Copyright © 2000 by the Genetics Society of America

In Vivo Structure/Function Analysis of the Drosophila *fat facets* Deubiquitinating Enzyme Gene

Xin Chen and Janice A. Fischer

Section of Molecular Cell and Developmental Biology, Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712

Manuscript received June 9, 2000
Accepted for publication August 23, 2000

ABSTRACT

The Drosophila Fat facets protein is a deubiquitinating enzyme required for patterning the developing compound eye. Ubiquitin, a 76-amino-acid polypeptide, serves as a tag to direct proteins to the proteasome, a protein degradation complex. Deubiquitinating enzymes are a large group of proteins that cleave ubiquitin-protein bonds. Fat facets belongs to a class of deubiquitinating enzymes called Ubps that share a conserved catalytic domain. Fat facets is unique among them in its large size and also because Fat facets is thought to deubiquitinate a specific substrate thereby preventing its proteolysis. Here we asked which portions of the Fat facets protein are essential for its function. *P*-element constructs that express partial Fat facets proteins were tested for function. In addition, the DNA sequences of 12 mutant *fat facets* alleles were determined. Finally, regions of amino acid sequence similarity in 18 Drosophila Ubps revealed by the Genome Project were identified. The results indicate functions for specific conserved amino acids in the catalytic region of Fat facets and also indicate that regions of the protein both N- and C-terminal to the catalytic region are required for Fat facets function.

DEUBIQUITINATING enzymes (DUBs) are a large group of proteins that cleave ubiquitin-protein bonds and whose physiological roles and mechanisms of function are poorly understood. Ubiquitin (Ub) is a 76-amino-acid polypeptide that can be linked covalently to other proteins via an isopeptide bond between an internal lysine on the substrate protein and the terminal glycine residue (G76) of Ub (Pickart 1998). Ub chains form through isopeptide linkages most often between an internal lysine residue (K48) of the first Ub and G76 of an incoming Ub monomer (Pickart 1998). Monoubiquitination can serve as a signal for endocytosis of a membrane protein (Hicke 1999; Strous and Govers 1999) or it can modulate protein activity (Chen *et al.* 1996). In contrast, Ub chains mark proteins for degradation by the proteasome, a multi-subunit proteolytic complex (Lupas and Baumeister 1998; Rechsteiner 1998). Once thought to be a mechanism only for disposing of damaged proteins, it is now well established that Ub-mediated proteolysis is widely used to modulate the levels of critical regulatory proteins (Koepp *et al.* 1999; Maniatis 1999).

There are two classes of DUBs (Wilkinson and Hochstrasser 1998): the Uch enzymes (*u*biquitin *C*-terminal *h*ydrolases) and the Ubp enzymes (*ub*iquitin *p*rocessing proteases). The functional distinction between Uchs and Ubps is ambiguous but the two enzyme families have structurally distinct catalytic domains (Wilkinson and Hochstrasser 1998). Ubps are the larger class of DUBs; yeast have only 1 Uch but 16 Ubps (Wilkinson and Hochstrasser 1998) and the *Drosophila* Genome Project has identified 4 Uchs and 19 Ubps (Rubin *et al.* 2000). Ubps are distinguished by their two conserved catalytic domains: the Cys domain, centered around the catalytic cysteine residue and the His domain, containing two catalytically important histidine residues (Baker *et al.* 1992; Papa and Hochstrasser 1993; Huang *et al.* 1995; Wilkinson and Hochstrasser 1998).

One likely function for DUBs is to generate Ub monomers from precursor proteins; Ub is synthesized in the cell as peptide-linked Ub polymers or Ub-protein fusions (Pickart 1998). Other general roles in the Ub pathway have been proposed for two yeast Ubps: Ubp4 (Doa4) and Ubp14 are thought to cleave isopeptide-linked Ub chains, either linked to remnants of degraded proteins (Ubp4) or free (Ubp14), thus preventing them from clogging the proteasome (Papa and Hochstrasser 1993; Amerik *et al.* 1997). An editing function has been proposed for one vertebrate DUB that may cleave specifically the terminal Ub from short isopeptide-linked chains, thereby deubiquitinating and preventing the degradation of proteins with short Ub chains (Lam *et al.* 1997).

The Drosophila *fat facets* (*faf*) gene encodes a Ubp that is essential specificially for patterning the developing eye and also for viability of the early embryo (Fischer-Vize *et al.* 1992). Flies with null mutations in the *faf* gene are viable and have only two obvious defects: their eyes are malformed and females lay eggs that un-

Corresponding author: Janice A. Fischer, The University of Texas at Austin, Moffett Molecular Biology Bldg., 2500 Speedway, Austin, TX 78712. E-mail: jaf@mail.utexas.edu

dergo several rounds of nuclear cleavage after fertilization but never cellularize (Fischer-Vize *et al.* 1992). In the eye, *faf* is required in a cell communication pathway that prevents particular precursor cells from misdetermination as neurons; in *faf* mutant eyes, each facet has more than the normal complement of eight photoreceptors (Fischer-Vize *et al.* 1992).

Faf is unique among Ubps in that it has been shown genetically to antagonize the Ub-mediated proteolysis pathway (Huang *et al.* 1995; Wu *et al.* 1999). Thus it has been proposed that Faf deubiquitinates a particular substrate or set of substrates thereby preventing their degradation. Genetic experiments have identified a single gene, called *liquid facets*, that is likely to encode the critical substrate for Faf in the eye (Cadavid *et al.* 2000). However, Faf may also have other substrates in the Drosophila eye (Li *et al.* 1997) and ovary.

At 2711 and 2778 amino acids, the two similar Faf proteins, generated by altenative splicing of the final exon, are the largest known Ubps (Fischer-Vize *et al.* 1992; Wilkinson and Hochstrasser 1998). One hypothesis to explain the large size of Faf is that it has several substrates and there may be distinct domains along the length of the protein for recognizing each. Yet, the results of genetic and biochemical experiments suggest that the catalytic region of Faf alone may be largely sufficient for its function (Taya *et al.* 1998, 1999; Wu *et al.* 1999).

Faf has mouse and human homologs, called Fam and DFFRX/Y, respectively (Jones *et al.* 1996; Wood *et al.* 1997) and Fam can substitute for Faf in Drosophila (Chen *et al.* 2000). The predicted amino acid sequences of these genes are highly conserved along most of their lengths, which precludes their comparison as a means of finding potential functional domains.

As a first step toward gaining insight into how the structure of Faf relates to its function, we generated six different deletion mutants of the *faf* gene and tested each for function in the developing eyes of *P*-element-transformed flies. In addition, the DNA sequences of 12 *faf* alleles with point mutations were determined. Finally, we identified conserved regions among the amino acid sequences of the Ubps in the Drosophila genome. There are two main conclusions from this work. First, distinct amino acid residues within the catalytic region of Faf, other than the catalytic residues themselves, have been defined as essential for Faf function. Second, we found unexpectedly that for its essential function in the eye alone, as well as for its ovary function, protein domains spanning nearly the entire Faf protein, both N-terminal and C-terminal to the catalytic region, are required.

MATERIALS AND METHODS

Drosophila genetics: All flies were grown on standard food at 25°. The alleles faf^{FO8} and faf^{BX4} are described in Fischer-Vize *et al.* (1992) and the P{*w*+, *ro-faf*+} transformants are described in Huang and Fischer-Vize (1996). *P*-element transformation was performed as described previously (Spradling 1986; Fischer-Vize *et al.* 1992). P{*w*+} insertions were introduced into a faf^{FO8}/faf^{BX4} background using standard crosses.

Plasmid constructions: Standard procedures (Sambrook *et al.* 1989) were used for all subcloning manipulations. Enzymes used for plasmid construction were obtained from New England Biolabs (Beverly, MA), Promega Biotech (Madison, WI), and Boehringer Mannheim (Indianapolis). All of the deletion constructs derive from a plasmid called pBA-Myc1-cDNA, which is a derivative of Bluescript (Stratagene, La Jolla, CA) with its *Sma*I site changed to *Asc*I (pB*Asc*I) and that has an ~8.5-kb *Asc*I fragment (described in Huang *et al.* 1995) containing a *faf* cDNA with a Myc epitope tag between amino acids 53 and 54 cloned into the *Asc*I site. For each of the fafΔ constructs, the DNA sequence at the deletion breakpoint was determined to check that the reading frame was restored. Subsequently, an *Asc*I fragment containing each deletion construct (in pB-fafΔ) was ligated into the *Asc*I site of the pRO transformation vector (Huang and Fischer-Vize 1996) and a plasmid with the fragment in the correct orientation was identified. *faf*Δ*1*: An ~0.55-kb 5′-end fragment of *faf* was isolated from pBA-Myc1-cDNA restricted with *Hin*dIII, treated with mung bean nuclease, and then restricted with *Asc*I. An ~7.2-kb 3′-end fragment of *faf* was isolated from pBA-Myc1-cDNA restricted with *Sca*I and *Asc*I. To generate pB-fafΔ1, the 5′-end and 3′-end fragments were ligated into pB*Asc*I. *faf*Δ*2*: pBA-Myc1-cDNA was restricted with *Eco*NI and *Stu*I, deleting an ~0.5-kb fragment, and then treated with Klenow. To generate pB-fafΔ2, the larger fragment was isolated and religated. *faf*Δ*3*: An ~3.1-kb 5′-end fragment of *faf* was isolated from pBA-Myc1-cDNA restricted with *Asc*I and *Dra*I. An ~3.8-kb 3′-end fragment of *faf* was isolated from pBA-Myc1-cDNA restricted with *Ahd*I, treated with T4 DNA polymerase, and then restricted with *Asc*I. To generate pB-fafΔ3, the 5′-end and 3′-end fragments were ligated into pB*Asc*I. *faf*Δ*4*: pBA-Myc1-cDNA was restricted with *Sna*BI and *Hpa*I, deleting an ~1.6-kb fragment. To generate pB-fafΔ4, the larger fragment was isolated and religated. *faf*Δ*5*: pBA-Myc1-cDNA was restricted with *Sph*I and *Nco*I, deleting an ~1.5-kb fragment, and then treated with T4 DNA polymerase. To generate pB-fafΔ5, the larger fragment was religated. *faf*Δ*6*: An ~2.3-kb fragment of *faf* was isolated from pB-fafΔ5 restricted with *Hpa*I and *Asc*I and ligated into pB*Asc*I restricted with *Asc*I and *Eco*RV. An ~2.3-kb 3′-end fragment of *faf* was isolated from the resulting plasmid restricted with *Hin*dIII and *Asc*I. An ~0.6-kb 5′-end *faf* fragment was isolated from pB-fafΔ5 restricted with *Asc*I and *Hin*dIII. To generate pB-fafΔ6, the 5′-end and 3′-end fragments were ligated into pB*Asc*I.

Western blot analysis: Protein extracts were prepared from adult transformants as follows. Twenty adult flies of each genotype were heat-shocked at 37° for 1 hr, allowed to recover at 25° for 1 hr, and then frozen in a dry ice/ethanol bath. The flies were thawed, homogenized in 200 ml of 2× Laemmli buffer, boiled for 5 min, and then spun in a microfuge at 4° for 5 min. The supernatants were boiled for another 5 min, size-separated by SDS-PAGE on a 5% gel, and transferred to nitrocellulose. SDS-PAGE, Western transfer, and hybridization of the blot were according to standard procedures (Sambrook *et al.* 1989). The primary antibody was mouse monoclonal anti-Myc (Santa Cruz Biochemical) used at 1:200 dilution and the secondary antibody was HRP-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA) used at 1:1000. Blots were developed with ECL Renaissance reagents (Amersham, Arlington Heights, IL) used according to the manufacturer's instructions.

Analysis of Drosophila eyes: Scanning electron micrographs

and 1-μm plastic sections of adult Drosophila eyes were prepared as described previously (Huang *et al.* 1995). The fraction of wild-type facets was calculated by scoring 100–250 facets per eye in one sectioned eye from at least three different flies of each genotype.

DNA sequence analysis: Mutant *faf* alleles were amplified by the polymerase chain reaction (PCR) using total genomic DNA prepared from a single fly homozygous or hemizygous [*in trans* to *Df(3L)faf*^{BP}; Fischer-Vize *et al.* 1992] for each mutant allele. Genomic DNA was prepared by adding the fly to a microfuge tube containing 50 μl of buffer (10 mM Tris pH 8.2, 1 mM EDTA, 25 mM NaCl) and 1 μl of proteinase K (20 mg/ml). The fly was homogenized with a pipet tip and then incubated at 37° for 1 hr and then at 100° for 2 min to inactivate the proteinase K. A 4-μl aliquot of this homogenate was used in a single PCR reaction. Fourteen primer pairs, each of which generated a PCR product ranging in size from 350 to 1000 bp, were used to amplify each *faf* allele in 14 pieces, each of which was sequenced directly by automated fluorimetric methods. The DNA sequences of each of the seventeen *faf* gene exons, all intron splice consensus sequences, and all introns except 1, 3, 4, and 16 were determined. To distinguish *bona fide* allele mutations from PCR-induced mutations, PCR products with non-wild-type DNA sequences were reamplified from genomic DNA a second time and their sequences were determined again. If the same mutation was found a second time, then it was considered to be amplified from the endogenous gene and not a PCR-induced mutation. In all cases, when more than one mutation was found in a single allele, only one reappeared in the second amplification. Details concerning the primer sequences and the PCR reaction conditions will be furnished on request.

Drosophila Ubp amino acid sequence analysis: The Ubp amino acid sequences were obtained by using Query GadFly (Flybase 1999) for proteins with UCH motifs. The amino acid sequences obtained were then subjected to BLOCKS analysis (www.blocks.fhcrc.org/blockmkr/make_blocks.html).

RESULTS

Construction of six *ro-faf*Δ transgenes: We have shown previously that single amino acid substitutions in the key Cys or His residues of the catalytic domain severely attenuated or abolished the function of Faf in the eye (Huang *et al.* 1995). In addition, a fragment of Faf containing mainly the catalytic domain can cleave synthetic peptide-linked Ub-protein substrates in bacteria efficiently (Huang *et al.* 1995). To test whether domains of Faf outside the catalytic region are also important for Faf function in the eye, five different deleted forms of the *faf* cDNA were constructed (*faf*Δ*1*–*faf*Δ*5*), each of which encodes a Faf protein with a block of 163 to 514 amino acids removed (Figure 1). In addition, a sixth construct (*faf*Δ*6*) containing mainly the catalytic domain was generated. Each of the deletion constructs was generated in the context of a *faf* cDNA that encodes the smaller of the two forms of Faf protein (2711 amino acids) as this cDNA was shown previously to complement completely the function of the endogenous *faf* gene in the eye (Huang *et al.* 1995).

Each deleted cDNA construct was cloned into a *P*-element transformation vector called pRO (Huang and Fischer-Vize 1996) that activates transcription using a

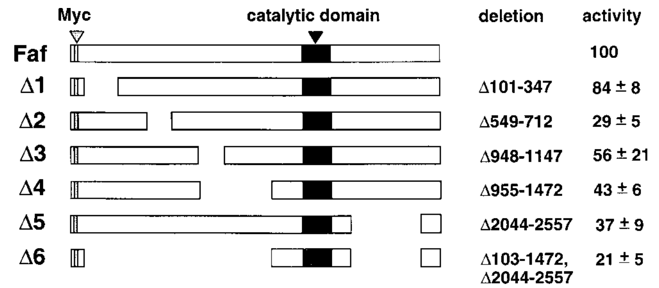

Figure 1.—FafΔ proteins. The structures of the Faf protein derivatives expressed by the six different *ro-faf*Δ genes are shown. Each Faf protein has a Myc-epitope tag inserted between residues 53 and 54 and the catalytic domain is approximately between residues 1670 and 2060 of the 2711 amino acid wild-type Faf protein. The locations of the amino acid residues deleted in each protein are shown and enumerated at right. The relative ability of each construct to substitute for the endogenous *faf* gene in the eye is also shown at right (see Figure 3A).

rough gene enhancer and a *heat shock protein 70* promoter. The pRO vector drives expression in a band of undifferentiated cells surrounding the facet preclusters early in eye development and also later in a subset of four of the eight photoreceptor cells (Kimmel *et al.* 1990; Heberlein *et al.* 1994; Dokucu *et al.* 1996). We have shown previously that due to their early expression in cells surrounding facet preclusters, transgenes in which the pRO vector drives expression of the wild-type *faf* cDNA (P{*ro-faf*+}) complement completely the eye defects in *faf* null mutants (Huang and Fischer-Vize 1996).

Complementation of the *faf* mutant eye phenotype by the *ro-faf*Δ transgenes: *P*-element transformant lines were generated with each of the six *ro-faf*Δ constructs, and to test each for function, each *P* element was introduced into a *faf*$^-$ background. The particular *faf*$^-$ background used, *faf*^{BX4}/*faf*^{FO8}, lacks all or nearly all endogenous *faf* activity; *faf*^{BX4} is a null mutation and *faf*^{FO8} is a strong mutant allele (Fischer-Vize *et al.* 1992). The eyes of flies with two copies of each *P*-element construct in several independent transformant lines were analyzed. Tangential sections of compound eyes revealing the anatomy of each facet (Figure 2) were scored for the fraction of normally developed as opposed to aberrantly assembled facets (Figure 3A).

Functional analysis of each *ro-faf*Δ construct in the eye leads to three main observations. First, none of the six deletion constructs retains wild-type activity (Figure 3A). Second, *faf*Δ*2* retains only slightly more activity than *ro-faf*Δ*6* (Figure 3A), suggesting that the 164 amino acids deleted in *ro-faf*Δ*2* (Figure 1) may be highly significant functionally. And finally, *ro-faf*Δ*6*, which expresses the smallest Faf protein derivative consisting mainly of the catalytic domain (Figure 1), retains only slight ability to complement *faf* mutations (Figure 3A).

We wanted to determine whether a small difference

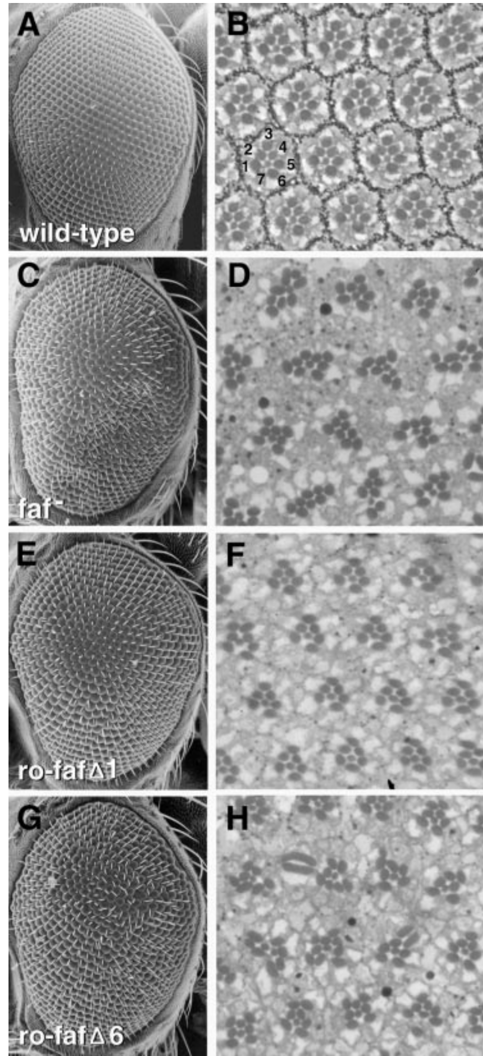

FIGURE 2.—Eyes of *faf* mutants expressing *ro-faf*Δ transgenes. Scanning electron micrographs (A, C, E, and G) and apical tangential sections (B, D, F, and H) of adult Drosophila compound eyes are shown. (A and B) Wild type; the external eye surface is regular and in each facet seven of the eight photoreceptors (1–7) are visible. (C and D) faf^{BX4}/faf^{FO8}; the external eye surface is irregular and in the majority of facets there are more than the wild-type number of photoreceptors. At least some of the ectopic photoreceptor cells arise from the "mystery cells" that are associated with photoreceptor precursors early in eye development (TOMLINSON and READY 1987); in *faf* mutants, the mystery cells are often misspecified as photoreceptors (FISCHER-VIZE *et al.* 1992). (E and F) P{*w*+, *ro-faf*Δ*1*}, faf^{BX4}/faf^{FO8}; one copy of the *P* element complements the *faf* mutant eye phenotype well. Most of the facets appear wild type. (G and H) P{*w*+, *ro-faf*Δ*6*}, faf^{BX4}/faf^{FO8}; one copy of the *P* element complements the *faf* mutant eye phenotype weakly. Most of the facets are malformed.

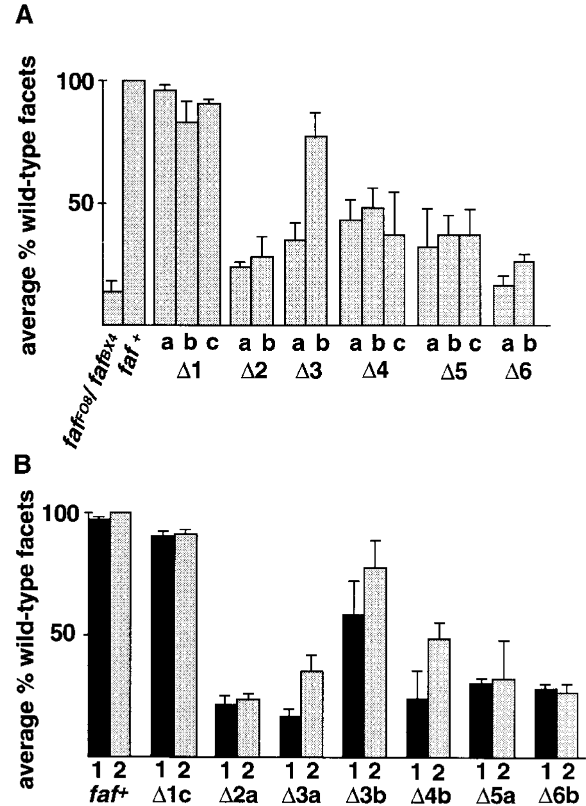

FIGURE 3.—Complementation of the *faf* mutant phenotype by *ro-faf*Δ transgenes. Histograms indicate the fraction of wild-type facets in *faf* mutant (faf^{BX4}/faf^{FO8}) flies transformed with *ro-faf*Δ transgenes. (A) The fraction of wild-type facets in *faf* mutants and in *faf* mutants containing two copies of a *P* element expressing *ro-faf*+ or each of the *ro-faf*Δ constructs (Δ1–Δ6) is shown. The fraction of wild-type facets is shown for each individual transformant line analyzed. Standard deviations represent variability between flies within a single transformant line. (B) The fraction of wild-type facets in flies as in A is shown. Flies contain one copy (1) or two copies (2) of the particular *P* element shown at bottom.

in expression level (for example, twofold) could have an effect on the ability of each *ro-faf*Δ construct to substitute for the endogenous *faf* gene in the eye. Thus, we analyzed the eyes of faf^- flies bearing single copies of each *ro-faf*Δ *P* element and compared their phenotypes with faf^- flies containing two copies of the same *P* element (Figure 3B). Significant differences in the abilities of *ro-faf*Δ genes to complement the *faf* mutant eye phenotype were observed only for *ro-faf*Δ*3* and *ro-faf*Δ*4* lines; for these two constructs, two copies complement the *faf* mutant eye phenotype significantly more effectively than one copy (Figure 3B).

Relative levels of wild-type and FafΔ proteins in transformed flies: To determine whether or not the six FafΔ proteins and wild-type Faf accumulate similarly when expressed as *ro-faf* transgenes, Faf protein in extracts from heat-shocked transformant flies were visualized on protein blots. The Faf proteins expressed by the transgenes are Myc-epitope tagged (Figure 1) so that by using anti-Myc antibodies to visualize the Faf proteins only Faf protein expressed by the transgenes was detected. We found that wild-type Faf and the FafΔ proteins accumulated to similar levels except for FafΔ6 (Figure 4); in extracts from each of the two *ro-faf*Δ*6* lines, significantly more Faf protein was detected than in any of the other extracts (Figure 4 and data not shown).

Thus we conclude that the differences in the ability

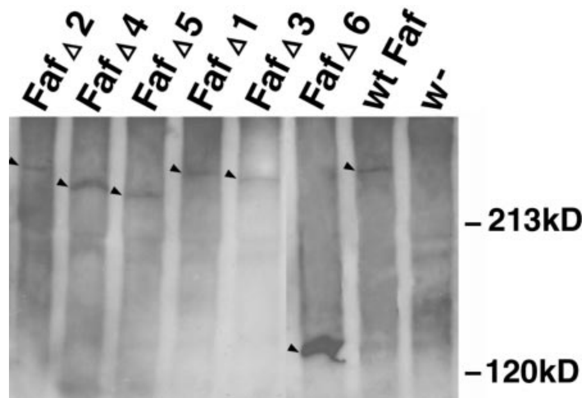

FIGURE 4.—Faf protein accumulation in transformed flies. Faf proteins expressed from *ro-faf* transgenes in protein extracts from heat-shocked adult flies were visualized on protein blots using antibodies to the Myc epitope. Wild-type (wt) Faf expressed from *ro-faf*+ and the six deletion derivatives (FafΔ1–Δ6) expressed from *ro-faf*Δ*1-6* are shown. No Myc-tagged Faf protein is detected in protein extracts from w^- flies containing no *P*-element construct. Each lane contains protein from one fly equivalent. Arrows indicate Faf and FafΔ protein bands.

of the *ro-faf*Δ constructs to complement the *faf* mutant eye phenotype are not due primarily to an effect on Faf protein accumulation, but rather to differences in the activities of the Faf proteins. Although FafΔ6 protein accumulates to much higher levels than wild-type Faf and all of the other FafΔ proteins, *ro-faf*Δ*6* retains nearly no ability to complement *faf* mutations (Figure 3A). This observation demonstrates clearly that the catalytic domain is insufficient *in vivo*, at least in the Drosophila eye, for Faf function.

In addition, the particular sensitivity to copy number of the *ro-faf*Δ*3* and *ro-faf*Δ*4* constructs is unlikely to be due to lower FafΔ3 and FafΔ4 protein accumulation than the other FafΔs or wild-type Faf. Instead, the particular nature of the effect that the deletions have on the activity of Faf could render FafΔ3 and FafΔ4 more sensitive to twofold concentration differences than the other FafΔ proteins with low activity.

DNA sequences of *faf* alleles with point mutations: The DNA sequences of 14 point mutant *faf* alleles were determined and the results are shown in Figure 5. All the mutants have a similar maternal effect lethal phenotype: females homozygous for each mutant allele produce embryos that never cellularize (FISCHER-VIZE *et al.* 1992). By contrast, the alleles fall into two groups on the basis of their mutant eye phenotypes: five of them have extremely weak mutant eye phenotypes (>90% wild-type facets) when homozygous or *in trans* to strong alleles and seven have strong mutant eye phenotypes (<5% wild-type facets; FISCHER-VIZE *et al.* 1992; Figure 5). Mostly likely, cellularization of the embryo simply requires a higher level of Faf activity than does eye patterning.

Eight of the mutant *faf* alleles have revealing molecular lesions that allowed them to be sorted into four groups. The first group contains only faf^{FO8}, in which the second catalytic His residue is changed to Tyr (Figure 5). As faf^{FO8} is a strong allele, this confirms the results of previous experiments that indicated the importance of this His residue for Faf function (HUANG *et al.* 1995).

The second group consists of three strong alleles, faf^{B8}, faf^{B7}, and faf^{BX1}, whose molecular lesions define amino acids within the catalytic region that may be essential for function. In faf^{B8}, a single Glu residue between the Cys and His domains, conserved in Fam (WOOD *et al.* 1997), is changed to Lys. This result could indicate a specific function for the Glu residue in catalysis, as the Glu residue is within a motif conserved among all Drosophila Ubps (BLOCK 3, consensus "D", see below). The faf^{BX1} allele contains a deletion of the amino acids MLFY, which are just C-terminal to the His domain and conserved among yeast Ubps (WILKINSON and HOCHSTRASSER 1998), Drosophila Ubps (BLOCK 8, see below), and Fam (WOOD *et al.* 1997). This result indicates a requirement for these conserved residues for Ubp function. Similarly, the mutation in faf^{B7} introduces a stop codon just prior to the MLFY residues. The faf^{BX1} lesion is within the deletion in *faf*Δ*5*, thus confirming the importance of this region for Faf function.

The third group consists solely of faf^{B4}, a weak mutant allele in which a Phe residue near the beginning of Faf protein is replaced by an insertion of six other amino acids (Figure 5). The Phe residue is within the region deleted in *faf*Δ*1*, thus confirming a role for the region upstream of the catalytic domain in Faf function.

The molecular lesions within mutations of the fourth group, consisting of three weak mutant alleles, faf^{B5}, faf^{BX5}, and faf^{BX3}, show that Faf protein regions well C-terminal to the His domain are also important for Faf function. The faf^{B5} and faf^{BX5} alleles have frameshift mutations near the C terminus of Faf; the mutation in faf^{BX5} destroys only the terminal ~250 amino acids. The faf^{BX3} allele has a 15-bp deletion in the middle of intron 11, which is normally only 60 bp in length. This intron may not be spliced at all, in which case a stop codon would be encountered within the intron and a truncated protein, similar to that produced by faf^{B5}, would be generated. Alternatively, the mutation may render the splicing of intron 11 less efficient, in which case the result would be that some wild-type Faf protein would be present, but less of it.

Comparison of Drosophila Ubp amino acid sequences: All of the protein sequences classified as Ubps in the GadFly database in Flybase were submitted to BLOCKS analysis (MATERIALS AND METHODS). The results indicated that Drosophila have at least 18, and probably 19, Ubps (Figure 6 legend). Eight blocks of sequence conservation within the catalytic region, including the Cys domain (Block 1) and the His domain (Block 6), were identified (Figure 6). The conserved Blocks are similiar in position and sequence to those found for the yeast Ubps (WILKINSON and HOCHSTRASSER 1998) but the motifs have diverged. In addition,

Faf is larger than any Ubp previously reported in any organism and is by far the largest Ubp in Drosophila; it is more than twice as large as the next largest Ubp (Figure 6). The size of Faf's catalytic region, however, is moderate. Thus, the bulk of Faf is unique sequence outside the catalytic region.

DISCUSSION

Faf is a Ubp required for patterning the Drosophila eye and for cellularization of the embryo. Faf is the largest known Ubp and the only deubiquitinating enzyme thought to counteract the proteolysis machinery by deubiquitinating particular substrates and thereby preventing their degradation. Six different deleted forms of the *faf* gene were expressed in Drosophila *P*-element transformants and tested for their ability to substitute for the endogenous *faf* gene in the eye. In addition, the DNA sequences of 12 point mutant *faf* alleles were determined. To aid analysis of these results, conserved sequences in the 18 Drosophila Ubps were identified. Unexpectedly, we found that protein domains along the entire length of the Faf protein are required for full activity of the protein.

In the eye, there is genetic evidence that Faf has one critical pathway, and the substrate in this pathway may be the Liquid facets protein (Cadavid *et al.* 2000). Thus, the *faf* mutant eye phenotype most likely reflects the ability of Faf to locate and deubiquitinate one substrate. The results presented here indicate that domains along virtually the entire primary structure of Faf are required for this one function. Thus, the unusually large size of Faf cannot be explained simply by the presence of multiple substrate-binding domains arranged in a linear fashion along the protein sequence. The same Faf protein domains are required for the eye and ovary functions of Faf; all of the mutants, whether they are weak or strong in the eye, have the same maternal effect lethal phenotype (Fischer-Vize *et al.* 1992). Thus, if there is a different substrate in the ovary, domains of Faf both N- and C-terminal to the catalytic region are also required for its recognition and deubiquitination, and these domains overlap those required in the eye.

The results of previous biochemical experiments suggest that only the catalytic domain of Faf may be re-

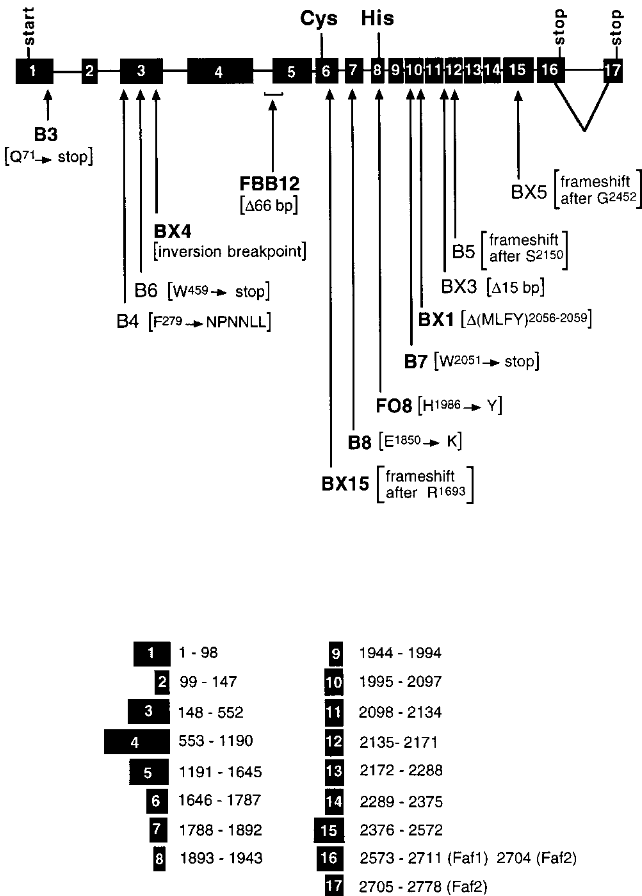

Figure 5.—Molecular mutations in *faf* alleles. At top are shown the 17 exons (black boxes) and introns (lines connecting the boxes) of the *faf* gene. The start codon and two alternate stop codons are indicated, as well as the locations of the catalytic domains containing the key cysteine (Cys) and histidine (His) residues. The positions of the DNA lesions in 13 different *faf* mutant alleles are indicated beneath the exons. The boldface allele names indicate strong mutants and the others are weak mutant alleles. At bottom, the amino acids within each exon are indicated by number. Five of the mutations are due to single base changes that result in altered codon identities: faf^{FO8} (CAC → TAC), faf^{B3} (CAG → TAG), faf^{B6} (TGG → TGA), faf^{B7} (TGG → TGA), faf^{B8} (GAA → AAA). One mutation involves two base changes: faf^{BX5} (GT → A just downstream of G^{2452}). Four mutations are small deletions: faf^{FBB12} (66-bp deletion including the 3′ splice acceptor site of intron 4 and part of exon 5), faf^{BX1} (12-bp in-frame deletion), faf^{BX3} (15-bp deletion in the middle of intron 11), faf^{BX15} [deletion of 4 bp (GGGT)]. Two mutations are insertions: faf^{B4} [deletion of TTT codon and insertion of 18 bp: (AATCCCAA CAATCTACTG)], faf^{B5} [deletion of AG and insertion of 15 bp (TAATTTTTTTTTTAA)]. The faf^{B6} allele sequence is surprising; it has a stop codon in exon 3 but imparts a weak mutant eye phenotype. The most likely explanation is that there is an alternative splice within intron 2 and exon 3, such that the part of exon 3 containing this lesion is not always used. Two alleles, faf^{BX8} and faf^{BX10}, have no lesions within any exon or any of the introns sequenced (see materials and methods). Most likely, their lesions lie within transcriptional control sequences. The faf^{BX4} allele is an inversion (Fischer-Vize *et al.* 1992). In the process of sequencing the mutations, we found two errors in the sequence of exon 17, which, when corrected, resulted in the larger form of Faf protein having a slightly longer open reading frame in exon 17 than reported previously (Fischer-Vize *et al.* 1992). These corrections agree with the Genome Project sequence data and have been sent to GenBank. Also, the numbers of the catalytic His residues were reported in error previously (Huang *et al.* 1995) due to a mistake in sequence numbering (Fischer-Vize *et al.* 1992). The correct numbers (His^{1978} and His^{1986}) are shown here. The *faf* alleles shown in this figure are the only ones that remain of the point mutations reported in Fischer-Vize *et al.* (1992), except for faf^{BX13}, which was not sequenced because it appears to have a cytological rearrangement.

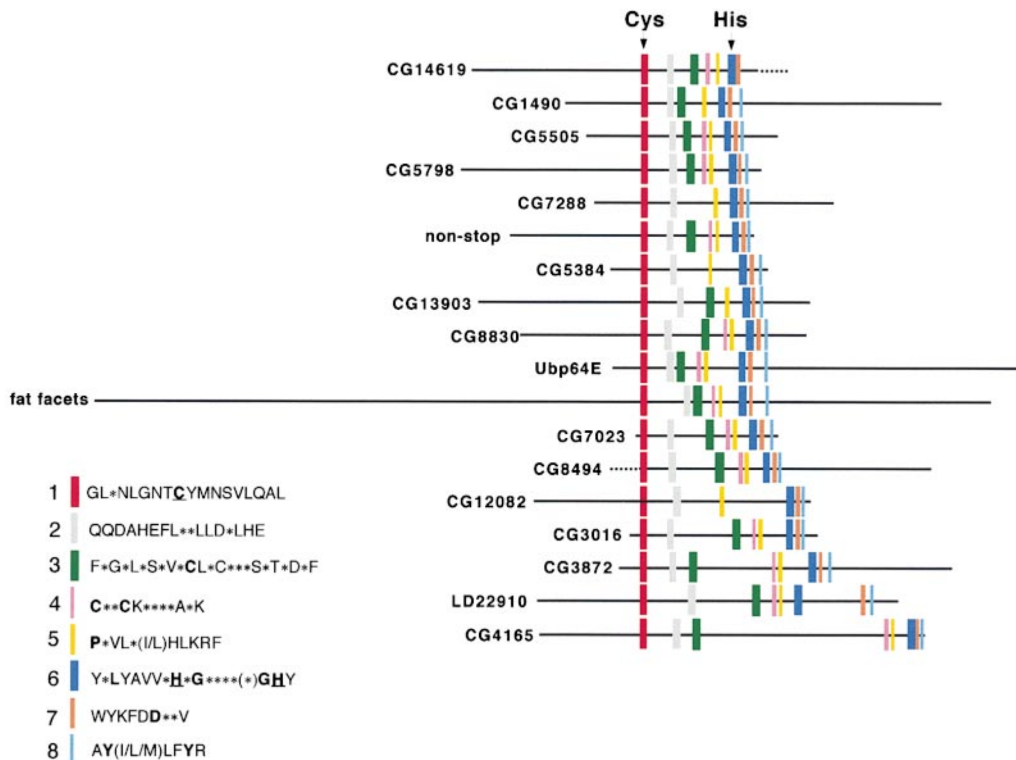

FIGURE 6.—Regions of similarity among the Drosophila Ubps. The 18 Drosophila Ubps are represented as black lines and the eight blocks of similarity, all within the catalytic region, are depicted as colored rectangles. The Ubps are aligned by Block 1, which contains the catalytic Cys residue. A consensus sequence for each block is shown at the bottom left. The amino acids in boldface are invariant and the catalytic Cys and His residues are underlined. The asterisks indicate that no clear consensus amino acids could be identified. The asterisk in parentheses indicates that an amino acid may or may not be present at that position. A probable nineteenth Ubp, CG8334, contains Blocks 1–3, but none of the others, perhaps due to a sequencing error. One protein, CG8232, listed as a Ubp in GadFly, does not have any of the conserved BLOCKS.

quired for its function. First, when expressed in bacteria, a fragment of Faf containing only the catalytic region can deubiquitinate artificial substrates in the form of peptide-linked Ub-protein fusions (Huang *et al.* 1995), suggesting that the catalytic domain has the intrinsic ability to recognize Ub. Other experiments suggest that the catalytic domain of Fam can also recognize a specific substrate, even when that substrate is not ubiquitinated. The catalytic domain of Fam specifically binds to particular domains of two proteins, AF-6 and β-catenin, *in vitro* and also in cultured cells (Taya *et al.* 1998, 1999). In addition, when expressed in cultured cells, AF-6 can be ubiquitinated and the catalytic domain of Fam can deubiquitinate it (Taya *et al.* 1998). The *in vivo* significance of the Fam/AF-6 and Fam/β-catenin interactions is not yet clear. Fam and AF-6 co-localize in some mouse tissues (Kanai-Azuma *et al.* 2000), suggesting that their interaction may be significant. However, genetic evidence (Chen *et al.* 2000) suggests that neither the Drosophila homolog of Af-6 (Canoe, Miyamoto *et al.* 1995) or β-catenin (Armadillo, Wieschaus *et al.* 1984) is an important Faf substrate in the eye. Nevertheless, in *in vitro* and cell culture assays, the catalytic domains of Faf/Fam alone can recognize Ub and specific substrates.

The results of genetic experiments also have suggested that the catalytic domain of Faf alone might be sufficient for its function. When expressed in the fly eye with the same expression vector used here, either of two different yeast Ubps, Ubp2 and Ubp3, can substitute for the endogenous Faf protein more effectively than any of the FafΔ proteins except for FafΔ1 (Wu *et al.* 1999). [In these experiments, Ubp2 and Ubp3 accumulate to levels similar to FafΔ6 and Faf+, respectively (Wu *et al.* 1999; Z. Wu and J. A. Fischer, unpublished data).] However, neither Ubp2 nor Ubp3 shows obvious amino acid sequence similarity with Faf outside the catalytic domain. One possible explanation for these results is that the catalytic domain of Faf, which is similar to that of Ubp2 and Ubp3, is the essential part of the protein. However, yeast Ubp4, which shares the conserved catalytic domain, cannot substitute for *faf* (Wu *et al.* 1999). There are several alternative explanations. For example, structural similarities between Faf and the two yeast Ubps may have escaped detection. Alternatively, Ubp2 and Ubp3 may recognize the substrate of Faf in a different way than Faf does; the yeast Ubps might have an enhanced ability, relative to Faf, to bind to Ub chains linked to some substrates. This idea seems plausible especially for Ubp2 as it may be a more promiscuous deubiquitinating enzyme than Faf. When overexpressed in yeast, Ubp2 inhibits proteolysis generally and deubiquitinates a variety of substrates (Baker *et al.* 1992). In addition, a high level of Ubp2 expression in the Drosophila eye disrupts eye development, presumably because it deubiquitinates proteins inappropriately (Wu *et al.* 1999).

We conclude that, *in vivo*, the large unique regions of Faf are required for full protein activity. The large

size of Faf appears to reflect a requirement for many Faf protein domains in the recognition of one substrate, rather than a linear array of domains for recognition of multiple substrates. We speculate that, *in vivo*, Faf might locate its substrate by binding to several different proteins in a complex. Perhaps when Faf/Fam and the substrate is overexpressed in cell culture assays or *in vitro*, the need for Faf/Fam interactions with other proteins in the complex is overcome by the nonphysiological increase in concentration of both proteins. Further experiments are required to test this idea.

We thank the University of Texas, Austin, DNA Analysis Center for performing the DNA sequencing. We thank Maya Zhang and Kerrie-Ann Smyth for helping to determine the DNA sequences of two alleles. We also thank J. Mendenhall for performing the scanning electron microscopy, J. Loera for technical assistance, and G. Gage for help in preparing the figures. This work was supported by a grant from the National Institutes of Health (HD30680) to J.A.F. Support for X.C. also came from the Institute for Cellular and Molecular Biology, the Center for Developmental Biology, and the Department of Zoology at the University of Texas at Austin.

LITERATURE CITED

Amerik, A. Y., S. Swaminathan, B. A. Krantz, K. D. Wilkinson and M. Hochstrasser, 1997 *In vivo* disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. EMBO J. **16:** 4826–4838.

Baker, R. T., J. W. Tobias and A. Varshavsky, 1992 Ubiquitin-specific proteases of *Saccharomyces cerevisiae*. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. J. Biol. Chem. **267:** 23364–23375.

Cadavid, A. L. M., A. Ginzel and J. A. Fischer, 2000 The function of the Drosophila Fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. Development **127:** 1727–1736.

Chen, Z. J., L. Parent and T. Maniatis, 1996 Site-specific phosphorylation to IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell **84:** 853–862.

Chen, X., E. Overstreet, S. A. Wood and J. A. Fischer, 2000 On the conservation of function of the Drosophila fat facets deubiquitinating enzyme and Fam, its mouse homolog. Dev. Genes Evol. (in press).

Dokucu, M. E., S. L. Zipursky and R. L. Cagan, 1996 Atonal, rough and the resolution of proneural clusters in the developing Drosophila retina. Development **122:** 4139–4147.

Fischer-Vize, J. A., G. M. Rubin and R. Lehmann, 1992 The *fat facets* gene is required for Drosophila eye and embryo development. Development **116:** 985–1000.

Flybase, 1999 The Flybase database of the Drosophila genome projects and community literature. Available from http://flybase.bio.indiana.edu/. Nucleic Acids Res. **27:** 85–88.

Heberlein, U., A. Penton, S. Falsafi, D. Hackett and G. M. Rubin, 1994 The C-terminus of the homeodomain is required for functional specificity of the Drosophila *rough* gene. Mech. Dev. **48:** 35–49.

Hicke, L., 1999 Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. Trends Cell Biol. **9:** 107–111.

Huang, Y., and J. A. Fischer-Vize, 1996 Undifferentiated cells in the developing Drosophila eye influence facet assembly and require the Fat facets deubiquitinating enzyme. Development **122:** 3207–3216.

Huang, Y., R. T. Baker and J. A. Fischer-Vize, 1995 Control of cell fate by a deubiquitinating enzyme encoded by the *fat facets* gene. Science **270:** 1828–1831.

Jones, M. H., R. A. Furlong, H. Burkin, I. J. Chalmers, G. M. Brown *et al.*, 1996 The Drosophila developmental gene *fat facets* has a human homologue in Xp11.4 which escapes X-inactivation and has related sequences on Yq11.2. Hum. Mol. Genet. **5:** 1695–1701.

Kanai-Azuma, M., J. S. Mattick, K. Kaibuchi and S. A. Wood, 2000 Co-localization of Fam and Af-6, the mammalian homologs of Drosophila *faf* and *canoe*, in mouse eye development. Mech. Dev. **91:** 383–386.

Kimmel, B. E., U. Heberlein and G. M. Rubin, 1990 The homeodomain protein Rough is expressed in a subset of cells in the developing Drosophila eye where it can specify photoreceptor cell subtype. Genes Dev. **4:** 712–727.

Koepp, D. M., J. W. Harper and S. J. Elledge, 1999 How the cyclin became a cyclin: regulated proteolysis in the cell cycle. Cell **97:** 431–434.

Lam, Y. A., W. Xu, G. N. Demartino and R. E. Cohen, 1997 Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature **385:** 737–740.

Li, Q., I. K. Hariharan, Y. Huang and J. A. Fischer, 1997 Genetic interactions with *Rap1* and *Ras1* reveal a second function for the *fat facets* gene in Drosophila eye development. Proc. Natl. Acad. Sci. USA **94:** 12515–12520.

Lupas, A., and W. Baumeister, 1998 The 20S proteasome, pp. 127–146 in *Ubiquitin and the Biology of the Cell*, edited by J.-M. Peters, J. R. Harris and D. Finley. Plenum Press, New York.

Maniatis, T., 1999 A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways. Genes Dev. **13:** 505–510.

Miyamoto, H., I. Nihonmatsu, S. Kondo, R. Ueda, S. Togashi *et al.*, 1995 *canoe* encodes a novel protein containing a GLGF/DHR motif and functions with Notch and *scabrous* in common developmental pathways in Drosophila. Genes Dev. **9:** 612–625.

Papa, F. R., and M. Hochstrasser, 1993 The yeast *doa4* gene encodes a deubiquitinating enzyme related to a product of the human *tre-2* oncogene. Nature **366:** 313–319.

Pickart, C. M., 1998 Polyubiquitin chains, pp. 19–63 in *Ubiquitin and the Biology of the Cell*, edited by J.-M. Peters, J. R. Harris and D. Finley. Plenum Press, New York.

Rechsteiner, M., 1998 The 26S proteasome, pp. 147–189 in *Ubiquitin and the Biology of the Cell*, edited by J.-M. Peters, J. R. Harris and D. Finley. Plenum Press, New York.

Rubin, G. M., M. D. Yandell, J. R. Wortman, G. L. G. Miklos, C. R. Nelson *et al.*, 2000 Comparative genomics of eukaryotes. Science **287:** 2204–2215.

Sambrook, G., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Spradling, A. C., 1986 P-element mediated transformation, pp. 175–197 in *Drosophila: A Practical Approach*, edited by D. B. Roberts. IRL Press, Oxford.

Strous, G. J., and R. Govers, 1999 The ubiquitin-proteasome system and endocytosis. J. Cell Sci. **112:** 1417–1423.

Taya, S., T. Yamamoto, K. Kano, Y. Kawano, A. Iwamatsu *et al.*, 1998 The Ras target AF-6 is a substrate of the Fam deubiquitinating enzyme. J. Cell Biol. **142:** 1053–1062.

Taya, S., T. Yamamoto, M. Kanai-Azuma, S. A. Wood and K. Kaibuchi, 1999 The deubiquitinating enzyme Fam interacts with and stabilizes β-catenin. Genes Cells **4:** 757–767.

Tomlinson, A., and D. F. Ready, 1987 Neuronal differentiation in the Drosophila ommatidium. Dev. Biol. **120:** 366–376.

Wieschaus, E., C. Nusslein-Volhard and G. Jurgens, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*: zygotic loci on the X-chromosome and the fourth chromosome. Roux's Arch. Dev. Biol. **193:** 296–307.

Wilkinson, K. D., and M. Hochstrasser, 1998 The deubiquitinating enzymes, pp. 99–125 in *Ubiquitin and the Biology of the Cell*, edited by J.-M. Peters, J. R. Harris and D. Finley. Plenum Press, New York.

Wood, S. A., W. S. Pascoe, K. Ru, T. Yamada, J. Hirchenhain *et al.*, 1997 Cloning and expression analysis of a novel mouse gene with sequence similarity to the Drosophila *fat facets* gene. Mech. Dev. **63:** 29–38.

Wu, Z., Q. Li, M. Fortini and J. A. Fischer, 1999 Genetic analysis of the role of the Drosophila *fat facets* gene in the ubiquitin pathway. Dev. Genet. **25:** 312–320.

Communicating editor: K. Golic