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The function of the *Drosophila* Fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis

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SUMMARY

Fat facets is a deubiquitinating enzyme required in a cell communication pathway that limits to eight the number of photoreceptor cells in each facet of the *Drososphila* compound eye. Genetic data support a model whereby Faf removes ubiquitin, a polypeptide tag for protein degradation, from a specific ubiquitinated protein thus preventing its degradation. Here, mutations in the *liquid facets* gene were identified as dominant enhancers of the *fat facets* mutant eye phenotype. The *liquid facets* locus encodes epsin, a vertebrate protein associated with the clathrin endocytosis complex. The results of genetic experiments reveal that *fat facets* and *liquid facets* facilitate endocytosis and function in common cells to generate an inhibitory signal that prevents ectopic photoreceptor determination. Moreover, it is demonstrated that the *fat facets* mutant phenotype is extraordinarily sensitive to the level of *liquid facets* expression. We propose that Liquid facets is a candidate for the critical substrate of Fat facets in the eye.

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

INTRODUCTION

Ubiquitin (Ub), a highly conserved 76-amino acid polypeptide that can be linked covalently to an internal lysine residue of a target protein, is an important regulator of molecular pathways in development (Maniatis, 1999; Koepp et al., 1999). Mono-ubiquitination can serve as a signal for modulation of protein activity (Chen et al., 1996; van Delft et al., 1997a) or endocytosis (Hicke, 1999; Strous and Govers, 1999). Alternatively, a poly-Ub chain, which serves as a recognition signal for the proteasome, a protein degradation complex, may be formed on the targeted protein (Hershko, 1998). In addition to the enzymes required to ubiquitinate target proteins (Scheffner et al., 1998), there is a large family of deubiquitinating enzymes that cleave Ub-protein bonds and play diverse roles in the Ub pathway (Wilkinson and Hochstrasser, 1998). One *Drosophila* deubiquitinating enzyme, encoded by the *fat facets* (*faf*) gene, is required in a cell communication pathway that determines cell fate during compound eye development (Fischer-Vize et al., 1992a). Unique among deubiquitinating enzymes, Faf has been shown genetically to antagonize ubiquitination and proteolysis (Huang et al., 1995; Wu et al., 1999). Thus, Faf is thought to remove a Ub chain from a specific substrate thereby preventing its degradation.

In a genetic screen for dominant enhancers of the *faf* mutant eye phenotype, designed to identify among other genes in the *faf* pathway the critical substrate of Faf in the eye (Fischer et al., 1997), we isolated mutants in a previously uncharacterized essential *Drosophila* gene named *liquid facets* (*lqf*). Here, cloning and molecular characterization of *lqf* show that it encodes two homologs of vertebrate epsin, a protein associated with the clathrin-mediated endocytosis complex.

There are three key components of the endocytosis complex (Robinson, 1994): (1) clathrin, which forms a cage structure engulfing the cell membrane, (2) AP-2, the core adaptor complex, which binds to clathrin and brings it to the cell surface and (3) dynamin, a GTPase required for vesicle formation. Additional proteins associated with AP-2 have been identified, many of which contain protein-protein interaction domains called EH-domains and EH-domain-binding motifs (De Fiore et al., 1997; Marsh and McMahon, 1999; Mayer, 1999). Epsin is an EH-domain-binding protein identified as a partner for Eps15 (Chen et al., 1998), an EH-domain protein that also binds AP-2 (Wong et al., 1995; Benmerah et al., 1996; Tebar et al., 1996; van Delft et al., 1997b; Iannolo et al., 1997; Carbone et al., 1997; Benmerah et al., 1998; Wendland and Emr, 1998). The large number of AP-2-binding proteins identified suggests that many of them may have temporal and/or tissue-specific functions (Marsh and McMahon, 1999). The precise roles of Eps15 and epsin in endocytosis are unknown.

Genetic interaction experiments with *faf*, *lqf* and other endocytosis and Ub pathway gene mutants reveal that both *faf* and *lqf* gene activities facilitate endocytosis and antagonize ubiquitination. In addition, phenotypic analysis of *lqf* mutants in the eye and genetic mosaic and transgene experiments indicate that *faf* and *lqf* function in common cells on the

signaling side of a biochemical pathway that prevents the formation of ectopic photoreceptors early in eye development. Moreover, it is observed that a subtle increase in the level of *lqf* expression obviates the need for *faf* during eye development. These results indicate that endocytosis, and epsin in particular, is required for the specific cell communication events in eye patterning that depend on *faf* activity. In addition, we propose that Lqf is a candidate protein for the critical substrate of Faf in eye development.

MATERIALS AND METHODS

Drosophila genetics

Drosophila were grown on standard food at 25°C unless noted otherwise. Standard cross schemes were used to generate the genotypes described. All marker mutations are described in Lindsley and Zimm (1992).

Recombination and physical mapping of *lqf*

The lqf^{FDD9} allele was localized to polytene position 66A on chromosome 3L by calculating recombination frequency with respect to four markers (*th cu sr e*) and then a series of P{w^+} insertions as described previously (Fischer-Vize et al., 1992b). Each *lqf* allele (Table 1) failed to complement the lethality of *Df(3L)pbl-X1* (65F3-66B10; Hime and Saint, 1992). Using a *th* lqf^{FDD9} chromosome as previously described (Fischer-Vize et al., 1992b), lqf^{FDD9} was localized 0.23% proximal to P{w^+} *l(3)j8A6* at 66A1-2 (Berkeley *Drosophila* Genome Project (BDGP)) and 0.29% distal to P{w^+} *l(3)S5277* at 66A8-10 (BDGP). Analysis of the breakpoints of the inversion associated with lqf^{AG} localized *lqf* precisely to 66A3-4.

Identification of *lqf* mutant alleles

The alleles lqf^{cE278}, lqf^{ARI} and lqf^{AG} were isolated in an F_1 screen for enhancers of *faf* performed as described (Fischer et al., 1997) using X-rays (4000 rads) as a mutagen. The lqf^{BT} allele was isolated in an F_2 screen for X-ray-induced lethal mutations in *trans* to lqf^{FDD9} as follows. Males (*st)* were exposed to X-rays (4000 rads) and then crossed to *TM3/TM6B* females. Individual male progeny (*st/TM3* or *st/TM6B*) were crossed with lqf^{FDD9} *th st cu sr e/ TM6B* females and the absence of *st*/lqf^{FDD9} *th st cu sr e* progeny identified an X-ray-induced *lqf* allele on the *st* chromosome. The allele referred to as lqf^{AX1} previously (Fischer et al., 1997) has been renamed lqf^{BT}.

Table 1. Mutant alleles of *lqf*

Allele	Homozygous phenotype	Special features	Induced by
lqf^{FDD9}	Viable; eye and wing defects	Temperature sensitive	EMS
lqf^{bE25}	Lethal	None	EMS
lqf^{bE428}	Lethal	None	EMS
lqf^{cE278}	Lethal	Df(3L)65F; 66B10-11	X-rays
lqf^{BT}	Lethal	None	X-rays
lqf^{AG}	Lethal	In(3L)66A3-4; 67D7-9	X-rays
lqf^{ARI}	Lethal	Small deletion	X-rays
$lqf^{P011027}$	Lethal	P-element insertion	P element

Identification of $lqf^{bE25}$and lqf^{bE428} was described previously (Fischer et al., 1997) and lqf^{FDD9} was isolated in the mutant screen described previously (Fischer-Vize et al., 1992b). The allele $lqf^{P011027}$, originally named *l(3)0110/27*, was identified as described (Deak et al., 1997). The four X-ray-induced alleles were generated in an F_1 mutagenesis screen for enhancers of *faf* or in an F_2 screen for lethal mutations in trans to lqf^{FDD9} (Materials and Methods). The name 'liquid facets' refers to the appearance of the sectioned retina in clones of cells homozygous for lqf^{BT} (Fischer et al., 1997).

Cloning of *lqf*

Standard techniques (Sambrook et al., 1989) were used for molecular biology procedures. The initial cloning strategy was to isolate a series of overlapping clones containing all of the genomic DNA between P{w^+}*l(3)j8A6* and *P*{w^+}*l(3)S5277*. Genomic DNA fragments adjacent to each P{w^+} element were isolated by plasmid rescue and used to initiate chromosomal walks in a *Drosophila* genomic DNA library in bacteriophage (Stratagene). After seventeen steps towards 66A3-4 were taken from each rescue fragment, polytene chromosome hybridization experiments indicated that the walk was yet incomplete. Meanwhile, the inversion allele lqf^{AG} was isolated and its breakpoint at 66D7-9 fused *lqf* gene sequences to genomic DNA contained within P1 clone DS01747 (BDGP); when used as a hybridization probe to lqf^{AG}/lqf^{+} polytene chromosomes, the P1 clone DNA was observed to cross the inversion breakpoint of lqf^{AG}. In DNA blotting experiments and by polytene chromosome hybridization, the inversion breakpoint was localized to a 4.0 kilobase (kb) *Sac*I fragment of the P1 clone. In order to isolate *lqf* genomic DNA sequences, a *Drosophila* genomic DNA library in bacteriophage was generated from lqf^{AG}/lqf^{+} flies and, using the 4.0 kb *Sac*I fragment as a hybridization probe, recombinant phage clones were isolated that contain the inversion breakpoint. A DNA fragment containing sequences from 66A adjacent to the breakpoint was identified and used to isolate phage from a wild-type *Drosophila* genomic DNA library. A 6.3 kb fragment of wild-type genomic DNA containing the inversion breakpoint was identified and used as a probe to an eye disc cDNA library (see Fischer-Vize et al., 1992a) resulting in the isolation of cDNA-2 and cDNA-3. Subsequently, the P element insertion allele $lqf^{P011027}$ (Deak et al., 1997) became available and 17 kb of genomic DNA adjacent to it was isolated by plasmid rescue. The position of the P element on the genomic DNA map was determined by sequencing ~400 base pairs (bp) of the 17 kb genomic fragment using a primer corresponding to the end of the plasmid vector adjacent to the P element insertion point. The DNA sequence obtained contained 118 nucleotides of novel genomic sequence, followed by 234 nucleotides of the 5′UTR sequence in cDNA-3, followed by novel genomic sequence that appears to be the 5′ end of an intron. Thus, it was concluded that the P element is inserted either in the 5′UTR or within the promoter of *lqf*. The approximate positions of the genomic DNA lesions in lqf^{ARI} and lqf^{AG} were determined in DNA blotting experiments.

DNA sequence analysis

The DNA sequences of cDNA-3 and cDNA-2 were determined on both strands using an automated fluorometric method and the sequences were compiled and analyzed using AssemblyLign and MacVector software. BLAST analysis (tblastn and blastp) was performed using the NCBI Web page. The DNA sequences of cDNA-3 and cDNA-2 have been submitted to GenBank (Accession numbers AF160975 and AF160976, respectively).

P element constructs and transformation

The ~20 kb genomic DNA fragment in phage 19G was subcloned as a *Not*I fragment into the *Not*I site of pCasper 3 (Thummel and Pirrotta, 1992). The *ro-lqf* plasmid was generated as follows. An *Eco*RI fragment containing cDNA-3 was subcloned into Bluescript (Stratagene) and the *Eco*RV site in the polylinker at the 5′ end of *lqf* was changed to *Asc*I. A FLAG tag was introduced into the 5′ end of the cDNA as follows. A 5′ end fragment was generated by PCR using cDNA-3 as a template and two primers: 5′-primer: 5′-TTGGCGCGCCCAACATGGGATCCCAGGTCAATGTCGCTGGT-3′ and 3′-primer: 5′-TTGCACTGCTGGGCGACC-3′. The resulting 315 bp fragment was subcloned into the *Sma*I site of a derivative of Bluescript with no *Bam*HI site. A FLAG tag was introduced into the *Bam*HI site just 3′ to the ATG start codon using the following two annealed 30-mers: 5′-GATCCGACTACAAGGACGACGATGAC-AAGG-3′ and 5′-GATCCCTTGTCATCGTCGTCCTTGTAGTCG-3′. An *Asc*I-*Ppu*MI fragment of the resulting plasmid, containing the

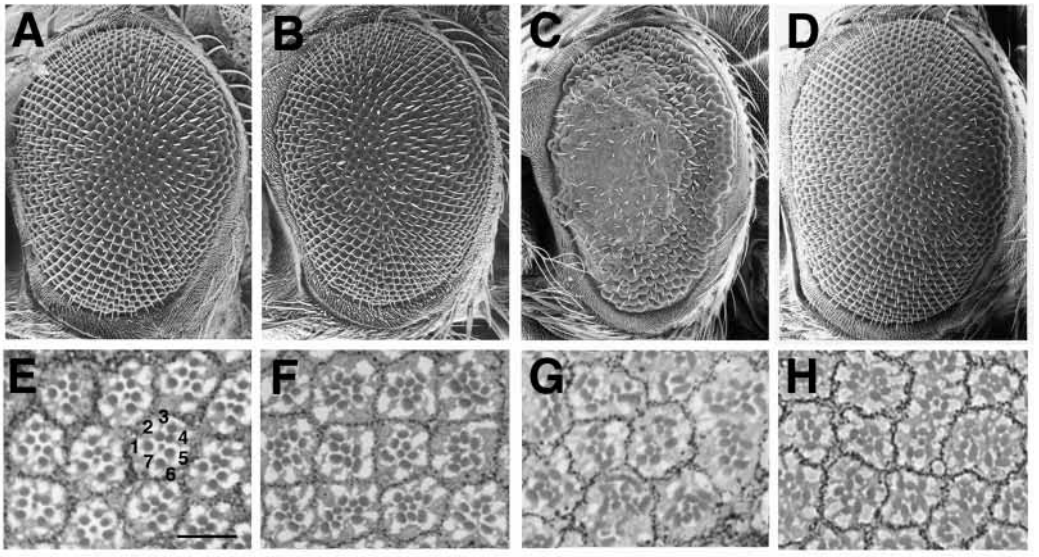

Fig. 1. Genetic interactions between *faf* and endocytosis mutants. Scanning electron micrographs (A-D) and apical tangential sections (E-H) of adult *Drosophila* eyes are shown. (A,E) faf^{BX3}/faf^{FO8}. The allele faf^{BX3} is weak and faf^{FO8} is strong (Fischer-Vize et al., 1992a). These eyes are nearly wild-type (compare to Fig. 2A,D) but have reduced *faf* activity and thus provide a sensitized background (Fischer et al., 1997). Seven of eight trapezoidally arranged photoreceptor cells (1-7) are visible in an apical section and these are labeled in a phenotypically wild-type facet in (E). (B,F) lqf^{bE25} faf^{BX3}/faf^{BX3}. (C,G) lqf^{AG} faf^{BX3}/faf^{FO8}. (D,H) chc^{1} faf^{BX3}/faf^{FO8}. The chc^{1} allele is null; no enhancement was observed with chc^{4}, which is hypomorphic (Bazinet et al., 1993). Both chc^{1} and chc^{4}, however, are strong dominant enhancers of the mutant phenotypes of lqf^{bE25} faf^{BX3}/faf^{BX3} flies and lqf^{FDD9} homozygotes (see Fig. 4). All of the mutant phenotypes shown are fully penetrant. Dominant enhancement similar to that seen with chc^{1} was observed with shi^{EM42} and shi^{EM45} (Grant et al., 1998) but with lower penetrance and slight but fully penetrant enhancement was observed with shi^{ts1} and shi^{ts2} (Grigliatti et al., 1973; Chen et al., 1991) homozygotes. No genetic interactions were observed between *α-Ada* (Gonzalez-Gaitan and Jackle, 1997) and *faf*. The scale bar in E represents 20 μm (E-H) and 120 μm (A-D).

FLAG-tagged 5′ end of *lqf* was exchanged with the *Asc*I-*Ppu*MI fragment of cDNA-3 in Bluescript. The *Sna*BI site at the 3′ end of *lqf* in the resulting plasmid was changed to *Asc*I and the *Asc*I fragment containing FLAG-tagged cDNA-3 was subcloned into the *Asc*I site of the pRO (Huang and Fischer-Vize, 1996) P element transformation vector. Four independent P{w^{+}, *ro-lqf*} insertion lines were generated and one copy of each of the three tested complemented completely the mutant eye phenotype of lqf^{FDD9} at 25°C. P element transformation of w^{1118} flies was performed as described previously (Spradling, 1986; Fischer-Vize et al., 1992a) and P{w^{+}} elements were localized to a chromosome using standard crosses.

Mosaic analysis

Clones of w^{-} cells homozygous for lqf^{FDD9} in the eyes of w^{+} heterozygotes (lqf^{+}/lqf^{FDD9}) were generated using the FLP/FRT technique (Xu and Rubin, 1993). After heat shock, w^{-} clones were observed in females of the genotype *w P{ry⁺, hs-FLP}/ w; lqf^{FDD9} P{ry⁺, neoFRT}80B/ P{w⁺}70C P{ry⁺, neoFRT}80B.*

Analysis of eyes, wings and legs

Larval eye disc staining was performed as described (Fischer-Vize et al., 1992a,b; Huang and Fischer-Vize, 1996) with PEMS + 4% paraformaldehyde fix and PBST washes. The primary antibody was rat mAb-Elav (Robinow and White, 1991; a gift of G. M. Rubin) used straight and the secondary antibody was HRP-anti-rat (Santa Cruz Biochemicals) used at 1:250. Scanning electron micrographs and tangential sections of adult eyes were produced as described previously (Huang et al., 1995). Wings and legs were dehydrated in 70% ethanol, mounted in DPX (Fluka) and photographed with a Zeiss Axioplan microscope.

RESULTS

lqf mutants are dominant enhancers of *faf*

The model for Faf function is that Faf deubiquitinates and thus protects from degradation a particular targeted substrate (Huang et al., 1995; Wu et al., 1999). We reasoned that decreasing the level of the substrate by mutagenizing one gene copy would magnify the eye defects in flies homozygous for hypomorphic *faf* mutations. Therefore, in an attempt to identify the gene encoding the substrate of Faf, mutagenesis screens for dominant enhancers of the *faf* mutant eye phenotype were performed and mutations in the *lqf* gene were identified (Fischer et al., 1997). The eyes of homozygotes for hypomorphic *faf* mutations are nearly wild-type (compare Fig. 1A,E with Fig. 2A,D) and flies with only one functional *lqf* gene copy also have wild-type eyes. However, when *faf* activity is compromised and the *lqf* gene dose is halved in the same flies, their eyes resemble those of flies with no *faf* gene function (*faf* null mutants, see below and Fischer-Vize et al., 1992a); nearly all of the facets (unit eyes) contain one or more ectopic photoreceptors in addition to the normal complement of eight (Fig. 1B,F). In a background with slightly less *faf* activity, halving the *lqf* gene dose results in severely malformed eyes (Fig. 1C,G), even more disrupted than *faf* null mutant eyes. The extreme sensitivity of the *faf* mutant eye phenotype to a decrease in the *lqf* gene dosage suggests that the two genes function in the same direction in a biochemical pathway.

The *lqf* mutant phenotype

The *lqf* gene itself is essential in *Drosophila*; in an otherwise wild-type background, *lqf* null mutants (lqf^{cE278}/lqf^{AG}, Table 1) die as embryos. Clones of cells in the eye in which there is little or no *lqf* gene function have severely disrupted eye morphology (Fischer et al., 1997) indicating that *lqf* is required also after embryogenesis for eye development.

The mutant phenotypes associated with two weak *lqf* mutant alleles (lqf^{FDD9} and lqf^{P11027}, Table 1) reveal specific roles for *lqf* in eye, wing and leg development (Fig. 2A-L). The lqf^{FDD9} allele is temperature sensitive; at 18°C lqf^{FDD9} homozygotes

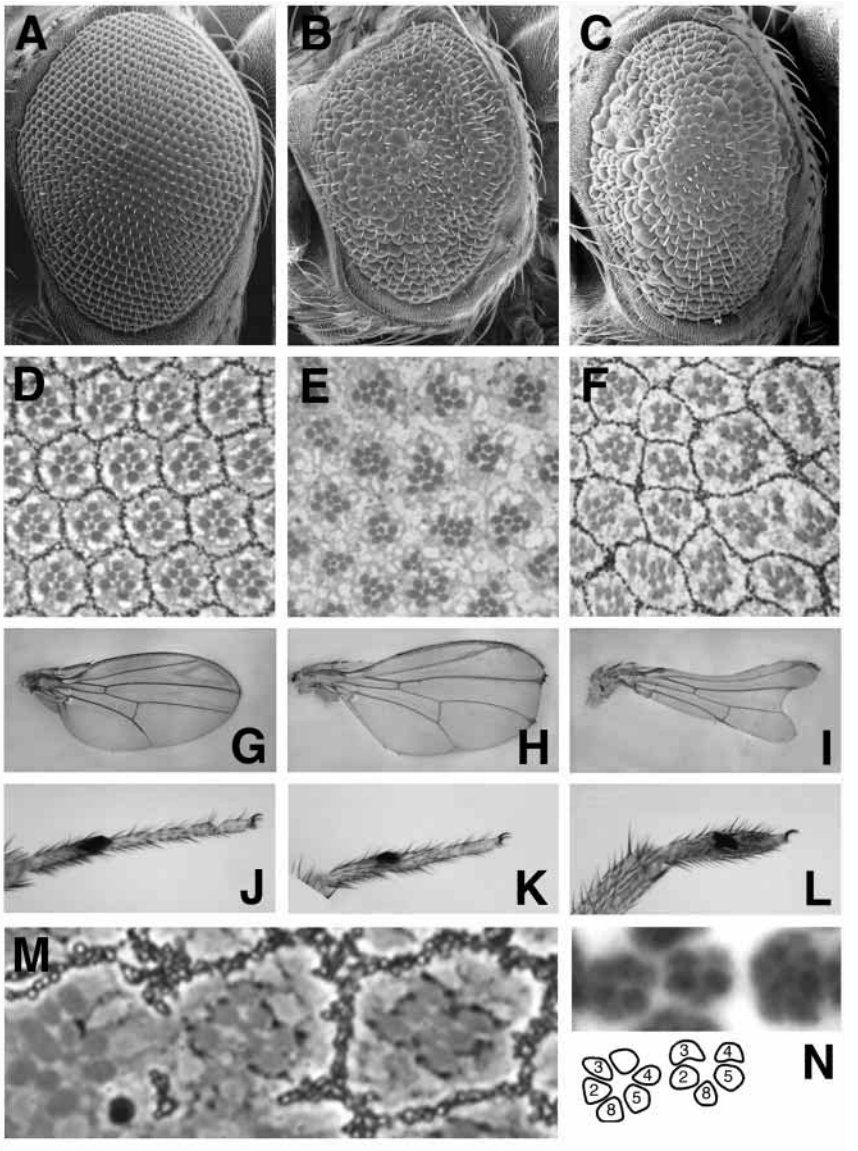

Fig. 2. Mutant phenotypes of viable *lqf* mutants. Eyes (A-C, scanning electron micrographs and D-F, apical tangential sections), wings (G-I) and first legs (J-L) of adult *Drosophila* grown at 25°C are shown. (A,D,G,J) Wild-type; (B,E,H,K) lqf^{FDD9}/lqf^{FDD9}. Mutant eyes (E) contain facets with ectopic photoreceptor cells. The eyes of lqf^{FDD9} and *faf* null mutants are similar (compare E to Fig. 5C). (H) Mutant wings have thickenings of the longitudinal veins and clumps of bristles and notching at the wing margin. (K) Mutant legs have fused and shortened tarsal segments. Mutant second and third legs have defects similar to those in the first legs and a variety of bristle duplications were also observed. (C,F,I,L) $lqf^{FDD9}/lqf^{P011027}$. Mutant phenotypes similar to but more severe than those in lqf^{FDD9} homozygotes are observed. (M) An apical tangential section of an adult eye containing a clone of w^{-} lqf^{FDD9} cells induced in a w^{+} lqf^{+} background (Materials and Methods). Facets mosaic for w^{+} lqf^{+} and w^{-} lqf^{FDD9} R-cells at the clone border are shown. The black dots associated with each R-cell are pigment granules and mark the w^{+} lqf^{+} cells. At right are two phenotypically mutant facets in which every R-cell is w^{+} lqf^{+}. (N) Facets at an early stage of development in a lqf^{FDD9} larval eye disc (larvae grown at 25°C) immunostained with an antibody that recognizes neural nuclei (Materials and Methods) are shown. Facets assemble stepwise beginning with the photoreceptors in a stereotyped order (Wolff and Ready, 1993). Early in facet assembly, preclusters of approximately seven cells form and resolve into five-cell preclusters containing the future photoreceptors 8, 2, 5, 3 and 4. The excluded cells, called the 'mystery cells' (M-cells), vary in number and in the immature precluster are located between the precursors to photoreceptors 3 and 4 (Tomlinson and Ready, 1987). In *faf* and also in lqf^{FDD9} mutants, the M-cells remain in the mature preclusters and become ectopic photoreceptors. A wild-type appearing facet (middle) is adjacent to one (left) with an ectopic photoreceptor between cells 3 and 4. Another facet (right) has far too many photoreceptors.

are nearly wild-type while at 25°C eye, wing and leg defects are observed (Fig. 2A-L). The eye defects in lqf^{FDD9} homozygous adults resemble those in *faf* null mutants (Fig. 2E and see below). As in *faf* mutants (Fischer-Vize et al., 1992a), the additional photoreceptors in *lqf* mutants arise from specific precursor cells (M-cells) present early during eye development (Fig. 2N). In contrast to *lqf* null mutants, *faf* null mutants are viable, have normal wings and legs and have less severe eye defects (Fischer-Vize et al., 1992a). Thus, *lqf* functions more broadly than *faf*, but both the *lqf* and *faf* genes are required during eye development in order to prevent the M-cells from becoming photoreceptors.

Cloning the *lqf* gene: *lqf* encodes *Drosophila* epsin

The *lqf* gene was localized between two closely linked *P*{*w*+} element insertions on chromosome 3 by meiotic and physical mapping (Materials and Methods). Genomic DNA corresponding to the *lqf* gene was cloned by chromosomal walking, using the breakpoints of lqf^{AG}, lqf^{ARI} and a P element insertion allele (Table 1) as indicators of the locus (Fig. 3A) (Materials and Methods). Two cDNAs in the region were identified in a *Drosophila* eye disc cDNA library and their complete DNA sequences determined. The two cDNAs appear to represent alternately spliced versions of the same primary transcript, which encode similar putative proteins of 784 and 640 amino acids (Fig. 3B). Comparison of the amino acid sequences with the sequence database (Materials and Methods) indicates that each cDNA encodes a *Drosophila* homolog of the rat epsin protein (Fig. 3B,C).

A 20 kb *Drosophila* genomic DNA fragment (19G, Fig. 3A) was cloned into a P-element transformation vector and used to generate P{w^{+}, lqf^{+}*19G*} transformants lines. One copy of the P{w^{+}, lqf^{+}*19G*} transgene in each of the four insertion lines tested complemented completely the mutant eye, wing and leg phenotypes of lqf^{FDD9} homozygotes at 25°C and also the lethality and all mutant phenotypes of *lqf* null mutants (lqf^{cE278}/lqf^{AG}, data not shown). In addition, one copy of a P element construct (*ro-lqf*) in which expression of the longer *lqf* cDNA (cDNA-3, Fig. 3A) is driven from an eye-specific promoter (see below) complements completely the eye defects

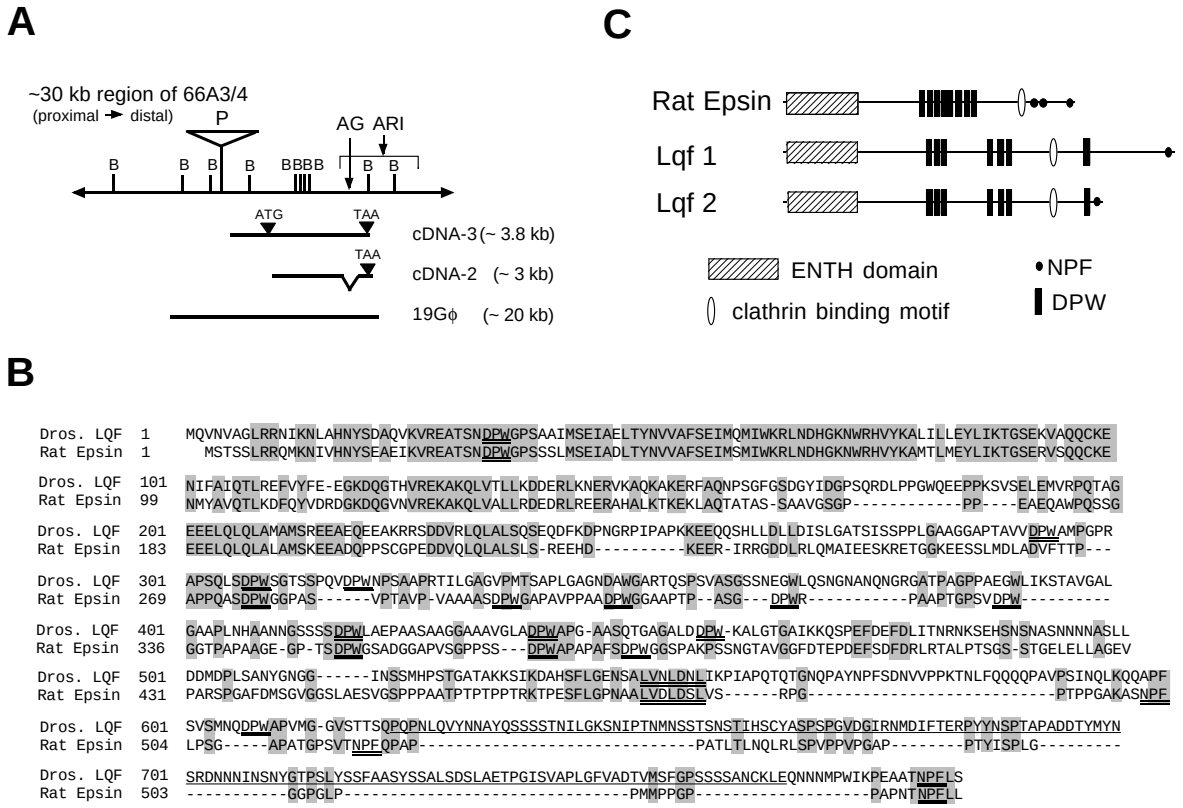

Fig. 3. Molecular analysis of the *lqf* gene. (A) A molecular map of the *lqf* locus is shown. The top line is a *Bam*HI (B) restriction map of the genomic DNA. AG and ARI indicate the *Bam*HI fragments to which the lesions in lqf^{AG} and lqf^{ARI} (Table 1) respectively, are localized and P indicates the approximate position of the P-element insertion in $lqf^{P011027}$ (Materials and Methods). The lines beneath the genomic DNA represent cDNAs and indicate the *Bam*HI fragments to which each cDNA corresponds; the precise exon/intron of the structure of the cDNAs was not determined. cDNA-3 is 3798 bp and contains a 2352 bp open reading frame. With respect to cDNA-3, cDNA-2 has a 5′ end truncated by 325 bp and also lacks 144 bp of coding sequence contained within the open reading frame of cDNA-3 (see below). The start codon (ATG) of cDNA-3 is at nuclotide 256 and is the first ATG in the cDNA sequence. When used as hybridization probes, each of the two cDNAs detects two mRNAs of approximately 3.8 and 3.6 kb in RNA blots of wild-type eye disc RNA. The line labeled 19Gϕ represents a genomic DNA fragment from a recombinant bacteriophage that contains the entire *lqf* gene. (B) Lqf and rat epsin (AF018261; Chen et al., 1998) amino acid sequences, aligned using ClustalW in MacVector, are shown. Shaded regions correspond to identical amino acids. The singly underlined region of Lqf (amino acids 621-767) is absent in cDNA-2. The DPW repeats bind α-adaptin (Chen et al., 1998; Owen et al., 1999) and the NPF repeats define domains for binding to proteins with EH-domains (Salcini et al., 1997; Paoluzi et al., 1998). The sequence LVDLDSL in rat epsin is the clathrin binding motif (Kay et al., 1999). The N-terminal region of high sequence similarity is the ENTH (Epsin N-terminal Homology) domain (Kay et al., 1999). BLAST analysis aligned Lqf with many proteins across species in addition to epsin that contain the ENTH domain but are otherwise unrelated or less related to Lqf than to epsin. (This group includes yeast Ent1 and Ent2 (Wendland et al., 1999; Kay et al., 1999). In addition, the BLAST search revealed two other proteins, *Xenopus* MP90 (U95102) and mouse Ibp2 (AF057286), that are likely to be epsin homologs (see Kay et al., 1999), rat Epsin 2 (AF062085) and three human epsins (AF073727, AF062085, AF062084). All of these proteins have DPW and NPF motifs in variable numbers and a clathrin binding domain. (C) A comparison of the structure of rat epsin and Lqf is shown. Lqf1 and Lqf2 are encoded by cDNA-3 and cDNA-2, respectively.

of lqf^{FDD9} homozygotes at 25°C (data not shown). Thus, we conclude that the *lqf* gene encodes *Drosophila* epsin.

Genetic interactions between *lqf*, endocytosis mutants and *faf*

The results of experiments in which epsin function was disrupted in cultured cells showed that rat epsin is required for endocytosis (Chen et al., 1998). The *S. cerevisiae* epsin-like genes *ent1* and *ent2* have also been shown genetically and biochemically to function in endocytosis in yeast (Wendland et al., 1999). In order to determine if Lqf1 and Lqf2 are involved in clathrin-mediated endocytosis in *Drosophila*, genetic interactions between *lqf* mutations and mutations in three other genes known to encode components of the endocytosis complex were tested: *chc* (*clathrin heavy chain;* Bazinet et al., 1993)), *α-Ada* (*α-adaptin;* Gonzalez-Gaitan and Jackle, 1997) and *shi* (*shibire;* Grigliatti et al., 1973; Chen et al., 1991; Grant et al., 1998). The *lqf* mutant phenotype is enhanced by decreasing the activity of each of these gene products, most notably by decreasing *chc* activity (Fig. 4). These results provide genetic evidence that the Lqf protein indeed facilitates endocytosis.

The strong genetic interactions between *faf* and *lqf* suggest that the endocytosis complex may be regulated by Ub-mediated protein degradation and Faf. Although the enhancement of the *faf* mutant phenotype by *lqf* mutations is the strongest effect (Fig. 1A-C,E-G), the observation that *chc* (Fig. 1D,H) and *shi* (Fig. 1 legend) mutations also act as dominant enhancers of *faf*

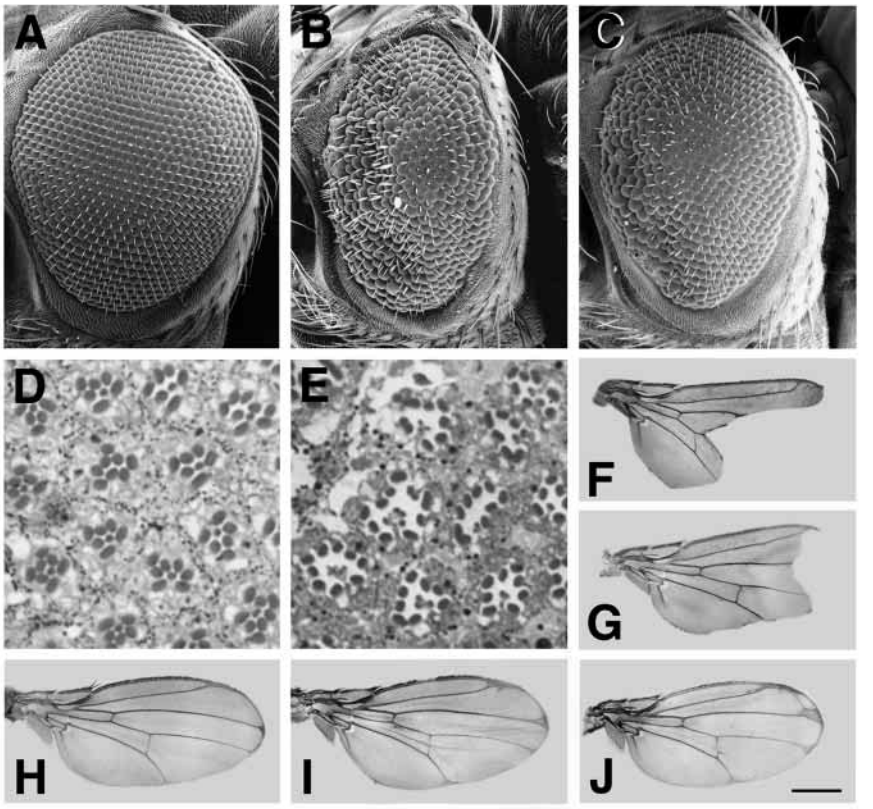

Fig. 4. Genetic interactions between *lqf* and mutants in endocytosis genes. Eyes (A-C, scanning electron micrographs and D,E, apical tangential sections) and wings (F-J) of adult *Drosophila* are shown. All flies were grown at 18°C except for those in F and G, which were grown at 25°C. (A,D,H) lqf^{FDD9}/lqf^{FDD9}. These eyes and wings are weakly mutant (compare with wild-type; Fig. 2A,D,G); some of the facets in D have too many R-cells and there is slight thickening of the longitudinal veins at the wing margins in H. (B,E) $chc^{4}/+$; lqf^{FDD9}/lqf^{FDD9}. The eye defects are enhanced relative to D. (I) $chc^{1}/+$; lqf^{FDD9}/lqf^{FDD9}. This genotype is lethal; these wings are from rare escapers that also have severely malformed eyes and tarsal fusions. The wing phenotype is enhanced relative to H. The *chc* alleles are described in Fig. 2 legend. (C,J) α-$Ada^{3}/+$; lqf^{FDD9}/lqf^{FDD9}. (C) The anterior of the eye (left) is rough (compare to A) and the mutant wing phenotype (J) is enhanced relative to H. The other existing α-*Ada* mutant allele is a hypomorphic P allele that is weaker than α-Ada^{3} (Gozalez-Gaitian and Jackle, 1997) and it shows no enhancement. (F,G) $chc^{1}/+$; lqf^{bE25} faf^{BX3}/faf^{BX3}. The wings have much more severe defects than those of lqf^{bE25} faf^{BX3}/faf^{BX3}, which are similar to those in H. All of the mutant phenotypes shown here are fully penetrant. Enhancement similar to that shown in B and E was observed with shi^{ts1} and shi^{ts2} homozygotes and this effect was not fully penetrant. Eyes and wings were prepared and photographed as described in Fig. 2. The scale bar in J is 20 μm (D,E), 120 μm (A-C) and 500 μm (F-J).

Table 2. Suppression of *lqf*:*faf* interactions by Ub pathway mutants

Genotype	% mutant facets
lqf^{bE25} faf^{BX3}/faf^{FO8}	100
lqf^{bE25} $faf^{BX3}/UbcD1^{XS347}$ faf^{FO8}	3±2
lqf^{bE25} $faf^{BX3}/UbcD1^{1462}$ faf^{FO8}	34±8
lqf^{bE25} $faf^{BX3}/l(3)73Ai^{1rv10e}$ faf^{FO8}	88±1
lqf^{bE25} $faf^{BX3}/l(3)73Ai^{1}$ faf^{FO8}	74±4

Recessive lethal mutations in *UbcD1* (Cenci et al., 1997; Neufeld et al., 1998) and *l(3)73Ai* (Saville and Belote, 1993) were tested for genetic interactions with *lqf*. *UbcD1* encodes a Ub conjugating enzyme (Treier et al., 1992) which is required for Ub chain formation (Scheffner et al., 1998) and *l(3)73Ai* encodes a subunit of the proteasome (Saville and Belote, 1993). Mutations in each gene were previously shown to act as strong dominant suppressors of the *faf* mutant eye phenotype (Huang et al., 1995; Wu et al., 1999). For each genotype, 100-200 facets in each of three eyes were analyzed.

supports this idea. In addition, the mutant eye phenotype resulting from *lqf* mutations in a weak *faf* mutant background was suppressed by debilitating the ubiquitination machinery genetically (Table 2), confirming a link between the Ub pathway and *lqf* function in the eye. Thus both *faf* and *lqf* gene activities antagonize ubiquitination and facilitate endocytosis.

lqf and *faf* function in the same cells

In its role of preventing the M-cells from becoming photoreceptors, *lqf*, like *faf*, appears to function at least in part from outside the M-cells. Facets mosaic for lqf^{+} and lqf^{-} cells were generated and phenotypically mutant facets containing ectopic photoreceptors were observed in which each photoreceptor, including the ectopic one, is lqf^{+} (Fig. 2M). Thus, the presence of cells with reduced levels of *lqf* gene function outside the M-cells and the photoreceptors in the facet can result in the misdetermination of the M-cells as photoreceptors. Similar results were observed in facets mosaic for faf^{+} and faf^{-} cells (Fischer-Vize et al., 1992a), indicating that both the *lqf* and *faf* gene products can influence M-cell fate from cells outside of the early facet precluster.

This conclusion is supported by the results of experiments described above in which a P{w^{+}, *ro-lqf*} transgene complements the eye defects of lqf^{FDD9} homozygotes at 25°C. The *ro* expression vector (pRO, Huang and Fischer-Vize, 1996) activates transcription in undifferentiated cells surrounding the facet preclusters early in eye development and also later in a subset of photoreceptors (Kimmel et al., 1990; Heberlein et al., 1994; Dokucu et al., 1996). A transgene that expresses *faf* with the identical promoter, P{w^{+}, *ro-faf*}, has been shown previously to complement completely the *faf* null mutant eye phenotype due to the early expression of the transgene (Huang and Fischer-Vize, 1996). Thus, we conclude that, in order to prevent misdetermination of the M-cells as photoreceptors, *lqf* and *faf* gene functions are essential in the *ro*-expressing cells surrounding the facet preclusters.

Slight overexpression of *lqf* obviates the need for *faf*

We have presented genetic data consistent with the idea that Lqf and Faf proteins function in the same cells to facilitate a

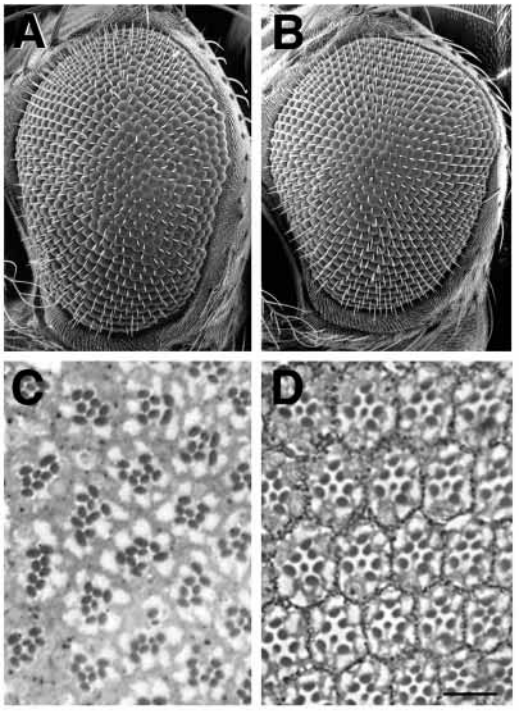

Fig. 5. Complementation of the *faf* mutant eye phenotype by *lqf* genes in P elements. Scanning electron micrographs (A,B) and apical tangential sections (C,D) of adult *Drosophila* eyes, prepared and photographed as in Fig. 2. (A,C) faf^{FO8}/faf^{BX4}. The faf^{FO8} allele is strong and faf^{BX4} is null (Fischer-Vize et al., 1992a). (B,D) P{w^+, lqf^+ *19G*} (1 copy); faf^{FO8}/faf^{BX4}. These eyes appear wild-type (compare to Fig. 2A and D). One copy of each of the five independent P{w^+, lqf^+ *19G*} lines or each of two independent P{w^+, *ro-lqf*} lines complement completely the faf^{FO8}/faf^{BX4} mutant eye phenotype. The scale bar in D is 20 μm (C,D) and 120 μm (A,B).

cell communication pathway regulated by Ub. The model for the function of Faf whereby it deubiquitinates and thus prevents proteolysis of its substrate predicts that overexpression of the substrate could eliminate the need for Faf in the eye. Thus, we tested whether additional copies of lqf^+ provided by P elements could substitute for *faf* in the eye. Remarkably, one additional copy of the *lqf* gene in a lqf^+ background, provided either by the P element containing the 20 kb fragment of genomic DNA from the *lqf* locus (P{w^+, lqf^+ *19G*}) or by the P element containing the *ro-lqf* cDNA construct (P{w^+, *ro-lqf*}) complements completely the eye defects in *faf* null mutants (Fig. 5). Thus, one extra copy of the *lqf* gene can substitute for the *faf* gene in eye development.

DISCUSSION

We have identified, characterized genetically and cloned the *Drosophila lqf* locus. We have shown that *lqf* is an essential gene in *Drosophila* and that it encodes two homologs of epsin, a protein identified originally as a vertebrate endocytosis complex component. Genetic interactions between *lqf* and *Drosophila* endocytosis pathway genes suggest that *lqf* facilitates endocytosis in vivo. In addition, the results of a variety of genetic experiments suggest that *lqf* and *faf* are required in common cells in a cell communication pathway that inhibits neural determination of the M-cells within the facet precluster. Finally, we have shown that the *faf* mutant eye phenotype is extraordinarily sensitive to *lqf* gene dosage.

Lqf is a candidate for the essential substrate of Faf in eye development

The *faf* mutant eye phenotype is unusually sensitive to a decrease in the dose of the *lqf* gene, suggesting strongly that the two genes function in a common pathway. Genetic interactions with endocytosis and Ub pathway mutants show that *faf* and *lqf* facilitate endocytosis and antagonize ubiquitination. In addition, although *lqf* is more broadly required than *faf* in the eye and elsewhere in the fly, weak *lqf* mutations reveal that like *faf*, *lqf* is required to prevent the misdetermination of M-cells as photoreceptors. Moreover, when expressed only in the ro^+ cells surrounding the facet preclusters, both *faf* and *lqf* genes rescue completely to wild-type their respective M-cell misdetermination mutant phenotypes in the eye. Finally, given the relationship between Faf and its substrate protein, it would be expected that increasing the dose of the substrate should suppress the *faf* mutant phenotype. We show here that slight overexpression of *lqf* completely obviates the need for *faf* in eye development. The simplest model consistent with all of this genetic data is that Lqf is the substrate of Faf. Other more complicated explanations are, of course, possible.

Another candidate in vivo substrate of Faf

There is biochemical evidence that AF-6, a scaffolding protein thought to modulate cell-cell junctions in response to Ras activation (Ponting, 1995; Ponting and Benjamin, 1996; Kuriyama et al., 1996; Matsuo et al., 1997; Zhadanov et al., 1999) may be an in vivo substrate of Fam, the mouse homolog of Faf (Wood et al., 1997); AF-6 and Fam bind each other in vitro and ubiquitinated AF-6 can be detected and deubiquitinated by Fam in cultured cells (Taya et al., 1998). Like *lqf*, the *Drosophila Af6* homolog, *canoe*, is required pleiotropically for *Drosophila* eye development (Miyamoto et al., 1995). In contrast to *lqf* mutations, however, *canoe* mutations do not act as strong dominant enhancers of the *faf* mutant eye phenotype (Q. Li and J. A. F., unpublished). Given the striking genetic interactions between *faf* and *lqf*, it seems that *canoe* is unlikely to play a significant role in the essential *faf* pathway in the eye.

While only one Faf/substrate interaction may be essential to normal eye development in *Drosophila*, Faf and Fam may have several substrates in vivo. Normally non-essential roles for *faf* later in eye development have been revealed in particular mutant backgrounds (Fischer et al., 1997; Li et al., 1997) and Faf could have different substrates for its critical role in M-cell fate determination than in its redundant roles. Moreover, in addition to its essential role in eye development, *faf* is required maternally for cellularization of embryos (Fischer-Vize et al., 1992a) and the critical maternal substrate of Faf is unknown. As *faf* has mouse and human (*DFFRX/Y*; Jones et al., 1996) homologs, the modes of regulation by Faf are likely to be conserved. However, it is possible that the critical substrate(s) of Faf in *Drosophila* may differ from those in vertebrates.

Regulation of endocytosis by Ub

If Lqf is the substrate of Faf, then epsin levels, determined by

the balance between its ubiquitination and deubiquitination, could regulate endocytosis. Mono-ubiquitination, however, has been shown previously to regulate endocytosis in two different ways. First, mono-ubiquitination of cell surface receptors can act as a signal for receptor endocytosis, which leads to lysosomal degradation (Hicke, 1999; Strous and Govers, 1999). Here, the Ub moiety is somehow recognized by the endocytosis machinery; this process has nothing to do with the proteasome. Second, Eps15, an endocytosis complex component in mammalian cells (Benmerah et al., 1996; Tebar et al., 1996; van Delft et al., 1997b; Carbone et al., 1997; Benmerah et al., 1998), is mono-ubiquitinated in response to EGF receptor activation and Eps15 may require this modification to stimulate receptor endocytosis (van Delft et al., 1997a). In addition, Pan1p a yeast protein similar to Eps15, is required for endocytosis in yeast (Wendland et al., 1996, 1998; Tang et al., 1997). Although it is unknown whether Pan1p is mono-ubiquitinated in yeast, there is evidence that ubiquitination of an endocytic complex component is required for endocytosis in yeast; Rsp5p, a component of the ubiquitination machinery called a ubiquitin-ligase, may bind to Pan1p and is required generally for endocytosis in yeast, even for endocytosis of proteins with non-Ub endocytosis signals (Galan et al., 1996; Zallodek et al., 1997; Wendland et al., 1998; Hicke, 1999).

As Eps15 binds to epsin (Chen et al., 1998), could a mono-ubiquitinated Drosophila Eps15 homolog be the substrate of Faf? Two of our experimental results are inconsistent with this model. First, it has been shown previously that the activity of Faf antagonizes proteolysis (Huang et al., 1995), not just ubiquitination (Wu et al., 1999); mutations in a gene encoding a proteasome subunit act as strong suppressors of the *faf* mutant eye phenotype. This result strongly suggests that Faf activity antagonizes proteolysis and thus that Faf deubiquitinates a protein containing a Ub chain targeting it for degradation, rather than a mono-ubiquitinated protein. Second, if mono-ubiquitination of Eps15 activates it, as the available data suggests, then deubiquitination of Eps15 by Faf would render Eps15 inactive and thus the function of Faf would antagonize endocytosis. The data presented here clearly indicate the opposite; mutations in endocytosis complex genes (particularly *lqf* and *chc*) act as strong dominant enhancers of *faf*, suggesting that the normal function of Faf is to facilitate endocytosis.

Regulation of cell communication through modulation of an endocytosis complex component?

Elevated levels of Lqf obviate the need for Faf, presumably by stimulating epsin-dependent endocytosis generally or stimulating endocytosis of a specific cell surface protein. How can the observation that Lqf and Faf function outside the M-cells to determine M-cell fate be reconciled with a role for Lqf in endocytosis? Endocytosis is known to modulate ligand/receptor interactions by a variety of mechanisms. One possibility is that M-cell fate is affected by a diffusible ligand which, like Wingless (Bejsovec and Wieschaus, 1995), travels via endocytosis through several cell distances. Alternatively, regulation of a membrane-bound receptor by endocytosis in cells adjacent to the M-cells could affect M-cell fate indirectly. For example, EGF receptor activity is downregulated by endocytosis following ligand binding (Wells et al., 1990; Viera et al., 1996; Wilde et al., 1999). By contrast, activity of the Notch receptor may be up-regulated by endocytosis of activated receptors whose intracellular domains have been cleaved off prior to their translocation into the nucleus. Membrane-bound Notch receptors lacking their intracellular domains display dominant negative activity (Lieber et al., 1993; Rebay et al., 1993) and endocytosis of cleaved Notch receptors may be required normally for precise modulation of Notch activity (Seugnet et al., 1997). Patterning of the photoreceptor preclusters in the developing eye may require that both Notch and the EGF receptor are activated in the *ro*-expressing cells surrounding the facet preclusters (Baker et al., 1996; Dominguez et al., 1998; Kumar et al., 1998). Thus, Faf could regulate the activity of one or both of these receptors. More experiments are necessary in order to test these specific hypotheses.

The genetic relationship between *faf* and *lqf* described here provokes us to speculate that perhaps through modulation of Lqf levels, Faf and the Ub-mediated proteolytic pathway may regulate the activity of specific cell surface receptors, with profound effects on cell determination.

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