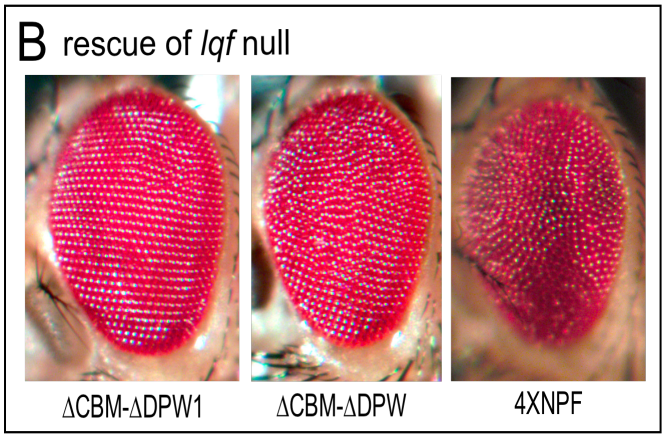


Figure 2.4. The CBMs, DPW motifs, and NPF motifs, are redundant with each other. (A) At left are diagrams of full-length (FL) Epsin-GFP protein and three deletion derivatives (described in text) expressed by P element transgenes. *4xNPF* was introduced using a PhiC31 integrase vector (A.1.1). See Fig.2.2 legend for explanation of the remainder of the table. (B) Shown are eyes of rescued *lqfARI/lqfL71* adults expressing the Epsin variant indicated from a single transgene copy.

containing only the ENTH domain and the UIMs (*ENTH-UIM*). *ENTH-UIM* has no ability to rescue the mutant phenotypes of *lqf-* mutants (Fig. 6A,C). As I have shown that each of the C-terminal module types is dispensable, the C-terminal module types must be redundant for Epsin's role in ligand signaling. To explore the nature of the redundancy, I generated two additional *lqf-gfp* transgenes, one with deletions of the CBMs and one DPW cluster (*ΔCBM–ΔDPW1*), and the other with the CBMs and both DPW clusters deleted (*ΔCBM–ΔDPW*). *ΔCBM–ΔDPW1* rescues *lqf* null mutants to wild-type, and *ΔCBM–ΔDPW* has just slightly less activity in that the null eye has a small number of mutant facets (Fig. 6). This result indicates that Epsin functions nearly normally in ligand cells with only one of the C-terminal module types intact – the NPFs.

I wondered whether like the NPFs, the DPW motifs or the CBMs would support Epsin function independent of the other two module types. Instead of testing that with deletion constructs, I attempted to generate "minimal Epsin-GFP" constructs containing the ENTH domain, UIM1, and four copies of either the CBMs (*4XCBM*), DPW clusters (*4XDPW*), or NPF motifs (*4XNPF*) in the context of the 16kb *lqf* genomic DNA fragment. I did this to address whether or not there are unknown motifs in the C-terminus that may be contributing to Epsin function. I detected no Epsin-GFP protein in flies transformed with *4xCBM* or *4XDPW* (data not shown). In contrast, the 4XNPF protein was expressed at high levels and the transgene rescued *lqf-* mutant phenotypes significantly (about as well as *ΔENTH* does) (Fig. 6A,B). A likely possibility is that the transgene is expressed at somewhat lower levels in the wing than in the eye or in early development, and that this expression level is beneath the threshold for detectable Cut expression (see Discussion.)

I conclude that there is functional redundancy among the C-terminal modules, and that the NPF motifs alone were sufficient to provide a significant portion of the function

of this region. Additional experiments are needed to determine whether the CBMs or DPW motifs are as significant to Epsin function in ligand cells as are the NPFs.

2.2.5. Deletion of UIMs, CBMs, DPWs or NPFs interferes with Epsin binding *in vitro* to Ubiquitin, Clathrin, α-adaptin, and Eps15, respectively

Protein-protein interactions between Epsin's various modules (UIMs, CBMs, DPWs, and NPFs) have been characterized previously in a variety of different contexts. Nevertheless, I wanted to test whether the deletions of modules are indeed disrupting the ability of the particular *Drosophila* Epsin variants I generated to interact with the expected *Drosophila* proteins. To this end, I generated GST fusion proteins in bacteria with Ubiquitin (GST-Ub), the Clathrin heavy chain "terminal domain" (GST-Chc-TD), the EH domain (NPF-binding region) of Eps15 (GST-Eps15-EH), and the ear domain of α-adaptin, the AP-2 subunit that binds Epsin (GST-α-Ada-ear). Purified GST fusion proteins were tested in GST "pull-down" experiments for interaction with wild-type *Drosophila* Epsin (FL) or Epsin deletion derivatives purified in bacteria as MBP fusions. As a control for artifactual results, Epsin and each derivative was also tested under similar conditions for binding to GST alone and none of the proteins bound GST (data not shown).

First, I found that under conditions in which FL bound to GST-Ub, ΔUIM2 (Fig. 1) bound to GST-Ub also, but ΔUIM (Fig. 1) lost the ability to interact with Ub (Fig. 7A). I conclude that the UIMs are required not only for Epsin function but also for Epsin to bind Ub *in vitro*. In addition, UIM1, which is sufficient for Epsin function without UIM2 in an otherwise intact protein, is also sufficient for Ub binding *in vitro*. Thus there is a correlation between Epsin's ability to bind Ub *in vitro* and its ability to function *in vivo*.

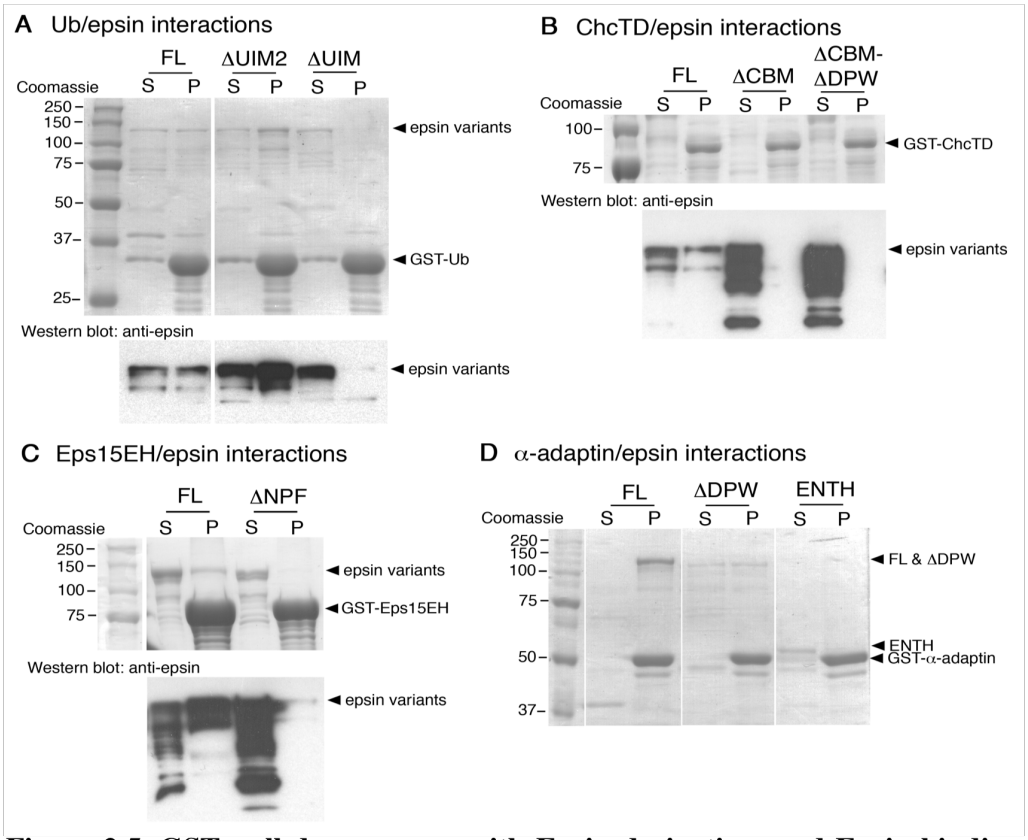


Figure 2.5. GST pull-down assays with Epsin derivatives and Epsin-binding proteins. Coomassie-stained protein gels and protein blots of the same gel hybridized with anti-Epsin are shown. (In D, only a Coomassie-stained gel is shown.) At left are size markers (units are kD). The GST fusion proteins indicated were immobilized on columns and their ability to bind the MBP-Epsin variants indicated (described in text) was tested. S=supernatant (unbound fraction), P=pellet (bound fraction). Equivalent amounts of each Epsin variant were used in each assay. See A.3 for complete experimental details. (A) An assay of GST-Ub binding to each of the MBP-Epsins indicated is shown. Each S lane represents 1/80 of the unbound fraction, and each P lane is 1/3 of the bound fraction. The panels below show only the top portion of the blot. GST-Ub=36 kD. Sizes of MBP fusion proteins: FL (Lqf2)=118 kD; ΔUIM2=116 kD; ΔUIM=113 kD. (B) An assay of GST-ChcTD binding to each of the MBP-Epsins indicated is shown. Each S lane represents 1/24 of the unbound fraction, and each P lane represents 2/5 of the bound fraction. The Epsin variants were not visible with Coomassie staining, but were detectable on the blot probed with anti-Epsin. GST-ChcTD is 92 kD. Sizes of MBP fusion proteins: ΔCBM=117 kD; ΔCBM-ΔDPW =110 kD. (C) An assay of GST-Eps15EH binding to each of the Epsins indicated is shown. Each S lane represents 1/24 of the unbound fraction, and each P lane represents 2/5 of the bound fraction. GST-Eps15EH is 84 kD, MBP-ΔNPF=111 kD. (D) An assay of GST-α-AdaEar binding to each of the MBP-Epsins indicated is shown. Each S lane represents 1/80 of the unbound fraction, and each P lane represents 1/4 of the bound fraction. GST-α-AdaEar is 56 kD. Sizes of MBP fusion proteins: ΔDPW=112 kD; ENTH =73 kD.

Next, I found that GST-ChcTD pulled down FL, but pulled down neither ΔCBM nor ΔCBMΔDPW (Fig.2.5B). Thus, deletion of the CBMs hampered the ability of Epsin to bind Clathrin *in vitro*. Similarly, I found that deletion of the NPF motifs (Fig. 1, ΔNPF) prevented Epsin from interacting *in vitro* with the EH domain of Eps15 (Fig. 6C), and deletion of the two clusters of DPW motifs (Fig. 1, ΔDPW) disrupted the *in vitro* interaction between α-adaptin and Epsin (Fig. 6D). I conclude that at least *in vitro*, deletion of the characterized motifs is indeed interfering with the ability of Epsin to bind the expected proteins.

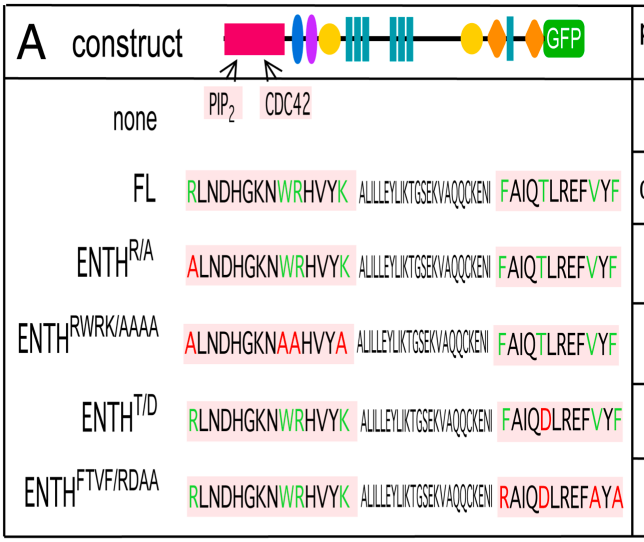
2.2.6. The lipid-binding function of the ENTH domain, but not the Cdc42 GAP interaction function, is required for Epsin's role in ligand cells

I found above that the ENTH domain, while not essential for Epsin function in ligand cells, is required for maximal Epsin activity (Fig. 1). Two functions have been proposed for the ENTH domain. The ENTH domain brings Epsin to the plasma membrane through interactions with PIP_2. Moreover, the ENTH domain may also insert into the plasma membrane and induce curvature. In addition, the ENTH domain binds Cdc42 GAP which may serve to coordinate cell actin dynamics and cell polarity with endocytosis. Distinct ENTH domain amino acids have been identified that are specific to each of the two functions (refs). To determine which of the two functions of the ENTH domain is required for maximal Epsin activity in ligand cells, I generated *lqf-gfp* transgenes that express proteins with mutations that alter amino acids key to one function or the other.

First, I generated *ENTH*$^{RWRK/AAAA}$ which produces an Epsin variant with four amino acids critical for ENTH domain binding to PIP_2 changed to Alanine. ENTH$^{RWRK/AAAA}$ protein accumulates to only a small fraction of wild-type levels (0.2) and it provides only

A

construct	protein level	rescue of *lqf-*	
		null	hypomorph
none	1.0	embryonic lethal	viable; Notch-like defects (10±1% wt facets)
FL	0.4-1.2	wild-type	wild-type
ENTH$^{R/A}$	0.8	nearly wild-type (64±14% wt facets)	wild-type
ENTH$^{RWRK/AAAA}$	0.2	embryonic lethal	viable; rescued slightly (16±5% wt facets)
ENTH$^{T/D}$	1.3	wild-type	wild-type
ENTH$^{FTVF/RDAA}$	0.9	wild-type	wild-type



B

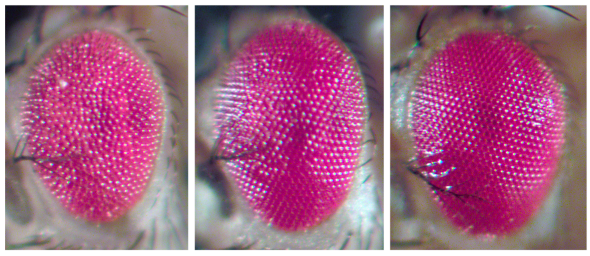


Figure 2.6. The lipid binding function of the ENTH domain affects Epsin's function in Notch signaling. (A) At left, alterations of the ENTH domain in three different Epsin variants indicated are shown. The pink shaded box at the left contains amino acids (green) important for plasma membrane lipid (PIP2) binding, and the pink box to the right contains amino acids important for binding Cdc42-GAP. The amino acids in red are altered. The transgenes in this experiment were all introduced into the same genomic location using a PhiC31 integrase vector (A.1.1). See Fig.2.2 legend for explanation of the remainder of the table. (B) Eyes of *lqf-* (*lqfAR1*/*lqfL71*) adults expressing the Epsin variant indicated from a single transgene copy.

a barely detectable amount of Epsin activity (Fig. 7A). The observation that *ENTH$^{RWRK/AAAA}$* has almost no activity, while expression of wild-type Epsin at levels only 2-fold greater rescues the *lqf* mutant phenotype completely (Fig. 7A) suggests that the activity of ENTH$^{RWRK/AAAA}$ is compromised. (*ENTH$^{RWRK/AAAA}$* provides much less Epsin activity than *ΔENTH*, which lacks the ENTH domain entirely (Fig. 1B), but ΔENTH

accumulates to levels four times as great as ENTH$^{RWRK/AAAA}$ does.) As low levels of ENTH$^{RWRK/AAAA}$ protein accumulation make these results difficult to interpret, I generated *ENTH$^{R/A}$*, which contains a mutation that changes only one key residue to Alanine (Fig. 7A). ENTH$^{R/A}$ protein accumulates to normal levels and although it retains more activity than ΔENTH (Fig. 1B), it is not quite wild-type (Fig. 7A,B). Thus, ENTH$^{RWRK/AAAA}$ protein activity is likely compromised, and ENTH$^{R/A}$ protein differs from wild-type Epsin (FL) by only one amino acid and yet has less activity than wild-type Epsin. In contrast, Epsins with ENTH domains containing alterations in either one or four amino acids required for the CDC42 regulation function (ENTH$^{T/D}$ or ENTH$^{FTVF/RDAA}$) function as well as wild-type (FL) Epsin (Fig. 7A,B). Taken together, the results are most consistent with the conclusion that the lipid-binding function, and not the CDC42-GAP binding function of the ENTH domain, is required for maximal Epsin activity in Notch signaling.

2.3. Discussion

Epsin is a complex multi-modular protein that functions differently in different contexts. In yeast, the essential function of yeast Epsin is actin polarization mediated by the ENTH domain alone (Aguilar et al., 2006). In contrast, in vertebrate cell culture, Epsin has been shown to function in endocytosis of a variety of proteins (Wendland et al., 1999). Moreover, in *C. elegans*, *Drosophila*, and mice, Epsin is needed specifically in Notch ligand cells (Fig1.7, Overstreet et al., 2004; Wang and Struhl, 2004; Tian et al., 2004; Chen et al., 2009). Epsin has been presumed to be facilitating endocytosis of the Notch ligands based mainly on Epsin's role in cell culture and on other indirect evidence. For example, *lqf* interacts genetically with endocytosis genes (Cadavid et al., 2000; Eun et al., 2008; Banks et al., 2011). Also, Notch ligands sometimes accumulate abnormally

at the plasma membrane in the absence of Epsin (Overstreet et al., 2004; Wang and Struhl, 2004). I performed a careful structure/function analysis of the Epsin protein by making systematic deletions in a genomic context. My data have allowed me to distinguish between several models of Epsin function in Notch signaling. I have shown that the lipid binding feature of Epsin, and not its ability to organize the actin cytoskeleton, is involved in Notch signaling. Furthermore, a UIM is critical for the function of Epsin in Notch signaling. Finally, the CBMs are dispensible for the role of Epsin in Notch signaling. Here I would like to discuss how these results might affect our understanding about ligand endocytosis in Notch signaling.

2.3.1. The lipid-binding function, and not the actin organizing function of the ENTH domain contributes to Epsin function in ligand cells

Until this work, the hypothesis that Epsin is required for ligand endocytosis was based on indirect evidence. One type of evidence is the accumulation of Delta or Serrate that plasma membrane in the absence of Epsin. In wing disc clones that overexpress both Delta and the Ub ligase Neuralized (Neur), Delta is cleared from the plasma membrane more efficiently in the presence of Epsin than in its absence (Wang and Struhl, 2004). Similarly, in eye discs higher than normal levels of Delta accumulate at the plasma membrane in cells simultaneously lacking Epsin and deficient in the Neur (Eun et al., 2007). Likewise, Serrate accumulates on the plasma membrane of *lqf-* clones in wing discs. A second line of evidence is that there are strong genetic interactions between *lqf* and two endocytosis genes, *Clathrin heavy chain* (*Chc*) and *auxilin* (*aux*) (Cadavid et al., 2000; Eun et al., 2007; Eun et al., 2008). Auxilin is required for uncoating Clathrin-coated vesicles (see Eun et al., 2008). *Chc* and *aux* are strong dominant enhancers of the *Notch*-like mutant phenotype of *lqf* hypomorphs, suggesting that all four genes work in

the same direction in a pathway. Thirdly, as ligand endocytosis requires the Ub ligases Neur and Mindbomb (Mib) and the ligands are ubiquitinated (reviewed in Weinmaster and Fischer, 2011), given that Epsin has two UIMs, the obvious inference is that Epsin is required for ligand internalization. Finally, most Epsin is detected at the plasma membrane in eye discs (Overstreet et al., 2004). However, a complicating factor in the interpretation of all of these experiments is that Epsin, especially when overexpressed, may be involved with ligand endocytosis that does not result in signaling and Notch activation in adjacent cells (Wang and Struhl, 2004, 2005).

My observation that the PIP_2 binding function of the ENTH domain is required for maximal Epsin activity in ligand cells suggests that in the context of Notch ligand cells, Epsin functions at the plasma membrane. The lack of requirement for the Cdc42 GAP binding function of the ENTH domain suggests that in this context the primary role of *Drosophila* Epsin, unlike yeast Ent1, is not regulation of actin dynamics. This observation is consistent with the lack of typical Notch signaling defects in *Drosophila cdc42* mutants (Genova et al., 2000).

2.3.2. A UIM is critical to Epsin activity in Notch ligand cells, and a single UIM is sufficient

There are two types of UIMs: single-sided UIMs that bind one Ubiquitin, and double-sided UIMs that bind two Ubiquitins simultaneously (Hirano et al., 2006). As the affinity between a UIM and Ubiquitin is low, successful interaction between a mono-ubiquitinated protein and a UIM-containing protein is thought to require either one double-sided UIM, or two single-sided UIMs (Hawryluk et al., 2006; Hirano et al., 2006; Barriere et al., 2006). Epsins have single-sided UIMs, and so the observation that only one single-sided UIM is required for *Drosophila* Epsin function in Notch signaling is

unexpected. The simplest explanation is that Notch ligands use multiple mono-Ubiquitins or Ubiquitin chains as a signal for Epsin-mediated internalization (Traub and Lukacs, 2007; Heuss et al., 2008). Two distinct Lysine residues in the intracellular domains of both Delta and Serrate have been implicated as important for the function of each ligand (Glittenberg et al., 2006; Parks et al., 2006). In the case of Serrate, mutation of both lysines results in a protein that can neither activate Notch nor be endocytosed in eye discs. In the case of Delta, mutation of either of the two lysines results in accumulation of protein at the cell surface of eye discs and failure to signal. Although Delta is thought to be mono-ubiquitinated (Heuss et al., 2008), these results suggest the possibility that Delta could be multiply mono-ubiquitinated. Alternatively, it's possible that mono-ubiquitinated ligands cluster to generate an environment where multiple Ubiquitins attract Epsin to Delta at the plasma membrane.

Another explanation is that the PIP2 binding of the ENTH domain greatly enhances the single UIM-Ub interaction. Since the Notch ligands are transmembrane proteins, PIP2 on the plasma membrane might be close enough be considered as extended region of the proteins. Alternatively, Epsin may have unknown sites that bind to the ligands directly. Although Epsin without the UIMs failed to provide significant activity, it still contains residual activity (Fig.2.2). One copy of the ΔUIM transgene rescued the *lqf* null phenotypes from embryonic lethality to pupal lethality. The rescue activity is further confirmed by the *lqf* hypomorph rescue assay (Fig.2.2). Deletion of the ENTH domain and the UIMs results in a transgene with no residual activity, while deletion of the UIMs along with other parts of the protein retain the residual activity (Table 1). Therefore it seems possible that the ENTH domain is responsible for the residual activity of the ΔUIM transgene, suggesting that the ENTH domain might contain sites to bind Notch ligands

directly. It will be interesting to test *in vitro* whether Epsin can bind to the Notch ligands directly or not.

2.3.3. The CBMs are dispensable to Epsin's function in ligand cells

Given the compelling evidence for a requirement for Clathrin in Notch ligand endocytosis and signaling for early embryogenesis, viability, and appendage development (Cadavid et al., 2000; Eun et al., 2007; Eun et al., 2008), and the lack of strong genetic interaction between *α-adaptin* (AP-2) and *lqf* (Epsin) (Cadavid et al., 2000), the simplest model for Epsin function in Notch signaling was as an adapter protein that links Clathrin and the plasma membrane. This model predicted that direct interaction between Epsin and Clathrin would be necessary, and thus the most surprising result of this work is that deletion of the CBMs had no detectable effect on Epsin activity. The dispensability of the CBMs means that Epsin-dependent Clathrin cages form without Epsin acting as a typical adapter protein linking the plasma membrane and Clathrin.

In the *Drosophila* female germ-line, Notch signaling requires Epsin but not Clathrin (Windler and Bilder, 2010). Similarly, Epsin has been shown to function in Clathrin-independent internalization of ubiquitinated transmembrane cargos in vertebrate cell culture (Sigismund et al., 2005). Epsin must function differently in the female germ-line than in somatic cells, and it will be interesting to determine which Epsin motifs are required in this context.

2.3.4. Epsin functions similarly in different Notch contexts

I began the experiments with the assumption that the same Epsin modules would be required for Epsin function in all developmental contexts in *Drosophila*. This idea is sensible as Epsin could be regarded as a core component of Notch signaling. Epsin appears to be required in every Notch signaling context tested. Thus, the expectation is

that Epsin would function in the same manner in all tissues. Although my experiments do not test this notion rigorously, the results presented here are most consistent with the idea that the requirements for the different Epsin modules are at least similar in different signaling contexts. The three assays we used for Epsin activity – rescue of lethality and eye morphology of a *lqf* null, rescue of eye morphology of a *lqf* hypomorph, and rescue of the ability of *lqf* null cells to activate Cut expression (a marker for Notch activation) in cells at the D/V boundary in the wing disc (Bomsoo Cho, personal communication) – may have different detection sensitivities and different response thresholds. In addition, I measured protein levels in eye discs, and it is possible that expression in different tissues may not be proportional due to position differentially effecting different cell types. Both of these effects likely contributed to the differences in behavior of a few of the constructs in the different assays. Yet, I have never observed even one case where a module appeared to be dispensable in one assay, and essential in another in Notch signaling.

2.3.5. A model for Epsin function in Notch ligand cells

Notch ligands require ubiquitination and (usually) Clathrin-dependent endocytosis, and formation of Clathrin-coated vesicles requires adapter proteins that link the plasma membrane with Clathrin (Owen et al., 2004). The absolute necessity of at least one UIM and the observation that the lipid-binding function of the ENTH domain plays a role in ligand cells suggests that Epsin indeed binds ubiquitinated Notch ligands at the plasma membrane. However, as an Epsin derivative lacking CBMs functions in Notch signaling, the essential role of Epsin in Notch signaling cannot be as a monomeric Clathrin adapter that links Clathrin directly to ligand at the plasma membrane. As any pair of the three types of modules is sufficient for Epsin function (CBMs+DPWs, CBMs+NPFs, or DPWs+ NPFs), Epsin must be able to link ligand to Clathrin in a variety

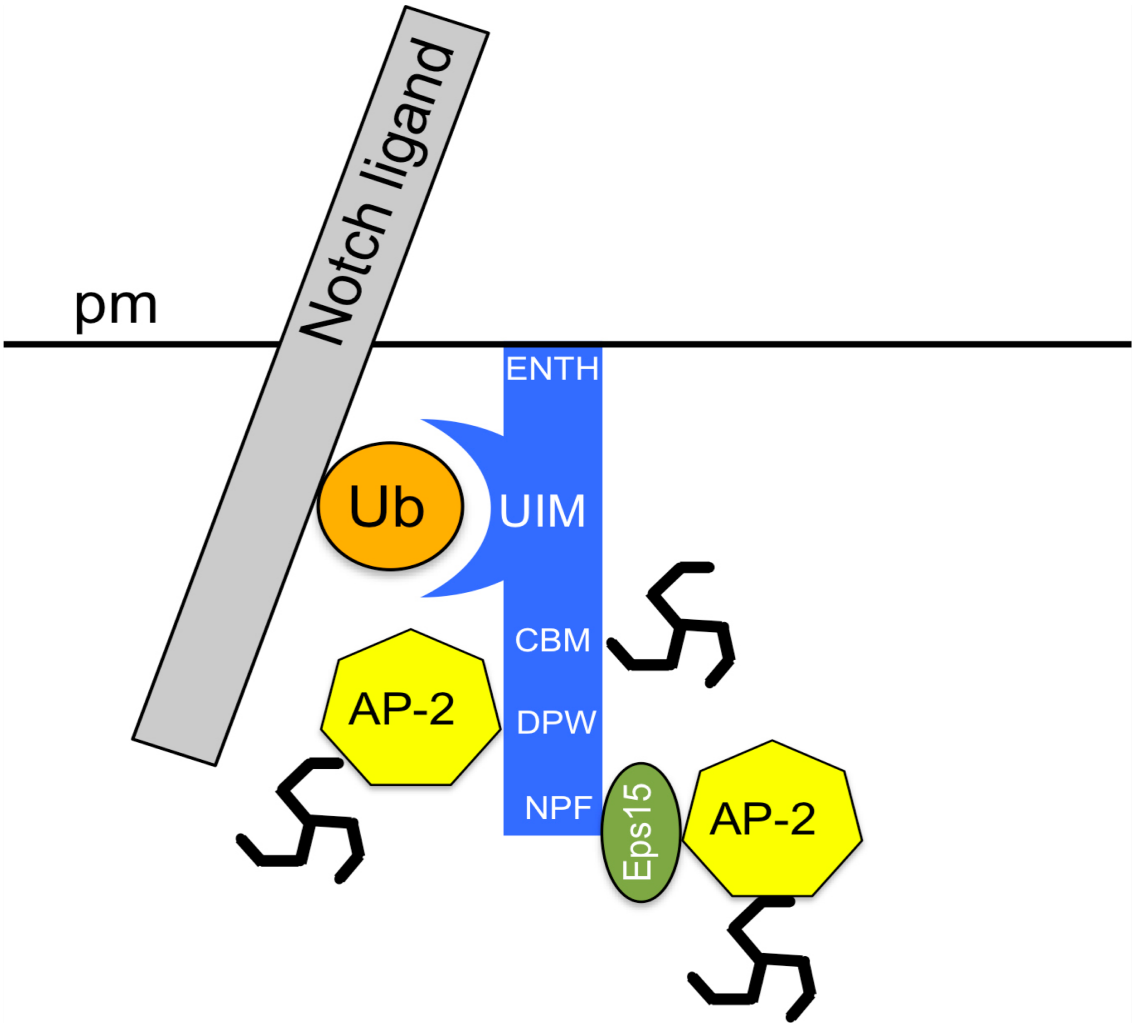


Figure 2.7. A model for Epsin function in Notch ligand cells. We propose that in its function in Notch ligand cells, Epsin serves as the cargo selection subunit of a Clathrin adapter. Through its UIMs, Epsin binds ubiquitinated Notch ligands at the plasma membrane. The ENTH domain aids in Epsin localization to the plasma membrane. The Epsin C-terminus binds Clathrin both directly and indirectly to promote Clathrin-dependent ligand endocytosis. Epsin cooperates with another Clathrin-binding protein, likely AP-2, and also with an EH-domain protein, likely Eps15.

of different ways (Fig 9). I speculate the involvement of Eps15 because Eps15 is the only one with motifs for a known Clathrin-binding protein (AP-2) among the three EH-domain proteins in *Drosophila*. From analysis of mutant phenotypes and genetic interaction studies, there is no evidence for involvement of Eps15 nor AP-2 in Notch signaling. The

results presented here suggest that Eps15 and AP-2 may be redundant with each other in the presence of intact Epsin. A test of this idea requires further experiments. In light of the evidence indicating a requirement for Clathrin in ligand cells, the results presented here suggest the idea that Epsin is required absolutely for Notch signaling because it is the only UIM-containing endocytic protein with the appropriate complement of interaction modules to target ubiquitinated Notch ligands to Clathrin-coated vesicles.

Chapter 3. Significance and future direction

3.1. The importance of using a genomic *lqf* DNA fragment to perform structure/function analysis of *Drosophila* Epsin in Notch signaling

Epsin contains five types of modules for interaction with phospholipids or proteins (Chen et al., 1998). It interacts with PIP2, Ubiquitin, Clathrin, AP-2, or EH-domain containing proteins. The different interaction motifs in Epsin suggest several possible functions of Epsin inside the cell. Some of the interacting proteins are essential to perform general functions of the cell, like Clathrin and AP-2 in Clathrin-dependent endocytosis. This makes it critical to control the level of Epsin in the cell. Over-expression of an Epsin fragment containing the DPW motifs disrupted the endocytosis of transferrin and EGFR in cultured cells (Chen et al., 1998). And over-expression of the full-length protein of *Drosophila* Epsin with an Act5C promoter kills the animal.

To precisely analyze how Epsin functions in Notch signaling, it is critical to express Epsin variants at endogenous levels in cells that endogenously express Epsin, and at stages in development when endogenous Epsin is expressed. Previous studies using a *ro-hs* promoter showed that either part (divided after the ENTH domain) of *Drosophila* Epsin functions in Notch signaling during eye development (Overstreet et al., 2003). This result is quite different from my results. When I conducted the functional analysis of Epsin using a genomic fragment containing the *lqf* gene, the ENTH domain failed to provide any detectable activity. Even an extended N-terminal protein containing the ENTH domain and the UIMs did not provide any activity. Epsin requires the ENTH domain, a UIM, and one of the C-terminal motifs to function in signaling cells. One way to explain the discrepancy is that the over-expression of the ENTH domain under the *ro-hs* promoter generated a gain-of-function effect, which enables the ENTH domain to

perform a novel function to promote ligand endocytosis. Alternatively, the ENTH domain may contain an unknown binding site for the Notch ligands. It can route the ligands to the endocytic vesicles when over-expressed. However, it fails to recruit the ligands to the vesicles when expressed close to the endogenous level efficiently. The vesicle binding could function through the single DPW motif in the ENTH domain, or the Cdc42-GAP interaction (see 1.4.2). The confusing results from the over-expression of partial Epsin proteins emphasize the importance of using a genomic fragment to conduct the functional analysis of *Drosophila* Epsin. This might be true to study any kind of gene.

Another advantage of using a genomic fragment is the possibility to study multiple developmental events under physiological conditions with the same set of transgenes. We have used the same transgenes to study Notch signaling in wing development. They could also be used to study Notch signaling during oogenesis, which does not require Clathrin. It will be interesting to determine which motifs of Epsin are important in that context. In addition to Notch signaling, Epsin has also been shown to be involved in synaptic vesicle endocytosis, mitosis, and autophagy (Bao et al., 2008; Csikos et al., 2009; Jakobsson et al., 2008; Liu and Zheng, 2009). The transgenes I generated can easily be used to determine which modules of Epsin are required in those biological events.

3.2. Epsin's role in "pulling" model or "recycling" model

Epsin has been indicated to define a specialized endosomal pathway that allows the ligands to be processed into active forms (Wang and Struhl, 2004). If this is the case, I expect that the Epsin-mediated endosomes are different from the general AP-2-mediated Clathrin-coated vesicles. Epsin may function as a Clathrin adapter to form endosomes without AP-2, or recruit unique proteins to form a recycling endosome. My

results showed that the CBMs are not required at all, which ruled out the possibility that Epsin functions as a Clathrin adapter. The ENTH domain functions to bind PIP2 to facilitate ligand endocytosis. And the DPWs or NPFs are not required individually. These results demonstrated that the ENTH domain, DPWs, or NPFs, are not likely to play an essential role to recruit special proteins to form recycling endosomes. The UIMs are the only indispensable modules for Epsin's role in ligand signaling. Since ligand ubiquitination is required to signal, the UIMs are likely to function to recruit the ubiquitinated cargos. Thus, my structure/function analysis of *Drosophila* Epsin does not favor the "recycling" model. Epsin can neither function as a Clathrin adapter, nor use one of its modules to define a "recycling" endosome. In stead, my work is consistent with the "pulling" model very well. Epsin uses the UIMs to bind the ubiquitinated ligands, and one of the C-terminal motifs to recruit the ligand-receptor complex to the CCVs to promote the ligand endocytosis. The ENTH domain facilitates the ligand endocytosis by binding to PIP2 on the plasma membrane. And the endocytosis of the ligands generates a pulling force to trigger S2 cleavage of the ligand-bound Notch receptor.

3.3. Epsin activity is regulated by ubiquitination

Epsin protein is regulated by ubiquitination. The UIMs are necessary and sufficient for ubiquitination of *Xenopus* Epsin and mouse Eps15 (Oldham et al., 2002; Polo et al., 2002). I decided to test whether the two UIMs of *Drosophila* Epsin are required for self-ubiquitination. *Drosophila* Fat Facets (Faf) up-regulates Epsin activity by deubiquitinating the protein (Chen et al., 2002). In protein extracts from *faf-* animals, ubiquitinated forms of Epsin are detectable, usually containing one-to-four ubiquitins (Chen et al., 2002). In order to stabilize ubiquitinated Epsins, I expressed the mutant Epsins in the *faf* null background. A GFP purification kit was used to purify and

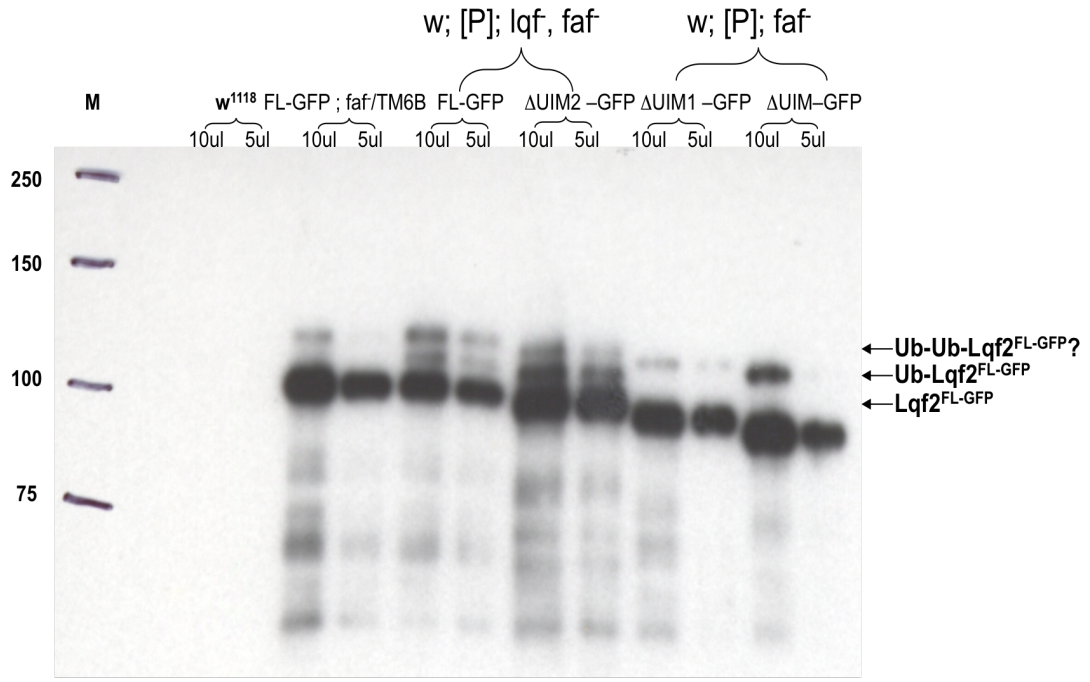


Figure 3.1. UIMs are required for the ubiquitination of *Drosophila* Epsin. A western blot assay to determine the ubiquitination of the Epsin variants. GFP tagged Epsin variants were purified by GFP purification kit (130-091-288) with Protease Inhibitor Cocktail. And the proteins was detected with a GFP antibody.

concentrate the Epsin variants. After that, western blot was used to determine whether or not the mutant Epsin proteins are ubiquitinated.

The results showed that a single UIM is necessary and sufficient for Epsin ubiquitination *in vivo* (Fig.3.1). Compelling genetic evidences indicate that ubiquitination of *Drosophila* Epsin inhibits Epsin function in Notch signaling (Cadavid et al., 2000; Chen et al., 2002; Overstreet et al., 2004). Thus, the correlation between function in Notch signaling and ubiquitination observed in mutant Epsins most likely does not reflect a requirement for Epsin ubiquitination for signaling, but rather suggests that a common aspect of the UIM activity, possibly ubiquitin binding, is necessary both for Epsin ubiquitination and Epsin function in Notch signaling.

Preliminary results suggested that a functioning UIM is needed for ubiquitination. However, this result does not help us to answer the question of whether or not ubiquitination is important for Epsin function. Whether Epsin is multiple mono-ubiquitinated, or is attached with ubiquitin chains, and how these chains might be linked, are unknown. It will be helpful to determine the position and nature of the ubiquitin tag on Epsin.

Ubiquitin molecule (8kDa) is small compared to the *Drosophila* Epsin protein (68.4kDa), especially when Epsin is tagged with GFP (95.7kDa). This makes it hard to separate the ubiquitinated and un-ubiquitinated Epsins. The UIMs from different proteins has been shown to promote ubiquitination with an orientation-dependent manner (Miller et al., 2004). If Drosophila Epsin is ubiquitinated with the same manner, then the ubiquitination sites are very likely to reside in the ENTH domain. And the ENTN-UIM protein should be ubiquitinated the same way as the full-length protein. Since the ENTH-UIM-GFP (57.4kDa) is much smaller than the full-length Epsin-GFP (95.7kDa), it should be much easier to isolate and purify the ubiquitinated proteins for MASS-SPEC analysis.

3.4. Why is Epsin the only protein that can trigger ligand endocytosis and Notch signaling

The systematic structure/function analysis I performed suggested that *Drosophila* Epsin functions to link the ubiquitinated Notch ligands to the endocytic vesicles to promote their endocytosis. If this is the case, any protein that contains modules to interact with ubiquitin and endocytic vesicle-residing proteins should enable the ligands to signal. There are lots of proteins that contain ubiquitin-interaction motifs (Mueller and Feigon, 2003; Miller et al., 2004; Hirano et al., 2006). Some of them also contain AP-2 interaction module, for example, Eps15. However, Eps15 has been indicated to

negatively regulate Notch signaling (Tang et al. 2005), not function positively in the signaling cells. It seems that Epsin is required for any Notch signaling events.

What makes Epsin unique in activating Notch signaling? One of the reasons could be the position of the UIMs. Epsin contains two UIMs close to the N-terminus, while Eps15 has two UIMs at the C-terminus. Since Notch ligands are likely to be ubiquitinated at specific lysines (Glittenberg et al., 2006; Parks et al., 2006), only correctly positioned UIMs can get access to the ubiquitins. Another reason might be the expression pattern and level of the proteins. To activate a Notch receptor, one molecule of the ligand needs to bind the receptor and be internalized through Epsin to trigger the S2 cleavage of the receptor. After that, the receptor is further processed and the NICD is trafficked into the nucleus to function as a transcription factor (see 1.1). To activate the target genes, the level of the NICD needs to build up to reach a threshold concentration. That requires lots of Notch processing and thus lots of ligand endocytosis. Epsin is an abundant protein that is ubiquitously expressed in all kinds of tissues (Chen et al., 1998). This makes it possible for Epsin to promote a large amount of ligand endocytosis, which is not feasible for other proteins that are not abundant or ubiquitous. One way to test this idea is to drive the expression of the candidate genes under the control of the regulatory elements of the 16kb *lqf* gene. Another way to test the function model of Epsin is to generate an artificial protein that contains the ligand-interaction motifs (for example, NHR in Neur, Fig1.3) and the C-terminus of Epsin. This experiment should help to determine whether the essential function of the UIMs of *Drosophila* Epsin is to bind the Notch ligands.

Appendices

Appendix 1. Generation of Epsin deletion P element and PhiC31 integrase transgenes and transformants

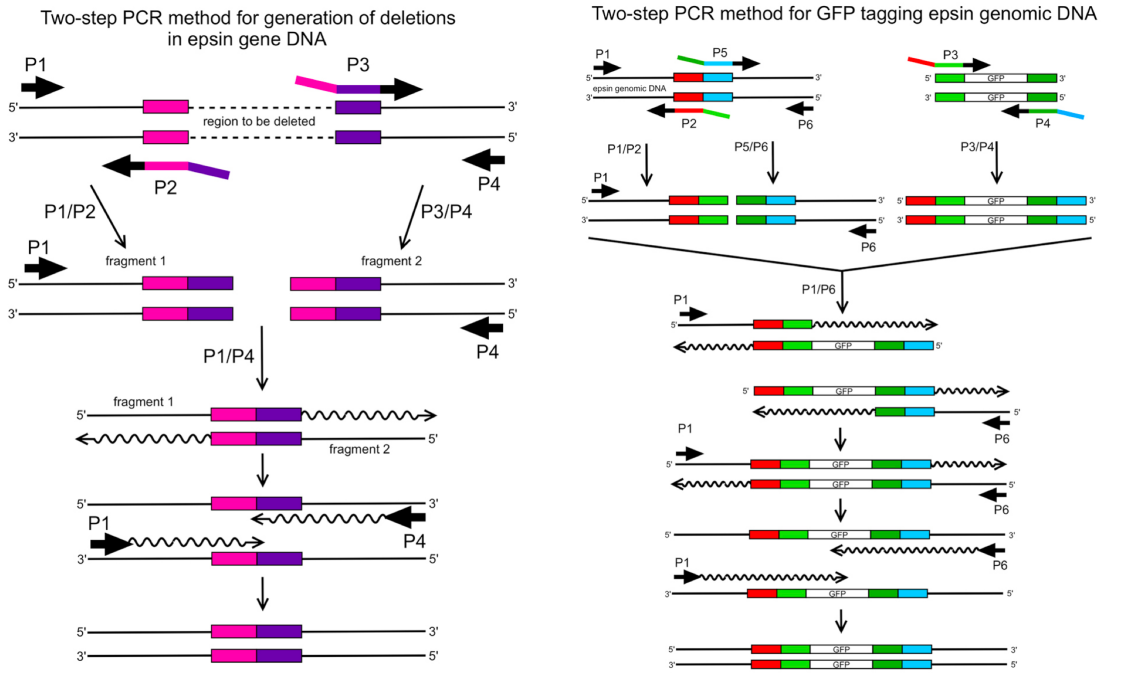


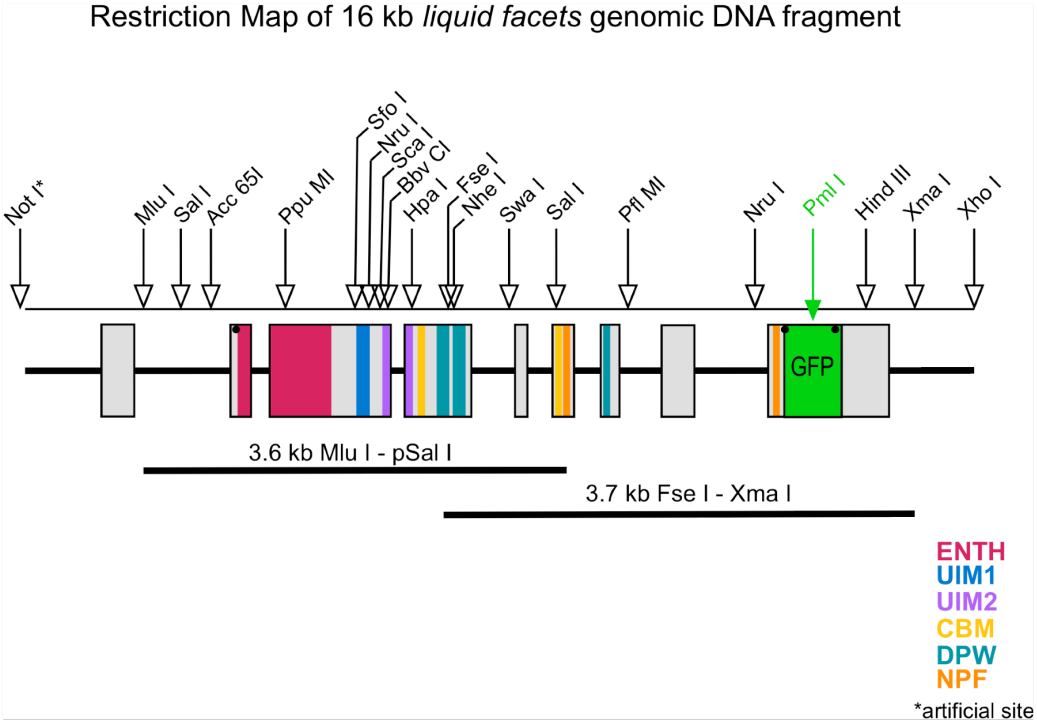
Fig.A.1. Methods for generation of deletions and insertion of GFP using PCR.
Left: A diagram of the PCR technique used to generate deletions in *lqf* genomic DNA is shown. The black lines represent single DNA strands. P1-P4 are PCR primers. The pink and purple bars are 20-29 nt long. Two separate PCR reactions using *lqf* genomic DNA as the template generate fragments 1 and 2, which are used as a mixed template for a second PCR reaction. The final amplification product is a portion of the *lqf* genomic DNA sequence that contains the deletion.
Right: A diagram of the PCR technique used to insert GFP coding sequences in-frame at the 3' end of the *lqf* coding region. The black lines represent single DNA strands. P1-P6 are PCR primers. The red, blue, light green, and dark green bars are 20-29 nt long. Three separate PCR reactions, the two at left using *lqf* genomic DNA as template, and the one at right using a *gfp*-containing plasmid as template, generate the three DNA fragments shown. All three fragments are used subsequently as a mixed template for a second PCR reaction with primers 1 and 6. The final amplification product is a portion of the *lqf* genomic DNA sequence containing *gfp* fused in-frame to the 3' end of *lqf*.

Deletion mutants (except for ΔUIM and ΔUIM2) were generated by a PCR-based method (Fig.A.1). This PCR method was also used to fuse GFP in-frame to the Epsin C-terminus (Fig.A.1). A list of primers used for each construct, complete construction details, and the amino acid content of each Epsin variant are shown below. All constructs were ligated ultimately as *Not I – Xho I* fragments into *pCaSpeR4* (Thummel and Pirrotta,

1992) or into an attB vector that I constructed called *pCaSper4-attB*. Complete details of the vector construction and a map are in Fig.A.2. P element transformation was by Genetic Services (Sudbury, MA) or Genetivision (Houston, TX) and PhiC31 integrase transformation (into site VK37 at polytene band 22A3) was by Genetivision.

The deletions in constructs *ΔUIM* and *ΔUIM2* were generated using a different method than the others. The general idea of the method was to generate two PCR amplification products that flank the deletion, where the 3'-end of the upstream fragment (UF) and the 5'-end of the downstream fragment (DF) each contain blunt-end introduced restriction sites. The restricted fragments are ligated together into Bluescript (Stratagene) to generate the deletion, and an *Mlu I – partial Sal I* fragment containing the deletion was substituted for wild-type gene sequences by inserting it into a plasmid containing construct A, restricted at unique *Mlu I* and *Sal I* sites, upstream and downstream of the deletion, respectively. Constructs *ΔUIM* and *ΔUIM2* were generated by substituting a *Sal I* fragment containing each deletion for a corresponding *Sal I* fragment in transgene *FL*.

A precursor to construct *FL* (construct A) without the GFP-tag was generated by ligating into *pCaSpeR4* restricted with *Not I* and *Xho I*, an ~16,240 bp *Not I – Xho I* fragment of *Drosophila* genomic DNA containing the *lqf* gene, obtained from a subclone called 19G (Cadavid et al., 2000). A map of the *Not I – Xho I* fragment showing restriction sites used to make the constructs is below. To generate construct *FL*, the GFP coding region was amplified from *ro-hs-GFP* (Overstreet et al., 2004). The PCR-generated deletions were each ligated first into one of two smaller *lqf* genomic DNA subclones, containing either a 3.6 kb *Mlu I – Sal I* fragment or a 3.7 kb *Fse I – Xma* fragment.



A.1.1. Genomic deletion constructs

pGEM-T-GFP:

P1 : 5'- CTATGAGCTGCTGAATGGGGAGTGC -3'

P2 : 5'-

ACTCATAGCAGCAGCAGCAGCAGCCGACAAAAACGGATTTGTTGCTGC-3'

P_G1:

5'-

TTGTCGGCTGCTGCTGCTGCTGCTATGAGTAAAGGAGGAGAACTTTTCACTGG

AGAAC-3'

P_G2: 5'- CGCCAGTTTATTTGTATAGTTCATCCATGCCATGTGTAATCC-3'

P3: 5'-ACTATACAAATAAACTGGCGTCGATCAATCACAACAATG-3'

P4: 5'- GCAAATGTGTTAGACTCAAGTTCAGTTTACTTGTAAGC -3'

I used Erin's pGEM-3'-GFP vector as template to amplify GFP sequence.

NruI + HindIII to make BD23-GFP

In the context of BD5_FseI-XmaI, add BD23-GFP_FseI-XmaI to make BD5-GFP

NotI + XhoI to make pCaSpeR4-FL-GFP

Maxi-prep for injection

pGEM-T-ENTH-GFP:

P1: 5'-CGCACAACTACTCCGATGCC-3'

P2: 5'-CAGCCGACAAGCCGGCCGTCTGG-3'

P3: 5'-GACGGCCGGCTTGTCGGCTGCTGCTGCTG-3'

P4: 5'-AGCACGTGTCTTGTAGTTCCCGTC-3'

In the context of BD5-FL-GFP_PpuMI-PmlI digestion fragment, add

pGEM-T-ENTH-GFP_PpuMI-PmlI fragment to make BD5-ENTH-GFP

NotI + XhoI to make pCaSpeR4-ENTH-GFP

Maxi-prep for injection

pGEM-T-ENTH-UIM-GFP:

P1: 5'-CGCACAACTACTCCGATGCC-3' (same with pGEM-T-ENTH-GFP-P1)

P2: 5'- CAGCCGACAAATGACTCTGTTGTTCCTCCTTCTTGG-3'

P3: 5'-ACAGAGTCATTTGTCGGCTGCTGCTGC-3'

P4: 5'-AGCACGTGTCTTGTAGTTCCCGTC-3' (same with pGEM-T-ENTH-GFP-P4)

In the context of BD5-FL-GFP_PpuMI-PmlI digestion fragment, add

pGEM-T-ENTH-UIM-GFP_PpuMI-PmlI fragment to make BD5-ENTH-UIM-GFP

NotI + XhoI to make pCaSpeR4-ENTH-UIM-GFP

Maxi-prep for injection

pGEM-T-ΔENTH:

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3'

P2: 5'-GCGCCTTCACCATATCGTCCTTTTGCTTTCTC-3'

P3: 5’-GGACGATATGGTGAAGGCGCAGAAGGCAAAG-3’

P4: 5'- CGTTCTCTACTCACTTGAAATCCTGCTC -3'

Acc65I + BbvCI to make BD28-ΔENTH

In the context of BD5 (or BD5-GFP)_SalI, add BD28-ΔENTH_SalI digestion fragment to make BD5-ΔENTH (or BD5-ΔENTH-GFP)

NotI + XhoI to make pCaSpeR4-ΔENTH (or pCaSpeR4-ΔENTH-GFP)

Maxi-prep for injection

pGEM-T-ΔCBM1:

P1: 5'-AGTGATGATGTGCGTCTGCAACTC -3'

P2: 5’- GAGAAATATCATGACTCTGTTGTTCCTCCTTCTTG-3’

P3: 5'-ACAGAGTCATGATATTTCTCTGGGGGCTACGAGC -3'

P4: 5'-CGACAGCAGTGGATTTTATCAGCCAG -3'

BbvCI + FseI to make BD28-ΔCBM1

In the context of BD5-GFP_SalI, add BD28-ΔCBM1_SalI digestion fragment to make BD5-ΔCBM1-GFP

NotI + XhoI to make pCaSpeR4-ΔCBM1-GFP

pGEM-T-ΔCBM2:

P1: 5'- CGGCAAATAATGGTAGCTCATCTTCG -3'

P2: 5’- TCGGTTTGATCGCAGAGTTCTCGCCGAG-3’

P3: 5'-GAACTCTGCGATCAAACCGATTGC -3'

P4: 5'- CACCGCAGACGCACGAAATC -3'

NheI + PflMI to make BD23-ΔCBM2-GFP

In the context of BD5_FseI-XmaI, add BD23-ΔCBM2-GFP_FseI-XmaI digestion fragment to make BD5-ΔCBM2-GFP

NotI + XhoI to make pCaSpeR4-ΔCBM2-GFP

pGEM-T-ΔNPF-GFP:

P1: 5'- CGGCAAATAATGGTAGCTCATCTTCG -3' (same as pGEM-T-ΔCBM2-P1)

P2: 5'- CAGCCGACAAGTACGCCGGCTGATTACCCG -3'

P3: 5'-GCCGGCGTACTTGTCGGCTGCTGCTGCTG-3'

P4: 5'- AGCACGTGTCTTGTAGTTCCCGTC -3' (same as ENTH-P4)

NheI + PmlI to make BD23-ΔNPF-GFP

In the context of BD5_FseI-XmaI, add BD23-ΔNPF-GFP_FseI-XmaI digestion fragment to make BD5-ΔNPF-GFP

In the context of BD5-ΔNPF-GFP_SalI, add BD28-ΔENTH_SalI digestion fragment to make BD5-ΔENTH−ΔNPF-GFP

NotI + XhoI to make pCaSpeR4-ΔNPF-GFP (or pCaSpeR4-ΔENTH−ΔNPF-GFP)

Maxi-prep for injection

pGEM-T-ΔDPW1:

P1: 5'-AGTGATGATGTGCGTCTGCAACTC -3' (same as pGEM-T-ΔCBM1-P1)

P2: 5'- CGGAGGGATTGACAACAGCCGTGG-3'

P3: 5'-GGCTGTTGTCAATCCCTCCGCTGCCCCAC-3'

P4: 5'-CGACAGCAGTGGATTTTATCAGCCAG -3'

BbvCI + FseI to make BD28- ΔDPW1

In the context of BD5-GFP_SalI, add BD28-ΔDPW1_SalI digestion fragment to make BD5-ΔDPW1-GFP

NotI + XhoI to make pCaSpeR4-ΔDPW1-GFP

pGEM-T-ΔDPW2:

P1: 5'-AGTGATGATGTGCGTCTGCAACTC -3' (same as pGEM-T-ΔCBM1-P1)

P2: 5'- CGAGTGCTTTCGAAGATGAGCTACCATTATTTGCCG-3'

P3: 5'- CTCATCTTCGAAAGCACTCGGAACTGG-3'

P4: 5'-CATTGTTGTTGTTGGAGGCGTTAG-3'

FseI + SwaI to make BD28-ΔDPW2

In the context of BD5-GFP_SalI, add BD28-ΔDPW2_SalI digestion fragment to make BD5-ΔDPW2-GFP

NotI + XhoI to make pCaSpeR4-ΔDPW2-GFP

pGEM-T-ΔCBM1-ΔDPW1:

In the context of pGEM-T-dCBM1, use the same primers to generate pGEM-T-ΔDPW1

P1: 5'-AGTGATGATGTGCGTCTGCAACTC -3' (same as pGEM-T-ΔCBM1-P1)

P2: 5'- CGGAGGGATTGACAACAGCCGTGG-3'

P3: 5'-GGCTGTTGTCAATCCCTCCGCTGCCCCAC-3'

P4: 5'-CGACAGCAGTGGATTTTATCAGCCAG -3'

In the context of BD28_BbvCI-FseI digestion fragment, add pGEM-T-ΔCBM1-ΔDPW1_ BbvCI-FseI fragment to make BD28-ΔCBM1-ΔDPW1

BD28-ΔDPW:

In the context of BD28-dDPW2_BbvCI-FseI digestion fragment, add pGEM-T-ΔDPW1_ BbvCI-FseI fragment to make BD28-ΔDPW

In the context of BD5-GFP_SalI, add BD28-ΔDPW_SalI digestion fragment to make BD5-ΔDPW-GFP

NotI + XhoI to make pCaSpeR4-ΔDPW-GFP

Maxi-prep for injection

BD5-ΔCBM-GFP (or BD5-ΔCBM-ΔDPW1-GFP):

In the context of BD5-ΔCBM2-GFP_SalI digestion fragment, add BD28-dCBM1 (or BD28-ΔCBM1-ΔDPW1)_SalI fragment to make BD5-ΔCBM-GFP (or BD5-ΔCBM-ΔDPW1-GFP)

NotI + XhoI to make pCaSpeR4-ΔCBM-GFP (or pCaSpeR4-ΔCBM-ΔDPW1-GFP)

Maxi-prep for injection

BD5-ΔCBM-ΔDPW-GFP:

In the context of BD28-ΔDPW2_BbvCI-FseI digestion fragment, add

pGEM-T-ΔCBM1-ΔDPW1_ BbvCI-FseI fragment to make BD28-ΔCBM1-ΔDPW

In the context of BD5-ΔCBM2-GFP_SalI digestion fragment, add

BD28-ΔCBM1-ΔDPW_SalI fragment to make BD5-ΔCBM-ΔDPW-GFP

NotI + XhoI to make pCaSpeR4-ΔCBM-ΔDPW-GFP

Maxi-prep for injection

BD5-ΔENTH-ΔCBM-GFP (BD5-ΔENTH-ΔDPW-GFP):

In the context of BD28-ΔCBM1 (or BD28-ΔDPW)_Acc65I-BbvCI digestion fragment,

add BD28-ΔENTH_Acc65I-BbvCI fragment to make

BD28-ΔENTH-ΔCBM1 (or BD28-ΔENTH-ΔDPW)

In the context of BD5-ΔCBM2-GFP (or BD5-GFP)_SalI digestion fragment, add

BD28-ΔENTH-ΔCBM1 (or BD28-ΔENTH-ΔDPW)_SalI fragment to make

BD5-ΔENTH-ΔCBM-GFP (or BD5-ΔENTH-ΔDPW-GFP)

NotI + XhoI to make pCaSpeR4-ΔENTH-ΔCBM-GFP (or pCaSpeR4-ΔENTH-ΔDPW-

GFP)

Maxi-prep for injection

pBS-ΔUIM1:

P1: 5'- ACGCGTGGTCTTACTTTG -3'

P2: 5'-GGC/GCCGGCCGTCTGGGGG

MluI + SfoI to make pBS−ΔUIM1-F1

P3: 5'- TCG/CGACGCAGTGATGAT-3'

P4: 5'-GTCGACGGATGCATACTG-3'

NruI + SalI to make pBS−ΔUIM1-F2

In the context of pBS−ΔUIM1-F1_SfoI-SalI digestion fragment, add

pBS−ΔUIM1-F2_NruI-SalI fragment to make pBS−ΔUIM1

In the context of BD5_MluI-SalI digestion fragment, add

pBS-ΔUIM1_MluI-SalI to make BD5−ΔUIM1

NotI + XhoI to make pCaSpeR4-ΔUIM1

Maxi-prep for injection

pBS-ΔUIM2:

P1: 5'- ACGCGTGGTCTTACTTTG -3' (same as pBS-ΔUIM1-P1)

P2: 5'- AGT/ACTGCGTCGCTTGGC-3'

MluI + ScaI to make pBS−ΔUIM2-F1

P3: 5'- GTT/AACGGACGACCTATT-3'

P4: 5'-GTCGACGGATGCATACTG-3' (same as pBS-ΔUIM1-P4)

HpaI + SalI to make pBS−ΔUIM2-F2

In the context of pBS−ΔUIM2-F1_Mlu-ScaI digestion fragment, add

pBS−ΔUIM2-F2_HpaI-SalI to make pBS−ΔUIM2

In the context of BD5_MluI-SalI digestion fragment, add

pBS-ΔUIM2_MluI-SalI to make BD5−ΔUIM2

NotI + XhoI to make pCaSpeR4-ΔUIM2

Maxi-prep for injection

pBS-ΔUIM:

In the context of pBS−ΔUIM1-F1_SfoI-SalI digestion fragment, add

pBS−ΔUIM2-F2_MluI-HpaI to make pBS−ΔUIM

In the context of BD5_MluI-SalI digestion fragment, add

pBS-ΔUIM_MluI-SalI to make BD5−ΔUIM

NotI + XhoI to make pCaSpeR4-ΔUIM

Maxi-prep for injection

In the context of BD5-FL-GFP_SalI digestion fragment, add

pBS-ΔUIM1 (or pBS-ΔUIM2, or pBS-ΔUIM)_SalI fragment to make

BD5-ΔUIM1-GFP (or BD5-ΔUIM2-GFP, or BD5-ΔUIM-GFP)

NotI + XhoI to make

pCaSpeR4-ΔUIM1-GFP (or pCaSpeR4-ΔUIM2-GFP, or pCaSpeR4-ΔUIM-GFP)

Maxi-prep for injection

In the context of BD28-ΔCBM1 (or BD28- ΔCBM1-ΔDPW)_Acc65I-BbvCI digestion fragment, add pBS−ΔUIM1_Acc65I-BbvCI fragment to make

BD28- ΔUIM1-ΔCBM1 (or BD28- ΔUIM1-ΔCBM1-ΔDPW)

In the context of BD5-FL-GFP_SalI digestion fragment, add BD28-ΔUIM1-ΔCBM1_ SalI fragment to make BD5-ΔUIM1-ΔCBM1-GFP

In the context of BD5-ΔCBM2-GFP_SalI digestion fragment, add BD28-ΔUIM1-ΔCBM1-ΔDPW)_ SalI fragment to make BD5-ΔUIM1-ΔCBM-ΔDPW-GFP

NotI + XhoI to make

pCaSpeR4-ΔUIM1-ΔCBM1-GFP (or pCaSpeR4-ΔUIM1-ΔCBM-ΔDPW-GFP)

Maxi-prep for injection

pGEM-T-ΔENTH-ΔUIM1

In the context of pBS−ΔUIM1, use the same primers to generate pGEM-T-ΔENTH

P1: 5'-GTAACACATTCCACATACGTTCCACAC-3'

P2: 5'-GCGCCTTCACCATATCGTCCTTTTGCTTTCTC-3'

P3: 5'-GGACGATATGGTGAAGGCGCAGAAGGCAAAG-3'

P4: 5'- CGTTCTCTACTCACTTGAAATCCTGCTC -3'

Acc65I + BbvCI to make BD28-ΔENTH-ΔUIM1

In the context of BD5_SalI digestion fragment, add BD28-ΔENTH-ΔUIM1_SalI fragment to make BD5-ΔENTH-ΔUIM1

NotI + XhoI to make pCaSpeR4-ΔENTH-ΔUIM1

Maxi-prep for injection

pGEM-T-ΔENTH-ΔUIM2 (or pGEM-T-ΔENTH-ΔUIM)

In the context of pBS−ΔUIM2 (or pBS−ΔUIM), use the same primers to generate pGEM-T-ΔENTH

P1: 5'-GTAACACATTCCACATACGTTCCACAC-3' (same as pGEM-T-ΔENTH-P1)

P2: 5'-GCGCCTTCACCATATCGTCCTTTTGCTTTCTC-3' (same as pGEM-T-ΔENTH-P2)

P3: 5'-GGACGATATGGTGAAGGCGCAGAAGGCAAAG-3' (same as pGEM-T-ΔENTH-P3)

P4: 5'-GGAGGGCCGGCCGGAG-3'

Acc65I + FseI to make BD28-ΔENTH-ΔUIM2 (or BD28-ΔENTH-ΔUIM)

In the context of BD5_SalI digestion fragment, add BD28-ΔENTH-ΔUIM2 (or BD28-ΔENTH-ΔUIM)_SalI fragment to make BD5-ΔENTH-ΔUIM2 (or BD5-ΔENTH-ΔUIM)

NotI + XhoI to make pCaSpeR4-ΔENTH-ΔUIM2 (or pCaSpeR4-ΔENTH-ΔUIM)

Maxi-prep for injection

pGEM-T-UIM1-to-UIM2

In the context of pBS−ΔUIM2, use the following primers

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'-ATCCTGCTCACTCTGGCTGAGGGCGAGTTGCAGACGCACATCATCCTCGCCGGCCGTC-3'

P3: 5'-

GATGATGTGCGTCTGCAACTCGCCCTCAGCCAGAGTGAGCAGGATGCGGAAC

AGGAGGAG-3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

Acc65I + FseI to make BD28-UIM1-to-UIM2

In the context of BD5-GFP_SalI digestion fragment, add BD28-UIM1-to-UIM2_SalI digestion fragment to make BD5-UIM1-to-UIM2-GFP

NotI + XhoI to make pCaSpeR4-UIM1-to-UIM2-GFP

Maxi-prep for injection

pGEM-T-UIM2-to-UIM1

In the context of pBS−ΔUIM1, use the following primers

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'-

TTCCTCTCGTGACATGGCCATGGCCAGCTGCAGTTGAAGTTCCTCACTGCGTC

GtCCaGC-3'

P3:5'-

GAGGAACTTCAACTGCAGCTGGCCATGGCCATGTCACGAGAGGAATTCAAGT

GAGTAGAGAACGTCTAATAAAAC-3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

Acc65I + FseI to make BD28-UIM2-to-UIM1

In the context of BD5-GFP_SalI digestion fragment, add BD28-UIM2-to-UIM1_SalI digestion fragment to make BD5-UIM2-to-UIM1-GFP

NotI + XhoI to make pCaSpeR4-UIM2-to-UIM1-GFP

Maxi-prep for injection

Construction of pCaSpeR4-attB

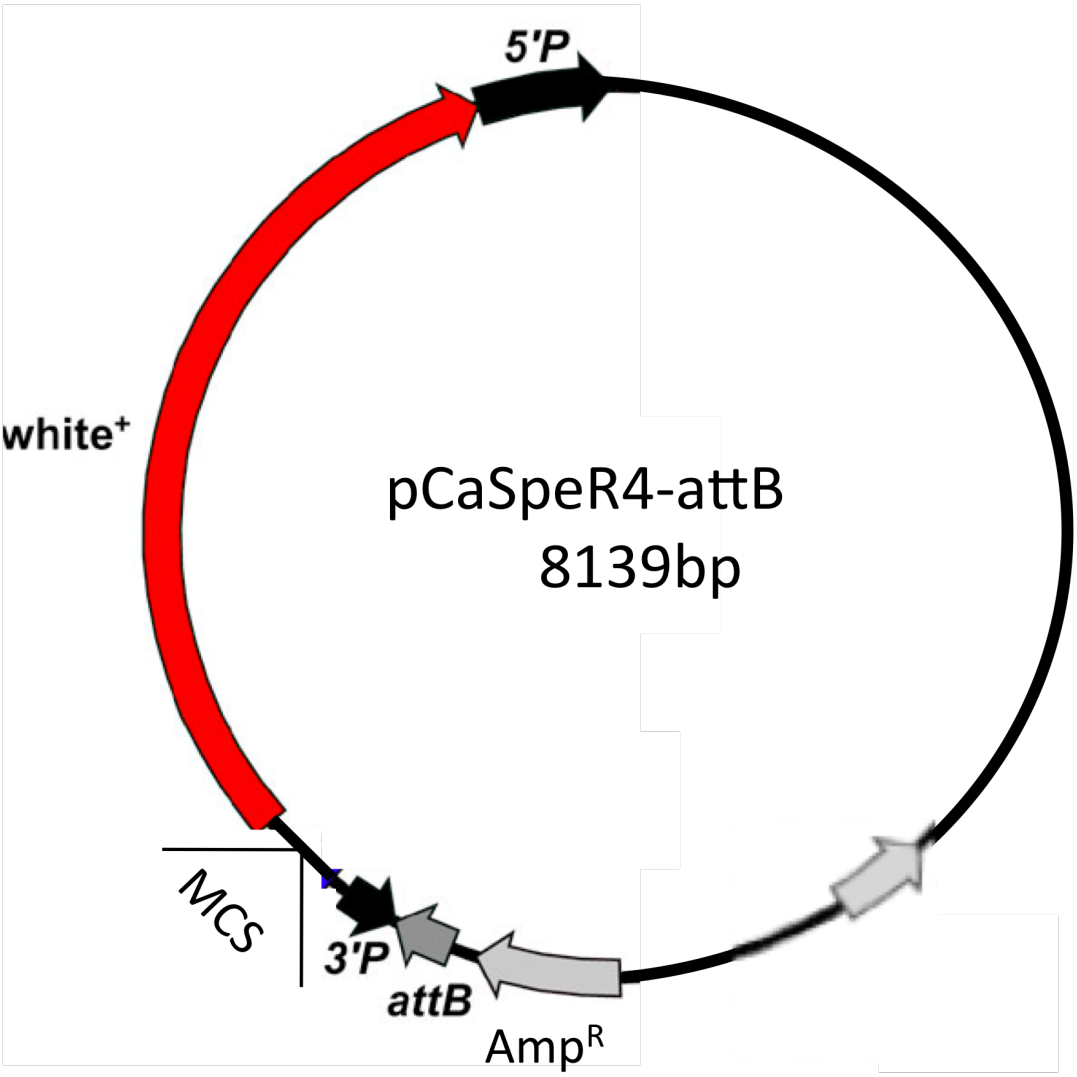


Figure A.2. attB-pCaSpeR4 vector. In the context of pCaSpeR4, use two-step PCR technique to add 284bp attB sequence (P[acman] from DGRC) between 3'P and AmpR sites.

The attB site was inserted into pCaSpeR4 using a two-step PCR protocol similar to that used for insertion of GFP into the *lqf* genomic DNA (see Fig.A.1).

• Use pCaSpeR4 as template and PCR primers P1-P2 and P3-P4 (below) to generate DNA fragments flanking the attB insertion site.

• Use P[acman] (DGRC) as a template and PCR primers attB_P1-attB_P2 to amplify attB sequences (284bp) with overhangs compatible with flanking fragments.

• Use primers P1-P4 and all three fragments generated above simultaneously as a template to amplify the pCaSpeR4-attB fragment, digest with Aat II + Not I.

• Ligate above fragment with pCaSpeR4 digested with Aat II-Not I.

In the context of pCaSpeR4, use the primers P1-P2 and P3-P4 to generate flanking

P1 : 5'- TGCCGCAAAAAAGGGAATAAGG -3'

P2 : 5'- CATCGTCGACGCTTCCGGGTGCTCGCATATC -3'

attB_P1: 5'- ACCCGGAAGCGTCGACGATGTAGGTCACG -3'

attB_P2: 5'- TCGACATGCCCGCCGTG -3'

P3: 5'- CACGGCGGGCATGTCGATCACGATGAGAATGGCCAG -3'

P4: 5'- CAAACGGTGGCGAAAGAGATAGC -3'

pCaSpeR4-attB-FL-GFP (or pCaSpeR4-attB–ΔENTH-GFP, or pCaSpeR4-attB-ΔUIM1-GFP, or pCaSpeR4-attB-ΔUIM2-GFP)

In the context of pCaSpeR4-attB_NotI-XhoI, add BD5-FL-GFP (or BD5-ΔENTH-GFP, or BD5-ΔUIM1-GFP, or BD5-ΔUIM2-GFP)_NotI-XhoI to make pCaSpeR4-attB-FL-GFP (or pCaSpeR4-attB–ΔENTH-GFP, or BD5-ΔUIM1-GFP, or BD5-ΔUIM2-GFP)

Maxi-prep for injection

BD5-mEpsin-CBM2X:

In the context of BD5-dUIM2-GFP, use the primers to generate mEpsin-CBM fragment, digest with PpuMI + PmlI

P1: 5'-GTAACACATTCCACATACGTTCCACAC-3' (same as pGEM-T-ΔENTH-P1)

P2: 5'

TGGCGCAATCGGTTTGATCAGATTGTCTAGGTTAACAAGCGCAGAGTTCTCG

CCGAGGAAAGAAGCCCCCAGAGAAATATCCAG-3'

P3: 5'-

TCTTTCCTCGGCGAGAACTCTGCGCTTGTTAACCTAGACAATCTGATCAAACC

GATTGCGCCATTGTCGGCTGCTGC -3'

P4: 5'- AGCACGTGTCTTGTAGTTCCCGTC -3' (same as ENTH-P4)

In the context of BD5-dUIM2-GFP_PpuMI + PmlI, add mEpsin-CBM_PpuMI + PmlI fragment to make BD5-mEpsin-CBM-GFP

In the context of BD5-dUIM2-GFP_PpuMI + FseI, add mEpsin-CBM-FseI_PpuMI + FseI digestion fragment to make BD5-mEpsin-CBM-FseI-GFP

P1: 5'-GTAACACATTCCACATACGTTCCACAC-3' (same as pGEM-T-ΔENTH-P1)

P2: 5' CCTCCCGGCCGGCCTGGCGCAATCGGTTTGATCAG 3'

In the context of BD5-mEpsin-CBM-FseI-GFP, use the primers P1-P2 to generate mEpsin-CBM2X-F1 fragment; In the context of BD5-GFP, use the primers P3-P4 to generate mEpsin-CBM2X-F2 fragment; use the primers P1-P4 to generate mEpsin-CBM2X fragment, digest with FseI + PmlI

P1: 5' GGAGGGCCGGCCCAAGAAGGAGGAACAAC 3'

P2: 5' CAGCCGACAATGGCGCAATCGGTTTGATCAG 3'

P3: 5'- GATTGCGCCATTGTCGGCTGCTGC -3'

P4: 5'- AGCACGTGTCTTGTAGTTCCCGTC -3' (same as ENTH-P4)

In the context of BD5-mEpsin-CBM-FseI-GFP_FseI-PmlI, add mEpsin-CBM2X_FseI-PmlI digestion fragment to make BD5-mEpsin-CBM2X-GFP

NotI + XhoI to make pCaSpeR4-attB-mEpsin-CBM2X-GFP

Maxi-prep for injection

BD5-mEpsin-DPW2X:

In the context of BD5-dUIM2-GFP, use the primers to generate mEpsin-DPW-FseI fragment, digest with PpuMI + PmlI

P1: 5'-GTAACACATTCCACATACGTTCCACAC-3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- CAACAGCCGTATGACTCTGTTGTTCCTCCTTCTTG-3'

P3: 5' ACAGAGTCATACGGCTGTTGTCGATCCCTG 3'

P4: 5' TACCATTATTGGCAGCGGAGGGATTCC 3'

P5: 5' CTCCGCTGCCAATAATGGTAGCTCATC 3'

P6: 5' CCTCCCGGCCGGCCTCCGAGTGCTTTCCATGGGTC 3'

In the context of BD5-dUIM2-GFP_PpuMI + PmlI, add mEpsin-DPW-FseI_PpuMI + PmlI fragment to make BD5-mEpsin-DPW-FseI-GFP

In the context of BD5-mEpsin-DPW-FseI-GFP, use the primers P1-P2 to generate mEpsin-DPW2X-F1 fragment; In the context of BD5-GFP, use the primers P3-P4 to generate mEpsin-DPW2X-F2 fragment; use the primers P1-P4 to generate mEpsin-DPW2X fragment, digest with FseI + PmlI

P1: 5' GGAGGGCCGGCCCAAGAAGGAGGAACAAC 3' (same as Minimal epsin_CBM2X_F1-P1)

P2: 5' CAGCCGACAATCCGAGTGCTTTCCATGGGTC 3'

P3: 5'- AGCACTCGGATTGTCGGCTGCTGCTGCTG-3'

P4: 5'- AGCACGTGTCTTGTAGTTCCCGTC -3' (same as ENTH-P4)

In the context of BD5-mEpsin-DPW-FseI-GFP_FseI-PmlI, add mEpsin-DPW2X_FseI-PmlI digestion fragment to make BD5-mEpsin-DPW2X-GFP

NotI + XhoI to make pCaSpeR4-attB-mEpsin-DPW2X-GFP

Maxi-prep for injection

BD5-mEpsin-NPF2X:

In the context of BD5-dUIM2-GFP, use the primers to generate mEpsin-NPF-FseI fragment, digest with PpuMI + PmlI

P1: 5'-GTAACACATTCCACATACGTTCCACAC-3' (same as pGEM-T-ΔENTH-P1)

P2: 5'-

CCTCCCGGCCGGCCAGCAGCAGCCGACAAAAACGGATTTGTGGCAGCCAAA AAGGGATTTGTATGACTCTGTTGTTCCTCCTTCTTGG -3'

In the context of BD5-dUIM2-GFP_PpuMI + PmlI, add mEpsin-NPF-FseI_PpuMI + PmlI fragment to make BD5-mEpsin-NPF-FseI-GFP

In the context of BD5-mEpsin-NPF-FseI-GFP, use the primers P1-P2 to generate mEpsin-NPF2X-F1 fragment; In the context of BD5-GFP, use the primers P3-P4 to generate mEpsin-NPF2X-F2 fragment; use the primers P1-P4 to generate mEpsin-NPF2X fragment, digest with FseI + PmlI

P1: 5' GGAGGGCCGGCCCAAGAAGGAGGAACAAC 3' (same as Minimal epsin_CBM2X_F1-P1)

P2: 5' AGCAGCAGCCGACAAAAACG 3'

P3: 5'- CGTTTTTGTCGGCTGCTGCTG-3'

P4: 5'- AGCACGTGTCTTGTAGTTCCCGTC -3' (same as ENTH-P4)

In the context of BD5-mEpsin-NPF-FseI-GFP_FseI-PmlI, add mEpsin-NPF2X_FseI-PmlI digestion fragment to make BD5-mEpsin-NPF2X-GFP

NotI + XhoI to make pCaSpeR4-attB-mEpsin-NPF2X-GFP

Maxi-prep for injection

BD5-mEpsin-DPW-NPF:

In the context of BD5-mEpsin-DPW-FseI-GFP_FseI-PmlI, add mEpsin-NPF2X_FseI-PmlI digestion fragment to make BD5-mEpsin-DPW-NPF-GFP

NotI + XhoI to make pCaSpeR4-attB-mEpsin-DPW-NPF-GFP

Maxi-prep for injection

BD5-UIM2-2X-GFP:

In the context of BD5-FL-GFP, use the following primers to generate UIM1-UIM2 fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'-
ATCCTGCTCACTCTGGCTGAGGGCGAGTTGCAGACGCACATCATCCTCGCCG
GCCGTC-3'

P3: 5'-
GATGATGTGCGTCTGCAACTCGCCCTCAGCCAGAGTGAGCAGGATGCGGAAC
AGGAGGAG-3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add UIM1-UIM2_PpuMI-FseI digestion fragment to make BD5-UIM2-2X-GFP

NotI + XhoI to make pCaSpeR4-attB-UIM2-2X-GFP

Maxi-prep for injection

BD5-FL-EEE/AAA-GFP (or BD5-ΔUIM2-EEE/AAA-GFP):

In the context of BD5-FL-GFP (or BD28-ΔUIM2), use the following primers to generate FL_EEE/AAA (or ΔUIM2_EEE/AAA) fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- CTGCAGTTGAAGAGCGGCAGCGCCGGCCGTC-3'

P3: 5'GCTGCCGCTCTTCAACTGCAGCTGGCCATGGCCATGTC -3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add FL_EEE/AAA (or ΔUIM2_EEE/AAA)_PpuMI-FseI digestion fragment to make BD5-FL-EEE/AAA-GFP (or BD5-ΔUIM2-EEE/AAA-GFP)

NotI + XhoI to make pCaSpeR4-attB-FL-EEE/AAA-GFP (or pCaSpeR4-attB-ΔUIM2-EEE/AAA-GFP)

Maxi-prep for injection

BD5-UIM1-AAAAA-GFP:

In the context of BD5-FL-GFP, use the following primers to generate UIM1-UIM2 fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'-

GGCAGCTCGGGCCATGGCCATGGCAGCCTGAGCTTGGGCAGCGGCAGCGCCG GCCGTC-3'

P3: 5'-

GCTGCCGCTGCCCAAGCTCAGGCTGCCATGGCCATGGCCCGAGCTGCCGCGG AACAGGAGG -3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add UIM1-AAAAA_PpuMI-FseI digestion fragment to make BD5-UIM1-AAAAA-GFP

NotI + XhoI to make pCaSpeR4-attB-UIM1-AAAAA-GFP

Maxi-prep for injection

BD5-FL-S/A-GFP:

In the context of BD5-FL-GFP, use the following primers to generate FL-S/A fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- TCGAGCCATGGCCATGGCCAG-3'

P3: 5'-CTGGCCATGGCCATGGCTCG-3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add FL-S/A_PpuMI-FseI digestion fragment to make BD5-FL-S/A-GFP

NotI + XhoI to make pCaSpeR4-attB-FL-S/A-GFP

Maxi-prep for injection

BD5−ΔUIM2-A/G-GFP:

In the context of BD28-ΔUIM2, use the following primers to generate ΔUIM2-A/G fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- TGACATGGCCATGCCCAGC-3'

P3: 5'-GGGCATGGCCATGTCACGAG-3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add ΔUIM2-A/G_PpuMI-FseI digestion fragment to make BD5−ΔUIM2-A/G-GFP

NotI + XhoI to make pCaSpeR4-attB−ΔUIM2-A/G-GFP

Maxi-prep for injection

BD5−ΔUIM2-EELQ/DDVR-GFP:

In the context of BD28-ΔUIM2, use the following primers to generate ΔUIM2-EELQ/DDVR fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- TGACATGGCCATGCCCAGC-3'

P3: 5'-GGGCATGGCCATGTCACGAG-3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add ΔUIM2-EELQ/DDVR_PpuMI-FseI digestion fragment to make BD5−ΔUIM2-EELQ/DDVR-GFP

NotI + XhoI to make pCaSpeR4-attB−ΔUIM2-EELQ/DDVR-GFP

Maxi-prep for injection

BD5−ΔUIM2-MAMSR/LSQSE-GFP:

In the context of BD28-ΔUIM2, use the following primers to generate ΔUIM2-MAMSR/LSQSE fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- TCCTCCTCACTCTGGCTGAGGGCCAGCTGCAGTTGAAG-3'

P3: 5'- TGGCCCTCAGCCAGAGTGAGGAGGAAGCGGAACAGGAGGAG-3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add ΔUIM2-MAMSR/LSQSE_PpuMI-FseI digestion fragment to make BD5−ΔUIM2-MAMSR/LSQSE-GFP

NotI + XhoI to make pCaSpeR4-attB−ΔUIM2-MAMSR/LSQSE-GFP

Maxi-prep for injection

BD5−ΔUIM2-EE/QD-GFP:

In the context of BD28-ΔUIM2, use the following primers to generate ΔUIM2-EE/QD fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- CCTGTTCCGCATCCTGTCGTG-3'

P3: 5'- CACGACAGGATGCGGAACAGG-3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add ΔUIM2-EE/QD_PpuMI-FseI digestion fragment to make BD5−ΔUIM2-EE/QD-GFP

NotI + XhoI to make pCaSpeR4-attB−ΔUIM2-EE/QD-GFP

Maxi-prep for injection

BD5-Ks/Rs-FL-GFP:

In the context of BD5-FL-GFP, use the primers to generate Ks/Rs-FL-FseI fragment, digest with PpuMI + FseI

P1: 5'-GTAACACATTCCACATACGTTCCACAC-3' (same as pGEM-T-ΔENTH-P1)

P2: 5' TCGTGCTCTCTGCGCTCTCACACGCTCATTC 3'

P3: 5' GTGAGAGCGCAGAGAGCAAGAGAAAGATTCGCCCAGAAC 3'

P4: 5' GGATACCGATCTAGGCGGCTCC 3'

P5: 5' GGAGCCGCCTAGATCGGTATCC 3'

P6: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add Ks/Rs-FL-FseI_PpuMI-FseI digestion fragment to make BD5-Ks/Rs-FL-GFP

NotI + XhoI to make pCaSpeR4-attB-Ks/Rs-FL-GFP

Maxi-prep for injection

BD5-Ks/Rs-dENTH-GFP:

In the context of BD5-dENTH-GFP, use the primers to generate Ks/Rs-dENTH fragment, digest with Acc65I + BbcCI

P1: 5'-GTAACACATTCCACATACGTTCCACAC-3' (same as pGEM-T-ΔENTH-P1)

P2: 5' TCGTGCTCTCTGCGCTCTCACCATATCGTCC 3'

P3: 5' GTGAGAGCGCAGAGAGCAAGAGAAAGATTCGCCCAGAAC 3'

P4: 5' GGATACCGATCTAGGCGGCTCC 3'

P5: 5' GGAGCCGCCTAGATCGGTATCC 3'

P6: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD28_Acc65I-BbcCI, add Ks/Rs-dENTH_Acc65I-BbcCI digestion fragment to make BD28-Ks/Rs-dENTH

In the context of BD5-GFP_SalI digestion fragment, add BD28-Ks/Rs-dENTH_SalI digestion fragment to make BD5-Ks/Rs-dENTH-GFP

NotI + XhoI to make pCaSpeR4-attB-Ks/Rs-dENTH-GFP

Maxi-prep for injection

BD5-R/A-GFP:

In the context of BD5-FL-GFP, use the following primers to generate R/A fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- CCGTGGTCATTAAGAGCCTTCCAG -3'

P3: 5'- GATCTGGAAGGCTCTTAATGACCACG -3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add R/A_PpuMI-FseI digestion fragment to make BD5-R/A-GFP

NotI + XhoI to make pCaSpeR4-attB-R/A-GFP

Maxi-prep for injection

BD5-RWRK/AAAA-GFP:

In the context of BD5-FL-GFP, use the following primers to generate RWRK/AAAA fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- CCGTGGTCATTAAGAGCCTTCCAG -3'

P3: 5'- GATCTGGAAGGCTCTTAATGACCACG -3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH−ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add RWRK/AAAA_PpuMI-FseI digestion fragment to make BD5-RWRK/AAAA-GFP

NotI + XhoI to make pCaSpeR4-attB-RWRK/AAAA-GFP

Maxi-prep for injection

BD5-T/D-GFP:

In the context of BD5-FL-GFP, use the following primers to generate T/D fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- CTCTCGCAGATCTTGAATGGCAAAG -3'

P3: 5'- CTTTGCCATTCAAGATCTGCGAGAG -3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH–ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add T/D_PpuMI-FseI digestion fragment to make BD5-T/D-GFP

NotI + XhoI to make pCaSpeR4-attB-T/D-GFP

Maxi-prep for injection

BD5-FTVF/RDAA-GFP:

In the context of BD5-FL-GFP, use the following primers to generate FTVF/RDAA fragment, digest with PpuMI + FseI

P1: 5'- GTAACACATTCCACATACGTTCCACAC -3' (same as pGEM-T-ΔENTH-P1)

P2: 5'- CCGTGGTCATTAAGAGCCTTCCAG -3'

P3: 5'- GATCTGGAAGGCTCTTAATGACCACG -3'

P4: 5' GGAGGGCCGGCCGGAG 3' (same as pGEM-T-ΔENTH–ΔUIM2-P4)

In the context of BD5-FL-GFP_PpuMI-FseI, add FTVF/RDAA_PpuMI-FseI digestion fragment to make BD5-FTVF/RDAA-GFP

NotI + XhoI to make pCaSpeR4-attB-FTVF/RDAA-GFP

Maxi-prep for injection

BD5-human Epsin 1-isoB-GFP:

In the context of BD5-FL-GFP, use the following primers to generate human epsin 1-isoB fragment, digest with MluI + PmlI

P1: 5'- GTGTTTATAGTGAATATAGTAACACACGTACTTTGC -3'

P2: 5'-ACGAGGTCGACATATCGTCCTTTTGCTTTCTCACTG-3'

P3: 5'- GGACGATATGTCGACCTCGTCCTTGAGG-3'

P4: 5' CAGCAGCAGCTAGGAGGAAGGGATTAGTGTTGG 3'

P5: 5'- CTTCCTCCTAGCTGCTGCTGCTGCTGCTATG-3'

P6: 5'- AGCACGTGTCTTGTAGTTCCCGTC -3' (same as ENTH-P4)

In the context of BD5-FL-GFP_MluI-PmlI, add human epsin 1-isoB_MluI-PmlI digestion fragment to make BD5-human epsin 1-isoB-GFP

NotI + XhoI to make pCaSpeR4-attB-human epsin 1-isoB-GFP

Maxi-prep for injection

BD5-ΔCBM-ΔDPW-ΔNPF-GFP

In the context of BD5-ΔCBM-ΔDPW-GFP, use the following primers to generate ΔCBM-ΔDPW-ΔNPF fragment, digest with FseI + PmlI

P1: 5'- GGCTGTTGTCAATCCCTCCGCTGCCCCAC -3'

P2: 5'- CAGCCGACAAGTACGCCGGCTGATTACCCG -3'

P3: 5'- GGACGATATGTCGACCTCGTCCTTGAGG-3'

P4: 5'- AGCACGTGTCTTGTAGTTCCCGTC -3' (same as ENTH-P4)

In the context of BD5-FL-GFP_FseI-PmlI, add ΔCBM-ΔDPW-ΔNPF_FseI-PmlI digestion fragment to make BD5-ΔCBM-ΔDPW-ΔNPF-GFP

NotI + XhoI to make pCaSpeR4-attB-ΔCBM-ΔDPW-ΔNPF-GFP

Maxi-prep for injection

A.1.2. Amino acid content of Epsin deletions expressed by each construct

Lqf1 and Lqf2 amino acid sequence

MQVNVAGLRRNIKNLAHNYSDAQVKVREATSNDPWGPSAAIMSEIAELTYNVVAFSEIM
QMIWKRLNDHGKNWRHVYKALILLEYLIKTGSEKVAQQCKENIFAIQTLREFVYFEEGK
DQGTHVREKAKQLVTLLKDDERLKNERVKAQKAKERFAQNPSGFGSDGYIDGPSQRDLP
PGWQEEPPKSVSELEMVRPQTAGEEELQLQLAMAMSREEAEQEEAKRRSDDVRLQLALS
QSEQDFKDPNGRPIPAPKKEEQQSHLLDLLDISLGATSISSPPLGAAGGAPTAVVDPWA
MPGPRAPSQLSDPWSGTSSPQVDPWNPSAAPRTILGAGVPMTSAPLGAGNDAWGARTQS
PSVASGSSNEGWLQSNGNANQNGRGATPAGPPAEGWLIKSTAVGALGAAPLNHAANNGS
SSSDPWLAEPAASAAGGAAAVGLADPWAPGAASQTGAGALDDPWKALGTGAIKKQSPEF
DEFDLITNRNKSEHSNSNASNNNNASLLDDMDPLSANYGNGGINSSMHPSTGATAKKSI
KDAHSFLGENSALVNLDNLIKPIAPQTQTGNQPAYNPFSDNVVPPKTNLFQQQQPAVPS
INQLKQQAPFSVSMNQDPWAPVMGGVSTTSQPQPNLQVYNNAYQSSSSTNILGKSNIPT
NMNSSTSNSTIHSCYASPSPGVDGIRNMDIFTERPYYNSPTAPADDTYMYNSRDNNNIN
SNYGTPSLYSSFAASYSSALSDSLAETPGISVAPLGFVADTVMSFGPSSSSANCKLEQN
NNMPWIKPEAATNPFLSAAAAAAMSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGD
ATYGKLTLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQE
RTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMA
DKQKNGIKANFKTRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNE
KRDHMVLLEFVTAAGITHGMDELYK

Lqf modules are red.
Deleted regions are shaded with grey.
Six-Alanine linker between Lqf and GFP is shaded with light green.
GFP tag is highlighted with dark green.
Additional amino acids in Lqf1 are marked with lighter letters shaded with grey.

General Information

Lqf1-GFP: 1028aa = amino acid 1-1028	111 kD
Lqf2-GFP: 884aa = amino acid 1-629, 774-1028	96 kD
Lqf1: 784aa = amino acid 1-784	84 kD
Lqf2: 640aa = 1-629,774-1028	68 kD
GFP: 238aa	27 kD

Construct Contents

As Lqf2 is expressed in the eye nearly exclusively, all of the amino acid numbers below and the molecular sizes are in the Lqf2 (or Lqf2-GFP) context (640aa).

Lqf2-GFP

MQVNVAGLRRNIKNLAHNYSDAQVKVREATSNDPWGPSAAIMSEIAELTYNVVAFSEIM
QMIWKRLNDHGKNWRHVYKALILLEYLIKTGSEKVAQQCKENIFAIQTLREFVYFEEGK
DQGTHVREKAKQLVTLLKDDERLKNERVKAQKAKERFAQNPSGFGSDGYIDGPSQRDLP
PGWQEEPPKSVSELEMVRPQTAGEEELQLQLAMAMSREEAEQEEAKRRSDDVRLQLALS
QSEQDFKDPNGRPIPAPKKEEQQSHLLDLLDISLGATSISSPPLGAAGGAPTAVVDPWA
MPGPRAPSQLSDPWSGTSSPQVDPWNPSAAPRTILGAGVPMTSAPLGAGNDAWGARTQS
PSVASGSSNEGWLQSNGNANQNGRGATPAGPPAEGWLIKSTAVGALGAAPLNHAANNGS
SSSDPWLAEPAASAAGGAAAVGLADPWAPGAASQTGAGALDDPWKALGTGAIKKQSPEF
DEFDLITNRNKSEHSNSNASNNNNASLLDDMDPLSANYGNGGINSSMHPSTGATAKKSI
KDAHSFLGENSALVNLDNLIKPIAPQTQTGNQPAYNPFSDNVVPPKTNLFQQQQPAVPS
INQLKQQAPFSVSMNQDPWAPVMGGVSTTSQQNNNMPWIKPEAATNPFLSAAAAAAMSK
GEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVT
TFSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVN
RIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKANFKTRHNIEDGGVQLAD
HYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK

FL: 1-640 (68 kD)

FL-GFP: 1-640, AAAAAA-GFP (96 kD)

ΔUIM2: 1-226, 246-640, AAAAAA-GFP (94 kD)

ΔCBM: 1-261, 267-543, 551-640, AAAAAA-GFP (94 kD)

ΔDPW: 1-291, 321-416, 458-640, AAAAAA-GFP (89 kD)

ΔNPF: 1-566, 639-640, AAAAAA-GFP (88 kD)

ΔCBMΔDPW1: 1-261, 267-291, 321-543, 551-640, AAAAAA-GFP (91 kD)

ΔENTH: 1, 146-640, AAAAAA-GFP (79 kD)

ΔCBMΔDPW: 1-261, 267-291, 321-416, 458-543, 551-640, AAAAAA-GFP(87kD)

ΔUIM: 1-200, 246-640, AAAAAA-GFP (90 kD)

ENTH-UIM: 1-261, 639-640, AAAAAA-GFP (57 kD)

A.1.3. Determine the rescue activities of the transgenes

		code	Level	*lqf*^{null}	*lqf*^{hypo}	*aux*^{hypo} 23^0C	*aux*^{hypo} 25^0C
NO construct		/	/	L, E	V, N	V, N	L, P
	lqf^{FL}	A=1B/Cyo	0.4	V, wt			V, N
	lqf^{FL}	A=1C/Cyo	0.8	V, wt	V, wt	V, N-S	V, N
	lqf$^{\Delta UIM2}$	C=4C	0.8	V, wt	V, wt	V, N-S	V, N
Group I	*lqf*$^{FL\text{-}GFP}$	B=10A	1.0	V, wt	V, wt	V, N-S	V, N
full	*lqf*$^{\Delta UIM2\text{-}GFP}$	D=29A	2.8	V, wt	V, wt	V, N-S	V, N
activity	*lqf*$^{\Delta CBM\text{-}GFP}$	G=14B	0.5	V, wt	V, wt		
	lqf$^{\Delta CBM\text{-}\Delta DPW1\text{-}GFP}$	L=12C	1.0	V, wt	V, wt		
	lqf$^{\Delta DPW\text{-}GFP}$	J=15B	0.9	V, wt	V, wt		
	lqf$^{\Delta NPF\text{-}GFP}$	K=16B	1.4	V, wt	V, wt		
	lqf$^{\Delta CBM\text{-}\Delta DPW\text{-}GFP}$	O=13A	0.7	V, N?	V, wt	V, N-S	V, N
Group II	*lqf*$^{\Delta ENTH}$	M=5A	0.3	SEMI-L	V, wt		
partial	*lqf*$^{\Delta ENTH}$	M=5BE	0.8	V, N	V, wt	L, P	L, P
activity	*lqf*$^{\Delta ENTH\text{-}GFP}$	N=11A	0.5	SEMI-L	V, wt	V, N	V, N
	lqf$^{\Delta ENTH\text{-}\Delta UIM2}$	U=7C	0.3	L, P	V, N-S	V, N-E	L, P
	lqf$^{\Delta UIM1}$	P=2A	0.7	L, P	V, N-S	V, N-E	L, P
	lqf$^{\Delta UIM}$	R=3d	0.9	L, P	V, N-S	V, N-E	L, P
	lqf$^{\Delta UIM1\text{-}GFP}$	Q=28A	2.1	L, P	V, N-S	V, N-E	L, P
Group III	*lqf*$^{\Delta UIM\text{-}GFP}$	S=30A/Cyo	2.4	L, P	V, N-S	V, N-E	L, P
residual	*lqf*$^{\Delta UIM1\text{-}\Delta CBM1\text{-}GFP}$	T=22C	0.9	L, P	V, N-S	V, N	L, P
activity	*lqf*$^{\Delta UIM1\text{-}\Delta CBM\text{-}\Delta DPW\text{-}GFP}$	23C	0.9	L, P	V, N-S	V, N	L, P
	lqf$^{\Delta ENTH\text{-}\Delta CBM\text{-}GFP}$	V=17A	0.5	L, P	V, N-S	V, N	V, Esc
	lqf$^{\Delta ENTH\text{-}\Delta DPW\text{-}GFP}$	W=18A/Cyo	0.5	L, P	V, N-S	V, N	V, Esc
	lqf$^{\Delta ENTH\text{-}\Delta NPF\text{-}GFP}$	X=19A	0.5	L, P	V, N-S	V, N	V, Esc
	lqf$^{\Delta UIM1\text{--}UIM2}$	CC=31A	1.7	L, P	V, N-S	V, N	L, P
	lqf$^{\Delta UIM2\text{--}UIM1}$	DD=33B	2.1	L, P	V, N-S	V, N	L, P
	lqf$^{\text{-}EU\text{-}GFP}$	Y=21B	4.8	L, P	V, N-S	V, N-E	L, P
Group IV	*lqf*$^{\text{-}ENTH\text{-}GFP}$	Z=20A/Cyo	3.9	L, E	V, N	V, N	L, P
no	*lqf*$^{\text{-}ENTH\text{-}GFP}$	Z=20B	3.7	L, E	V, N		L, P
activity	*lqf*$^{\Delta ENTH\text{-}\Delta UIM1}$	AA=6A	0.6	L	V, N-E	V, N-E	L, P
	lqf$^{\Delta ENTH\text{-}\Delta UIM}$	BB=8A	0.4	L	V, N-E	V, N-E	L, P

	attB-GFP constructs	code	Level	*lqf*^{null}	*lqf*^{hypo}
	NO construct	/	/	L, E	V, N
Group I full activity	*lqf*^{FL}	40A	1.2	V, wt	V, wt
	lqf$^{\Delta UIM2}$	46A	1.9	V, wt	
	lqf$^{Ks/Rs\_FL}$	58AB	0.8	V, wt	
	lqf$^{T/D}$	62A	1.3	V, wt	
	lqf$^{FTVF/RDAA}$	63A	0.9	V, wt	
Group II partial activity	*lqf*$^{mEpsin\text{-}NPF\text{-}2X}$	43A	4.5	SEMI-L	V, wt
	lqf$^{mEpsin\text{-}NPF\text{-}2X}$	43B	/	SEMI-L	
	lqf$^{UIM1\_EEE/AAA\_FL}$	49A	1.8	V, N	
	lqf$^{UIM1\_S/A\_FL}$	52AB	3.4	V, N	
	lqf$^{UIM1\_EE/QD\_\Delta UIM2}$	56AB	1	V, N	
	lqf$^{\Delta ENTH}$	57AB	0.9	L, P	
	lqf$^{R/A}$	60A	0.8	V, N	
	hEpsin	64A	6.3	L, P	V, wt
Group III residual activity	*lqf*$^{\Delta UIM1}$	45A	2.9	L, P	V, N-S
	lqf$^{UIM2\text{-}2X}$	48AB	1.5	L, P	V, N-S
	lqf$^{UIM1\_EEE/AAA\_\Delta UIM2}$	50A	1.2	L, P	V, N-S
	lqf$^{UIM1\_AAAAA\_FL}$	51A	1.4	L, P	V, N-S
	lqf$^{UIM1\_A/G\_FL}$	53ABC	1.9	L, P	V, N-S
	lqf$^{UIM1\_EEEQ/DDVR\_\Delta UIM2}$	54AB	0.9	L, P	V, N-S
	lqf$^{UIM1\_MAMSR/LSQSE\_\Delta UIM2}$	55A	0.7	L, P	V, N-S
	lqf$^{Ks/Rs\_\Delta ENTH}$	59A	0.7	L, P	V, N-S
	lqf$^{RWRK/AAAA}$	61AB	0.2	L, P	V, N
Group IV no activity	*lqf*$^{mEpsin\text{-}CBM\text{-}2X}$	41A	<0.1	L, E	V, N-E
	lqf$^{mEpsin\text{-}CBM\text{-}2X}$	41B	<0.1	L, E	V, N-E
	lqf$^{mEpsin\text{-}DPW\text{-}2X}$	42A	<0.1	L, E	V, N-E
	lqf$^{mEpsin\text{-}DPW\text{-}NPF\text{-}2X}$	44ABC	<0.1	L, E	V, N-E
	lqf$^{\Delta CBM\text{-}\Delta DPW\text{-}\Delta NPF}$	65AB	<0.1	L, E	V, N

Table 1. Quantitative analysis and rescue activities of the genomic Epsin variants. The protein levels were determined directly by comparing one copy of transgene with one copy of endogenous gene. Alternatively, they were determined by comparing one copy of transgene with one copy of *lqfFL-GFP* (10A) transgene. Based on the rescue activities, the transgenes are divided into four groups. L, E: Lethal at Embryonic stage; L, P: Lethal at Pupae stage; Semi-L: a few adult escapers with Notch-like mutant phenotypes; V, wt: Viable with wild-type phenotypes; V, N: Viable with Notch-like mutant phenotypes; V, N-S: Viable with suppressed Notch-like mutant phenotypes.

Based on the rescue activities in the *lqf* null and hypomorphic backgrounds, the transgenes are divided into four groups. Group I transgenes have complete Epsin activity; they complemented the lqf_{L71}/lqf_{ARI} (and lqf_{FDD9}) mutant phenotypes fully, meaning that the flies are viable and have no apparent morphological defects. Group II transgenes retain significant Epsin activity; they complement lqf_{L71}/lqf_{ARI} partially (the flies are at least semi-viable and have morphological defects typical of *Notch* pathway mutants), and complement lqf_{FDD9} completely. Group III transgenes have only residual activity; they enable lqf_{L71}/lqf_{ARI} to survive only until they are pupae, and they complement the morphological mutant phenotype of lqf_{FDD9} partially. Group IV transgenes have no apparent Epsin activity; they fail to complement lqf_{L71}/lqf_{ARI} or lqf_{FDD9} detectably.

Appendix 2. Western blot analysis

A.2.1. Quantitative Western analysis

Epsin-GFP variants expressed by transgenes were quantified using Western blots using anti-GFP and anti-β-tubulin, and compared to endogenous Epsin indirectly through one copy of *FL*. Eye disc protein extracts were generated and analyzed on Western blots as described (Chen et al., 2002), probed with guinea pig-anti-Lqf (1:1000) or mouse-anti-GFP (1:1000) from Santa Cruz Biotechnology, and mouse mAbE7 (anti-β-tubulin from DSHB) at 1:100. Secondary antibodies were HRP-anti-guinea pig (Jackson) at 1:20,000, HRP-goat-anti-mouse at 1:2000 (Sigma) and HRP-anti-mouse (Santa Cruz Biotechnology) at 1:500. The results were quantified using NIH Image J.

A.2.2. Purify Epsins to detect ubiquitinated forms

This assay is based on the stabilization of ubiquitinated epsin in *fat facets* (*faf*) mutant flies (Chen et al., 2002). Protein extracts were generated from 40 third instar larvae by grinding in buffer containing a protease inhibitor cocktail (Sigma P2714),

according to the instructions provided with the Miltenyi Biotec GFP purification kit. Purified GFP-tagged protein was subjected to 8% PAGE (Bio-Rad) and transferred to Westran CS membrane (Whatman). Signals were obtained by treatment with mouse anti-GFP (Santa Cruz Biotechnology sc-9996, 1:1000), HRP-anti-mouse (Sigma A4416, 1:2000), and developed with ECL Plus Detection Reagents (Amersham RPN2132), and Sigma (Z370371) photographic film. Fig. S2: guinea pig- anti-Lqf (1:4000), HRP-rabbit-anti-guinea pig (1:20,000; Sigma), mouse-anti-GFP, HRP-anti- mouse-HRP (1:2000).

Appendix 3. Protein interaction experiments

A.3.1. *In vitro* expression constructs

MBP-Epsin variants

• Total RNA prepared using RNA-Easy (Qiagen) from flies transformed with each Epsin construct used to make MBP-Epsin protein in Fig. 8.

• cDNA generated by RT-PCR using SuperScript First Strand Synthesis System (Invitrogen).

• Epsin cDNA from the transgene only was amplified using the following two primers – a common 5' primer and a common 3' primer to GFP:

pENTR-D-TOPO_lqfs

P1 : 5'-CTCCGCGGCCGCCCCCTTCACCATGCAGGTCAATGTCGCTG-3'

P2 : 5'- GGGTCTCGAGCATTTAAGCAGCAGCAGCAGCCGAC -3'

P1_ΔENTH: 5'-CTCCGCGGCCGCCCCCTTCACCATGGTGAAGGCGCAGAAGGC-3'

P1_S/A: 5'-CACCATGGTGAAGGCGCAGAAGGC-3'

In the context of pENTR-D-TOPO_NotI-XhoI, add lqf_cDNA-PCR_NotI-XhoI to make pENTR-D-TOPO_lqf constructs (S/A use the vector from the kit), LR reaction to transfer

lqf fragments into pVP-13 expression vector (or pDEST15 expression vector), transform into BL21_Rosetta cells and use IPTG to induce expression of the proteins.

pENTR-D-TOPO_Dm-CHC_TD579aa

P1: 5'-CACCATGACGCAACCACTGCCCATC-3'

P2: 5'- GAGTTAACCCTCGGCGGGACGGTTATG-3'

Use the pENTR-D-TOPO vector from the kit to clone the CHC_TD579aa-cDNA_PCR product, LR reaction to transfer CHC_TD579aa fragments into pVP-13 expression vector (or pDEST15 expression vector), transform into BL21_Rosetta cells and use IPTG to induce expression of the protein.

pENTR-D-TOPO_α-Adaptin_isoB_249aa out of 952aa

P1: 5'-CTCCGCGGCCGCCCCCTTCACCATGTACGGCAGCAATAGTAACAAC-3'

P2: 5'-GGGTCTCGAGCCATGATTGTTGTGGCTTAGAATTGATCC-3'

In the context of pENTR-D-TOPO_NotI-XhoI, add α-Adaptin_isoB_249aa_cDNA-PCR_NotI-XhoI to make pENTR-D-TOPO_α-Adaptin_isoB_249aa construct, LR reaction to transfer α-Adaptin_isoB_249aa fragments into pVP-13 expression vector (or pDEST15 expression vector), transform into BL21_Rosetta cells and use IPTG to induce expression of the protein.

pENTR-D-TOPO_Eps15_isoA_515aa out of 1254aa

P1: 5'-CTCCGCGGCCGCCCCCTTCACCATGAATGTGGACTTTGCGAG-3'

P2: 5' GGGTCTCGAGGGTCGCGAATCTTCTACACCTGCTC 3'

In the context of pENTR-D-TOPO_NotI-XhoI, add Eps15_isoA_515aa_cDNA-PCR_NotI-XhoI to make pENTR-D-TOPO_Eps15_isoA_515aa construct, LR reaction to transfer Eps15_isoA_515aa fragments into pVP-13 expression vector (or pDEST15 expression vector), transform into BL21_Rosetta cells and use IPTG to induce expression of the proteins.

pENTR-D-TOPO_Ub-63E-first Ub_76aa

P1: 5'-CACCATGCAGATCTTTGTGAAGACTTTGAC-3'

P2: 5'-CGAAGATCTGTTAACCACCACGGAG-3'

Use the pENTR-D-TOPO vector from the kit to clone the Ub-63E-first Ub_76aa-cDNA_PCR product, LR reaction to transfer Ub-63E-first Ub_76aa fragments into pVP-13 expression vector (or pDEST15 expression vector), transform into BL21_Rosetta cells and use IPTG to induce expression of the protein.

A.3.2. *In vitro* pull-down assay

The vectors *pDEST15* (GST fusions) or *pVP13* (MBP fusions) and BL21 Rosetta cells were used to express proteins in bacteria according to manufacturer's instructions (Invitrogen). Details of the plasmid constructions are shown below. The MBP fusion proteins were induced with IPTG and purified with Amylose Resin (BioLabs #E8021S) from BL21 Rosetta Cells according to the procedure at http://wolfson.huji.ac.il/purification/TagProteinPurif/MBP_Tag_nature.html. Proteins were quantified on Coomassie stained gels with Precision Plus Protein Unstained Standards (Bio-Rad #161-0363). GST-tagged proteins were immobilized on glutathione Sepharose 4B GE Healthcare #17-0756-01) by mixing 1 ml of bacterial cell lysate with 200 ul Sepharose prewashed with GST binding buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 0.5% NP-40; just before use, 10μl 1M DTT and 1 tablet Protease Inhibitor Cocktail (Roche: cat#11836170001) added per 10 ml buffer). A 30 μl aliquot of the GST-fusion loaded Sepharose was used for protein quantification as described above. The GST pull-down procedure used is modified from Drake et al., 2000. Aliquots (30 μl) of loaded Sepharose were mixed with MBP-Epsin proteins (the same weight as GST-fusion protein) and assay buffer (25 mM Hepes-KOH ph 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 1 mM DTT) added to make the total volume 100

μl. After overnight incubation at 4^{O}C with continuous gentle mixing, the Sepharose beads were recovered by centrifugation at 10,000 g for 1 min. The supernatant was removed (the "S" fraction) and a portion of that was mixed with SDS-PAGE buffer (see below). The Sepharose pellets were then washed 5X with 1 ml ice-cold PBS and centrifugation, the final supernatants aspirated and some of each pellet (the "P" fraction) resuspended in SDS-PAGE buffer.

GST-*Dm*-Ub (0.6mg/ml) + MBP-Epsins (0.2mg/ml):

IP-S: 10ul aliquots of supernatant were removed and adjusted to 80 ul with SDS-PAGE sample buffer. 10ul were used to load the gel, equivalent to 1⁄80 of each supernatant.

IP-P: pellet was resuspended in 30ul SDSPAGE sample buffer. 10ul were used to load the gel, equivalent to 1⁄3 of each pellet.

After coomassie blue staining, the same gel was used to do western blot.

GST-*Dm*-clathrin-TD (0.1mg/ml) + MBP-Epsins (1mg/ml):

IP-S: 25ul aliquots of supernatant were removed and adjusted to 120 ul with SDS-PAGE sample buffer. 20ul were used to load the gel, equivalent to 1⁄24 of each supernatant.

IP-P: pellet was resuspended in 50ul SDSPAGE sample buffer. 20ul were used to load the gel, equivalent to 2⁄5 of each pellet.

After coomassie blue staining, the same gel was used to do western blot.

GST-*Dm*-α-adaptin_isoB-ear domain (0.2mg/ml) + MBP-Epsins (0.2mg/ml):

St (starting epsin variants): 5ul aliquots of purified epsin variants were adjusted to 80 ul with SDS-PAGE sample buffer. 20ul were used to load the gel, equivalent to 1⁄80 of the starting material.

IP-S: 5ul aliquots of supernatant were removed and adjusted to 80 ul with SDS-PAGE sample buffer. 20ul were used to load the gel, equivalent to 1⁄80 of each supernatant.

IP-P: pellet was resuspended in 80ul SDSPAGE sample buffer. 20ul were used to load the gel, equivalent to 1⁄4 of each pellet.

Western blot did not work.

GST-*Dm*-Eps15-EH domain (0.4mg/ml) + MBP-Epsins (0.4mg/ml):

IP-S: 25ul aliquots of supernatant were removed and adjusted to 120 ul with SDS-PAGE sample buffer. 20ul were used to load the gel, equivalent to 1⁄24 of each supernatant.

IP-P: pellet was resuspended in 50ul SDSPAGE sample buffer. 10ul were used to load the gel, equivalent to 2⁄5 of each pellet.

After coomassie blue staining, the same gel was used to do western blot.

Appendix 4. Molecular Biology

Enzymes were from Promega Biotech, New England BioLabs, and Boehringer Mannheim. Herculase polymerase (Stratagene) was used for PCR. DNA sequences of all PCR amplification products were verified. Automated fluorometric DNA sequencing was performed in the DNA analysis facility of the Institute for Cell and Molecular Biology (ICMB) at UT Austin.

Appendix 5. Analysis of eyes

Adult external eyes were photographed with an Olympus SZX12 microscope equipped with a SPOT idea (Diagnostic Instruments) camera. Plastic sectioning of adult eyes was as described (Tomlinson and Ready, 1987). Eye sections were photographed with a Zeiss Axioplan equipped with an Axiocam Hrc. For immunostaining, eye discs were fixed in PEMS and antibody incubations and washes were in PBST (Fischer-Vize et al., 1992). The antibodies were: guinea pig anti-Lqf (1:1000) (Chen et al., 2002) and 488-donkey anti-guinea pig (1:200) (Jackson Laboratories), rat anti-Chc (1:100) (Wingen et al., 2009) and 647-goat anti-rat (1:200; Molecular Probes); (C, D) chicken anti-GFP

(1:1000) (AbCam) and 488-goat anti-chicken (1:800) (Jackson Laboratories); anti-Chc as in (A, B). The eye discs were immunostained as follows. Discs were fixed in PEMS buffer with 1.0% NP-40 for 15 min. Antibody treatment was as described previously (Lim et al., 2007) with modifications. Fixed discs were blocked for 2 hrs at 4°C in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40 and 5 mg/ml BSA, and then incubated in primary antibody diluted in blocking solution overnight at 4°C. Discs were washed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5% NP-40 three times for 5 min., and incubated with secondary antibodies in washing solution for 2 hrs at room temperature, and then washed three times for 5 min. Primary antibodies were: mouse monoclonal anti-Cut at 1:100 from [Developmental Studies Hybridoma Bank (DSHB)], mouse monoclonal anti-β-galactosidase at 1:50 from the DSHB, rabbit anti-Svp at 1:100. Secondary antibodies (1:200; Molecular Probes) were: Alexa^{568}-anti-rabbit, Alexa^{568}-anti-mouse, Alexa^{568}-anti-guinea pig, Alexa^{633}-anti-mouse, Cy5-anti-rabbit. Phalloidin treatment of eye and wing discs (568-phalloidin (Invitrogen)) was as described (Chen et al., 2002). Eye and wing discs were mounted in Vectashield (Vector) and photographed with a Leica TCSSP2 or SP2AOBS confocal microscope. Images were processed with Adobe Photoshop CS3. MARCM clones (Lee and Luo, 1999) were generated by heat shocking first or second instar larvae at 37°C for 60 min.

Appendix 6. Fly Stocks

Transformant lines containing each of the deletion constructs in Table 1 are kept in w; [P]/CyO; MKRS/TM6B or [P]/[P]; MKRS/TM6B backgrounds. All the RNAi lines tested are included in Table 2. The following mutant alleles of *lqf* and *faf*, maintained in our laboratory, were used.

lqf^{FDD9} (FBal0104483),

lqf^{P011027} (FBal0100180),

lqf^{ARI} (FBal0104485),

lqf^{L71} (FBal0147029),

faf^{FO8} (FBal0031258)

faf^{B3} (FBal0031239)

faf^{BX4} (FBal0028189).

Appendix 7. An *in vivo* RNAi assay to investigate possible roles of endocytic factors in Notch signaling

A.7.1. Introduction

The functional analysis of Epsin demonstrated that it functions as an accessory factor to internalize Notch-bound ligands. It seems possible that other endocytic factors could also be involved in the process. Clathrin and the general Clathrin adaptor, AP-2, are good candidates. Since the essential role of Epsin is to link the receptor-bound ligands to the endocytic machinery, any proteins involved in the formation of Clathrin-coated vesicles or Clathrin-independent vesicles might be involved. The possible roles of these proteins in Notch signaling are largely unknown partly due to the fact that these proteins are mostly pleiotrophic. Except for the possible roles in Notch signaling, they also function in other essential events. Thus, their functions in Notch signaling are masked by other phenotypes. One way to get around that problem is to conditionally knock-out the proteins in specific cells, for instance, in the signal-sending cells.

Notch signaling is involved in several steps during *Drosophila* eye development. Studies using a temperature sensitive *Notch* allele showed that disruption of Notch signaling at different stages resulted in different phenotypes. Disruption of Notch signaling in all eye cells results in failure of proneural enhancement, which gives a “no

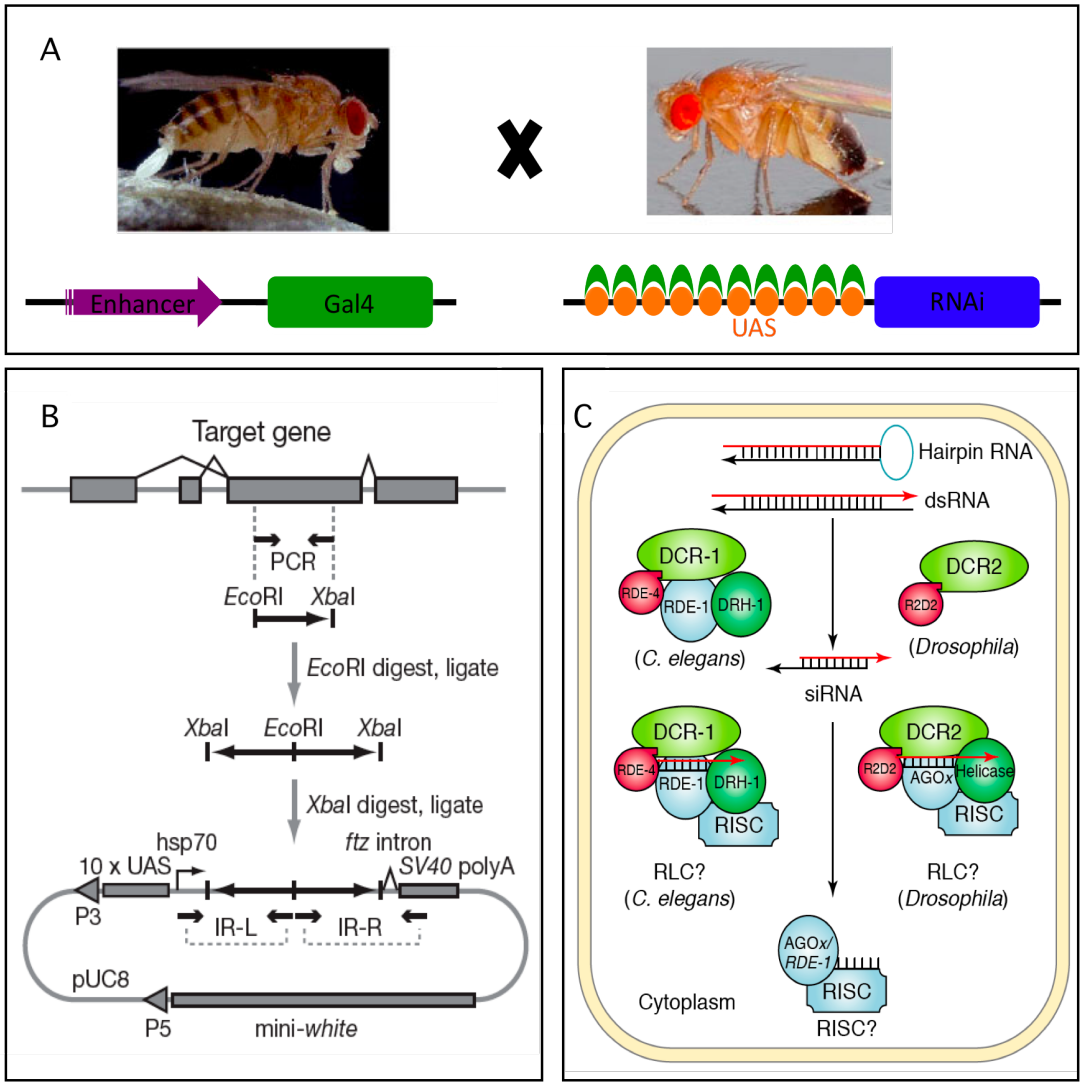


Figure A.3. Gal4>UAS-RNAi techinque (modified from Dietzl et al, 2007). A. A cross between a femail containing the enhancer-Gal4 gene and a male containing the UAS-RNAi transgene results in the expression of the small RNAi molecules in the progenies. B. The RNAi sequence is designed so that two complementary sequences are pointing out toward each other. C. The formed hairpin RNA is processed by the RISC complex to block the expression of the target mRNA.

eye" phenotype. Disruption of Notch signaling in lateral inhibition-related cells generates fused ommatidia. Disruption in R2/5/3/4 and surrounding cells causes extra-photoreceptors. Disruption in R3/4 only may result in both extra and symmetric photoreceptors. This provides a unique opportunity to study the possible roles of different

genes in Notch signaling. Knock-out a specific gene in some of the cells may result in specific Notch-related mutant phenotypes. This assay may also help to distinguish between the genes involved in Notch signal-sending and signal-receiving cells.

The RNA interfering (RNAi) technique has been widely used to knock down the expression of specific proteins both *in vivo* and *in vitro*. Dickson group initiated a project to generate a genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Basically, they tried to clone specific sequences from every single gene in *Drosophila*, put it into a UAS vector to produce loopless hairpins, which are processed by the RNAi machinery. The Vienna *Drosophila* RNAi Center (VDRC: http://stockcenter.vdrc.at/control/main) keeps the fly stocks that cover 93% of the *Drosophila* genome. What I need is a unique promoter to drive the expression of Gal4 proteins in specific cells of the eye.

ey-Gal4 drives gene expression in all kinds of cells during eye development. *GMR-Gal4* is expressed in all kinds of cells 2 rows after morphogenetic furrow. *Elav-Gal4* is specifically expressed in all the neural cells: photoreceptors. *ro-hs-Gal4* can be expressed in the morphogenetic furrow and R2/5/3/4. *sevEP-Gal4* is mostly in R3/4 photoreceptors. And *mδ0.5-Gal4* is mainly in R4. *ey-Gal4* was used to test the UAS-RNAi lines for each candidate gene. Then I used *ro-hs-Gal4* or *sevEP-Gal4* to knock-out a specific gene in various photoreceptors to determine whether the gene is involved in Notch signal-sending or signal-receiving cells.

Photoreceptors 2/5/3/4 are related to two events: R cell restriction and R3/R4 asymmetric determination. During R cell restriction, R2/5/3/4 send signals to the surrounding "mystery cells" to prevent them becoming neural cells. For R3/R4 asymmetric determination, R3 sends signals to R4 to form asymmetric pattern in the facets. When Notch signal-sending activity is disrupted in R2/5/3/4, both developmental

Transformant ID	ON	OFF	V	chr	*ey-GMR*	*ro-hs*	*ro+DCR*	*sev*	*mδ0.5*
Notch, 3C									
1112	1	0	V	2	L-noH	M2		M2	
27228	1	0	L	3	L-noH	M4			
27229	1	0	V	3	L-noH	M3		M3	
100002	1	0	V	2	L-noH	M1		M1	M
Dl, 92A									
3720	1	0	V	2	M2	M2		M2	
37287	1	0	V	3	M1-s-E	M1		M1	M
37288	1	0	V	3	M1	M1		M1	M
109491	1	0	V	2	M3	M3		M3	
Serrate, 97E									
27174	1	0	V	1	M1			wt	
108348	1	0	V	2	M2			wt	
Neurized, 85C									
10662	1	0	V	3	wt	wt	wt		
108239	1	1	V	2	M	M	M		
Mib, 72C									
27525	1	0	V	2	M	wt	wt	M	
27526	1	0	V	2	M	wt	wt	M	M
chc, 13F5~F7									
23666	1	0	V	2	L-noH	Lethal		M	
24789	1	0	V	2	L-noH	Lethal		M	M
103383	1	0	V	2				M	
Rab5, 22E									
34094	1	2	V	1					
34096	1	2	V	3	Lethal	Lethal		M2	M
103945	1	1	V	2	Lethal	Lethal		M1	L
Rab11, 93B									
22198	1	3	V	3	Lethal	Lethal		M1	
108382	1	0	V	2	Lethal	Lethal		M2	
Rab7, 95D								M2	
40337	1	0	V	3	wt	wt	wt	M1	M
40338	1	0	V	3	wt	wt	wt	M1	M
Shibire, 13F18								M3	
3798	1	1	L	3	M-s-L	M			
3799	1	1	S	3	L	M			
105971	1	1	V	2	M?	M	wt		
lqf, 66A									
35948	1	1	L	3	M2	wt	wt		
35949	1	1	V	2	M3?	wt	wt		
107300	1	0	V	2	M1	wt?	M		
aux, 82A									
16182	1	0	V	2	M?	wt			
103426	1	1	V	2	M-s-L	wt	M		

Transformant ID	ON	OFF	V	chr	*ey-GMR*	*ro-hs*	*ro+DCR*	*sev*	*mδ0.5*
Dmel\Csk / C-terminal Src kinase / CG42317, 86E									
32877	1	0	V	3	M2			wt	
109813	1	0	V	2	M1			M?	
Dmel\trio / Rho guanyl-nucleotide exchange factor activity, 61E									
40137	1	0	S	3	M?			wt	
40138	1	0	V	3	M-F			wt	
Dmel\Rala / Ras-related protein, 3E									
105296	1	0	L	2	M-F			M?	
Dmel\spen / split ends, 21B									
49543	1	1	V	2	M			M	
108828	1	1	V	2	M			M	
Sanpodo, 99F									
6856	1	0	V	3	wt			M?	
104092	1	0	V	2	wt			wt	
Presenilin, 77C									
43083	1	0	L	3	M2			wt	
101379	1	0	V	2	M1			wt	
Avalanche, 78D									
5413	1	0	V	3	L			M	
107264	1	0	V	2	L			M?	
Tsg101 / Erupted / VPS23, 73D									
23944	1	0	S	2	M-T			M?	
VPS25, 44D									
38821	1	0	V	3	M2			M?	
108105	1	0	V	2	M1-T			M?	
4267	1	2	V	2	M?	wt	wt		
VPS20, 58F									
26388	1	0	V	1	M-T			M?	
103944	1	1	V	2	L			M	
Big brain, 30F									
46675	1	**727**	V	1	L			M	
103327	1	2	V	2	M			wt	
Rumi, 94C									
14480	1	1	V	2	M?			wt	
14481	1	1	V	3	M?			wt	
Bearded, 71A4									
107929	1	1	V	2	M			M?	
Tom, Twin of m4, 71A3									
36613	1	0	V	2	wt			wt	
Ero1L, 64A									
51169	1	0	V	3	M1			M?	
110454	1	0	V	2	M2			M?	

Transformant ID	ON	OFF	V	chr	*ey-GMR*	*ro-hs*	*ro+DCR*	*sev*	*mδ0.5*
Egghead, 3A									
10137	1	0	V	1	wt			wt	
45160	1	0	V	2	M?			M?	
Brainiac, 4A									
44939	1	0	S	3	wt-S			wt	
45457	1	0	L	3	wt-S			M?	
UbcD1 / Dmel\eff / effete, 88D									
26011	1	0	V	3				M?	
26012	1	0	V	3	M?			wt	
105731	1	0	V	2	M			M?	
Dmel\CHIP / CG5203, 31E									
34124	1	1	V	2	M?			M?	
34125	1	1	V	2	M?			wt	
107447	1	0	V	2	M			M	
β–Arrestin									
104029	1	0	V	2	M?			M?	

Table 2. RNAi analysis from VDRC stocks. Each RNAi line was driven by one of the four Gal4 enhancers: ey-Gal4, GMR-Gal4; ro-hs-Gal4; sevEP-Gal4; and mδ0.5. The RNAi effect was analyzed based on the phenotypes of the flies. L-noH: Lethal without head; M-T: Mutant with Tumor-like eyes; M-s-L: semi-lethal with mutant phenotypes; M-s-E: Mutant with small eyes.

events will be affected. The facets will contain extra photoreceptors and show symmetric pattern. When Notch signal-receiving activity is disrupted in R2/5/3/4, R cell restriction can still happen. However, R3/4 asymmetry will be affected. The facets will become symmetric with normal numbers of photoreceptors. The *ro-hs* promoter drives expression of proteins mainly in R2/5/3/4. I decided to use *ro-hs*-Gal4 to knock-down specific genes in those cells. The adult eyes were dissected to determine the pattern of the photoreceptors. Based on the mutant phenotypes, I can decide firstly if that gene is involved in Notch signaling, and secondly if the gene is involved in Notch signal-sending or receiving during *Drosophila* eye development.

A.7.2. Results

A.7.2.1. Choose the genes interested

To test the efficiency of the Gal4/UAS system and determine whether the enhancer traps work as described in the literatures, I started with three control genes: the Notch receptor itself, and the two ligands Delta and Serrate. Then, I tested the genes that have been shown to function in Notch signaling. Finally, I investigated some of the general endocytic factors in this assay cause endocytic trafficking has been shown to regulate Notch signaling pathway in various contexts. In total, I tested 36 different genes with 80 transgenic lines (Table 2).

A.7.2.2. Find a good UAS-RNAi line from VDRC

There are two general concerns about RNAi analysis: the targeting is not specific to one single gene and the knock-down is not efficient to show mutant phenotypes. To get around these two problems, I tried to use several transgenic UAS-RNAi lines for each gene. Results from different lines driven by *ey-Gal4 - GMR-Gal4* were used to determine the best line to continue the experiment with *ro-hs-Gal4* or *sevEP-Gal4*.

A.7.2.3. Choose the appropriate Gal4 line for each RNAi line

Three Gal4 lines have been shown to drive protein expression in some of the photoreceptor 2/5/3/4: *ro-hs-Gal4* in all; *sevEP-Gal4* in R3/4; and *mδ0.5-Gal4* in R4. The primary data using Notch RNAi lines showed that none of the four Gal4 lines display restricted patterns as documented. All four lines killed the fly when driven by the *ey-Gal4 - GMR-Gal4*. There is essentially no head when I opened the dead pupal cases. These results indicate that the *ey-Gal4 - GMR-Gal4* driver is not specific to the eye. When I drove the four Notch lines with *ro-hs-Gal4*, the eyes showed all kinds of mutant phenotypes. Some of them gave symmetric facets as expected, whereas others generated extra-photoreceptor facets. There are even some facets containing fewer photoreceptors. These results showed that the *ro-hs-Gal4* promoter has leaky expression during early eye development. So I switched to a *sevEP-Gal4* line to drive three of the Notch RANi lines.

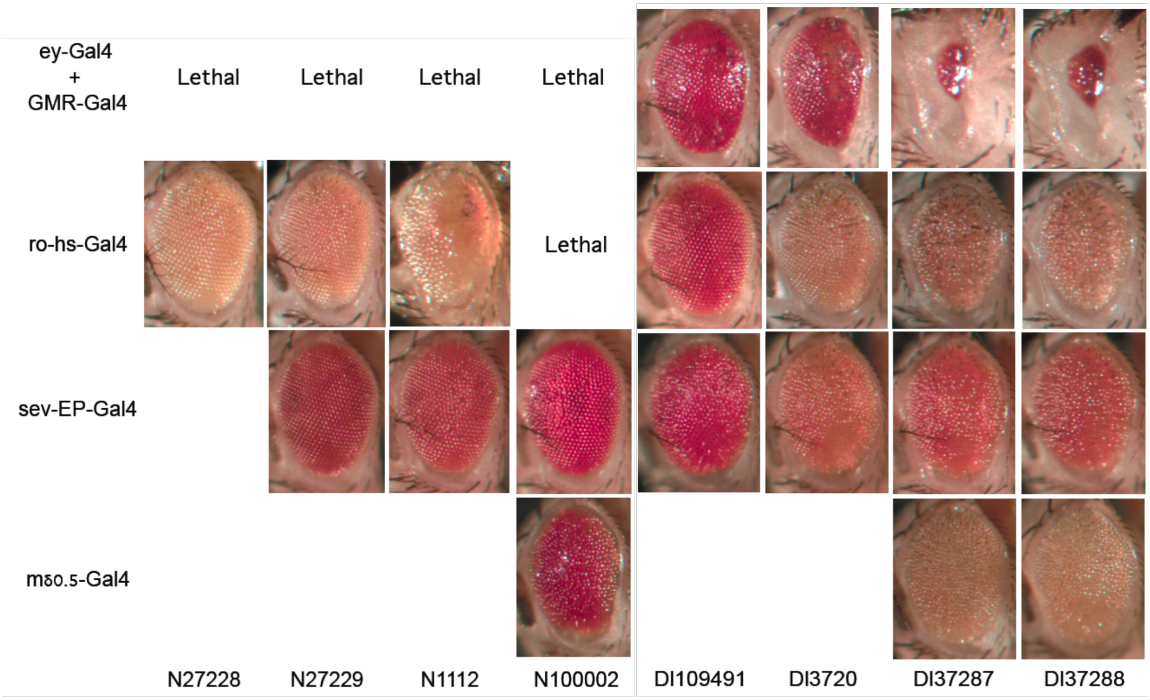


Figure A.4. Choose the appropriate Gal4 lines. Four drivers were used to express four RNAi lines of Notch and four RNAi lines of Delta in the eye. Adult eye images were taken and adult eyes were sectioned when necessary.

It seems this Gal4 line is more specific than the *ro-hs-Gal4* promoter. Most of the mutant facets showed symmetric facets, resulting from the failure of R3/4 asymmetric determination. In one of the lines all the mutant facets showed the symmetric phenotype only, so I decided to use the *sevEP-Gal4* driver to continue the experiments.

A.7.3. Discussion

Although all the drivers showed leaky expression, the RNAi results in the Notch receptor and ligands produced expected results. This indicates that it is possible to combine the Gal4/UAS system and the RNAi technique to conduct gene function analysis for Notch signaling in the eye. However, most of the analysis with other genes failed to generate clear results. There might be several reasons to explain these results.

A.7.3.1. The proteins are very stable

Notch signaling depends solely on the formation of the Notch intracellular domain (NICD) in the cytosol through serial cleavages and transport of the NICD into the nucleus. To get constant Notch signaling, it is important for the cells to constantly express Notch receptor. The same phenomenon might apply to the ligands too. This makes the protein levels of the Notch receptor and ligands extremely sensitive to the translational levels of the related genes. However, most of the other proteins may be very stable. Chc and Epsin could be recycled over and over, as suggested in the Auxilin analysis. Auxilin is an enzyme to catalyze the uncoating event. In the end-division cells like the photoreceptors 2/5/3/4, these protein activities can last for a long time. That may explain why the RNAi experiments with the receptor and the ligands gave better results. Alternatively, the RNAi lines I used might be not strong enough to show the expected mutant phenotypes.

A.7.3.2. The genes have pleitrophic functions

One reason that I decided to use the RNAi technique to analyze the possible roles of the endocytic factors in Notch signaling is that most of those genes have pleiotrophic functions. Unfortunately, this is still the case even when I restricted the knock-down effect only in two cells R3/4 by *sevEP-Gal4*. Rab5, Rab11 showed a strong effect in rhabdomere formation, which makes it hard to visualize the photoreceptors in the adult eyes. One way to get around the problem is to check out the earlier developmental events during eye development, for example, in the third instar larva eye discs.

Appendix 8.

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PLoS one

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

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Abstract

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

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Introduction

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

Several results support the pulling model. First, when separated from its transmembrane domain and secreted, the extracellular domain of Delta blocks Notch activation [9]. Second, the extracellular domains of Notch and Delta are sometimes found together in endosomes inside signaling cells [10,11]. Third, structural studies suggest that the ADAM protease site on the Notch extracellular domain, which must be cleaved to activate the receptor, is exposed by ligand binding [12]. Finally, there is evidence that ligand internalization into signaling cells depends on the presence of Notch in adjacent cells [13]. There is also evidence in favor of the recycling model. For example, in some epithelial cells, the GTPase Rab11, which is required for endosomal recycling [14], is needed in signaling cells for signaling and for

Delta recycling [15–17]. In addition, the ligand intracellular domain, which is normally ubiquitinated by specific ubiquitin ligases that are necessary for signaling and ligand endocytosis [18–26], may be replaced by the internalization and recycling signals from the vertebrate LDL receptor [27]. Finally, Delta transcytosis has been observed, and it is thought to relocate ligand to a site on the plasma membrane near Notch in the adjacent cell [15–17,28].

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

Epsin and auxilin are two endocytic proteins required in signaling cells for ligand endocytosis and signaling [27,30–36]. Epsin, which has been shown to be an essential component of the Notch pathway in *C. elegans* [36] and vertebrates [37], as well as in *Drosophila* [27,30], has binding sites for the plasma membrane, ubiquitin, clathrin, and other proteins present in clathrin-coated vesicles [38]. Although the mechanism of epsin function in Notch signaling is not well understood, studies of epsin in other contexts suggest that epsin probably links ubiquitinated ligand with endocytic vesicles [38]. Another endocytic protein, auxilin, is also required in Notch signaling cells in all *Drosophila* tissues tested [31–34]. Auxilin brings the ATPase Hsc70 to clathrin cages, and stimulates Hsc70 to uncoat clathrin coated vesicles [39]. At first glance, it would appear that the requirement for auxilin supports the recycling model; uncoating of newly internalized clathrin-coated vesicles containing ligand is prerequisite for trafficking of ligand through an endosomal pathway for recycling. However, it is also possible that auxilin is required only to maintain the pool of free clathrin, and not for production of uncoated vesicles [33]. In addition, it was shown recently that to send Delta signals, *Drosophila* female germline cells require epsin-mediated endocytosis, but not clathrin [40]. Vertebrate epsin is known to function in both clathrin-dependent and clathrin-independent endocytosis [41–43]. However, this result suggests the possibility that epsin function in Notch signaling is generally clathrin-independent, and thus the function of auxilin in signaling cells might be other than its characterized role in clathrin dynamics.

Here, we performed genetic experiments in *Drosophila* to test the roles of *Rab11* and *auxilin* in Notch signaling, and ultimately to test the recycling model. First, we found that *Rab11* is not required for Notch signaling events in the eye disc that require both epsin and auxilin. Second, we found that female germline cells that do not require clathrin in order to signal also do not require auxilin. Finally, we found that overexpression of both clathrin heavy chain and epsin suppress nearly completely the lethality and severe eye morphology defects of *auxilin* mutants. Taken together, the results argue strongly that in many cell types, ligand recycling is not the primary function of epsin-dependent ligand endocytosis by Notch signaling cells.

Results

Rab11 was dispensable for Notch signaling events in the eye disc

We wanted to determine whether or not ligand recycling is required for Notch signaling during eye development. If so, it would be expected that the two GTPases Rab5 and Rab11 would both be required in signaling cells. Rab5 mediates fusion of early endosomes with the sorting endosome, an event required for trafficking through any endosomal pathway, and Rab11 is required for subsequent routing of an endosome through the recycling pathway [14]. First, we asked about one characterized event early in eye development, called R-cell restriction [30]. Photoreceptors R2/R5 and R3/R4 in early ommatidial preclusters signal via Delta to other precluster cells, preventing them from becoming ectopic photoreceptors (R-cells). When this signaling event fails (for example in hypomorphic *lqf* or *aux* mutants), ommatidia have one or several extra photoreceptors [30,32,33,44]. When dominant negative *shibire* (encodes *Drosophila* dynamin) or *Delta* genes are expressed specifically in R2/R5 and R3/R4 using a *rough* (*ro*) gene expression vector, ommatidia in adult eyes have extra R-cells due to failure of R-cell restriction [30]. Using the same *ro* expression vector, we generated transgenes expressing dominant negative forms of *Rab5* or *Rab11* (*ro-Rab5*^{N142I} and *ro-Rab11*^{N124I}). Rab11^{N124I} has been shown to act as a dominant negative late in eye development, where it blocks transport of rhodopsin to rhabdomeres and formation of multivesicular bodies in late endosomes [45]. Neither transgene had an effect on eye development, even when present in as many as four copies (data not shown). These results suggest that neither *Rab5* nor *Rab11* is required for this Notch signaling event, but there are other plausible explanations for the failure of these transgenes to interfere with Notch signaling. For example, expression levels that are too low for effective competition with wild-type proteins.

To overcome the problem in interpreting results obtained with dominant negative transgenes, we wanted to generate *Rab5-* or *Rab11-* (null) clones in the eye disc. *Rab5* null clones have an overgrowth phenotype that would obscure a Notch signaling defect [46]. *Rab11* null clones in the eye have not been reported, but we were able to generate them, and they were not hypertrophic (see below). The *Rab11* null allele we used, *Rab11*^{FRT}, has a deletion of the promoter and first two exons, and produces no protein [47]. We used *Rab11* null clones to ask whether or not well-characterized signaling events in the eye disc required Rab11. The adult *Drosophila* eye develops from the larval eye imaginal disc, a monolayer epithelium [48]. Rows of ommatidia assemble stepwise posterior to the morphogenetic furrow, as it moves from the posterior to the anterior of the disc. The first cells to join the facets are the eight photoreceptors (R1-R8), and they do so in an invariant order in every ommatidium. Nearly every step of ommatidial assembly involves Notch signaling [49,50], and so elimination of the Notch pathway in clones of mutant cells is catastrophic to eye development. In *Notch-* clones, no cells are specified as photoreceptors because Notch signaling is required anterior to the furrow to give cells neural potential, a process called proneural enhancement [51]. In *Delta-* clones, there are no photoreceptors in the middle of the clone. At the clone border, however, *Delta-* cells do become photoreceptors because they receive Notch signals from adjacent wild-type cells. Discrete ommatidia do not form within the clone because subsequent lateral inhibitory signaling cannot occur between adjacent *Delta-* cells, and the result is that too many cells adopt neural fate [51]. Clones of either *lqf-* (*liquid facets* [*lqf*] is the *Drosophila* epsin gene [44]) or *auxilin-* (*aux-*) cells in the eye disc appear identical to *Delta-* clones, consistent with the idea that epsin and auxilin are required in the signaling side of the Notch pathway [30,33]. In accord with the developmental mutant phenotype, reporters for Notch activation are not expressed at all in *N-* cell clones, and are expressed in *Dl-*, *lqf-*, or *aux-* clones only in cells at the clone border, adjacent to wild-type cells that can signal [30,33,34,51, and see below].

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

immunostained with anti-Elav, which labels photoreceptor nuclei [52]. We found that compared with the calamitous effect on development in *N-*, *Dl-*, *lqf-*, or *aux-* clones [30,33,34,51], ommatidial assembly was not obviously disrupted within the *Rab11-* clones; discrete ommatidia were present in the middle of the clone and at the clone borders (Fig. 1E, E2-E3'). This is consistent with results of similar experiments performed with *Rab11* hypomorphs, where eye morphology defects observed were due mainly to late events: cell death and the failure to form light-gathering rhabdomeres [45,53]. These eye discs also contain a reporter transgene called *mδ-lacZ*, which is transcribed in R4 when Notch is activated in response to Delta signaling by R3 [54,55]. This Notch signaling event distinguishes R3 and R4 [54–56]. No cells in *Notch-* (null) clones expressed *mδ-lacZ* (Fig 1A, A'), while *Delta-* (null) cells did express *mδ-lacZ*, but only when they were adjacent to wild-type cells at the clone edges (Fig. 1B, B'). We found that like *Delta-* cells, *lqf-* (null) or *aux-* (null) cells at the clone edge activated *mδ-lacZ* (Fig. 1C–D'). This result is consistent with other evidence that *lqf+* and *aux+* function in the signaling cells [27,31,33,34,40], and was important to show here because the marker used to assess Notch activation in *lqf-* or *aux-* eye clones previously [31,33,34,40] was sometimes expressed in the absence of Notch activation [51]. In *Rab11-* cells, the pattern of *mδ-lacZ* expression was undisrupted; Notch was activated in the middle of the clone as well as at the edges (Fig. 1E–E2). The *mδ-lacZ* marker also revealed that the clusters inside *Rab11-* clones were at least normal enough that R4s were neatly spaced within the clone (Fig. 1E1, E1'). We conclude that *Rab11* is not required for several Notch signaling events in the eye disc – proneural enhancement, lateral inhibition, and R3/R4 signaling – all of which require epsin.

Although *Rab5* and *Rab11* are required for Notch signaling in *Drosophila* sensory organ precursor cells [15−17], it has been shown recently that female germline cells signal without either GTPase [40]. The observation here that *Rab11* is not required for several Notch signaling events in somatic cells indicates that the ability of a cell to signal independent of *Rab11* is not peculiar to the germline. Moreover, the eye disc is an epithelium, and thus the requirement for Rab11 in Notch signaling is not a general feature of epithelial cells. In addition, as the germline experiments were performed with a *Rab11* dominant negative transgene, residual *Rab11+* activity could potentially have accounted for the results. Here, we remove all doubt that cells devoid of Rab11 may activate Notch in their neighbors.

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

Auxilin is known to be required for Notch signaling in the eye, wing, and embryo [31–34]. Strong genetic interactions between *clathrin heavy chain* (*chc*) and *lqf* [44], and the requirement for *aux* in signaling cells [31–34] suggested that epsin promotes clathrin-mediated endocytosis of ligand in signaling cells. Therefore, we were puzzled by the observation that for signaling by female germline cells, epsin is needed, but clathrin is dispensable [40]. One possibility suggested by this observation is that epsin likewise promotes clathrin-independent endocytosis of ligand in imaginal discs, and that in imaginal discs and embryos, auxilin and possibly also clathrin perform functions other than clathrin-mediated endocytosis. Alternatively, as epsin is known to facilitate both kinds of endocytic pathways [41–43], epsin may promote ligand endocytosis through a clathrin-independent pathway in female germline cells, and through a clathrin-dependent pathway in imaginal discs. In this scenario, auxilin would perform its known function in clathrin dynamics, which is uncoating clathrin-coated vesicles after internalization [39].

One way to distinguish between these two alternatives is to determine if the function of auxilin in Notch signaling is separable from the function of clathrin, and so we tested whether or not *aux+* was required in the female germline. In the ovary, the sixteen germline cells in the nurse cell/oocyte complex signal to surrounding somatic follicle cells at stage 6 of oogenesis, and Notch receptor activation may be monitored by expression of the target gene Hindsight (Hnt) (Fig. 2A) [40,46]. In wild-type ovaries, Hnt is present in the nuclei of all surrounding follicle cells following stage 6 (Fig. 2B,B') [40,46]. In mosaic ovaries in which the follicle cells are *aux+* and the germline cells are *aux-* (null), the follicle cells nevertheless express Hnt (Fig. 2C–D'). Identical results were observed previously in ovaries mosaic for *Chc+* and *Chc-* cells [46] (see legend to Fig. 2). The same results were obtained using two different *aux-* backgrounds: aux^{136}/aux^{727} or aux^{F956*} homozygotes. aux^{136} [32,33] and aux^{F956*} [34] have nonsense mutations positioned between the codons for the clathrin binding domain and the J domains, which binds Hsc70. Thus, C-terminally truncated auxilin proteins that could in theory be produced would lack the J domain, which is essential for auxilin function in Notch signaling [33,34]. aux^{727} has a nonsense mutation early in the open reading frame, and an N-terminally truncated protein containing both the clathrin binding and J domains, produced by translation reinitiation, could function in Notch signaling [33,34]. No auxilin protein from aux^{727} was detectable with immunofluorescence using an auxilin antibody, and the genetic behavior of aux^{727} was indistinguishable from that of aux^{136} [34]. Thus, we conclude that the germline cells, which do not require clathrin for signaling, also do not require auxilin. This result indicates that germline and eye and wing disc cells simply internalize ligand through different endocytic pathways. Thus, the requirement for clathrin and auxilin in eye and wing discs most likely means that auxilin regulates clathrin dynamics in Notch signaling cells in the eye disc.

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

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

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

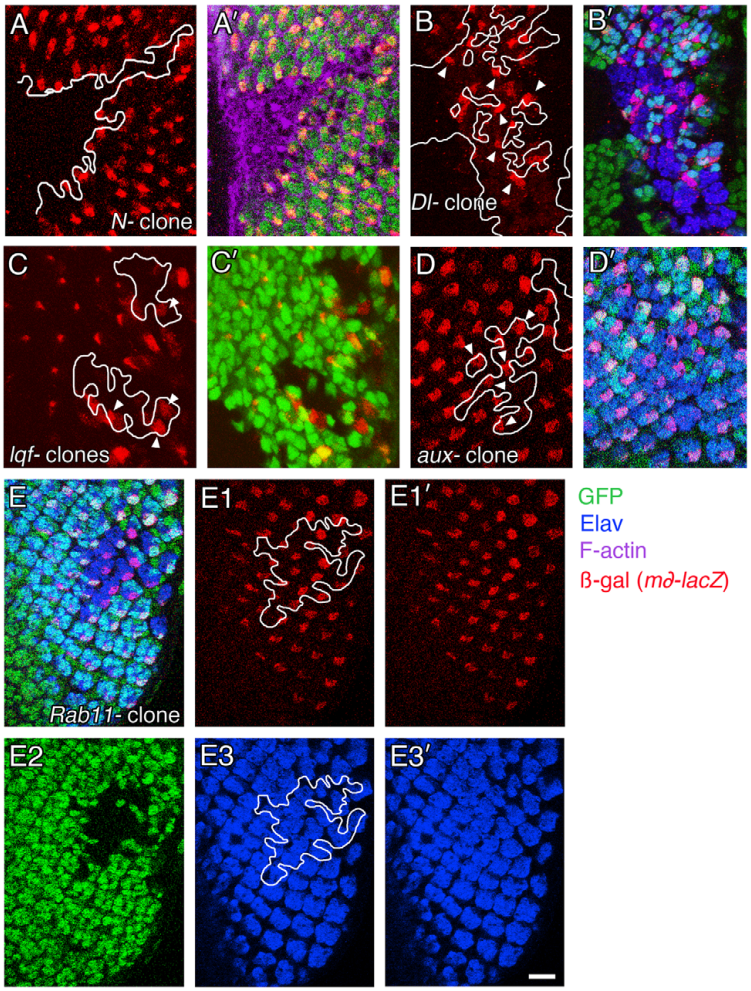

Figure 1. *Rab11* is not required for Notch signaling in eye discs. Confocal microscope images of third instar larval eye discs with clones of mutant cells are shown. The discs are immunolabeled to reveal Notch activation (anti-ßgal), photoreceptor cell nuclei (anti-Elav), and F-actin (phalloidin). Homozygous mutant cell clones are marked by the absence of nuclear GFP expression. Clones are outlined in white. Arrow heads point to some of the mutant cells within the clones that express ß-gal, indicating that Notch is activated. (A,A′) A *Notch* null (*N*-) clone was generated in larvae of the genotype N^{55e11} *FRT19A/ubi-ngfp FRT19A; ey-gal4, UAS-flp/+; mə-lacZ/+*. (B,B′) A *Delta* null (*Dl*-) clone was generated in larvae of the genotype *ey-flp;mə-lacZ/+; FRT82B* Dl^{rev10}*/FRT82B ubi-ngfp* (C,C′) *lqf*- clones generated in larvae of the genotype *eyflp; mə-lacZ/+;* lqf^{4RI} *FRT80B/ubi-ngfp FRT80B*. (D,D′) *aux*- clones were generated in larvae of the genotype *ey-flp; mə-lacZ/+;* $FRT^{5\text{-}5Z3515}$ aux^{F956*}*/*$FRT^{5\text{-}5Z3515}$ *tub-ngfp*. (E-E3′) The same *Rab11*- clone is shown in all panels, generated in larvae of the genotype *ey-flp; mə-lacZ/+;* $Rab11^{\Delta FRT}$*/FRT5377 Hrb98DE::GFP*. Scale bar 20 µm.
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nonsense mutation in *aux* (aux^{K47}/aux^{D128}) [32] rarely reach adulthood when grown at 25°C (Table 1). In addition, adult escapers have severely malformed imaginal disc-derived structures [32], including their eyes (Fig. 3A,B,F–H). Addition to the aux^{K47}/aux^{D128} flies of a transgene containing a genomic DNA copy of the *Chc*+ gene (*PgChc*+) that can substitute for the endogenous *Chc*+ gene [33] increases the eclosion frequency of adults markedly (Table 1). Also, as reported previously [33], the mutant eye phenotype of those rescued adults was suppressed somewhat (from 8% to 28% wild-type ommatidia) (Fig. 3D, J).

Next, we wondered whether epsin overexpression, either alone or in combination with *Chc*+ overexpression, would suppress the aux^{K47}/aux^{D128} mutant phenotype. We reasoned that if epsin links ligand to clathrin, it may be freed along with clathrin when auxilin uncoats clathrin from newly endocytosed vesicles. Alternatively, increased epsin levels in *aux* mutants may result in more efficient plasma membrane localization of the remaining free clathrin. We found that a transgene with a genomic DNA copy of the *lqf*+ gene (*Pglqf*+) that complements *lqf* null mutants (similar to the transgene in ref. 44; X. X. and J.A.F., manuscript in preparation) rescued the

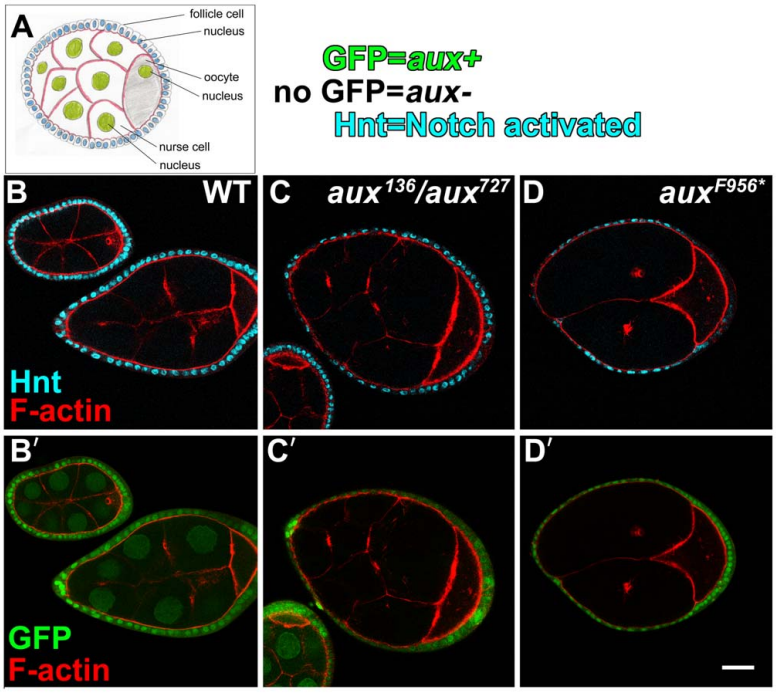

Figure 2. Female germline cells do not require *auxilin* to send Delta signals to follicle cells. (A) A diagram of an oocyte/nurse cell complex (stage 6–7) is shown. The fifteen nurse cells are diploid, and the cytoplasms of the nurse cells and the oocyte are interconnected by cytoplasmic bridges. (B–D′) Confocal microscope images of oocyte/nurse cell complexes are shown. The complexes were immunolabeled to reveal Notch activation in the follicle cells (anti-Hnt) and F-actin (phalloidin). Homozygous mutant cell nuclei are marked by the absence of GFP. (B,B′) Wild-type (WT) complexes are shown. Notch is activated in the follicle cells. (C,C′) A mosaic complex with *aux*- germ-line cells and *aux*+ follicle cells is shown. Notch was activated in the follicle cells. The clone was generated in females of the genotype *hs-flp/+; ubi-gfp tub-aux FRT40A/FRT40A; aux*^{136}*/aux*^{727}. (D,D′) As in (C,C′), except the genotype was *hs-flp/+; FRT*$^{5\text{-}5Z3515}$*, aux*^{F956*}*/FRT*$^{5\text{-}5Z3515}$*, ubi-ngfp*. Reduced levels of Hnt were seen at the poles of the *aux+/aux*- mosaic oocyte/nurse cell complexes, as was also observed in *Chc+/Chc*- mosaics [SLW and DB, unpublished observation]. This is quite distinct, however, from the absence of Hnt throughout the follicle epithelium observed with *lqf*- or *Dl*- germ line clones [40]. Scale bar 20 μm.
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lethality of *aux*^{K47}*/aux*^{D128} mutants (Table 1) and suppressed their mutant eye phenotype even better than *PgChc*+ did (62% wild-type ommatidia) (Fig. 1E,K). Moreover, *aux*^{K47}*/aux*^{D128} flies carrying both *Pglqf*+ and *PgChc*+ had remarkably normal-appearing eyes (97% wild-type ommatidia) (Fig. 3C,I). However, no increase in viability was detected in these flies above the level observed with *Pglqf*+ alone (Table 1; see also legend).

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

Discussion

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

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

genotype[a]	# flies[c]	# expected[d]
w; +/CyO; aux^{K47}/aux^{D128}	2	0
w; Pglqf+/+; aux^{K47}/aux^{D128}	84	61
w; PgChc+/CyO; aux^{K47}/aux^{D128}	44	61
w; PgChc+/Pglqf+; aux^{K47}/aux^{D128}	52	61
w; +/CyO; aux^{b}/TM6B	69	122
w; P glqf+/+; aux/TM6B	114	122
w; PgChc+/CyO; aux/TM6B	193	122
w; PgChc+/Pglqf+; aux/TM6B	114	122
total	672	671

[a]The flies of the genotypic classes listed were obtained from crosses of three *w; gChc+/+; aux^{K47}/TM6B* males with eight *w; glqf+/CyO; aux^{D128}/TM6B* virgin females, kept at 25°C, and transferred to new food vials every 2–3 days for 5 days. Flies with *glqf+* only were differentiated from *gChc+/glqf+* flies by the latter having darker eye color.

[b]*aux* means either *aux^{K47}* or *aux^{D128}*

[c]The important comparison is between the first row and the three rows beneath. Addition of either or both *Pglqf+* or *PgChc+* transgenes increases drastically the viability of *aux^{K47}/aux^{D128}* adults. It is not clear why the effect of both transgenes is not greater than the effect of a single transgene. One possibility, suggested by the expected frequency of adults (see d below) is that each transgene rescues viability completely. In this case, the differences from expectation would be due to the effects of other aspects of the genotype, such as the presence or absence of *CyO*, and transgene insertion sites.

[d]The expected numbers were calculated making three simplifying assumptions: (1) *aux^{K47}/aux^{D128}* is completely lethal; (2) one copy of either transgene rescues viability fully; (3) no aspect of the genotype other than *aux^{K47}/aux^{D128}* affects viability.

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free epsin). Taken together, these results argue strongly that at least in some cell types, the fundamental role of Notch ligand endocytosis is not ligand recycling.

Is it possible that the fundamental mechanism of Notch signaling is so completely distinct in different cell types, that ligand endocytosis serves *only* to activate ligand via recycling in some cellular contexts, and *only* for exerting mechanical force on the Notch receptor in others? While formally possible, this is not parsimonious. Thus, we favor a model where the fundamental role of ligand endocytosis is to exert mechanical force on the Notch receptor. In addition, some cell types will also require ligand recycling. As no altered, activated form of ligand has yet been identified, while ligand transcytosis has been well-documented [15–17,28], the most likely role of recycling is to relocalize ligand on the plasma membrane prior to Notch receptor binding.

Materials and Methods

Drosophila mutants and transgenes

The alleles and transgenes used are listed below. FlyBase id numbers (http://flybase.org/) are provided when available. Chromosomes and genotypes used in particular experiments are indicated in Figure Legends. Mutant alleles: *aux^{F956}** (FBal0240439), *aux^{K47}* (FBal0197315), *aux^{D128}* (FBal0197310), *aux^{136}* (FBal0197311), *aux^{727}*(FBal0197308), *Rab11$^{\Delta FRT}$* [47], *Df^{ewF10}* (FBal0029366), *N^{55e11}* (FBal0012701). Transgenes: *PgChc+* [33], *tub-aux* [33], *ey-flp* (FBti0015982), *mδ-lacZ* (on 2 and 3; FBtp0010977), *hs-flp^{122}* (on X), *ubi-ngfp* (on X,2L,3R), *Hrb98DE::GFP* [47], *FRT82B* (FBti0002074), *FRT18A* (FBti0002070), *FRT40A* (FBti0002071), *FRT5377* [47], *FRT$^{5\text{-}5\sim3515}$* [34], *ey-gal4* (on 2), *UAS-flp* (on 2), *UAS-Rab11^{N124I}* (FBal0190955). Transgenes generated in this work: *Pglqf+* (on 2), *ro-Rab5^{N142I}* (multiple lines), *ro-Rab11^{N124I}* (multiple lines).

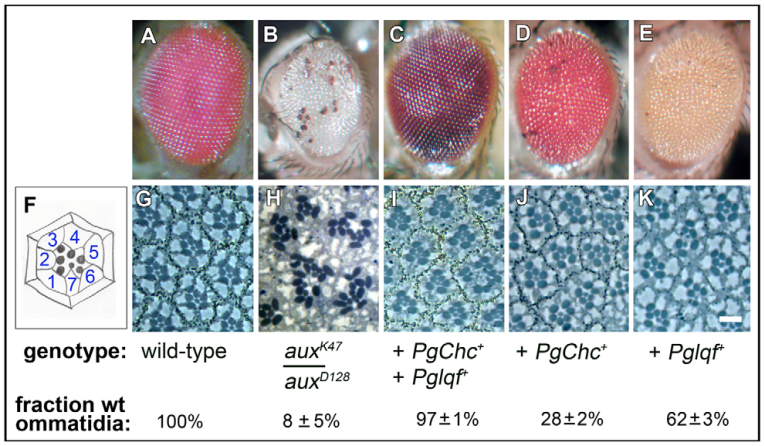

Figure 3. Overexpression of clathrin heavy chain and/or epsin suppresses the adult eye defects in *aux* loss-of-function mutants. (A–E) Light micrographs of adult external eyes of the genotypes indicated beneath are shown. (F) A diagram of an apical tangential section of a single ommatidium is shown.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

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Transgene construction

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

ro-Rab5^{N142I}. Total RNA from 5 w^{1118} females was isolated using TRI reagent (Molecular Research Center), and 5 µg was used for reverse transcription with SuperScriptII (Invitrogen). The primers used were Rab5F (5′-AAAGGCGCGCCATGGCAAC-CACTCCACGC-3′) and Rab5R (5′-AAAGGCGCGCCTCA-CTTGCAGCAGTTGTTCG-3′). The cDNA was diluted to 200 µl, and 2 µl was used as the template for the following PCR reactions. The mutant *Rab5* cDNA was generated in two steps. First, two PCR reactions were performed with mutagenic primers, Rab5CF (5′-GGCCGGCA**T**CAAGGCAG-3′) and Rab5NR (5′-CTGCCTTG**A**TGCCGGCC-3′). One reaction used the primer pairs F and NR, and the other used R and CF. Next, the amplification products from each reaction were mixed, and used together as a template for PCR with primers F and R. The resulting amplification product was ligated as an *Asc I* fragment into *BluescriptIIKS+* (Stratagene) with its *Bam HI* site changed to *Asc I*, an its DNA sequence was verified. Finally, an ~660 bp *Asc I* fragment containing the *Rab5^{N142I}* cDNA was ligated into *pRO* [59].

ro-Rab11^{N124I}. The mutant *Rab11* cDNA was obtained by PCR using as template genomic DNA from flies containing *UAS-Rab11^{N124I}* [45], and the primers Rab11F (5′-AAAGGCGCG-CCATGGGTGCAAGAGAAGACGA-3′) and Rab11R (5′-A-AAGGCGCGCCTCACTGACAGCACTGTTTGC-3′). The resulting ~660 bp amplification product was ligated as an *Asc I* fragment into *pUAS$_t$ –XA* [57] restricted with *Asc I*.

Analysis of eyes

Plastic sectioning of adult eyes was performed as described [60], and sections were viewed and photographed with a Zeiss Axioplan equipped with an Axiocam HRc. Eyes were photographed in whole flies using an Olympus SZX12 microscope equipped with a SPOT idea (Diagnostic Instruments) camera. For immunostaining, eye discs were fixed in PEMS and antibody incubations and washes were in PBST as described [61]. Primary antibodies were from the Developmental Studies Hybridoma Bank (DSHB): rat monoclonal anti-Elav (1:1), and mouse monoclonal anti-ßgal (1:50). Secondary antibodies were: 568-AlexaFluor goat anti-mouse (1:200) (Invitrogen), and Cy5-AffiniPure goat anti-rat (1:200) (Jackson ImmunoResearch). 633-AlexaFlour phalloidin (Invitrogen) was also used (15 µl of a 300 U/1.5 ml methanol stock solution). Immunofluorescent eye discs were photographed with a Leica TCSSP2 or SP2AOBS confocal microscope. Images were processed with Adobe Photoshop.

Analysis of germline clones

aux- germ line clones were generated by heat shocking first to third instar larvae at 37°C for 2 hours on 2 consecutive days. Adult females of the appropriate genotype were collected upon eclosion. The females were fed on yeast in the presence of males for 2 days, flipped onto fresh yeast for 2 more days, and then their ovaries dissected. Egg chambers were fixed in 4% formaldehyde in 1X phosphate-buffered saline (PBS) for 15 minutes and washed with PBS. Primary antibody staining was performed in 1X PBS + 0.3% Triton-100 (PBT3) containing 5% normal goat serum overnight at 4°C, followed by washing with PBT, staining with secondary antibodies, and mounting in antifade reagent (Invitrogen). The following antibodies were used: mouse anti-Hindsight at a dilution of 1:50 (DSHB) and 647-AlexaFluor donkey anti-mouse (Invitrogen). Cells were also labeled with TRITC-phalloidin (Sigma) at 1:200 to detect F-actin. Images were collected using a Leica TCS confocal microscope and assembled using Adobe Photoshop. Single sections are shown for each sample.

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Author Contributions

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

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Appendix 9.

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Research Paper

On the roles of the Drosophila KASH domain proteins Msp-300 and klarsicht

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Abbreviations: *klar*, *klarsicht*; *Msp-300*, *muscle specific protein-300*; KASH, klarsicht, anc-1, syne-1 homology; R-cell, photoreceptor

Key words: *Msp-300*, *klarsicht*, nuclear positioning, eye development, oocyte/nurse cell complex, KASH domain, actin, oogenesis

KASH (Klarsicht, Anc-1, Syne-1 homology) domain-containing proteins anchor the nucleus to the actin cytoskeleton or to microtubules. KASH proteins thus play pivotal roles in a variety of developmental processes where nuclear positioning is critical. Two KASH proteins have been identified in Drosophila: Muscle-specific protein-300 (Msp-300) and Klarsicht (Klar). Msp-300 anchors nuclei to actin, and has been reported to be essential for positioning of nurse cell nuclei during oogenesis, and thus production of mature ooctyes. Klar is required for positioning of photoreceptor and cone cell nuclei in the developing eye, which is critical for proper eye morphology. Here, we asked whether KASH domain-containing forms of Msp-300 are required for nuclear positioning in the eye, and we found that they are not. Moreover, in the course of this work, we discovered that contrary to previous reports, KASH domain-containing forms of Msp-300 are not required for viability, nor for oogenesis. However, we did find that Msp-300 has a function in egg laying, normally redundant with a function of Klar.

Introduction

The KASH domain is a small (~60 amino acid) C-terminal protein module that inserts into the outer nuclear envelope, allowing the N-terminal region of the protein to project into the cytoplasm.[1,2] There are two well-characterized kinds of KASH domain-containing proteins: those that connect the nucleus to actin, and those that connect the nucleus to the microtubule organizing center.[1,2] KASH domain proteins in vertebrates, *C. elegans* and Drosophila have been shown to play critical roles in nuclear positioning in a variety of contexts, including human brain development.[1-3]

In Drosophila, there are two identified KASH proteins, Msp-300 and Klar.[4-6] Msp-300 has N-terminal actin-binding domains,[5] and the N-terminal region of Klar, probably indirectly, binds microtubules.[7-11] Klarsicht is not essential for Drosophila viability, but is required for positioning of neural and non-neural nuclei that is critical for normal cell shape and eye morphology.[12,13] Msp-300 was reported to be required for viability, and for nuclear positioning during oogenesis.[14,15] Proper nuclear positioning of nurse cells in the oocyte/nurse cell complex prevents nuclei from blocking the cytoplasmic connections between the nurse cells and the oocyte, which are needed for transfer of RNAs and proteins to the oocyte.[16,17] In addition, tethering of the oocyte nucleus is critical as its position defines the ventral side of the oocyte and the dorsal/ventral axis of the resulting embryo.[18] Msp-300 was reported to be essential for proper positioning of nurse cell nuclei, and also possibly for oocyte nuclear positioning.[15] A role for Msp-300 in the eye has not been investigated previously.

We were interested in whether Msp-300 might play a role in nuclear positioning during eye development. One idea is that Klarsicht might drive nuclei to migrate apically along with the microtubule organizing center,[8] and then Msp-300 might tether the nucleus to actin at the apical plasma membrane. This hypothesis is analogous to roles suggested for the *C. elegans* Msp-300 homolog, Anc-1 and another *C. elegans* KASH protein, Unc-83.[1] To test this hypothesis, we generated *Msp-300* mutants that lack the KASH domain. We could not detect nuclear positioning or other defects in the eyes of the resulting mutants, even in the absence of KASH domain-containing forms of Klar protein. In the course of these experiments, we also determined that contrary to a previous report, KASH domain-containing forms of Msp-300 are not required for viability, female fertility or for nuclear positioning in during oogenesis. We did find, however, a role for KASH domain-containing forms of Msp-300 in egg laying that is normally redundant with Klar function.

Results and Discussion

A KASH domain-containing form of Msp-300 is expressed in larval and pupal eye discs. In order to determine if a KASH-containing form of Msp-300 is expressed during eye development, we tested first for the presence of transcripts. We performed PCR using cDNA prepared from third instar larval eye disc RNA or embryo RNA (a positive control) as the template and primers that flank the KASH domain coding sequences. In both the eye disc and embryo samples, we detected PCR products of the size corresponding to spliced mRNA (Fig. 1A and B).

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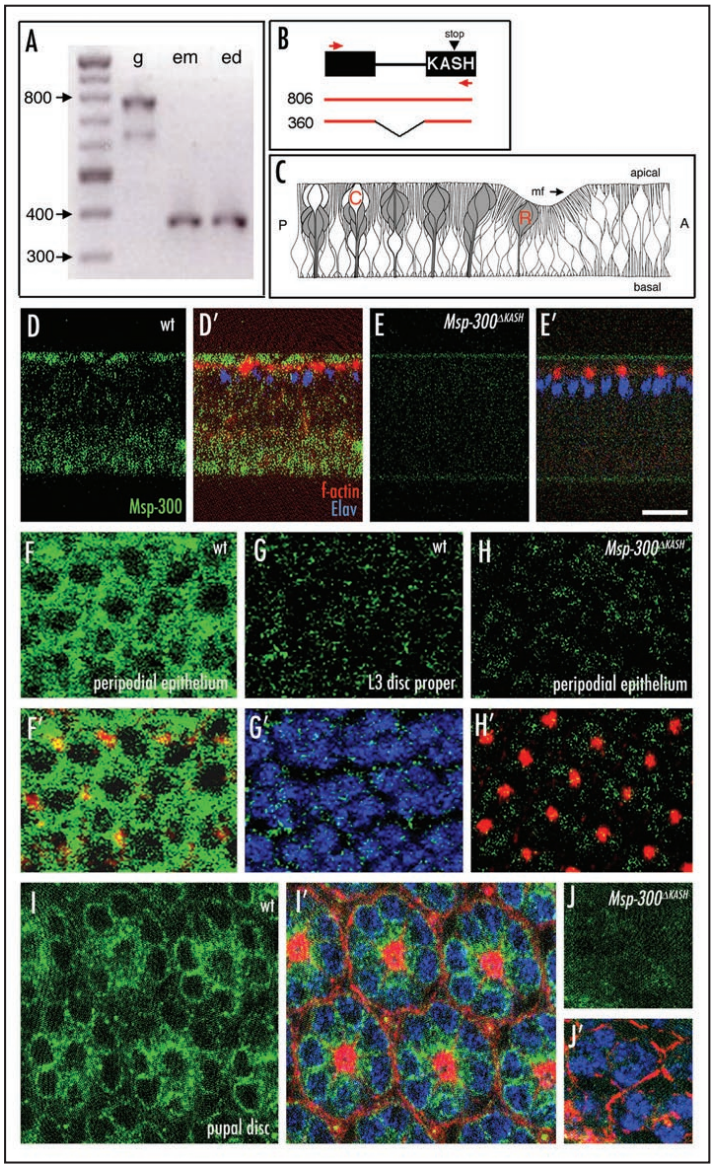

Figure 1. Expression of KASH domain-containing forms of Msp-300 in the Drosophila eye. (A) Ethidium bromide stained agarose gel showing PCR products from wild-type (w^{1118}) genomic DNA (g), embryo cDNA (em) or eye disc cDNA (ed). (B) Diagram showing the expected sizes of PCR products in (A). Red arrows are PCR primers. Black boxes are C-terminal *Msp-300* exons separated by an intron (black line), one of which includes KASH domain coding sequences and the stop codon. The solid red line represents the 806 bp amplification product expected if genomic DNA is the PCR template, and the red line broken by the intron represent the 360 bp amplification product expected if spliced mRNA, used to generate cDNA, is the PCR template. (C) Diagram (Z-section) of third instar larval eye disc: R-cells (R) are grey; cone cells (C) are white. The morphogenetic furrow (mf) is moving in the direction of the arrow. A, anterior; P, posterior. Modified from ref 30. (D–J′) Confocal images of third instar larval eye discs labeled with anti-Msp-300, anti-Elav (in R-cell nuclei) and phalloidin (marks f-actin at plasma membrane). The discs are either wild-type (wt) or mutant (*Msp-300*$^{\Delta KASH}$). (D and D′) Z-section of disc as in (C). (E and E′) Z-section; negative control for anti-Msp-300 signal. (F and F′) Apical XY-section. (G and G′) Slightly more basal XY-section of same disc in (F and F′). (H and H′) Apical XY-section as in (F and F′); negative control for anti-Msp-300 signal. (I and I′) Apical XY-section of pupal eye disc. (J and J′) Disc as (I and I′); negative control for anti-Msp-300 signal. The scale bar in (E′) is ~20 μm in (D–E′), and ~10 μm in all other panels.

Next, we used a polyclonal antibody generated against a 225 amino acid region including the entire KASH domain and ~160 amino acids N-terminal to it,[15] to detect KASH-containing protein in whole-mount third instar larval and pupal eye discs. In larval discs, the morphogenetic furrow is advancing and rows of ommatidia are assembling posterior to it, starting with the photoreceptor cells (R-cells) and followed by the non-neural cone cells[26] (Fig. 1C). The R-cell and cone cell nuclei rise apically as the cells are determined,[27] and their apical nuclear position depends on a KASH-containing form of Klar called Klara.[6,13,28] We find that Msp-300 protein is concentrated mainly in the peripodial epithelium, a cell layer that overlies the disc proper (Fig. 1D, D', F and F'). Notably, no perinuclear Msp-300 is observed in the disc proper (Fig. 1G and G'). During pupal eye development, the accessory cells (pigment and bristle cells) that form the hexagonal lattice surrounding the photoreceptors and cone cells are recruited into developing ommatidia.[26] The nuclei of the R-cells and cone cells normally remain apical (ref). In contrast to what was observed in third instar larval eye discs, perinuclear Msp-300 is observed in R-cells of late pupal eye discs (Fig. 1I and I'). We know that the antibody signals we detect in wild-type discs are specific for Msp-300 as no signal is detected in larval or pupal eye discs homozygous for an *Msp-300* allele (*Msp-300*$^{\Delta KASH}$) that contains no KASH domain coding sequences (Fig. 1E, E', H, H', J and J'; and see below). We conclude that most of the KASH-containing Msp-300 expressed in the third instar larval eye disc is in the peripodial epithelium, and it is cytoplasmic but not perinuclear there. However, low levels of perinuclear KASH-containing Msp-300 in the disc proper may have escaped detection.

Msp-300^{ez75} **homozygotes are viable and have no obvious morphological defects in the eye.** We wanted to know if flies that

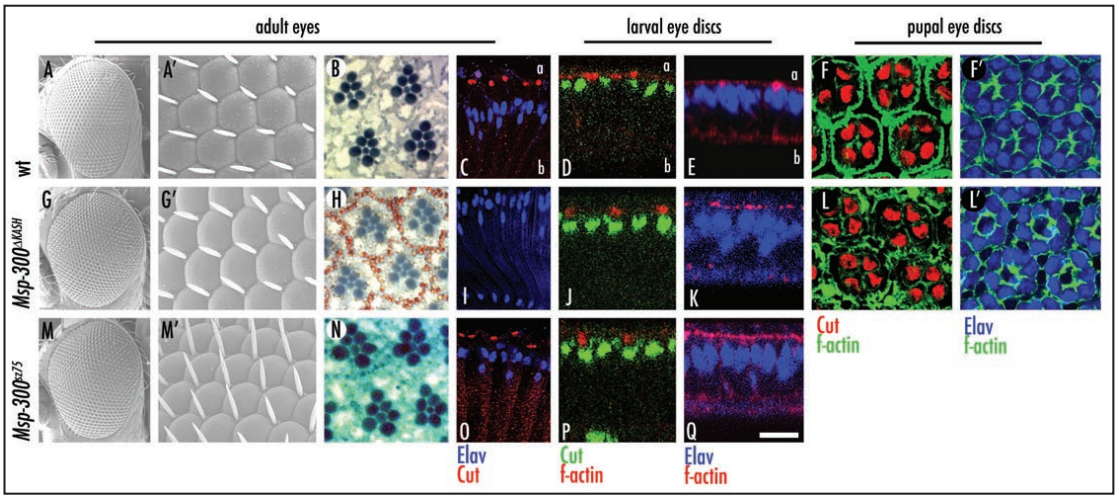

Figure 2. Eye morphology of *Msp-300* mutants. (A, G and M) Scanning electron micrographs of adult eyes. (A′, G′ and M′) Enlargements of the panels at left. (B, H and N) Light micrographs of apical tangential sections of retina. Trapezoids corresponding to the rhabdomeres (light-gathering organelles) of 7 R-cells are visible. (C, I and O) Confocal images (Z-sections) are shown. a, apical; b, basal. Elav is in R-cell nuclei, and Cut is in cone cell nuclei. Nuclei of R-cells 1–7 and cone cell nuclei are apical. In (I), the normally basal R8 nuclei are shown. (D, E, J, K, P and Q) Confocal images (Z-sections) are shown. R-cell and cone cell nuclei are apical. (F, F′, L and L′) Confocal images of apical XY-sections are shown. (F′ and L′) show slightly more basal planes than (F and L). Cone cell and R-cell nuclei are apical. The scale bar in (Q) is ~20 μm in (A′, B, G′, H, M′, N, E, K and Q), ~200 μm in (A, G and M), ~40 μm in (C, I and O) and ~10 μm in (F and L).

lack KASH-containing forms of Msp-300 have nuclear positioning defects in the eye. *Msp-300*^{sz75}, which was induced by EMS and is a single nucleotide change that introduces a stop codon in an early exon,[14,15] was the only existing mutant allele prior to the present study. This allele is reported to be homozygous lethal,[14,15] to reduce or eliminate expression of KASH forms of Msp-300,[15] and to result in nuclear positioning defects in the oocyte/nurse cell complex.[15] We wanted to test if *Msp-300*^{sz75} homozygotes also result in nuclear positioning defects in the eye. We planned to use mitotic recombination to generate mosaic flies with *Msp-300*^{sz75} homozygous eyes in an otherwise *Msp-300*^{sz75}/+ animal. As mitotic recombination would result in all of chromosome arm 2L bearing the *Msp-300*^{sz75} allele becoming homozygous in the eye, by several rounds of outcrossing with a wild-type chromosome, we generated *Msp-300*^{sz75} chromosomes cleaned of other mutations (Materials and methods). We found that *Msp-300*^{sz75} homozygotes with cleaned chromosomes are viable and have no obvious morphological mutant phenotypes. The eyes of *Msp-300*^{sz75} homozygotes were examined in detail. The external eyes and the retinal tissue are indistinguishable from wild-type (Fig. 2A, B, M and N). Moreover, the R-cell and cone cell nuclei are positioned normally in larval eye discs and adult eyes (Fig. 2C–E and O–Q).

***Msp-300* mutants that lack KASH domain exon sequences (*Msp-300*$^{\Delta KASH}$) are viable and fertile with no obvious external morphological defects.** The *Msp-300* locus is large (it spans ~90 kilobases) and there are predicted to be several different promoters and splice forms,[20] but they are poorly characterized. The KASH domain exon is at the very 3' end of the locus, and exactly which upstream exons it does and does not connect to in particular tissues is uncertain. Thus, it is possible that KASH domain-containing forms of Msp-300 are expressed in *Msp-300*^{sz75} homozygotes. As we wanted to know whether KASH-containing forms of Msp-300 play a role in nuclear positioning in the eye, we used ends-out homologous recombination to generate an *Msp-300* allele, *Msp-300*$^{\Delta KASH}$, in which the KASH domain coding region is deleted (Fig. 3). We find that like *Msp-300*^{sz75} mutants, *Msp-300*$^{\Delta KASH}$ homozygotes are viable, fertile (see below), and have externally normal eyes (Fig. 2G and H).

KASH domain-containing forms of Msp-300 are not required for nuclear positioning in the eye. Although the adult eyes of *Msp-300*$^{\Delta KASH}$ homozygotes appear normal, there could be defects in nuclear positioning. We first examined the positions of R-cell and cone cell nuclei in third instar larval eye discs and found no difference between *Msp-300*$^{\Delta KASH}$ homozygotes and wild-type (Fig. 2D, E, J and K). As perinuclear KASH-containing Msp-300 protein does not appear in R-cells until later during pupal development, this result is not surprising. However, R-cell and cone cell nuclei appear normally positioned in late pupal *Msp-300*$^{\Delta KASH}$ eye discs as well (Fig. 2F, F', L and L'), and R-cell nuclei are normally positioned in adult eyes (Fig. 2C and I). We conclude that KASH-containing forms of Msp-300 are not necessary for nuclear positioning in the eye.

KASH domain-containing forms of Msp-300 are not required for nuclear positioning in the oocyte/nurse cell complex. Each Drosophila oocyte derives from a single diploid cystoblast, which is a daughter of a germ-line stem cell.[16,29] A cystoblast undergoes four mitotic divisions to generate a sixteen cell cyst where the cells are connected by cytoplasmic bridges.[16,29] One of the sixteen cells develops a microtubule organizing center and becomes the oocyte, which undergoes meiosis.[16,29] Throughout oogenesis, the nurse cells

supply the oocyte with RNAs and proteins, and ultimately empty their cytoplasms into the oocyte in a process called "cytoplasmic dumping".[16,17,29] Throughout oogenesis, and particularly during "dumping", the nurse cell nuclei must be prevented from clogging the ring canals through a mechanism thought to require actin.[17,29] KASH domain-dependent nuclear positioning defects in nurse cells, resulting in failure of "cytoplasmic dumping" and female sterility, were reported for females with *Msp-300*^{ez75} homozygous germ-lines.[15] Defects in positioning of the oocyte nucleus were also reported in these females.[15] Thus, we were surprised to find that *Msp-300*$^{\Delta KASH}$ homozygous females are as fertile as wild-type controls (Table 1) and that nuclear positioning in both nurse cells and oocytes appears normal (Table 2 and Fig. 4A–D). Moreover, no cytoplasmic dumping defects, nurse cell or oocyte nuclear positioning defects, nor female sterility was observed in any *trans*-heterozygous combination of the following four chromosomes, which include two deficiencies that delete complementary portions of the *Msp-300* locus:[20] *Msp-300*$^{\Delta KASH}$, *Msp-300*^{ez75}, *Df(2L)BSC109*, *Df(2L)Exel6011* (data not shown). In contrast, in females homozygous for the "cleaned" *Msp-300*^{ez75} chromosomes, we did observe the completely penetrant dumpless phenotype (Fig. 4G) and sterility reported previously for *Msp-300*^{ez75} homozygotes.[15] However, unlike what was reported previously, we found that the dumpless phenotype and sterility are not complemented by *Dp(2:1)B19*, an X chromosome that contains the entire *Msp-300* locus (Fig. 4F). The *Dp(2:1)B19* chromosome does, however, restore Msp-300 protein expression to the ovary (Fig. 4E and E'). Moreover, we find that Msp-300 is perinuclear only in the oocyte, not in the nurse cells (Fig. 4A, A', E and E'). We conclude that KASH domain-containing forms of Msp-300 are not required for nuclear positioning in the oocyte/nurse cell complex.

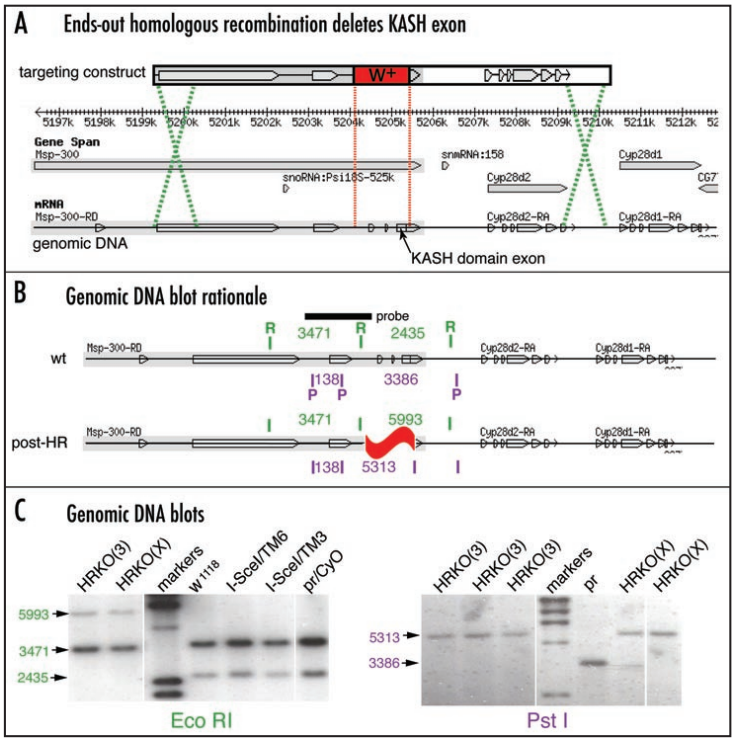

Figure 3. Generation of *Msp-300*$^{\Delta KASH}$ by homologous recombination. (A) A diagram of the targeting construct used to replace 3' exons of *Msp-300* with the *w+* gene is shown. At top, the extent of Drosophila genomic DNA ligated upstream and downstream of *w+* sequences is shown. The green dotted lines indicate the region where homologous recombination takes place. The red dotted lines indicate the region of *Msp-300* that is replaced by *w+*. The picture of the genomic region is from FlyBase.[20] (B) *Eco RI* (R) and *Pst I* (P) sites in wild-type (wt) genomic DNA and genomic DNA after homologous recombination (post-HR) are shown. The red squiggle is the inserted *w+* gene. The probe is 951 bp. (C) Blots, probed as shown in (B), of genomic DNA restricted with *Eco RI* (left) or *Pst I* (right). At left, HRKO(3) and HRKO(X) are examples of homologous recombination knock-out lines generated by a targeting construct initially present on chromosome 3 or the X chromosome, respectively. The four right-most lanes are different *Msp-300+* lines. At right, three different HRKO(3) lines and two different HRKO(X) lines are shown. The *Msp-300+* control is a *pr* line.

How can these results be reconciled with those reported by Yu et al.? [15] The simplest explanation is that the dumpless phenotype and sterility associated with the *Msp-300*^{ez75} chromosome is caused by a closely linked mutation other than the *Msp-300*^{ez75} mutation. Alternatively, *Msp-300*^{ez75} may be neomorphic, and produce a dumpless phenotype only when homozygous.

The KASH proteins Klarsicht and Msp-300 do not have redundant essential roles in the fly or redundant roles in development of the eye or ovary. So far, our experiments reveal no role for KASH forms of Msp-300 for viability, eye development, oogenesis or female fertility. In contrast, a KASH domain-containing form of Klar is required for eye development, but not for nurse cell or oocyte nuclear positioning (Table 2). We were curious to know if KASH domain-containing forms of Msp-300 and Klar might function redundantly in oogenesis or eye development. If so, *Msp-300*$^{\Delta KASH}$; *klar*^{CD4} double mutants would be expected to display nuclear positioning defects in the oocyte/nurse cell complex, a dumpless phenotype and female sterility. In addition, the phenotype of *klar*^{CD4} eyes would be more severe. We find that as in either single mutant, there are no nuclear positioning defects (Table 2) and there is no dumpless phenotype (data not shown) in double mutant ovaries. In addition, the double mutant eye phenotype is like *klar*^{CD4} alone; double mutant eyes are not more severly malformed that *klar*^{CD4} eyes, and R-cell and cone cell nuclei are misplaced similarly in both (Fig. 5). Unlike *Msp-300*$^{\Delta KASH}$ mutants, *klar*^{CD4} mutants have reduced fertility as compared with wild-type: they lay fewer eggs and fewer of the eggs laid hatch (Table 1). The egg laying

Table 1 **Fertility of *Msp-300* and *klar* mutant females**

Female genotype	Expt #	Average fraction eggs hatched (%)	Average fraction eggs hatched in mutants/wt (%)	Average number eggs laid	Average number eggs laid in mutants/wt (%)
w^{1118}	1	94 ± 1	100	485 ± 52	100
	2	84 ± 2	100	689 ± 40	100
	3	88 ± 3	100	320 ± 20	100
$Msp\text{-}300^{\Delta KASH}$	1	89 ± 1	95	483 ± 77	100
	2	91 ± 2	108	562 ± 70	82
$klar^{CD4}/Df(3L)emc^{E12}$	1	17 ± 4	18	378 ± 71	78
	2	30 ± 3	36	465 ± 58	67
$Msp\text{-}300^{\Delta KASH}$;	1	26 ± 15	27	19 ± 12	4
$klar^{CD4}/Df(3L)emc^{E12}$	2	19 ± 9	22	77 ± 36	11
$Msp\text{-}300^{\Delta KASH}$;	1	92 ± 1	98	402 ± 79	83
$klar^{CD4}/TM6B$	2	93 ± 1	110	535 ± 41	78
$Msp\text{-}300^{\Delta KASH}/CyO$;	1	10 ± 1	11	90 ± 26	19
$klar^{CD4}/Df(3L)emc^{E12}$	2	14 ± 7	17	98 ± 75	14
$Df(3L)emc^{E12}/+$	3	77 ± 8	88	285 ± 50	89

Three separate experiments were performed (Expt. #1–3). In each experiment, three replicate crosses were set up, each containing eight virgin females of the genotype indicated and six w^{1118} males. After collection, the flies were aged separately for 48 hours, allowed to mate in vials for 48 hours, and then transferred to egg-laying chambers. Eggs were collected at 24 hour intervals three times. The data shown reflect the number of eggs produced over the three day period, averaged for the three replicate crosses. Standard error was calculated for the data in each of the three replicates.

defect is enhanced in females heterozygous for $Msp\text{-}300^{\Delta KASH}$, and females homozygous both $Msp\text{-}300^{\Delta KASH}$ and $klar^{CD4}$ lay only a small fraction of the wild-type number of eggs (Table 1). The fraction of the eggs from double mutant mothers that hatch, however, is similar to the fraction from $klar^{CD4}$ mothers (Table 1). We conclude that KASH forms of Msp-300 do not play a redundant role with KASH forms of *klar* in nuclear positioning in the eye or in the ovary. However, KASH forms of Klar and Msp-300 function in egg laying. The egg-laying defect could be related to a reported function of Msp-300 in muscle development.[4]

Table 2 **Nuclear positioning in *Msp-300* and *klar* mutant oocyte/nurse cell complexes**

Genotype	Number complexes with a mispositioned nurse cell nucleus/number assayed
w^{1118}	7/229 = 3%
$Msp\text{-}300^{\Delta KASH}$	2/48 = 4%
$klar^{CD4}/Df(3L)emc^{E12}$	2/43 = 5%
$Msp\text{-}300^{\Delta KASH}$; $klar^{CD4}/Df(3L)emc^{E12}$	1/51 = 2%

Oocyte/nurse cell complexes were labeled with TOPRO-3, anti-Lamin and phalloidin. Mispositioned nurse cell nuclei were stuck in ring canals between a nurse cell and the oocyte. The oocyte nucleus was visible with anti-Lamin in about half of the complex, and its position was always normal.

Materials and Methods

Drosophila strains. *y w* (our laboratory stock)
Sco/CyO (our laboratory stock)
CyO, *gfp* (our laboratory stock)
FRT40A (FBti0002071; Bloomington)
$Msp\text{-}300^{sz75}/BCL$ (FBal0008339; T. Volk)
$Msp\text{-}300^{sz75}$ *FRT40A* (D. Starr)
Df(2L)sc19-8/SM6b; Dp(2;1)B19, y^1 ed^1 dp^{o2} cl^1 (Dp is FBab0010523; Bloomington)
Df(2L)sc19-4/In(2L)Cy L^{t^R} *In(2R)Cy,* Cy^1, Roi^1, cn^2, sp^2; *Dp(2;1)B19,* y^1 ac^1 sc^1 pn^1 ed^1 dp^{o2} cl^1 (Bloomington)
w^{1118}; *Df(2L)BSC109/CyO* (FBab0038758; Bloomington)
w^{1118}; *Df(2L)Exel6011/Cy*O (FBab0037853; Bloomington)
$Df(3L)emc^{E12}$ (FBab0002367; Bloomington)
$klar^{CD4}$ *st/TM6B* (FBal0039645; our laboratory stock)
y w; *P{70FLP}23 P{70I-SceI}4A/TM6* (FBti0026978, FBti0026981)
w; *P{70FLP}10* (FBti0026979)
w; *GMR-hid cl FRT40A ey-FLP* (S. Stowers)

Drosophila genetics. All fly crosses were performed using standard methods at 25°C unless otherwise noted. Particularly important fly crosses are described in detail below. P element transformation of w^{1118} embryos was performed using standard methods and plasmids purified using Qiagen maxi-preps.

Molecular biology reagents. All molecular biology procedures were performed using standard methods. Restriction enzymes and common modifying enzymes came from New England Biolabs. PCR was performed using Platinum PCR Supermix (Invitrogen) or Herculase (Stratagene) according to the manufacturers' instructions. Oligonucleotide primers were from Integrated DNA Technologies. DNA sequencing was performed using automated fluorimetric methods. Important details of procedures are provided below.

Cleaning the $Msp\text{-}300^{sz75}$ chromosome. Males of the genotype $Msp\text{-}300^{sz75}/BCL$ were crossed with *FRT40A* females, and virgin female progeny of the genotype $Msp\text{-}300^{sz75}/FRT40A$ were crossed with *Sco/CyO* males. Thirty-three *CyO* male progeny were crossed individually with *Sco/CyO* virgin females and stocks of 33 independent recombined chromosomes balanced with *CyO* were established. Males of each stock were crossed with *GMR-hid cl FRT40A ey-FLP/CyO* virgins, and 19 stocks with *FRT40A*-containing chromosomes were identified (eyes of non-*Cy* flies from the cross do not have the

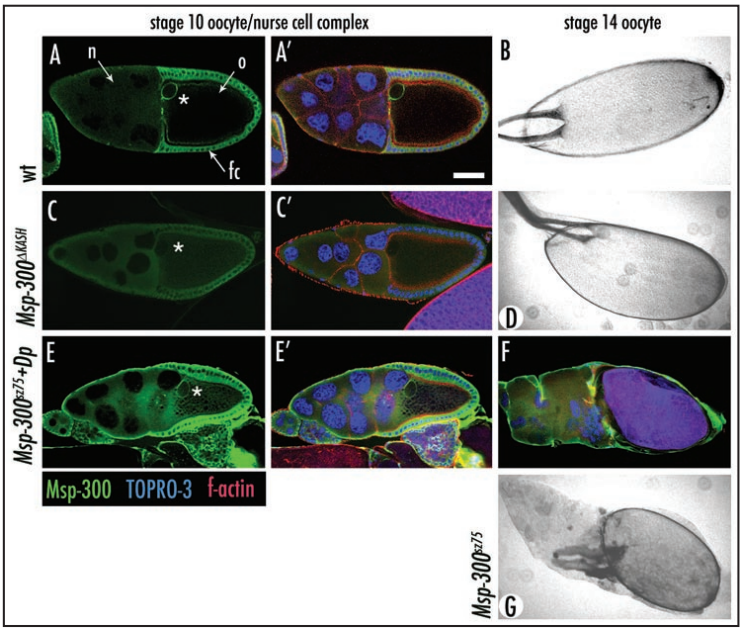

Figure 4. Oocyte/nurse cell complexes in *Msp-300* mutants. (A, A′, C, C′, E and E′) Confocal images are shown: o, oocyte; n, nurse cells; fc, follicle cells. (A and A′) In wild-type (wt), Msp-300 is expressed in the somatic follicle cells that surround the complex, is perinuclear in the oocyte, and also present at low levels in the nurse cell cytoplasm. The oocyte nucleus is marked with an asterisk. (C and C′) Negative controls for anti-Msp-300. Note that the nurse cell and oocyte nuclei are positioned as in wild-type. (E and E′) The genotype is *Dp(2;1)B19/+; Msp-300^{sz75}*. Note that Msp-300 expression is wild-type in the presence of *Dp(2;1)B19*. TOPRO-3 labels nurse cell nuclei, but the oocyte nucleus. (B, D and G) Light micrographs and (F) confocal image. The oocytes in (F) and (G) have a "dumpless" phenotype. The scale bar in (A′) is ~60 μm in all panels.

GMR-hid phenotype) and saved. The second chromosomes in these 19 stocks showed a variety of different homozygous phenotypes, including viability and female sterility, lethality and rough eyes in clones generated using the *GMR-hid* technique,[19] semi-lethality, and others had a wild-type phenotype. Four independent chromosomes were tested for the presence of the *Msp-300^{sz75}* mutation using PCR and the primers 5'-TTGGATGAACTGGAGCGTCG-3' and 5'-CGAGGTGGTTATGGCACTTAGG-3', and determining the DNA sequence of the amplified product. All four lines (two homozygous viable and female sterile and two homozygous lethal with rough eyes in clones) contained the *Msp-300^{sz75}* mutation. (After we determined that the female sterility is not complemented by *Dp(2;1)B19* (see Results), we wanted to test the chromosomes that were homozygous viable and female fertile but we had discarded them.)

Testing *Dp(2;1)B19* for complementation of *Msp-300^{sz75}*-associated female sterility. In three separate crosses, females homozygous for *Dp(2:1)B19* (*Df(2L)sc19-8/SM6b; Dp(2;1)B19, y^{1} ed^{1} dp^{o2} cl^{1}*) were crossed with males containing one of three different *Msp-300^{sz75}* chromosomes: *Msp-300^{sz75}/BCL* (from T. Volk), *Msp-300^{sz75} FRT40A* (from D. Starr) or our cleaned *Msp-300^{sz75} FRT40A* chromosome (see above). Male progeny of the genotype *Dp(2;1)B19, y^{1} ed^{1} dp^{o2} cl^{1}/Y; Msp-300^{sz75}/SM6b* were crossed with virgin females containing the same *Msp-300^{sz75}* chromosome as the males, and female progeny heterozygous for the duplication and homozygous for *Msp-300^{sz75}* (*Dp(2;1)B19/+; Msp-300^{sz75}*) were obtained. In all three cases, these females were sterile. These results were confirmed by performing the same crosses with a different stock containing *Dp(2;1)B19* (*Df(2L)sc19-4/In(2L)Cy^{L}t^{R} In(2R)Cy, Cy^{1}, Roi^{1}, cn^{2}, sp^{2}; Dp(2;1)B19, y^{1} ac^{1} sc^{1} pn^{1} ed^{1} dp^{o2} cl^{1}*).

RNA analysis. To obtain eye disc RNA for RT-PCR, ~500 eye disc pairs were dissected into RNAlater (Ambion). Crude total RNA was isolated using TriReagent (Molecular Research Center) according to manufacturer's instructions. For embryo RNA, 0.5–1.0 g of 0–24-hour embryos were dechorionated in 50% bleach for 2 minutes, and then ground with a mortar and pestle in 5–10 volumes TriReagent. After precipitation, the crude RNA pellet was dissolved in nuclease-free water (Ambion) and treated with TurboDNase (Ambion). After phenol/chloroform extraction, DNA-free RNA was precipitated. All RT reactions were performed using standard methods according to manufacturer's instructions (SuperScript II, Invitrogen). The finished RT reaction products were used as template in PCR reactions according to manufacturer's instructions. PCR products were run on a 1% TAE/agarose gel with ethidium bromide. The PCR primers used for the *Msp-300* KASH domain sequences were 5'-GGATCCGGCTACGAGGGCGACAATCTC-3' and 5'- gaattCTTTACGTGGGTGGCGGTCC -3'.

Ends-out homologous recombination. A homologous recombination P element construct was generated, as follows, that would delete 1086 bp of *Msp-300* intron and exon sequences (nucleotides 5204338–5205423 [R5.5 coordinates][20]), spanning

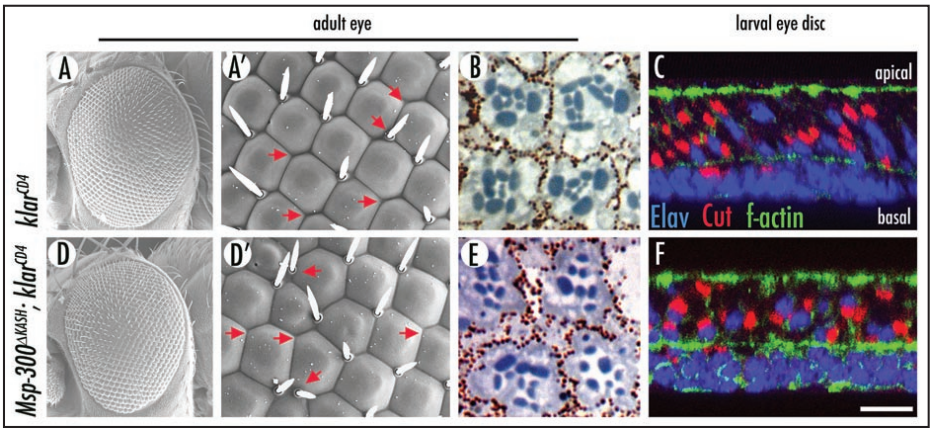

Figure 5. Eye phenotypes of *Msp-300$^{\Delta KASH}$; klar^{CD4}* double mutants. (A and D) Scanning electron micrographs of adult eyes. (A′ and D′) Enlargements of the panels at left, showing misplaced or missing bristles (arrows). (B and E) Apical tangential sections of adult retinas showing malformed rhabdomeres. (C and F) Confocal Z-sections. Elav is in R-cell nuclei, and Cut is in cone cell nuclei. Both R-cell and cone cell nuclei are mispositioned. (Compare with Fig. 2D, E, J, K, P and Q.) The scale bar in F is ~200 μm in (A and D), ~20 μm in (A′, B, D′ and E), ~10 μm in (C and F).

the coding region for 150 amino acids that includes the entire KASH domain. An ~5.5 kb genomic DNA fragment 5' to the KASH sequences and an ~4.4 kb 3' fragment were amplified by PCR using Herculase (Stratagene), BACR06K07 (BacPac Resources) as template and the following primers: 5' fragment (5'-GCGGCCGCATTTCATTGATAGGTGGTGGCATAC-3'/ 5'-GCATGCCGCAACAAAAACGGCAACAC-3') and 3' fragment (5'-CCTGCAGGAACCCCCTATGAAAAACGCC-3'/ 5'-ACTAGTCTCTGGACAAACAATGTGTGTAGCAC-3'). The amplified products were ligated separately into pGEM-T-easy (Promega), and as there are two genes downstream of *Msp-300* contained within it, the sequence of the 3' fragment was verified. The 5' genomic fragment was excised with *Not I* and *Sph I* and ligated into those sites in pW35[21] to generate *pW35-5'mKASH*, and the 3' genomic fragment was excised with *Spe I* and *Sbf I* and ligated into pW35 restricted with *Avr II* and *Pst I* to generate *pW35-3'mKASH*. A pW35 vector containing both fragments (*pW35-ΔmKASH*) was generated by ligating an ~9.9 kb *Sac II-Rsr II* fragment of *pW35-5'mKASH* containing the 5' genomic fragment and the *w+* gene with an ~8.7 kb *Rsr II-Sac II* fragment of *pW35-3'mKASH* containing the 3' genomic fragment, the *I-Sce I* site, the FRTs, and the rest of the plasmid. Four independent P element transformants (*P{w+HRKO$^{\Delta MKASH}$}*) were obtained: two on X, one on chromosome 2, and one on chromosome 3. Homologous recombination was performed using *P{w+HRKO$^{\Delta mKASH}$}* lines on the X and 3rd chromosomes. For *P{w+HRKO$^{\Delta mKASH}$}* on chromosome 3, virgin females of the genotype *w; P{w+HRKO$^{\Delta mKASH}$}* were crossed with *y w; P{70FLP}23 P{70I-SceI}4A/TM6B* males. Embryos and larvae (0–72 hours old) were heat shocked for 1 hour at 38°C by immersion of the food vial in a water bath, and then allowed to mature to adulthood at 25°C. Eighty crosses were set up with non-TM6B virgin females (*w; P{HRKOΔmKASH}/P{70FLP}23 P{70I-SceI}4A*) and *w; P{70FLP}10* males. Among the progeny, a male with solid red eyes, considered a knock-out candidate, was found in 21 of the 80 crosses. These males were crossed individually with *w; Sco/CyO* virgin females and the progeny were used for segregation tests to determine if the *w+* gene was on chromosome 2 (this was the case for 14/17 lines), and also to balance *w+* 2nd chromosomes with *CyO*. Similar crosses were performed simultaneously with the *P{w+HRKO$^{\Delta mKASH}$}* line on X and the results were similar: 16 of 80 crosses gave a solid red-eyed male, and in 12 of the 15 males tested, the *w+* gene segregated on chromosome 2.

Genomic DNA blots. Genomic DNA was prepared from ten flies of each genotype as described.[22] The entire preparation was restricted and size-separated on a 1% agarose gel, transferred to BrightStar-Plus nylon membrane (Ambion). A 951 bp digoxygenin-labeled probe was prepared using the PCR DIG Probe Synthesis Kit (Roche), BACR06K07 (BacPac Resources) as the template, and the primers 5'-CCACCAAATGGCAAGGTCTTC-3'/ 5'-AGGGTTAGAATGGGCGGACG-3'. The DIG wash and block buffer (Roche) and ULTAhyb (Ambion) were used according to manufacturer's instructions. The blot was developed with the DIG Luminescent Detection Kit (Roche).

Immunohistochemistry of eye discs. Third instar larval eye disc dissection, fixation, antibody or phalloidin staining were with the standard PEMS/PBST procedure.[23] Pupal retinas were prepared according to: http://www.bioprotocol.com/protocolstools/protocol.jhtml;jsessionid=IC5Z1ADQNFN2FR3FQLMSFEWHUWBNQIV0?id=p19. Primary antibodies were used diluted in PBST as follows: polyclonal rat anti-Msp-300,[15] at 1:50, mouse monoclonal anti-Elav at 1:10, and rat monoclonal anti-Elav at 9:1, mouse monoclonal anti-Cut at 1:100. All antibodies were obtained from the Developmental Studies Hybridoma Bank (DSHB), except for anti-Msp-300 which was from D. Starr. Secondary antibodies (Molecular Probes) were used at 1:200 in PBST: 488-goat-anti-rat, 647-goat-anti-rat, 568-goat-anti-mouse, 647-goat-anti-mouse. 488-, 568- or 647-phalloidin (stains f-actin at plasma membranes; Molecular Probes) was 0.03 U/ul (3U in 15ul methanol died and

resuspended in 100ul PBST). After antibody incubation, some discs were incubated in phalloidin solution for 15 minutes at 25°C.

Immunohistochemistry of ovaries. Ovaries were dissected, fixed and labeled with antibodies as described.[24] Antibodies used were polyclonal rat anti-Msp-300 at 1:50 and 488-goat-anti-rat (Molecular Probes) at 1:200. 568-phalloidin was used at 0.03 U/ml, TOPRO-3 (a pan-nuclear stain, Molecular Probes) was used at 1:1000, and mouse anti-Lamin (mADL84.12 from DSHB) at 1:100.

Analysis of adult eyes. Scanning electron microscopy was as described.[25] Fixation, embedding and sectioning of adult eyes was as described.[6] For immunofluorescence, adult eyes were prepared as follows. Bisected heads were dipped in 70% ethanol for 30 secs., incubated im PEMP fixative (0.1 M PIPES pH 7.0, 1.0 mM $MgCl_2$, 4% paraformaldehyde, 2.0 mM EGTA) for 1 hr on ice, during which time the cuticle was removed. Eyes were washed 5 times with 1 ml PBST, then blocked overnight with 10% goat serum, followed antibody staining as for eye discs.

Microscopy and imaging. Light microscope images were acquired with a Zeiss Axioplan microscope and AxioCam. Confocal images were obtained using a Leica SP2AOBS or a Leica TCSSP2. Vectashield (Vector Laboratories) mounting medium for fluorescence was used. Images were processed with Adobe Photoshop software.

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