Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells

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Summary

Endocytosis modulates the Notch signaling pathway in both the signaling and receiving cells. One recent hypothesis is that endocytosis of the ligand Delta by the signaling cells is essential for Notch activation in the receiving cells. Here, we present evidence in strong support of this model. We show that in the developing *Drosophila* eye Fat facets (Faf), a deubiquitinating enzyme, and its substrate Liquid facets (Lqf), an endocytic epsin, promote Delta internalization and Delta signaling in the signaling cells. We demonstrate that while Lqf is necessary for three different Notch/Delta signaling events at the morphogenetic furrow, Faf is essential only for one: Delta signaling by photoreceptor precluster cells, which prevents recruitment of ectopic neurons. In addition, we show that the ubiquitin-ligase Neuralized (Neur), which ubiquitinates Delta, functions in the signaling cells with Faf and Lqf. The results presented bolster one model for Neur function in which Neur enhances Delta signaling by stimulating Delta internalization in the signaling cells. We propose that Faf plays a role similar to that of Neur in the Delta signaling cells. By deubiquitinating Lqf, which enhances the efficiency of Delta internalization, Faf stimulates Delta signaling.

Key words: Eye, *Drosophila*, Notch, Delta, *fat facets*, *liquid facets*, Epsin, Endocytosis, Deubiquitinating enzyme, Ubiquitin

Introduction

Endocytosis controls cell signaling through a variety of different mechanisms (Seto et al., 2002; Gonzalez-Gaitan and Stenmark, 2003). For example, signaling by the epidermal growth factor receptor following ligand binding is attenuated by receptor endocytosis and lysosomal degradation. Endocytosis of epidermal growth factor receptor also enhances signaling by transporting activated receptor to its targets. In addition, endocytosis plays a variety of roles in establishing gradients of morphogens like Hedgehog, Decapentaplegic and Wingless. Moreover, several different aspects of Notch pathway function rely on endocytosis.

Two proteins required for pattern formation in the *Drosophila* eye, the deubiquitinating enzyme Fat facets (Faf) and its substrate Liquid facets (Lqf), are linked to both cell signaling and clathrin-mediated endocytosis (Fischer-Vize et al., 1992; Huang et al., 1995; Cadavid et al., 2000; Chen et al., 2002; Overstreet et al., 2003). Lqf protein levels in the *Drosophila* eye are controlled by the balance between ubiquitination, which targets the protein for proteasomal degradation, and deubiquitination by Faf, which prevents Lqf degradation (Huang et al., 1995; Wu et al., 1999; Chen et al., 2002). Faf and Lqf mediate a cell communication event that prevents overneuralization of the compound eye. Accordingly, *faf* or *lqf* mutant eyes contain more than the normal complement of eight photoreceptors in each facet (or ommatidium) of the eye. As mosaic experiments demonstrate that faf^+ and lqf^+ function outside of the ectopic photoreceptors, the extra photoreceptors must result from a failure of cell signaling (Fischer-Vize et al., 1992; Cadavid et al., 2000). Several observations suggest that Faf and Lqf facilitate endocytosis. First, Lqf is the *Drosophila* homolog of epsin, a multi-modular protein that binds phosphoinisitol lipids at the cell membrane, the adapter complex AP2, clathrin, ubiquitin and other endocytic accessory factors (Kay et al., 1998; De Camilli et al., 2001; Wendland, 2002). Epsin is required for endocytosis in yeast and in mammalian cells (Wendland et al., 1999; Itoh et al., 2001; Shih et al., 2002). In addition, *faf* and *lqf* mutations show dramatic genetic interactions with mutations in the *clathrin heavy chain* gene, which indicate that all three genes function in the same direction in a pathway (Cadavid et al., 2000). Finally, the Notch ligand Delta fails to be internalized normally in *lqf* mutant eye discs (Overstreet et al., 2003).

The overneuralization phenotype in *faf* and *lqf* mutants, and the altered Delta localization in *lqf* mutants suggest a role for Faf and Lqf in Notch/Delta signaling. The Notch pathway is highly conserved in metazoans and participates in a wide range of cell communication events that determine cell fate. Mutants in the Notch receptor and in other genes in the signaling pathway (‘neurogenic’ genes) were first isolated on the basis of their role in inhibiting neural cell fate determination in *Drosophila* embryos (Lehmann et al., 1981). It is now apparent that Notch receptor activation, in different cellular contexts, can result in either inhibition or promotion of a variety of cell fates (Artavanis-Tsakonas et al., 1999). The mechanism of Notch signaling is unusual in that upon ligand binding, a

fragment of the Notch intracellular domain is cleaved, travels into the nucleus, and acts a transcriptional regulator (Artavanis-Tsakonas et al., 1999). Although details of the events that lead to nuclear translocation of the Notch intracellular domain are contentious, there is a consensus model where binding of ligand to the Notch extracellular domain induces two cleavages of Notch. The first cleavage (called S2) detaches the extracellular domain from the remainder of the Notch protein, and is prerequisite for the second cleavage (S3) that releases the transcription factor domain (Baron, 2003).

Endocytosis controls Notch signaling in both the signaling and receiving cells. The first evidence for this idea came from analysis of *Drosophila shibire* mutants. *shibire* encodes the *Drosophila* homolog of dynamin, a GTPase required for scission of endocytic vesicles (Chen et al., 1991). *shibire* mutant phenotypes resemble *Notch* loss-of-function phenotypes, and the results of mosaic experiments suggest that *shibire* is required in both the signaling and receiving cells (Poodry, 1990; Seugnet et al., 1997). A model for the dual function of *shibire* was formulated for Notch signaling during lateral inhibition, where both the signalers and receivers express both Notch and Delta. In this case, selective internalization of either Notch or Delta could bias cells to become either the signaler or the receiver. Recent experiments with *Drosophila* sensory organ precursors support the idea that Notch internalization may bias a cell to become the signaler. The Numb protein, which binds Notch and the endocytic protein α-adaptin, is asymmetrically distributed between two daughter cells and the Numb-containing cell becomes the signaler (Rhyu et al., 1994; Lu et al., 1998; Santolini et al., 2000; Berdnik et al., 2002; Le Borgne and Schweisguth, 2003). Thus, by stimulating Notch internalization, Numb may bias one sensory organ precursor cells to become the signaler.

In addition to preventing a cell from displaying either Notch or Delta at the cell membrane, endocytosis has also been proposed to play a positive role in Notch receptor activation (Parks et al., 2000). The idea is that the Notch extracellular domain, bound to Delta, is trans-endocytosed into the Delta-expressing (signaling) cell. This trans-endocytosis event is prerequisite for S2 cleavage, and therefore for S3 cleavage and activation of Notch in the receiving cell. Evidence for this model comes from experiments in the developing *Drosophila* eye using two non-neural cell types: cone cells and pigment cells (Parks et al., 1995; Parks et al., 2000). *Delta* is transcribed in cone cells, and *Notch* is transcribed in pigment cells. Yet, the extracellular domain of Notch (N^{ECD}) is detected with Delta in endosomes inside the cone cells. Moreover, in *shibire* mutants, Notch and Delta both accumulate at cone cell plasma membranes. In addition, in *Delta* mutants, there are significantly fewer N^{ECD}-containing vesicles in cone cells. In addition, in temperature-sensitive *Delta* loss-of-function mutants, Delta accumulates on cone cell membranes. Finally, in cell culture, cells expressing *Delta* alleles that encode endocytosis-defective ligands do not trans-endocytose N^{ECD}.

Consistent with the trans-endocytosis model, the ubiquitin-ligases Neuralized (in *Drosophila* and *Xenopus*) and Mindbomb (in zebrafish) modulate Delta endocytosis and Delta signaling. Neuralized (Neur) and Mindbomb ubiquitinate Delta thereby stimulating Delta internalization (Itoh et al., 2003; Yeh et al., 2001; Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001). The results of several studies suggest that Neur and Mindbomb are required in the Delta signaling cells to promote Notch activation in the receiving cells (Pavlopoulos et al., 2001; Itoh et al., 2003; Le Borgne and Schweisguth, 2003; Li and Baker, 2004). However, the role of Neur is unclear, as other reports suggest that Neur is required for Delta internalization in the receiving cells, perhaps to bias those cells to become the receivers (Yeh et al., 2000; Lai et al., 2001; Lai and Rubin, 2001a; Lai and Rubin, 2001b).

Here, we report a unique mechanism for regulating Notch/Delta signaling. We show that the deubiquitinating enzyme Faf, through its substrate Lqf, promotes Delta internalization and Delta signaling by the signaling cells. The signaling cells, photoreceptor precursors R2/3/4/5, thus activate Notch in surrounding undifferentiated cells, preventing recruitment of ectopic photoreceptors (R-cells). We call this event R-cell restriction. In addition, we show that while Faf is required only for R-cell restriction, Lqf is needed also for two earlier events in the eye that require Notch/Delta signaling: proneural enhancement and lateral inhibition. We also provide evidence that Neur functions with Faf and Lqf in R-cell restriction. There are three main conclusions of this work. First, the results provide strong support for the model where Delta internalization by the signaling cell is required for Notch activation in the receiving cell. Second, the results support a model where Neur stimulates Delta internalization in the signaling cells rather than in the receiving cells. Finally, we demonstrate that deubiquitination by Faf of the endocytic factor Lqf is a novel mechanism for regulating Delta signaling. We propose that by elevating Lqf activity, Faf enhances the efficiency of Delta endocytosis and promotes Delta signaling.

Materials and methods

Drosophila lines

Our laboratory maintains stocks of lqf^{ARI} *FRT80B* (Overstreet et al., 2003), lqf^{FDD9} (Cadavid et al., 2000) and faf^{FO8} (Fischer-Vize et al., 1992; Chen and Fischer, 2000). *FRT82B* Dl^{rev10} (Baker and Yu, 1996) was obtained from N. Baker. The following lines were obtained from the Bloomington *Drosophila* Stock Center: $neur^{1}$ and $neur^{11}$ (Lehmann et al., 1993); *Ub-GFP FRT80B* and *FRT82B Ub-GFP* (FlyBase, 2003; Xu and Rubin, 1983); *ey-FLP* on X (Newsome et al., 2000); and *EGUF; FRT82B GMR-hid l(3)CL-R* (Stowers and Schwarz, 1999).

Although $neur^{1}$ and $neur^{11}$ are reported to be null alleles, several results presented here suggest that $neur^{11}$ retains some $neur^{+}$ activity. As described below, $neur^{1}$ enhances the lqf^{FDD9} phenotypes much more strongly than does $neur^{11}$, and the eye disc patterning defects in $neur^{1}$ are more severe than in $neur^{11}$.

Eye disc clones

lqf^{ARI} eye disc clones were generated in larvae of the following genotypes: *ey-FLP; lqf^{ARI} FRT80B/Ub-GFP FRT80B*. Dl^{rev10} eye disc clones were generated in larvae of the following genotype: *ey-FLP; FRT82B Dl^{rev10}/FRT82B Ub-GFP*. $neur^{1}$ eye disc clones were generated in larvae of the following genotype: *ey-FLP; FRT82B neur/FRT82B Ub-GFP*. $neur^{11}$ eye discs were generated in larvae of the following genotype: *EGUF/RO-GFP; FRT82B $neur^{11}$/FRT82B GMR-hid l(3)CL-R*.

Analysis of adult eyes

Sectioning, light microscopy and photography of adult eyes was as described (Huang et al., 1995). Flies with $neur^{11}$ eyes were: *EGUF/+; FRT82B $neur^{11}$/FRT82B GMR-hid l(3)CL-R*. The faf^{FO8}/faf^{+} mosaic

ommatidia are those described (Fischer-Vize et al., 1992) and they were reanalyzed here using different criteria. The faf^{BX4}/faf^{+} mosaic ommatidia were generated and prepared for microscopy exactly as described (Fischer-Vize et al., 1992).

Immunocytochemistry of eye discs

Primary antibodies used were rabbit polyclonal anti-Ato at 1:2000 (Jarman et al., 1994) from Y. N. Jan; anti-Boss mouse ascites at 1:2000 (Kramer et al., 1991) from H. Kramer; anti-E(spl) mAb323 supernatant at 1:2 (Jennings et al., 1994) from S. Bray; anti-Dl mAb202 supernatant at 1:10 (Parks et al., 1995) from H. Kramer; and rat monoclonal anti-Elav supernatant at 1:9 (O'Neill et al., 1994) from the Developmental Studies Hybridoma Bank. Secondary antibodies (Molecular Probes) were $Alexa^{633}$-anti-mouse, $Alexa^{568}$-anti-mouse, $Alexa^{633}$-anti-rat and $Alexa^{633}$-anti-rabbit, all used at 1:500. In addition, $Alexa^{568}$- and $Alexa^{633}$-phalloidin were used as described (Chen et al., 2002). Eye discs immunostaining and confocal microscopy were as described (Chen et al., 2002).

P element constructs and transformation

RO-GFP

A DNA fragment containing *GFP* flanked by *Asc*I sites was generated by PCR, using a GFP-containing plasmid (Siemering et al., 1996) as a template and the following primers: 5′GGCGCGCCATGAGTAAAG-GAGAAGAAC3′ and 5′GGCGCGCCTTATTTGTATAGTTCATC-CC3′. The PCR product was ligated into pGEM-T-Easy (Promega) to generate pGEM-GFP. The *GFP* DNA sequence in pGEM-GFP was determined, and the *Asc*I fragment containing *GFP* was isolated and ligated into the *Asc*I site of pRO (Huang and Fischer-Vize, 1996). A plasmid, pRO-GFP, with the *Asc*I fragment in the appropriate orientation was isolated.

RO-GFP-lqf

An *Asc*I-*Nde*I DNA fragment containing *GFP* was generated by PCR using a GFP-containing plasmid (Siemering et al., 1996) as a template and the following primers: 5′CAGATGGGCGCGCCATGAGTA-AAGGAGAAC3′, 5′CATATGTTTGTATAGTTCATCC3′. The PCR product was ligated into pGEM-T-Easy to generate pGEM-GFP-AN. The *GFP* DNA sequence in pGEM-GFP-AN was determined and the ~700 bp *Asc*I-*Nde*I *GFP* fragment was isolated and ligated into a plasmid containing the *lqf* cDNA called pMoPac-lqf-cDNA3. pMoPac-lqf-cDNA3 was constructed as follows: the *lqf* cDNA was generated in two parts by PCR using as a template a plasmid containing *lqf* cDNA-3 (Cadavid et al., 2000). The 5′ part of *lqf* was generated as an *Nde*I-*Hpa*I fragment using the primers 5′AT-GCAGGTCAATGTCGCTGG3′ and 5′CGGTTTGATCAGATTGTC-TAGG. The PCR product was ligated into pGEM-T-Easy to generate pGEM-Lqf5′ and the *lqf* DNA sequence in the plasmid was determined. The 3′ part of *lqf* was generated as an *Hpa*I-*Asc*I fragment using the primers 5′TTTCCTCGGCGAGAACTC3′ and 5′TTACGA-CAAAAACGGATTTGTTG3′. The PCR product was ligated into pGEM-T-Easy to generate pGEM-cDNA3-3′ and the *lqf* DNA sequence in the plasmid was determined. A ~1650 bp *Nde*I-*Hpa*I fragment of pGEM-Lqf5′ and ~800 bp *Nde*I-*Asc*I fragment of pGEM-cDNA3-3′ were isolated and ligated into pMoPac (Hayhurst et al., 2003) restricted with *Nde*I and *Asc*I.

RO-shi^{DN}

An *Spe*I-*Sal*I fragment of pTM1 containing shi^{K44A} (Moline et al., 1999) (obtained from A. Bejsovec) was ligated into pBSKII (Stratagene) restricted with *Spe*I and *Sal*I to generate pBSK-shi^{DN}. *Asc*I sites flanking the shi^{K44A} gene were added as follows: pBSK-shi^{DN} was restricted with *Spe*I, treated with Klenow fragment, and an *Asc*I linker ligated in. A second *Asc*I linker was ligated similarly into the *Sal*I site. The resulting *Asc*I fragment of shi^{K44A} was purified and ligated into pRO. A plasmid, pRO-shi^{DN}, with the *Asc*I fragment in the appropriate orientation, was isolated.

RO-Dl^{DN}

A DNA fragment of *Delta* lacking the cytoplasmic domain and flanked by *Asc*I sites was generated by PCR, using as a template pG1C (Fehon et al., 1990) (obtained from M. Muskavitch), which contains a complete *Delta* cDNA and the following primers and also inserted a stop codon: 5′GGCGCGCCCACACACACACACAGCCCTG3′ and 5′GGCGCGCCTTACACCGCATTCTGTTC3′. The PCR product was ligated into pGEM-T-Easy to generate pGEM-Dl^{DN}. An *Asc*I fragment containing the truncated *Delta* gene was purified from pGEM-Dl^{DN} and ligated into pRO. A plasmid, pRO-Dl^{DN}, with the *Asc*I fragment in the appropriate orientation was isolated.

P-element transformants were generated by injection of w^{1118} embryos using standard techniques.

Results

faf+ and *lqf*+ are required for Delta endocytosis in R-cell preclusters

Drosophila eye development is controlled by a complex network of cell signaling pathways, which includes many roles for Notch/Delta signaling (Mlodzik, 2002; Nagaraj et al., 2002). The *Drosophila* compound eye is composed of hundreds of identical ommatidia. The eye develops in larval and pupal stages from a cellular monolayer called the eye disc (Wolff and Ready, 1993). In third instar larvae, a wave of morphogenesis, initiated at the posterior of the disc by the morphogenetic furrow, moves anteriorly through the monolayer of undifferentiated cells. A column of organized preclusters emerges from the furrow (column 0) (Fig. 1G). A few cells are excluded from the initial preclusters and the remainder differentiate into five of the eight photoreceptors (R-cells; R8/2/3/4/5). These clusters then recruit R1/6/7, the four cone cells, and finally the pigment and bristle cells. As the furrow moves forward by one column approximately every 2 hours, each more posterior column is one step more mature and the sequence of ommatidial assembly can be observed in a single disc.

The pattern of *Delta* expression in wild-type eye discs has been well-characterized. *Delta* transcription is ubiquitous in the morphogenetic furrow, and then resolves to the R-cell preclusters as they emerge from the furrow (Parks et al., 1995). Delta protein is detected in a similar pattern of cells and its subcellular localization is intriguing. Although Delta is expected to function at the membrane, an antibody to the Delta extracellular domain detects most of the protein in endosomal vesicles posterior to the furrow (Fig. 1D) (Parks et al., 1995). Delta-containing vesicles first accumulate in preclusters emerging from the furrow, then in R-cells as they differentiate, and remain detectable in some R-cells until at least column 14 (Parks et al., 1995). Using unusual tissue preparation conditions (no detergent), low levels of membrane-bound Delta are observed in the same pattern as Dl transcripts (Baker and Yu, 1998). These observations suggest that in some cells, most of the Delta at the cell surface is internalized and that endosomal Delta is not degraded rapidly.

In lqf^{FDD9} eye discs, which produce low levels of wild-type Lqf protein, Delta accumulates on cell membranes in columns 0-3 posterior to the furrow (Fig. 1F) (Overstreet et al., 2003). Like lqf^{FDD9}, *faf* mutant discs have decreased levels of Lqf protein (Chen et al., 2002). In order to determine if Delta internalization is defective in *faf* mutant discs and in which cells, we double-labeled faf^{FO8} third instar larval eye discs

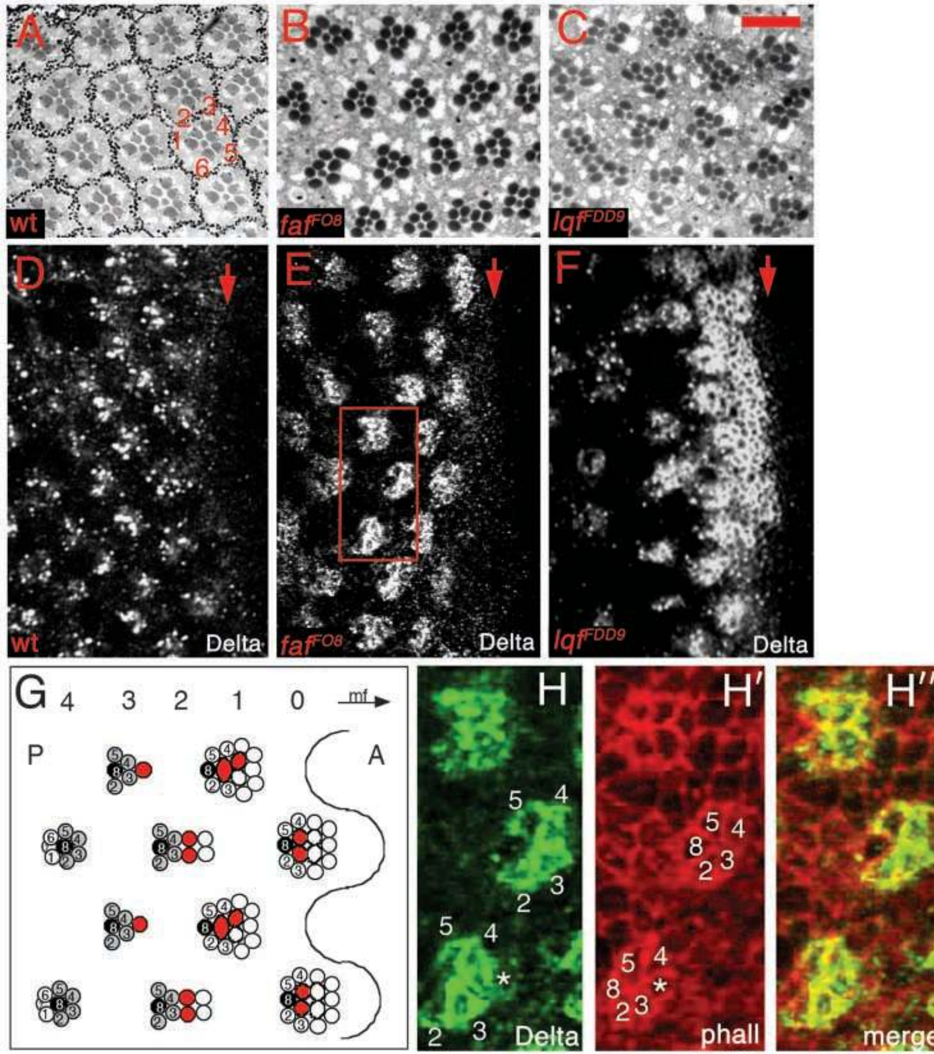

Fig. 1. Delta localization in eye discs. (A-C) Tangential sections through adult eyes are shown. The numbers in A refer to the outer R-cells, R1-R6. (D-F) Confocal images of eye discs labeled with anti-Delta are shown. Anterior is towards the right and the arrows indicate the position of the furrow. (G) A diagram of the early stages of ommatidial assembly. A is anterior, P is posterior; 0-4 at the top indicate columns emerging from the furrow (mf). R-cell identities are indicated by the numbers inside the circles. The red cells may be those that become ectopic R-cells in *faf* mutants. (H-H″) Enlargement of the boxed region in E. Numbers indicate R-cells and asterisks indicate an ectopic R-cell. In H″, both membrane-bound Delta (yellow) and vesicular Delta (green) are present. Scale bar: 20 μm in A-C; 10 μm in D-F; 5 μm in H-H″.

[faf^{FO8} is a strong mutant allele (Fischer-Vize et al., 1992; Chen and Fischer, 2000)] with antibodies to the Delta extracellular domain and with phalloidin to outline the apical membranes of the ommatidial cluster cells. We find that Delta is present on the membranes of R2/3/4/5 and the ectopic R-cells in columns 0-3 of faf^{FO8} discs (Fig. 1E,H-H″). Some vesicular Delta is also observed (Fig. 1H″). We conclude that both faf^{+} and lqf^{+} are required for Delta endocytosis in R-cell clusters in columns 0-3.

The observation that similar Delta internalization defects occur in *faf* and *lqf* mutant discs supports the idea that the *faf* mutant phenotype results from a decrease in the level of Lqf protein. However, more Delta-expressing cells emerge posterior to the furrow in lqf^{FDD9} discs than in wild-type or *faf* discs. The difference in *Delta* expression between *faf* and lqf^{FDD9} discs reflects a broader requirement for lqf^{+} in early developmental decisions (see below).

faf⁺ and *lqf*⁺ function in R2/3/4/5 precursors

In *faf* mutants, the R2/3/4/5 precursors display Delta endocytosis defects. In order to determine whether faf^{+} and lqf^{+} function in these cells, we investigated the expression pattern of the vector pRO (Huang and Fischer-Vize, 1996). pRO transgenes that drive expression of a *faf* cDNA (*RO-faf*) can substitute for the endogenous *faf* gene (Huang and Fischer-Vize, 1996). Likewise, a *RO-lqf* transgene rescues to wild type the mutant eye phenotype of lqf^{FDD9} or *faf* (Cadavid et al., 2000). We generated a *RO-GFP* transgene and observed the pattern of GFP expression in eye discs from three independent transformant lines. We find that GFP is expressed in R2/3/4/5 beginning in column1 (Fig. 2A,B). The same results were obtained with a *RO-GFP-lqf* transgene which also complements the *faf* and lqf^{FDD9} mutant phenotypes (data not shown). We conclude that expression of faf^{+} or lqf^{+} in R2/3/4/5 is sufficient to substitute for the endogenous *faf* gene or to compensate for the lower levels of Lqf protein in lqf^{FDD9}.

To investigate further the requirement for faf^{+} in R2/3/4/5, we analyzed adult ommatidia mosaic for marked faf^{+} and faf^{-} cells generated by mitotic recombination. Two types of genetically mosaic facets were observed and analyzed: phenotypically mutant ommatidia with more than six outer (R1-6) R-cells, and phenotypically wild-type ommatidia. The genotype of each outer R-cell (including ectopic cells) was scored in both types of mosaic facets (Fig. 2C-E). In assigning R-cell identities, we assumed that the ectopic R-cells arise between R3 and R4. If faf^{+} is required in all or a subset of R2/3/4/5, then we would

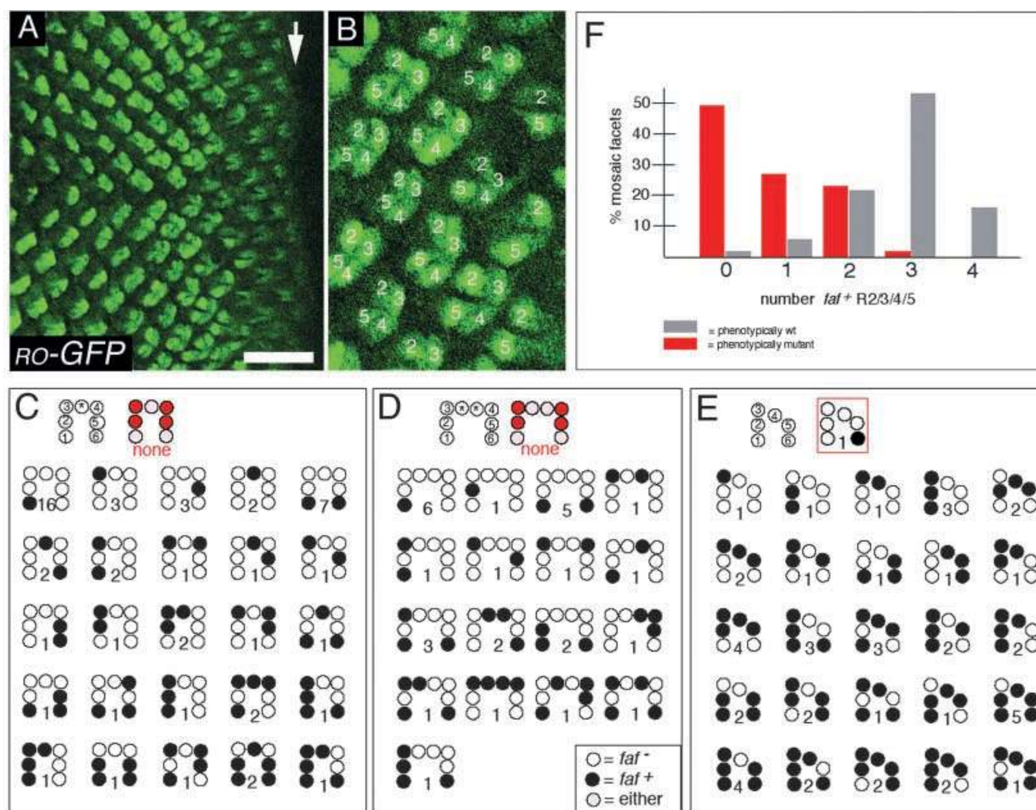

Fig. 2. faf^+ functions in R2/3/4/5. (A,B) Confocal images of GFP expression from a *RO-GFP* transgene in an eye disc. In A, anterior is towards the right and the arrow indicates the position of the furrow. (B) An enlargement of part of A is shown, the numbers indicating R-cells R2/3/4/5. (C,D) Tabulation of the different phenotypically mutant faf^+/faf^{FO8} mosaic facets with one (C) or two (D) ectopic R-cells are shown. (E) Tabulation of the different phenotypically wild-type faf^+/faf^{BX4} mosaic ommatidia are shown. Numbers beneath each diagram refer to the number of facets with that particular mosaic pattern observed. The faf^+ R-cells are $white^+$ (have pigment granules) and the faf^- R-cells are $white^-$ (do not have pigment granules). (F) Aspects of the data in C-E are displayed graphically. Scale bar: 20 μm in A; 10 μm in B.

expect to find no phenotypically mutant facets where R2/3/4/5 are all faf^+. As expected, in not one of 86 mutant mosaic facets at the borders of 30 different faf^{FO8} clones were R2/3/4/5 all faf^+ (Fig. 2C,D). Moreover, in nearly half of the mutant mosaic ommatidia (42/86), none of the R2/3/4/5 group is faf^+ and in only 2/88 mutant mosaics are three of the R2/3/4/5 group faf^+ (Fig. 2C,D,F). Conversely, we expected that R2/3/4/5 would not all be faf^- in phenotypically wild-type facets. For this analysis, we used faf^{BX4}, which is a null allele (Fischer-Vize et al., 1992). In only 1/51 phenotypically wild-type mosaic facets in 13 different clones were R2/3/4/5 all faf^- (Fig. 2E). Moreover, although no particular R-cells in the R2/3/4/5 cell group were always faf^+, at least three of them were faf^+ in 36/51 mosaic facets, and at least two of them were faf^+ in 47/51 of the mosaic facets (Fig. 2E,F). The wild-type mosaic ommatidia where not one R-cell (1/51) or only one R-cell (3/51) of the R2/3/4/5 group is faf^+ can be explained by the observation that in faf^{BX4} homozygotes, ~10% of the facets are phenotypically wild type. These results show that as more of the R-cells in the R2/3/4/5 group are faf^+, there is an increasing tendency for the ectopic R-cells to be excluded.

Endocytosis is required in R2/3/4/5 precursors to prevent ectopic R-cell recruitment

faf^+ and lqf^+ activities are linked to endocytosis and Delta endocytosis fails in precluster cells with decreased lqf^+ activity (faf^{FO8} or lqf^{FDD9}). Is a failure of endocytosis the cause of the *faf* and lqf^{FDD9} mutant eye phenotypes? If so, then disrupting endocytosis in R2/3/4/5 through a mechanism other than blocking faf^+ or lqf^+ gene activity should result in an eye phenotype similar to that of *faf* or lqf^{FDD9}. We interfered with endocytosis in R2/3/4/5 by expressing a dominant-negative form of Shibire (Moline et al., 1999) using the pRO vector ($RO\text{-}shi^{DN}$). We find that otherwise wild-type flies expressing $RO\text{-}shi^{DN}$ display adult retinal defects similar to those in *faf* or lqf^{FDD9} mutants (Fig. 3A, Fig. 1A-C). The ectopic R-cells in $RO\text{-}shi^{DN}$ join the clusters in columns 0-3 as in *faf* or lqf^{FDD9} discs (Fig. 3B-D). Moreover, Delta internalization defects similar to those in *faf* or lqf^{FDD9} are observed in $RO\text{-}shi^{DN}$ eye discs (Fig. 3B-D, Fig. 1E,F). We conclude that R2/3/4/5 precursors require endocytosis to prevent inappropriate recruitment of neighboring precluster cells as R-cells.

Delta signaling and endocytosis in R2/3/4/5 precursors is required to prevent ectopic R-cell recruitment

Does the failure of Delta signaling in R2/3/4/5 cause the *faf* and lqf^{FDD9} mutant phenotypes? If so, then specifically interfering with Delta endocytosis and signaling in R2/3/4/5

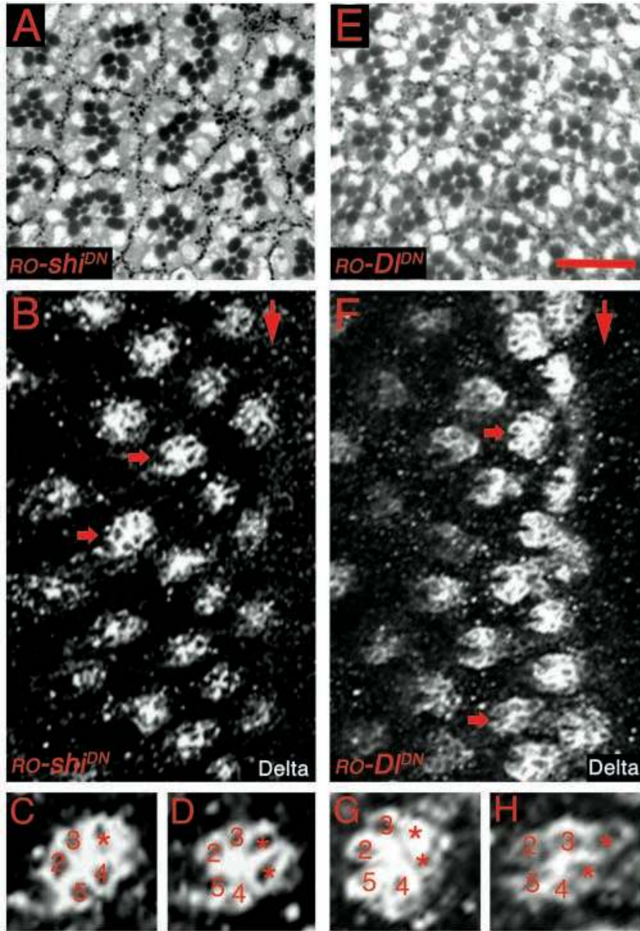

Fig. 3. *RO-shi*^{DN} (A-D) or *RO-Dl*^{DN} (E-H) phenocopy *faf* mutant eyes. (A,E) Shown are tangential sections through adult eyes of flies expressing the indicated transgenes. (B,F) Confocal images of eye discs labeled with anti-Delta are shown. Anterior is towards the right and large arrows indicate the position of the furrow. (C,D) Enlargements of clusters in B indicated by small arrows. (G,H) Enlargements of clusters in F indicated by small arrows. In C,D,G,H, numbers refer to R-cells and asterisks are ectopic R-cells. Scale bar: 20 μm in A,B,E,F; 10 μm in C,D,G,H.

should phenocopy *faf* and *lqf*^{FDD9} mutants. To test this, we used the pRO vector to express in R2/3/4/5 a dominant-negative form of Delta (Dl^{DN}) (Sun and Artavanis-Tsakonas, 1996). In *RO-Dl*^{DN} transformant eye discs, ectopic R-cells join the clusters in columns 0-3 (Fig. 3F-H) and are present in adult eyes (Fig. 3E). In addition, Delta protein accumulates on R-cell membranes near the furrow (Fig. 3F). The Dl^{DN} protein has a truncated intracellular domain and if Delta endocytosis is required for Delta signaling, the dominant-negative activity of Dl^{DN} is probably due to its failure to be internalized. Thus, the membrane-associated Delta protein observed in *RO-Dl*^{DN} discs may be a mixture of Dl^{DN} protein and wild-type Delta that is prevented by Dl^{DN} from interacting with Notch. We conclude that specific disruption of Delta signaling and endocytosis in R2/3/4/5 results in the same developmental consequences as does interfering with *faf* or *lqf* function.

lqf^{+} is required in the signaling cells for two *faf*$^{+}$-independent Delta signaling events at the morphogenetic furrow

We have shown that in order to prevent recruitment of ectopic R-cells into the ommatidia, *faf*$^{+}$ and *lqf*$^{+}$ are required for Delta signaling by R-cell precursors just posterior to the furrow. *faf*$^{+}$ appears to be essential only for this one Delta signaling event: in *faf*^{FO8} (strong) mutants, Delta is on the membrane in R-cell preclusters, ectopic R-cells are recruited just posterior to the furrow and the adult eye phenotype (ectopic R-cells) reflects these events. By contrast, *lqf*$^{+}$ appears to be necessary also for earlier patterning processes. In *lqf* mutant eye discs [*lqf*^{FDD9} or discs with small *lqf*^{ARI} (null) clones], all cells emerging from the furrow express Delta (Fig. 1F) (Overstreet et al., 2003) (also see below), whereas in wild-type discs Delta is expressed in distinct clusters (Fig. 1D) (Parks et al., 1995). In addition, in the adult eye, the phenotype of *lqf*^{ARI} clones is much more severe than that of *faf* mutants (Fischer et al., 1997).

Prior to the *faf*$^{+}$-dependent signaling event, two discrete Notch/Delta signaling processes are required for the evolution of expression of the proneural protein Atonal (Baker and Yu, 1996; Baker et al., 1996; Baker, 2002). First, Notch activation in groups of cells anterior to the furrow upregulates Atonal expression; this event is referred to as proneural enhancement. Elevated Atonal levels are necessary for neural determination of these cells. Second, Notch/Delta signaling is essential for lateral inhibitory interactions that resolve Atonal expression to one cell by column 0. The one Atonal-expressing cell becomes R8, the founder R-cell of each ommatidium (Baker and Yu, 1998).

In order to determine whether *lqf*$^{+}$ is required for Delta signaling during proneural enhancement and/or lateral inhibition, we analyzed the phenotypes of large *lqf*^{ARI} (null) clones using a number of different antibodies and compared them with the phenotypes of large *Dl*^{rev10} (null) clones. We find that the *lqf*^{ARI} clone phenotypes closely resemble those of *Dl*^{rev10} clones described earlier (Baker and Yu, 1996). Upregulation of *atonal* (proneural enhancement) does not occur in the *Dl*^{rev10} or *lqf*^{ARI} clone centers (Fig. 4); although the cells in the middle of the clone are *Notch*$^{+}$, there are no *Delta*$^{+}$ cells adjacent to them to activate Notch. As would be expected, *Dl*^{rev10} or *lqf*^{ARI} mutant cells at the clone borders adjacent to *Delta*$^{+}$ cells do upregulate *atonal* (Fig. 4). In the absence of proneural enhancement, no R-cells are expected to be determined posterior to the furrow. Consistent with this, R-cells are absent from the centers of *Dl*^{rev10} or *lqf*^{ARI} clones (Fig. 5A,A′,C,C′). By contrast, at the clone borders where mutant cells undergo proneural enhancement, R-cells are present (Fig. 5A,A′,C,C′). Lateral inhibition also fails in *Dl*^{rev10} and *lqf*^{ARI} clones. The R-cells at the *Dl*^{rev10} or *lqf*^{ARI} clone borders are not organized into discrete ommatidia; instead, it appears that all of the mutant border cells are R-cells (Fig. 5A,A′,C,C′). As these cells cannot send Delta signals, lateral inhibition fails. Consistent with this idea, there are clusters of R8s at the borders of the clones (Fig. 5B,B′,D,D′). We conclude that *lqf*$^{+}$ is required in the Delta signaling cells for proneural enhancement and lateral inhibition.

lqf-null mutant cells can function as receivers but not as signalers

The results so far suggest that *faf*$^{+}$ and *lqf*$^{+}$ are required for

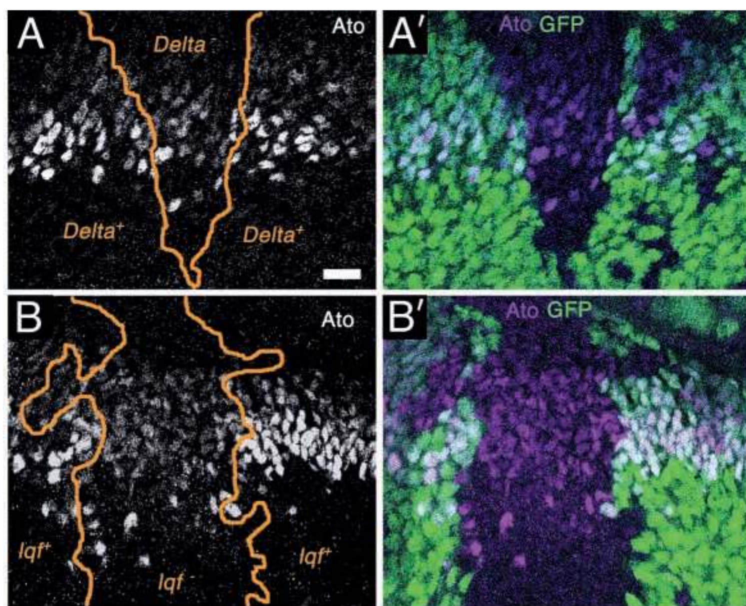

Fig. 4. Atonal expression in *Delta* and *lqf*-null eye disc clones. Eye discs labeled with anti-Atonal are shown. Anterior is upwards. (A,A′) A clone of *Delta*^{rev10} cells marked by the absence of GFP. (B,B′) A clone *lqf*^{ARI} cells marked by the absence of GFP. Clone borders are outlined in A and B. Scale bar: 10 μm.

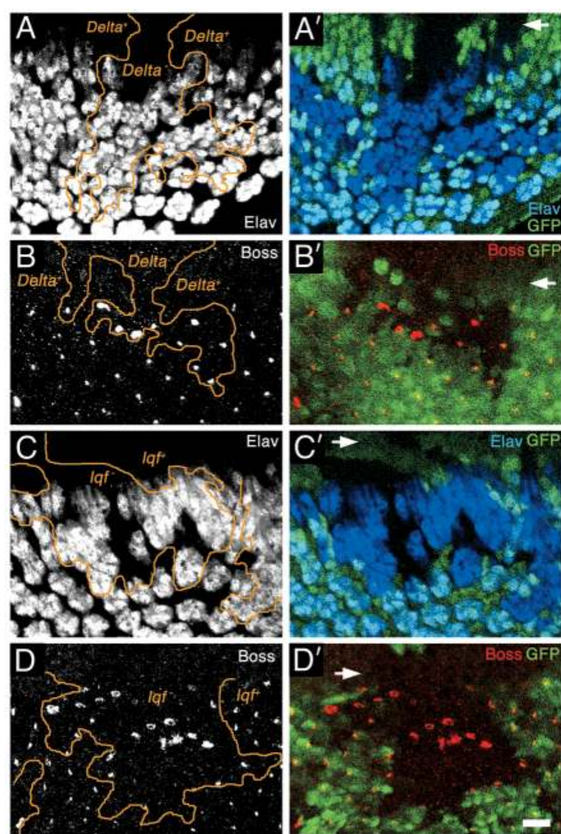

Fig. 5. R-cell determination in *Delta* and *lqf*-null eye disc clones. Confocal images of eye discs are shown. Anterior is upwards in all panels and the arrows indicate the position of the furrow. The discs contain *Delta*^{rev10} clones (A,A′,B,B′) or *lqf*^{ARI} clones (C,C′,D,D′) marked by the absence of GFP. The discs are labeled with anti-Elav in (A,A′,C,C′) and with anti-Boss in (B,B′,D,D′). In A-D, the clone borders are outlined. The Elav and Boss-expressing cells can be seen several cell distances in from the edge of the clone. This is probably due to long-range Delta signaling, a phenomenon that is not well understood (De Joussineau et al., 2003). Scale bar: 10 μm.

Delta internalization and Delta signaling. One prediction of this model is that *faf*$^+$ and *lqf*$^+$ should function non-autonomously; *faf*$^+$ or *lqf*$^+$ cells adjacent to mutant cells should fail to have their Notch pathways activated and should be misdetermined as R-cells. Ectopic R-cells present in *faf*$^+$/*faf*$^-$ mosaic ommatidia in adult eyes are often *faf*$^+$ (Fig. 2C,D) (Fischer-Vize et al., 1992). The same phenomenon was observed in *lqf*$^+$/*lqf*$^-$ mosaic facets (Cadavid et al., 2000). Thus, *faf*$^+$ and *lqf*$^+$ function non-autonomously. Conversely, if *lqf*$^+$ functions in the Delta-signaling cells as opposed to the receiving cells, it should be possible to activate Notch in *lqf*-null mutant cells that are adjacent to *lqf*$^+$ cells. To test this, we generated *lqf*^{ARI} clones and *Dl*^{rev10} (null) clones as a control in eye discs and labeled them with mAb323, which recognizes several different Enhancer of split [E(spl)] proteins expressed in response to Notch activation (Jennings et al., 1994). There is little Notch activation in the middle of the *Dl*^{rev10} clones (Fig. 6A,A′) or the *lqf*^{ARI} clones (Fig. 6B,B′) (see also legend). Thus, like *Delta*$^+$, *lqf*$^+$ is required for Notch activation in neighboring cells. At the borders of the *Dl*^{rev10} clones near the furrow, *Delta*$^+$ *Notch*$^+$ cells outside the clone can signal the *Dl*^{rev10} *Notch*$^+$ cells inside the clone. Thus, E(spl) protein is detected in many *Dl*^{rev10} cells at the clone borders (Fig. 6A,A′). The same phenomenon is observed the borders of *lqf*^{ARI} clones (Fig. 6B,B′). Thus, the Notch signaling pathway may be activated in *lqf*$^-$ cells. We conclude that cells lacking *lqf*$^+$ activity can activate their own Notch pathway in response to signals from neighboring cells, but cannot signal to activate Notch in their neighbors.

Membrane accumulation of Delta is cell autonomous in *lqf* null mutant cell clones

If the effect of *lqf*$^+$ on Delta endocytosis is direct, then when *lqf*$^+$ and *lqf*$^-$ cells are juxtaposed, Delta should accumulate only on the membranes of *lqf*$^-$ mutant cells. In small *lqf*^{ARI} (null) clones in eye discs, Delta accumulates on the membranes of all cells emerging from the furrow (Fig. 7) (Overstreet et al., 2003). At the clone borders, high levels of membrane-bound Delta are observed only in the *lqf*^{ARI} mutant cells (Fig. 7). We conclude that the effect of Lqf on Delta internalization is cell autonomous.

neur$^+$ functions with *faf*$^+$ and *lqf*$^+$ in R2/3/4/5

Neur is required for Delta internalization in wing and eye discs (Lai et al., 2001; Pavlopoulos et al., 2001). However, the only specific functions demonstrated for *neur*$^+$ in the eye are a weak requirement in proneural enhancement and lateral inhibition (Lai and Rubin, 2001a; Li and Baker, 2001; Li and Baker, 2004). The observation that the *neur* adult eye mutant phenotype resembles that of *faf* and *lqf*^{FDD9} mutants (Fig. 8A) (Lai and Rubin, 2001a) and that *neur*$^+$ is expressed specifically in R-cells that emerge from the furrow (Pavlopoulos et al.,

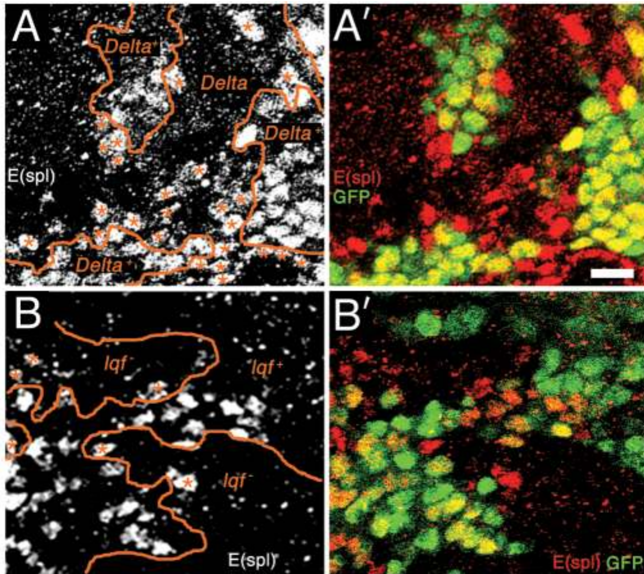

Fig. 6. Notch activation in *Delta* and *lqf*-null eye disc clones. Confocal images of eye discs in the region of the furrow are shown. Anterior is upwards in all panels. Eye discs are labeled with mAb323, which recognizes E(spl) proteins. (A,A′) An eye disc containing a $Delta^{rev10}$ clone marked by the absence of GFP is shown. In A, the clone is outlined and the asterisks indicate $Delta^{rev10}$ cells that express E(spl). (B,B′) An eye disc containing a lqf^{ARI} clone marked by the absence of GFP is shown. In B, the clone is outlined and the asterisks indicate lqf^{ARI} cells that express E(spl). The clones were examined throughout the depth of the eye disc and most E(spl)-expressing cells are adjacent to clone borders at all levels. Some E(spl)-positive cells are several distances from the clone border (as in A,A′). This may be evidence for long-range Delta signaling, a process that is not well understood (De Joussineau et al., 2003). Scale bar: 10 μm.

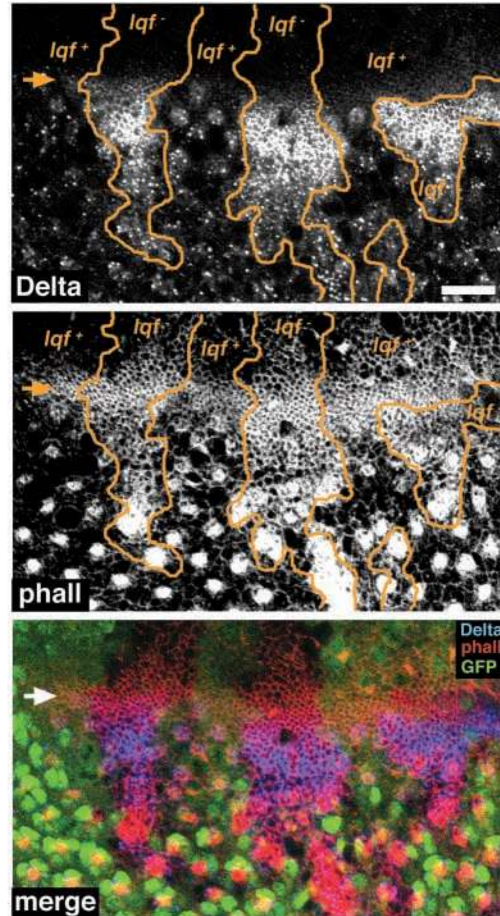

Fig. 7. Cell autonomy of Delta mislocalization in *lqf* null eye disc clones. Confocal images of an eye disc (anterior upwards) containing lqf^{ARI} clones, marked by the absence of GFP, is labeled with anti-Delta and with phalloidin, which marks f-actin at cell membranes. The top panel shows Delta localization, the middle panel shows phalloidin, and the bottom panel is a merge of Delta, phalloidin and GFP. Arrows indicate the position of the furrow. Scale bar: 20 μm.

2001; Lai and Rubin, 2001b) led us to test whether $neur^+$ is also required for faf^+-dependent Delta signaling by R2/3/4/5 precursors.

In order to determine if $neur^+$ is required in R2/3/4/5 precursors for Delta internalization and signaling, we performed three experiments. We first tested *neur* for genetic interactions with *faf* and *lqf*. We find that two strong mutant *neur* alleles ($neur^1$ and $neur^{11}$) are powerful dominant enhancers of lqf^{FDD9}. $neur^1$ lqf^{FDD9}/lqf^{FDD9} animals die as larvae. $neur^{11}$ lqf^{FDD9}/lqf^{FDD9} are viable and their retinal defects are more severe than lqf^{FDD9}/lqf^{FDD9} (compare Fig. 8B with Fig. 1C). In eye discs, *neur* enhances the lateral inhibition defects in lqf^{FDD9}; the clusters of Delta-expressing cells are larger in lqf^{FDD9} $neur^1/lqf^{FDD9}$ discs (Fig. 8C,C′) than in lqf^{FDD9} (Fig. 1F) and Delta is on the cell membrane. *neur* mutants enhance the *faf* mutant phenotype weakly (data not shown). The genetic interactions are consistent with the idea that $neur^+$, lqf^+ and faf^+ function in the same direction in a pathway. Second, we monitored the distribution of Delta in *neur* eye discs. In $neur^{11}$ eye discs that express *RO-GFP* we find that, similar to *faf* mutants, Delta accumulates on membranes of the R-cell clusters (Fig. 8D,D′). The Delta mislocalization phenotype of $neur^1$ eye discs is stronger than $neur^{11}$ and similar to lqf^{FDD9} (Fig. 8E,E′). Finally, we asked what effect *neur* mutant cells have on Notch activation near the furrow. We find that $neur^-$ cells behave similarly to lqf^- cells; Notch is activated in $neur^1$ cells at clone borders that are adjacent to $neur^+$ cells, but not in $neur^1$ cells in the center of mutant clones (Fig. 8F,F′). These results suggest that an important function of $neur^+$ in the eye is in R-cell restriction and that $neur^+$ functions with faf^+ and lqf^+ in the Delta signaling cells.

Discussion

Delta signaling requires Lqf-dependent endocytosis of Delta

Cells with decreased lqf^+ activity accumulate Delta on apical membranes and fail to signal to neighboring cells. We examined three Notch/Delta signaling events in the eye: proneural enhancement, lateral inhibition and R-cell restriction (Fig. 9A). We find that loss of lqf^+-dependent endocytosis during all three events has identical consequences to loss of Delta function in the signaling cells. We conclude that lqf^+-dependent endocytosis of Delta is required for signaling, supporting the notion that endocytosis in the signaling cells

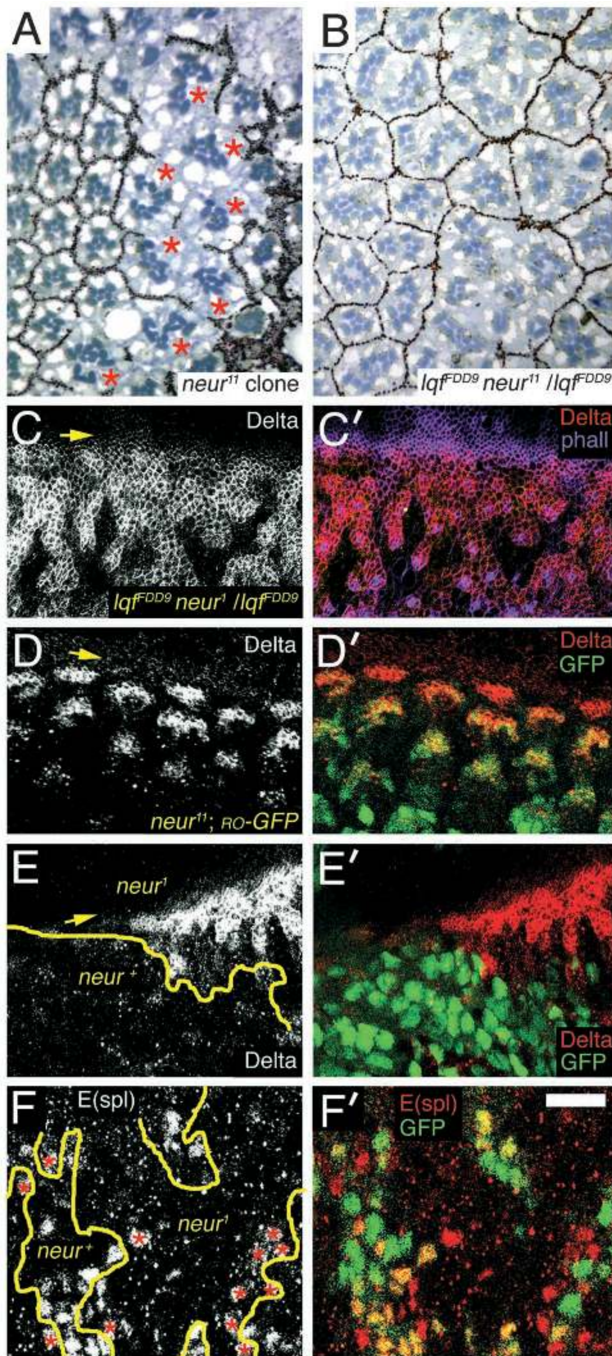

Fig. 8. Role of *neur*+ in eye patterning. (A,B) Tangential sections of adult eyes are shown. In A, ommatidia with ectopic R-cells (indicated by asterisks) within a clone of *neur*^{11} cells. In B, the entire eye is the genotype indicated. (C,C′) Eye discs labeled with anti-Delta and phalloidin. (D,D′) Eye disc expressing a *RO-GFP* transgene and labeled with anti-Delta. (E,E′) Eye disc containing a clone of *neur*^{1} cells marked by the absence of GFP. In E, the clone border is outlined. The arrows in C-E indicate the position of the furrow. (F,F′) An eye disc labeled with mAb323 [recognizes E(spl) proteins] containing *neur*^{1} clones near the furrow, which are marked by the absence of GFP. In F, the clone borders are outlined and *neur*^{1} cells that express E(spl) are marked with asterisks. Discs were observed at depths throughout the apical/basal plane and a few E(spl)-positive cells were found at a distance from the clone borders. Scale bar: 20 μm in A-C′; 15 μm in D-F′.

activates Notch in the receiving cells. However, Lqf is not required absolutely for all Delta internalization in the eye. Even in *lqf*-null cells, which are incapable of Delta signaling, some vesicular Delta is present (see Fig. 7). Perhaps not all of the vesicular Delta present in wild-type discs results from signaling.

Deubiquitination of Lqf by Faf increases Lqf activity

Genetic studies in *Drosophila* indicate clearly that deubiquitination of Lqf by Faf activates Lqf activity (Wu et al., 1999; Cadavid et al., 2000). Moreover, genetic and biochemical evidence in *Drosophila* suggests that Faf prevents proteasomal degradation of Lqf (Huang et al., 1995; Chen et al., 2002). In vertebrates, however, it is thought that epsin is mono-ubiquitinated to modulate its activity rather than poly-ubiquitinated to target it for degradation (Oldham et al., 2002; Polo et al., 2002). If Lqf regulation by ubiquitin also occurs this way in the *Drosophila* eye, the removal of mono-ubiquitin from Lqf by Faf would activate Lqf activity.

Whatever the precise mechanism, given that both Faf and Lqf are expressed ubiquitously in the eye (Fischer-Vize et al., 1992; Chen et al., 2002), two related questions arise. First, why is Lqf ubiquitinated at all if Faf simply deubiquitinates it everywhere? One possibility is that Faf is one of many deubiquitinating enzymes that regulate Lqf, and expression of the others is restricted spatially. This could also explain why Faf is required only for R-cell restriction (see below). Another possibility is that Faf activity is itself regulated in a spatial-specific manner in the eye disc. Alternatively, Lqf ubiquitination may be so efficient that Faf is needed to provide a pool of non-ubiquitinated, active Lqf. Similarly, Faf could be part of a subtle mechanism for timing Lqf activation. Second, why is Faf essential only for R-cell restriction? One possibility is that there is a graded requirement for Lqf in the eye disc, such that proneural enhancement requires the least Lqf, lateral inhibition somewhat more and neural inhibitory signaling by R2/3/4/5 the most. The mutant phenotype of homozygotes for the weak allele *lqf*^{FDD9} supports this idea, as R-cell restriction is most severely affected. Alternatively, Lqf may be expressed or ubiquitinated with dissimilar efficiencies in different regions of the eye disc. More experiments are needed to understand the precise mechanism by which the Faf/Lqf interaction enhances Delta signaling.

Neur stimulates Delta internalization in the signaling cells

In *neur* mutants, Delta accumulates on the membranes of signaling cells and Notch activation in neighboring cells is reduced. These results support a role for Neur in endocytosis of Delta in the signaling cells to achieve Notch activation in the neighboring receiving cells, rather than in downregulation of Delta in the receiving cells. Because *neur* shows strong genetic interactions with *lqf* and both function in R-cells, Neur and Lqf might work together to stimulate Delta endocytosis. Lqf has ubiquitin interaction motifs (UIMs) that bind ubiquitin (Polo et al., 2002; Oldham et al., 2002). One explanation for how Neur and Faf/Lqf could function together is that Lqf facilitates Delta endocytosis by binding to Delta after its ubiquitination by Neur (Fig. 9B). This is an attractive model that will stimulate further experiments.

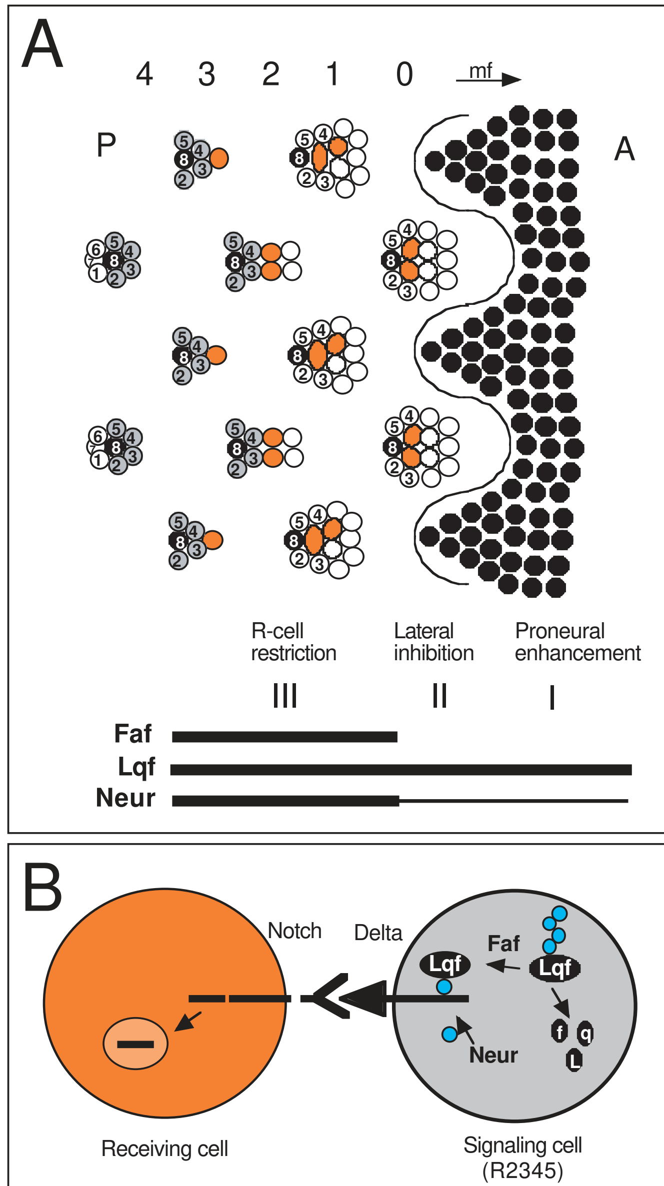

Fig. 9. Model for Faf, Lqf and Neur function. (A) Early events in ommatidial assembly (see Wolff and Ready, 1993). The morphogenetic furrow (mf) moves in the direction of the arrow. A is anterior and P is posterior. The first several columns (0-4) of developing ommatidia are shown. Atonal-expressing cells are black. R1-R8 are indicated. Three processes (I, II, III) that require Notch/Delta signaling are shown. (I) Proneural enhancement: Atonal expression is upregulated. (II) Lateral inhibition: Atonal expression is limited to groups of ~10 cells and ultimately to R8s in column 0. (III) R-cell restriction: R2/3/4/5 precursors signal their neighbors to prevent excessive neural determination. As the ectopic cells in *faf* mutants appear to arise between R3/4, they may be the orange cells. As depicted by the black bars, Faf is essential only for event III, Lqf is essential for events I, II and III, and Neur is essential for event III but is required to a lesser extent for the events I and II. (B) A model showing how Faf/Lqf may function with Neur in the Delta signaling cells is shown. The blue circles are ubiquitin. Lqf is deubiquitinated by Faf, which increases Lqf levels. Ubiquitination of Delta by Neur may stimulate interactions between Delta and Lqf and thereby facilitate Delta internalization.

Specificity of Lqf for Delta endocytosis

One exciting observation is that the endocytic adapter Lqf may be essential specifically for Delta internalization. Although we have not examined these signaling pathways directly, *hedgehog*, *decapentaplegic* and *wingless* signaling appear to be functioning in the absence of Lqf. These three signaling pathways regulate movement of the morphogenetic furrow (Lee and Treisman, 2002) and are thought to require endocytosis (Seto et al., 2002). The furrow moves through *lqf*-null clones and at the same pace as the surrounding wild-type cells (Fig. 7) (Overstreet et al., 2003). Moreover, all mutant phenotypes of *lqf*-null clones can be accounted for by loss of *Delta* function. Further experiments will clarify whether this apparent specificity means that Lqf functions only in internalization of Delta, or if the process of Delta endocytosis is particularly sensitive to the levels of Lqf.

Endocytic proteins as targets for regulation of signaling

Lqf expands the small repertoire of endocytic proteins that are known targets for regulation of cell signaling. In addition to Lqf, the endocytic proteins Numb and Eps15 (EGFR phosphorylated substrate 15) are objects of regulation. In vertebrates, asymmetrical distribution into daughter cells of the α-adaptin binding protein Numb may be achieved through ubiquitination of Numb by the ubiquitin-ligase LNX (Ligand of Numb-protein X) and subsequent Numb degradation (Nie et al., 2002). In addition, in vertebrate cells, Eps15 is phosphorylated and recruited to the membrane in response to EGFR activation and is required for ligand-induced EGFR internalization (Confalonieri et al., 2000). Given that endocytosis is so widely used in cell signaling, endocytic proteins are likely to provide an abundance of targets for its regulation.

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