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Determining the role of a small GTPase, Ral, and an endocytic factor, epsin, in *Drosophila* Notch signaling

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Dedication

To my family for their love and faith in me

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Determining the role of a small GTPase, Ral, and an endocytic factor, epsin, in *Drosophila* Notch signaling

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Cell-cell communication events are crucial to determine the fate of each cell during development. Notch signaling is involved in many different contexts in determining cell fate by mediating cell-cell communication. Furthermore, regulation of the Notch transduction pathway is critical for normal cellular function, which is implicated in various diseases, including cancers. At a certain developmental time point, intrinsic or extrinsic developmental cues induce biases in ligands and Notch receptors between neighboring cells. These initial biases are further amplified by various cellular factors which eventually dictate cell fates. In *Drosophila*, two Notch ligands, Delta and Serrate, trigger Notch receptor activation in nearby cells by virtue of numerous regulating factors. One important question in this area is how cells become Notch signal sending or receiving cells for cell fate decisions. I show evidence about a distinct mechanism for biasing the direction of Notch signaling that depends on a small GTPase, Ral, during *Drosophila* photoreceptor cell development. Investigations described here indicate that Fz signaling up-regulates *Ral* transcription in a signal sending fate cell, the R3 precursor, and Ral represses ligand-independent activation of Notch in the R3 precursor. This event

ensures R3 to become a signaler and contributes to asymmetric Notch activation in the neighboring cell, R4. Ral is a small Ras-like GTPase that regulates membrane trafficking and signaling. Here, possible Ral effector pathways that are important for Notch regulation will be proposed. To trigger Notch activation in adjacent cells, Notch ligand endocytosis by the signaling cells is necessary. Recently, it was suggested that control of membrane trafficking is important not only for ligand signaling, but also for Notch receptor activation. Furthermore, Notch receptor trafficking regulates critical cellular functions, including proliferation, which is implicated in tumors. Therefore, another important question in Notch signaling is about the role of membrane trafficking in regulation of the Notch transduction pathway. *Drosophila* endocytic epsin, Liquid facets [Lqf], is a key component necessary for ligand endocytosis, thereby triggering Notch activation in adjacent cells. However, its function in signal receiving cells for Notch activation has not been studied. In this dissertation, I provide evidence that epsin is also required in signal receiving cells for Notch activation in developmental contexts. Furthermore, genetic and molecular evidence suggests that epsin regulates Notch receptor trafficking via Rab5-mediated endosomal sorting pathway for Notch activation. These studies support the idea that Notch activation at the plasma membrane is not the only way to transduce Notch signaling, but the Notch receptor must enter through an epsin-mediated endocytic pathway into subcellular compartments to be activated, at least in some contexts.

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Chapter 1: *General Introduction*

I have studied Notch signaling using *Drosophila* as a model system. As a general introduction, I will begin by providing a general overview about Notch signaling.

The Notch signaling pathway is highly conserved in most multicellular organisms, by which cell fates are controlled through local cell-cell interaction. Furthermore, in combination with other factors, Notch receptor signal influences differentiation, proliferation, and apoptotic cell death at all developmental stages. The Notch protein sequence was initially obtained from *Drosophila melanogestar* about 20 years ago and the Notch signaling pathway has been intensively studied in *Drosophila*.

Drosophila Notch is a 300-KD single-pass transmembrane receptor (Kidd et al., 1986), in which the extracellular domain plays a role in ligand binding and this event triggers serial proteolytic cleavage of the Notch receptor. Once Notch proteins are sythesized in endoplasmic reticulums, polysaccharides are added onto the receptors (O-glycosylation by unknown enzymes and fucosylation by O-Fucosyl transferase, O-Fut1) for generating functional Notch (Okajima and Irvine, 2002). At the Golgi apparatus, Notch receptors are further modified by the glycosyltransferase, Fringe (Brückner et al., 2000), and the first Notch cleavage event occurs by Furin at the S1 cleavage site (Logeat et al., 1998); in *Drosophila*, the requirement of S1 cleavage for the production of functional Notch protein is controversial (Kidd and Lieber, 2002). After S1 cleavage, calcium dependent non-covalent bond links two Notch parts which are targeted to the plasma membrane (Rand et al., 2000). Ligand binding to the Notch receptor triggers ADAM family metalloprotease-mediated second cleavage at the extracelluar domain (Lieber et al., 2002), followed by γ-secretase-dependent third cleavage at the

transmembrane domain of the Notch receptor (Struhl and Greenwald, 1999; Ye et al., 1999). These serial Notch cleavage events release the Notch intracellular domain (Nicd) that is transported into the nucleus, and the Nicd in the nucleus binds to the CSL (CBF-1/Suppressor of Hairless/Lag-1) transcriptional repressor complex, derepressing transcription of downstream target genes (Figure 1-1) (Kidd et al., 1998; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998).

In *Drosophila*, two Notch ligands, Serrate and Delta, were identified (Fleming, 1998). Both are single-pass transmembrane proteins, but their binding affinity to Notch is different; modified (by Fringe) and unmodified Notch extracellular domains are recognized preferentially by Delta and Serrate, respectively (Brückner et al., 2000). To generate active ligands, mono-ubiquitination of ligands is necessary and two E3 ubiquitin ligases, Neuralized (Lai et al., 2001; Pavlopouos et al., 2001; Yeh et al., 2001) and Mind-bomb (Lai et al., 2005; Le Borgne et al., 2005; Wang and Struhl, 2005), have been identified as essential enzymes for ligand ubiquitination in *Drosophila*. In *Drosophila*, these two E3 ligases function in different tissues or different developmental stages rather than exert redundant roles (Lai et al., 2005; Le Borgne et al., 2005; Wang and Struhl, 2005). More detailed description about ligand ubiquitination will follow below.

Notch ligand endocytosis is necessary for sending signal to adjacent Notch receptor cells. *Drosophila* dynamin, Shibire, was identified as a crucial component for sending signal (Seugnet et al.,1997), and our and other groups showed that *Drosophila* endocytic epsin, Liquid facets [Lqf], is required for ligand signaling (Overstreet et al., 2004; Wang and Struhl, 2004). Given that mono-ubiquitination of Notch ligands is necessary for sending signal, it has been proposed that epsin recognizes and endocytoses ubiquitinated ligands, which is an essential step for sending signal (Barriere et al.,2006; Hawryluk et al., 2006). However, it is unclear why ligand endocytosis is required, and

several ideas for explaining the requirement of ligand endocytosis are still controversial.

Even though intensive studies about Notch signaling have been carried out, our current understanding about Notch signaling is limited, and further research is definitely required. Two main questions in this area are how cells become Notch signal sending or receiving cells for cell fate decisions, and what is the role of membrane trafficking in Notch activation. My study is about understanding these two questions, and I will mainly introduce and discuss studies related to these two questions in this chapter.

In a previous genetic screen carried out by our group, a mutation of *Drosophila* Ral GTPase gene (*Ral*) was identified as a dominant enhancer of the rough eye caused by epsin overexpression. In the same genetic screening, we found mutations in Notch signaling components, *auxilin* and clathrin heavy chain, and a negative regulator of Notch, *spen*, as enhancers (Eun et al., 2007), implying that epsin overexpression can reduce Notch activity and also increase Notch activity in certain circumstances. As Ral involvement in Notch signaling had not been characterized, I decided to study Ral function in Notch signaling.

My dissertation work is about how Ral is involved in Notch signaling and identifying possible Ral effector loops for Notch regulation, and finally, determining and clarifying the role of epsin in membrane trafficking and Notch signaling. Results presented here propose 1) Ral dictates an extrinsic developmental cue and contributes to establish Notch asymmetry by down-regulating ligand-independent Notch activation in the signal sending cell in the eye, 2) Ral-mediated control of the endocytic pathway could be important for Notch regulation, 3) epsin has distinct cell-autonomous roles, one of which is promoting Notch activation by regulating Notch trafficking. Therefore, as an introduction to the work that I have carried out, first, I will describe Notch asymmetry establishment with examples well studied in *Drosophila*. Second, as I study Notch

signaling using *Drosophila* tissues as model systems, Notch signaling events in developing eye, wing, and ovarian cells will be summerized. Third, given that the interaction between ubiquitinated ligands and epsin could be crucial for sending signal and also provide a distinct epsin function, ligand ubiquitination and endocytosis will be described. Fourth, as a novel function of epsin for Notch activation in signal receiving cells has been studied in my dissertation, regulation of Notch activity via Notch trafficking will be introduced. Finally, I will provide an overview of non-canonical ligand-independent Notch signaling that Ral could control, and Ral functions characterized from other studies.

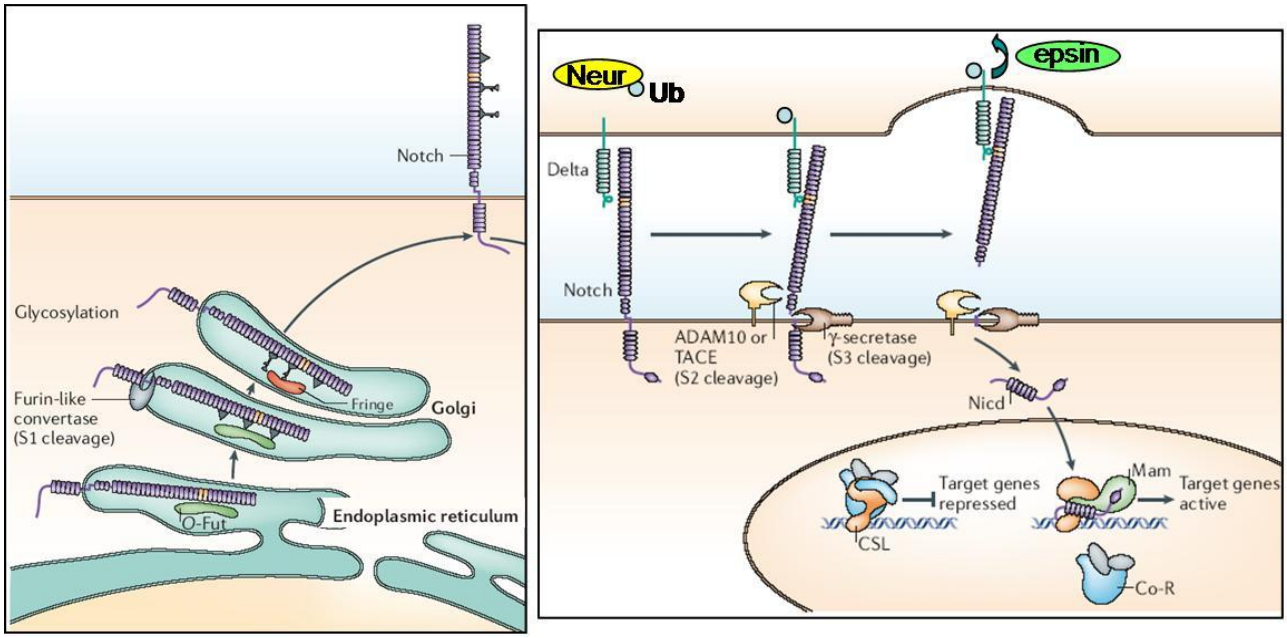


Figure 1-1. Maturation of Notch receptor and Notch activation by ligand endocytosis (adapted from Bray, S. J., 2006). Notch is synthesized and modified by O-fut1 at ER. At Golgi, Notch is processed by the Furin like convertase (S1 cleavage) and modified further by the Fringe enzyme. For Notch activation, ligand ubiquitination and endocytosis in nearby cells are required for inducing ADAM family metalloprotease-mediated second cleavage, followed by γ-secretase-dependent third cleavage. Released Nicd is transforted into the nucleus and binds to the CSL transcriptional repressor complex, derepressing transcription of downstream target genes.

NOTCH ASYMMETRY ESTABLISHMENT IN *DROSOPHILA*

From my *Drosophila Ral* and previous studies, establishment of the initial Notch signal-directionality between cells is critical for correct Notch signal amplification and final cell fate decisions. Therefore, previous studies about mechanisms which cause the initial signal bias between cells will be described in this section.

NOTCH ASYMMETRY IN SOP CELL DEVELOPMENT

Asymmetric division of Sensory Organ Precursor (SOP) Cells in the *Drosophila* peripheral nervous system is a good example about how segregation of proteins produces directional signaling between daughter cells to decide cell fates. Asymmetric division of SOP gives rise to two different daughter cells, pIIb and pIIa (Figure 1-2A). pIIb gives rise to a neuron and a sheath cell, whereas pIIa gives rise to a socket cell and a hair cell (Lu et al., 1999; Roegiers et al., 2001). The different cell fates between pIIb and pIIa are achieved by asymmetric Notch activation. pIIb activates Delta ligand signaling and sends signal to pIIa, which induces different transcriptional events in these cells (Figure 1-2).

Despite Notch and Delta being expressed in both cells, asymmetric distribution of regulatory proteins, Numb (Rhyu et al., 1994) and Neuralized (Neur) (Le Borgne and Schweisguth, 2003) in pIIb, enable it to send signal to pIIa. Both Numb and Neur localize to the anterior cortex of SOPs during mitosis and extensively segregate into pIIb cells (Shen et al., 1998; Schaefer et al., 2001; Bellaiche et al., 2001; Le Borgne and Schweisguth, 2003). Numb, with α-adaptin, downregulates Notch activity in pIIb, which ensures the cell to become a signal sender (Berdnik et al., 2002). The transmembrane protein Sanpodo is needed for Notch signaling during pIIa and pIIb generation (Skeath and Doe, 1998). Because Sanpodo is present at the plasma membrane in pIIa cells and localizes on endosomes in pIIb cells, it was suggested that Sanpodo localization at the plasma membrane in pIIa is required for Notch activation (Hutterer and Knoblich, 2005).

Indeed, without Numb or α-adaptin, Sanpodo was observed at the plasma membrane in both pIIb and pIIa cell, which causes two pIIa-like cells, similar to the *Numb* mutant itself (Hutterer and Knoblich, 2005). Therefore, Numb and α-adaptin are required for Sanpodo endocytosis, reducing plasma membrane Sanpodo, thereby diminishing Notch activation in pIIb cells. Neur monoubiquitinates Delta and promotes endocytosis of Delta in pIIb cells, which triggers Notch activation in pIIa cells (Le Borgne and Schweisguth, 2003).

In addition, recent papers suggested that asymmetric distribution of endosomes during mitosis also exerts Notch asymmetry, although their exact roles need to be clarified. For example, most Rab11 recycling endosomes are assorted in pIIb and this, with Sec15, an exocyst component, iduces signal sending property of pIIb (Emery et al., 2005). Furthermore, Sara early endosomes were also found to be partitioned asymmetrically in pIIa, which induces asymmetric Notch activation between pIIb and pIIa (Coumailleau et al., 2009).

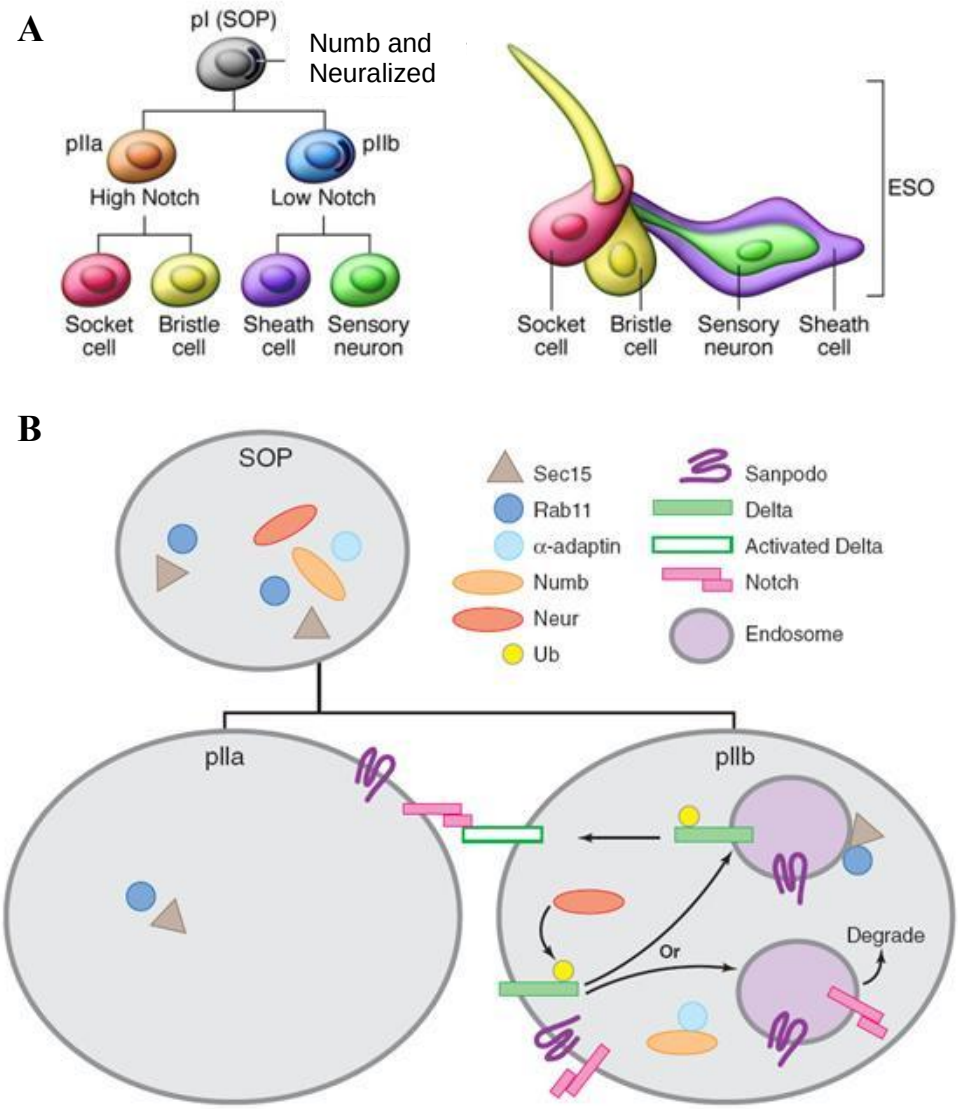


Figure 1-2. Notch asymmetry in SOP cell development (adapted from Pear, W. S., 2010 and Fischer, J. A. et al., 2006). **(A)** The SOP cell gives rise to four different types of cells (Socket, bristle, sheath, and sensory neuron cells) after two asymmetric cell division events. **(B)** Asymmetric localization and segregation of Numb and Neur induces Notch directionality from pIIb to pIIa. Sanpodo maintains Notch activity at the plasma membrane in the pIIa cell and Numb-mediated Sanpodo trafficking in the pIIb cell down-regulates Notch receptor in the pIIb. Rab11-mediated recycling of Delta via Sec15 in pIIb potentiates signal sending property in the pIIb cell. (ESO; Epithelial Sensory Organ).

NOTCH ASYMMETRY IN R3/4 PHOTORECEPTOR CELL DEVELOPMENT

Different from SOP development, in which intrinsic molecular biases induces signaling direction between daughter cells during mitosis, fates of *Drosophila* photoreceptor neural cells are not coupled with mitosis. Instead, neural fate cells are initially choosen and recruited in groups of cells, called ommatida, in a stepwise manner (Wolff and Ready, 1993). During these processes, an extrinsic signal governs Notch asymmetry particularly in the R3/R4 cell determination process (Figure 1-3). Planar Cell Polarity (PCP) signal from the equator of the developing eye activates Frizzled receptor in equatorial cells and induces cell-fate specification of the R3/R4 pairs through regulation of Notch/Delta signaling, where R3 signals and activates the Notch receptor in R4 (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). This signaling event is important not only for asymmetric Notch activation in R3/R4 pairs, but also for ommatidial rotation. In the *Drosophila* eye, ommatidia differentiate as two chiral forms, one in the dorsal and one in the ventral half, generating a line of mirror symmetry. The chiral forms are distinguished by the specific arrangement of the eight R-cells in an asymmetric trapezoid (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). Asymmetry is caused by the relative positions of the R3 and R4 cells. PCP in the eye is specified in the third instar imaginal disc, in the five-cell precluster posterior to the morphogenetic furrow (Fanto and Mlodzik, 1999; Cooper and Bray, 1999). The precluster consists of the R8, R2/R5, and R3/R4 precursors, the latter pair being critical for polarity specification (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). The precursor of the pair that is closer to the equator (the D/V midline of the eye field) has higher Fz activity and adopts the R3 fate (the equatorial cell), while the neighboring cell (the polar cell) becomes R4. Subsequently, the

clusters rotate 90° to generate the final mirror-image arrangement (Figure 1-3) (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999).

Fz/PCP signaling controls the fates of R3 and R4 by regulating Notch signaling (Fanto and Mlodzik, 1999; Cooper and Bray, 1999). Although the precise mechanism for this regulation is not at all clear, two observations suggest mechanisms that are not mutually exclusive. First, transcription of *Delta* and *neuralized* is up-regulated in R3 (Fanto and Mlodzik, 1999; Del Alamo and Mlodzik, 2006), and in experiments where R3/R4 are mosaic, the *Dl*+ R3/R4 precursor always become R3 (Tomlinson and Struhl, 1999). The result is somewhat more complicated for *neur*. Instead of the *neur*+ cell always becoming R3, loss of *neur*+ in R4 had no effect, but loss of *neur*+ in R3 resulted in equalization of R3/R4 cell fate in most cases (Del Alamo and Mlodzik, 2006). Because it is thought that ubiquitination of Delta is absolutely required for its endocytosis, one would expect the result of removing *neur* function to be the same as the result for Delta. The difference observed is likely due to the fact that higher Delta ligand is still present on the plasma membrane in *neur* mutant R3 precusor cells. Although the ligand cannot signal, it can inhibit Notch receptors on the same cell membrane from being activated, a process termed "cis-inhibition'' (Miller et al., 2009). Second, activated Fz moves, along with Dsh, from the equatorial side of R3 and R4 precursors, to the polar side (Strutt et al., 2002). Dsh may thus inhibit Notch function at the side of the R3 that is adjacent to R4 (Strutt et al., 2002). Both Fz relocalization and *Delta* and *neur* up-regulation may contribute to R3 becoming the signaling cell, or Fz relocalization could have nothing to do with it. Alternatively, it is possible that Fz relocalization is the defining event, and *Delta* and *neur* up-regulation occur downstream. There is some evidence that *Delta* and *neur* transcription are controlled by Fz directly, rather than as a secondary effect of Notch

activation in R4, which argues that *Delta* and *neur* are primary determinants of biasing R3 to be the signaling cell (Fanto and Mlodzik, 1999; Del Alamo and Mlodzik, 2006).

Notch signaling in R3/4 pairs has been relatively well undersood and provides a useful system to study components for directionality of Notch signal between cells. However, the factors involved in Fz-mediated Notch down-regulation are not well characterized. In this study, Ral function in Notch asymmetry establishment was analyzed in the R3/4 developmental context and how Ral mediates the Fz receptor pathway for down-regulating Notch will be described.

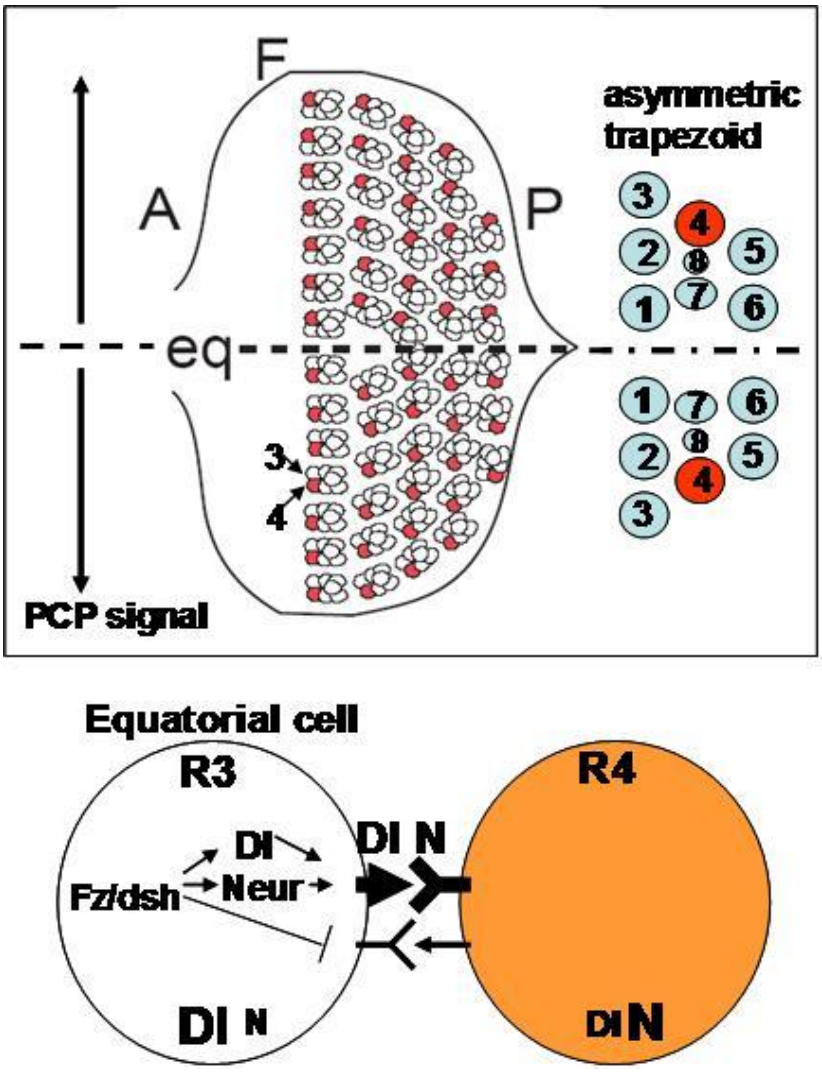


Figure 1-3. Asymmetric Notch activation in R3/4 photoreceptor pairs in *Drosophila* eye. During eye development, the PCP (Planar Cell Polarity) signal from the equator of the developing eye activates Frizzled receptor signaling in equatorial cells, R3 precursors. Activated Frizzled signaling induces the expression of *Delta* and *Neur*, or down-regulates Notch receptor activation (or both) in R3 precursors, which activates the Notch receptor in the R4 precursors. These signaling events determine cell fates of R3 and R4, asymmetric photoreceptor arrangement, and rotation of ommatidia in the adult eye. (A; Anterior, P; Posterior, F; Morphogenetic furrow, eq; Equator).

NOTCH SIGNALING IN FATE DETERMINATION OF *DROSOPHILA* PHOTORECEPTOR NEURAL CELLS

Drosophila eye provides a powerful model system to study all kinds of cellular and developmental events. In *Drosophila* eye, cell proliferation and differentiation occur at distinct developmental stages and various evolutionarily conserved signaling events regulate eye formation. As I have used eye tissues for studing *Ral* and *epsin* functions in the Notch signaling pathway, Notch signaling events, particularly during photoreceptor development, will be introduced in this section.

The *Drosophila* adult eye is developed from a monolayer cell tissue, an eye-antenna imaginal disc, which starts to be formed at first instar larval stage. Notch signaling is required during numerous steps in eye development. In earlier stages before neural cell differentiation, Notch signaling is required for eye homeostasis and Dorsal/Ventral (D/V) axis determination (Cho and Choi, 1998; Dominguez and de Celis, 1998; Kumar and Moses, 2001). Therefore, without Notch signaling in early eye developmental steps, the eye is malformed or transformed into an antenna. Reduced Notch activity in early developmental stages also induces inverted ommatidial orientation at the dorsal or ventral plane of the eye, indicating abnormal D/V axis formation (Cho and Choi, 1998; Dominguez and de Celis, 1998).

The *Drosophila* compound eye is composed of approximately 800 ommatidia, or facets, each of which contains 22 cells. In the larval eye imaginal disc, serial cell communication events pattern the eye field and recruit cells into ommatidia (Wolff and Ready, 1993). In the eye disc, posterior to a wave of morphogenesis, known as the morphogenetic furrow, ommatidia form stepwise, starting with the eight photoreceptors, or R-cells (Wolff and Ready, 1993). At first, R8s are determined, and a few rows later, the fates of R2/5 and R3/4 are determined (Wolff and Ready, 1993; Overstreet et al.,

2004). Finally, R1/6 cells and then R7 are recruited to join the R2/3/4/5 pre-clusters (Figure 1-4) (Nagaraj et al., 2002). At first, anterior to the morphogenetic furrow, Notch signaling is required for proneural cell promotion (proneural enhancement) (Overstreet et al., 2004; Nagaraj et al., 2002; Wolff and Ready, 1993). Second, at around the morphogenetic furrow, R8 cells laterally inhibit surrounding cells from becoming R8 cells by Notch activation (lateral inhibition) (Overstreet et al., 2004; Nagaraj et al., 2002; Wolff and Ready, 1993). Third, during R2/3/4/5 determination, Delta signaling by R3 differentiates the R3/4 pair (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999) and once the fates of R2/3/4/5 are determined, they prevent pre-cluster cells that have not become R2/5 and R3/4 from becoming ectopic R-cells, just posterior to the furrow (R-cell restriction) (Overstreet et al., 2004). After R1/6 cells are recruited, R1/6 send weak Notch signals to R7 precursors to recruit them as R7s. R1/6 cells also send strong Notch signals to cone cell precursors to recruit them as non-neural cone cells (Cooper and Bray, 2000). These events are tightly regulated as the strength and timing of Notch/Delta signaling is important to the outcome.

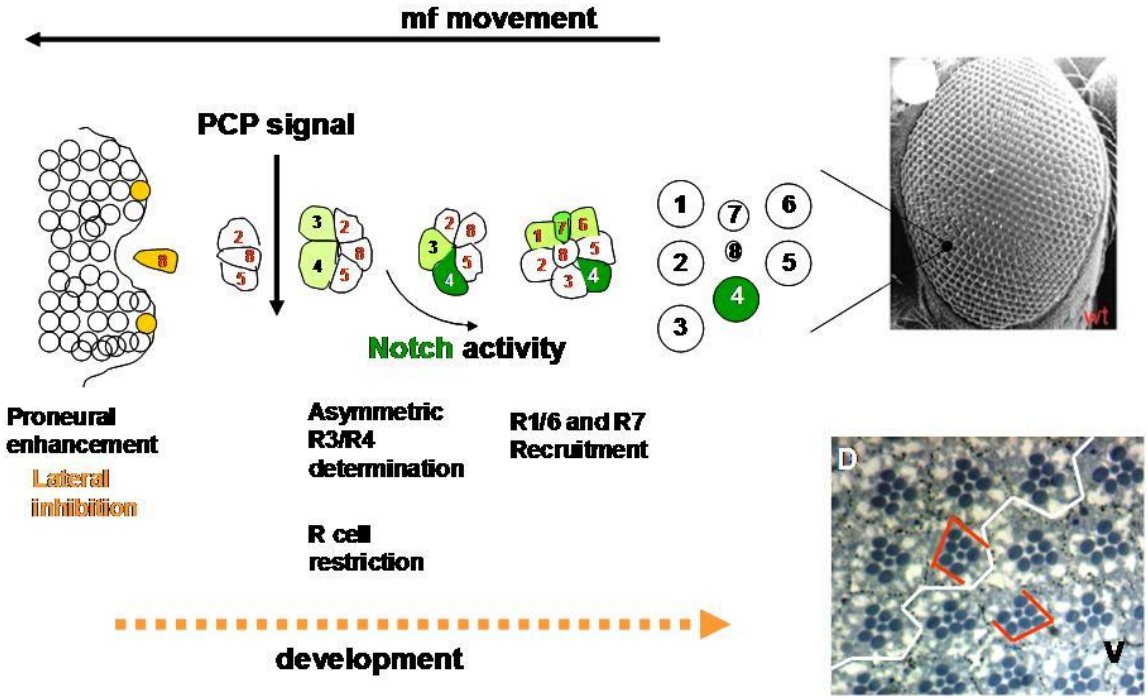


Figure 1-4. Notch signaling events in *Drosophila* photoreceptor development. In the eye imaginal disc, following the movement of a morphogenetic wave, called a morphogenetic furrow (mf), more differentiated cells are left behind during eye development. Photoreceptor neural cells are chosen from proneural cell clusters and recruited as a stepwise manner (see the text for the detail). Notch signaling is involved in multiple steps of the photoreceptor development, including proneural enhancement, lateral inhibition, asymmetric R3/4 determination, R cell restriction, and R7 determination steps.

NOTCH SIGNALING AT D/V BOUNDARY IN *DROSOPHILA* WING DISCS

Notch signaling also plays a role in *Drosophila* wing margin development. In the fly wing primordium, the D/V boundary is initially formed in the middle of the tissue and the line of cells at the D/V axis moves toward the margin of the wing tissue, which becomes the boundary in the adult wing. Notch is activated at this line of D/V boundary cells and loss-of or gain-of-function mutations of *Notch* cause notched wing or additional wing margin, respectively. In my epsin experiments, Notch activation was monitored at the D/V boundary in the wing disc.

Initially, a homeodomain transcription factor, Apterous (AP), determines dorsal cell fates by inducing the expression of a *Drosophila* glycosyltransferase, *Fringe*, and *Serrate* in dorsal cells (Figure 1-5) (Diaz-Benjumea and Cohen, 1993; Diaz-Benjumea and Cohen 1995, Irvine and Wieschaus 1994, Kim et al., 1995). Fringe-mediated Notch modification confers Notch to be insensitive to Serrate, but Serrate can activate unmodified Notch in ventral cells (Diaz-Benjumea and Cohen 1995; Couso et al., 1995). Notch activation in the ventral stripe of cells promotes the expression of a secretory ligand, Wingless (Wg), the ligand for Wg signal (Diaz-Benjumea and Cohen 1995), which activates transcription of *Serrate* and *Delta* near the *Wg*-expressing stripe of cells at the D/V boundary (de Celis and Bray, 1997; Klein and Martinez Arias, 1998). Initially, *Delta* is expressed at both D and V planes of the wing tissue, which is different from the the pattern of *Serrate* expression. However, Notch activation and *Wg* expression confines *Delta* expression to the D/V boundary (de Celis and Bray, 1997; Klein and Martinez Arias, 1998; Micchelli et al., 1997). The modified Notch receptor is sensitive to Delta and Delta in ventral cells activates the Notch receptor in dorsal cells (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; Panin et al., 1997). These initial events and signal amplification by feedback mechanisms restrict Notch activation,

thearby *Wg* expression, to the stripe of D/V boundary cells. Wg promotes proliferation of cells at each D/V site, during which Notch-activating (thereby Wg expressing) D/V boundary cells function as an organizing center for regulating wing size. Cut, another homeodomain containing transcription factor, is the direct target of Notch and regulates *Wg* transcription (Micchelli et al., 1997).

Since Notch ligand and receptor cells are designated depending on ligand expression near the D/V midline, the epsin requirement in Notch receptor cells which express no or less ligands (Figure 1-5), was analyzed by monitoring *cut* expression as a read-out for Notch activity in my epsin study. Moreover, as Serrate and Delta signaling events occur concomitantly in the wing tissue, modules of epsin for different ligands, Serrate and Delta, have been studied, which were not characterized before.

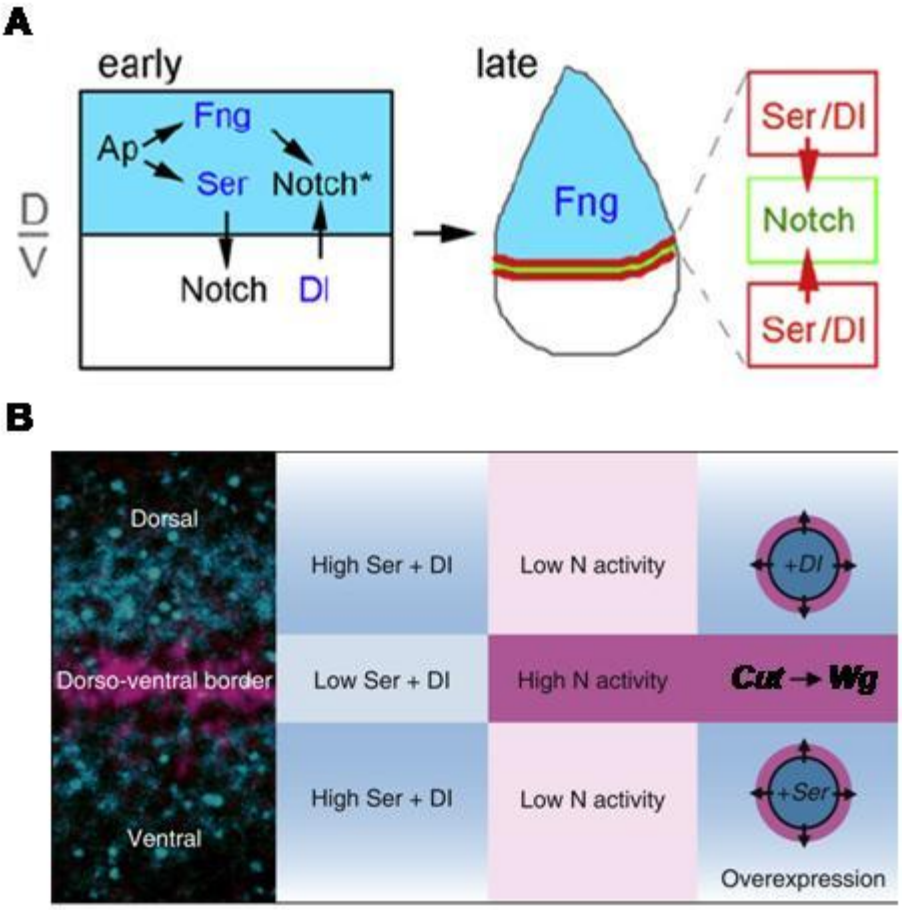


Figure 1-5. Notch signaling at D/V boundary in *Drosophila* wing discs (adapted from del Álamo et al., 2011 and Becam et al., 2010). **(A)** In early wing developmental stages, a transcription factor, AP (Apterous), induces *Serrate* and *Fng* expression in the dorsal plane, in which Fng modifies Notch at dorsal side and Serrate activates unmodified Notch at the ventral part. Ventral Delta activates modified Notch in the dorsal side. **(B)** In later stages, signal amplification and feedback regulation restrict the expression of *Serrate* and *Delta* to cells near the D/V boundary where Notch activating cells lose ligand expression. *Delta* or *Serrate* overexpression induces ectopic Notch activation outside the over-expressing cells at the dorsal or ventral side, respectively.

NOTCH SIGNALING IN *DROSOPHILA* FOLLICLE CELLS

Drosophila egg chamber is another ideal model system to study Notch signal, because Notch is temporally regulated through a series of distinct stages (Figure 1-6). Furthermore, distinction between signal sending cells (germ line cells) and signal receiving cells (follicle cells) is unambiguous, providing an ideal environment to study factors required in signal sending or receiving cells.

During oogenesis, Notch regulates multiple processes, including specifying the terminal follicle cells, dorsal anterior follicle cells, and polar cells (Ruohola et al., 1991; Xu et al., 1992; Larkin et al., 1996; Keller Larkin et al., 1999; Gonzalez-Reyes and St Johnston, 1998). Follicle cells surround germ line cells and two rounds of cell cycle transition govern proliferation and gene transcription of the follicle cells. Follicle cells divide and proliferate up until stage 5 of oogenesis (mitotic cell cycle) and from stage 6 through stage 10B/11, cell division stops and genomic contents of follicle cells increase without dividing (endocycle) (Royzman and Orr-Weaver, 1998). Later, after stage 10B/11, follicle cells overaying on the oocyte proceed through second cell cycle transition from endocycle to gene amplification in a specific genome loci (Royzman and Orr-Weaver, 1998). Regulation of Notch signal is critical for the transition of each cell cycle in follicle cells (Figure 1-6).

Delta starts to be transcribed in germ line cells at stage 6, reaches highest level at stage 7, and sends signal to activate the Notch receptor pathway in follicle cells (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). In *Drosophila*, String, a Cdc25 phosphatase, promotes mitotic cell cyle (Edgar et al., 1994), and Notch activation downregulates the expression of *String* at thease stages, which induces mitotic to endocycle transition (Deng et al., 2001). Follicle cell Notch activation proceeds up until

stage 10B/11 oocytes and then, after this stage, the Notch pathway is repressed in conjunction with ecdysone signal (Calvi et al., 1998; Sun et al., 2008).

Notch activation upregulates expression of a zinc-finger transcription factor, *Hindsight* (*Hnt*) (Sun and Deng, 2007), and downregulates expression of the immature fate marker, *cut*, in follicle cells (Sun and Deng, 2005) (Figure 1-6). In my works, I analyzed epsin requirement for receiving Notch signal in follicle cells by testing expression of these markers.

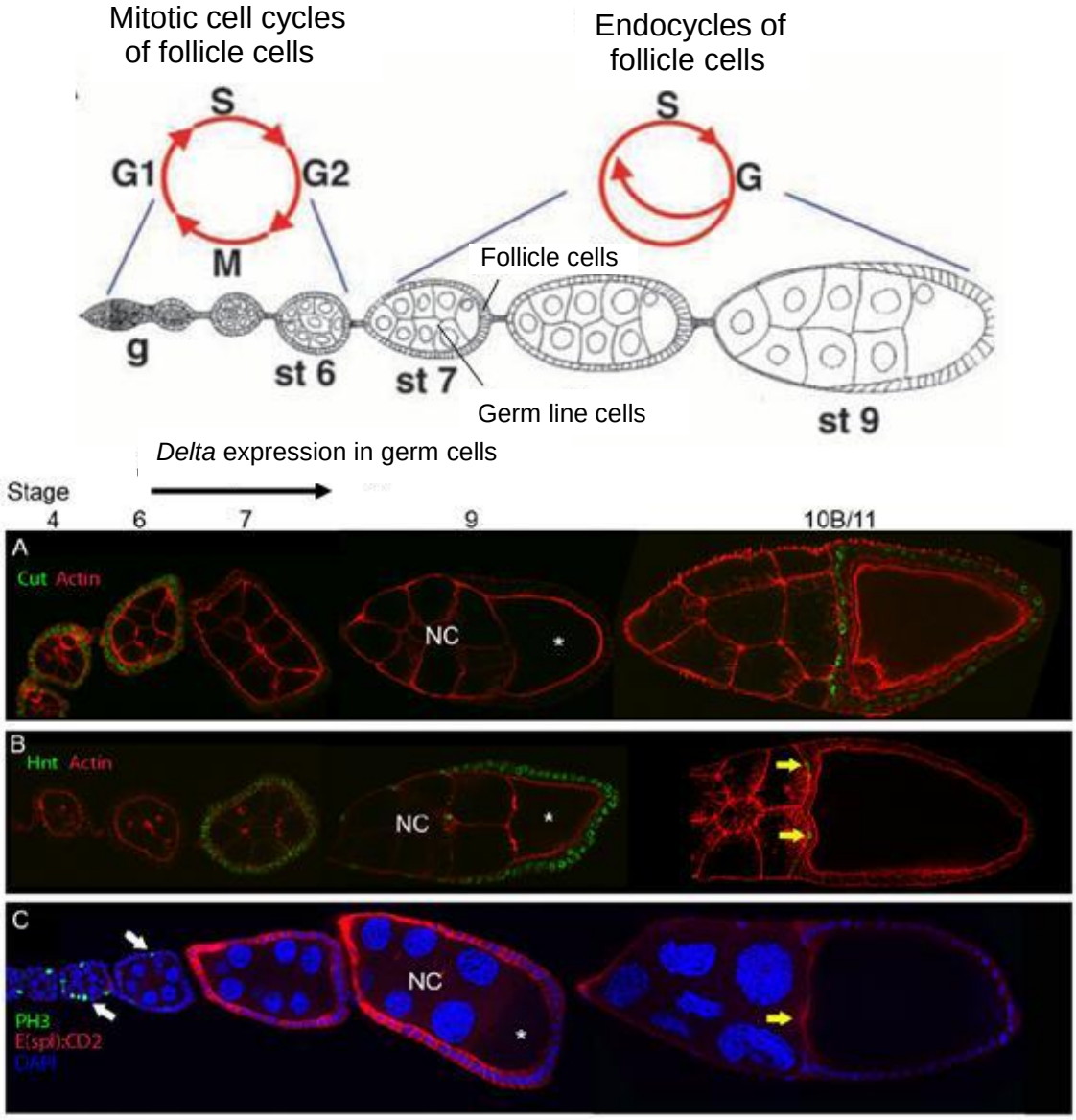


Figure 1-6. Notch signaling-mediated cell cycle controls of follicle cells in *Drosophila* egg chambers (adapted from Deng et al., 2001 and Poulton et al., 2011). During oogenesis, follicle cells proceed through two rounds of the cell cycles; mitotic- and endocycles. The expression of *Delta* is induced in germ line cells at stage 6/7 and activates Notch in follicle cells which represses the mitotic cell division of follicle cells. After stage 10B/11, Notch signaling is down-regulated in follicle cells overaying the oocyte. Follicle cell Notch activation turns off the expression immature fate marker, *cut*, and turns on *hnt* expression. Mitotic phosphorylation of Histone H3 (PH3) is also reduced by Notch activation.

UBIQUITINATION OF NOTCH LIGANDS IS NECESSARY FOR NOTCH ACTIVATION

So far, I have described establishment of initial Notch directionality and Notch signaling events in *Drosophila* tissues. As I mentioned in previous sections, initial biases (of ligands or of Notch receptors) appear to be required for all cell-cell communication events, where cell fates need to be determined. Then, what factors activate Notch signaling for signal amplification and final cell fate decisions? Endocytosis of ubiquitinated Notch ligands is one of the essential steps for Notch activation in nearby cells, where epsin plays a role. Since the apparent role of epsin is in endocytosis of ubiquitinated Notch ligands (description followed), ligand ubiquitination is critical for epsin function. In this section, I will describe two essential enzymes for ligand ubiquitination and the meaning of ligand ubiquitination in Notch signaling.

Ubiquitination of Notch ligands is necessary for sending a signal to neighboring cells by triggering ligand endocytosis. A chimeric form of Delta substituted by Ub at the intracellular domain was enough to send signal (Wang and Struhl, 2005), and truncation of intracellular domain or mutation in putative E3 Ub ligase binding sites of Delta reduced signal sending activity (Wang and Struhl, 2005; Glittenberg et al., 2006; Parks et al., 2006). *Drosophila* Mind bomb (Mib) and Neuralized (Neur) are both RING domain-containing E3 Ub ligases; one RING domain for Neur (Lovering et al., 1993) and three RING domains for Mib (Itoh et al., 2003), and monoubiquitinate Delta and Serrate (Glittenberg et al., 2006; Parks et al., 2006). Whereas Mib is the main E3 ligase in vertebrates (Barsi et al., 2005; Koo et al., 2005), both Mib and Neur are required in *Drosophila* development. Although their functions are similar, their expression is dependent on tissues and they are required in different developmental stages in *Drosophila* (Lai et al., 2005; Le Borgne et al., 2005; Wang and Struhl, 2005). Indeed, expression of one E3 enzyme, Neur, compensates for the absence of the other, Mib, in the

wing tissue (Wang and Struhl, 2005). However, in some contexts, such as neurogenic embryo phenotypes caused by *neur* mutation, *mib* expression failed to fully rescue defects caused by *neur* mutaion, indicating the presence of a distinct function of Neur, even though the unique function of Neur need to be studied further (Le Borgne et al., 2005).

Why is ubiquitination required for ligand signaling? It was suggested that endocytosis of ubiquitinated ligands generates enough activity for Notch activation in nearby cells, which is distinguished from general endocytosis for regulating the level of ligands at the plasma membrane (Wang and Struhl, 2005). This may require a specific adaptor protein that recognizes Ub on the ligand and internalize it for enough force generation, or the Ub on the ligand may signal for futher activation of the ligand, once internalized into a specific cellular compartment. *Drosophila* endocytic epsin has Ub-interacting motifs and is necessary for ligand signaling, but not for general endocytosis of ligands (Wang and Struhl, 2005), suggesting that epsin could be the adaptor exerting the specific role.

LIGAND ENDOCYTOSIS BY SIGNALING CELLS IS REQUIRED FOR NOTCH SIGNALING

Much experimental evidence supports the idea that ligand endocytosis is required for Notch signaling. For instance, mutant Delta proteins that cannot be internalized cannot signal (Parks et al., 2000) and the ubiquitin ligases, Neur or Mib are required in the signaling cells for ligand ubiquitination, which promotes ligand internalization and signaling (Pavlopoulos et al., 2001). More recently, we and others have shown that the endocytic proteins, epsin and auxilin, are required in signaling cells for ligand endocytosis and signaling (Overstreet et al., 2004; Wang and Struhl, 2004; Eun et al.,

2008; Kandachar et al., 2008). There are two popular models to explain why ligand endocytosis is required for signaling (Figure 1-7). In the pulling model, endocytosis of ligands delivers physical force to the Notch receptor and thereby exposes a Notch proteolytic site (Parks et al., 2000) or dissociates the Notch heterodimer (Nicolas et al., 2007). After a second Notch cleavage event, the Notch intracellular domain translocates into the nucleus where it interacts with transcription factors to control gene expression. Generally, this Notch activation event induces repressors (ie, Enhancer of split proteins) to inhibit cells from neural fate determination (Cooper et al., 2000). In the recycling model, an inactive form of ligand is expressed on the cell surface and, through epsin-dependent endocytosis, ligand is internalized and recycled back to the membrane in an active state to trigger Notch cleavage events (Wang and Struhl, 2004; Emery et al., 2005). In this scenario, active forms of ligands might be internalized for the force generation in an epsin-dependent manner, which, in part, supports the pulling model. A very recent paper suggested that endocytosis and recycling of Delta moves Delta from a membrane domain where it cannot interact with Notch to another membrane domain where it can bind and activate Notch (Benhra et al., 2010). In a third version of the recycling model, recycled ligand is secreted as exosomes to induce Notch signaling in adjacent cells (De Joussineau et al., 2003). These models are each supported by some data; all of the data indicate that epsin-dependent ligand endocytosis is needed for signaling.

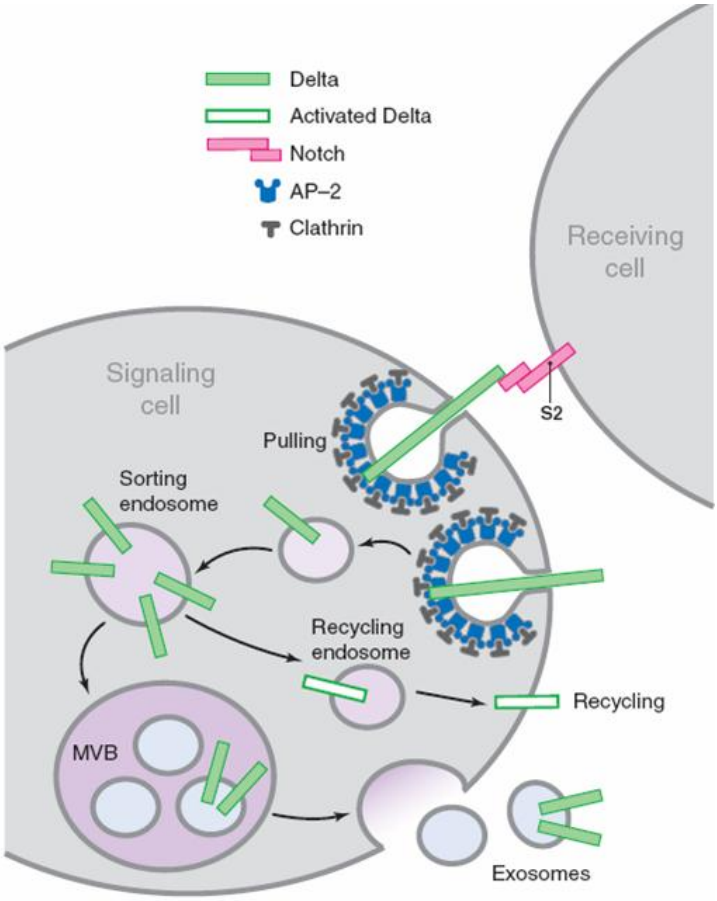


Figure 1-7. Models for the requirement of ligand endocytosis in Notch activation (Adapted from Fischer, J. A. et al., 2006). Delta internalization may generate the physical force for exposing S2 cleavage site of Notch (pulling model). Alternatevely, Delta internalization and recycling as an active form of ligand, or exosomes, may induce Notch activation (recycling).

LQF, *DROSOPHILA* ENDOCYTIC EPSIN, IS REQUIRED FOR LIGAND ENDOCYTOSIS

Epsins are evolutionarily conserved modular proteins that function in vesicle formation at the plasma membrane and trans-Golgi network (Chen et al., 1998; Wendland, 2002; Hirst et al., 2003). Lqf is the *Drosophila* homolog of endocytic epsin, which works at the plasma membrane for vesicle formation (Chen et al., 2002). In mammalian cells and yeast, endocytic epsin has been suggested to have a variety of different functions: a clathrin adaptor for clathrin-mediated endocytosis of transmembrane proteins that use ubiquitin as an internalization signal (Chen et al., 1998), or a factor for clathrin-independent endocytosis of those proteins, or an accessory factor for AP-2 in clathrin-dependent endocytosis of transmembrane proteins with AP-2 binding sites (Hirst et al., 2003). However, regardless of these various functions of epsin, the only apparent essential role of epsin in *Drosophila* is in ligand endocytosis for Notch signaling (Overstreet et al., 2004; Wang and Struhl, 2004). Epsin is multi-ubiquitinated, and its deubiquitination by Fat facets (Faf), a deubiquitinating enzyme, promotes epsin activity, possibly by preventing epsin degradation (Cadavid et al., 2000; Chen et al., 2002).

As an endocytic factor, epsin has motifs and domains for promoting cargo internalization. Its' N-terminus has the epsin N-terminal homology (ENTH) domain (Kay et al., 1999; Rosenthal et al., 1999; De Camilli et al., 2002), which binds PIP_2 (phosphatidylinositol 4,5-bisphosphate) at the plasma membrane (Itoh et al., 2001; Ford et al., 2002; Aguilar et al., 2003). The C-terminal region of epsin has four different protein-protein interaction motifs varying in numbers between animal species (Kay et al., 1999; De Camilli et al., 2002). *Drosophila* has two epsin isoforms generated by alternative splicing of *lqf* gene mRNA (Cadavid et al., 2000). Each 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., 2004; 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 adaptor complex (Owen et al., 1999), and two NPF motifs that bind EH domain-containing factors (Figure 1-8) (Aguilar et al., 2003; Salcini et al., 1997; Paoluzi et al., 1998).

As mentioned above, even though epsin is a well-conserved endocytic factor throughout animal kingdoms, the mechanism of epsin function is still controversial. More importantly, cell-autonomous roles of epsin in multicellular organisms have not been studied, whereas epsin was suggested as a general endocytic factor for controlling various signals in previous yeast and mammalian cell culture studies. My dissertation work includes studies about the roles of the ENTH domain and each motif of epsin in Notch signaling. Particularly, UIMs were observed to be critical for sending signal, but not for receiving signal or general roles of epsin. I will provide evidence about distinct motifs of epsin used in sending and receiving Notch signal. Finally, evidence about the epsin function as both a specific adaptor for Notch and an accessory factor for curvature formation, by which Notch is routed to a specific early endosome, will be provided.

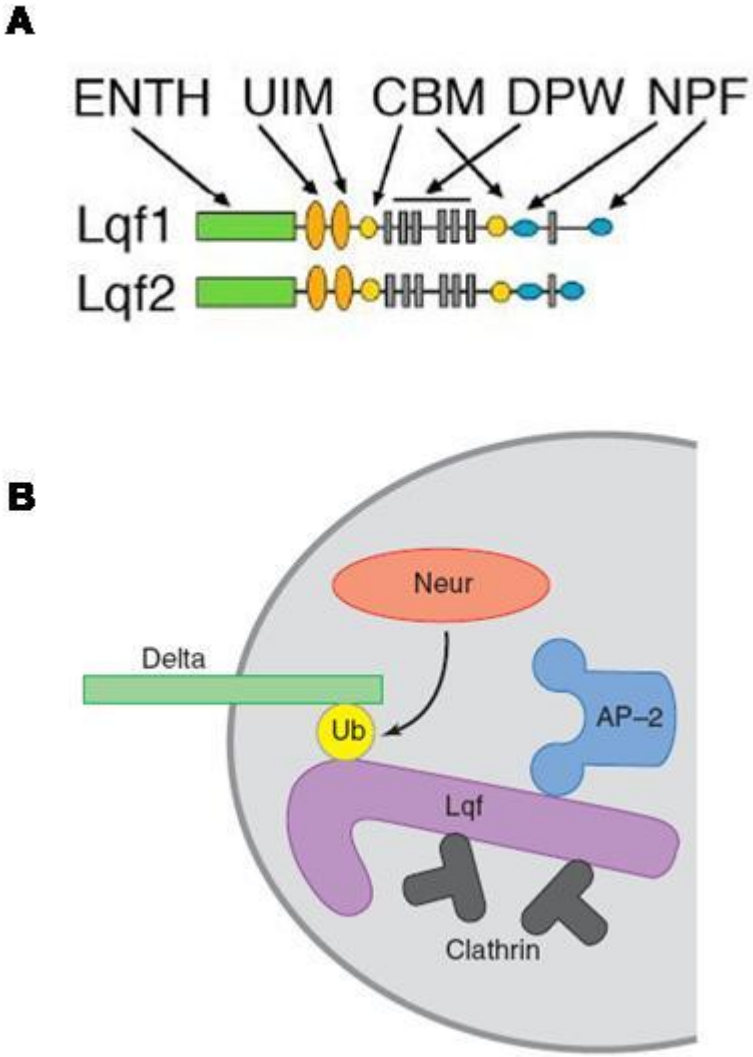


Figure 1-8. Motifs and domains of *Drosophila* endocytic epsin, Lqf, and working model of Lqf in ligand endocytosis (Adapted from Fischer, J. A. et al., 2006). **(A) Structure of *Drosophila* epsin, Lqf.** *Drosophila* has two splicing varients of the *lqf* gene and each has an ENTH domain and four types of motifs; UIMs, CBMs, DPWs, and NPFs (see text for details). **(B) Epsin working model for ligand endocytosis for Notch activation.** Neur (or Mib) ubiquitinates ligands and epsin (Lqf) recognizes ubiquitinated ligands and internalize it. It is still unclear whether epsin-mediated ligand internalization requires clathrin and (or) AP-2.

CONTRLOL OF NOTCH ACTIVATION VIA NOTCH TRAFFICKING

Much evidence supports that ligand endocytosis is necessary for Notch activation. Intriguingly, recent studies argue that Notch endocytosis is also required for Notch activation. In agreement with this, my experimental evidence showed epsin requirement in Notch trafficking and Notch activation *in cis*, which was an unexpected result based on epsin's role in ligand endocytosis. Therefore, I will describe the biological meaning of Notch trafficking suggested by previous studies in this section.

In multiple aspects, regulation of Notch trafficking is important to regulate Notch signaling (Figure 1-9). Notch endocytosis controls the membranous Notch level by routing Notch to the lysosomal pathway. In this process, several E3 ubiquitin ligases are reported to play roles in the lysosomal degradation of Notch, thereby down-regulating Notch signal; the RING finger E3 ligase c-Cbl (Jehn et al., 2002), the two HECT (homologous to E6-AP carboxyl terminus) domain containing E3 ligases Suppressor of Deltex [Su(dx)] (Fostier et al., 1998) and Nedd4 (Wilkin et al., 2004). Nedd4 and c-Cbl are involved in the sorting and lysosomal degradation of unactivated Notch and gain-of-function of *Su(dx)* and *Nedd4* showed a loss-of-function Notch wing phenotype, indicating that these E3 ligases are negative regulators of Notch signaling (Fostier et al., 1998; Wilkin et al., 2004). However, direct biochemical evidence is still missing and the function of these E3 ligases relys on specific Notch events (context-or tissue-dependent), not all Notch signaling events.

Shibire (Shi), a drosophila dynamin, was found to be required for sending and receiving Notch signal (Seugnet et al., 1997). Given that dynamin plays a role in pinching off membrane curvatures, endocytosis could be required not only for ligand signaling, but also for Notch receptor activation. Paradoxically, expression of full-length uncleaved Notch was epistatic to *shi* mutation in the same study (Seugnet et al., 1997). Therefore,

they interpretated that Shi-dependent endocytosis is not prerequisite for Notch activation, but *Drosophila* dynamin might have an additional role in promoting ligand/Notch interaction; Notch overexpression overcomes the *shi* mutation by increasing the chance of ligand/Notch interaction. However, recent studies showed that clathrin heavy chain is required in signal receiving follicle cells (Windler and Bilder, 2010), and endosomal factors, such as Rab5, are required in signal receiving cells in the eye, wing, and ovarian tissues, for Notch activation (Vaccari et al., 2008; Windler and Bilder, 2010). All these support the idea that Notch receptor endocytosis, thereby Notch routing to certain endosomal compartments, is required for the Notch receptor transduction pathway. They suggested that Notch cleavage occurs in certain endosomes and the acidic endosomal condition might promote Notch cleavage by activating proteolytic enzymes.

Then why does uncleaved Notch rescue *Shi* mutants? There are two possible explanations. First, Shi-dependent Notch endocytosis could be the dominant pathway in normal condition, but there might be minor Shi-independent Notch endocytosis which could result in rescuing Notch activity without *Shi* in high Notch expressing condition. However, because there are no known factors that substitute for dynamin function in *Drosophila*, this explanation may not be plausible. Second, Shi-dependent Notch endocytosis may play a role in more efficient Notch cleavage than the cleavage at the plasma membrane. In this scenario, Notch overexpression might increase Notch cleavage at the plasma membrane, albeit inefficiently in the absence of *Shi*.

More complexity comes from the study carried out by Windler and Bilder (Windler and Bilder, 2010). In this paper, patterns of cellular Notch accumulation in mutants of various endosomal components are similar or different in terms of Notch accumulation in cellular compartments. Furthermore, in some mutants, patterns of Notch activation were different; one mutation affects Notch activation, but the other does not,

albeit defects in Notch trafficking patterns being similar. These results suggest that endosomal sorting components are functionally different, even between early endosomes or late endosomes. Moreover, these results propose the presence of specific Notch trafficking routes for Notch activation, separable from general Notch trafficking for controlling the membranous Notch level.

Why is Notch internalization required? And how is signaling Notch internalization regulated to exert separate roles from simple control of the membranous Notch level? Part of my dissertation proposes that epsin-dependent Notch compartmentalization to Rab5 endosomes could be the distinct route for Notch activation.

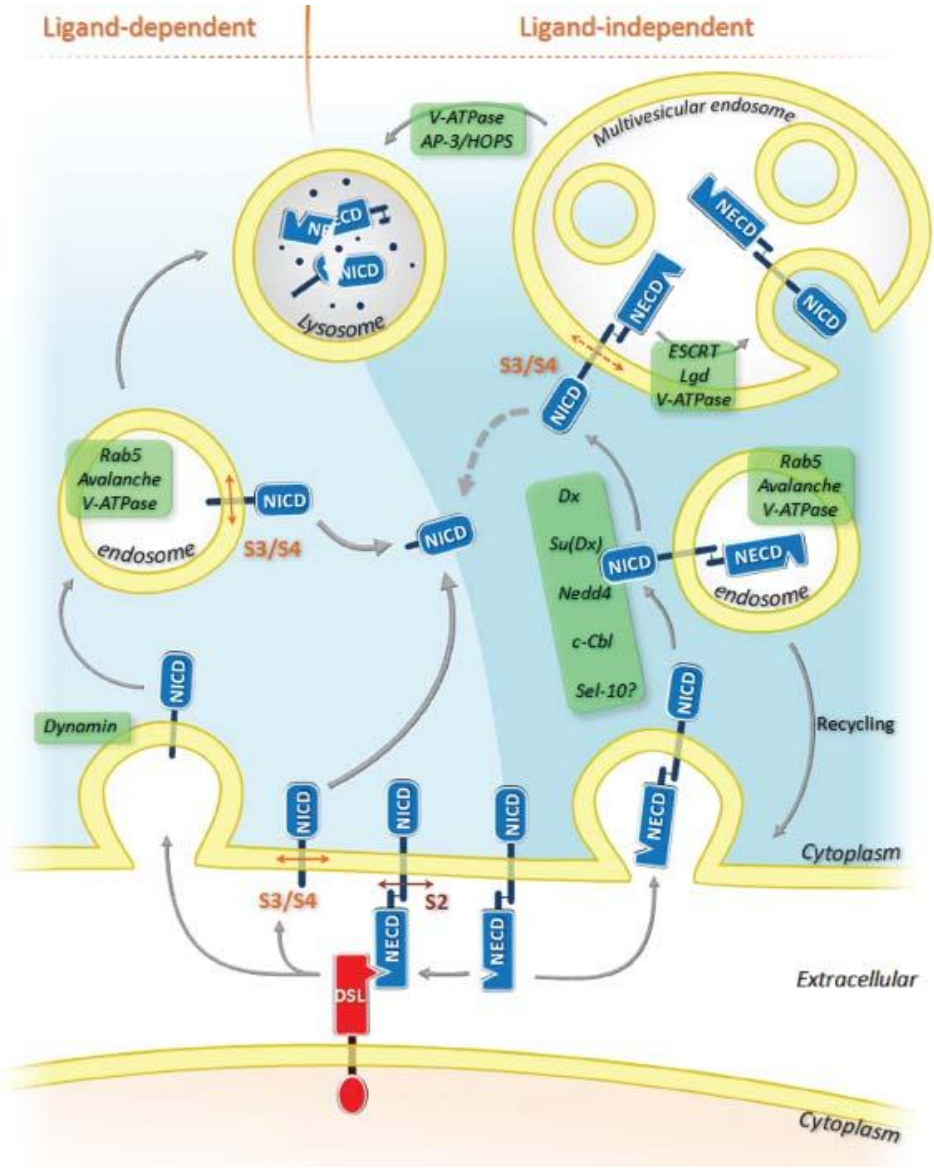


Figure 1-9. Control of Notch activation via Notch trafficking (Adapted from Le Bras et al., 2010). Ubiquitinated Notch receptors are routed to the lysosomal degradation pathway, where ligand-independent Notch activation may be induced (ex, Deltex-mediated ubiquitination and ligand-independent Notch activation, see the text for the detail). Mutations in components of ESCRT and Lgd cause accumulation of Notch, which induce ligand-independent ectopic Notch activation. Dynamin- and Rab5-mediated Notch endosomal sorting is required for ligand-dependent Notch activation, even though it is not clear whether processed or unprocessed Notch is routed to Rab5 endosomes. All these models are challenging to the classical ligand-depedent Notch activation at the plasma membrane.

LIGAND-INDEPENDENT NOTCH SIGNALING

As mentioned above, control of Notch trafficking is not a simple constitutive cellular process for Notch level control at the plasma membrane, but a way for productive signal transduction. On the other hand, recent studies showed that regulation of Notch trafficking plays a role in prevention of ligand-independent Notch activation which causes inappropriate Notch activation once the regulatory mechanism is disrupted (Figure 1-9). In my dissertation work about *Ral*, repressing ligand-independent Notch activation appeared to be involved in determining the signal directionality of Notch between cells. Therefore, in this section, I will introduce the ligand-independent Notch activation mechanisms that Ral could control.

In a previous study, mutation in the C2 domain containing protein Lethal giant discs (Lgd) showed Notch endosomal accumulation and ectopic ligand-independent Notch activation (Childress et al., 2006; Jaekel and Klein, 2006). Furthermore, an E3 ligase, Deltex, also has been shown to prevent ligand-independent Notch activation, even though it works in specific Notch signaling events (Mukherjee et al., 2005; Wilkin. et al., 2008). Recently, mutations of the ESCRT components, which sort ubiquitinated proteins from early endosomes to multivesicular bodies, were found to result in Notch accumulation in identified or unidentified early endosomes, causing ligand-independent Notch activation (Vaccari et al., 2008; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Whereas early endocytic factors such as *Shi* and *Rab5* induce productive Notch trafficking, where Notch is activated, it is proposed that ESCRT components sort Notch to the lysosomal degradation pathway to prevent attenuation of Notch in certain endosmes, in which Notch cleavage might be able to occur (Vaccari et al., 2008). However, it is unclear whether two Notch trafficking pathways (productive and unproductive) share common cellular compartments, and how internalized Notch is

correctly sorted for these distinct processes. Moreover, there might be mechanisms that distinguish endocytic events of active (ligand bound) and inactive Notch (ligand unbound) at the plasma membrane. More importantly, whether the control of ligand-independent Notch signaling is implicated in cell fate specification is unknown. In this dissertation, possible roles of Ral GTPase in ligand-independent Notch regulation and cell fate specification will be described. In my epsin study, I will propose that epsin is involved in endocytosis of active (ligand bound) Notch, which promotes Notch ativation.

CELLULAR FUNCTIONS OF A SMALL GTPASE, RAL

In a previous genetic screen, a *Ral* mutant was isolated by our group as an enhancer of eye phenotypes caused by epsin overexpression (Eun et al., 2007). Numerous Ral studies suggest that Ral functions are critical for multiple cellular events, albeit no obvious functional studies in multicellular organisms. In this section, I will describe previous studies about cellular functions of Ral which may be implicated in cellular events of multicellular organisms and developmental processes.

Ral (Ras-related) proteins are those of the Ras branch of small GTPases (Chardin and Tavitian, 1986). In mammals, Ral GTPases are regulated by Ras signaling, even though it is controversial in *Drosophila* (reviewed in Feig et al., 1996; Mirey et al., 2003). Ras regulates multiple downstream effectors, including specific Ral GEFs that make Ral GTPases unique in their cellular functions (reviewed in Feig et al., 1996). As other GTPases, Ral GTPases mediate cellular functions by cycling between a GTP-bound (active) and a GDP-bound (inactive) state; the GTP-bound form of Ral recruits or alters downstream target proteins that mediate cellular functions (Takai et al., 2001; Feig, 2003). Mammalian cells have two Ral GTPases, RalA and RalB, and their cellular

functions are different, even if they share 85% similarity (Chardin and Tavitian, 1986; Feig, 2003). Various cellular functions of Ral, including regulation of endocytosis, exocytosis, and several signal transduction pathways have been reported (Figure 1-10). For example, Ral binds to RalBP1 (Ral binding protein 1, also known as RLIP76) which in turn, binds to Eps homology (EH) domain containing proteins, POB1 and Reps1, indicating a Ral function in endocytosis, given that EH domain proteins participate in endocytosis (Cantor et al., 1995; Ikeda et al., 1998; Yamaguchi et al., 1997). In a mammalian cell culture study, Ral GTPase is thought to regulate epsin in a manner that depends on the cell-cycle; right before mitosis, epsin is phosphorylated by a mitotic Cdk activated by Ral, thus rendering epsin inactive (Kariya et al., 2000). In addition, Ral also binds to the exocyst complex via direct interaction with Sec5 and regulates transport of basolateral membrane proteins (Sugihara et al., 2002; Moskalenko et al., 2002). Therefore, Ral regulates endocytosis of receptors, such as EGFR (Epidermal Growth Factor Receptor) and insulin receptor (Shen et al., 2001), excocytosis of membrane proteins, and secretion of neurotransmitters. Previously, a tyrosine kinase Src was reported to be activated by the Ral pathway, partly promoting cell proliferation by regulating Jun-kinase (JNK) and Stat (Goi et al., 2000). Furthermore, with unkown mechanisms, Ral activates transcription factors, including TCF, NF-κB, and AFX (Wolthuis et al., 1997; Kops et al., 1999; Henry et al., 2000). Very recently, Ral was reported to regulate the JNK pathway through binding to the exocyst complex to regulate immune response (Chien et al., 2006), and the autophagy process by regulating autophagosome formation (Bodemann et al., 2011).

All cellular functions of Ral proteins described above have been studied using cell culture systems and functions of Ral in multicelluar organisms are barely understood. Unlike mammals, the *Drosophila* genome encodes a single *Ral* gene which is proposed to

generate two transcription variants by alternative splicing (Sawamoto et al. 1999b). The Ral GTPase-centered network is well conserved between vertebrates and *Drosophila*. Particularly, *Drosophila* proteins downstream of Ral GTPase in the receptor endocytic pathway are well conserved (Mirey et al., 2003). Most studies of *Drosophila Ral* have been carried out by over-expressing either a dominant negative form, or a constitutively active form of Ral in the eye and notum (Sawamoto et al. 1999a; Sawamoto et al. 1999b; Mirey et al., 2003). Recently, using *Ral* hypomorphs and lethal mutants, the roles of Ral GTPase have been studied in more physiological conditions in *Drosophila*. In these studies, Ral signaling was found to be a negative regulator of JNK-mediated apoptosis (Balakireva et al., 2006). Same *Ral* lethal mutants also showed defects in maintaining the Jak/Stat pathway in ovarian cells in a defferent *Drosophila* study (Ghiglione et al., 2008). Recently, Ral was proposed to play a role in cell fate specification events in a *C. elegans* study (Zand et al., 2011).

Considering Ral studies published before, functions of Ral proteins could be very important in multicellular organisms by mediating multiple cellular processes which are indispensable for developmental processes. Our group identified a mutation in the *Drosophila Ral* gene and mutants of regulatory genes involved in Notch signaling as dominant enhancers of the rough eye caused by epsin over-expression (Eun et al., 2007). Because the essential function of epsin is in Notch signaling, I started to study Ral function in Notch signaling. As mentioned above, although the relationship between Ral and epsin was described in a mammalian cell culture study, it is not characterized *in vivo*. Their relationship in cell fate specification events will be described and possible mechanisms about how Ral regulates Notch signaling will be discussed in this dissertation.

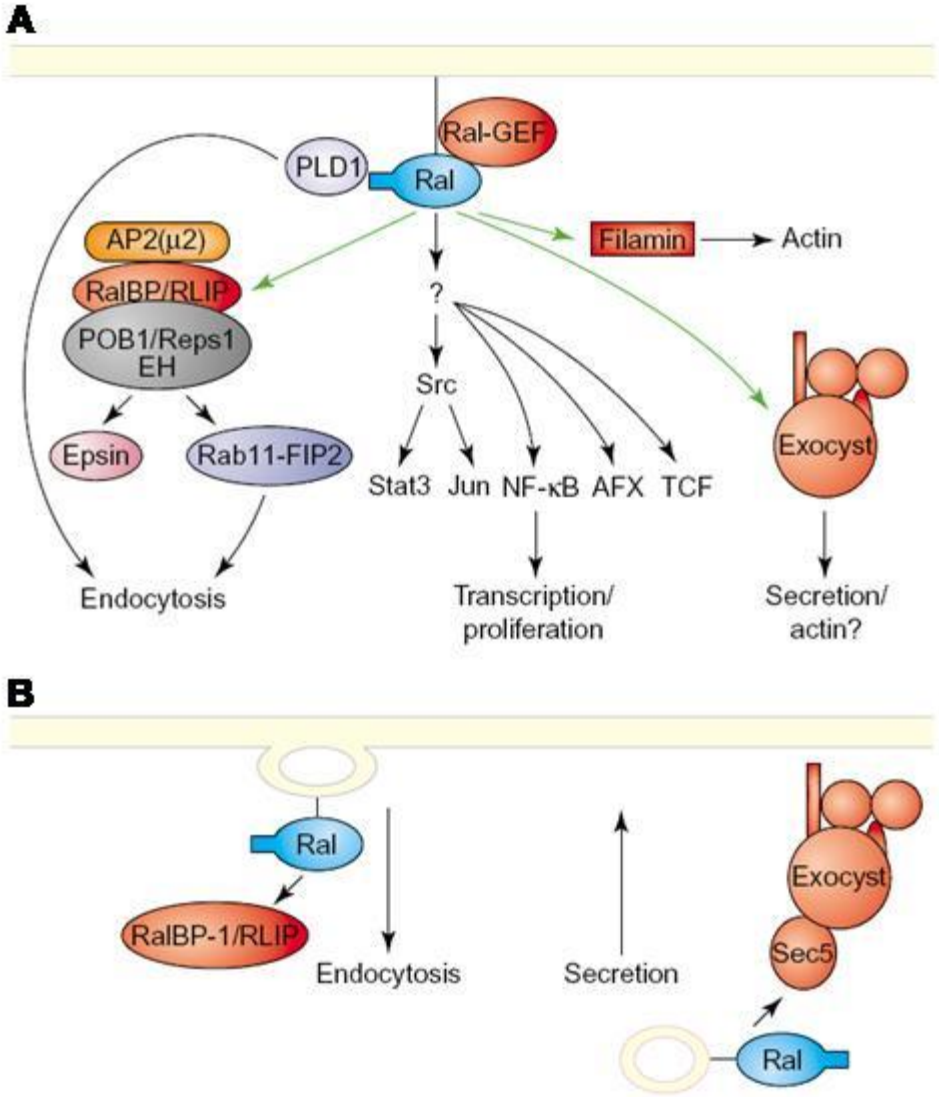


Figure 1-10. Multiple Ral effector pathways in mammalian cells (adapted from Feig, 2003). **(A) Ral effector pathways.** Ral is activated by Ral GEF and recruits RalBP (RLIP) for regulating endocytic machinaries. Active form of Ral recruits exocyst complex via binding to Sec5 and regulates basolateral membrane protein targeting. With unknown mechanisms, Ral also activates several transcription factors and promotes proliferation of cells. **(B) Cellular function of Ral in membrane trafficking.** Cellular localization of Ral may be important for regulating endocytosis and exocytosis. At the plasma membrane, Ral recruits and regulates endocytic components and at intracellular compartments, Ral may regulate exocytosis. However, the precise study about localization of Ral has not been carried out.

Chapter 2: *Ral GTPase promotes asymmetric Notch activation in the Drosophila eye in response to Frizzled/PCP signaling by repressing ligand-independent receptor activation**

INTRODUCTION

Functions for Ral (Rala – FlyBase), a small Ras-like GTPase, are only beginning to be discovered. Ral has a well-characterized role in secretion (Moskalenko et al., 2002; Sugihara et al., 2002), and is also implicated in other membrane trafficking and remodeling events (Feig, 2003; van Dam and Robinson, 2006; Chen et al., 2006; Cascone et al., 2008; Wu et al., 2008; Spiczka and Yeaman, 2008; Lalli, 2009; Hase et al., 2009). Ral also regulates Rheb-dependent nutrient sensing in vertebrate cells (Maehama et al., 2008), Jak/Stat- and JNK-dependent apoptotic pathways in *Drosophila* (Balakireva et al., 2006; Ghiglione et al., 2008), and vertebrate tumor cell survival (Camonis and White, 2005; Chien et al., 2006). Here, I describe a specific role for Ral in PCP-dependent Notch signaling that patterns the *Drosophila* eye.

The *Drosophila* eye exhibits PCP in the arrangement of its ommatidia, or facets (Wolff and Ready, 1993). There are two chiral forms of ommatidia, dorsal and ventral, reflected through the dorsal/ventral midline, or equator. Ommatidial polarity is governed by the Fz/PCP signaling pathway, which has common core components in vertebrates and *Drosophila* (Strutt and Strutt, 2005; Klein and Mlodzik, 2005; Lawrence et al., 2007; Strutt and Strutt, 2009; Wu and Mlodzik, 2009; Axelrod, 2009; Simons and Mlodzik, 2008). Ommatidial chirality is defined by a pair of photoreceptors, R3 and R4, at the apex of a trapezoidal arrangement of eight photoreceptors. The presumptive R3 is closer to the equator, and thus, early in eye development, has higher levels of Fz activation than the presumptive R4. Fz asymmetry results in the pre-R3 cell, via the ligand Delta, activating the Notch receptor in the pre-R4 cell (Fanto and Mlodzik, 1999; Cooper and Bray, 1999;

Tomlinson and Struhl, 1999). Asymmetric Notch activation in the R3/R4 pair ultimately determines the chirality of the ommatidium. Two different mechanisms, which are not necessarily mutually exclusive, have been proposed to explain how the difference in Fz activation leads to asymmetric Delta/Notch signaling. In one model, elevated Fz activation in pre-R3 leads directly to elevated transcription of *Delta* and *neuralized* (*neur*), which promote Delta signaling in R3 (Fanto and Mlodzik, 1999; del Alamo and Mlodzik, 2006). *neur* encodes a ubiquitin ligase that ubiquitylates Delta, and is required for Delta signaling. Subsequently, Notch activation in pre-R4 suppresses *Delta* and *neur* expression, which leads to less Notch activation in pre-R3 via a feedback loop. Alternatively (or in addition), Fz activation polarizes cells by localizing a Fz/Disheveled complex to one side of the plasma membrane (Strutt et al., 2002) (see also Tomlinson and Struhl, 1999). Disheveled (Dsh) is a cortical cytoplasmic protein required for transducing the Fz signal. At the interface between pre-R3 and pre-R4, Fz/Dsh is at the pre-R3 plasma membrane, where Dsh may directly inhibit Notch receptor activation in R3. The asymmetrically localized Fz/Dsh complex may also amplify the difference in Fz activation between the two cells through a feedback loop.

Here, I discover a unique Ral-dependent pathway by which Fz/PCP signaling leads to asymmetric Notch activation in R4. I show that in direct response to Fz activation, *Ral* expression in pre-R3 represses Notch activation, thereby biasing pre-R3 to become the Delta signaling cell. Moreover, I found unexpectedly that Ral prevents Notch activation that occurs independent of ligand binding. Thus, Ral regulates Notch signaling, and ligand-independent Notch activation is a target of regulation during cell patterning.

* This study was published in *Development* 2011

RESULTS

Ral is required for R-cell specification and PCP in the eye

Three *Ral* alleles were used in this work: Ral^{EE1}, Ral^{PG69} and Ral^{PG89}. Ral^{EE1} is a mis-sense mutation that alters a nucleotide-binding site ($Ser^{154}\rightarrow Leu^{154}$) (Eun et al., 2007). Ral^{PG69} and Ral^{PG89} are P-element insertions (*gal4*-expressing enhancer traps) in the 5′-UTR and the first intron of *Ral*, respectively (Ghiglione et al., 2008). Ral protein expression levels from the P alleles are reduced relative to wild type.

Ral^{EE1}/Y males or Ral^{EE1} homozygous females are viable with morphological abnormalities, including reduced rough eyes, curved wings, and missing hairs and bristles (Eun et al., 2007). Ral^{EE1} behaves like a hypomorphic allele. All aspects of the Ral^{EE1} mutant phenotype in hemizygous males are complemented by *Act5C-gal4;UAS-*Ral^{wt} (Eun et al., 2007). In addition, heterozygotes for Ral^{EE1} and either of the lethal hypomorphic alleles Ral^{PG69} or Ral^{PG89} have a mutant phenotype similar to Ral^{EE1} hemizygotes or homozygotes, that is complemented by *Ral* ^{PG69}*; UAS-*Ral^{wt}. Moreover, flies that express Ral^{RNAi} in the eye have defects similar to those in Ral^{EE1} flies, and the defects are rescued to wild-type by overexpression of wild-type *Ral* (Figure 2-1).

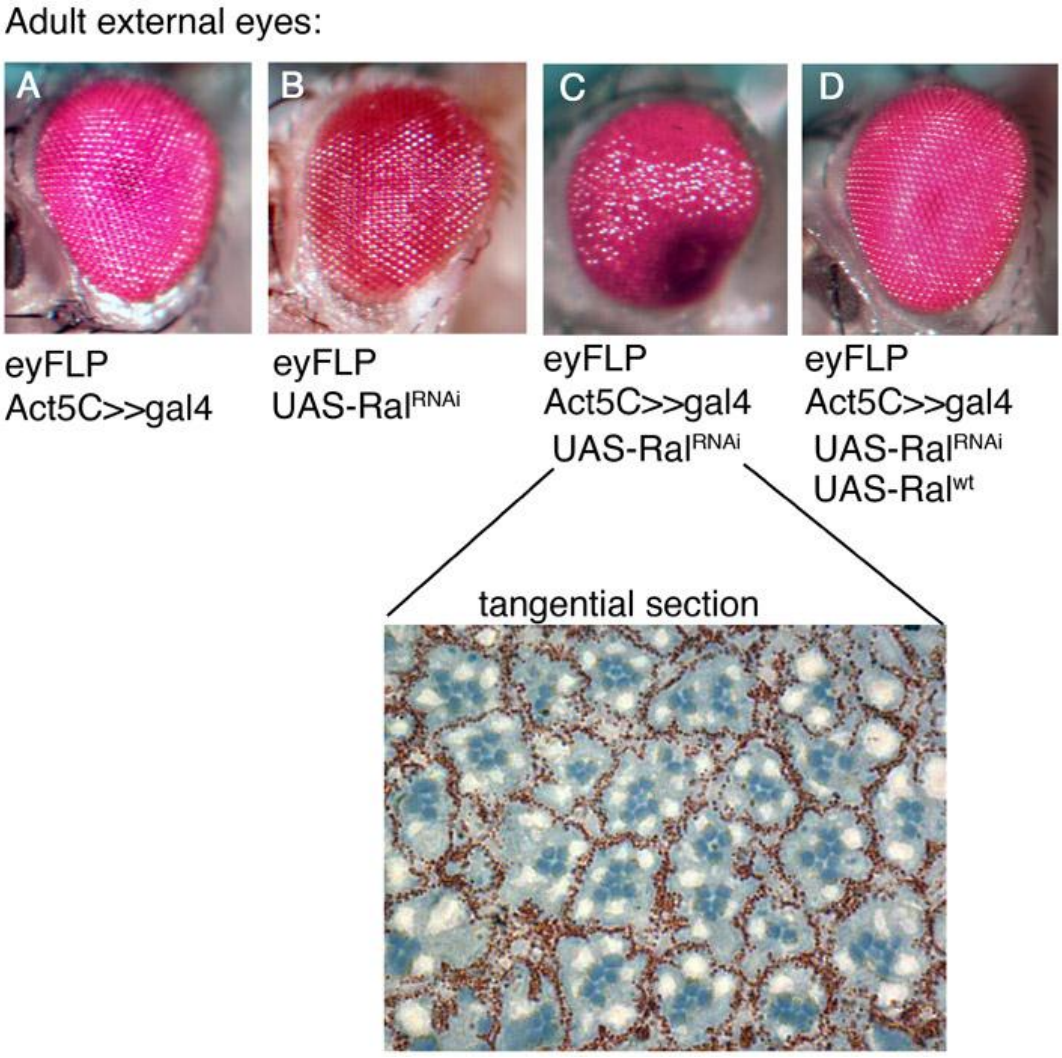


Figure 2-1. **Ral^{RNAi} eyes have defects similar to *Ral^{EE1}* eyes.** (**A**) *eyflp2*/+; *Act5C>stop>gal4, UAS-gfp*/+. (**B**) *eyFLP2*/+; *UAS-Ral^{RNAi}*. (**C**) *eyflp2*/+; *Act5C>stop>gal4, UAS-gfp*/ *UAS-Ral^{RNAi}*. (**D**) *eyflp*/+; *Act5C>stop>gal4, UAS-gfp*/ *UAS-Ral^{RNAi}*; *UAS-Ral*/+.

I analyzed the eyes of Ral^{EE1}/Y flies in detail. Wild-type adult eyes have ~800 facets, or ommatidia, each with eight photoreceptors (R cells) arranged in a trapezoid that is asymmetrical owing to the positions of R3 and R4 (Figure 2-2A,D). In wild-type eyes, the trapezoids are perfectly aligned with one another. There are two chiral forms of ommatidia, mirror-image symmetrical through the equator that divides the eye into dorsal and ventral halves (Figure 2-2A). Adult ommatidia of Ral^{EE1}/Y eyes had a variety of defects, including loss of R cells, loss of R3/R4 asymmetry and defects in orientation with respect to the equator (Figure 2-2B,C). Adult eyes of Ral^{EE1}/Ral^{PG69} and Ral^{EE1}/Ral^{PG89} were similar to Ral^{EE1}/Y. I examined Ral^{EE1}/Y eye discs to determine whether or not the adult eye abnormalities were due to defects in early development. The eye disc is a monolayer epithelium in which ommatidia assemble stepwise posterior to the morphogenetic furrow as it travels across the disc from posterior to anterior (Wolff and Ready, 1993). Five R-cell precursors (R8, R2/5, R3/4) emerge as a pre-cluster, and then R1/6 and R7 are recruited from the remaining pool of undifferentiated cells (Figure 2-2D). Assembling ommatidia normally rotate in mirror-image reflection with respect to the equator (Figure 2-2D). In Ral^{EE1}/Y eye discs, many ommatidia rotate either too much or too little, and R1 or R6 are frequently absent (Figure 2-2E,F). Similar observations were made with Ral^{EE1}/Ral^{PG69} and Ral^{EE1}/Ral^{PG89} eye discs. We conclude that *Ral* is required for patterning early in eye disc development.

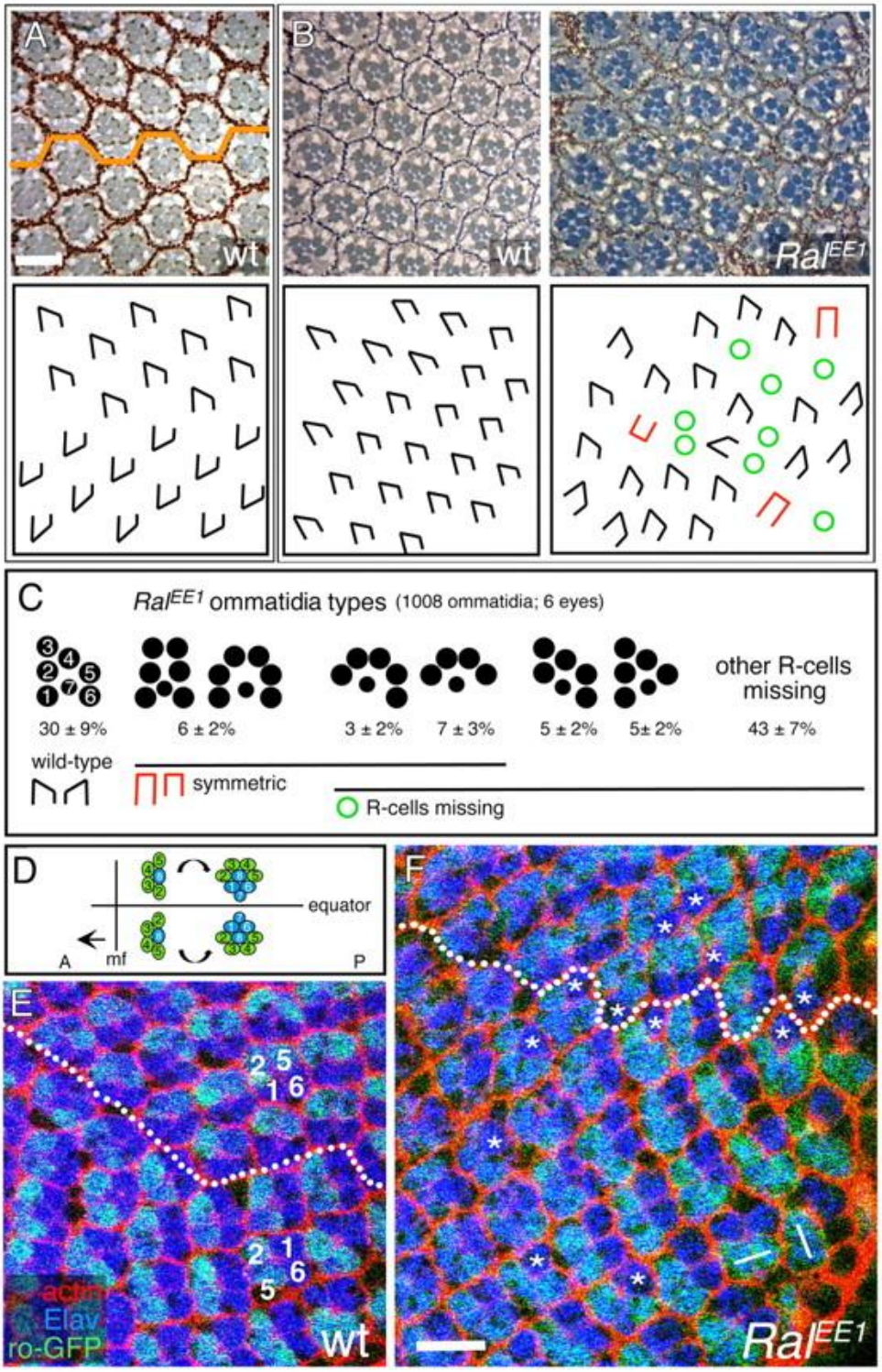


Figure 2-2. *Ral* mutant eye phenotype.

Figure 2-2 continued. (**A**) An apical tangential section through a wild-type adult eye is shown at the top. The orange line marks the equator. The diagram beneath indicates the facet orientations. (**B**) (Top) Sections of wild-type and Ral^{EE1}/Y eyes. The diagrams beneath indicate facet orientations and mutant phenotypes. Symbols are defined in C. (**C**) Quantification of classes of Ral^{EE1}/Y ommatidia. Reverse orientation facets were not scored because in the context of all of the aberrant ommatidia, it was often difficult to locate the equator. (**D**) Diagram of five R-cell pre-clusters in a third instar larval eye disc and rotation with respect to the equator. A, anterior; P, posterior; mf, morphogenetic furrow, moving in the direction of arrow. (**E**,**F**) Third instar larval eye discs expressing GFP in R2/5 and R3/4, and immunolabeled with anti-Elav (R-cell nuclei) and phalloidin (actin). The genotypes are *ro-gfp* (wild-type) and Ral^{EE1}/Y; *ro-gfp*. The dotted line is the equator, and the morphogenetic furrow is leftward. Numbers indicate R2/R5 and R1/R6. Asterisks are ommatidia in which one of the R1/R6 pair are absent. The lines indicate misrotated ommatidia. Scale bar: in A, 20 μm for A,B; in F, 10 μm for E,F.

***Ral* eye defects and genetic interactions suggest that Ral regulates Notch signaling**

Ral^{EE1} was identified in a mutagenesis screen for dominant enhancers of the rough eye caused by epsin overexpression (Eun et al., 2007). Epsin is an endocytic protein required in Notch signaling cells for ligand endocytosis and signaling (Overstreet et al., 2004; Wang and Struhl, 2004; Wang and Struhl, 2005). Loss-of-function alleles of genes acting in Notch signaling both positively and negatively were identified in the screen (Eun et al., 2007). To determine whether *Ral* plays a role in Notch signaling normally, I tested for genetic interactions between *Ral* and *lqf* loss-of-function mutations. Ral^{-} mutations were dominant suppressors of *lqf* hypomorphic eye phenotypes (Figure 2-3A), and the Ral^{EE1} eye phenotype was suppressed by *lqf* (Figure 2-3B). These results imply that *Ral* regulates Notch signaling, and further suggest that *Ral* activity opposes Notch activation.

Two additional observations supported the idea that *Ral* is a negative regulator of Notch signaling. First, N^{5419} (a null allele) was a dominant enhancer of the eye defects caused by expression of constitutively active Ral (Sawamoto et al., 1999) during eye development (*ey-gal4, GMR-gal4; UAS-*Ral^{CA}) (Figure 2-4). Second, the Ral^{EE1} ommatidial defects described above are similar to those observed when there is too much Notch activity. Normal R3/R4 asymmetry (and rotation) results from Notch activation in the R4 precursor by Delta in the R3 precursor (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). Notch activation in both R3/4 precursors results in equivalent symmetric R3/4 cells (Fanto and Mlodzik, 1999; Cooper and Bray, 1999), and overactive Notch in R1/6 precursors results in their failure to differentiate as R cells (Cooper and Bray, 1999). Too little Notch activation early in eye development likewise results in symmetric R3/4 cells (Fanto and Mlodzik, 1999; Cooper and Bray, 1999) and also extra R3/4 cells due to the failure of Notch activation in surplus precluster

cells (Cagan and Ready, 1989; Overstreet et al., 2004). Extra R3/4 cells are not observed in Ral^{EE1} ommatidia (Figure 2-2B), suggesting that in *Ral* mutants, the Notch pathway is generally overactive.

In contrast to the suppressive interactions between Ral^{EE1} and lqf^{-}, we were surprised to find that the Ral^{EE1} eye roughness was dominantly enhanced by loss-of-function mutations in two other Notch pathway genes, *Dl* and *neur* (Figure 2-3B). These results suggest that Ral promotes Notch signaling. Taken together, the genetic interactions led us to conclude that Ral regulates Notch signaling, but in a complex manner. Further experiments described below that illuminated the role of *Ral* in Notch signaling also suggested resolutions to this paradox, and this will be explained below.

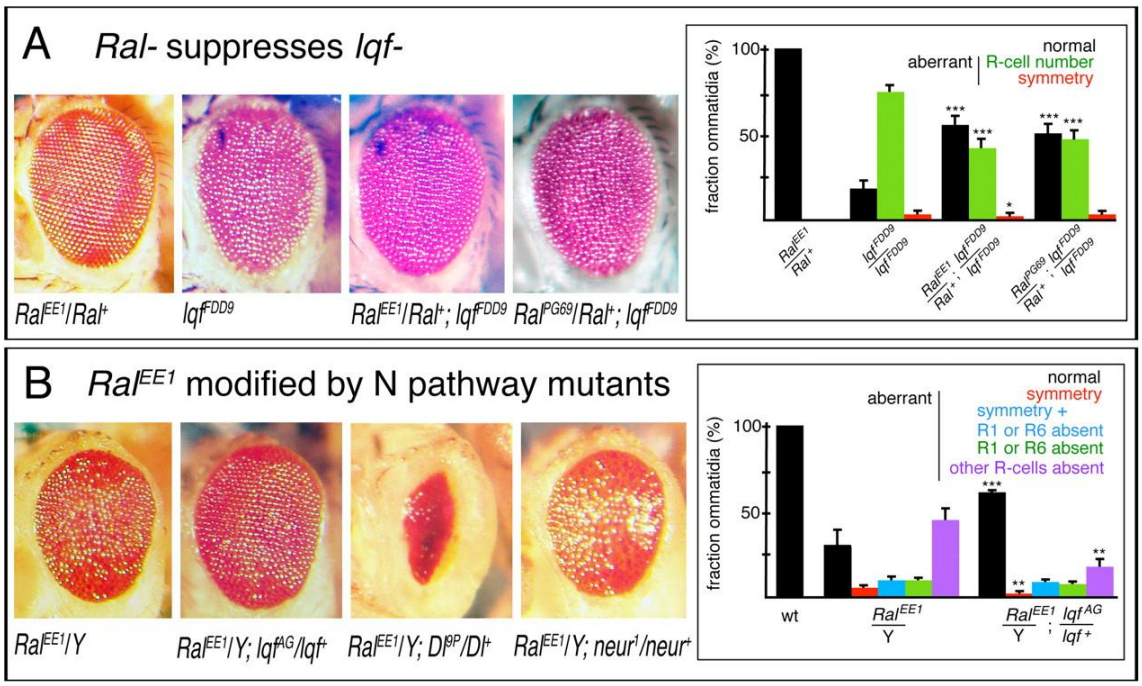


Figure 2-3. Genetic interactions between *Ral* and Notch pathway mutants. (A) External adult eyes of the genotypes indicated are shown. Ral^{-} heterozygous eyes are like wild type (smooth, left), lqf^{FDD9} eyes are rough and although this is not obvious in the photographs, eyes with both mutations are more like wild type than like lqf^{FDD9}. The graph shows quantitative analysis of the wild-type and mutant ommatidia observed in tangential adult eye sections. For each genotype, the data were obtained from five eyes and 600-900 ommatidia. The symmetric ommatidia were scorable only in ommatidia with normal numbers of R cells. ***P<0.0001, *P<0.02; unpaired t-test. Data are mean + s.e.m. **(B)** External adult eyes of the indicated genotypes are shown. Ral^{-} hemizygous eyes (left) are rough. They are made smoother by heterozygous loss-of-function of *lqf*, and rougher by heterozygous loss-of-function of *Dl* or *neur*. The suppressive effect of *lqf* is quantified on the right. Ral^{EE1}/Y data are from Fig. 1 and Ral^{EE1}/Y; lqf^{AG}/lqf^{+} data were obtained from tangential sections of 732 ommatidia in four eyes. ***P<0.0005, **P<0.003; unpaired t-test. Data are mean + s.e.m.

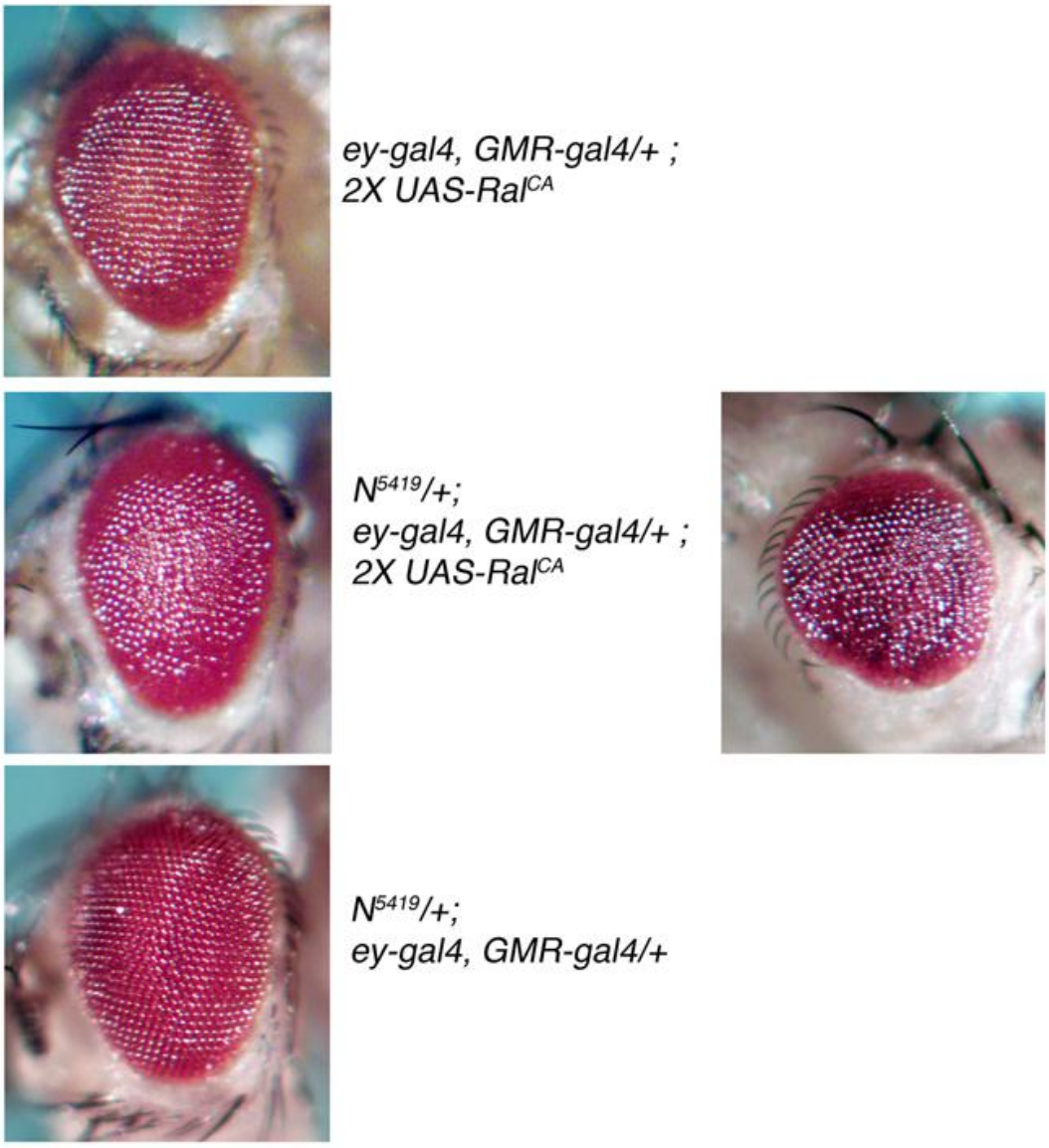


Figure 2-4. N^{-} enhances the rough eye due to expression of Ral^{CA}. Adult external eyes of the genotypes indicated are shown.

Ral prevents ligand-independent Notch activation

To examine further the idea that Ral represses Notch activation, we tested for genetic interactions between Ral^{EE1} and transgenes that overexpress two different forms of the Notch receptor, called N^{+}-GV3 and N^{Sev11}-GV3, under heat-shock control (Struhl and Adachi, 1998) (Figure 2-5A). When Notch is activated, two proteolytic cleavages occur; an ADAM metalloprotease cleaves the extracellular domain, and then Presenilin cleaves within the transmembrane domain to generate a cytoplasmic fragment called N^{icd} (icd, intracellular domain) that travels to the nucleus and derepresses target gene transcription (Bray, 2006). N^{+}-GV3 contains Gal4/VP16 within its N^{icd} fragment (N^{icd}-GV3). N^{+}-GV3 functions in the same way as wild-type Notch in that the transgene complements N^{-} mutations, and also N^{icd}-GV3 activates transcription of *UAS-lacZ* in response to ligand binding (Struhl and Adachi, 1998). N^{Sev11}-GV3 is an altered version of N^{+}-GV3, in which the Notch extracellular and transmembrane domains were replaced by a truncated version of those domains of the Sevenless receptor. N^{Sev11}-GV3 thus cannot bind Notch ligands, and therefore it does not normally activate *UAS-lacZ* (Struhl and Adachi, 1998). I used both forms of the Notch receptor in order to determine whether the interaction between Ral and Notch required the Notch extracellular domain and, thus, ligand binding.

If Ral represses Notch activation, then N^{+}-GV3 overexpression would be expected to enhance the Ral^{EE1} rough eye, and I found that it does (Figure 2-5B). Remarkably, I also found that N^{Sev11}-GV3 overexpression had a similar effect (Figure 2-5B). These results suggest that in Ral^{EE1} cells, both N^{+}-GV3 and N^{Sev11}-GV3 receptors are activated. To test this, I assayed the expression of *UAS-ngfp* in Ral^{+} or Ral^{EE1} larvae that express either N^{+}-GV3 or N^{Sev11}-GV3. I observed little or no GFP in Ral^{+} larvae (Figure 2-5C, parts c,e), but in Ral^{-} larvae, there were high levels of GFP in the midgut

(Figure 2-5C, parts d,f) (see also Figure 2-6). *Ral* expression is reportedly elevated in the larval gut (Tweedie et al., 2009), and Ral is required for intestinal antibacterial immunity (Cronin et al., 2009). Consistent with these reports, I observed high levels of GFP in the midguts of Ral^{PG69}*; UAS-ngfp* larvae (Figure 2-5C, part a). We conclude that Ral blocks ligand-independent activation of Notch.

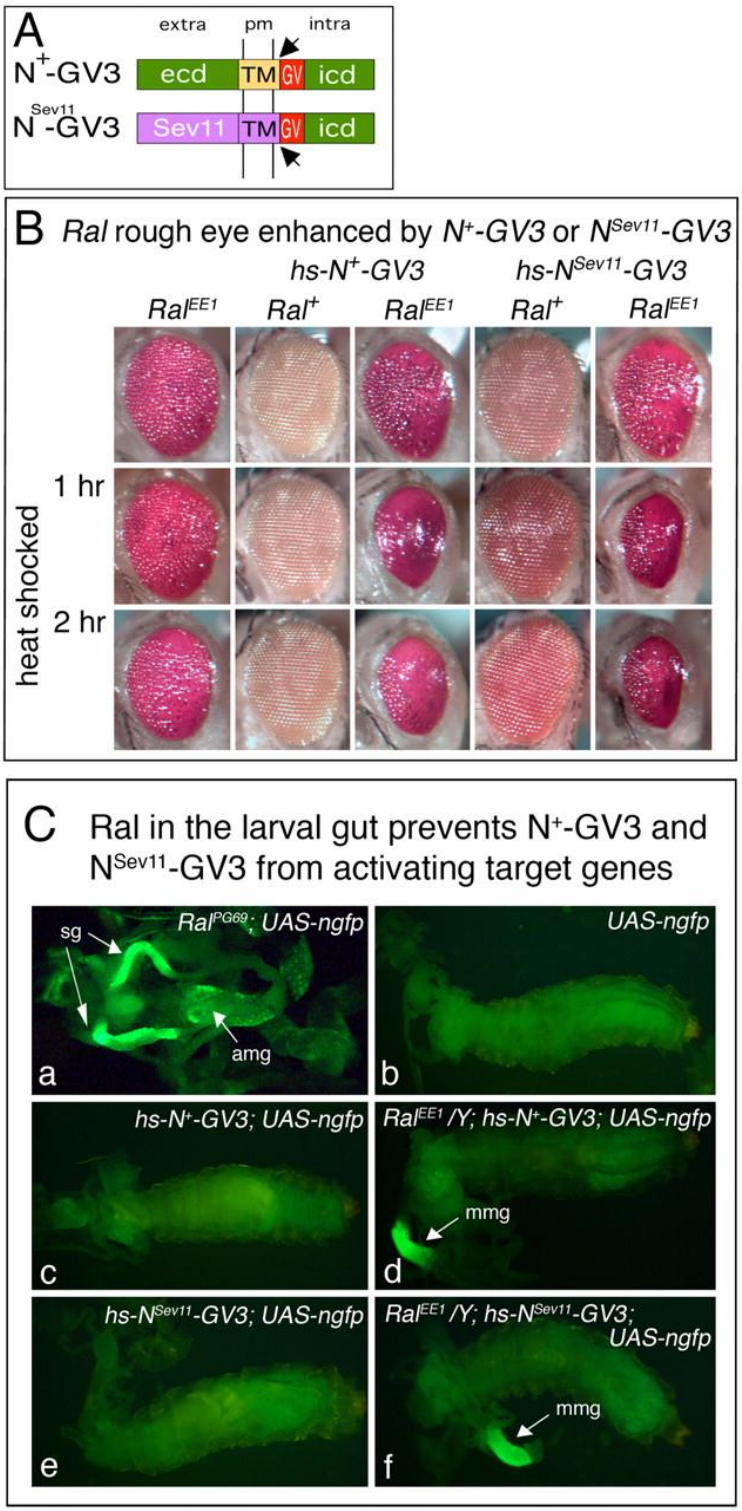
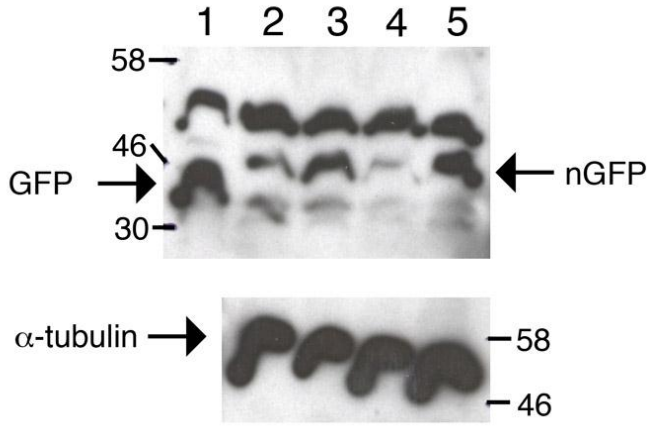


Figure 2-5. Interactions between *Ral*, *N^{+}-GV3* and *N^{Sev11}-GV3*.

Figure 2-5 continued. (A) Diagrams of the protein products of the *hs-N^{+}-GV3* and *hs-N^{Sev11}-GV3* transgenes (Struhl and Adachi 1998). ecd, extracellular domain; icd, intracellular domain; TM, transmembrane domain; pm, plasma membrane; GV, Gal4/VP16; Sev11, truncated extracellular and TM domains of Sevenless receptor; arrow, cleavage site that generates N^{icd}-GV3. **(B)** External eyes of the genotypes indicated at the top are shown. The flies were either not heat-shocked, or else heat-shocked as third instar larvae for 1 or 2 hours at 37^{o}C to express the *hs-N^{+}-GV3* or *hs-N^{Sev11}-GV3* transgene. Neither N^{+}-GV3 nor N^{Sev11}-GV3 causes eye roughness, but each enhances the roughness of *Ral^{EE1}/Y* eyes. **(C)** Dissected third instar larvae with gut extruded from cuticle, photographed to visualize GFP fluorescence. *Ral*-driven GFP expression (part a) is visible in the salivary glands (sg) and anterior midgut (amg), and mainly further posterior in the middle midgut (mmg) in parts d and f. GFP expression was quantified on protein blots.



1: *ubi-gfp/TM6B*
2: *N^{+}-GV3/+; UAS-ngfp/+*
3: *Ral^{EE1}/Y; N^{+}-GV3/+; UAS-ngfp/+*
4: *N^{Sev11}-GV3/+; UAS-ngfp/+*
5: *Ral^{EE1}/Y; N^{Sev11}-GV3/+; UAS-ngfp/+*

Figure 2-6. Quantitation of GFP expression activated by N^{icd}-GV3. Western blots of protein extracts of heat-shocked male larvae of the genotypes indicated, hybridized with anti-GFP (top) or anti-tubulin (bottom) are shown. Size markers (kDa) are as indicated. The unlabeled bands in the top panel are cross-reacting species. There is approximately twice the amount of GFP in lane 3 than in lane 2, and approximately four times the amount of GFP in lane 5 than in lane 4.

Ral activity in R3 promotes R3/R4 asymmetry

To understand the developmental context in which Ral activity is important for eye patterning, I focused on R3/R4 asymmetry, which is affected in Ral^{-} mutants (Figure 2-2B,C). Notch activation in the polar cell (pre-R4) by Delta in the equatorial cell (pre-R3) distinguishes R3 and R4 (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). As Ral activity antagonizes Notch activation, the simple expectation is that Ral is required in the signaling cell, R3. To determine whether there is a requirement for *Ral* in either R3 or R4, I used FLP/FRT-induced mitotic recombination to induce white^{-}-marked (w^{-}) Ral^{EE1} homozygous clones in Ral^{EE1}/Ral^{+} eyes, thereby generating ommatidia mosaic for w^{-} Ral^{EE1} and w^{+} Ral^{+} cells at the clone borders (Figure 2-7A). I scored the phenotypes of ommatidia at the clone borders in which one of the R3/R4 pair was Ral^{+} and one was Ral^{EE1}, and also of ommatidia in which both cells of the R3/4 pair were either Ral^{+} or Ral^{EE1} (Figure 2-7B). When both of the R3/R4 pair were Ral^{+}, all of the facets were asymmetrical and had normal chirality; the equatorial cell always became R3 and the polar cell always became R4. When both were Ral^{EE1}, ~10% of ommatidia were symmetrical, meaning that R3 and R4 were not distinguished. In ommatidia where one of the R3/R4 pair was Ral^{+} and the other Ral^{EE1}, different results were observed depending on whether the equatorial cell or the polar cell was Ral^{+}. When the equatorial cell was Ral^{+} (and the polar cell Ral^{EE1}), all of the ommatidia were wild type. By contrast, when the polar cell was Ral^{+} (and the equatorial cell Ral^{EE1}), ~7% were symmetrical and ~2% had reversed chirality, meaning that the polar cell became R3 and the equatorial cell became R4. These results lead to two conclusions. First and most significantly, Ral^{+} functions in the equatorial cell (pre-R3) to promote asymmetric R3/R4 differentiation. We know this because whenever the equatorial cell was Ral^{+}, R3 and R4 were always properly asymmetrical, but when the equatorial cell was Ral^{-}, symmetrical

R3/R4 cells were sometimes observed. Second, having higher Ral^{+} activity (at least the difference between Ral^{EE1} and Ral^{+}) is insufficient to ensure that an R3/R4 precursor will always become R3. We know this because in ommatidia where the polar cell was Ral^{+} and the equatorial cell was Ral^{EE1}, polar cells did occasionally reverse their normal differentiation and become R3, but more often the R3/R4 pair was either symmetrical or wild type.

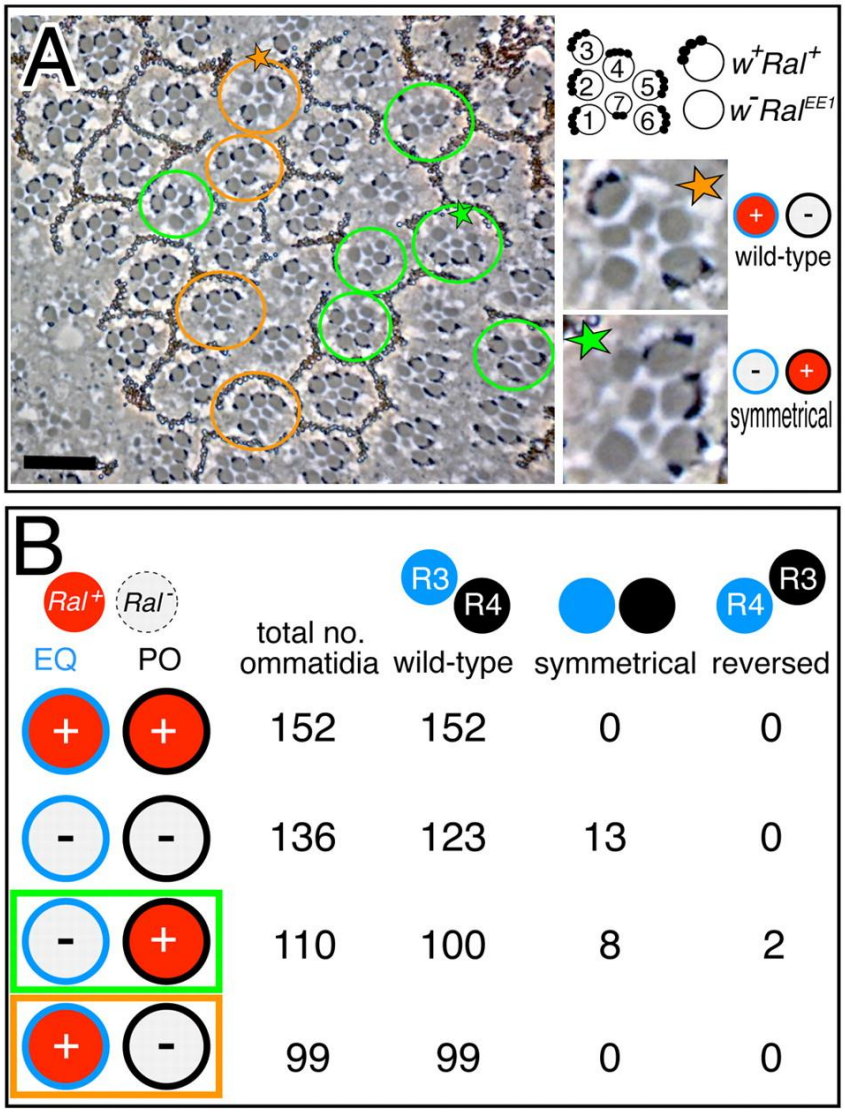


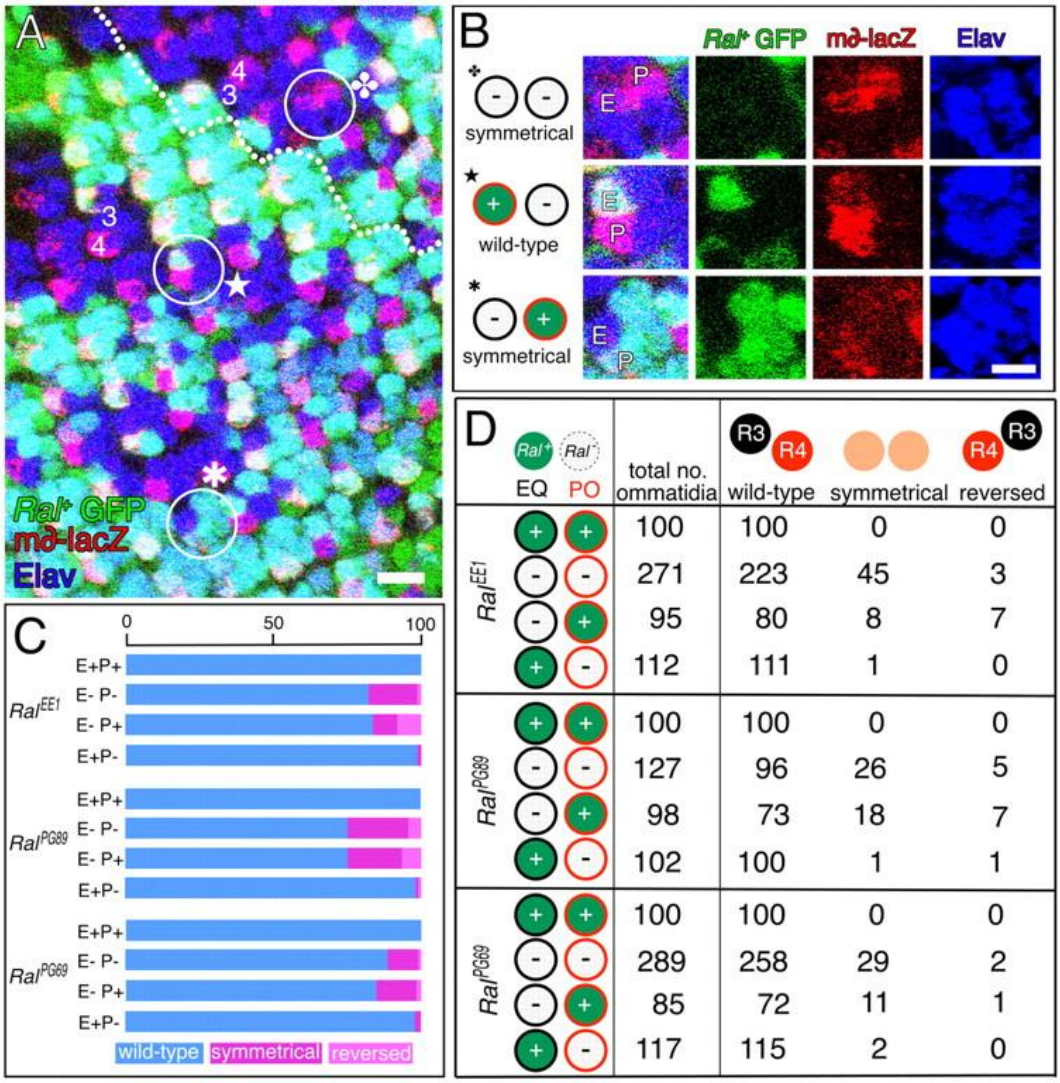
Figure 2-7. Analysis of adult ommatidia where pre-R3/pre-R4 are mosaic for *Ral*$^{+}$ or *Ral*$^{-}$. (**A**) An apical tangential section through a w^{-} *Ral*$^{-}$/w^{-} *Ral*$^{-}$ clone in a w^{-} *Ral*$^{-}$ / w^{+} *Ral*$^{+}$ eye. The genotype is: *w Ral*^{EE1} *FRT19A/FRT19A; eyFLP/+*. Circled ommatidia have mosaic R3/R4 cells; orange are EQ+/PO− (all asymmetrical) and green are EQ−/PO+ (the starred one is symmetrical). Scale bar: 20 μm. (**B**) Pooled results from 13 different eye clones are shown. EQ, equatorial cell (pre-R3); PO, polar cell (pre-R4).

***Ral* activity in R3 promotes asymmetric Notch activation in R4**

I have shown above that Ral^+ activity in R3 influences R3/R4 asymmetry, but does it do so through an effect on Delta/Notch signaling? Notch activation in the R3/R4 pair may be monitored by the expression of a transgene called *m∂-lacZ*, in which the transcriptional control sequences of the Notch target gene *E(spl)m∂* drives expression of *lacZ* (Cooper and Bray, 1999). In wild-type third instar larval eye discs, *m∂-lacZ* is expressed mainly in the polar cell, which becomes R4 (Cooper and Bray, 1999) (Figure 2-8A). To determine whether Ral^+ activity in either the equatorial (pre-R3) or polar cell (pre-R4) affects Notch activation (*m∂-lacZ* expression), I generated developing ommatidia mosaic for gfp^+ Ral^+ and gfp^- Ral^- cells by inducing gfp^- Ral^- clones (Ral^{EE1}, Ral^{PG69} or Ral^{PG89}) in gfp^+ Ral^+/Ral^- eye discs. I scored *m∂-lacZ* expression in mosaic facets in which the polar and equatorial cells were both Ral^+, both Ral^-, or where one of the R3/R4 pair was Ral^- and one was Ral^+ (Figure 2-8B-D). I found that when both the equatorial and polar cells were Ral^+, *m∂-lacZ* was expressed in R4. By contrast, when both cells of the R3/R4 pair were Ral^-, *m∂-lacZ* expression was often symmetrical (absent or at low levels in both cells), or occasionally the pattern of *m∂-lacZ* expression was reversed, meaning that the equatorial cell expressed *m∂-lacZ* and the polar cell did not. These results indicate that Ral^+ activity in the R3/R4 pair does affect Notch activation. In addition, the pattern of *m∂-lacZ* expression was almost always wild-type when the equatorial cell was Ral^+ and the polar cell Ral^-. By contrast, when the equatorial cell was Ral^- and the polar cell Ral^+, *m∂-lacZ* expression was often symmetrical or reversed. We conclude that Ral^+ activity in the equatorial cell (pre-R3) promotes asymmetric Notch activation in the polar cell (pre-R4).

The role of Ral in R3 clarifies how different loss-of-function mutations in genes that promote Delta signaling, *Delta* and *neur* versus *lqf*, can have opposite effects on the

Ral mutant phenotype. *Ral, Delta* and *neur* are all required in the pre-R3 cell, where they bias pre-R3 to become the Delta signaler. Pre-R3 is sensitive to the levels of activity of all three genes, and so the observation that *Delta* or *neur* mutations enhance *Ral* mutations makes sense in this context. Why does *lqf* interact with *Ral* in the opposite way? One possibility is that pre-R4 is more sensitive to Lqf levels than pre-R3 is, and so the major effect of *lqf* mutation is not in pre-R3, but in pre-R4. Unlike *Delta* and *neur, lqf* is not upregulated in pre-R3 (B.C. and J.A.F., unpublished observations). Perhaps the lower levels of Delta and Neur in pre-R4 render pre-R4 more sensitive than pre-R3 to the levels of Lqf. If so, the negative effect of lowering the *lqf* gene dose on the ability of a cell to become the signaler would be more significant in pre-R4 than in pre-R3. In this scenario, Ral^{-} or lqf^{-} mutations would have opposite effects on R3/R4 asymmetry, and would be expected to suppress each other. Alternatively, Lqf might antagonize Ral activity directly in R3 by promoting ligand-independent Notch activation. The latter possibility may be tested with additional experiments. If the role of Ral in other cell fate decisions in the eye is similar to its role in R3/R4, then this kind of logic could explain the effects on overall eye roughness observed in various mutant combinations.



	EQ	PO	total no. ommatidia	wild-type	symmetrical	reversed
Ral^{EE1}	+	+	100	100	0	0
	−	−	271	223	45	3
	−	+	95	80	8	7
	+	−	112	111	1	0
Ral^{PG89}	+	+	100	100	0	0
	−	−	127	96	26	5
	−	+	98	73	18	7
	+	−	102	100	1	1
Ral^{PG69}	+	+	100	100	0	0
	−	−	289	258	29	2
	−	+	85	72	11	1
	+	−	117	115	2	0

Figure 2-8. Analysis of developing larval ommatidia where pre-R3/pre-R4 are mosaic for *Ral*$^{+}$ or *Ral*$^{-}$. (**A**) A Ral^{EE1}/gfp^{+} eye disc with Ral^{EE1} homozygous (gfp^{-}) clones is shown. The disc expresses *mə-lacZ* and is immunolabeled with anti-β-gal (Notch activation) and anti-Elav (R-cell nuclei). The genotype is: Ral^{EE1} *FRT19A/ubi-gfp FRT19A; ey-gal4, UAS-flp/+; mə-lacZ/+*. The broken line is the equator, the furrow is on the left. The numbers are R3/R4 cells. Circled ommatidia are enlarged in B. Scale bar: 10 μm. (**B**) Enlargements of a Ral^{-}/Ral^{-} (E/P) ommatidium, and two kinds of mosaics are shown. E, equatorial cell; P, polar cell. Scale bar: 5 μm. (**C**) Fractions of normally constructed (wild-type) and mutant (symmetrical or reversed) ommatidia in each of the four genotypic classes indicated. The scale at the top is in percent. E, equatorial cell; P, polar cell; + is Ral^{+}; − is Ral^{-}. (**D**) Raw data for graph in C. EQ, equatorial cell; PO, polar cell.

Ral expression is enriched in R3 and depends on Frizzled near the morphogenetic furrow

The results above indicate a requirement for Ral^{+} in the equatorial cell (pre-R3). We were curious to know whether *Ral* expression is ubiquitous or spatially restricted. To monitor *Ral* transcriptional activation, I used Ral^{PG69}, the *gal4*-expressing enhancer trap in the 5′-UTR of *Ral*, driving expression of *UAS-ngfp*. I found that GFP was expressed in all R-cells, but in the majority of ommatidia, GFP was enriched in R3 beginning at approximately row 4 posterior to the furrow (Figure 2-9A). In ~12% of ommatidia, GFP levels were higher in R4 than in R3 (Figure 2-9A), but all of these ommatidia were posterior to row 7. As *m∂-lacZ* expression in R4, which is indicative of R3/R4 specification, normally begins at row 3 or 4 (Fanto and Mlodzik, 1999; Cooper and Bray, 1999), Ral^{PG69} activity is generally elevated in pre-R3 at the time when R3 and R4 are specified. (We expect a delay of about one row in Ral^{PG69} due to the Gal4 intermediate.)

Specification of the equatorial cell as R3 depends on Fz activation (Zheng et al., 1995; Tomlinson and Struhl, 1999; Fanto and Mlodzik, 1999; Cooper and Bray, 1999). In fz^{-} eyes, ommatidia are symmetrical, or their chirality is randomized (wild-type or reversed). Fz activation increases transcription of *Delta* and *neur* (Fanto and Mlodzik, 1999; del Alamo and Mlodzik, 2006) and may also repress Notch receptor activation in the equatorial cell (Strutt et al., 2002). To determine whether Ral^{PG69} activity depends on Fz signaling, I monitored β-galactosidase (β-gal) in fz^{-} clones with Ral^{PG69}*; UAS-nlacZ*. β-Gal expression was reduced in fz^{-} clones, most severely near the morphogenetic furrow, where R3 and R4 are first distinguished (Figure 2-10A, parts a-b′). We also observed non-autonomous repressive effects of fz^{-} clones on *Ral* expression outside of the clones (Figure 2-10A, parts a-b′). Moreover, at the borders of clones, when one cell of a mosaic (fz^{+}/fz^{-}) R3/R4 pair expressed β-galactosidase, it was usually the fz^{+} cell (15/21

pairs in eight clones) (Figure 2-10A, parts b,b′), and the six exceptions were at the posterior of the eye disc.

As expression of *UAS-Ral*^{wt} under *Ral*^{PG69} control complements *Ral*$^{-}$ mutants, the activity of *Ral*^{PG69} observed probably mirrors, at least in part, the normal *Ral* transcription pattern. To test this assumption, I examined Ral protein in eye discs using a polyclonal antibody to human RalB [*Drosophila* Ral and human RalB are identical in 148/201 amino acids, and a different antibody to human Ral B was used to recognize *Drosophila* Ral in ovaries and on protein blots (Balakireva et al., 2006; Ghiglione et al., 2008)]. In wild-type eye discs, the antibody labeled puncta posterior to the furrow (Figure 2-9C, parts a,a′). Although a *Ral* protein null allele to use as a control is unavailable, several experiments lead us to conclude that the antibody recognizes Ral specifically in the eye. First, the antibody signal was strikingly lower in *Ral*^{PG89} homozygous clones than in surrounding heterozygous tissue (Figure 2-9B). Second, in eye discs that overexpress Ral in a subset of R cells (R2/5, R3/4) using *ro-gal4; UAS-Ral*^{wt}, highly elevated signal was detected in R2/5 and R3/4 (Figure 2-9C, parts b,b′). Third, the pattern of antibody labeling resembles closely the pattern of expression of GFP from *Ral*^{PG69}*; UAS-ngfp*. Ral antibody signal begins posterior to the furrow in approximately row 3 or 4 (Figure 2-9C, parts a), and appears generally elevated in R3 (Figure 2-9D). In wild-type eye discs, or in eye discs that overexpress *Ral* using *Ral*^{PG69}*; UAS-Ral*^{wt}, Ral protein is in basal puncta (Figure 2-9C, parts a′,a″), that in most ommatidia appear concentrated in R3 (Figure 2-9D and legend).

The results above indicate that near the morphogenetic furrow, Ral expression is controlled by Fz. I tested this idea further by overexpressing *fz* in the R3/R4 pair using a *sevenless* expression vector construct, *sev-fz*. Expression of *sev-fz* results in R3/4 symmetry or random chirality, because the equalization of and/or excess of Fz activity in

the R3/4 pair disrupts asymmetric Notch activation in the polar cell (pre-R4) (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). I found that the normal pattern of *Ral*^{PG69}*; UAS-ngfp* expression was disrupted in eye discs expressing *sev-fz* (Figure 2-10B,C). *Ral*^{PG69}*; UAS-ngfp* expression was enriched in the equatorial cell (pre-R3) less often than in wild-type discs, and enriched in the polar cell (pre-R4) more often than in wild type (Figure 2-10C). We conclude that *Ral* transcriptional control is downstream of Fz.

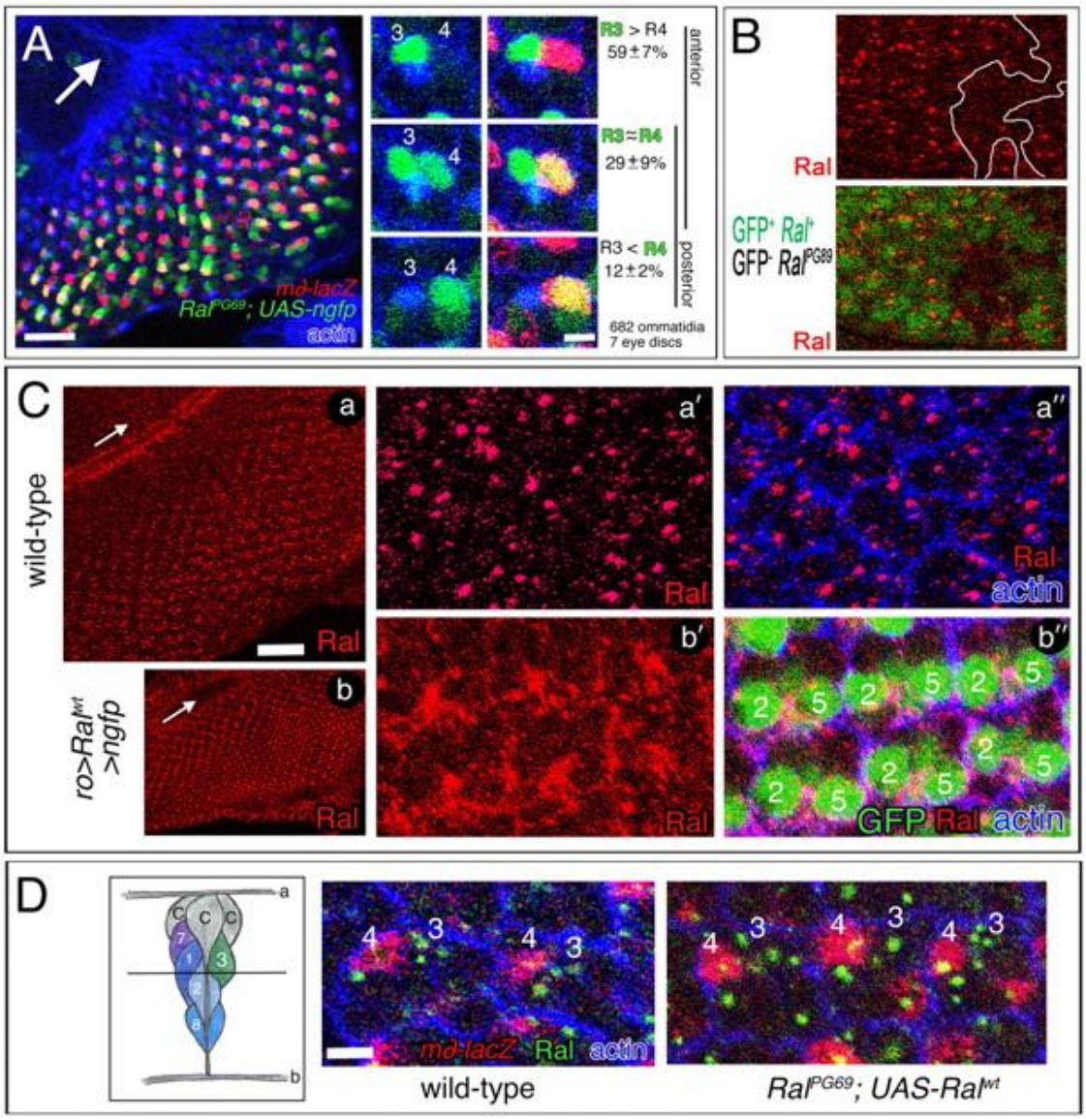
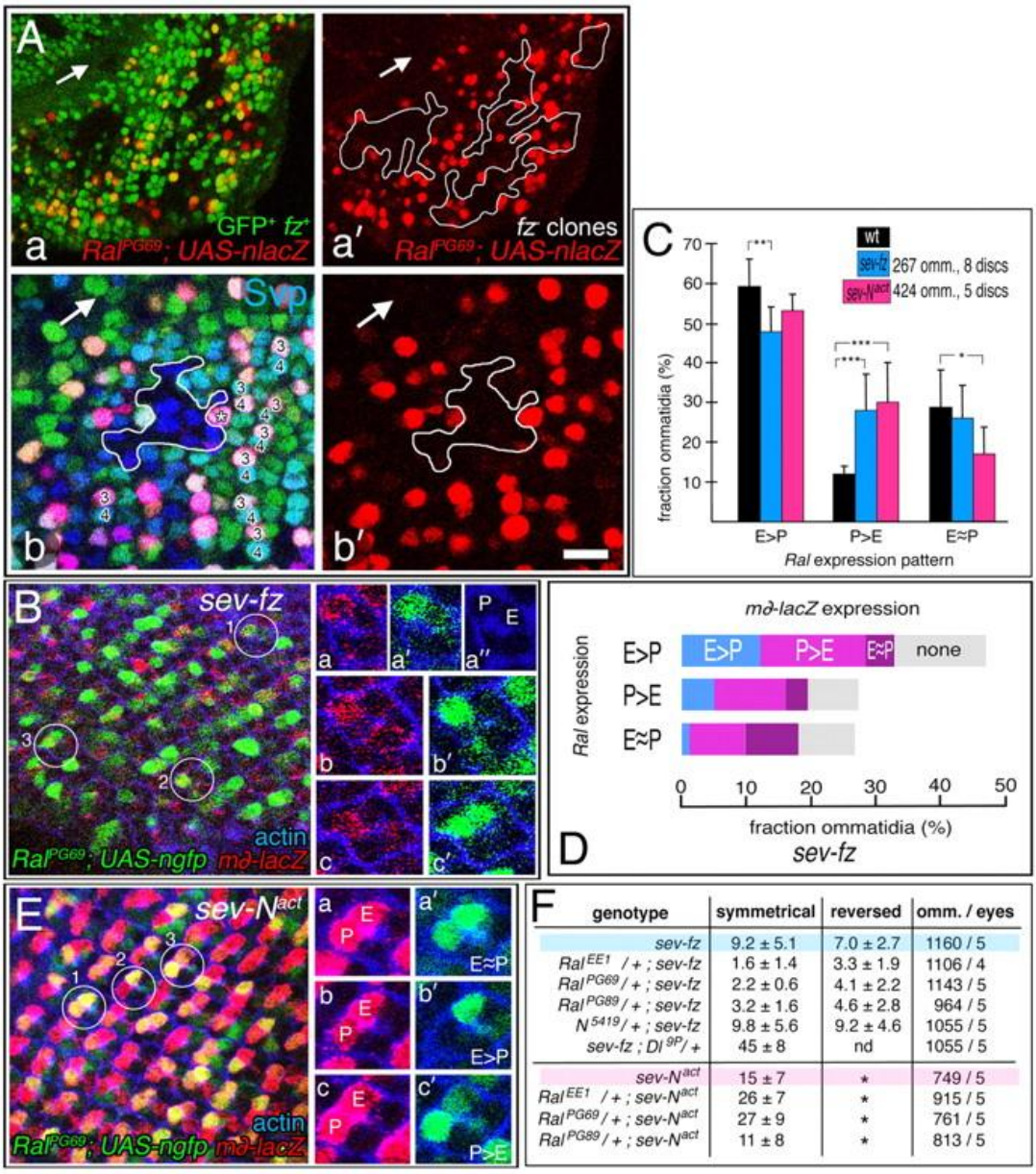


Figure 2-9. Ral expression in eye discs.

Figure 2-9 continued. (**A**) An eye disc is shown on the left that expresses *m∂-lacZ* and *ngfp* under the control of a *Ral* enhancer trap, and immunolabeled with anti-β-gal and phalloidin. The genotype is: Ral^{PG69}*/+; m∂-lacZ/UAS-ngfp.* Enlargements of the three different classes of ommatidia indicated are on the right. Scale bars: in the large panel and in B, 20 μm; in the small panels, 5 μm. Arrow indicates morphogenetic furrow. (**B**) A Ral^{PG89}/Ral^{+} eye disc containing a clone of Ral^{PG89}/Ral^{PG89} cells (outlined at top), immunolabeled with anti-Ral. The genotype is Ral^{PG89} *FRT19A/ubi-gfp FRT19A; ey-gal4, UAS-flp/+*. (**C**) Wild-type eye discs and eye discs that overexpress *Ral* and *ngfp* in R2/R5 and R3/R4 under the control of *ro-gal4* are shown. The genotype is: *ro-gal4/+; UAS-Ral/UAS-ngfp*. The discs are immunolabeled with anti-Ral and phalloidin. Scale bar: 50 μm in a,b; 5 μm in a′,a″,b′,b″. Arrows indicate morphogenetic furrow. (**D**) A *z*-section of a developing ommatidium. A, apical membrane; b, basal membrane; c, cone cell; numbers are R-cells. The horizontal line represents the depth of the *xy* images on the right. A Ral^{+} (wild-type) eye disc that expresses *m∂-lacZ* and an eye disc that also overexpresses *Ral* under control of a *Ral* enhancer trap (Ral^{PG69}*; UAS-Ral*) are shown. The genotypes are: *m∂-lacZ/+* (wild type) and Ral^{PG69}*/+; UAS-Ral/m∂-lacZ*. Each is immunolabeled with anti-β-gal, anti-Ral and phalloidin. The numbers are R3 and R4. We counted the number of R3/R4 pairs in which there were more Ral^{+} puncta in R3 (R3>R4), where the numbers were similar (R3~R4), and where there were more in R4 (R4>R3) in wild-type and Ral^{PG69}*; UAS-Ral* eye discs. In five wild-type discs: R3>R4 (211/290), R3~R4 (31/290), R4>R3 (48/290). In six Ral^{PG69}*; UAS-Ral* discs: R3>R4 (161/219), R3~R4 (21/219), R4>R3 (37/219). Scale bar: 5 μm.

Frizzled control of asymmetric Ral expression is not through Notch

Enrichment of *Ral* transcription in R3 versus R4 near the furrow could be a direct effect of Fz signaling, and a reflection of more Fz signaling in R3 than R4. Alternatively, asymmetric *Ral* expression could be downstream of Notch activation. If so, *Ral* enrichment in R3 could reflect that Notch activation in R4 represses *Ral* transcription directly in R4 and/or that Notch activation in R4 activates *Ral* transcription in R3 through a feedback mechanism. One observation suggests that *Ral* expression could be controlled by Notch; in *sev-fz* discs, *m∂-lacZ* (Notch activation) was generally depressed (Figure 2-10B), and *Ral* had an increased tendency to be expressed in the polar cell (pre-R4) (Figure 2-10D). However, we observed further that in *sev-fz* discs, *m∂-lacZ* and Ral^{PG69}*; UAS-ngfp* were often expressed in the same cell (Figure 2-10D). Moreover, there was no tendency for cells that express *m∂-lacZ* not to express Ral^{PG69}*; UAS-ngfp* (Figure 2-10D). I also generated discs in which the R3/R4 pair both express constitutively active Notch (*sev-*N^{act}), which renders them symmetrical (Fanto and Mlodzik, 1999; Cooper and Bray, 1999). Although the pattern of Ral^{PG69}*; UAS-ngfp* is disrupted somewhat, *Ral* expression is not depressed (Figure 2-10E), and *Ral* has a greater tendency than in wild type to be expressed asymmetrically (Figure 2-10C). Finally, I find that Ral^{-} mutations suppress *sev-fz* defects, but fail to suppress (and in fact enhance) *sev-*N^{act} defects (Figure 2-10F). This means that Fz in the R3/4 pair requires Ral even when Notch signaling is depressed and the R3/4 pairs are symmetrical, but Notch does not. We conclude that Fz activation controls *Ral* transcription directly, not through Notch activation.



genotype	symmetrical	reversed	omm. / eyes
sev-fz	9.2 ± 5.1	7.0 ± 2.7	1160 / 5
Ral^{EE1} / + ; *sev-fz*	1.6 ± 1.4	3.3 ± 1.9	1106 / 4
Ral^{PG69} / + ; *sev-fz*	2.2 ± 0.6	4.1 ± 2.2	1143 / 5
Ral^{PG89} / + ; *sev-fz*	3.2 ± 1.6	4.6 ± 2.8	964 / 5
N^{5419} / + ; *sev-fz*	9.8 ± 5.6	9.2 ± 4.6	1055 / 5
sev-fz ; Dl^{9P} / +	45 ± 8	nd	1055 / 5
sev-N^{act}	15 ± 7	*	749 / 5
Ral^{EE1} / + ; *sev-N*^{act}	26 ± 7	*	915 / 5
Ral^{PG69} / + ; *sev-N*^{act}	27 ± 9	*	761 / 5
Ral^{PG89} / + ; *sev-N*^{act}	11 ± 8	*	813 / 5

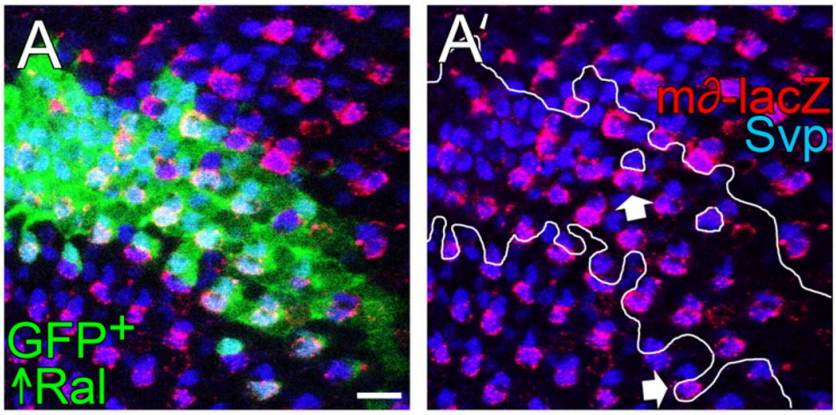
Figure 2-10. Control of *Ral* expression by Fz and Notch.

Figure 2-10 continued. (**A**) Eye discs are shown containing fz^{-} gfp^{-} clones (white outlines). The discs express nuclear β-gal under *Ral* control and were immunolabeled with anti-β-gal and anti-Svp (labels R3 and R4). The genotype is: Ral^{PG69}*/eyFLP; UAS-nlacZ/+;* fz^{P21} $fz2^{C1}$ *FRT2A/ubi-gfp FRT2A.* Arrows indicate the furrow. The disc in a,a′ contains several clones. In b,b′, a single clone from a different disc is shown enlarged. R3 and R4 are indicated. The asterisk indicates a fz^{+} R4 (β-gal^{+}) of a mosaic pair where the R3 is fz^{-} (β-gal^{-}). (**B**) An eye disc that expresses *sev-fz*, *m∂-lacZ* and *ngfp* under *Ral* control, immunolabeled with anti-β-gal and phalloidin, is shown. The genotype is: Ral^{PG69}*/+; sev-fz/+; m∂-lacZ/UAS-ngfp.* Enlargements of the three circled ommatidia are on the right. The furrow is on the right. E, equatorial cell; P, polar cell. In a and b, *Ral* expression is *P*>E, and in c, it is E~P. (**C**) Quantification of the three types of *Ral* expression patterns in ommatidia in *sev-fz* or $sev\text{-}N^{act}$ discs. ***P<0.001, **P<0.01, *P<0.04. Data are mean + s.e.m. (**D**) Quantification of the four types of *m∂-lacZ* expression pattern within each of the three types of *Ral* expression pattern in the *sev-fz* discs in C. (**E**) An eye disc is shown that expresses $sev\text{-}N^{act}$, *m∂-lacZ* and *ngfp* under *Ral* control, immunolabeled with anti-β-gal and phalloidin. Circled ommatidia representing each of the three classes of *Ral* expression pattern are enlarged on the right. The furrow is on the left. The genotype is: Ral^{PG69}*/+;* $sev\text{-}N^{act}$*/+; m∂-lacZ/UAS-ngfp*. (**F**) A table showing quantification of dominant genetic interactions between *Ral* mutants, and also null alleles of *Notch* and *Delta*, with *sev-fz*, and *Ral* mutants with $sev\text{-}N^{act}$. Data were obtained from sections of adult eyes. omm., number of ommatidia; nd, not determined; *, reversed ommatidia could not be scored because the eye field was too disorganized. Scale bar: 40 μm in A, parts a,a′; 20 μm in A, parts b,b′, and in B,E; 10 μm in the enlargements in B,E.

Ral-mediated Notch inhibition is one of several Fz-dependent pathways that control R3/R4 asymmetry

The effects on R3/R4 asymmetry of losing *Ral* in one or both cells are small; symmetry defects were observed in these experiments in 10-30% of the R3/R4 pairs (Figure 2-2C; Figure 2-7; Figure 2-8). In contrast to the results with *Ral* (Figure 2-7), in ommatidia where R3/R4 are mosaic for *Delta*, the $Delta^{+}$ cell always becomes R3 (Tomlinson and Struhl, 1999). The small effects observed for *Ral* could result, at least in part, from the Ral^{+} function remaining in the three hypomorphic mutants we used. However, similar weak effects were observed in analogous experiments using a strong *neur* loss-of-function allele (del Alamo and Mlodzik, 2006). This suggests that Ral^{EE1} may have an incompletely penetrant effect on R3/R4 asymmetry primarily because Ral works in only one or a subset of distinct Fz-dependent pathways that bias the pre-R3 cell to become the Delta signaler. Consistent with this interpretation, we find that overexpression of *Ral* (or Ral^{CA}, not shown) in both R3 and R4 in clones of otherwise wild-type eye disc cells had no effect on the pattern of Notch activation; *m∂-lacZ* is still expressed specifically in the polar cell (pre-R4) (Figure 2-11A,A′). Even in mosaic R3/R4 pairs at the clone border in which Ral^{CA} is overexpressed in pre-R4 and not in pre-R3, *m∂-lacZ* was nearly always expressed in the polar cell (Figure 2-11B). Similarly, *Ral* or Ral^{CA} overexpression in both R3 and R4 with *ro-gal4; 2XUAS-Ral* had only a very subtle effect on R3/4 asymmetry in the adult eye (Table 1). If *Ral* functioned in the sole pathway or in all Fz-dependent pathways for R3 specification, we would expect reversal of the normal *Ral* expression pattern in mosaics to reverse R3/R4 polarity, and equalization of *Ral* expression in R3 and R4 to result in R3/R4 symmetry. These effects were observed but they were very subtle. Strikingly similar results were obtained in analogous experiments with *neur* (del Alamo and Mlodzik, 2006). Moreover, we find that

in *fz*$^{-}$ cell clones, where all the pathways downstream of Fz that normally bias R3 to become the signaling cell are absent, Ral overexpression now does determine R3 cell fate. In clones of *fz*$^{-}$ cells in the eye disc, both cells of the R3/R4 pair express *m∂-lacZ*, at reduced levels compared with wild-type R4 (the cells are both R4 or some fate in between R3 and R4) (Figure 2-12A-B′). *Ral* overexpression in *fz*$^{-}$ R3/R4 precursor pairs abolished *m∂-lacZ* expression (both cells are now R3) (Figure 2-12C-D″). We conclude that Ral functions in one of several Fz-dependent pathways that control R3/R4 asymmetry.



EQ pre-R3 / PO pre-R4	EQ PO wild-type	EQ PO / EQ PO symmetrical		EQ PO reversed
○ ○	25	0	0	0
● ○	17	0	1	0
○ ●	21	3	0	1
● ●	19	4	0	2

B ● GFP+RalCA+ ● β-gal+

Figure 2-11. *Ral* overexpression in pre-R4 has only a subtle effect on R3/R4 determination. (**A**,**A′**) An eye disc is shown containing a *gfp*$^{+}$ cell clone (outlined in A′) that overexpresses *Ral*. The eye disc also expresses *mδ-lacZ* and is immunolabeled with anti-Svp and anti-β-gal. The arrows indicate *Ral*-overexpressing (*gfp*$^{+}$) R4s (β-gal+), the R3s of which (β-gal^{-}) do not overexpress *Ral* (*gfp*$^{-}$). The genotype is *hs-flp, tub-gal4, UAS-gfp/+; UAS-Ral*^{wt}*/mδ-lacZ; FRT82B/FRT82B tub-gal80.* Larvae (2nd and 3rd instar) were heat-shocked for 1 hour at 37°C. (**B**) An analysis of R3/R4 determination in pairs mosaic for wild-type and *Ral*^{CA}-overexpressing cells. Pooled results from six mosaic eye discs are shown. EQ, equatorial cell; PO, polar cell. The genotype is *hs-flp; Act5C>stoP>gal4, UAS-gfp/+; UAS-Ral*^{CA}*/mδ-lacZ.* Scale bar: 10 μm.

Table 1. Subtle effects of Ral or Ral^{CA} overexpression in R4

Ommatidial phenotypes (five eyes)				
Genotype		Number symmetrical	Number reversed	Number wild type
(R3>R4)	*Ral^{PG69}; 1XUAS-Ral*	3	0	1089
	Ral^{PG69}; 2XUAS-Ral	4	0	1144
(R3~R4)	*ro-gal4; 1XUAS-Ral*	4	0	1037
	ro-gal4; 2XUAS-Ral	33	3	1073
(R3>R4)	*Ral^{PG69}; 1XUAS-Ral^{CA}*	*Lethal		
(R3~R4)	*ro-gal4; 1XUAS-Ral^{CA}*	2	0	1077
	ro-gal4; 2XUAS-Ral^{CA}	32	6	1006

*Overexpression of *Ral^{CA}* outside of the eye with *Ral^{PG69}* is lethal. Expression of *Ral^{CA}* with *ro-gal4* is eye specific, and thus viable flies are obtained.

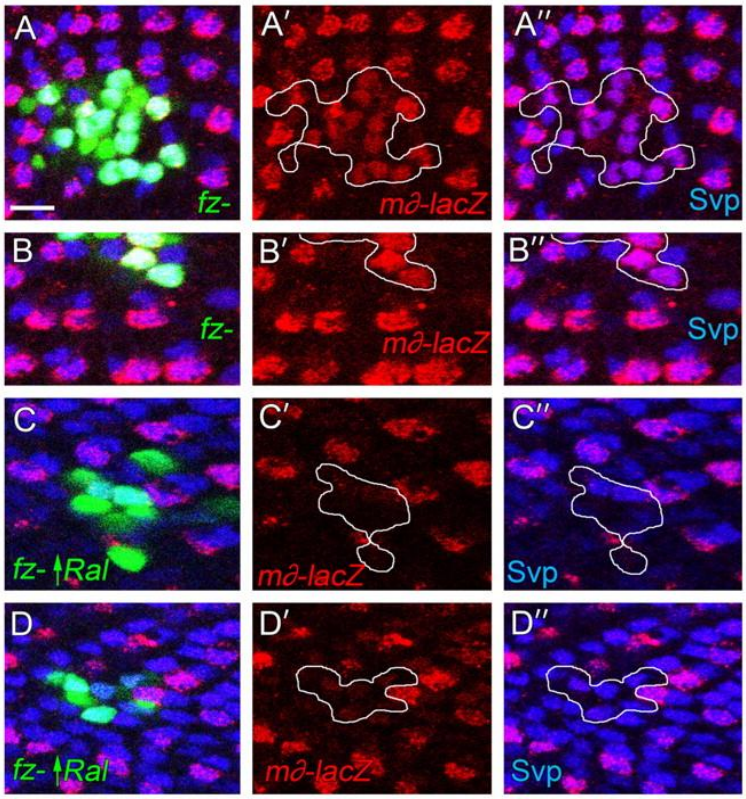


Figure 2-12. *Ral* controls R3/R4 cell fate in *fz*$^{-}$ cells. (**A-B″**) Two separate *gfp*$^{+}$ *fz*$^{-}$ clones (outlined) in an eye disc are shown in A-A″ and B-B″. The eye disc also expresses *mδ-lacZ* and is immunolabeled with anti-β-gal and anti-Svp. The genotype is *hs-flp tub-gal4, UAS-gfp/+; mδ-lacZ/+; fz^{P21} fz2^{C1} FRT2A/tub-gal80 FRT2A*. (**C-D″**) Two separate *gfp*$^{+}$ *fz*$^{-}$ clones that overexpress *Ral* (outlined) in eye discs are shown in C-C″ and D-D″. The eye disc also expresses *mδ-lacZ*, and is immunolabeled with anti-β-gal and anti-Svp. The genotype is *hs-flp tub-gal4, UAS-gfp/+; mδ-lacZ/UAS-Ral^{wt}; fz^{P21} fz2^{C1} FRT2A/tub-gal80 FRT2A*. Scale bar: 10 μm. To generate clones, larvae (2nd and 3rd instar) were heat-shocked for 1 hour at 37°C.

DISCUSSION

The results presented support the model for Ral function shown in Figure 2-13. *Ral* transcription is upregulated in response to Fz activation. As Fz is activated more in the equatorial cell than the polar cell, Ral is enriched in the equatorial cell. Ral activity represses ligand-independent Notch activation, and thus biases the equatorial cell to become R3. One way in which ligand-independent Notch activation occurs is an accident when normal Notch trafficking is disrupted (Fortini and Bilder, 2009; Fortini, 2009). Notch receptor undergoes endocytosis and endosomal trafficking continually and mutations that block trafficking of late endosomes to the lysosome block Notch degradation and result in endosomal accumulation of Notch and ligand-independent activation (Fortini and Bilder, 2009; Fortini, 2009). One possibility is that the endosomal environment promotes production of N^{icd} by Presenilin cleavage. Ligand-independent Notch activation may also occur normally in the lysosomal membrane (Wilkin et al., 2008; Fortini and Bilder, 2009). Ral GTPase activity might block ligand-independent Notch activation by regulating Notch trafficking to the lysosome, or by inhibiting another process, such as endosomal acidification (Yan et al., 2009), N^{icd} production or nuclear translocation. The punctate appearance of Ral protein suggests the possibility that Ral may play a role in endosomal trafficking. Although further experiments are required to determine the precise mechanism of Ral function, I have shown that Ral, a protein that prevents ligand-independent Notch activation, is a target for regulation during pattern formation. Fz/PCP signaling upregulates Ral expression to ensure that ligand-independent Notch activation does not tip the scales in favor of pre-R3 becoming the signal receiving cell. Moreover, I have shown that prevention of ligand-independent

Notch activation is not simply a constitutive process, but one that is modulated to ensure specific developmental outcomes.

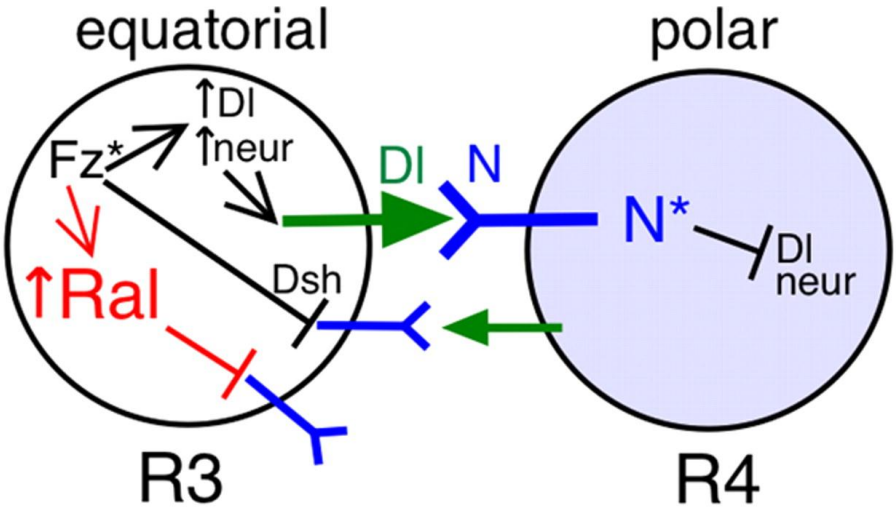


Figure 2-13. Model for Ral function in R3/R4 cell fate decision. Fz activation in the equatorial cell results in asymmetric Notch activation through two proposed mechanisms: (1) promotion of Delta signaling through transcriptional upregulation of *Delta* and *neur*, and their repression in the polar cell; and (2) direct repression of the Notch receptor through relocalization of a Fz/Dsh complex to the side of the equatorial cell plasma membrane adjacent to the polar cell. We have presented evidence for a distinct Ral-dependent mechanism in the equatorial cell. *Ral* transcription is upregulated in response to Fz activation, and Ral activity represses ligand-independent Notch activation. Notch activation in R4 does not repress *Ral* transcription in the polar cell.

MATERIALS AND METHODS

Drosophila strains

The following alleles were used in this work. FlyBase id numbers, when available, are in parentheses. Chromosomes used are indicated in the figure legends. Ral^{EE1} (FBal0197295); Ral^{PG69} (FBal0130802); Ral^{PG89} (FBal0130801); fz^{P21} (FBal0004937); $fz2^{C1}$ (FBal0102708); faf^{FO8} (FBal0031258); $neur^{1}$ (FBal0012940); Dl^{9P} (FBal0002474); N^{5419} (FBab0000564); lqf^{FDD9} (FBal0104483); lqf^{AG} (FBal0104486); *Act5C-gal4* (FBti0012293); *ey-gal4* (FBti0012711); *ro*-gal4 (E. Overstreet, PhD thesis, University of Texas at Austin, 2005); *GMR-gal4* (FBti0072862); *Act>stop>gal4* (from N.-S. Moon, McGill University, Quebec, Canada); *tub-gal4* (from G. Struhl, Columbia University, New York, USA); *tub-gal80* (FBti0012693, FBti0012683), UAS-*flp* (FBti0012285); $UAS\text{-}Ral^{wt}$ (FBal0101574); $UAS\text{-}Ral^{CA}$ (FBal0101576); $UAS\text{-}Ral^{RNAi}$ (VDRC# 105296); *UAS-ngfp* (on X, FBti0012492, FBti0012493); *UAS-nlacZ* (FBtp0001611); *ubi-ngfp* (FBti0015575, FBti0016102); *sev-fz* (FBal0082914); *m∂-lacZ* (FBtp0010977); *ro-gfp* (Overstreet et al., 2004); $hs\text{-}N^{+}\text{-}GV3$ (FBal0090683); $hs\text{-}N^{Sev11}\text{-}GV3$ (Struhl and Adachi, 1998); *FRT19A* (FBti0000870); *FRT82B* (FBti0002074); *FRT2A* (FBti0002046); *eyFLP* (FBti0015984, FBti0015982); *eyFLP2* (from B. Dickson, IMP, Vienna, Austria); *hs-FLP* (FBti0002044); and *FM7, gfp* (FBst0005193).

ro-gal4

gal4 DNA sequences were amplified from *GMR-gal4* flies using primers that inserted *Asc*I sites upstream of the start codon and downstream of the stop codon: 5′-ggcgcgccATGAAGCTACTGTCTTCTATCG-3′ and 5′-ggcgcgccTTACTCTTTTTTTGGGTTTGG-3′. The 2.7 kb amplification product was

ligated into pGEM (Stratagene, Santa Clara, CA, USA), its sequence verified, and the *Asc*I restriction fragment was ligated into the *Asc*I site of pRO (Huang and Fischer-Vize, 1996) (E. Overstreet, PhD thesis, University of Texas at Austin, 2005). The resulting plasmid was used to transform w^{1118} flies.

Imaging of eyes and larvae

Immunohistochemistry of third instar larval eye discs was performed as follows. Discs were fixed in PEMS buffer with 1.0% NP-40 for 15 minutes. Antibody treatment was as described previously (Lim et al., 2007) with modifications. Fixed eye discs were blocked for 2 hours 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 minutes, and incubated with secondary antibodies in washing solution for 2 hours at room temperature, and then washed three times for 5 minutes. Phalloidin was used as described previously (Chen et al., 2002). Eye discs were mounted in Vectashield (Vector, Burlingame, CA, USA) and viewed with a Leica TCSSP2 confocal microscope. Primary antibodies were: rat monoclonal anti-Elav supernatant at 1:2 [Developmental Studies Hybridoma Bank (DSHB)], mouse monoclonal anti-β-galactosidase at 1:50 from the DSHB, rabbit polyclonal anti-RalB (Proteintech Group, Chicago, IL, USA) at 1:100, rabbit anti-Svp at 1:100. Secondary antibodies (Molecular Probes, Carlsbad, CA, USA) used at 1:200 were: Alexa^{568}-anti-rabbit, Alexa^{568}-anti-mouse, Alexa^{488}-anti-rabbit, Alexa^{633}-anti-rat, Cy5-anti-rabbit. Plastic sectioning of adult eyes was as described (Tomlinson and Ready, 1987). Eye sections were photographed with a Zeiss Axioplan and Axiocam HRc. Adult external eyes were photographed with an Olympus SZX12 microscope equipped with a SPOT

idea (Diagnostic Instruments, Sterling Heights, MI, USA) digital camera. Larvae expressing GFP were viewed with a Leica M165FC microscope and photographed with a Leica DFC420C camera. Images were processed with Adobe Photoshop CS3. Statistical analysis of eye sections was using the unpaired *t*-test, Prism 3.0 software.

GFP protein blot

For quantitation of GFP (see Fig. S3 in the supplementary material), animals were grown at 29°C, heat shocked as third instar larvae for 1 hour at 37°C, and allowed to recover for 2 hours at 29°C. To generate protein extracts, five males were homogenized in 100 µl ice-cold PBS and 100 µl of 2× loading buffer was added. The extract was boiled for 10 minutes and then microfuged for 10 minutes. Supernatants (13 µl) were subjected to 10% SDS-PAGE and western blotted. Primary antibodies were rabbit anti-GFP (ABchem) at 1:5000 and mAbE7 (DSHB) at 1:1000. Secondary antibodies were HRP-anti-mouse and HRP-anti-rabbit (Santa Cruz Biochem) at 1:2000. Signals were quantified with NIH Image J software.

Chapter 3: *Dissecting Ral GTPase pathways for determining Ral-mediated Notch regulation pathway*

INTRODUCTION

I have shown that Ral is required in R3 for asymmetric R3/R4 specification likely by repressing ligand-independent Notch receptor activation. As I mentioned in chapter I, previous mammalian studies suggest that Ral is involved in membrane trafficking and other signaling events. Therefore, trafficking of the Notch receptor itself, or components necessary for Notch receptor activation, may be regulated by Ral. As I described in chapter I and II, by regulating Notch trafficking, Notch activity was shown to be regulated; Notch can be internalized and subsequently degraded, or cleaved and activated, dependent of ligand or independent of ligand in a certain cellular compartment(s). Proteases for Notch cleavage also need to properly localize for Notch processing and activation. Ral could be involved in trafficking of these enzymes. However, in mammalian studies, Ral is involved not only in membrane trafficking, but also in various signaling pathways to control downstream transcriptional events. Therefore, Ral may regulate Notch-signaling related transcriptional events.

In this chapter, I will present experimental results which suggest an important Ral-mediated pathway, possibly required for repressing Notch activation. As I demonstrated before, Notch can access the nucleus independent of the Notch extracellular domain when *Ral* is mutated, suggesting that Ral represses ligand-independent Notch activation. Several factors were previously reported to repress ligand-independent Notch activation. Most of them function in conrolling membrane trafficking. Mutation in the C2 domain containing protein Lethal giant discs (Lgd) induced Notch endosomal accumulation and ectopic ligand-independent Notch activation (Childress et al., 2006;

Jaekel and Klein, 2006). Recently, mutations of the ESCRT components such as *tsg101* and *vps25*, were found to result in Notch accumulation in identified or unidentified early endosomes, causing ligand-independent Notch activation (Vaccari et al., 2008; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Because ESCRT components sort cargos to lysosomal degradation pathway, it is proposed that these components prevent attenuation of Notch in certain endosmes where Notch cleavage could occur (Vaccari et al., 2008). Therefore, Ral may be involved in trafficking events where ESCRT components are involved. Furthermore, there may be mechanisms by which ligand-unbound Notch receptors are internalized and down-regulated for cell fate decisions, which Ral may control. For example, the adaptor protein Numb down-regulates Notch by internalizing Notch receptors to endosomal compartments in pIIb cells, by which Notch directionality is controlled (Hutterer and Knoblich, 2005).

It was shown that the constitutively active form of Ral inhibits endocytosis and exocytosis, probably sequestering downstream effectors (Sugihara et al., 2002; Moskalenko et al., 2002; Feig, 2003), due to the failure of being cycled to the GDP-bound form. Interestingly, Ral uses the same motif to bind endocytic machineries and exocyst complexes and the active form of Ral bearing a single amino acid change exclusively affects either endocytosis or exocytosis (Sugihara et al., 2002; Moskalenko et al., 2002). The amino acid sequence of *Drosophila* Ral is highly conserved (~80% amino acid similarity with human), and the downstream effector pathways are also conserved (Mirey et al., 2003). Therefore, I tried to dissect Ral pathways by testing complementation of defects caused by *Ral* mutations using different sets of Ral point mutant constructs.

The cellular localization of Ral may be important for Ral function and studying Ral cellular localization may give us a clue for Ral function(s) in multicellular organisms.

However, intracellular compartments where Ral resides have not been studied in multicelluar organisms. In a previous *Drosophila* study, overexpression of GFP-tagged Ral in ovarian cells localized the proteins mainly to the plasma membrane (Ghiglione et al., 2008). However, as discussed in chapter II, endogenous Ral proteins were mainly observed in certain cellular compartments, even though Ral proteins were observed near the plasma membrane when Ral was overexpressed in the eye. These results imply that Ral proteins could traffic through membranous compartments (from the plasma membrane to intracellular compartments), where Ral can regulate cellular events.

RESULTS

Ral genetically interacts with components required for Notch trafficking control.

In chapter II, Ral appeared to repress ligand-independent Notch activation for generating biases in Notch signal between cells. As mentioned before, components involved in controlling Notch trafficking are important for regulating the Notch signaling output. Rab5 is known to regulate ligand-dependent Notch activation by regulating Notch trafficking. Vps25, a component in the ESCRT complex, was reported to repress ligand-independent Notch activation by sorting Notch receptors to the lysosomal pathway. In both cases, the precise mechanisms were not proposed. If Ral is involved in controlling Notch trafficking, thereby repressing Notch activation, *Ral* would genetically interact with components required in activating or repressing Notch signal through Notch trafficking control.

To determine whether *Ral* plays a role in Notch trafficking, I tested for genetic interactions between *Ral* and loss-of-function mutations of endocytic factors known for controlling Notch trafficking. *rab5*$^{-}$ and *vps25*$^{-}$ dominantly suppressed the *Ral*^{EE1}

hypomorphic eye phenotype; *vps25*$^{-}$ is a stronger suppressor than *rab5*$^{-}$ (Figure 3-1B, D compared with A). However, the *avl* mutation failed to show the modification of *Ral*^{EE1} hypomorphic eye phenotype and the *vps20* mutation was a dominant enhancer of the eye phenotype (Figure 3-1C, E and compare with A). An early endosomal marker, Avl (Avalnche), and an ESCRT component, Vps20, were shown to regulate Notch endosomal sorting, but their roles in Notch activation are not known. These results suggest the possibility that *Ral* regulates membrane trafficking by which Notch receptors are routed and sorted, but not all Notch trafficking routes. Because Ral is known to control endocytosis, *rab5* and *vps25* mutations could modify abnormal trafficking events caused by *Ral* mutation, where Notch could be involved.

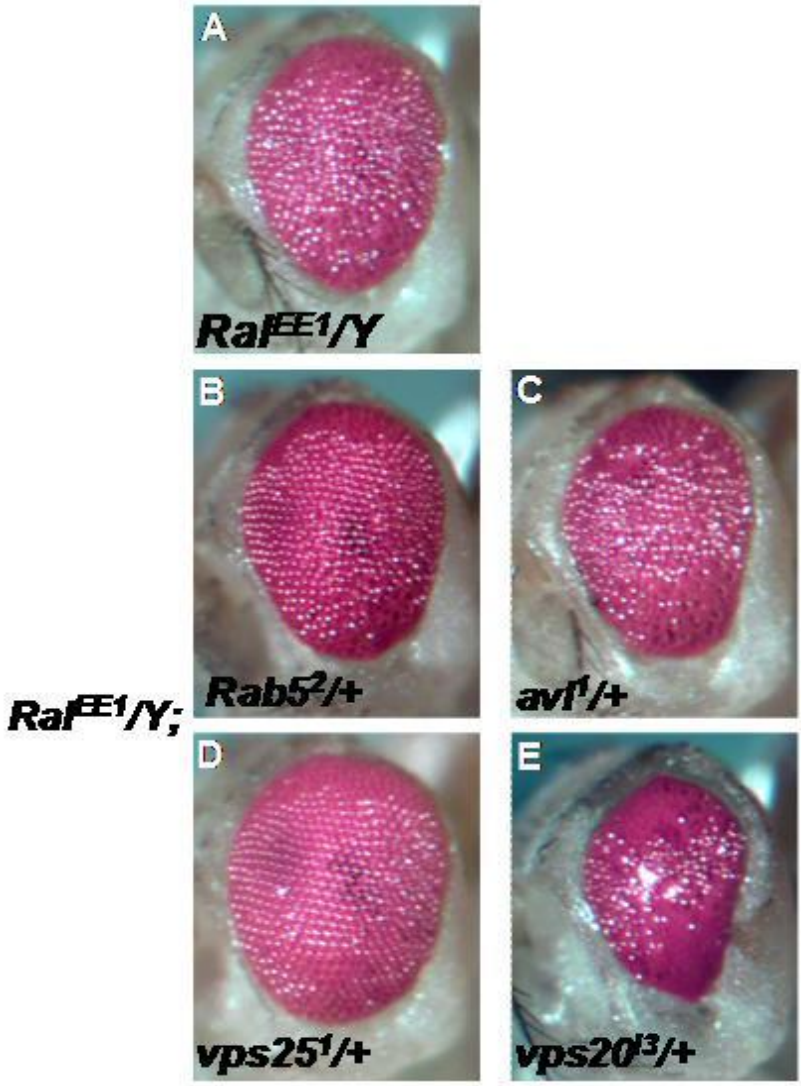


Figure 3-1. Genetic interactions of *Ral* with endocytic components required in Notch trafficking. Adult eyes of **(A)** Ral^{EE1}/Y. **(B)** Ral^{EE1}/Y; $Rab5^{2}/+$. **(C)** Ral^{EE1}/Y; +/+; $avl^{1}/+$. **(D)** Ral^{EE1}/Y; $vps25^{1}/+$. **(E)** Ral^{EE1}/Y; $vps20^{I3}/+$. One copy of *vps25* null mutation is the strongest suppressor of morphological defects of *Ral* mutation.

Ral-mediated endocytic control is important for rescuing *Ral* mutant defects.

Tangential sections of *Ral* mutant adult eyes show ommatidial defects observed in eyes with excess Notch activation (Figure 2-2B and 3-2A), as discussed in chapter II. These defects were generally suppressed by loss-of-function *lqf* mutants. Given that epsin is mainly implicated in ligand endocytosis and Notch activation, this suppressive genetic interaction suggests that control of Notch signaling could be the main role of Ral, at least in eye developmental processes. Ral was reported to have multiple cellular functions for regulating various cellular events. Ral-mediated multiple cellular processes could be involved in controlling Notch signaling, or a specific Ral downstream pathway(s) may be important for regulating Notch signal. Therefore, I decided to dissect Ral-mediated pathways to identify the important Ral downstream pathway(s) which is implicated in the excess Notch-related ommatidial phenotypes of *Ral* mutant eyes.

Ral shares a common binding site for RalBP1 and Sec5, each of which mediating endocytosis and exocytosis, respectively. Alteration of a single amino acid in the binding site exclusively affects endocytosis or exocytosis, but not other signaling pathways, when combined with the point mutation generating the active form of Ral (Moskalenko, S. et al, 2002). This specific motif is also conserved in *Drosophila* Ral and its binding to RalBP1 and Sec5 was tested in a previous study (Mirey et al., 2003). In this motif, the amino acid Asp^{49} (Asp^{46} for *Drosophila*) is important for binding to both RalBP1 and Sec5. A sigle amino acid change ($Asp^{49}\rightarrow Asn^{49}$) of human Ral excludes the RalBP1 binding property (not Sec5), and the amino acid change ($Asp^{49}\rightarrow Glu^{49}$) of human Ral excludes binding to Sec5 (not RalBP1). Based on the amino acid information from these cell culture studies, *Drosophila* versions of point mutant *Ral* constructs were generated (generated by a former lab rotation student, Lee, C. H.); the amino acid change ($Asp(D)^{46}\rightarrow Asn(N)^{46}$) to exclude RalBP1 (not Sec5) binding and ($Asp(D)^{46}\rightarrow Glu(E)^{46}$)

change to exclude Sec5 (not RalBP1) binding. We reasoned that each *Ral* mutant construct would rescue Ral downstream pathways perturbed by *Ral* mutation, except for either Ral-mediated endocytosis with (D^{46}→N^{46}; Ral^{46N}) or exocytosis with (D^{46}→E^{46}; Ral^{46E}) construct (diagrams in Figure 3-3).

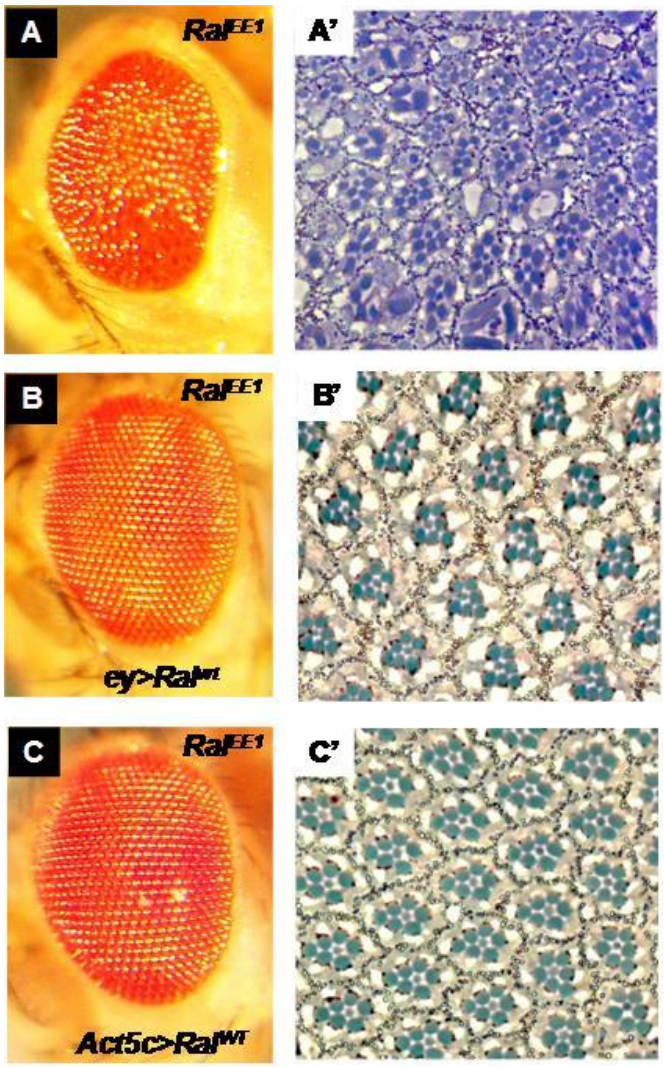


Figure 3-2. Overexpression of wild-type *Ral* rescues defects caused by *Ral* mutation. Adult eye morphology and sectioned eye of (A) Ral^{EE1}/Y. (B) Ral^{EE1}/Y; *ey-GAL4/+*; *UAS-*Ral^{WT}*/+*. (C) Ral^{EE1}/Y; *Act5c-GAL4/+*; *UAS-*Ral^{WT}*/+*. These are control experiments for Figure 3-3.

All aspects of ommatidial mutant phenotypes were complemented by low level (*ey-gal4;UAS-Ral^{wt}*) or high level (*Act5C-gal4;UAS-Ral^{wt}*) of *Ral^{wt}* expression (Figure 3-2). If Ral-mediated exocytosis is important for eye development, rescuing endocytosis and other pathways, by expressing *Ral^{46E}*, would fail to complement the ommatidial defects (the diagram on the left-hand side in Figure 3-3). However, the Sec5 binding defect, thereby defects in exocytosis, appeared not to be deleterious in complementing eye defects of *Ral^{EE1}*; low or high level expression of *Ral^{46E}* completely rescued eye defects of *Ral^{EE1}* (Figure 3-3A, C). In contrast, *ey-gal4* driven *Ral^{46N}* expression, thus rescuing Ral-mediated exocytosis and other pathways, failed to completely rescue eye defects of *Ral^{EE1}*, even though it rescued eye defects significantly (Figure 3-3B and compare with Figure 3-2A'). This incomplete rescue might not be caused by low level expression of the construct, because high level expression of the construct with a strong *gal4* driver (*Act5c-gal4*) also failed to show better rescue of the eye defects (Figure 3-3D). Therefore, I concluded that Ral without the RalBP1 binding property, losing Ral-mediated endocytic regulation, is insufficient to rescue defects of *Ral^{EE1}* completely (Figure 3-3B, D). Furthermore, ommatidial defects failed to be complemented, were similar to those of excess Notch; loss of rhabdomeres and symmetric R3/4s (Figure 3-3B'). These results were confirmed by using the *gal4*-expressing *Ral* enhancer trap line, *Ral^{PG69}*. Whereas *Ral^{PG69};UAS-Ral^{wt}* and *Ral^{PG69};UAS-Ral^{46E}* rescued the lehality of *Ral^{PG69}* male and the eyes are normal, *Ral^{PG69};UAS-Ral^{46N}* failed to complement the lethality (Figure 3-4A, B).

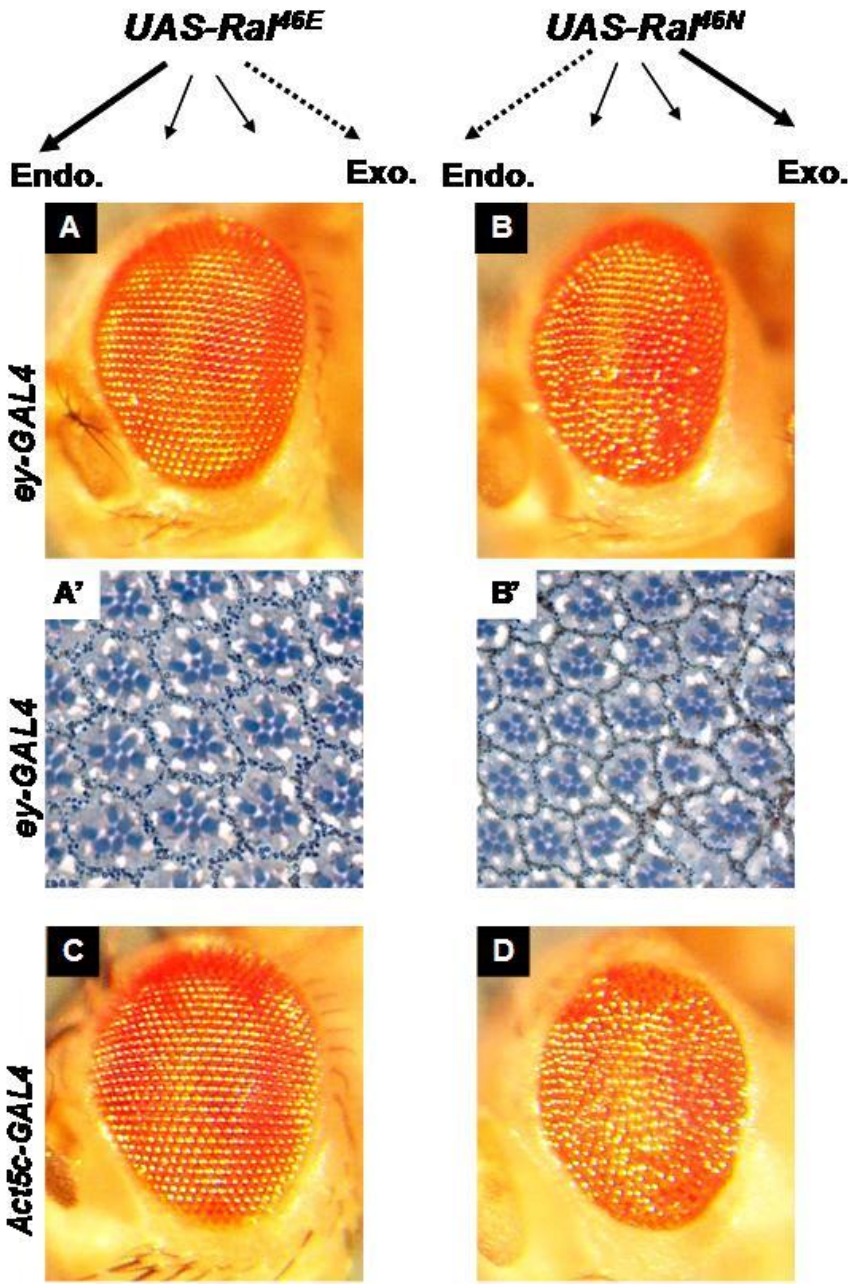


Figure 3-3. Rescuing activity of point mutant constructs of *Ral*. **(A)** *Ral*^{EE1}/*Y*; *ey-GAL4*/+; *UAS-Ral*^{46E}/+. **(B)** *Ral*^{EE1}/*Y*; *ey-GAL4*/+; *UAS-Ral*^{46N}/+. **(C)** *Ral*^{EE1}/*Y*; *Act5c-GAL4*/+; *UAS-Ral*^{46E}/+. **(D)** *Ral*^{EE1}/*Y*; *Act5c-GAL4*/+; *UAS-Ral*^{46N}/+. Diagrams on top of the images represent the pathways that each *Ral* construct can rescue. Note that rescuing exocytosis is not enough to restore eye defects of *Ral* mutants regardless of weak (*ey-GAL4*) or strong (*Act5c-GAL4*) drivers.

Overexpression of constitutively active form of Ral (*Act5C-gal4;UAS-Ral^{20V}*) killed the flies. This lethality might be caused by too much activation of Ral downstream pathways or too much sequestering of effector molecules, blocking downstream events. Previous studies showed that the constitutively active form of Ral inhibits and affects either endo- or exocytosis depending on the binding property of Ral to Sec5 or RalBP1. Results so far suggest that Ral-mediated endocytic control is more important than other Ral downstream pathways. Therefore, I hyposized that gain of function Ral activity in endocytosis, would cause more deleterious effects than that in excoytosis and other Ral downstream pathways. Indeed, dominant effects on Ral-mediated exocytosis by expressing *UAS-Ral^{20V46N}* under the control of *Ral^{PG69}* rescued the lehality of *Ral^{PG69}* male and the eyes had no defects (Figure 3-4C). On the contrary, dominant effects on endocytosis caused by *Ral^{PG69};UAS-Ral^{20V46E}* failed to rescue the lethality. Both *UAS-Ral^{20V46N}* and *UAS-Ral^{20V46E}* killed the flies when overexpressed by the *Act5c-gal4* driver, suggesting that dominant active forms of Ral are harmful to flies when they are highly overexpressed. Nonetheless, the dominant effects on exocytosis appeared not to be deleterious in the mild expression condition when using the *gal4*-expressing *Ral* enhancer trap line. It is possible that the residual activity for endocytosis was provided, thereby rescuing the lethality with no phenotypical defects (Figure 3-4C). Again, these results support the idea that Ral-mediated endocytic control is more important than other Ral downstream pathways in flies.

All these *Ral* mutant experiments suggest the possibility that Ral-mediated endocytic regulation is the critical process during eye development where Notch signaling is involved. Furthermore, as the ommatidial defects (not complemented by *Ral^{46N}*) resemble excess Notch defects, the Ral-mediated endocytic control pathway could be the key effector loop of Ral for Notch regulation.

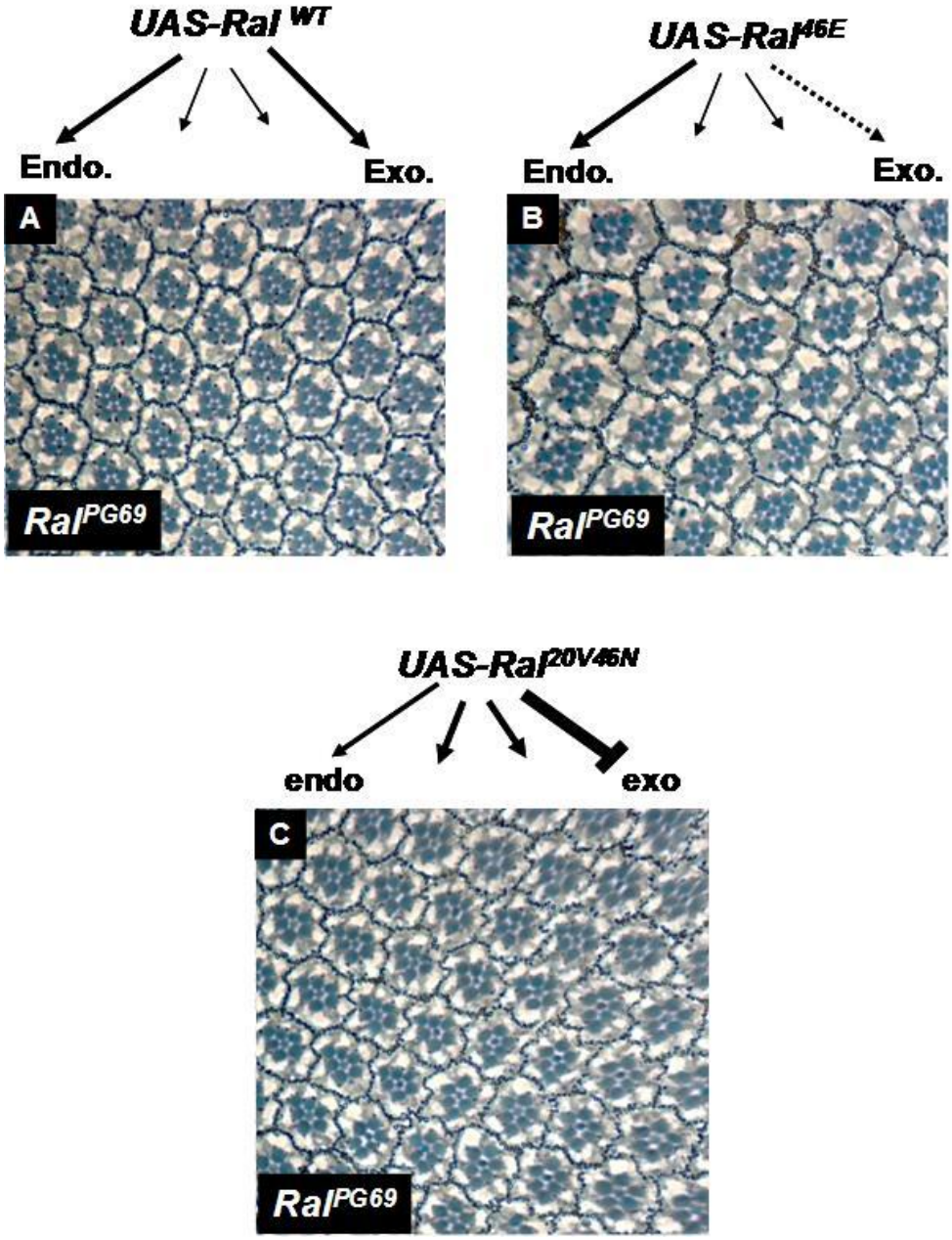


Figure 3-4. Activity tests of mutant *Ral* constructs under control of a *Ral* enhancer trap. Sectioned adult eyes of **(A)** Ral^{PG69}/Y; +/+; $UAS\text{-}Ral^{WT}$/+. **(B)** Ral^{PG69}/Y; +/+; $UAS\text{-}Ral^{46E}$/+. **(C)** Ral^{PG69}/Y; $UAS\text{-}Ral^{20V46N}$/+. Diagrams on top of (A) and (B) represent the pathways that each *Ral* construct can rescue. The constitutively active form of *Ral* with an additionl point mutation, which affects exocytosis, rescues lethality and ommatidial defects in **(C)**.

Ral localizes in distinct intracellular compartments and partially co-localizes with Rab5 and Rab7 endosomes.

Ral controls endocytosis and Ral-mediated endocytic control appeared to be critical in experiments described above. If Ral is an important regulator in endocytic events, Ral may localize to endosomal compartments, where Ral regulates membrane trafficking events. To test this possibility, I examined endogenous Ral proteins in eye discs with various endosomal markers, using a polyclonal antibody to human RalB, described in chapter II, and other antibodies detecting endosomal markers.

Ral positive puncta were barely merged with Hrs or Rab11 positive puncta, suggesting that most Ral proteins do not reside in Hrs or Rab11 endosomes. On the other hand, Ral positive puncta are sometimes also positive for YFP-Rab5 or Rab7-GFP puncta, implying that some of Ral proteins are present in Rab5 and Rab7 endosomes (Figure 3-5). Because the frequency of co-localization of Ral with either Rab5 or Rab7 positive puncta is not significant (Figure 3-5), still it is possible that Ral mainly localizes in other endosomes or cellular compartments; possibly exocyst complexes. Neverthless, these results suggest the possibility that some of the Ral protein population could be involved in controlling of Rab5- or Rab7-mediated endosomal sorting events. However, main cellular locations where Ral is functioning still need to be clarified.

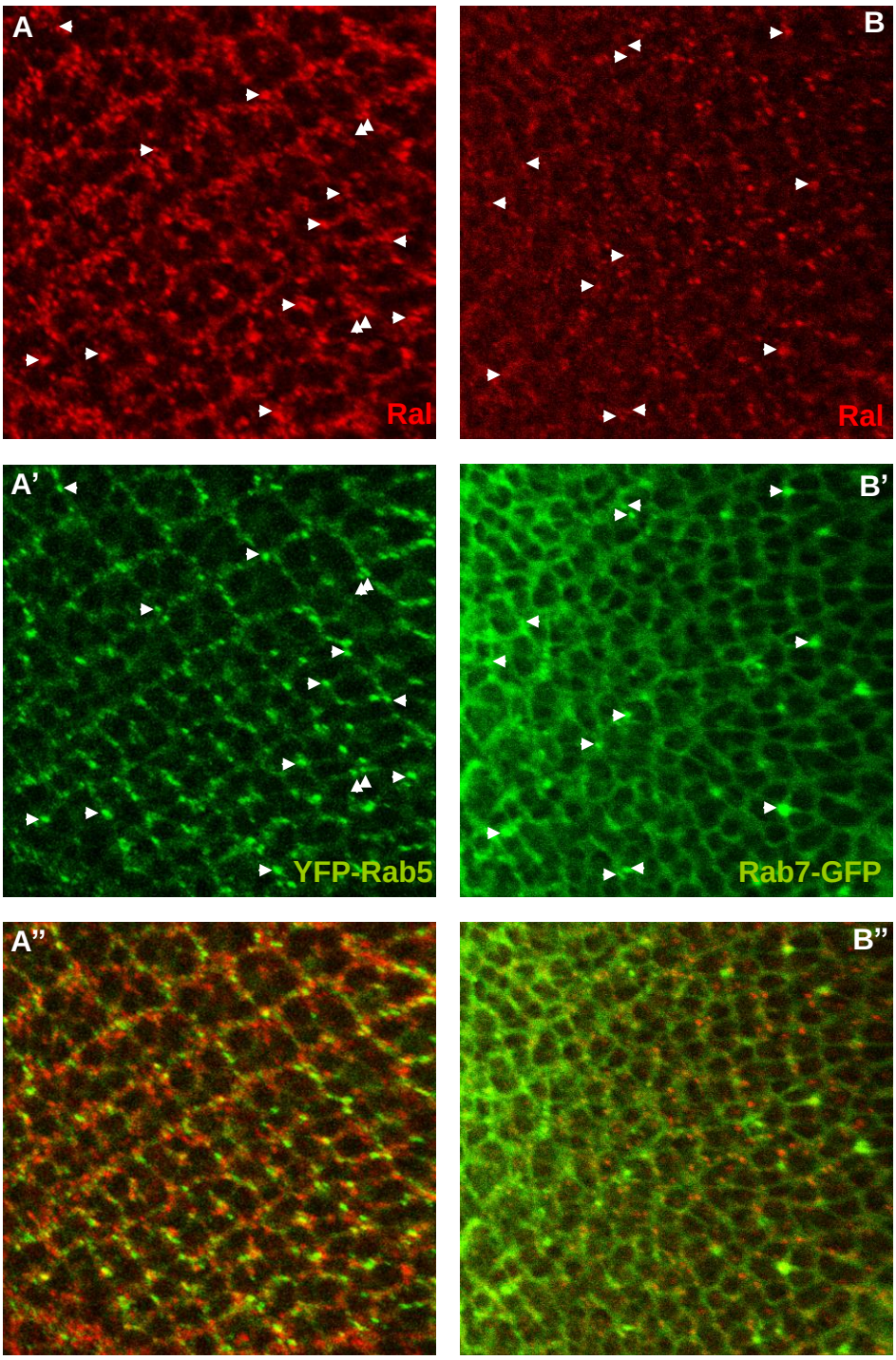


Figure 3-5. Ral localization in the eye disc. (A) *ey,GMR-gal4/UAS-yfp-rab5* **(B)** *Act5c-gal4,UAS-rab7-gfp/+*. Eye discs with each genotype were isolated and immunolabeled by anti-Ral antibody. Ral^{+} puncta merged with each endosome are indicated by arrow heads.

DISCUSSION

Possible mechanisms of Ral-mediated Notch signaling control.

In this chapter, I have shown that Ral could regulate membrane trafficking events in multicellular organisms and particularly, Ral-mediated control of endocytosis could be important during eye developmental processes. *Ral* genetically interacts with components necessary for regulating Notch trafficking and activation. Given that Notch trafficking control exerts various outcomes in Notch activation, Ral function in these events could be important for regulating Notch signaling. Interestingly, *rab5* and *vps25* mutations were the dominant suppressors of *Ral* mutation in this study. As Rab5 and Vps25 are important Notch regulators, Ral-mediated control of Notch signaling could be related to these components.

Why is Ral-mediated control of endocytosis required for proper Notch activation? As discussed in chapter I, several components were identified as regulators for Notch trafficking and Notch activation. However, the mechanisms by which trafficking and proteolytic cleavage of Notch are controlled are completely unknown. Vps25 was reported to repress ligand-independent Notch activation. Given that Ral appeared to repress ligand-independent Notch activation, the suppressive genetic interaction of the *vps25* mutant with *Ral* was unexpected. These results may imply that Vps25 has another role for promoting Notch activation, which is opposite to the Ral function in repressing Notch activation. A previous study showed that Notch cleavage can occur at the lysosomal compartment, a downstream cellular compartment of the multivesicular body where Vps25 functions, depending on Deltex (Wilkin et al, 2008). Furthermore, during this process, late endosomal marker, Rab7 was shown to be involved. Based on these studies, the idea that Ral represses Deltex-mediated ligand-independent Notch activation may be proposed. In this scenario, the *vps25* mutant reduces Notch transit to the

lysosome, which suppresses *Ral* mutant phenotypes. Moreover, partial co-localization of Ral with Rab7 endosmes (in my study) may propose that Ral controls Rab7-mediated Notch routing to the lysosome.

Finally, Ral-mediated control of endocytosis may regulate Notch cleavage events by regulating locations of proteolytic enzymes. Presenilin, a component of the γ-secretase complex, was reported to be targeted to endocytic compartments in order to cleave substrates (Lah and Levey, 2000). Furthermore, γ-secretase activity was observed in the lysosomal membrane, where presenilin and nicastrin, another component of γ-secretase complex, are localized (Pasternak et al., 2003). Therefore, it is possible that regulating localization of proteolytic enzymes implicated in Notch cleavage provides another level of Notch regulation. Even though the relationship between trafficking of the proteolytic enzymes and Notch activation is poorly understood, it will be interesting to test whether mislocalization of those enzymes causes abnormal Notch activation and whether *Ral* mutation causes the mislocalization of those enzymes.

MATERIALS AND METHODS

Drosophila strains

The following alleles were used in this work. FlyBase id numbers, when available, are in parentheses. Chromosomes used are indicated in the figure legends. Ral^{EE1} (FBal0197295); Ral^{PG69} (FBal0130802); *Act5C-gal4* (FBti0012293); *ey-gal4* (FBti0012711); *UAS-*Ral^{wt} (FBal0101574); *UAS-*Ral^{CA} (FBal0101576); *FM7, gfp* (FBst0005193); $Rab5^{2}$, avl^{1}, $vps25^{1}$, and $vps20^{I3}$ (from Bilder, D. at UC Berkely); *UAS-yfp-rab5* (FBst0009775); *UAS-rab7-gfp* (from González-Gaitán, M., Germany).

Ral mutant constructs

To generate mutant forms of *Ral* cDNA, two-step PCR reactions were carried out with genomic DNA containing wild-type *Ral* cDNA or the constitutively active form of *Ral* cDNA (Ral^{G20V}) as templates. To generate Ral^{46N} (D to N change in the amino acid sequence), primers, forward; 5'-ggcgcgccATGAGCAAGAAGCCGAC-3' (the *AscI* site at the 5' of the start codon) and reverse; 5'-TTTTTCCTATAGCTAT**T**GGCCTTGGT-3' (bold nucleotide; point mutation) were used to amplify the 5' cDNA fragment with the point mutation. Primers, forward; 5'-ACCAAGGCC**A**ATAGCTATAGGAAAAA-3' (mutation; bold) and reverse; 5'-ggcgcgccCTAAAGTAGGGTACACTT-3' (the *AscI* site at the 5' of the stop codon) were used to amplify the 3' cDNA fragment with the point mutation. For these first PCR reactions, geneomic DNA containing wild type *Ral* cDNA was used as the template. Two amplified fragments were used as templates for the second PCR, using primers, forward; 5'-ggcgcgccATGAGCAAGAAGCCGAC-3' (the *AscI* site at the 5' of the start codon) and reverse; 5'-ggcgcgccCTAAAGTAGGGTACACTT-3' (the *AscI* site at the 5' of the stop codon). To generate Ral^{46E} (D to E change in the amino acid sequence), primers, reverse; 5'-TCCTATAGCT**T**TCGGCCTTGGT-3' and forward; 5'-ACCAAGGCCGA**A**AGCTATAGGA-3' were used as mutant primers for amplifying 5' and 3' mutant cDNA fragments. The second PCR was carried out using same primers as before. To construct Ral^{20V46N} and Ral^{20V46E}, the same sets of primers were used for the first and second PCR, but genomic DNA containing the constitutively active form of *Ral* cDNA (Ral^{G20V}) was used as the template. After the second PCR reactions, full length mutant form of *Ral* cDNA fragments were digested by the *AscI* restriction enzyme and ligated into the *Asc*I site of pUASt (E. Overstreet et al., 2004). The resulting plasmid was used to transform w^{1118} flies.

Imaging of eyes

Immunohistochemistry of third instar larval eye discs was performed as follows. Discs were fixed in PEMS buffer with 1.0% NP-40 for 15 minutes. Antibody treatment was as described previously (Lim et al., 2007) with modifications. Fixed eye discs were blocked for 2 hours 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 minutes, and incubated with secondary antibodies in washing solution for 2 hours at room temperature, and then washed three times for 5 minutes. Phalloidin was used as described previously (Chen et al., 2002). Eye discs were mounted in Vectashield (Vector, Burlingame, CA, USA) and viewed with a Leica TCSSP2 confocal microscope. Rabbit polyclonal anti-RalB (Proteintech Group, Chicago, IL, USA) was used for Ral protein staining at 1:100. Secondary antibody, Alexa^{568}-anti-rabbit (Molecular Probes, Carlsbad, CA, USA) was used at 1:200. Plastic sectioning of adult eyes was as described (Tomlinson and Ready, 1987). Eye sections were photographed with a Zeiss Axioplan and Axiocam HRc. Adult external eyes were photographed with an Olympus SZX12 microscope equipped with a SPOT idea (Diagnostic Instruments, Sterling Heights, MI, USA) digital camera.

Chapter 4: *Cell-autonomous roles of epsin in Notch signaling; Notch routing to the Rab5 endosome and Notch activation requires epsin*

INTRODUCTION

So far, I have described how Ral GTPase biases Notch activity between two equivalent cells for cell fate decisions. In chapter III, experimental evidence suggests that Ral-mediated regulation of Notch trafficking could be important for modifying Notch activity, which initiates the bias in the signal directionality. As mentioned in chapter I, previous studies suggest that the regulation of membrane trafficking is important not only for ligand signaling, but also for Notch receptor signaling, and this might be applied to Ral-mediated Notch receptor regulation. Despite various studies from yeast and mammalian cells suggesting that epsin is a general endocytic factor regulating multiple endocytic events, thereby internalizing multiple cargos, the only apparent role of epsin in *Drosophila* is in Notch ligand endocytosis and ligand signaling. In this chapter, I will provide the experimental evidence that suggests novel cell-autonomous roles of epsin, including promoting Notch signal receiving.

Epsin is an evolutionarily conserved endocytic factor from yeast to human. As described in chapter I, epsin has an ENTH domain and motifs, which contribute to cargo endocytosis and membrane curvature formation. Epsin has an epsin N-terminal homology (ENTH) domain which is conserved in various endocytic factors (Kay et al., 1999; Rosenthal et al., 1999; De Camilli et al., 2002) in its N-terminus. The ENTH domain binds PIP_2 (phosphatidylinositol 4,5-bisphosphate) at the plasma membrane (Itoh et al., 2001; Ford et al., 2002; Aguilar et al., 2003). The C-terminal region of epsin has four different protein-protein interaction motifs varying in numbers between animal species (Kay et al., 1999; De Camilli et al., 2002). Is the ENTH domain required for Notch

signaling? Overexpression of the ENTH domain or the ENTH-deleted epsin C-terminus rescued eye defects caused by *epsin* mutation (Overstreet et al., 2003). However, requirement of these modules for Notch signaling were not precisely demonstrated. *Drosophila* has two epsin isoforms generated by alternative splicing of the *lqf* gene mRNA (Cadavid et al., 2000). Each 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., 2004; 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 adaptor complex (Owen et al., 1999), and two NPF motifs that bind EH domain-containing factors (Aguilar et al., 2003; Salcini et al., 1997; Paoluzi et al., 1998). All these motifs may imply that epsin-mediated endocytosis requires various factors, albeit no direct evidence about whether some of (or all of) these factors are involved in Notch signal sending or not. Regardless of these conserved motifs and domain, the only known essential role of epsin in multicellular organisms is promoting Notch ligand endocytosis and activating Notch in nearby cells. How does epsin exert this specific role in ligand endocytosis? It has been proposed that epsin recognizes an active form of ligand which is ubiquitinated by E3 enzymes (Wang and Struhl, 2004) and epsin UIMs were reported to recognize ubiquitinated proteins (Polo et al., 2002; Shih et al, 2002). Therefore, epsin UIMs could be important motifs for the role of epsin in ligand endocytosis, providing epsin with the specificity for recognizing ubiquitinated ligands. However, the role of UIMs is still controversial. In a previous cell cultue study, the UIM was shown to cause multiple mono-ubiquitinations on epsin and reduce epsin binding to other ubiquitinated proteins (Miller et al., 2004). In a very recent paper, the UIM was not restricted to bind to ubiquitinated receptors in yeast (Dores et al., 2010). Moreover, there is no study about the role of UIMs in Notch signaling in multicellular organisms.

Before finding *Drosophila* dynamin Shibire (Shi) as a key factor for receiving and sending Notch signal (Seugnet et al., 1997) , Notch activation events had been believed to occur at the plasma membrane where the interaction between ligands and receptors takes place. Even after finding the *Shi* mutants, it is still controversial whether Notch endocytosis is required for activating downstream Notch signaling events or not. Furthermore, in the original *Shibire* paper, the requirement of Notch endocytosis for activating the signal was not fully addressed; in their paper, *Notch* overexpression itself was epistatic to the *Shi* mutation, which drove them to the interpretation that *Shi* has a novel function for promoting ligand/receptor interaction and that Notch overexpression increases the chance of ligand/Notch interaction, not Notch endocytosis (Seugnet et al., 1997). However, recent papers strongly argue that Notch receptor endocytosis is required for activating Notch signal. Clathrin is required in signal receiving follicle cells (Windler and Bilder, 2010), and endosomal factors such as Rab5 are required in signal receiving cells in the eye, wing, and ovarian tissues, for Notch activation (Vaccari et al., 2008; Windler and Bilder, 2010). All of these support the idea that Notch receptor endocytosis and Notch routing to certain endosomal compartments, is required for Notch receptor transduction pathway. It was proposed that Notch cleavage occurs in certain endosomes and the acidic endosomal environment might promote Notch cleavage by activating proteolytic enzymes, indicating that there are Notch trafficking pathways seperable from simple membranous Notch level control.

Why is Notch internalization required? And how is internalization of signaling Notch, once bound by the ligand, regulated to exert separate roles from simple membranous Notch level control? In this study, I will provide some evidence supporting novel cell-autonomous roles of epsin in Notch signaling. Experimental results will show

that signaling ligand-bound Notch receptors travel through a specific trafficking pathway, the Rab5 endosomal pathway, for Notch activation. Furthermore, I will show evidence that epsin UIMs are necessary only for ligand signaling, providing epsin with the specificity for ligands, which is distinct from epsin's role in Notch receptor regulation and activation.

RESULTS

Epsin is required for receiving as well as sending Notch signal in wing discs

In the fly wing primordium, Notch is activated at the D/V boundary, forming a stripe of signal receiving cells. Fringe modifies Notch in D cells, which leads to make D cells more sensitive to Delta (Dl) and less sensitive to Serrate (Ser). Serrate in D cells activates Notch in the opposite direction, activating unmodified Notch in V cells, and Delta in V cells activates modified Notch in D cells.

Even though epsin requirement in signal sending cells for sending Notch signal was shown in a previous *Drosophila* wing study, epsin requirement in signal receiving cells at the D/V boundary was not clearly addressed under physiological condition. To address this issue, the MARCM technique was used to generate *lqf-* clones in wing discs and expression of the Notch target *cut* was analyzed. For the control experiments, *ser- Dl-* double mutant clones were generated and *cut* expression at D/V boundary cells was monitored. If epsin is required only in signal sending cells, the pattern of *cut* expression in D/V boundary cells in *lqf-* clones would be similar to that of ligand mutant cells. As previous studies showed, in *ser- Dl-* double mutant clones spanning the D/V boundary, *cut* expression was abolished at the D/V midline. Furthermore, *ser- Dl-* double mutant

cells at the clone border ectopically expressed *cut*, which was explained by ligand-mediated cis-inhibition of Notch (Figure 4-1A, A'). We further analyzed *ser- Dl-* double mutant clones generated in either the dorsal or ventral side contacting the D/V boundary. All of the clones analyzed received Notch signal and expressed *cut* at the D/V midline, suggesting ligand mutant cells can receive signal from wild-type cells on the opposite sides (Figure 4-1B, B'). Therefore, in ligand mutant clones, *cut* expression failure at the D/V boundary cells (Figure 4-1A, A') is due to signal sending failure from both the D and V sides.

Interestingly, *lqf* - clones in either the dorsal or ventral side contacting the D/V boundary often failed to receive signal from opposite sides (18 out of 21 *lqf-* clones, Figure 4-1D, D' and Figure 4-4) except for a couple of *lqf-* cells expressing *cut*. These results suggest the idea that Notch signaling failure at the D/V boundary in *lqf-* mutant clones (Figure 4-1C, C') is not only due to signal sending failure, but also to signal receiving failure. To clarify this idea further, *lqf-* clones were generated with one copy of a genomic *lqf* construct (Figure 4-1E, F). In these conditions, the genomic *lqf* construct rescued the signal receiving as well as signal sending properties (Figure 4-1F, F' and compare with Figure 4-1D, D').

The different outcomes between ligand and *lqf* mutant clones might be explained by ligand-mediated cis-inhibition of Notch; without ligands, the cells are more sensitive to receive signal as they are free from cis-inhibition. In the wing disc, dMib, an E3 ubiquitin ligase, is required for ligand activation and sending signal. In *dmib* mutant cells, Serrate proteins are accumulated at the plasma membrane, which may increase ligand-mediated cis-inhibition of Notch and show similar results to *lqf-* clonal analysis. However, a previous study clearly showed that *dmib* mutant cells contacting the D/V boundary can receive signal from the opposite side as normal cells do, arguing against the

idea that the ligand inhibits Notch from receiving signal. Furthermore, the level of ligand expression was reported to be very low at the D/V midline cells, meaning that ligand-mediated cis-inhibition could be absent or low at the D/V boundary cells. Moreover, ligand accumulation in *lqf* mutant cells was not as dramatic as in *dmib* mutant cells in wing discs. All these results argue that ligand-mediated cis-inhibition is not the cause of the failure to receive signal in *lqf*- cells contacting the D/V boundary. Taken together, these results suggest that epsin has a "cell-autonomous" role for receiving Notch signal, as well as a "cell-nonautonomous" role for sending signal in the wing disc, which is a distinct function from Notch ligands or Mib.

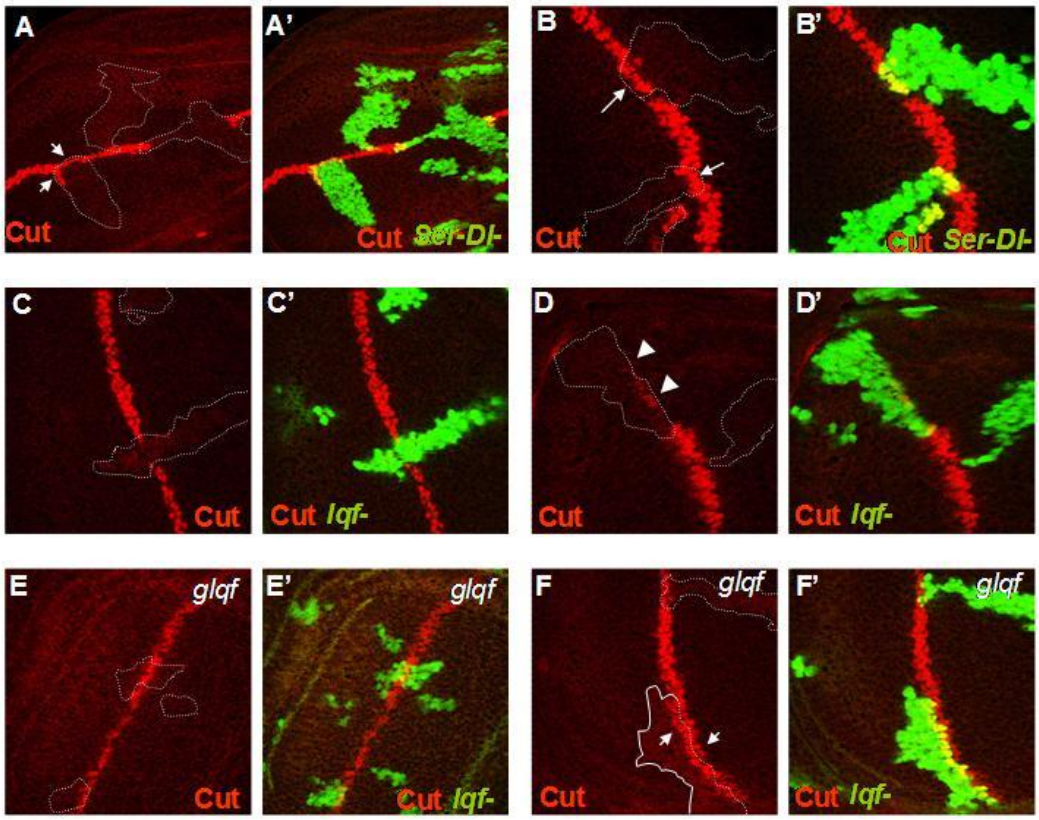


Figure 4-1. epsin requirement for Notch signal at the D/V boundary in wing disc.

Figure 4-1 continued. (**A-B**) *gfp*$^{+}$ *ser*$^{-}$*Dl*$^{-}$ clones (outlined) in two individual wing discs are shown. The genotype is *hs-flp tub-gal4, UAS-gfp/+; +/+; FRT82B ser*$^{-}$*Dl*^{REV}*/FRT82B tub-gal80.* (**C-D**) Two separate wing discs with *gfp*$^{+}$ *lqf*$^{-}$ clones (outlined). The genotype is *hs-flp tub-gal4, UAS-gfp/+; +/+; lqf*^{L71} *FRT80B/tub-gal80 FRT80B*. **(E-F)** The genotype is *hs-flp tub-gal4, UAS-gfp/+; glqf-gfp/+; lqf*^{L71} *FRT80B/tub-gal80 FRT80B*. To generate MARCM clones, larvae (1st and 2nd instar) were heat-shocked for 1 hour at 37°C and wing discs were isolated from 3rd instar larvae and immunolabeled with anti-Cut.

Epsin is required in R4s as well as in R3s for receiving and sending Notch signal in eye discs.

To determine whether epsin function is required for receiving Notch signal in different developmental contexts, Notch activation was analyzed in R3/R4 pairs of eye discs. During eye development, *Delta* expression is up-regulated in equatorial R3 precursors by Frizzled receptor signaling, then, the R3 precursor sends signal to the R4 precursor, which makes the R4 precursor a signal receiving cell. *lqf-* mosaic R3/R4 precursors were generated and the epsin requirement in R3 or R4 precursors was analyzed by monitoring R4-specific Notch reporter (*mδ-lacZ*) expression.

In adult eyes, Delta or Neur function is not required in R4, but in R3, for asymmetric positioning of the R3/R4 pair, suggesting that Delta or Neur function is not required in R4 for receiving Notch signal. However, the detailed molecular analysis for Notch activity in this context has not been carried out. I monitored *mδ-lacZ* expression in *ser- Dl-* or *neur-* mosaic R3/R4 precursor pairs as controls. Even though Serrate was shown not to be required in R3/R4 determination steps, this was based on phenotypic results, not molecular analysis. Therefore, to ensure no possible redundancy between ligands in the Notch activation process during R3/R4 determination, *ser- Dl-* double mutant clones were generated as controls. As another E3 ligase, Mib, was not required for Notch activation in R3/R4 pairs, clones of a *neur* mutation alone were generated for another control. We reasoned that if epsin is required only in signal sending R3 cells, such as ligands and Neur, Notch activation defects in *lqf* mutant clones would be the same as ligand or *neur* mutant clones; epsin activity is required only in R3 precursors.

Due to the general disorder inside the mutant clones, R3/R4 precursors, marked by Svp antibody (Figure 4-2), could not always be unambiguously assigned. Therefore, R3/R4 precursors were assigned in relatively small clones and mutant cells at the clone

border, according to the relative position compared to nearby wild type R3/4 precursor pairs. *ser- Dl-* double mutant R4 precursors always received signal from *ser+Dl+* R3 precursors as monitored by *lacZ* expression (Figure 4-2A, D). Similarly, *mδ-lacZ* was always expressed in *neur-* R4 precursors, if R3 precursors were *neur+* (Figure 4-2B, D). These results support the idea that in an R3/R4 context, ligand or Neur is not required in signal receiving R4s for asymmetric Notch activation in R4. Furthermore, these confirm previous studies showing that Delta or Neur is not required in R4s for asymmetric R3/4 development in adult eyes. On the contrary, if R4 precursors are *lqf-*, β-Gal signal was either weak or absent; in around 25% of the *lqf-* R4 precursors, β-Gal staining was undetectable even though R3 precursors were *lqf+* (Figure 4-2C, D). I analyzed *mδ-lacZ* expression in R3/4 precursor pairs with mutant R3s for each mutant. Mutant R3s paired with mutant or with wild-type R4s showed absolute epsin requirement in R3 precursors, as seen in ligand or *neur* mutants (Figure 4-2E, F).

The effect of an *lqf* mutation on R4 precursors for receiving Notch signal was not dramatic compared with the effect observed in wing discs (Figure 4-1). Proliferation of wing disc cells is known to start from the D/V boundary and the size of *lqf-* clones analyzed near the D/V boundary is relatively bigger than that of eye discs. This might argue that the *lqf* mutant cells analyzed lack epsin proteins from heterozygous parents in the wing discs. However, Lqf is expressed in the entire eye disc (from proliferating cells ahead of MF to differentiating cells posterior of the MF). The presence of epsin proteins in small clones (even in single cell clones, Figure 4-2) from heterozygous parents may need to be considered in eye discs, although *lqf* null alleles were used in this assay; in this aspect, R3 is more sensitive to the amount of epsin proteins than R4. On the other hand, *Delta* and *neur* are not transcribed prior to the Second Mitotic Wave (SMW) at third instar larval stages. Therefore, homozygous ligand or *neur* mutant R3 or R4 precursors

are predicted to lack wild-type protein, arguing that *mδ-lacZ* expression in R4 precursors is independent of the presence of wild type ligands or Neur. Considering all these together, these results indicate that epsin, not ligand or Neur, is required in R4 precursors for receiving Notch signal, as well as in R3 for sending signal.

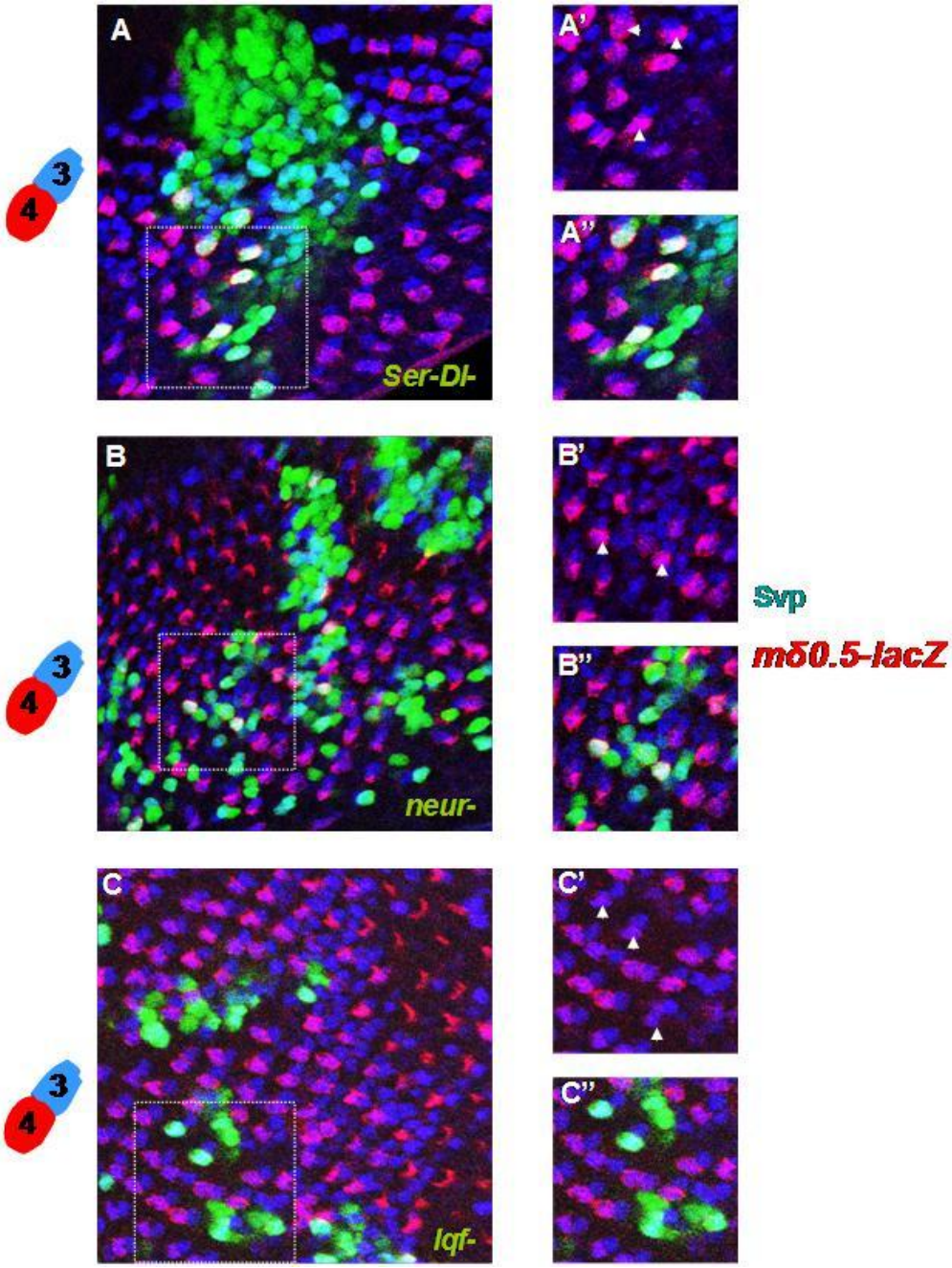


Figure 4-2. epsin requirement in signal receiving R4 precursors and signal sending R3 precursors for Notch signaling.

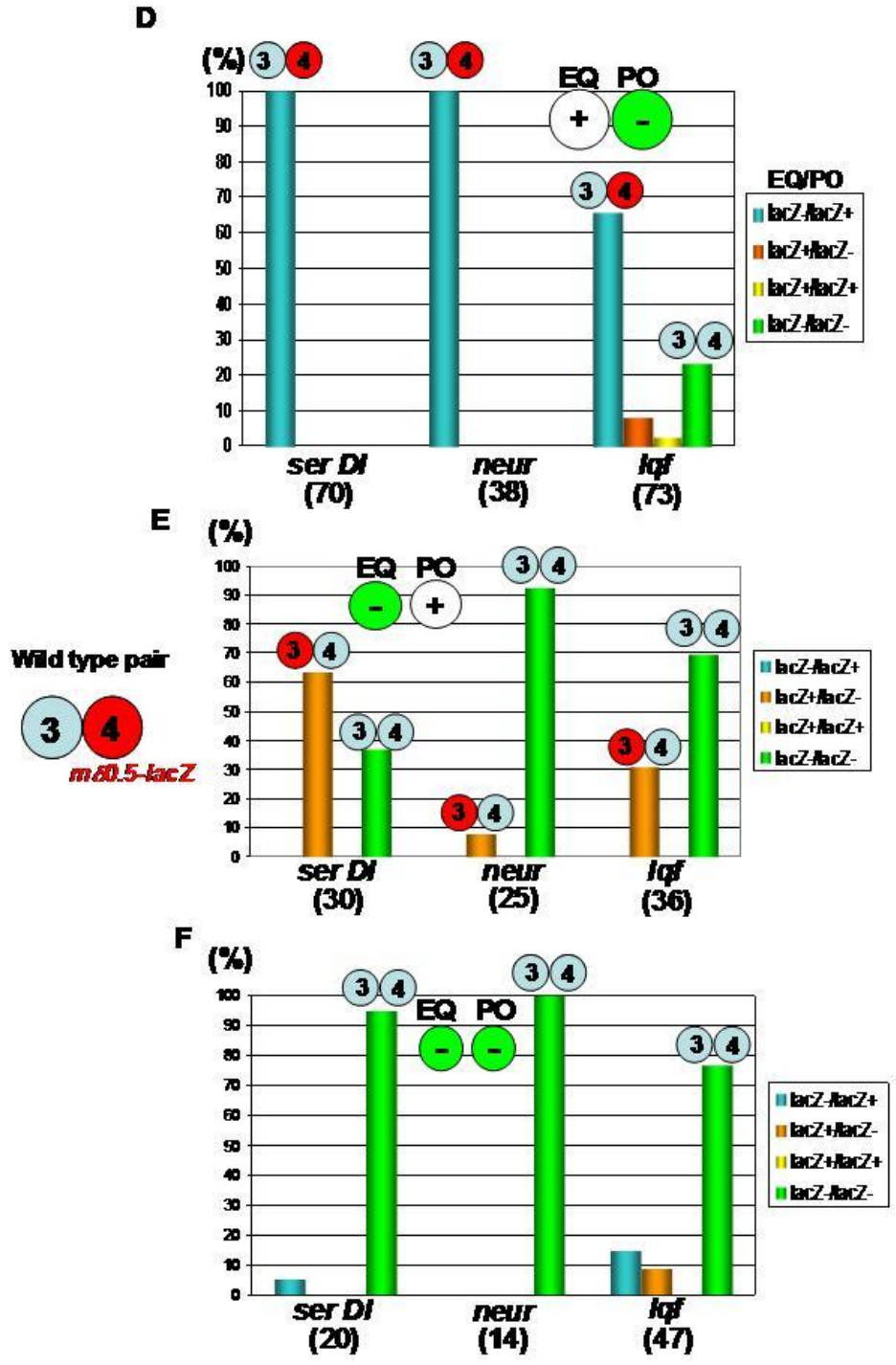


Figure 4-2 continued. epsin requirement in signal receiving R4 precursors and signal sending R3 precursors for Notch signaling.

Figure 4-2 continued. The genotype for (**A**) *hs-flp tub-gal4, UAS-gfp/+; mδ-lacZ/+; FRT82B ser⁻ Dl^{REV}/FRT82B tub-gal80.* (**B**) *hs-flp tub-gal4, UAS-gfp/+; mδ-lacZ/+; FRT82B $neur^{11}$/FRT82B tub-gal80.* **(C)** *hs-flp tub-gal4, UAS-gfp/+; mδ-lacZ/+; lqf^{L71} FRT80B/tub-gal80 FRT80B.* To generate MARCM clones, larvae (1^{st} and 2^{nd}) were heat-shocked for 1 hour at 37°C and eye discs were isolated from 3^{rd} instar larvae and immunolabeled with anti-β-gal and anti-Svp for testing Notch activity and labeling R3/4 pairs. Arrow heads indicate mutant R4s paired with wild-type R3s. **(D-F)** Quantificaion analysis of *mδ-lacZ* expression in $R3^+/R4^-$ mosaic pairs **(D)**; in $R3^-/R4^+$ mosaic pairs **(E)**; in $R3^-/R4^-$ mosaic pairs **(F)** for mutation of each gene. EQ; equatorial cell, R3 precursor, PO; polar cell, R4 precursor. Numbers at the bottom of each graph represent the number of mosaic R3/4 pairs analyzed.

Epsin promotes Notch receptor activation upstream of Notch cleavage in wing discs

I have shown that Notch receptor activation is diminished cell-autonomously in *lqf* mutants, although adjacent signal sending cells are normal, in wing and eye discs. *cut* expression in wing discs is abolished without Notch in the presence of *lqf*, suggesting that epsin does not play a redundant role in *cut* expression and regulates events upstream of Notch-mediated transcriptional control.

To determine the step in which epsin regulates Notch activation, *lqf*- clones that express wild-type Notch under Gal4 control (Figure 4-3A) were generated using the MARCM technique. Clones of cells that express Notch under Gal4 control activate Notch, as monitored by *cut* expression, particularly at the clone border (Figure 4-3D). As a control, *ser- Dl-* clones that over-express Notch were generated and I could not detect any difference when comparing *ser- Dl-* clones themselves in terms of *cut* expression (Figure 4-3B and compare with Figure 4-1A, B).

Notch over-expression restored signal receiving in the clones of *lqf*- cells generated near the D/V boundary, either at the D or V, as shown by *cut* expression (Figure 4-3E, F and compare with Figure 4-3C). This result indicates that signal receiving property is reduced in *lqf*- cells and excess Notch overcomes this defect. Furthermore, epsin might not be involved in Fng-mediated Notch modification, because Notch expression rescues receiving signal both D and V sides. Finally, these results show that Notch cleavage events occur properly in *lqf*- cells, suggesting that epsin is required upstream of Notch cleavage events for the signal receiving properties of the cell.

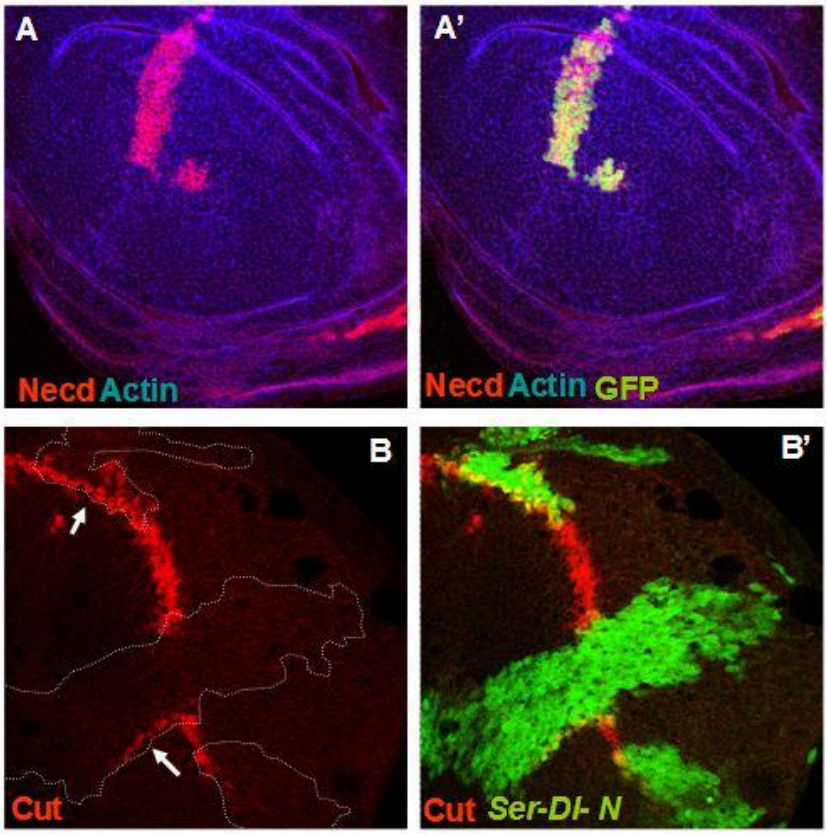


Figure 4-3. The effect of Notch overexpression on Notch signaling in ligand mutant cells at the D/V boundary in wing discs. (**A**) *hs-flp tub-gal4, UAS-gfp/+; UAS-N/+; FRT82B/FRT82B tub-gal80.* (**B**) *hs-flp tub-gal4, UAS-gfp/+; UAS-N/+; FRT82B ser*$^{-}$*Dl*^{REV}*/FRT82B tub-gal80.* To generate MARCM clones, larvae (1st and 2nd instar) were heat-shocked for 1 hour at 37°C and wing discs were isolated from 3rd instar larvae and immunolabeled with anti-Necd and anti-Cut. Actin was also labeled with phalloidin in **(A)**. Arrows indicate *cut* expression in the clones.

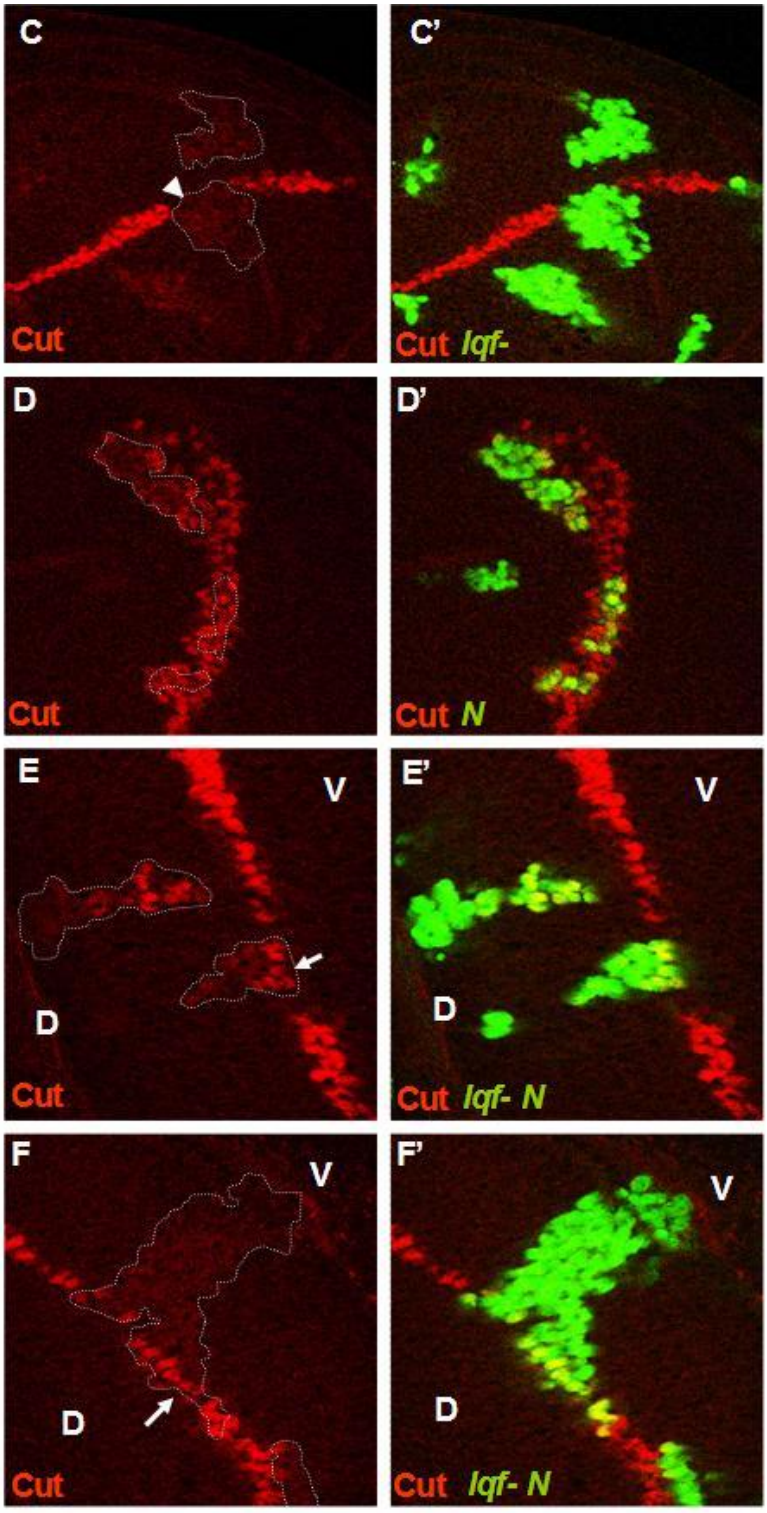


Figure 4-3 continued. The effect of Notch overexpression on Notch signaling in *epsin* mutant cells at the D/V boundary in wing discs.

Figure 4-3 continued. (C) *hs-flp tub-gal4, UAS-gfp; +/+; lqf^{L71} FRT80B/tub-gal80 FRT80B*. **(D)** *hs-flp tub-gal4, UAS-gfp/+; UAS-N/+; FRT80B/tub-gal80 FRT80B*. **(E,F)** *hs-flp tub-gal4, UAS-gfp/+; UAS-N/+; lqf^{L71} FRT80B/tub-gal80 FRT80B*. MARCM clonal wing discs were immunolabeled with anti-Cut. The arrow head in **(C)** indicates failure of *cut* expression in *lqf* mutant cells at the D/V line. Arrows indicate *cut* expression in the clones. D; dorsal, V; ventral.

Epsin uses different modules for sending and receiving Notch signal; UIMs are distinct motifs only for signal sending, not for receiving.

As an endocytic factor, epsin has multiple functional modules to route plasma-membrane cargos into cellular compartments. For sending Notch signal, ligand endocytosis is critical and epsin's function in this process was shown before. Given that epsin is required for sending and receiving Notch signal in this study, epsin might use different modules for these two distinct events. One of our lab member, Xuanhua Xie, has generated various deleted versions of genomic *lqf* constructs and tested critical motifs for giving full rescuing activity of *lqf* hypomorph and null mutations. However, direct experiments to test the effect of each module on Notch activity have not been carried out. Furthermore, these constructs might provide ways to discriminate which epsin modules are required for sending and receiving Notch signal.

To validate these ideas, *lqf-* clones were generated in wing discs with one copy of the genomic *lqf* constructs with deletions for the ENTH domain and each motif, using the MARCM technique (Figure 4-4). The wild-type *lqf* genomic construct fully recovered sending and receiving properties of *lqf-*, as monitored by *cut* expression (Figure 4-1E, F), while it rescued lethality and morphological defects caused by *lqf* null mutation in our study. The ENTH domain is known to help epsin binding to the plasma membrane and in cargo recognition. A previous study by our group showed that the ENTH deletion results in significant epsin activity, rescuing lethality of an *lqf* null, but still having mild morphological defects. Consistent with this result, the ENTH domain deletion construct rescued *cut* expression significantly (Figure 4-4A) and it appeared not to be critical for sending and receiving Notch signal (Figure 4-4B).

Drosophila epsin has two Ubiquitin Interacting Motifs (UIM1 and 2). UIM1 was shown to be critical for rescuing lethality and phenotypic defects; an epsin genomic

construct with a UIM1 deletion shows lethality at pupal stages in an *lqf* null background. *cut* expression was not recovered with the UIM1-deleted genomic construct (Figure 4-4I), but was recovered with the UIM2-deleted genomic construct (Figure 4-4B) in the *lqf-* clones spanning the D/V line. Even though *cut* expression was not recovered in *lqf-* clones spanning the D/V boundary without UIM1 and -2 (Figure 4-4C), significant *cut* expression was observed in the line of *lqf-* cells adjacent to the D/V boundary (Figure 4-4D and compare with Figure 4-1D and Figure 4-3A), suggesting that epsin UIMs are not necessary for receiving, but for sending Notch signal.

During *Drosophila* embryogenesis, follicle cells surrounding germ line cells receive Notch signal from germ line cells. In this specific developmental context, clathrin is not required for germ cells to send Notch signal, but is required for follicle cells to receive Notch signal. Given that epsin has Clathrin Binding Motifs (CBMs), clathrin and epsin binding through this motif might be required for receiving Notch signal in wing discs. However, *cut* expression was fully recovered without CBMs (Figure 4-4E), and cells in *lqf-* clones adjacent to the opposite plane and the line of normal cells facing the clones at the D/V boundary, expressed *cut* with a CBM-deleted *lqf* construct (Figure 4-4F). This suggests that CBM is not a critical epsin module for sending and receiving Notch signal in wing discs.

To further test whether different epsin modules have redundant roles for clathrin binding and signal receiving, a CBM and DPW double deletion construct was also analyzed in the same way. The CBM and DPW double deletion construct rescued *cut* expression significantly (Figure 4-4G), rescuing sending and receiving properties of *lqf-* (Figure 4-4H), suggesting that at least the DPW motif is not the redundant motif for Clathrin binding and signal receiving.

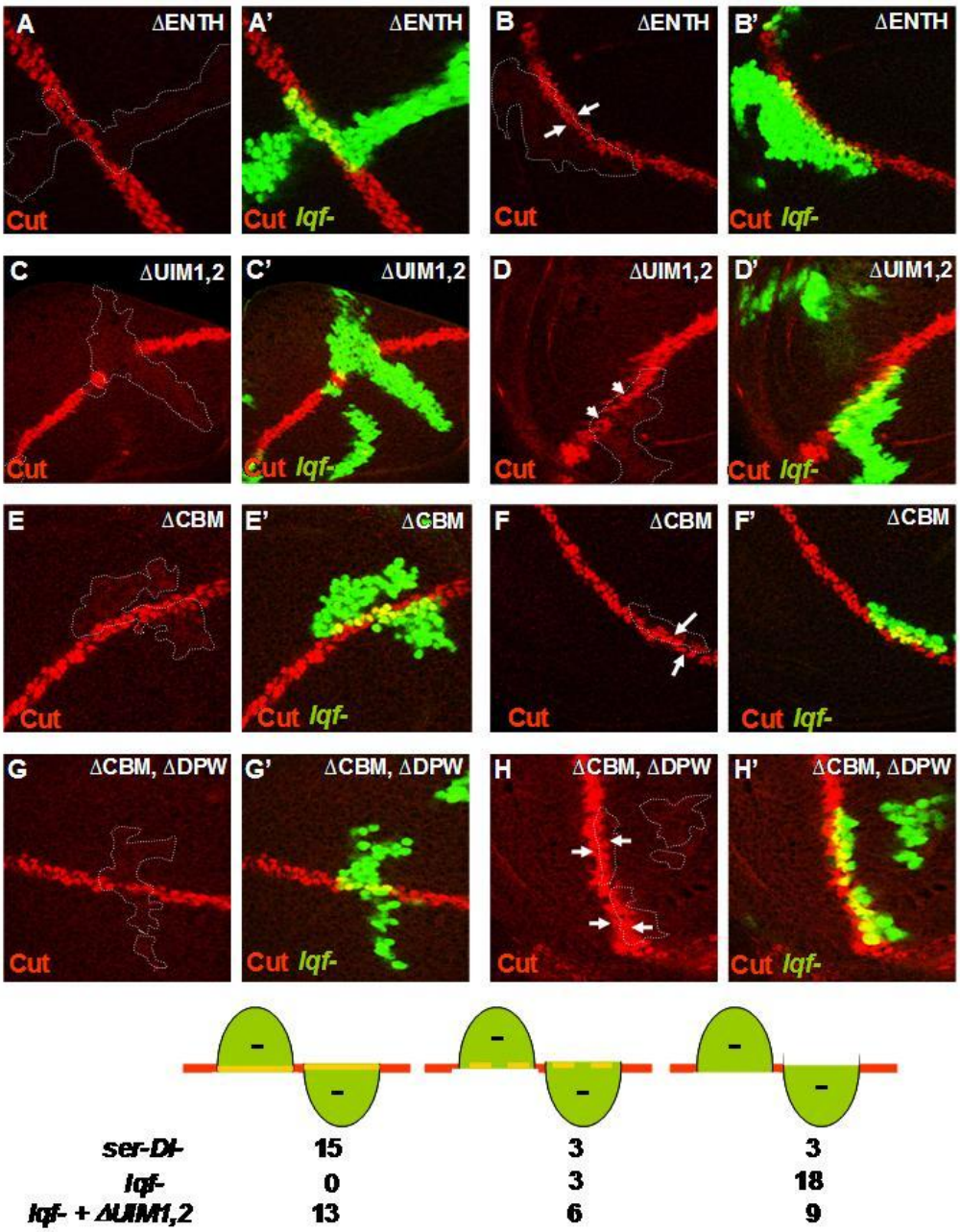


Figure 4-4. The rescuing activities of *lqf* deletion mutations in Notch signaling at the wing D/V boundary.

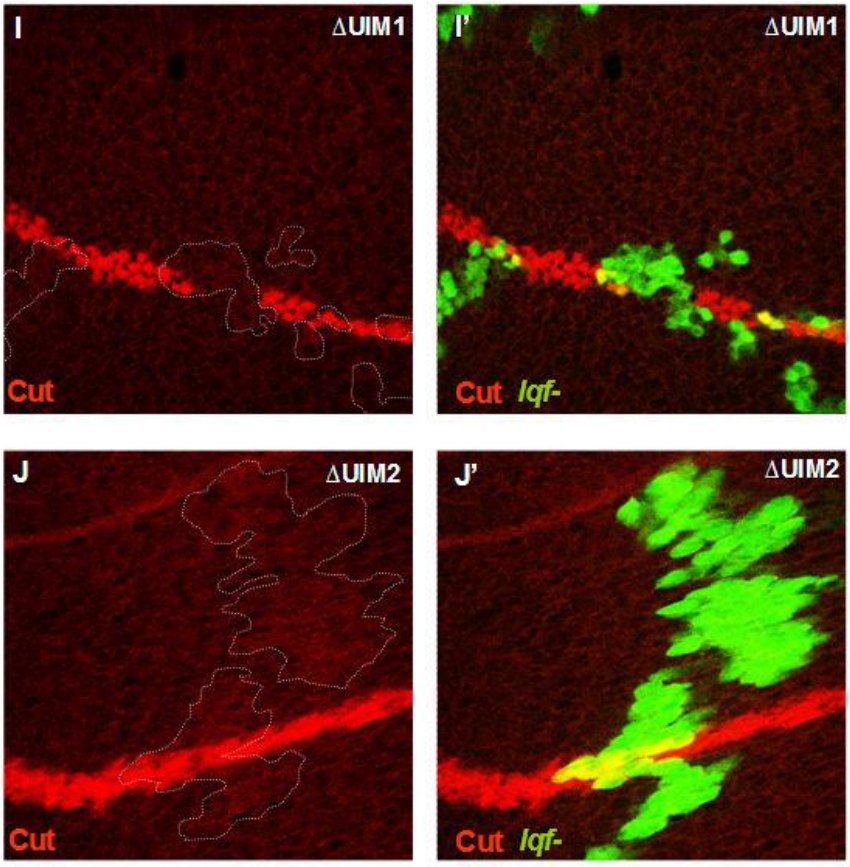


Figure 4-4 continued. The rescuing activities of *lqf* deletion mutations in Notch signaling at the wing D/V boundary. (A-J) All genotypes are the same except for the genomic *lqf* deletion constructs supplemented; *hs-flp tub-gal4, UAS-gfp/+; glqf*Δ*-gfp/+; lqf*^{L71} *FRT80B/tub-gal80 FRT80B*. Deletion geneomic constructs supplemented are **(A,B)** ENTH deletion; **(C,D)** UIM1 and -2 deletion; **(E,F)** CBM deletion; **(G,H)** CBM and DPW deletion; **(I)** UIM1 deletion; **(J)** UIM2 deletion. *lqf-* MARCM clones were generated as in previous experiments and wing discs were immunolabeled by anti-Cut. The cartoon below (G,H) represents the quantification of rescuing activity for the signal receiving property in the mutant clones contacting the D/V boundary (yellow lines; *cut* expression in mutant cells, red lines; *cut* expression in normal cells. Numbers; the number of mutant clones analyzed). Arrows indicate rescued *cut* expression in mutant and wild-type cells and the arrow heads indicate rescuing *cut* expression only in mutant cells.

UIMs (particularly UIM1), were shown to be necessary for sending, but not for receiving Notch signal in wing discs. Furthermore, other deletion genomic constructs of *lqf*, including ΔCBM, ΔDPW, ΔNPF, and ΔENTH, provided significant sending and receiving properties; only ENTH or ENTH-UIMs failed to rescue sending and receiving property of *lqf* mutants, as *cut* expression was not restored inside and outside *lqf*- clones (Figure 4-4). As UIMs appear to be the sole motifs necessary for sending Notch signal, I wanted to confirm this further by generating *Serrate* or *Delta* overexpressing *lqf* mutant clones supplemented by one copy of the UIMs-deleted genomic construct. As shown in Figure 4-5A and D, *Serrate* or *Delta* overexpression itself induced *cut* expression outside the clone boarder at the ventral or dorsal plane, respectively. However, the UIMs-deleted *lqf* genomic construct failed to rescue cut expression outside the ligand-overexpressing *lqf* mutant clones in both D/V planes (Figure 4-5B, E). I further analyzed the requirement of UIM2 in the same assay and it appeared that epsin can rescue *cut* expression without UIM2, thus UIM2 is not necessary for ligand signaling (Figure 4-5C, F). Therefore, these results suggest that epsin uses UIM1 as the main motif for both Serrate and Delta signaling.

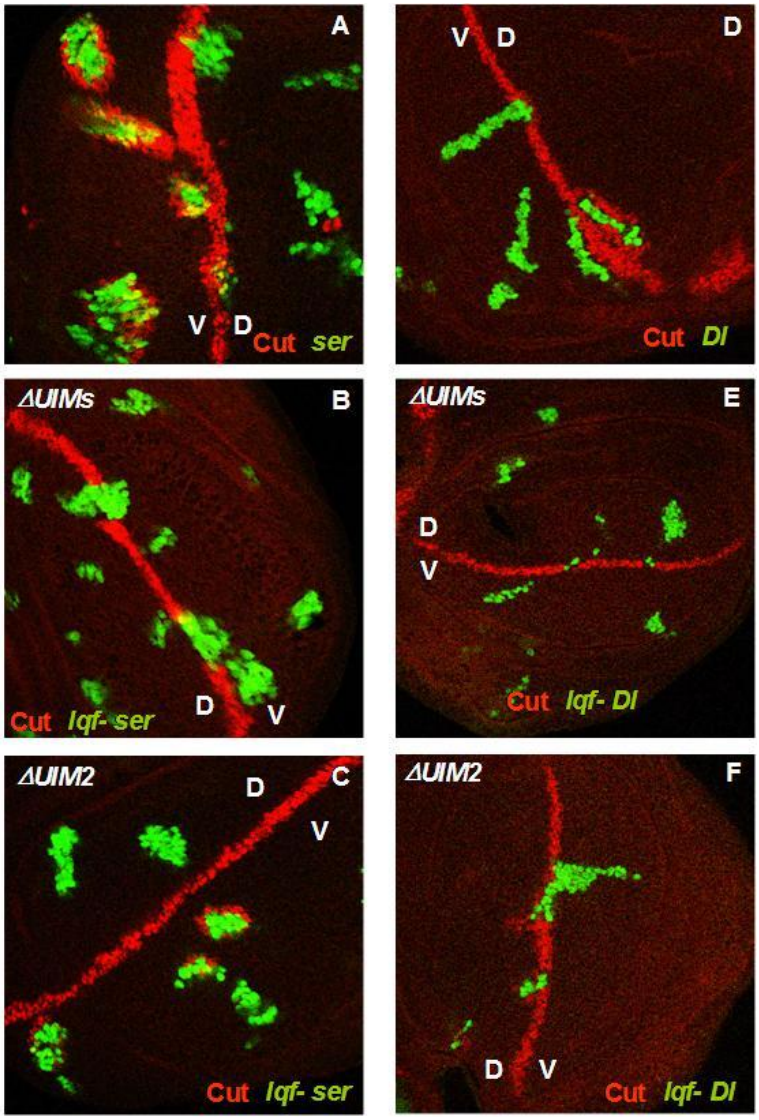


Figure 4-5. The requirement of epsin UIMs for Serrate and Delta signaling in wing discs. (A) *hs-flp tub-gal4, UAS-gfp/+; UAS-ser/+; FRT80B/tub-gal80 FRT80B*. **(B)** *hs-flp tub-gal4, UAS-gfp/+; UAS-ser/glqfΔUIMs-gfp; lqf^{L71} FRT80B/tub-gal80 FRT80B*. **(C)** *hs-flp tub-gal4, UAS-gfp/+; UAS-ser/glqfΔUIM2-gfp; lqf^{L71} FRT80B/tub-gal80 FRT80B*. **(D)** *hs-flp tub-gal4, UAS-gfp/+; UAS-Dl/+; FRT80B/tub-gal80 FRT80B*. **(E)** *hs-flp tub-gal4, UAS-gfp/+; UAS-Dl/glqfΔUIMs-gfp; lqf^{L71} FRT80B/tub-gal80 FRT80B*. **(F)** *hs-flp tub-gal4, UAS-gfp/+; UAS-Dl/glqfΔUIM2-gfp; lqf^{L71} FRT80B/tub-gal80 FRT80B*. Cut is ectopically expressed outside the border of *Serrate* and *Delta* overexpressing clones at the ventral and dorsal planes, respectably **(A,D)**. The UIM2 deletion *lqf* construct rescues ectopic *cut* expression for both cases **(C,F)**, but UIM1 and -2 deletion does not **(B,E)**. *cut* expression was analyzed as previous experiments. D; dorsal, V; ventral.

Next, I decided to analyze the activity of mutant forms of *lqf* in eye discs, focusing on the R3/R4 context. Significant numbers of *lqf* null R4 cells (~25%) lost Notch activation, as monitored by *mδ-lacZ* expression, even if R3s are *lqf*+ (Figure 4-2C, D). If the UIMs are necessary for sending but not receiving Notch signal, the UIMs-deleted *lqf* genomic construct would rescue signal receiving in *lqf* null R4s, but not rescue signal sending in *lqf* null R3s. Indeed, UIMs-deleted genomic *lqf* rescued the receiving property in *lqf*- R4, when R3 is *lqf*+, as much as the wild–type *lqf* genomic construct does (Figure 4-6A, B, C). However, whereas the wild–type *lqf* genomic construct almost completely rescued sending property in *lqf*- R3s (Figure 4-6A, B, D), UIMs-deleted genomic *lqf* failed to rescue the sending activity, as seen by loss of *mδ-lacZ* expression in R4 precursors; almost same pattern with *lqf*- control or more reversal pairs in *mδ-lacZ* expression (Figure 4-6A, B, D).

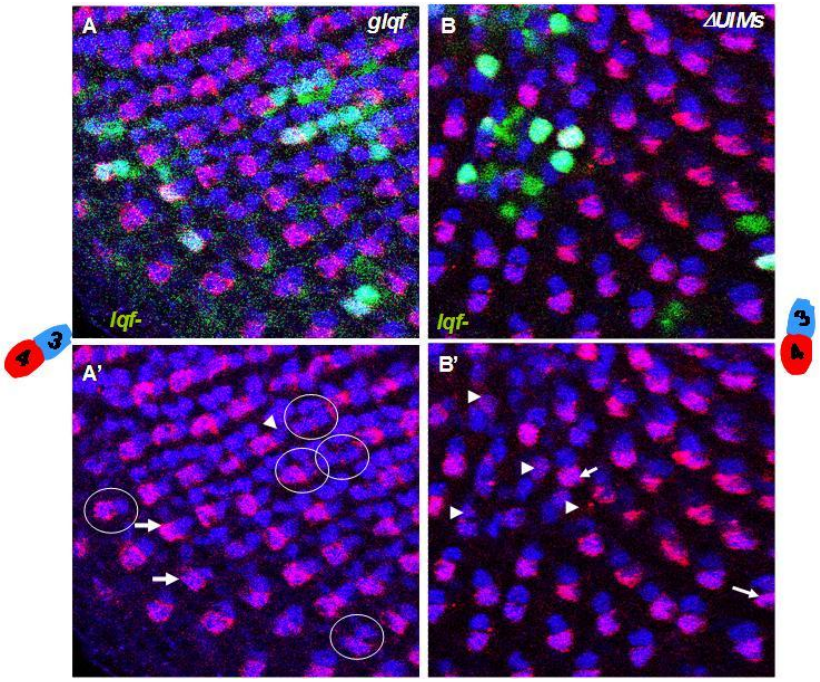


Figure 4-6. The requirement of epsin UIMs for Notch activation in R3 and R4 precursors.

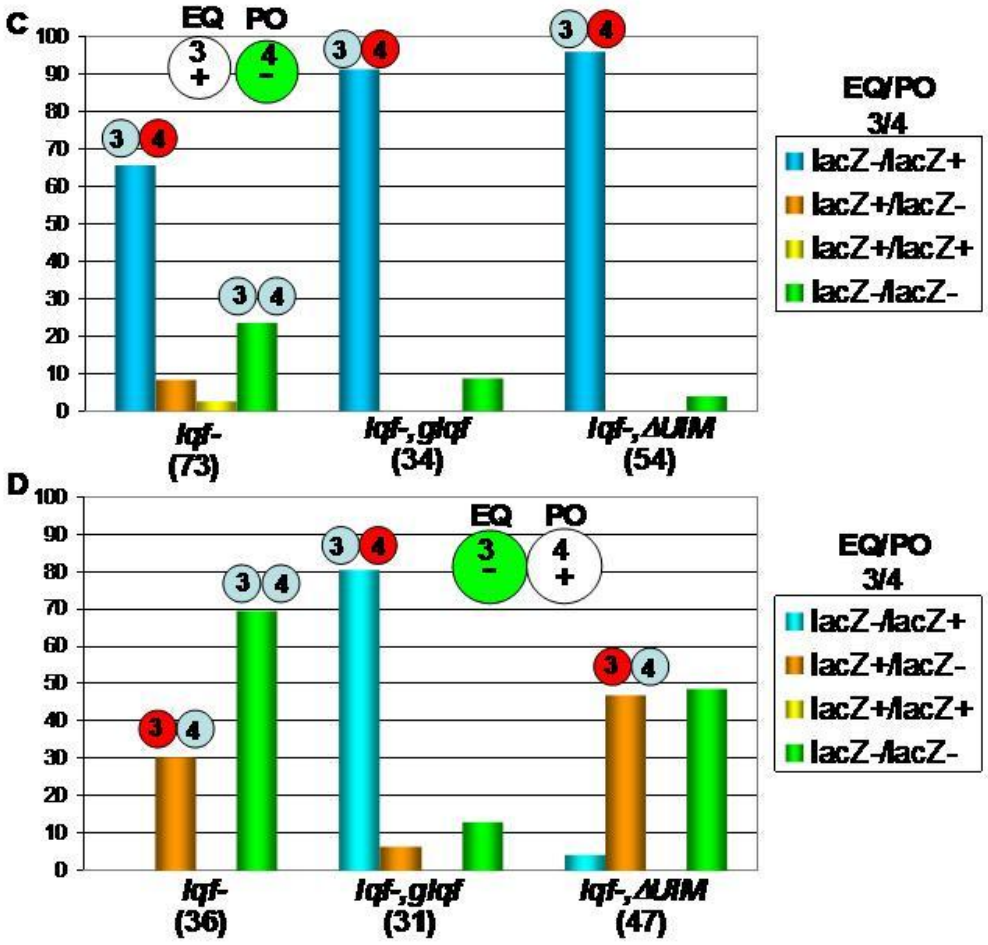


Figure 4-6 continued. The requirement of epsin UIMs for Notch activation in R3 and R4 precursors. (A) *hs-flp tub-gal4, UAS-gfp/+; glqf-gfp/+; lqf*^{L71} *FRT80B/tub-gal80 FRT80B*. **(B)** *hs-flp tub-gal4, UAS-gfp/+; glqfΔUIMs-gfp/+; lqf*^{L71} *FRT80B/tub-gal80 FRT80B*. For **(A,B)**, eye discs with *lqf-* MARCM clones were immunolabeled with anti-β-gal for testing Notch activity and anti-Svp for labeling R3/4 pairs. *mδ-lacZ* expression was analyzed in *lqf+/lqf-* mosaic R3/R4 pairs **(C),** and *lqf-/lqf+* mosaic R3/R4 pairs **(D)**. Numbers at the bottom of each graph represent the numbers of mosaic R3/4 pairs analyzed.

During oogenesis, *Delta* is expressed in germ line cells and sends signal to follicle cells to activate Notch in follicle cells. In a previous study, epsin was shown to be necessary for sending signal in germ line cells. However, epsin requirement in follicle cells for receiving signal is unknown. Because the pattern of epsin expression in ovarian cells has not been analyzed, the *lqf* expression pattern was assayed using Lqf antibody, after *lqf*- follicle cell clones were generated. Interestingly, Lqf was stained more strongly in follicle cells than in germ line cells that have the signal sending property (Figure 4-7I). Furthermore, Lqf signal was enriched in the apical surface of the follicle cells contacting germ line cells; we know this because *lqf* mutant follicle cells loose this polarity as well as overall signal intensity (Figure 4-7I), suggesting that there may be cell-cell communication events through epsin activity between germ line and follicle cells. Next, to test whether epsin is required in follicle cells for receiving a Notch signal, Notch activity was analyzed in *lqf*- follicle cells by generating *lqf*- follicle cell clones and monitoring Notch target *Hnt* (*Hindsight*) expression. *Hnt* expression was often reduced in *lqf*- follicle cells at stage 7 in which *Delta* expression, and thereby signaling activity, is highest in germ line cells (Figure 4-7A, B). These effects are likely to be caused by the reduced signal receiving property in *lqf*- follicle cells because *cut* expression was not fully repressed at the same stage in the reciprocal experiments (Figure 4-7C, D). These defects were rescued by wild-type or UIMs-deleted *lqf* constructs, suggesting that UIM motifs are not necessary for receiving Notch signal in follicle cells (Figure 4-7E-H).

Therefore, all of this experimental evidence proposes that epsin promotes Notch signal receiving as well as sending, and the signal sending property is acquired by the distinct UIM motifs which are not required for signal receiving.

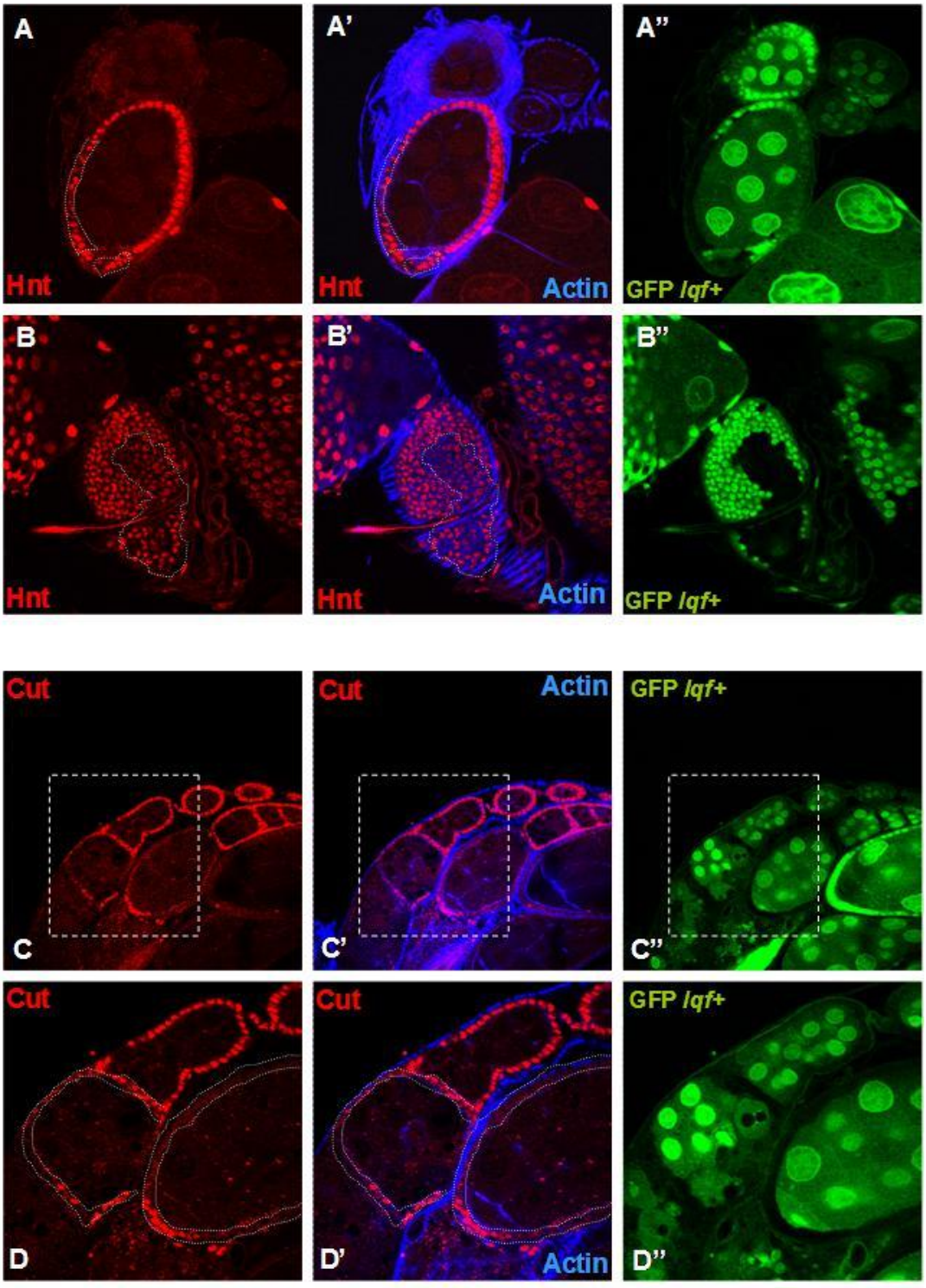


Figure 4-7. Epsin requirement in follicle cells for receiving Notch signal.

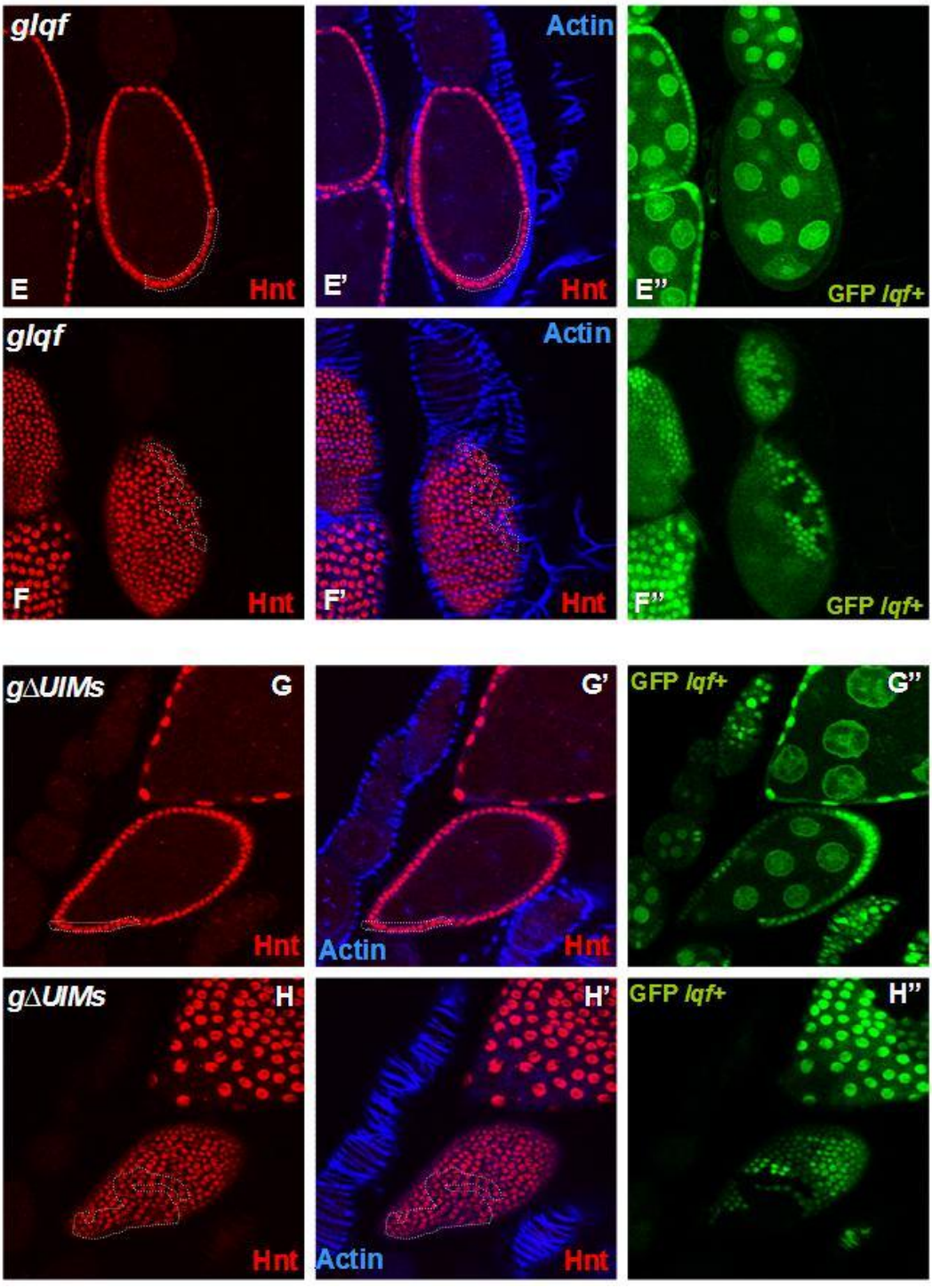


Figure 4-7 continued. Epsin requirement in follicle cells for receiving Notch signal.

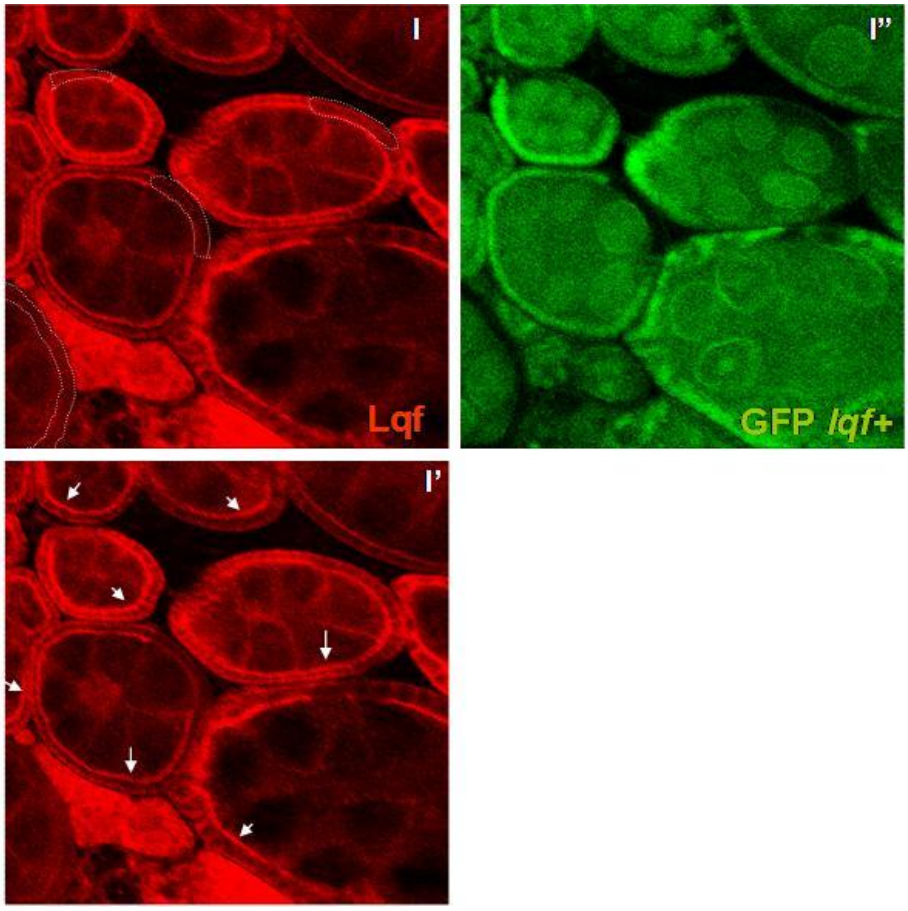


Figure 4-7 continued. Epsin requirement in follicle cells for receiving Notch signal. **(A-D, I)** *e22c-GAL4 UAS-FLP/+; lqf^{L71} FRT80B/Ubi-ngfp FRT80B*. **(E,F)** *e22c-GAL4 UAS-FLP/glqf-gfp; lqf^{L71} FRT80B/Ubi-ngfp FRT80B*. **(G,H)** *e22c-GAL4 UAS-FLP/glqfΔUIMs-gfp; lqf^{L71} FRT80B/Ubi-ngfp FRT80B*. Ovaries for each genetype were isolated and immunolabeled by anti-Hnt **(A,B,E-H)**, and anti-Cut antibody **(C,D)**. Actin was also labeled by phalloidin staining **(A-H)**. For **(I)**, ovaries were immunolabeled by anti-Lqf antibody. **(D)** Magnified images for **(C)**. Arrows in **(I)** indicate Lqf accumulation at the apical plasma membrane of follicle cells. For each image, *lqf-* follicle cell clones are marked by dotted lines.

Epsin is required for Notch routing to the Rab5 endosome and maintenance of Rab5 endosomes.

Notch overexpression recovered Notch receiving and thereby *cut* expression in *lqf* mutant cells at the D/V boundary in wing discs (Figure 4-3). This suggests that epsin could be required upstream of Notch cleavage events. In a previous study, Notch overexpression also rescued *Shi* mutation and Notch activity was rescued cell-autonomously. These results may indicate that Notch endocytosis is required for Notch cleavage and activation. Notch overexpression could provide a chance for Notch cleavage at the plasma membrane, which rarely happens under normal condition. Because clathrin and Rab5 were shown to be required in signal receiving cells for proper Notch trafficking and Notch activation, epsin may be involved in Notch trafficking events for Notch activation.

To test these ideas, the integrity of Rab5 endosomes was evaluated in *lqf*- cells compared with *lqf*+ cells by generating *lqf* mutant MARCM clones and staining Rab5 endosomes in the eye disc. At the same time, N^{+}-*GV3* (a Gal4-VP16 fused wild type Notch construct) was overexpressed by 1hr heat shock to analyze epsin dependency for Notch trafficking to Rab5 endosomes. Even though *hrs* and *Rab11* were not required for Notch activation in previous studies, integrity of these endosomes and Notch co-localization to these endosomes were also analyzed in the similar way. Interestingly, the size of Rab5 endosomes was consistently reduced in *lqf* mutant cells compared to surrounding wild type cells; in *lqf* mutant clones, the average size of Rab5 endosomes is only half of that in *lqf*+ area and small Rab5 endosmes are prevalent in the population of Rab5 endosomes (Figure 4-8). These results imply that epsin is required for the maintenance of Rab5 endosomes.

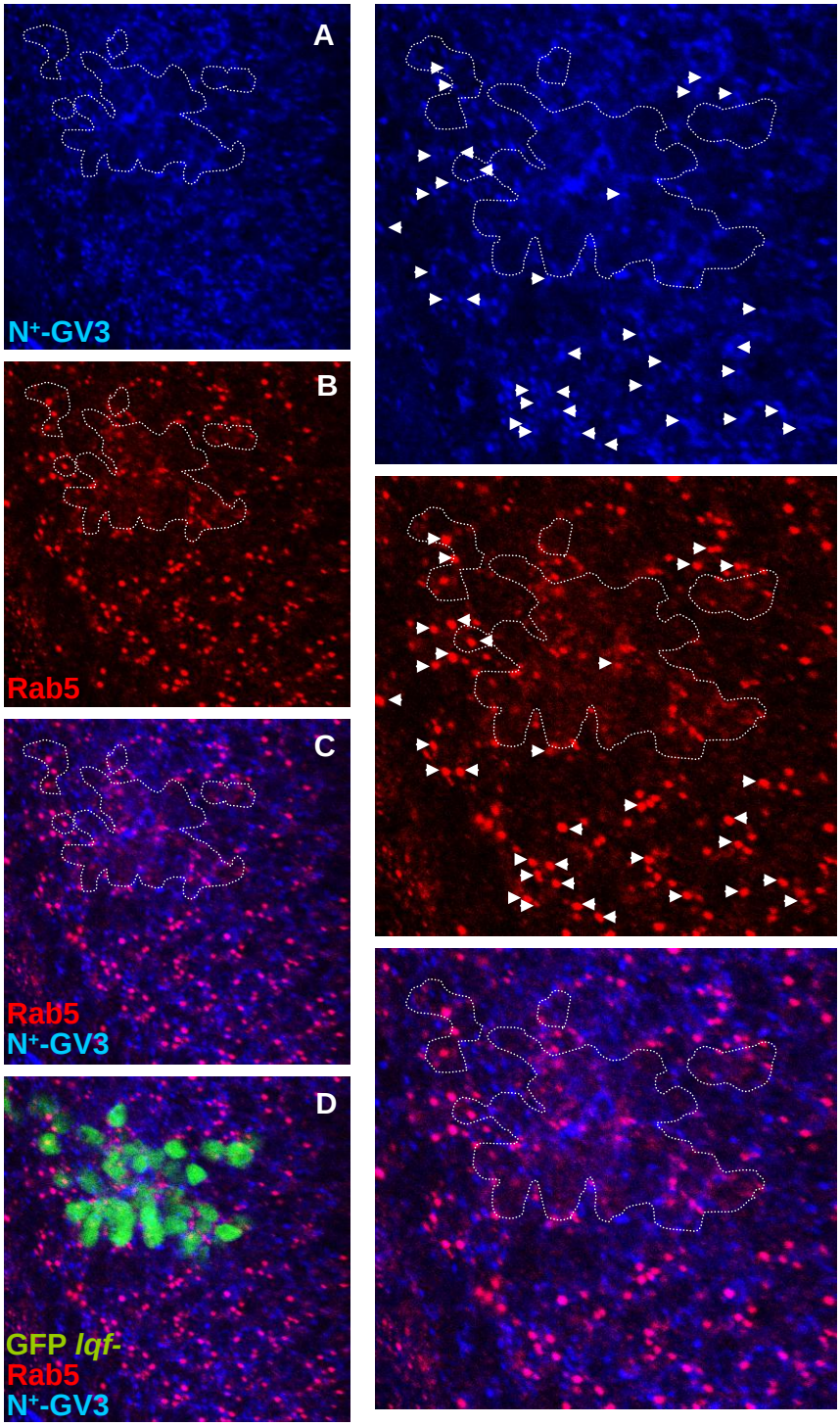


Figure 4-8. The requirement of epsin in maintaining the integrity of Rab5 endosomes.

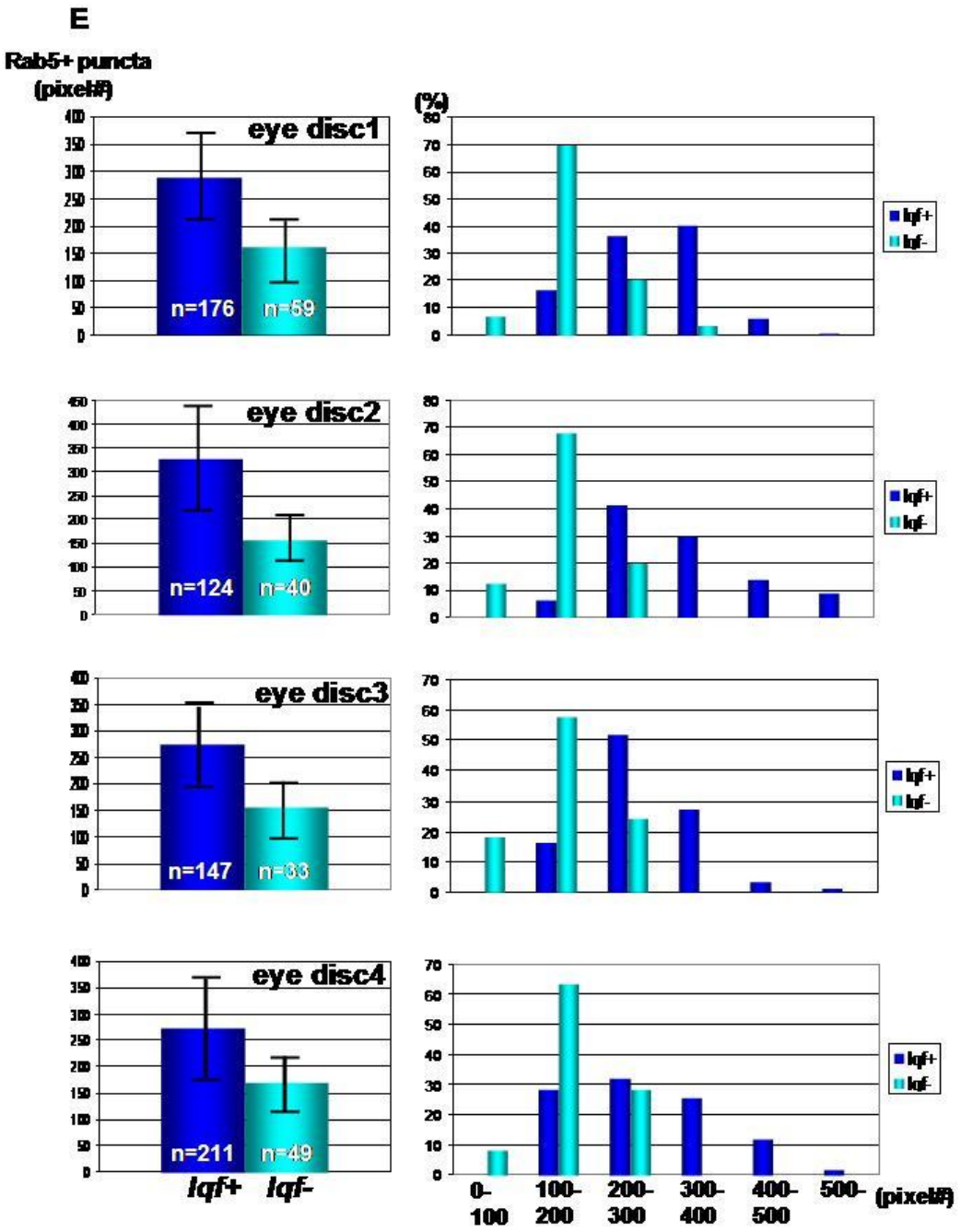


Figure 4-8 continued. The requirement of epsin in maintaining the integrity of Rab5 endosomes.

Figure 4-8 continued. (A-D) *hs-flp tub-gal4, UAS-gfp/+; hs-N^{+}-GV3/+; lqf^{k71} FRT80B/tub-gal80 FRT80B*. *lqf-* MARCM clones were generated in eye discs and the *N^{+}-GV3* construct was overexpressed by 1hr heat shock (see the material and method for details). Clonal eye discs were immunolabeled by anti-Rab5 and anti-Necd. Enlarged images are shown at the right-hand side. Arrow heads indicate Notch- and Rab5- double positive puncta (see also Figure 4-10 for quantification analysis). **(E)** Quantification of the Rab5 endosome size using the Adobe Photoshop software. The average pixel number for four different clonal eye discs are in the left side. Graphs in the right side represent the percentage of the Rab5 population by the pixel differences for each eye disc (more detailed statistical analysis will be carried out and see the material and method for details).

Next, I analyzed the number of Notch positive puncta co-stained with Rab5 endosomes compared to the total number of Rab5 endosomes, with or without *lqf*. In *lqf* mutant clones, fewer Notch positive puncta were merged with Rab5 puncta in each individual *lqf* clonal eye disc, suggesting that Notch routing to the Rab5 endosomes is dependent on epsin function (Figure 4- 8 and Figure 4-10E, F). Ubiquitin Interacting Motifs (UIMs) were not critical for receiving Notch signal and Notch activation (Figure 4-3). Therefore, these epsin dependencies might be dispensable for ligand binding to epsin, and UIMs of epsin may not be required for Rab5 endosome integrity and Notch routing to the Rab5 endosome. Indeed, one copy of the UIMs-deleted *lqf* genomic construct rescued the size of Rab5 endosomes and the efficiency of co-localization of Notch to Rab5 endosomes (Figure 4-9 and Figure 4-10E-G). Based on the epsin function in cargo internalization and curveture formation at the plasma membrane, these and the above results indicate that epsin promotes Notch endocytosis and vesicle formation of Rab5 endosomes. Furthermore, these results propose the possibility that epsin interacts with the Notch intracellular domain (directly or indirectly) to internalize Notch (a specific role of epsin as an adaptor) as well as promote membrane curvature formation for Rab5 endosome integrity (I failed to see epsin-dependency in similar experiments with Hrs or Rab11 endosomes, including Notch localization to these endosomes).

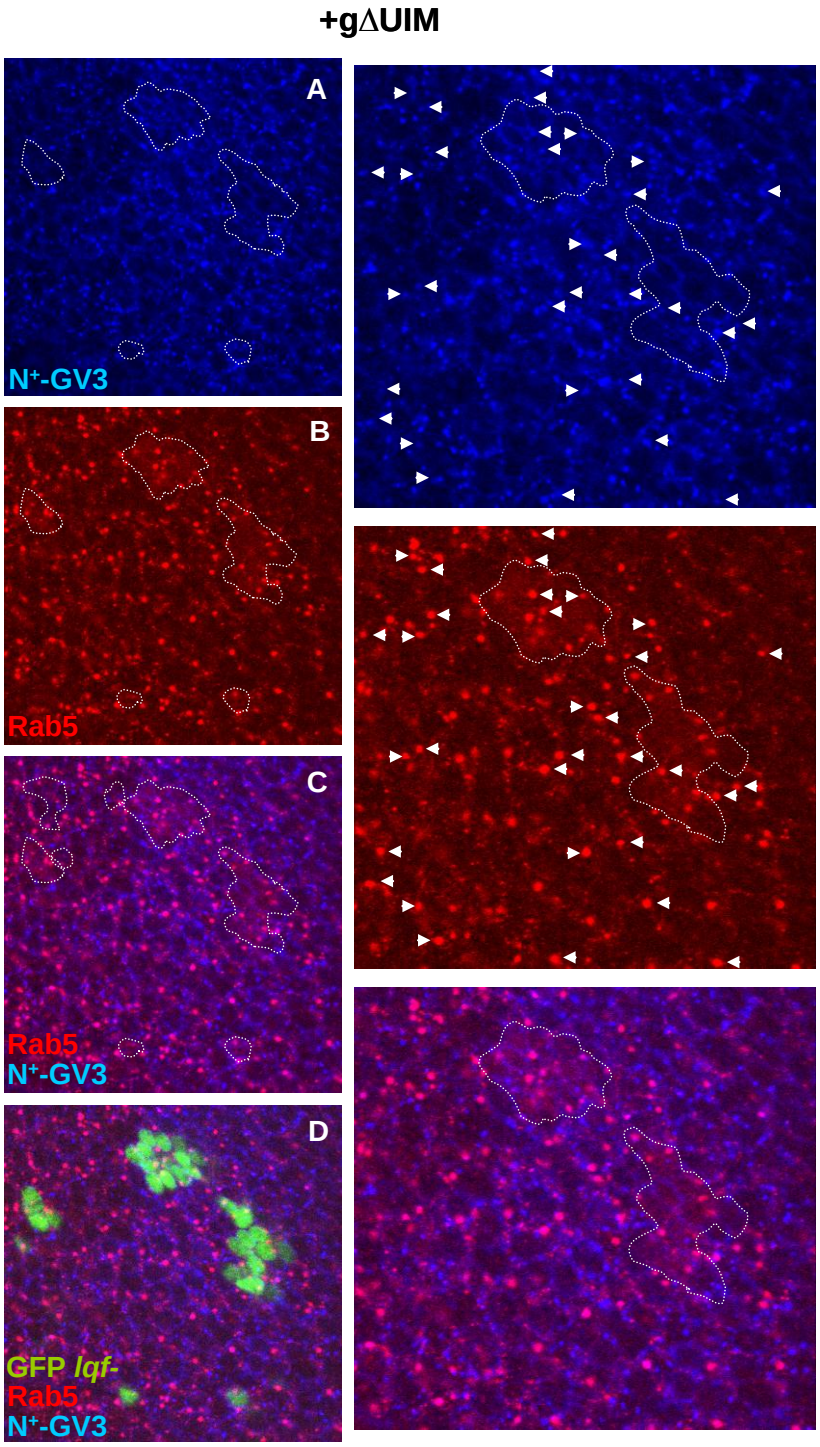


Figure 4-9. The requirement of epsin UIMs in maintaining the integrity of Rab5 endosomes.

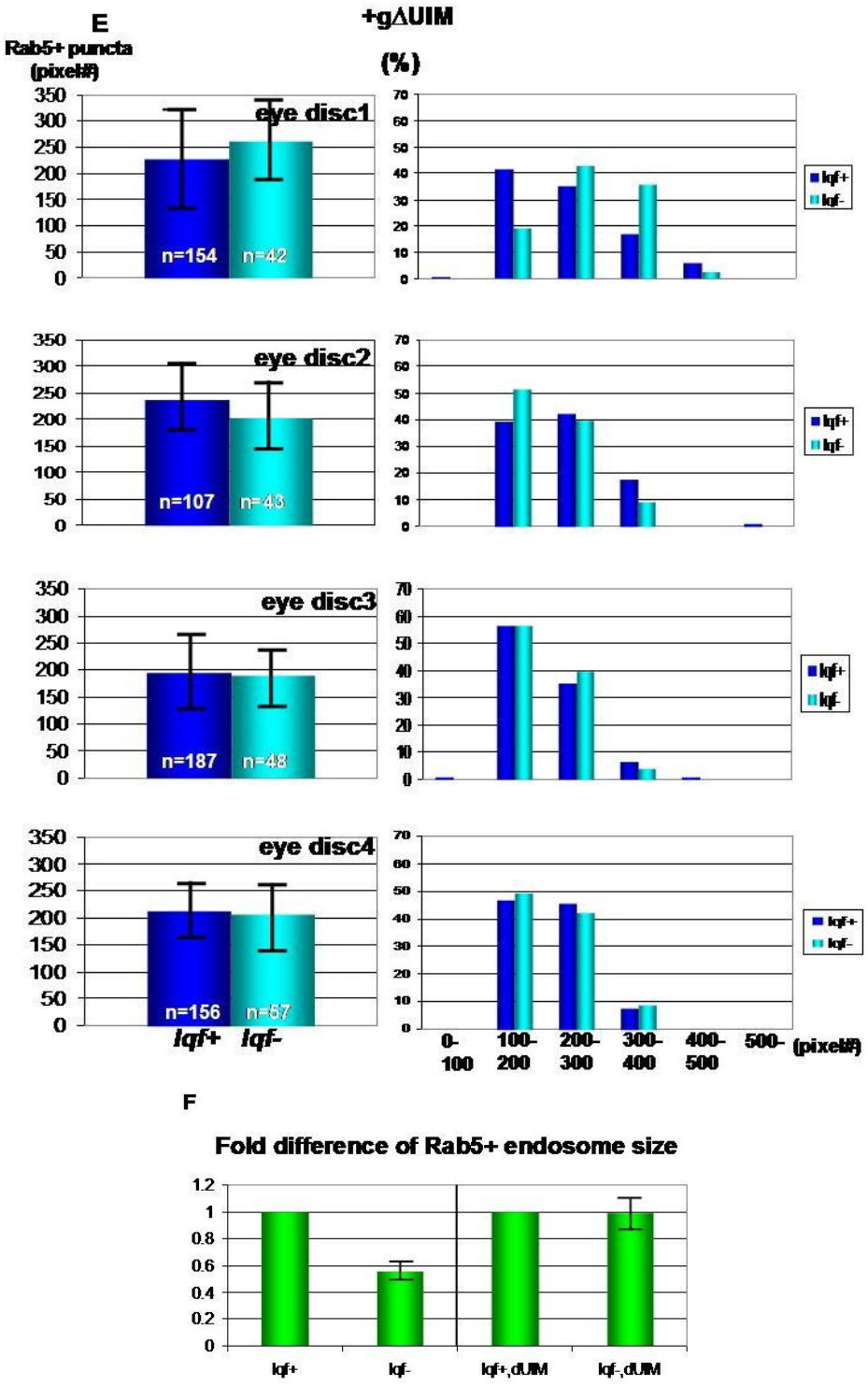


Figure 4-9 continued. The requirement of epsin UIMs in maintaining the integrity of Rab5 endosomes.

Figure 4-9 continued. (A-D) *hs-flp tub-gal4, UAS-gfp/+; hs-N^{+}-GV3/glqfΔUIMs-gfp; lqf^{k71} FRT80B/tub-gal80 FRT80B*. *lqf-* MARCM clonal eye discs were generated as in Figure 4-8 and one copy of the UIMs-deleted genomic *lqf* construct was provided. Clonal eye discs were immunolabeled by anti-Rab5 and anti-Necd. Enlarged images are shown at the right hand side. Arrow heads indicate Notch- and Rab5- double positive puncta. **(E)** Quantification of the Rab5 endosome size using the Adobe Photoshop software. The average pixel number for four clonal eye discs is in the left side. Graphs in the right side represent the percentage of the Rab5 population by the pixel differences for each eye disc (see the material and method for details). **(F)** The fold deference of the Rab5+ endosme size. The UIMs-deleted genomic *lqf* construct restores the Rab5 endosome size (more detail statistical analysis will be carried out).

If epsin plays a role as a direct or indirect adaptor for the Notch receptor, the Notch intracellular domain (Nicd) would be required for epsin-mediated endocytosis and Notch targeting to the Rab5 endosome. To validate this, epsin dependency on the trafficking of N^{+}-GV (Gal4-VP16 fused intracellular domain truncated form of Notch, Struhl and Adachi, 2000)) was analyzed. In this experiment, the size of Rab5 endosomes was reduced in *lqf-* clones as much as seen in experiments above (Figure 4-10A-D), showing epsin dependency again. However, routing of N^{+}-GV to Rab5 endosomes was not affected in the absence of *lqf* (Figure 4-10). These results and results above indicate that the Nicd is required for epsin-dependent trafficking of Notch, which is targeted to Rab5 endosomes. Previously, Notch/ligand cis-complexes were reported to be targeted to the endosomal degradation pathway. In this scenario, if epsin endocytoses Delta bound to Notch, the intracellar truncated version of Notch would be endocytosed by epsin indirectly. However, in this experiment, no epsin dependency on routing the intracellular truncated Notch to Rab5 endosome was observed, meaning that this event is independent from epsin-mediated ligand endocytosis for the cis-complex trafficking.

Taken together, these results suggest that epsin exerts its roles as a specific adaptor (via direct or indirect binding to Notch) for the Notch receptor as well as a general factor for vesicle formation, which contributes the integrity of Rab5 endosomes and Notch routing to the Rab5 endosme.

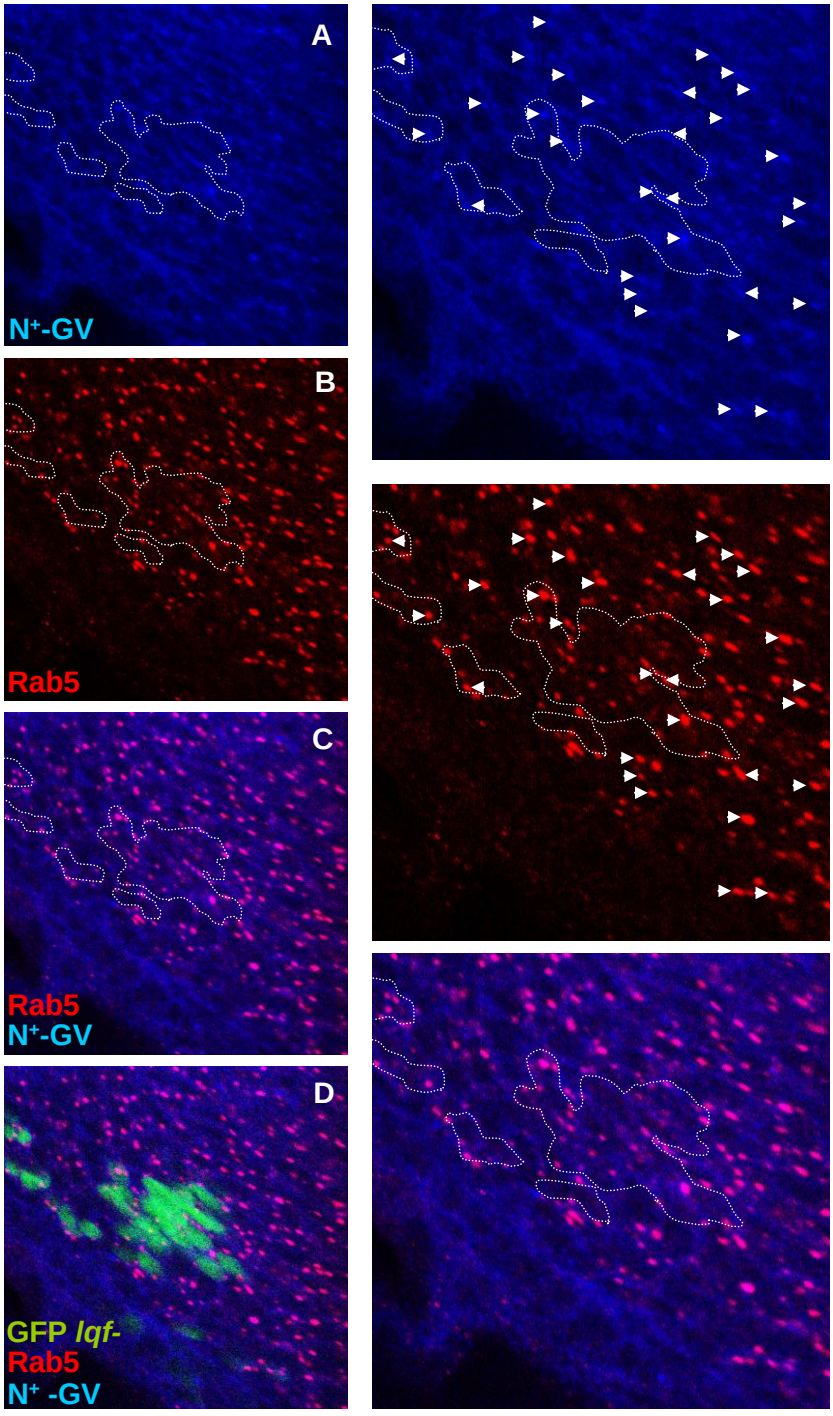


Figure 4-10. Requirement of the Nicd in epsin-dependent Notch routing to Rab5 endosomes.

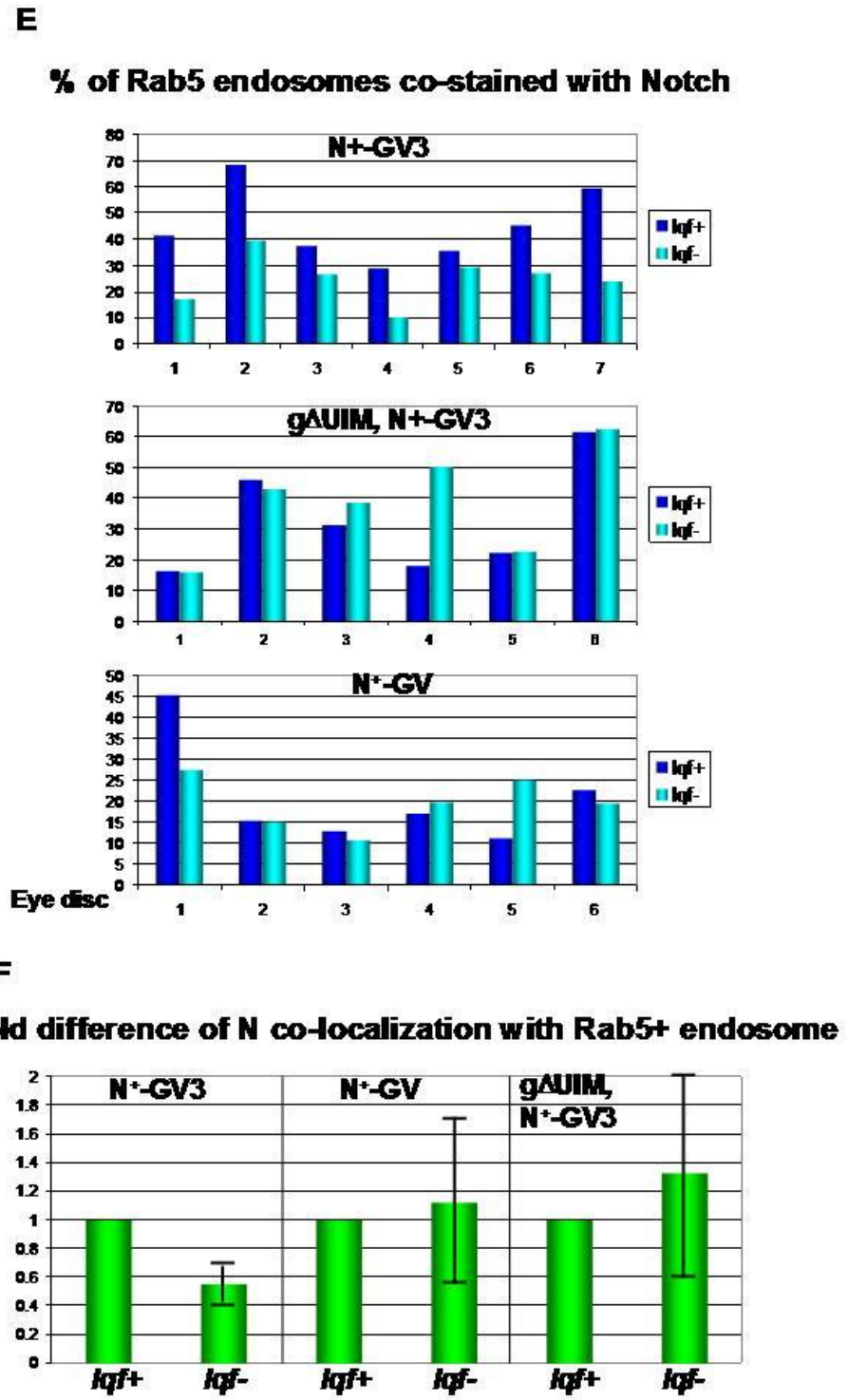


Figure 4-10 continued. Requirement of the Nicd in epsin-dependent Notch routing to Rab5 endosomes.

Figure 4-10 continued. (A-D) *hs-flp tub-gal4, UAS-gfp/+; hs-N^{+}-GV/+; lqf$^{\Delta 71}$ FRT80B/tub-gal80 FRT80B*. *lqf-* MARCM clonal eye discs were generated as in Figure 4-8, but the Nicd truncated form of the Notch construct, *N^{+}-GV*, was overexpressed. Clonal eye discs were immunolabeled by anti-Rab5 and anti-Necd. Enlarged images are shown at the right hand side. Arrow heads indicate Notch- and Rab5-positive puncta. **(E)** The percentage of Notch+ puncta co-stained with Rab5 out of total Rab5+ puncta in *lqf*+ and *lqf-* cells for the individual eye disc with the given genotype. **(F)** The fold deferences for the co-localization of Notch with Rab5 endosomes compared by *lqf*+ cells. The UIMs-deleted genomic *lqf* construct restores the co-localization efficiency and Notch localization to Rab5 endosomes is epsin-independent if Nicd is deleted (with the *N^{+}-GV* construct) (more detailed statistical analysis will be carried out).

***lqf* genetically interacts with *rab5* for regulating Notch signaling.**

Experimental results so far suggest that epsin is required for internalizing Notch receptors which are compartmentalized to Rab5 endosomes. These events might be simply related to continuous membrane trafficking events, not restricted to the Notch activation process accompanied by the Rab5 endosomal pathway. Because cellular functions of epsin are poorly understood, we can not rule out the possibility that defects in Notch trafficking and the Rab5 endosome integrity, caused by *lqf* mutation, are simply by-products due to general cell-architectural disorders in *lqf* mutant cells. To test whether epsin is involved in the Rab5-mediated Notch activation pathway, genetic interactions between these two genes were analyzed.

If *lqf* mutations affect the Rab5-mediated pathway, reducing gene dosages of both *lqf* and *rab5* would cause more severe defects than reducing one gene's dosage. Indeed, one copy of a *rab5* null allele, $rab5^{2}$, dominantly enhanced the lethality of *lqf* hypomorph. Furthermore, one escaper and the flies in the pupal cases have enhanced morphological defects compared to *lqf* hyphomorphs themselves (Figure 4-11C, D). With a weak *rab5* allele, I also observed morphological enhancement, not lethality (Figure 4-11B).

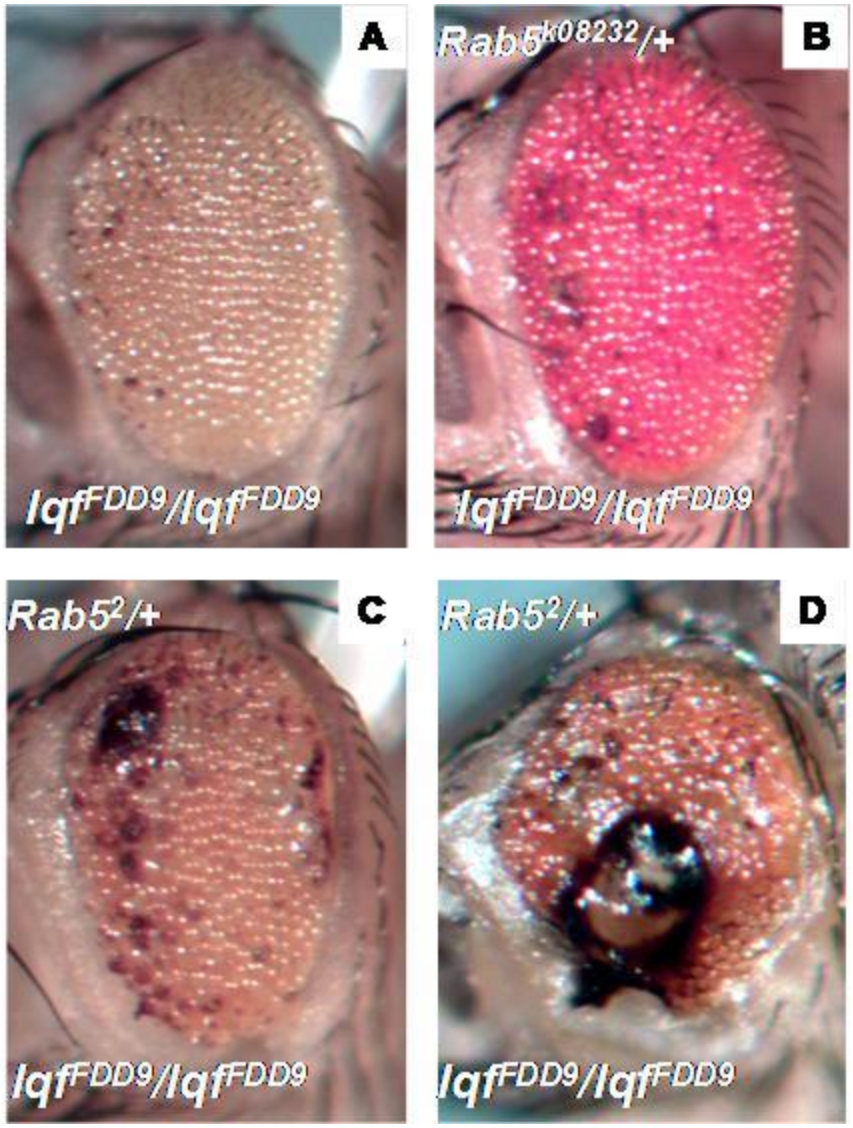


Figure 4-11. Genetic interactions of *lqf* with loss-of-function *rab5* mutants. (A-D) One copy of a hypomorphic *rab5* allele, $rab5^{k08232}$, and a null allele, $rab5^{2}$, were tested for genetic interaction with *lqf*. As an *lqf* mutant background, the homozygous lqf^{FDD9} mutation was used. **(A)** $+/+;\ lqf^{FDD9}/lqf^{FDD9}$, **(B)** $rab5^{k08232}/+;\ lqf^{FDD9}/lqf^{FDD9}$, **(C)** an escaper of $rab5^{2}/+;\ lqf^{FDD9}/lqf^{FDD9}$ **(D)** the pupal case of $rab5^{2}/+;\ lqf^{FDD9}/lqf^{FDD9}$ fly was eclosed and the eye image was taken.

To confirm these genetic interactions, I decided to analyze whether a gain-of-function *rab5*, a constitutively active form of *rab5* (*yfp-rab5*^{CA24}), also genetically interacts with *lqf*. The *UAS-yfp-rab5*^{CA24} construct was overexpressed by the *ey-GAL4* driver from early to late eye developmental stages, and the effects of loss of *lqf* activity in this background were tested. Constitutively active forms of small GTPases were reported to sequester binding partners, which causes similar effects to loss-of-function mutations, as well as showing gain-of-function effects. If epsin and Rab5 share the same pathways, defects caused by overexpression of the constitutively active form of Rab5 would be modified by the gene dosage of *lqf*; either enhanced or suppressed. As controls, null mutations of lignads, *neur*, and *Notch* were analyzed in the *yfp-rab5*^{CA24} overexpressing background to test whether Notch signaling events are modified. Eye specific overexpression of the constitutively active form of Rab5 caused subtle defects in the eye morphology; slightly disorganized ommatidia (Figure 4-12B). One copy of *Dl*, or *ser* and *Dl* double mutants dominantly enhanced the eye morphology caused by the constitutively active form of Rab5; small and rough eyes (Figure 4-12D, E). In rare cases, one of two eyes are not properly developed in these flies (Figure 4-12E'), which is also observed by *Notch* mutation in the same genetic background (Figure 4-12H), suggesting that the constitutively active form of Rab5 purterbs Notch signaling events in the eye. However, the reduced *neur* gene dosage failed to cause obvious morphological enhancement (Figure 4-12F, G); possibly because *neur* is required in late eye differentiation, but not in early eye fate determination stages, and the single copy of *neur* gives enough activity for ligand signaling. Interestingly, one copy of *lqf* null mutation enhanced the morphological defects, but in different ways from ligand mutations (Figure 4-12I, J); the overall eye shape and ommatidial organization were changed, but not in the eye size, or in rare cases, the eye was not formed (Figure 4-12J). Remarkably, further reducing Lqf activity by

using the homozygous *lqf* hypomorph caused loss of eyes, and sometimes the antenna was formed instead of the eye in the eye position (Figure 4-12K), which may imply a homeotic transformation. Reduced Notch activity caused the eye-to-antenna homeotic transformation in previous studies.

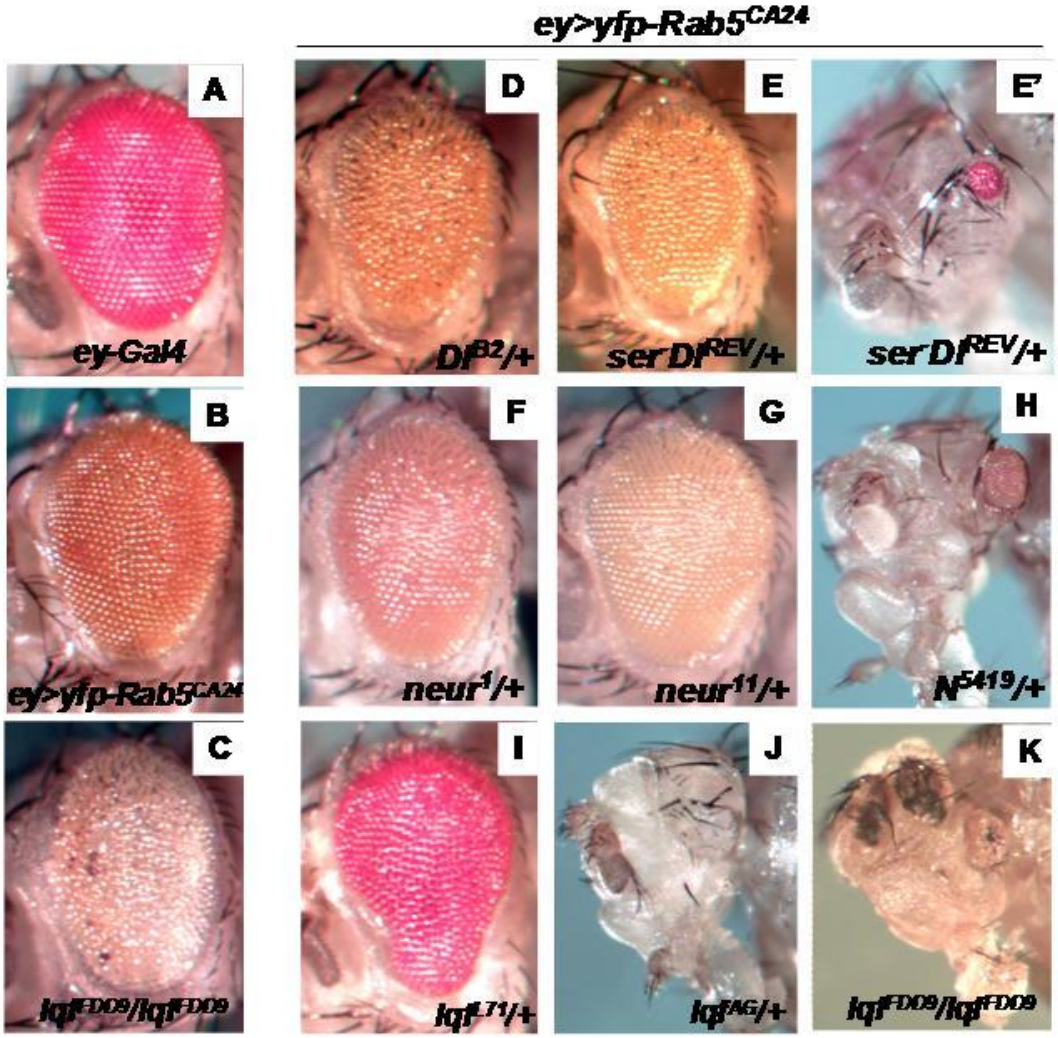


Figure 4-12. Genetic interactions of *lqf* and Notch signaling components with a gain-of-function *rab5* mutant. (A) *ey-gal4/+*, **(B)** *ey-gal4/UAS-yfp-rab5^{CA24}*, **(C)** *lqf^{FDD9}/lqf^{FDD9}*, **(D)** *ey-gal4/UAS-yfp-rab5^{CA24}; Dl^{B2}/+*, **(E, E')** *ey-gal4/UAS-yfp-rab5^{CA24}; ser Dl^{REV}/+* **(F)** *ey-gal4/UAS-yfp-rab5^{CA24}; neur^{1}/+* **(G)** *ey-gal4/UAS-yfp-rab5^{CA24}; neur^{11}/+* **(H)** *N^{5149}/+; ey-gal4/UAS-yfp-rab5^{CA24}*, **(I)** *ey-gal4/UAS-yfp-rab5^{CA24}; lqf^{L71}/+*, **(J)** *ey-gal4/UAS-yfp-rab5^{CA24}; lqf^{AG}/+*, **(K)** *ey-gal4/UAS-yfp-rab5^{CA24}; lqf^{FDD9}/lqf^{FDD9}*.

To clarify this, eye discs were isolated from third instar larvae with the given genotypes, and the pattern of Elav, an eye neural cell marker, was analyzed. In many cases, overexpression of the constitutively active form of *rab5* in a homozygous *lqf* hypomorphic background caused the eye to anntena transformation; no eye disc, but two antenna discs without Elav staining (Figure 4-13C', compare with A and B). All of these results suggest that Rab5 plays a role in Notch activation and the relationship between epsin and Rab5 is distinct from those between ligands (or neur) and Rab5; epsin and Rab5 are also involved in developmental organ shape control and sometimes the interaction is stronger than those with other Notch components.

The constitutively active form of Rab5 failed to show defects in Notch signaling in the R3/R4 pairs, when the overexpressing clones were generated; the construct we used might have weak activity, not that the expression level matters, because a strong Gal4 driver (*tub-GAL4*) was used in the clonal assay. The effects on Notch signaling in R3/R4 pairs might be detectable if we used the *rab5* loss-of-function mutation. However, *rab5* null clones failed to be generated in our and previous studies. Furthermore, *rab5* null eyes generated by the *minute* technique lose neural cells, but, proliferating cells were overgrown in our and previous studies. *rab5* null germ line cells still sent signal as normal germ cells do, but *lqf* null germ line cells completely failed to send signal in previous studies. This suggests that they are neither in a linear pathway nor in a redundant pathway in signal sending cells for signal sending. Furthermore, the relationship between *lqf* and *rab5* showed different outcomes from those between ligands and *rab5*. Therefore, the genetic interaction causing the typical Notch defect (the eye-to-antenna transformation) observed in this study is likely due to the combinatorial effects between the cell-nonautonomous role of epsin and the *cis* role of epsin related to the Rab5 pathway even though further studies are required.

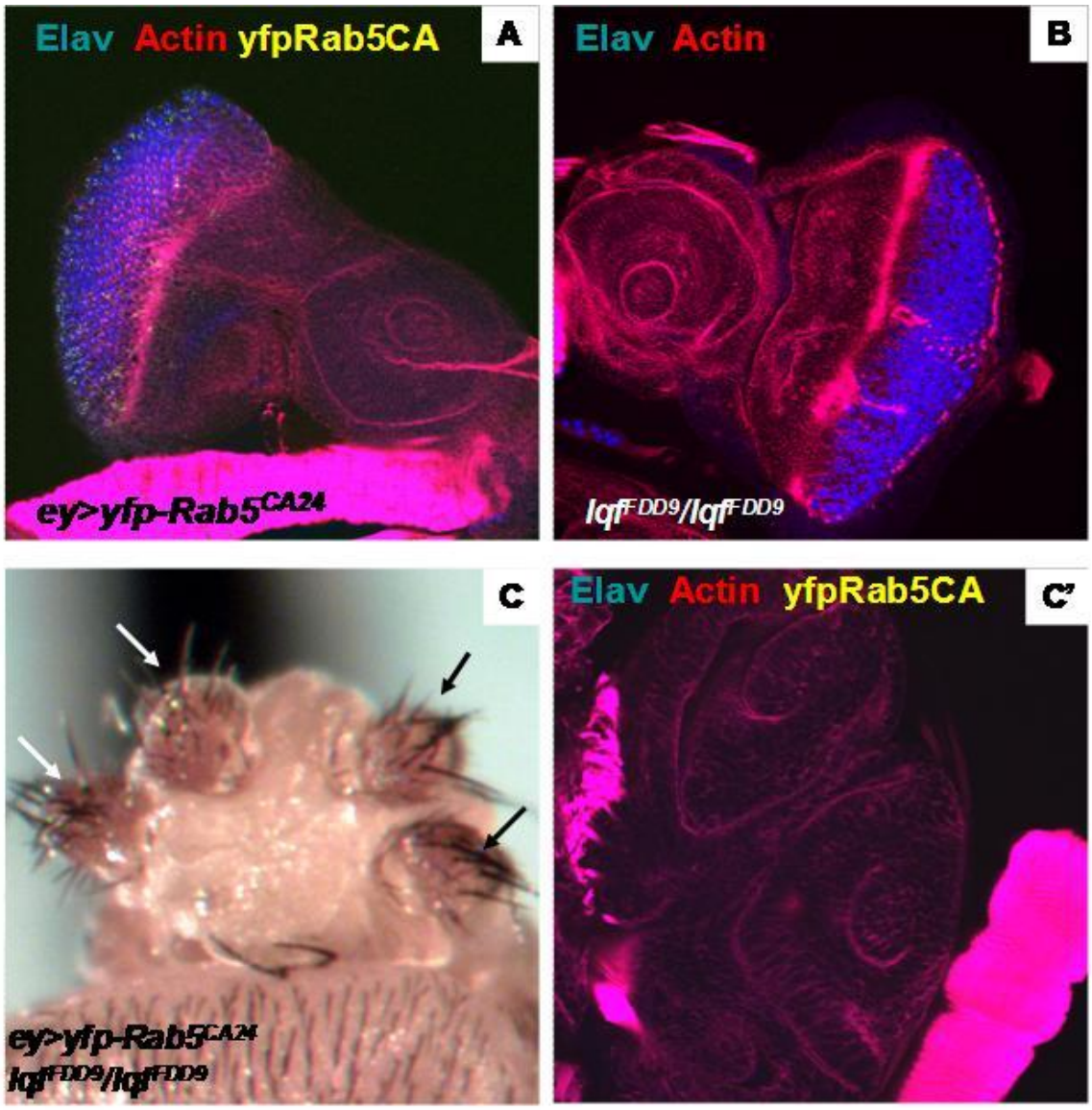


Figure 4-13. Genetic interactions of *epsin* with a gain-of-function *rab5* mutant. Eye-antenna discs from 3rd instar larvae with genotype **(A)** *ey-gal4/UAS-yfp-rab5^{CA24}*, **(B)** *lqf^{FDD9}/lqf^{FDD9}*, and **(C')** *ey-gal4/UAS-yfp-rab5^{CA24}; lqf^{FDD9}/lqf^{FDD9}* were isolated, and labeled with anti-Elav for photoreceptor neural cells and phalloidin for Actin. **(C)** A dorsal view of *ey-gal4/UAS-yfp-rab5^{CA24}; lqf^{FDD9}/lqf^{FDD9}* fly. Arrows indicate antenna, including two extra ones.

DISCUSSION

The roles of *Drosophila* endocytic epsin in Notch signaling.

Endocytic epsin is an evolutionarily conserved factor for vesicle trafficking. However, its cellular functions in multicellular organisms have been poorly understood until our and other groups found epsin function in Notch signaling-mediated developmental processes in *Drosophila*. Furthermore, the role of epsin in Notch signaling appears to be conserved in higher organisms, such as mammals. In previous *Drosophila* studies, epsin was shown to be a very specific factor for Notch ligand endocytosis and Notch activation in nearby cells. However, despite strong evidence suppoting the requirement of epsin in signal sending cells, its role in signal receiving cells for Notch activation has not been clarified. In a previous *Drosophila* wing study, ligand overexpression near *lqf* mutant cells, or *fng* overexpression in *lqf* mutant cells showed Notch activation in the *lqf* mutant cells by receiving signal from nearby lqf^{+} cells, suggesting that epsin is not the critical factor for receiving the Notch signal. Again, in my study, the effect of *lqf* mutation is greater in signal sending cells than in signal receiving cells. Nevertheless, in my and the previous data (even though they did not point it out), *lqf* mutant cells often failed to receive Notch signaling under physiological conditions although nearby signal sending cells were normal. Moreover, in my study, Notch defects were not observed in ligand or *neur* mutant cells *in cis*, suggesting epsin has a distinct function(s) seperable from the cell-nonautonomous role as seen in functions of ligands and Neur.

Even if no *in vivo* study was previously carried out, the Ubiquitin Interacting Motifs (UIMs) of epsin were suggested to exert internalization of ubiquitinated cargos, and other studies showed that the UIMs are important for the activity control of epsin

itself by regulating the ubiquitination status of epsin. If the UIM induces ubiquitination of epsin, thereby reducing epsin activity, as shown in previous studies, the UIMs-deleted *lqf* genomic construct would increase epsin activity and rescue both signal sending and receiving. In my *Drosophila* epsin study, UIMs-deleted epsin rescued Notch receiving, but failed to rescue signal sending. These indicate that epsin UIMs are likely to provide the signal sending property of epsin, not related to control of epsin activity. These propose that UIMs of epsin provide the specificity for ligand signaling of epsin, which is dispensable for epsin's cell-autonomous roles.

For the working mechanism of epsin in cargo internalization, several ideas have been suggested; as an adaptor for cargo recognition, or as an accessory factor for promoting AP2-dependent or independent membrane vesicle formation, either clathrin-dependently or independently. In this study, I showed that epsin works as both an adaptor and an accessory factor, in which epsin promotes Notch internalization to the Rab5 endosome, and maintains the integrity of Rab5 endosomes, likely by promoting vesicle formation. Neverthless, experimental results also showed that epsin might not be the sole factor for Notch internalization. Taken together, I propose that part of Notch trafficking events are regulated by epsin, by which the Notch signal transduction pathway can be regulated.

Meaning of epsin-mediated Notch internalization in Notch activation

As I mentioned before, control of Notch trafficking is important for regulating Notch signaling in various developmental contexts. The membranous Notch level is controlled by this process as other receptors are, and Notch is cleaved and activated in certain cellular compartments after it is internalized as recent papers have shown. This idea has been challenging a decade the old model in which Notch is cleaved and activated

at the plasma membrane. However, several recent papers strongly argue that Notch internalization is required for Notch activation *in cis*. Particularly, clathrin and Rab5 are required in signal receiving cells during ligand-dependent Notch signaling events, proposing that Notch activated by the ligand is cleaved downstream of the Rab5-mediated trafficking pathway. How does the Rab5 pathway recognize and activate the Notch receptor once activated by the ligand at the plasma membrane? One possible scenario is that the Notch S2 cleavage happens at the plasma membrane after the Notch receptor interacts with the ligand, and the cleaved Notch receptor (known as a NEXT complex) only is further cleaved by S3 proteolytic enzymes through the Rab5-mediated endosomal pathway. However, no one has really shown the endosomally-localized cleaved Notch (NEXT) in *Drosophila*, even though Notch extracelluar domain trans-endocytosed by the ligand was shown in a specific developmental context. Therefore, Notch cleavage may occur at the plasma membrane as a context-dependent manner, and in different contexts, Notch cleavages happen in a specific cellular compartment. If no NEXT complex is present in these contexts, how do cells distinquish ligand-bound or ligand-unbound Notch? In this case, a specific endosomal route(s) may provide the way to discriminate between them and the Rab5-mediated endosomal pathway could be the one. In this study, experimental evidence suggests that epsin internalizes Notch receptors and some of them are routed to Rab5 endosomes; epsin-independent Notch internalization was also observed in this study.

Based on the experimental results I showed, ligand signaling from neighboring cells is necessary for epsin-mediated Notch activation *in cis*; if the signal sending cell is *lqf* , no Notch activation was observed in the lqf^{+} neighboring cell. Therefore, epsin could recognize ligand-bound Notch receptors specifically for Notch activation *in cis* and internalize all of them, or at least some of them; *lqf* mutant cells still show a certain

amount of signal receiving activity, this might be caused by epsin proteins remaining after mutant clones were generated, and further research is required to solve this issue. Furthermore, genetic interactions of *lqf* with *rab5* suggest that the role of epsin in membrane trafficking is further mediated by the Rab5 endosomal pathway for some celluar events, including Notch signaling. As Notch routing to the Rab5 endosome is affected by *lqf* mutants, these genetic interactions propose that epsin and Rab5 could be in a linear pathway for Notch activation, even though further research is definitely required to understand the developmental stages where they interact and the mechanisms by which ligand-bound Notch is activated via the epsin-Rab5 pathway.

All together, this study shows the novel cell-autonomous function of epsin that is required for receiving Notch signal and possibly, for other cellular events. Furthermore, this study supports the idea that Notch internalization is required for Notch activation in some developmental contexts and the epsin-Rab5 endosomal route plays a role in this process.

MATERIALS AND METHODS

Drosophila strains

The following alleles were used in this work. FlyBase id numbers, when available, are in parentheses. Chromosomes used are indicated in the figure lege nds. Dl^{B2} (FBst0005602); Dl^{REV} (from N. E. Baker, Albert Einstein College of medicine, Bronx, NY, USA); $Ser^{RX82}Dl^{REV}$ (FBst0006300); $neur^{11}$ (FBst0002747); $neur^{1}$ (FBal0012940); N^{5419} (FBab0000564); N^{55e11} (FBst0028813); lqf^{FDD9} (FBal0104483); $lqf^{\Delta G}$

(FBal0104486); *lqf*^{L71} (our lab. Stock); *ey-gal4* (FBti0012711); *Act>stop>gal4* (from N.-S. Moon, McGill University, Quebec, Canada); *tub-gal4* (from G. Struhl, Columbia University, New York, USA); *tub-gal80* (FBti0012693, FBti0012683), UAS-*flp* (FBti0012285); *UAS-ngfp* (on X, FBti0012492, FBti0012493); *ubi-ngfp* (FBti0015575, FBti0016102); *hs-N*$^{+}$*-GV3* (FBal0090683); *hs-N*$^{+}$*-GV* (Struhl and Adachi, 2000); *FRT80B* (FBst0001988); *UAS-N* (FBst0026820); *UAS-ser* (from G. Struhl, Columbia University, New York, USA); *UAS-Dl* (FBst0026694); *FRT82B* (FBti0002074); *eyFLP* (FBti0015984, FBti0015982); *eyFLP2* (from B. Dickson, IMP, Vienna, Austria); *hs-FLP* (FBti0002044); *e22c-gal4* (from D. Bilder, UC Berkly, CA, USA); *UAS-yfpRab5*^{CA24} (FBst0009774); *Rab5*^{2} (from D. Bilder, UC Berkly, CA, USA); *Rab5*^{K08232} (FBst0010786); transgenic flies with *lqf* genomic constructs (generated by Xie, X).

Imaging of eyes and wings

Immunohistochemistry of third instar larval eye and wing discs was performed as follows. Discs were fixed in PEMS buffer with 1.0% NP-40 for 15 minutes. Antibody treatment was as described previously (Lim et al., 2007) with modifications. Fixed discs were blocked for 2 hours 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 minutes, and incubated with secondary antibodies in washing solution for 2 hours at room temperature, and then washed three times for 5 minutes. Phalloidin was used as described previously (Chen et al., 2002). Eye and wing discs were mounted in Vectashield (Vector, Burlingame, CA, USA) and viewed with a Leica TCSSP2 confocal microscope. 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, rabbit polyclonal anti-Rab5 at 1:200 (abcam, Cambridge, MA, USA), mouse monoclonal anti-N^{ECD} at 1:10 from the DSHB, rat monoclonal anti-Elav at 1:2 from DSHB, ginea pig polyclonal anti-Lqf at 1:400. Secondary antibodies (Molecular Probes, Carlsbad, CA, USA) used at 1:200 were: $Alexa^{568}$-anti-rabbit, $Alexa^{568}$-anti-mouse, $Alexa^{568}$-anti-ginea pig, $Alexa^{633}$-anti-mouse, Cy5-anti-rabbit. Adult external eyes were photographed with an Olympus SZX12 microscope equipped with a SPOT idea (Diagnostic Instruments, Sterling Heights, MI, USA) digital camera.

MARCM clone generation

Clones were generated by heat shocking first or second instar larvae with indicated genotypes at 37°C for 60 minutes.

Rab5 endosome analysis

lqf⁻ MARCM clones were generated as described above and to overexpress *Notch* constructs, additional 60 minutes of heat shock was given to the third instar larvae bearing the clones. After a 2hr recovery, eye discs were isolated and immunolabeled by anti-Rab5 and anti-N^{ECD} antibodies. To analyze the size of Rab5 endosomes, confocal images from two different horizontal sections bearing the same *lqf*- clone area were used for each eye disc. From the images, pixel numbers of the Rab5 positive endosomes in *lqf*⁻ and lqf^{+} areas were obtained using the Adobe Photoshop software; to rule out the possible difference in the staining efficiency between eye discs, each individual eye disc was analyzed seperately and the epsin-dependency on the size of Rab5 endosome was monitored. To analyze co-localization of Notch with Rab5 endosomes, all Rab5 endosomes inside and outside of *lqf*- clones from the images (described above) were

counted and the percentage of Notch positive Rab5 endosomes was calculated inside and outside the clones from the images of the individual eye disc.

Chapter 5: *Overall Conclusions*

The data presented in this dissertation show ways for establishment of initial Notch directionality and amplification of the initial bias, each mediated by Ral and epsin, respectively, for correct Notch signaling and cell fate decisions.

An interesting question for developmental biologists has been: how does a long distance signal become integrated into a local signal for cell fate decisions, leading to proper organization of tissues and organs. Experimental evidence in this dissertation shows a way that a long distance signal, such as PCP (Planar Cell Polarity), is dictated by the local Notch signal, mediated by the small GTPase, Ral. Furthermore, Ral appears to down-regulate ligand-independent Notch activation to ensure the signal sending property, providing Notch directionality between equivalent cells. *Drosophila* Numb, an adapter protein, also down-regulates Notch receptors before interacting with ligands. This is crucial for the signal directionality during SOP cell developmental processes. Ral may be a regulatory factor involved in this (or similar) processe. Because Numb and other regulatory factors down-regulating ligand-independent Notch activation are involved in Notch receptor trafficking, Ral-mediated regulation of membrane trafficking may control the Notch receptor and thus, the direction of signaling. I have also shown the possibility that Ral is a new PCP effector molecule that regulates organization of groups of cells in the plane of the *Drosophila* eye tissue. As Ral is a highly conserved factor in the animal kingdom, the evidence I have shown suggest a role for Ral in control of vertebrate PCP and Notch signaling. It will be valuable to test whether Ral is a downstream effector molecule of core PCP components, since downstream effector molecules for PCP control are not well-understood in vertabrates.

Once the Notch directionality is established, Notch signaling is needed to be activated and regulated for final cell fate decisions. Regulation of membrane trafficking is one of the ways to activate and regulate Notch signaling; as shown in the Ral study, control of membrane trafficking may also be involved in establishment of the initial Notch bias, as Numb regulates Notch receptor trafficking. Particularly, endocytosis of Notch ligands is necessary for Notch receptor activation in adjacent cells, which is accomplished by an endocytic factor, epsin (a "cell-nonautonomous role" of epsin). As epsin was shown to be a general endocytic factor in previous yeast and cell culture studies, it is interesting that epsin has been shown to have a role only in Notch ligand endocytosis in multicellular organisms. Experimental data in this dissertation suggests that epsin has "cell-autonomous" roles, including Notch receptor endocytosis and Notch activation. Even though Shibire, clathrin, and Rab5 were shown to be required for Notch receptor activation in signal receiving cells, it was still unclear how Notch receptors for signaling are selectively routed to Rab5 endosomes to be activated. In this dissertation, I provide some evidence suggesting that epsin is the factor for internalizing signaling Notch receptors into Rab5 endosomes for further activation of Notch. Notch receptors are also needed to be internalized for control of membranous level and epsin may provide a way to separate signaling Notch endocytosis from the simple Notch endocytosis (for controlling the membranous Notch level). In my dissertation, I also provide evidence that the UIMs are critical for the signal sending (not receiving) activity of epsin. Because ubiquitination of Notch ligands is a prerequisite for ligand activation and internalization, the UIMs could be involved in recognizing ubiquitinated ligands, as has been speculated in previous studies. This leads to the question: why are the UIMs not required for Notch receptor internalization and activation? There is no evidence about the requirement of ubiquitination of Notch receptors for internalization of Notch at the plasma membrane,

albeit ubiquitination of Notch required for degradation of the receptors in certain intracellular compartments. Therefore, Notch internalization at the plasma membrane may be mediated by a specific adaptor(s) independent of Notch ubiquitination. This factor may also provide distinction between productive and unproductive Notch internalization simultaneously. The results of my epsin study opens up new questions about how epsin selectively recognizes signaling Notch molecules for internalization.

Finally, a *Ral* mutation was isolated as an enhancer of eye defects caused by epsin overexpression in previous genetic screening. *Ral* and *lqf* mutations were mutual dominant suppressors of each other, which is different from other Notch signaling components, such as *neur* and *Delta*; *neur* and *Delta* mutations were dominant enhancers of *Ral* mutants. The Ral and epsin study I present here show that they are able to work in the same cell, but have opposing functions in terms of Notch receptor activation: Ral down-regulates and epsin promotes Notch receptor activation. Because my epsin study shows that epsin has a cell-autonomous role for Notch activation, the suppressive relationship with *laf* mutations may be explainable.

All together, my dissertation work may contribute to answering the questions about Notch directionality and the role of endocytosis in Notch signaling and expending our knowledge about Notch signaing.

Appendix: *Characterization of genes that function with lqf in Notch signaling*

RESULTS AND DISCUSSION

Screen for modifiers of *lqf* hypomorphic phenotype

A large screen (~30,000 F1) for enhancers of the *lqf* hypomorphic phenotype has been performed and 15 chromosome 2-linked enhancers and 66 chromosome 3-linked modifiers were identified by a former graduate student, Ben Doolan, in the laboratory.

Sorting mutants into lethal Complementation grouping

All of the chromosome 2-linked enhancers are homozygous viable with no morphological phenotypes. Therefore, I started to put 66 chromosome 3-linked modifiers (they reside on homozygous lethal chromosomes, and so could be mutations in essential genes) in a complementation matrix to address which are alleles of same gene. Thirty six enhancers fell into nine complementation groups (Table 2).

Table 2. Nine lethal enhancer groups of *lqf* hypomorph

group	# of alleles	function mapped meiotically	marker interval	physical interval	gene	comments
1	4	enhancer & lethal	sr/e			physical mapping in progress
2	2	lethal	th/cu	81F3-82F7 Df(3R)ME15		each allele fails to complement faf alleles but not faf
3	2				neur	each allele fails to complement neur alleles
4	3	enhancer & lethal	near cu	86E13-86E16 Df(3R)Exel7309	Csk	fails to complement two Csk alleles
5	14	lethal	distal to e	98E-98F5 Df(3R)Exel6210		region contains 29 genes
6	3	lethal	ru/h	64C5-64C10 Df(3L)Exel6103		region contains 19 genes
7	2	lethal	ru/h	61C9-61F7 Df(3L)ED202	trio	each allele fails to complement one trio null allele
8	2	lethal	distal to e			physical mapping in progress
9	4	enhancer & lethal	h/cu	73B5-73D1 Df(3L)Exel6130	lasp?	region contains 19 genes

Steps for identifing enhancer genes

At first, two members of each complementation group were tested for complementation of genes on chromosome 3 encoding known components of epsin-mediated Delta endocytosis: *neur*, *faf*, and *Dl*. Group 3 alleles failed to complement *neur* and group 2 alleles failed to complement *faf* mutant eye defects. Even though group 2 alleles failed to complement *faf* mutants, group 2 is unlikely to be *faf* based on its chromosome location (see below). For group 3, mapping experiments were performed to determine if it is *neur* or not, and the lethality was mapped in the *neur* loci. Second, using multiply marked 3rd chromosomes, meiotic mapping was carried out and each group was localized between markers. Third, using deficiency chromosomes that cover the interval between markers, complementation was tested for each complementation group. Finally,

candidate genes were tested using available mutant alleles of genes in the region. For six complementation groups, deficiency mapping has been finished and Gerrit van der Ende, a member of our group, is trying to find deficiency chromosomes that do not complement the other three complementation groups (Table 2). Particularly, group 4 alleles were identified as *Csk* (C-terminal Src kinase) mutants and showed characteristic *Csk* mutant phenotypes (dead large pupae) (Figure A1). Group 7 alleles were identified as *trio* mutants (Figure A2).

Identifying the nine complementation groups (results summarized in Table 1)

For group 1, mutation was localized to the interval *sr* and *e*, and complementation of the lethality will be tested for each allele using deficiency chromosomes which cover the interval between these markers.

Group 2, as described above, failed to complement *faf* null alleles and flies showed rough eyes with group 2 alleles in trans to *faf* null alleles. However, one deficiency chromosome, Df(3R)ME15, failed to complement group 2 alleles and therefore the locus was roughly mapped between polytene position 81F3 and 82F7 on the third chromosome. To obtain fine map for group 2, small deletion chromosomes covering this interval is being tested to check if they complement this group.

Group 3 alleles failed to complement *neur* null alleles; trans-heterozygous lethal. To confirm if this group is *neur*, meiotic mapping was carried out to address if this mutation is intragenic noncomplementation, or if group 3 is actually *neur*. The lethal chromosome loci from meiotic mapping and deficiency mapping was correspond to the *neur* loci.

As described above, group 4 was identified as *Csk* (c-terminal Src kinase) and DNA lesions will be molecularly characterized to confirm that the mutants are *Csk*.

For group 5, all 14 alleles showed complex complementation behavior. The chromosome region of group 5 is mapped between 98E and 98F5 (defined molecularly) using deficiency mapping and 29 known genes are included in this region. Therefore, mutations on candidate genes will be tested using already known mutant alleles.

For group 6, the chromosome region is mapped between 64C5 and 64C10 (defined molecularly) and 19 known genes, many of which are not characterized, are included in this region. FRT-containing transgene insertions will be used to make small deletion to narrow down the region.

Group 7 was identified as *trio* and DNA lesions will be molecularly characterized to confirm that the mutants are *trio*.

Group 8 localizes distal to *e* on 3R and physical mapping will be carried out.

For group 9, the lethality was uncovered by a small deficiency chromosome that is defined molecularly (73B5-73D1) and 19 known genes are included in this region. Mutants of *Dab (Disabled)* and *Nrt (Neurotectin)* showed complementation for this group and so, genomic DNA sequencing was carried out for another candidate gene *lasp*. And no mutation in *lasp* exons was found. If this group is not *lasp*, same mapping strategy with that of group 6 will be applied to narrow down the region.

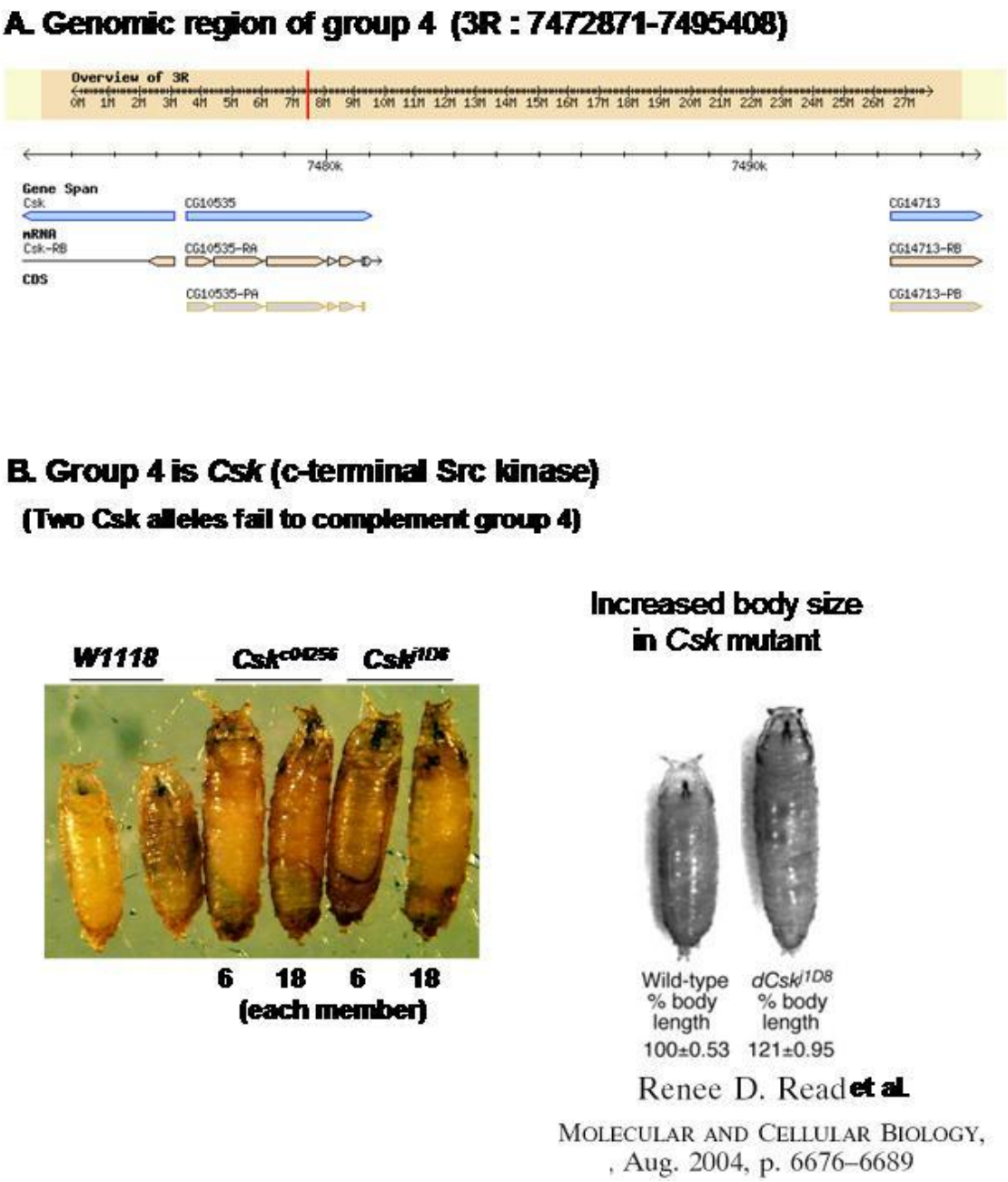
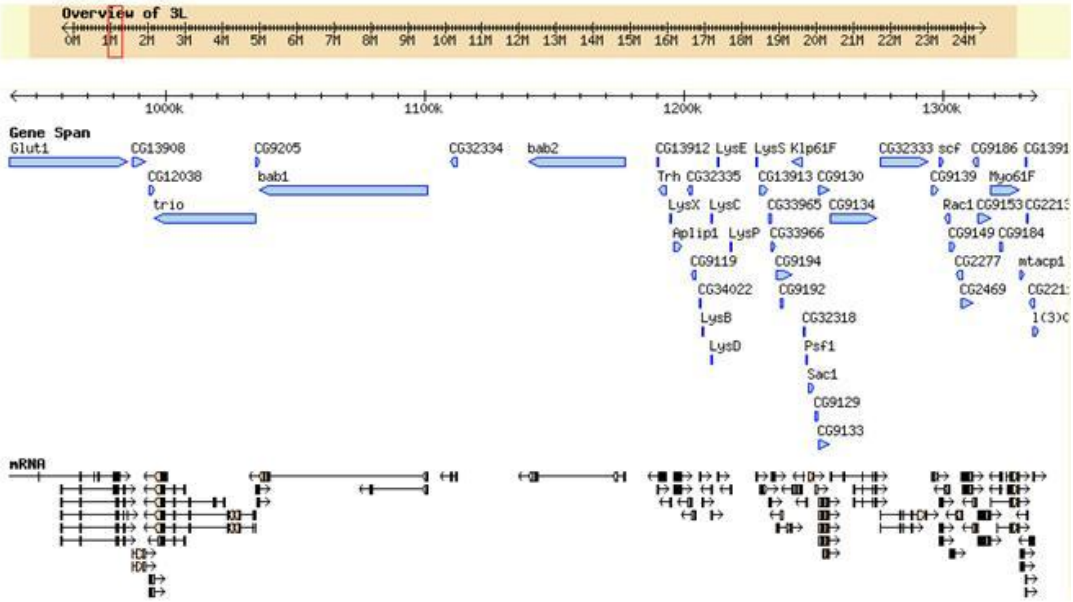


Figure A1. Group4 alleles are *Csk* mutants. (A) A Chromosomal region mapped for group 4 alleles includes three genes. **(B)** Two group 4 members failed to complement *Csk* mutant alleles, showing increased body size and pupal lethality.

A. Genomic region of group 7 (3L:940281-1336381)



B. Group 7 is *trio*

(*trio*^{2} allele fails to complement group 4)

	14	42
trio^1	V	V
trio^2	L	Ø
bab1^1	V	V
bab1^2	V	V

Ø: vestigial wing and leg phenotype

***trio*^{1}: *Trio[s137203]*(#8594) – semi-lethal**

***trio*^{2}: *Trio[6A]*(#8595) - lethal**

***bab1*^{1}: *bab1[Agal4-5]*(#6802) - lethal**

***bab1*^{2}: *bab1[Pgal4-2]*(#6803) - lethal**

***#42trio*^{2}**

Figure A2. Group7 alleles are *trio* mutants. (A) A Chromosomal region mapped for group 7 alleles includes about 30 genes. **(B)** Two group 7 members failed to complement *trio* mutant, not *bab* mutant alleles. For the strong *trio* mutant allele, *trio*^{2}, each group 7 member showed lethality and phenotypical defects.

Which genes are involved in epsin-mediated N/Dl signaling?

As described above, nine lethal complementation groups have been identified as dominant enhancers of the eye phenotypes of *lqf* hypomorph mutants. Two of them have been identified as *Csk* and *trio* alleles, and mapping and additional complementation test are being carried out to identify rest of them. These genes could function in common or parallel pathways with *lqf*. Even though the major non-redundant role of epsin is in Delta ligand endocytosis, epsin may have additional normally redundant functions. Therefore, seven complementation groups will be identified additionally by Gerrit van der Ende and their likely involvement in N/Dl signaling will be determined.

Enhancement phenotypes are similar to other enhancement phenotypes of N/Dl phenotypes

As mentioned before, some of the enhancers may work with epsin in redundant epsin pathways independent from N/Dl signaling. The first approach for testing which enhancers are involved in N/Dl signaling was to compare the enhancement phenotype of each enhancer with those of other N/Dl signaling mutants on *lqf* hypomporphic phenotypes (Figure A3). As shown in Figure A3, all of them show similar enhancement with that of a *neur* mutant on *lqf* hypomorphic phenotypes (Overstreet at al., 2004); more facet fusion events and more mutant facets than in *lqf* hypomorphs.

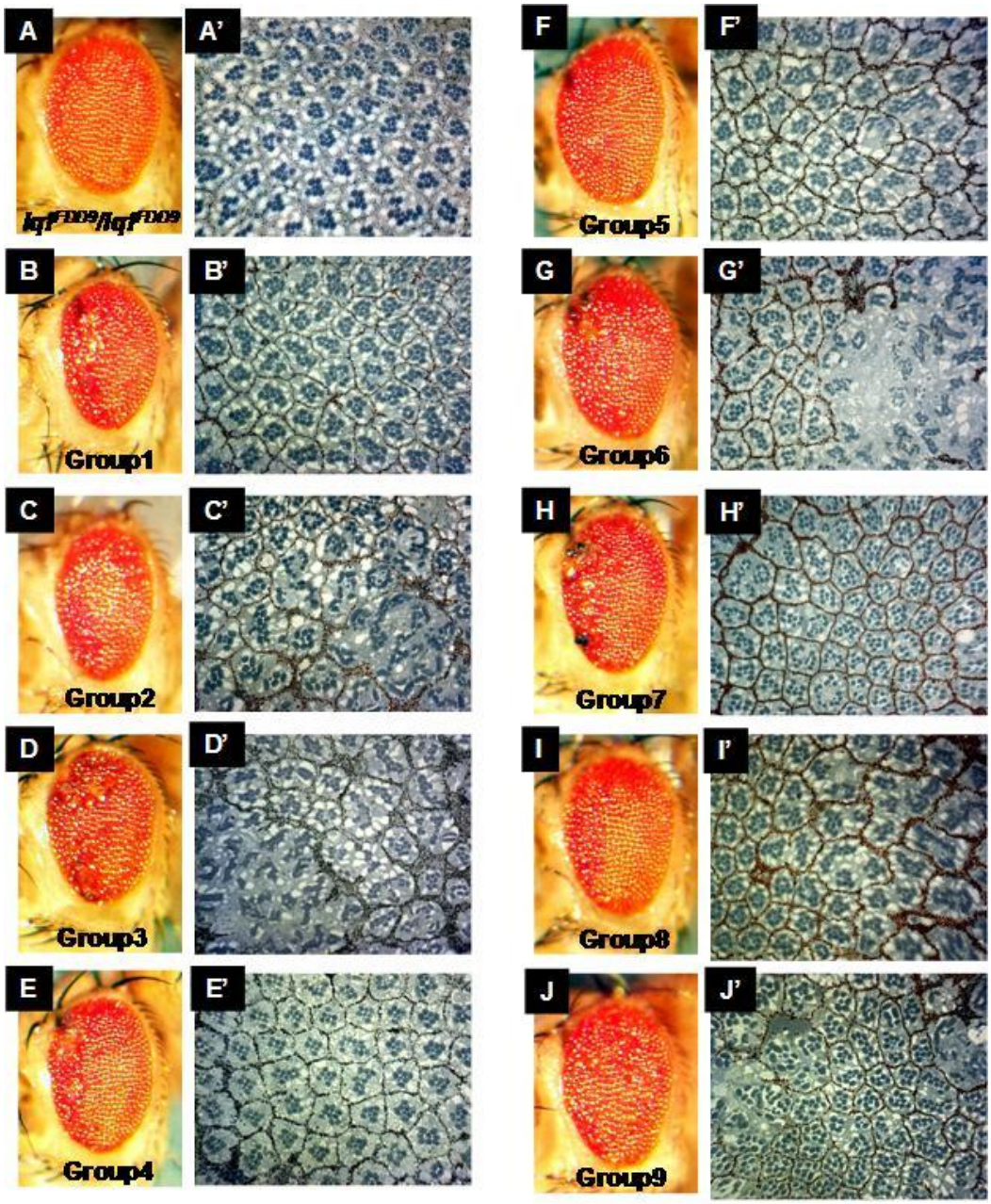


Figure A3. Enhancement of *lqf* hypomorph eye defects by each complementation group. Adult eyes with genotype, lqf^{FDD9}, E/lqf^{FDD9} (E; an ehancer mutation for each group) were sectioned and visualized **(B-J)**. More facet fusions are seen in **(B-J)** than lqf^{FDD9}/lqf^{FDD9} itself **(A)**.

Are their phenotypes similar with mutants of Delta signaling?

Because all enhancers showed enhancement phenotypes of N/Dl phenotypes, we need to determine if they have a non-redundant function in N/Dl signaling or not. To address this, because they are all homozygous lethal, Gerrit van de Ende is generating flies which have homozygous mutant eyes for each enhancer in an otherwise wild-type background using FLP/FRT GMR-hid mitotic recombination method. The adult homozygous mutant eyes are being sectioned and the phenotypes is being compared with mutant eyes of Delta signaling components. If the eye defects are similar with those of N/Dl signaling mutants, this would suggest that those enhancers have a non-redundant function in N/Dl signaling. If the eye defects are different from the defects of N/Dl signaling mutants, those enhancers would be considered as redundant genes in N/Dl signaling.

Is Csk involved in N/Dl signaling redundantly?

Csk is known as a negative regulator of non receptor tyrosine kinase, Src, and therefore, its function has been suggested as a controller of organ growth and proliferation (Read et al., 2004). Furthermore, even though *Csk* mutants show the increased eye disc size, neural cell patterns of the eye disc are normal (Read et al., 2004; Stewart et al., 3003). Therefore, Csk may not be directly involved in N/Dl signaling. However, because Src kinase is known to regulate Clathrin assembly and Dynamin function in mammals (Wilde et al., 1999; Ahn et al., 2002), the negative regulatory role of Csk on Src could be involved in the control of endocytosis, especially in Delta ligand endocytosis. To test this possibility, at first, genetic interactions between *Src* and *lqf*, or other Delta signaling components, can be observed. Furthermore, recent studies suggest that Csk activates tumor suppressor kinase, Warts, by phosphorylation. Interestingly, one

of the tumor suppression mechanisms of activated Warts is destabilizing DIAP (*Drosophila* Inhibitor of Apoptosis Protein) E3 ubiquitin ligase (Stewart et al., 2003; Hipfner and Cohen, 2004). Therefore, alternatively, genetic interaction between *warts* (and *DIAP*) and *lqf* may be tested. It will be interesting to test the possibility of DIAP as the E3 ubiquitin ligase of epsin, because epsin activity is known to be controlled by ubiquitination and deubiquitination process.

Is Trio GEF involved in N/Dl signaling redundantly?

Mainly, Trio GEF is known to be involved in axon steering mediated by non-canonical Notch signaling and actin remodeling by regulating Rac GTPases (Crowner et al., 2003). In previous studies, Trio and three Rac GTPase mutant eyes didn't show defects on neural cell differentiation which are commonly seen in *lqf* hypomorphs (Newsome et al., 2000; Hakeda-Suzuki et al., 2002). Therefore, this suggests that *trio* may act with *lqf* in other signaling, independently with canonical Notch signaling. However, there is evidence which suggest that actin is an important component in endocytosis (Rocca et al., 2008). Therefore, it will be interesting to test if a *trio* mutation enhances Notch phenotypes by checking the genetic interaction with other Delta signaling components, *Dl*, *faf*, and *neur*. If actin remodeling controlled by Trio is involved in N/Dl signaling, similar phenotypes would be observed with double mutants of *lqf* and other Delta signaling components.

Potential problems and alternative approaches

If there is no enhancer functioning non-redundantly in N/Dl signaling, it may be required to determine which enhancers are most plausible modulators of N/Dl signaling redundantly. For instance, once the gene identity is defined, a hypothesis about how they

are involved in N/Dl signaling redundantly could be generated and someone may test the hypothesis. Even though similar enhancements with those of N/Dl mutations were observed, previous studies about *Csk* and *trio* suggest that these genes may not have a non-redundant function in N/Dl signaling.

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Vita

Bomsoo Cho was born in Jeju, South Korea. After completing his study for Bachelor of Science degree in Department of Biology at Korea University, South Korea in 1999, he entered Graduate School of Life sciences and Biotechnology, Korea University in 1999. He received a Master of Science in Biochemistry in February 2001 (Thesis Title: rpS3/UV endonucleaseIII is involved in the Xeroderma Pigmentosum Group D phenotype) (Advisor: Kim, Joon, Ph.D). From January 2001 to November 2003, he had worked as a researcher at In2gen Co. Ltd., Seoul, South Korea, carrying out a cancer vaccine project. He was a researcher at Cancer Research Center, Seoul National University, College of Medicine, Seoul, South Korea from November 2003 to May 2004, and a research technician at Genome Regulation Center, Department of Biochemistry, College of Science, Yonsei University, Seoul, Korea, from July 2004 to July 2005. In September 2005, he started his graduate education in molecular biology at the Institute for Cell and Molecular Biology at the University of Texas at Austin to continue his education and training. He joined Dr. Janice Fischer's laboratory in the summer of 2006 to begin work on his Ph.D. dissertation studying the roles of a small GTPase, Ral, and an endocytic factor, epsin, in Notch signaling using *Drosophila* as a model system.