RESEARCH COMMUNICATION

A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets

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Eukaryotic genomes encode large families of deubiquitinating enzymes (DUBs). Genetic data suggest that Fat facets (Faf), a *Drosophila* DUB essential for patterning the compound eye, might have a novel regulatory function; Faf might reverse the ubiquitination of a specific substrate, thereby preventing proteasomal degradation of that protein. Additional genetic data implicate Liquid facets (Lqf), a homolog of the vertebrate endocytic protein epsin, as a candidate for the key substrate of Faf. Here, biochemical experiments critical to testing this model were performed. The results show definitively that Lqf is the key substrate of Faf in the eye; Lqf concentration is Faf-dependent, Lqf is ubiquitinated in vivo and deubiquitinated by Faf, and Lqf and Faf interact physically.

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Ubiquitin (Ub) is a highly conserved 76-amino-acid polypeptide, whose main role in eukaryotic cells is to target proteins for degradation by a proteolytic complex called the proteasome (Weissman 2001). Ub becomes covalently attached to an internal lysine residue of a substrate protein via an isopeptide bond, in a series of reactions requiring several enzymes and ATP. Through its C-terminal glycine residue, another Ub monomer may be linked to an internal lysine residue of the first one. Several rounds of Ub addition result in an isopeptide-linked chain; a chain of four or more Ub residues constitutes a degradation tag. In contrast, monoubiquitination can regulate protein function, in a manner analogous to phosphorylation (Hicke 2001).

Deubiquitinating enzymes (DUBs) cleave Ub–protein bonds; DUBs cleave Ub from peptide-linked translational fusion proteins and/or isopeptide-linked Ub chains (Wilkinson and Hochstrasser 1998). All DUBs fall into one of two classes: Ub–C-terminal hydrolases (Uchs) or Ub–specific processing proteases (Ubps). Although they may have overlapping functions, Uchs and Ubps have distinct, conserved catalytic regions (Wilkinson and Hochstrasser 1998). Ubps, the larger class of DUBs, have characteristic Cys and His domains, which center around a key catalytic cysteine residue and two catalytic histidine residues (Baker et al. 1992; Huang et al. 1995; Wilkinson and Hochstrasser 1998). Large families of DUB proteins have been revealed by functional screening (Baker et al. 1992) and genome sequencing (Wilkinson and Hochstrasser 1998; Chen and Fischer 2000), but the biological roles of only a few are well understood. The DUBs with known functions play housekeeping roles in the Ub-mediated protein degradation pathway; they generate Ub monomers by processing peptide-linked precursors (Ub polymers or Ub-protein translational fusions) or by recycling isopeptide-linked Ub chains (Wilkinson and Hochstrasser 1998).

The notion was advanced years ago that some DUBs may remove Ub chains from specific protein targets, and thus may act as substrate-specific regulators of ubiquitination and proteolysis (Hershko et al. 1980; Ellison and Hochstrasser 1991). Several DUBs could potentially perform such regulatory roles, as they have been shown to be associated with the control of a wide variety of biological processes, including tumor suppression, transcription, cell growth, chromosome condensation, neural pathfinding, and memory storage (Wilkinson and Hochstrasser 1998; Wilkinson 2000; Weissman 2001). However, in none of these cases has it been possible to identify a specific ubiquitinated substrate for a DUB or to determine its role in the Ub pathway.

In contrast, in the case of Fat facets (Faf), a *Drosophila* DUB required for patterning the compound eye, there is compelling genetic evidence that Faf activity antagonizes both ubiquitination and proteasomal proteolysis, and Faf was therefore hypothesized to cleave a degradation tag from a specific targeted protein (Huang et al. 1995; Wu et al. 1999). Subsequently, genetic experiments identified Liquid facets (Lqf), an endocytic protein homologous to vertebrate epsin, as a candidate for the critical substrate of Faf in the eye (Fischer et al. 1997; Chen et al. 1998; Cadavid et al. 2000). Four genetic observations are consistent with a model in which the function of Faf is to prevent Lqf degradation: (1) *lqf* loss-of-function mutations are strong dominant enhancers of the *faf* mutant eye phenotype, (2) *faf* and *lqf* loss-of-function mutations have similar mutant eye phenotypes, (3) the faf^+ and lqf^+ genes are required in the same group of cells in the eye, and (4) one extra copy of the lqf^+ gene obviates the need for the faf^+ gene in the eye.

The genetic data show definitively that lqf^+ functions downstream of faf^+ in a common pathway. Here, we report the results of critical biochemical tests of the model wherein Faf is a substrate-specific regulator of ubiquitination and proteolysis, and its key substrate is Lqf. We generated an antibody to Lqf and used it to show: (1) there is less Lqf protein in the developing *Drosophila* eye in the absence of functional Faf protein, (2) Lqf is ubiquitinated in developing eyes and deubiquitinated by Faf, and (3) Lqf and Faf interact physically.

Results and Discussion

Lqf protein colocalizes with endocytic proteins in Drosophila *eye discs*

To detect Lqf protein levels in developing eyes, we generated an antibody to Lqf (see Materials and Methods). Eye discs were double-labeled with anti-Lqf and antibod-

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ies to the endocytic protein Shibire (Shi; Estes et al. 1996), which shows that Lqf and Shi colocalize at cell membranes; Lqf and Shi are concentrated apically in cells within the morphogenetic furrow, an indentation that marks the onset of differentiation (Wolff and Ready 1993), and also in developing photoreceptors where their membranes meet (Fig. 1A–J). Similar results were obtained with antibodies to two other endocytic proteins (Dap160 and α-Adaptin [α-Ada]), and with phalloidin, which labels f-actin at cell membranes (data not shown).

Faf activity increases the level of Lqf protein in Drosophila *eye discs*

One prediction of the hypothesis that Faf activity prevents the degradation of Lqf is that in the developing eyes (larval eye discs) of *faf* null mutant flies, there should be less Lqf protein than in wild-type eyes. We expected there to be less Lqf protein, as opposed to no Lqf protein, because the *lqf* null mutant eye phenotype is much more severe than the *faf* null mutant eye phenotype (Fischer et al. 1997; Cadavid et al. 2000).

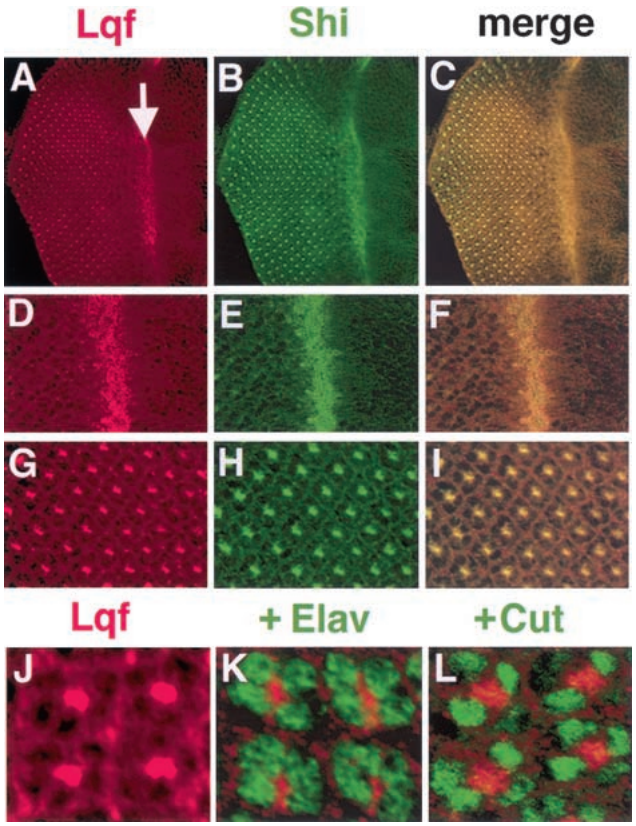

Figure 1. Colocalization of Shi and Lqf proteins in eye discs. (*A–J*) Apical views of a third instar larval eye disc, double-labeled with anti-Shi and anti-Lqf. (*A–C*) Lqf and Shi colocalize in cells within and posterior to the morphogenetic furrow, indicated by the arrow in *A*. (*D–F*) An enlargement of the area near the furrow shows that Lqf and Shi are concentrated at the apical tips of cells within the furrow. (*G–I*) An enlarged view of the area posterior to the furrow shows that Lqf and Shi are concentrated in dots, which are the apical membranes of the photoreceptors (R-cells), where they meet (see below). In addition, Lqf and Shi colocalize in a lattice, which is made up of the membranes of the surrounding cells. (*J*) A further enlargement of *G*, showing Lqf membrane localization in four adjacent facets. (*K,L*) An apical view of four adjacent facets in eye discs double-labeled with anti-Lqf and anti-Elav (*K*), which labels R-cell nuclei, or anti-Lqf and anti-Cut (*L*), which labels cone cell nuclei. There is no overlap in the localization of Lqf protein and either Elav or Cut; Lqf is outside the nuclei (which fill the apical cytoplasm) and in the central region of the developing facet where the photoreceptor cell membranes meet.

To test whether the level of Lqf is affected by faf^+ gene activity, first, using confocal microscopy, we compared the levels of Lqf in adjacent groups of faf^+ and faf^- cells in the eye disc. We generated clones of homozygous faf^- cells in faf^+/faf^- heterozygous eye discs, marked by the absence of β-galactosidase (β-gal) expression (see Materials and Methods). The eye discs containing clones were triple-labeled with antibodies to β-gal (to outline the clones), to Lqf (to detect the level of Lqf protein), and to Shi (as a negative control). We found that throughout the eye disc, the level of Lqf protein, reflected in the strength of the signal from antibody labeling, is lower within the faf^- clones than in the faf^+/faf^- heterozygous cells surrounding them (Fig. 2). In contrast, the levels of Shi protein are the same within and outside the clone boundaries (Fig. 2).

To quantify the difference in Lqf protein levels in faf^+ and faf^- cells, we assayed the levels of Lqf in eye disc protein extracts prepared from wild-type and faf^- flies in Western blot experiments (see Materials and Methods). We used homozygotes for two different mutant *faf* alleles that behave genetically as strong loss-of-function mutations: faf^{BX4} is an inversion that makes no functional Faf protein, and faf^{FO8} encodes an Faf protein with histidine residue 1986, which is critical for DUB catalytic activity, changed to tyrosine (Fischer-Vize et al. 1992; Huang et al. 1995; Chen and Fischer 2000). We found that there is two- to threefold less Lqf in eye disc protein extracts of faf^- homozygotes than in wild-type extracts (Figs. 3A, 4A). We also performed the experiment by adding faf^+ gene function back to faf^- flies (Figs. 3B, 4B). A transgene containing faf^+ genomic DNA (P{gfaf^+}), which when introduced into faf^- homozygotes complements the mutant eye phenotype (Fischer-Vize et al. 1992; Huang et al. 1995), results in a two- to threefold increase in Lqf protein level in eye disc extracts (Figs. 3B, 4B). A nearly identical transgene (P{gfaf^{C1677S}}) that fails to complement the faf^- mutant phenotype because it has a point mutation in the codon for cysteine 1677, which is critical to the DUB activity of Faf (Huang et al. 1995), fails also to increase the level of Lqf protein in eye disc extracts (Figs. 3B, 4B). We conclude that faf^+ activity results in an increase in the level of Lqf protein.

We asked whether the effect of faf^+ gene activity on the level of Lqf is specific to Lqf by performing the Western blot assay using antibodies to four other endocytosis complex proteins: Shi, α-Ada, Dap160, and Lap (see Materials and Methods). The levels of these proteins are unaffected by the absence of faf^+ activity (Fig. 3C).

Lqf is ubiquitinated in eye discs and deubiquitinated by Faf

A second prediction of the model wherein Faf prevents proteolysis of Lqf by deubiquitinating it, is that there should be Lqf protein linked to Ub chains present in eye discs. Ubiquitinated proteins are usually detected on Western blots as ladders of protein bands of higher molecular weight than the protein in question, in increments of ~8 kD; each "rung" on the ladder represents a

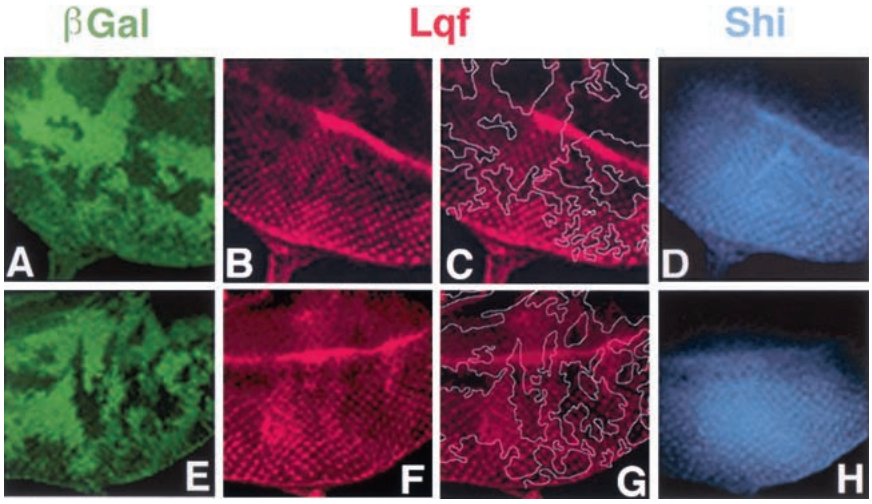

Figure 2. Detection of Lqf and Shi proteins in *faf*$^{-}$ clones in eye disc. Apical views of two different third instar larval eye discs are shown in *A–D* and *E–H*. (*A*,*E*) The *faf*$^{-}$ clones are labeled by the absence of β-gal protein. (*B*,*F*) The clone shapes are apparent as areas with lower levels of Lqf protein. (*C*,*G*) The clone shapes in *A* and *E* were outlined in white and layered over the panels in *B* and *F*. (*D*,*H*) The levels of Shi protein are unaffected in the *faf*$^{-}$ clones. We know that detection of the Lqf protein signal is unaffected by the β-gal protein signal, as clones are visible as areas of lower levels of Lqf signal in discs labeled only with anti-Lqf. A few of the clone areas in *A* and *E* are not obviously mirrored in *B* and *F*. This is because of the subtlety of the Lqf concentration difference (<twofold) often being detected; although there is only two- to threefold less Lqf in *faf*$^{-}$/*faf*$^{-}$ eye discs than in wild-type (*faf*$^{+}$/*faf*$^{+}$), the clones are *faf*$^{-}$/*faf*$^{-}$, but the surrounding cells are often *faf*$^{-}$/*faf*$^{+}$ (the clone twin spots are *faf*$^{+}$/*faf*$^{+}$). Slight variability in antibody penetration and so on within the disc can affect the staining and obscure concentration differences in parts of the disc. Nevertheless, it is clear that the clone shapes are generally present in the Lqf-stained discs (*B*,*F*), but not in the Shi-stained discs (*D*,*H*).

protein species with a Ub chain that is one Ub residue longer than the previous rung. Proteins with Ub chains are rapidly degraded, and thus difficult to detect; usually, inhibition of proteasome and/or DUB activity is required to detect them. Here, inhibition of the DUB activity of Faf, genetically, stabilizes ubiquitinated forms of Lqf.

In all of the Western blot experiments described above, which we performed to quantify Lqf levels in *faf*$^{+}$ and *faf*$^{-}$ eye discs, a ladder of higher-molecular-weight forms of Lqf is present consistently in the *faf*$^{-}$ eye disc protein extracts (Fig. 4A [faf^{BX4}], 4B [faf^{FO8}/faf^{BX4} and faf^{FO8}/faf^{BX4} + P{gfaf^{C1677S}}]). In contrast, higher-molecular-weight forms of Lqf are not detected in extracts prepared from *faf*$^{+}$ eye discs (Fig. 4A [wild-type], 4B [faf^{FO8}/faf^{BX4} + P{gfaf^{+}}]).

Three lines of evidence indicate that the ladders represent ubiquitinated forms of Lqf. First, consistent with the idea that the higher molecular forms of Lqf have degradation tags, the presence of the ladders in the extracts correlates with a decrease in Lqf protein level; the *faf*$^{-}$ protein extracts, which show two- to threefold lower than wild-type levels of Lqf protein (Fig. 3A,B), are the ones that contain the higher-molecular-weight forms of Lqf. Second, the incremental size differences between wild-type Lqf protein and each of the rungs of the ladder are consistent with the size of a Ub monomer (~8 kD; Fig. 4A,B; see legend). Third, the higher molecular forms are stabilized in the absence of Faf protein with its catalytic cysteine and histidine residues intact; we have shown previously that Faf deubiquitinates synthetic Ub–protein substrates in bacteria, and that this ability depends on its cysteine residue 1677 (Huang et al. 1995).

We conclude that in eye discs, Lqf is ubiquitinated, and subsequently either deubiquitinated by Faf or degraded. The observation that considerable amounts of nonubiquitinated Lqf protein remain in *faf*$^{-}$ eye discs indicates either that only a fraction of the Lqf protein in the eye disc is ubiquitinated, and/or that DUBs other than Faf also deubiquitinate some Lqf protein.

Faf and Lqf interact physically

A third prediction of the model wherein Lqf is the substrate of Faf is that the proteins should, either directly or indirectly, interact. We used anti-Lqf to immunoprecipitate Lqf from protein extracts prepared from embryos, and tested for the presence of Faf in the immunoprecipitates on Western blots (see Materials and Methods). Embryos were used because sufficient protein could not be obtained from eye discs. In addition, to facilitate detection of Faf, the embryos were transformed with a P{*hs-myc-faf*$^{+}$} transgene, which expresses a fully functional, myc-tagged Faf protein upon heat shock, that can be detected on Western blots with anti-myc (Huang et al. 1995; Huang and Fischer-Vize 1996). myc-Faf was detected in the anti-Lqf immunoprecipitate of the protein extract from heat-shocked transformant embryos, but not in the immunoprecipitates from non-heat-shocked embryos or from heat-shocked embryos when preimmune serum or no antibody was used instead of anti-Lqf (Fig. 4C). We conclude that myc-Faf and endogenous Lqf proteins interact physically in *Drosophila* embryos. Bacterially produced or in vitro translated partial Faf and full-length Lqf proteins do not bind to each other in GST pull-down assays (Cadavid 2000). One possible explanation is that only full-length Faf can bind to Lqf in these assays. Alternatively, Faf and Lqf may require other proteins for their interaction.

Conclusions

The experiments presented here provide critical biochemical evidence for a model in which a DUB called Faf specifically deubiquitinates Lqf protein, thereby preventing its proteolysis. We have shown that there is less Lqf protein in the developing eye in the absence of catalytically functional Faf protein, that Lqf is ubiquitinated and subsequently deubiquitinated by Faf, and that Faf and Lqf interact physically. Taken together with previous genetic evidence that provides strong support for the model, we conclude that Faf is a substrate-specific regulator of ubiquitination, a novel function for a DUB.

The eyes of *faf* null or *lqf* hypomorphic mutants have more than the normal complement of eight photoreceptor cells in each facet, owing to the failure of a cell communication pathway early in eye development (Fischer-

Vize et al. 1992; Huang and Fischer-Vize 1996; Cadavid et al. 2000). The Faf/Lqf interaction is essential in only a small number of cells in the eye disc, which must be particularly sensitive to the levels of Lqf, and in these cells, Lqf presumably controls the frequency or specificity of endocytosis. Although the precise mechanism of epsin function is unknown, vertebrate epsin binds to the endocytosis complex and also to PIP_2 at the cell membrane, and is required for endocytosis (Chen et al. 1998; Itoh et al. 2001). Apparently, appropriate endocytosis in this small group of cells is essential for successful communication with their neighbors; increased Lqf levels either enables these cells to send a signal to their neighbors that inhibits neural determination, or else prevents them from sending their neighbors a positive differentiation signal.

Through a variety of mechanisms, endocytosis is proposed to regulate ligand/receptor interactions during development (Cagan et al. 1992; Moline et al. 1999; Entchev et al. 2000; Parks et al. 2000; Dubois et al. 2001; Greco et al. 2001). How Lqf and endocytosis regulate *faf*$^+$-dependent cell signaling remains to be determined. As *faf* has vertebrate homologs (Jones et al. 1996; Wood et al. 1997), this mode of regulation is likely to be conserved. The finding that Lqf is the key substrate of Faf in the *Drosophila* eye shows not only that a DUB can regulate ubiquitination and thus proteolysis, but also that an endocytosis complex protein can be a target for the control of a cell communication event critical to cell determination.

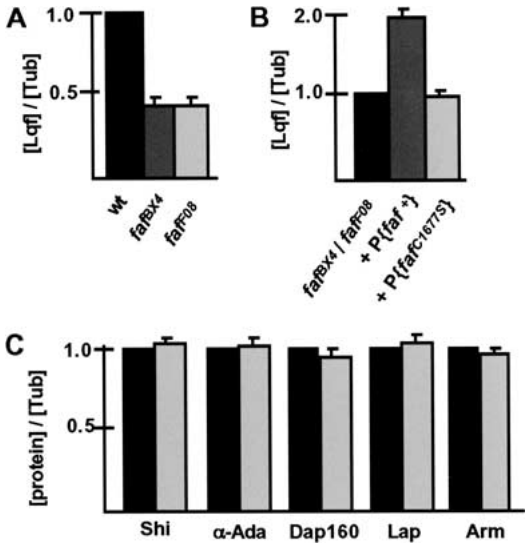

Figure 3. Comparison of protein levels in wild-type and *faf*$^-$ eye discs. (*A*) A histogram showing the level of Lqf protein, normalized to tubulin, in wild-type and *faf*$^-$ eye discs. The wild-type value was arbitrarily set to 1.0. Examples of Western blots used to generate this data are shown in Figure 4A below. (*B*) A histogram showing the level of Lqf protein, normalized to tubulin, in *faf*^{BX4} eye discs, and in *faf*^{BX4} eye discs with a copy of either a *faf*$^+$ transgene or a *faf*$^-$ transgene. The *faf*^{BX4} value was arbitrarily set to 1.0. An example of a Western blot used to generate these data is shown in Figure 4B below. (*C*) A histogram showing the levels of four different endocytosis complex proteins and Armadillo (Arm), normalized to tubulin, in wild-type (wt; black bars) and *faf*^{BX4} (gray bars) eye discs. The wild-type value was arbitrarily set to 1.0. Standard errors in *A–C* were calculated from differences in repeated experiments.

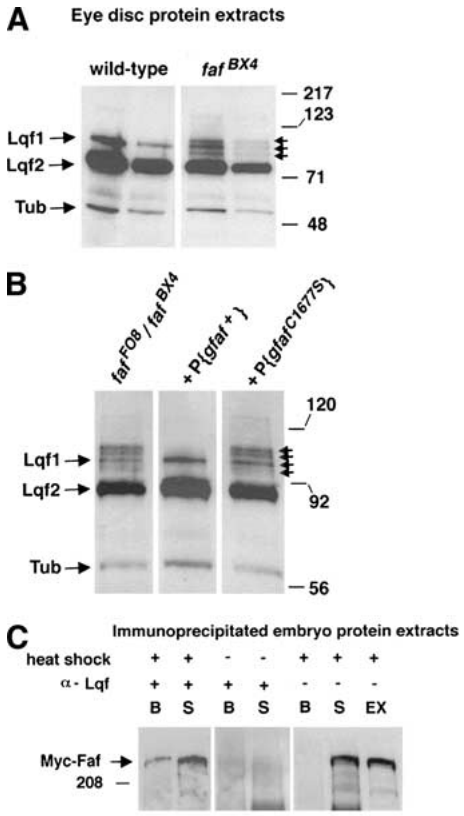

Figure 4. Deubiquitination and binding of Lqf by Faf. (*A*) Western blots of eye disc protein extracts, labeled with anti-Lqf and anti-tubulin, are shown. The *lqf* gene encodes two different proteins by alternate mRNA splicing, of predicted molecular weights ~86 kD (Lqf1) and ~70 kD (Lqf2; Cadavid et al. 2000); Lqf2 is the predominant form in eye discs. The small arrows indicate the rungs of the ladder of higher-molecular-weight forms of Lqf2. The size of the second rung of the ladder corresponds precisely to the size of Lqf1, which is the size predicted for Ub–Ub–Lqf2 (70 + 8 + 8 = 86). The two lanes shown for each genotype are different amounts of the same protein extract. These experiments were repeated 10 times, sometimes using *faf*^{FO8}, and identical results were always obtained. (*B*) A Western blot of eye disc protein extracts, labeled with anti-Lqf and anti-tubulin. Ubiquitinated forms of Lqf2 are stabilized in *faf*$^-$ extracts, disappear when a *faf*$^+$ transgene is introduced, but remain stabilized in the presence of a *faf*$^-$ transgene. Results similar to these were obtained in 3/3 repetitions. (*C*) Western blots of an immunoprecipitation experiment, labeled with anti-myc. The extracts are from embryos transformed with the P{*hs-myc-faf*} transgene that were heat-shocked and thus express myc-Faf (*left* and *right* panels), or not heat-shocked (*middle* panel). The extracts were immunoprecipitated with anti-Lqf (*left* and *middle* panels), or with no antibody as a control (*right* panel). Preimmune serum also failed to immunoprecipitate Lqf or myc-Faf (data not shown). Coomassie-stained gels of heat-shocked and non-heat-shocked embryo extracts appeared identical. The predicted molecular weight of myc-Faf is ~300 kD. (EX) 1/15 of a 150-µL crude extract from 150 µL of embryos, (S) 1/15 of the supernatant protein from 150 µL of crude extract, which was not immunoprecipitated by anti-Lqf, (B) total protein from the 150-µL extract bound to anti-Lqf beads.

Materials and methods

Lqf antibodies

A polyclonal antibody to Lqf was generated in guinea pigs (Cocalico Biologicals) from a bacterially produced partial Lqf protein, containing the region of Lqf1 C-terminal to the ENTH domain (Cadavid et al. 2000). A cDNA fragment encoding the C-terminal portion of Lqf1 was ligated into pET-28a (Novagen), and the resulting plasmid was used to transform

BL-21 cells (Stratagene). Protein expression was induced and the protein purified using a Ni^{2+} column according to the procedures recommended by Novagen. Western blots of protein extracts prepared from eye disc extracts or from 0–20-h embryos, labeled with anti-Lqf serum, showed only one or two bands, which correspond to the expected sizes of Lqf1 (~86 kD) and/or Lqf2 (~70 kD; Cadavid et al. 2000). Labeling with preimmune serum resulted in no signal on Western blots of bacterially produced antigen, eye disc, or embryo protein extracts, nor in eye disc whole mounts.

Generation of faf *mutant clones in the eye disc*

Clones of homozygous *faf*$^{-}$ (null mutants *faf*^{BX4} or *faf*^{B3}; Fischer-Vize et al. 1992; Chen and Fischer 2000) cells were induced in *faf*$^{-}$/*faf*$^{+}$ larval eye discs using P{*ry*$^{+}$*; hs-neo; FRT*} (Xu and Rubin 1993), P{*conD*}96A (Tio and Moses 1997), and P{*ey-FLP.D*}2 (Newsome et al. 2000) chromosomes. Eye discs were dissected from female third instar larvae of the genotype P{*ey-FLP*}/+; P{*FRT*}82B *faf*$^{-}$/P{*FRT*}82B P{*conD*}96A.

Antibody labeling of eye discs

For Figure 1, wild-type (*w*^{1118}) third instar larval eye discs were immunostained essentially as described previously, using PLP fixation and 0.1M NaP/0.2% saponin wash and incubation solutions (Fischer-Vize et al. 1992). The primary antibodies were guinea pig anti-Lqf at 1:1000, rabbit anti-Shi at 1:200, rat anti-Elav (mAb7E8A10; Developmental Studies Hybridoma Bank, DSHB) undiluted, and mouse anti-Cut (mAb2B10; DSHB) at 1:100. The secondary antibodies used (Molecular Probes) were Texas Red anti-guinea pig at 1:400, Alexa^{594}-anti-rabbit at 1:400, Alexa^{488}-anti-rat at 1:600, and Alexa^{488}-anti-mouse at 1:200. The eye discs with *faf*$^{-}$ clones (Fig. 2) were immunostained and photographed as described above. The primary antibodies used were mouse anti-β-gal (mAb40-1a; DSHB) at 1:10, guinea pig anti-Lqf at 1:1000, and rabbit anti-Shi at 1:200. The secondary antibodies used (Molecular Probes) were Alexa^{488}-anti-mouse (1:100), Texas Red anti-guinea pig (1:400), and Alexa^{350}-anti-rabbit (1:50). Stained eye discs were mounted in Vectorshield (Vector Laboratories) mounting medium. Images were produced using a Leica TCS 4D confocal microscope, and processed using Adobe Photoshop.

Western blot analysis of eye disc protein extracts

To generate eye disc protein extracts, five pairs of eye discs, dissected from third instar larvae, were homogenized in 50 µL of 2× Laemmli buffer in a microfuge tube with a Teflon pestle, and then centrifuged at 13,000 rpm at 4°C for 5 min. Four aliquots of the supernatant (15 µL, 10 µL, 7.5 µL, and 5 µL) were distributed into microfuge tubes and their volumes increased to 15 µL with 2× Laemmli buffer. Each aliquot was boiled for 5 min and then subjected to SDS-PAGE. The proteins were transferred to nitrocellulose (Bio-Rad), then labeled with primary and secondary antibodies (see below) using standard procedures. The HRP signal was detected with ECL Renaissance reagents (NEN). The signals were quantified using NIH Image 1.62 Software (http://vsb.info.nih.gov/nih-image). To obtain measurements within the linear range of detection of the software, we used exposures of the images such that measurements of the signals in at least three of the four lanes gave the same value for [Lqf]/[tubulin]; only Lqf2 was measured. The primary antibodies used were mouse anti-tubulin (mAbE7; DSHB) at 1:10, guinea pig anti-Lqf at 1:4000, rabbit anti-Shi (Estes et al. 1996) at 1:1000, rabbit anti-α-Ada (Gonzalez-Gaitan and Jackle 1997) at 1:1000, rabbit anti-Dap160 (Roos and Kelly 1998) at 1:200, guinea pig anti-Lap (Zhang et al. 1998) at 1:200, and mouse anti-Arm (monoclonal N2 7A1; DSHB) at 1:50. The secondary antibodies used were HRP-anti-guinea pig (Jackson) at 1:4000–20,000, HRP-anti-mouse (Santa Cruz) at 1:500, and HRP-anti-rabbit (Santa Cruz) at 1:500.

Immunoprecipitation

Anti-Lqf (2 µL) was added to 30 µL of protein A-agarose (Bio-Rad); the mixture was incubated on ice for 1 h, and the soluble protein extract from 150 µL of heat-shocked embryos was added in 150 µL. (Embryo extract preparation and heat-shock conditions were as described previously; Huang et al. 1995; Huang and Fischer-Vize 1996.) The mixture was incubated at 4°C on a nutator for 12 h, then the beads were spun down (8000 rpm in a microfuge for 2 min) and the supernatant was removed. The beads were then washed 3 times for 5 min each, with 1.5 mL of buffer (15 mM Hepes-KOH at pH7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 0.35 M sucrose), resuspended in 2× Laemmli buffer, and boiled for 5 min prior to loading on an SDS-PAGE gel. Three negative controls were performed; the immunoprecipitation reactions were carried out without the addition of anti-Lqf, with preimmune serum, or the protein extracts were prepared from P{*hs-myc-faf*} transformant embryos that were not heat-shocked. Western blots were prepared and developed with anti-myc (mAb9E10; Santa Cruz) at 1:100 as described above.

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