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Genetics

Genetic interactions with *Rap1* and *Ras1* reveal a second function for the Fat facets deubiquitinating enzyme in *Drosophila* eye development

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ABSTRACT **The *Drosophila* fat facets gene encodes a deubiquitinating enzyme that regulates a cell communication pathway essential very early in eye development, prior to facet assembly, to limit the number of photoreceptor cells in each facet of the compound eye to eight. The Fat facets protein facilitates the production of a signal in cells outside the developing facets that inhibits neural development of particular facet precursor cells. Novel gain-of-function mutations in the *Drosophila Rap1* and *Ras1* genes are described herein that interact genetically with fat facets mutations. Analysis of these genetic interactions reveals that Fat facets has an additional function later in eye development involving Rap1 and Ras1 proteins. Moreover, the results suggest that undifferentiated cells outside the facet continue to influence facet assembly later in eye development.**

Assembly of the *Drosophila* compound eye requires a complex series of positive and inhibitory, extracellular and intracellular, signals (1–3). The eight photoreceptors (R1–R8) and four cone cells that make up each of the 800 identical facets, or ommatidia, as well as the hexagonal lattice of pigment cells surrounding each facet, assemble within the eye imaginal disc, a cellular monolayer, and they do so in a strict order (4). After the first photoreceptor cell R8 is determined, first R8 and then the subsequently determined photoreceptors recruit the remaining cells from the surrounding undifferentiated cell pool into the growing facet (1). The major players in these inductions are the *Drosophila* epidermal growth factor receptor homolog (DER) and its ligand, Spitz (5, 6).

Patterning of the eye disc prior to R8 determination is poorly understood. Some aspect of this process requires the fat facets (*faf*) gene (7). The morphogenetic furrow marks the beginning of facet assembly; rows of facets assemble posterior to the furrow as it moves anteriorly through the eye disc (4). The *faf* gene is essential in an uncharacterized inhibitory cell communication pathway that operates anterior to the morphogenetic furrow and in cells outside of those that will become photoreceptors, to limit the number of photoreceptors in each facet to eight (7, 8).

The *faf* gene encodes one member of a family of deubiquitinating enzymes, also called ubiquitin (Ub) proteases, or Ubps (9). Ub is a 76-amino acid polypeptide that when covalently attached to proteins, targets them for degradation by a multisubunit protein complex called the proteasome (10). Deubiquitinating enzymes cleave Ub-protein bonds (10) and Faf is thought to remove Ub from a specific protein or proteins, thereby preventing degradation by the proteasome (9). Faf is of interest because it reveals a mechanism for regulation of the Ub-proteasome pathway and also because it defines a cell communication pathway critical for patterning the eye disc.

Herein, gain-of-function mutations in two *Drosophila* genes, *Ras1* and *Rap1*, are described that were identified because of their genetic interactions with *faf* mutants. Ras1 is a GTP-binding protein that can switch from an inactive (GDP-bound) to an active (GTP-bound) state (11). In its active form, Ras1 initiates a phosphorylation cascade, resulting ultimately in the activation or inactivation of particular transcriptional regulators (2). Ras1 relays the signals from DER and is thus required for the determination of all cells within the facet (12, 13). Rap1 is part of the Ras superfamily of GTP-binding proteins (14) and its normal role in signal transduction is not clear. Analysis of the genetic interactions among *faf* and *Rap1* and *Ras1* reveals that in addition to its critical role anterior to the morphogenetic furrow, Faf has a function in undifferentiated cells later in eye development that involves, probably indirectly, Ras1 and Rap1. These results suggest that cells outside the facet influence cell fates within the facet.

MATERIALS AND METHODS

Drosophila Strains. The *faf* alleles used (faf^{BX3}, faf^{FO8}, faf^{BX4}) were described previously (7). The *Rap1* loss-of-function mutants used are $Rap1^{rv(R)B1}$, $Rap1^{rv(R)B3}$ (15), and $Rap1^{CD3}$ [obtained from T. Sliter (Southern Methodist University) and described in H. Asha and I.K.H., unpublished results]. The $Rap1^{R}$ allele and $Df(3L)R^{E}$ are described in ref. 15. To generate the *GMR-Rap1* gene, the coding region of *Rap1* (15) was cloned into the pGMR vector (16) and transformant lines were generated. The *Ras1* loss-of-function mutations, $Ras1^{e1B}$ and $Ras1^{e2F}$ (12), were obtained from M. Simon (Stanford University). The deficiency chromosomes *Df(3R)by62* (85D11–14; 85F6) and *Df(3R)by10* (85D8–12; 85E7/F1), described in ref. 12, were obtained from the Bloomington Stock Center. The Gal4-expressing lines *sca-Gal4* (*T3*; ref. 17), *h-Gal4* (*H10*; ref. 8) and *elav-Gal4* (18) are described in ref. 8. The *hs-Gal4* line (unpublished line) was obtained from A. Brand (Wellcome/CRC Institute). The transformant lines $P[w^{+}, faf^{+}]$ (7) and $P[w^{+}, faf^{Ser1677}]$ (9) were previously described and the *ro-faf, sev-faf, glrs-faf,* and *UAS-faf* lines are described in ref. 8. The $P[w^{+}]$ at 70C (12) used for mosaic analysis was obtained from M. Simon. Other marker mutations and balancer chromosomes are described in ref. 19. The *Rap1* and *Ras1* alleles described herein originally had different names (20): $Rap1^{V153M}$ is *E(faf)E8*, $Rap1^{T58M}$ is *E(faf)E127* and $Ras1^{E63K}$ is *E(faf)bE382*.

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Abbreviation: Ub, ubiquitin.

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Mapping *Rap1* and *Ras1* Mutant Alleles. The meiotic recombination scheme used to localize the *E(faf)* mutations to an approximate position on the third chromosome are described in detail elsewhere (20). $Rap1^{V153M}$ and $Rap1^{T58M}$ were found to be near the tip of chromosome 3L. *In trans* to $Df(3L)R^E$, both $Rap1^{V153M}$ and $Rap1^{T58M}$ resulted in rough-eyed flies. $Ras1^{E63K}$ was found to be closely linked to *cu*. Although $Ras1^{E63K}/Df(3R)by62$ flies were wild-type, $Ras1^{E63K}/Df(3R)by10$ flies were inviable, thus localizing $Ras1^{E63K}$ to 85D8–11. *In trans* to loss-of-function *Ras* alleles, $Ras1^{E63K}$ resulted in rough-eyed flies.

Cloning and Analysis of Mutant *Rap1* and *Ras1* Genes. The *Rap1* gene was cloned by PCR of genomic DNA prepared from homozygous $Rap1^{V153M}$ or $Rap1^{T58M}/Df(3L)R^E$ flies. The PCR primers used were identical to those as described (15). The *Ras1* gene was cloned by PCR of genomic DNA from $Ras1^{E63K}/TM6B$ flies by using the primers 5′-CGAAAACG-GACGCCACAGCC and 5′-CGCCGACGCACATACA-GACA. The PCR products were cloned into Bluescript (Stratagene) plasmid vectors and the sequences was determined on both strands by fluorimetric automated sequencing. For $Rap1^{V153M}$ and $Rap1^{T58M}$, the sequences of two clones, one from each of two PCRs were determined and the identical nucleotide change was found in each (see Fig. 3). For $Ras1^{E63K}$, the DNA sequences of five different clones from a single PCR were determined: two of the clones had the wild-type sequence and three had the identical single nucleotide change (see Fig. 3).

Analysis of Eyes. Adult eyes were prepared for scanning electron microscopy and photographed or were fixed, sectioned, stained, and photographed as described (7).

Mosaic Analysis. Clones of homozygous w^- $Rap1^+$ cells in the eyes of *w*; $Rap1^+/Rap1^R$ $P[w^+]$ flies were induced with x-rays as described (7). Eyes containing clones were fixed and sectioned as described (7).

Detection of Faf Protein Produced by Promoter-faf Constructs. To be sure that the *UAS-faf* and *sev-faf* transformants produce Faf protein, protein extracts were prepared from 20 pairs of transformant eye discs by simply dissecting them into protein gel loading buffer. The transformants used were *sev-faf*, *GMR-Gal4* (5)/*UAS-faf*, and *glrs-faf*, which rescues *faf* mutant eye phenotypes and is thus a positive control. Myc-tagged Faf protein in the extracts was visualized in Western blot experiments precisely as described in ref. 9. Bands corresponding to the approximate size of Faf protein were observed in all extracts (data not shown).

RESULTS

Identification of $Rap1^{V153M}$, $Rap1^{T58M}$, and $Ras1^{E63K}$. Two homozygous viable mutant alleles of *Rap1* ($Rap1^{V153M}$ and $Rap1^{T58M}$) and one mutant allele of *Ras1* ($Ras1^{E63K}$) were isolated in genetic screens (20) for dominant enhancers of the abnormal eye phenotypes of *faf* mutants. The screens were performed in genetic backgrounds (faf^{BX3}/faf^{FO8} or faf^{BX3}/faf^{BX3}) whose mutant eye phenotypes are thought to be sensitive to changes in the levels of proteins in the *faf*-dependent cell communication pathway; faf^{BX3} is a weak mutant allele and faf^{FO8} is a strong mutant allele (7). The eyes of faf^{BX3}/faf^{FO8} and faf^{BX3}/faf^{BX3} flies are nearly wild type (Fig. 1) as are those of $E(faf)/+$ heterozygotes (not shown). However, when the mutations are combined, $E(faf)$ $faf^{BX3}/faf^{BX3\ or\ FO8}$ flies have malformed eyes, apparent externally as a rough eye surface (Fig. 1).

By using meiotic recombination followed by physical mapping, $Rap1^{V153M}$ and $Rap1^{T58M}$ were localized to a region on the left arm of the third chromosome defined by $Df(3L)R^E$, between polytene position 62B8–9 and 62C1. As *Rap1* is within this region, complementation tests were performed with three different *Rap1* loss-of-function mutations ($Rap1^-$). Each *Rap1* mutant allele fails to complement the function of $Rap1^{V153M}$ and $Rap1^{T58M}$ in the eye; $Rap1^{V153M}/Rap1^-$ and $Rap1^{T58M}/Rap1^-$ have externally rough eyes (Fig. 1*E*). The *Rap1* genes were isolated from $Rap1^{V153M}$ and $Rap1^{T58M}$ flies and their DNA sequences were determined. Different single amino acid mutations were found in each *Rap1* gene (Fig. 2), confirming that the *E(faf)* mutations are in *Rap1*.

Similarly, $Ras1^{E63K}$ was localized to polytene position 85D8–11, which includes *Ras1*, and $Ras1^{E63K}/Ras1^-$ flies have rough eyes (Fig. 1*Q*). DNA sequence analysis of the *Ras1* gene in $Ras1^{E63K}$ mutant flies revealed a single amino acid mutation (Fig. 2), confirming that $Ras1^{E63K}$ is a mutant *Ras1* allele.

***faf* Is a Genetic Enhancer of $Rap1^{V153M}$, $Rap1^{T58M}$, and $Ras1^{E63K}$.** The morphological defects in $Rap1^{V153M}$ or $Rap1^{T58M}$ faf^{BX3}/faf^{FO8} and $Ras1^{E63K}$ faf^{BX3}/faf^{FO8} eyes suggest that the combination of mutant *faf* alleles enhances the *Rap1* and *Ras1* mutant phenotypes. In the eyes of faf^{BX4} homozygotes [faf^{BX4} is a null allele (7)], most of the facets contain more than the normal complement of eight photoreceptor cells, many facets have extra R7-like cells and a small proportion of facets are missing R7 (refs. 7 and 9 and Fig. 1*T*). The eyes of faf^{BX3}/faf^{FO8} and faf^{BX3}/faf^{BX3} flies show the same defects, but in far fewer facets than in faf^{BX4} eyes (Fig. 1*D*).

Flies homozygous for $Rap1^-$ mutations die as larvae and clones of $Rap1^-$ cells in the eye produce scars due to cell death (15). However, flies heterozygous for a dominant mutant allele called $Rap1^R$ ($Rap1^R/+$) are viable and have roughened external eye morphology; internally, most facets are missing R7 and sometimes outer photoreceptor cells are also missing (ref. 15; Fig. 1 *I* and *J*). The mutant eye phenotype of $Rap1^{V153M}$ or $Rap1^{T58M}$ faf^{BX3}/faf^{FO8} flies resembles that of $Rap1^R/+$ flies rather than that of faf^- flies (Fig. 1 *F* and *J*). Thus, in a background where Faf activity is compromised (faf^{BX3}/faf^{FO8}), $Rap1^{V153M}$ or $Rap1^{T58M}/+$ flies display a mutant eye phenotype similar to that of $Rap1^R/+$ flies (Fig. 3). Similarly, because $Ras1^-$ homozygotes die during embryogenesis and clones of $Ras1^-$ cells in the eye are missing photoreceptors (12), the mutant phenotype of $Ras1^{E63K}/+$ is enhanced by faf^{BX3}.

***$Rap1^{V153M}$* and *$Rap1^{T58M}$* Have Genetic Properties Similar to the Dominant Allele *$Rap1^R$*.** The mutant phenotypes of $Rap1^{V153M}$ or $Rap1^{T58M}/+$ in a faf^{BX3}/faf^{FO8} background suggest that the two *Rap1* alleles may have genetic properties similar to $Rap1^R$. The eye morphology of $Rap1^{V153M}$ and $Rap1^{T58M}$ homozygotes in an otherwise wild-type background supports this idea, because the mutant retinas have phenotypes similar to $Rap1^R/+$ (Fig. 1 *J* and *L*). Thus, $Rap1^{V153M}$ and $Rap1^{T58M}$ behave in some sense as recessive versions of the dominant mutation $Rap1^R$.

Like $Rap1^R$ (15), $Rap1^{V153M}$ and $Rap1^{T58M}$ proteins must have some wild-type function in the eye and elsewhere because $Rap1^R$, $Rap1^{V153M}$, and $Rap1^{T58M}$ homozygotes all are viable but $Rap1^-$ homozygotes are larval-lethal and clones of mutant cells result in scars in the adult eye due to death of most $Rap1^-/Rap1^-$ photoreceptor cells (H. Asha and I.K.H., unpublished results).

Are *$Rap1^{V153M}$* and *$Rap1^{T53M}$* Simple Hypermorphs? One possibility is that $Rap1^{V153M}$ and $Rap1^{T53M}$ are slightly hypermorphic (overactive) proteins that display no mutant phenotype unless present in two copies. If so, flies in which the only Rap1 protein comes from one $Rap1^{V153M}$ or $Rap1^{T58M}$ gene copy [$Rap1^{V153M}$ or $Rap1^{T58M}$ *in trans* to a chromosome in which *Rap1* is deleted ($Df(3L)R^E$) or *in trans* to a $Rap1^-$ mutation] should be wild type. However, $Rap1^{V153M}/Df(3L)R^E$ or $Rap1^{V153M}/Rap1^-$ eyes are indistinguishable from $Rap1^{V153M}$ homozygous eyes (Figs. 1 *K* and *L* and 4) and $Rap1^{T58M}/Df(3L)R^E$ or $Rap1^{T58M}/Rap1^-$ eyes are much more mutant than those of $Rap1^{T58M}$ homozygotes (Figs. 1 *G* and *H* and 4). Similar results were obtained with $Rap1^R$ (data not shown). Thus, like $Rap1^R$, $Rap1^{V153M}$ and $Rap1^{T58M}$ are not simple hypermorphic mutations.

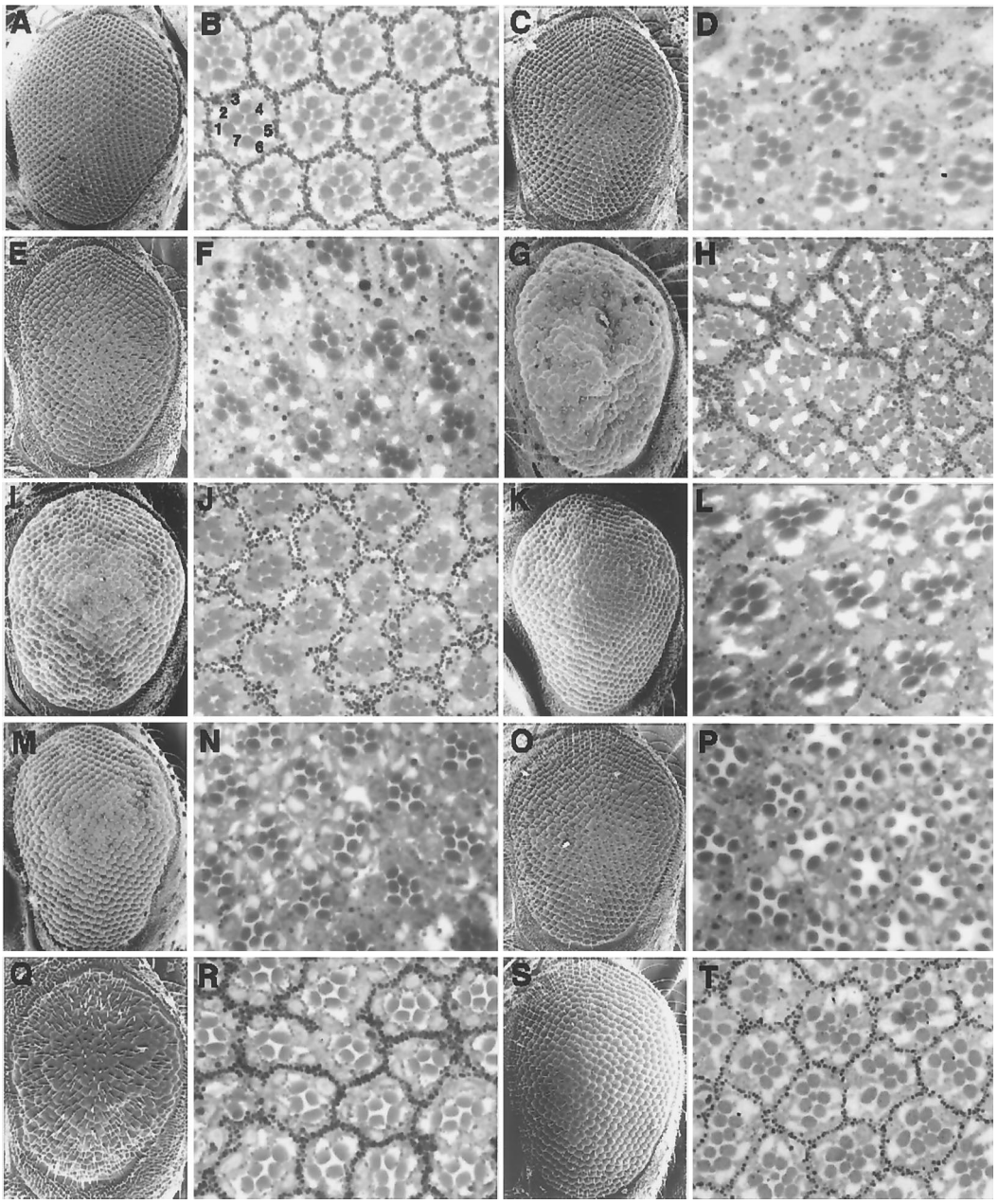

FIG. 1. Mutant eye phenotypes. Scanning electron micrographs and tangential sections through eyes of the following genotypes are shown. (*A* and *B*) Wild-type. (*C* and *D*) faf^{BX3}/faf^{FO8}. (*E* and *F*) $Rap1^{T58M}$ faf^{BX3}/faf^{FO8}. (*G* and *H*) $Rap1^{T58M}/Rap1^{-}$. (*I* and *J*) $Rap1^{R}/+$. (*K* and *L*) $Rap1^{V153M}/Rap1^{V153M}$. (*M* and *N*) $Rap1^{-}$ faf^{FO8}/faf^{BX3}. (*O* and *P*) $Ras1^{E63K}$ faf^{BX3}/faf^{FO8}. (*Q* and *R*) $Ras1^{E63K}/Ras1^{-}$. (*S* and *T*) faf^{BX4}.

Are $Rap1^{V153M}$ and $Rap1^{T53M}$ Simple Hypomorphs? Like $Rap1^{R}$ (15), $Rap1^{V153M}$ and $Rap1^{T58M}$ are not simple hypomorphic (weak loss-of-function) mutations. If so, it would be expected that $Rap1^{-}$ faf^{BX3}/faf^{FO8} eyes would be more mutant than $Rap1^{V153M}$ or $Rap1^{T58M}$ faf^{BX3}/faf^{FO8} eyes. However, precisely the opposite result was obtained (Figs. 1 *E*, *F*, *M*, and *N* and 4). Similarly, the mutant phenotype of $Rap1^{R}/+$ is enhanced by faf^{BX3}/faf^{FO8} (data not shown).

Are $Rap1^{V153M}$ and $Rap1^{T53M}$ Simple Antimorphs? Like $Rap1^{R}$, $Rap1^{V153M}$ and $Rap1^{T58M}$ have some antimorphic (antagonistic to wild-type) properties. In a *P* element transformant line containing a *GMR-Rap1* gene, *Rap1* is overexpressed ubiquitously posterior to the morphogenetic furrow. The *GMR-Rap1* gene suppresses the mutant eye phenotype of $Rap1^{R}/+$ flies to nearly wild type, suggesting that $Rap1^{R}$ protein may be antagonizing wild-type Rap1 (data not shown). Similarly, *GMR-Rap1* suppresses the eye defects in $Rap1^{V153M}$ or $Rap1^{T58M}$ faf^{BX3}/faf^{FO8} flies (Fig. 4).

In summary, $Rap1^{V153M}$ and $Rap1^{T58M}$ proteins, like $Rap1^{R}$, provide some wild-type function and also antagonize wild-type function in some manner.

Genetic Properties of $Ras1^{E63K}$. The $Ras1^{E63K}$ allele has genetic properties similar to those of the *Rap1* alleles described above. (*i*) Although $Ras1^{E63K}$ has some wild-type function [$Ras1^{-}$ homozygotes die as embryos (12) but $Ras1^{E63K}/Ras1^{-}$ flies are viable (see above)], it is not a simple hypomorph: the eyes of $Ras1^{E63K}$ faf^{BX3}/faf^{FO8} flies (Fig. 1 *O* and *P*) are more severely mutant than those of $Ras1^{-}$ faf^{FO8}/faf^{BX3} flies (Fig. 4), which are nearly wild-type. (*ii*) $Ras1^{E63K}$ is not hypermorphic, as the eyes of $Ras1^{E63K}/Ras1^{-}$ flies (Fig. 1 *Q* and *R*) are much more mutant than those of $Ras1^{E63K}/+$ flies, which are wild-type. Finally, $Ras1^{E63K}$ is not a simple antimorph, because it retains some wild-type function (above).

$Rap1^{R}$ Functions Mainly Within R7. To investigate the nature of the interaction between *faf* and *Rap1*, we sought to determine in which cells the mutant Rap1 proteins function to

Rap1

MREYKIVVL GSGGVGKS ALTVQFVQCIFVEKYDPTIEDSYRKQVEV

M (58)

DGQQCMLEIL DTAGTE QFTAMRDLYMKNGQGFVLVYSITAQSTFN

DLQDLREQILRVKDTDDVPMV LVGNKCDL EFERVVGKELGKNLATQ

M (153) L (R)

FNCAFM ETSAK AKVNVNDIFYDLVRQINKKSPEKKQKKPKKSLCVLL

Ras1

MTEYKLVVV GAGGVGKS ALTIQLIQNHFVDEYDPTIEDSYRKQVVIDG

K (63)

ETCLLDIL DTAGQE EYSAMRDQYMRTGEGFLLVFAVNSAKSFEDIGT

YREQIKRVKDAEEVPMV LVGNKCDL ASWNVNNEQAREVAKQYGIPYI

ETSAK TRMGVDDAFYTLVREIRKDKDNKGRRGRKMNKPNRRFKCKML

FIG. 2. Amino acid alterations in Rap1 and Ras1 mutant proteins. The amino acid sequences of *Drosophila* Rap1 (15) and Ras1 (12) are shown. The GTP-binding regions are boxed (21) and the region thought to bind effector is underlined (22, 23). The amino acid changes in $Rap1^{R}$ (15), $Rap1^{V153M}$, $Rap1^{T58M}$, and $Ras1^{E63K}$ mutant proteins are indicated. The codon changes are V153M (GTG → ATG), T58M (ACG → ATG), and E63K (GAG → AAG).

exert their detrimental effects on development. To determine in which cells the $Rap1^{R}$ protein functions, facets mosaic for $Rap1^{+}$ and $Rap1^{R}/Rap1^{+}$ photoreceptors were generated and analyzed (Table 1). The data in Table 1 show that in facets with wild-type photoreceptor cell arrangements, R7 has the strongest tendency to be $Rap1^{+}$. Thus, the $Rap1^{R}$ protein appears to exert its effect mainly within R7. The data also indicate a possible weaker requirement for $Rap1^{+}$ in R6. As outer R cells are also sometimes missing in $Rap1^{R}/+$ retinas, it is possible that one of these may be R6.

In Its Interactions with Rap1 and Ras1, Faf Functions Posterior to the Morphogenetic Furrow and in Cells Outside the Facet. The *faf* gene is expressed both anterior to and posterior to the morphogenetic furrow (7) and *Rap1* expression is ubiquitious (ref. 24 and H. Asha and I.K.H, unpublished results). Although the strongest expression of *faf* is anterior to the furrow (7), expression of *faf* within the furrow is sufficient to complement the eye defects in faf^{BX4} flies (8). To determine where in the eye disc Faf performs the function involving *Rap1*, transformant lines containing transgenes that express *faf* in a variety of patterns in the developing eye (Table 2) were tested for their ability to complement the mutant eye phenotypes in $Rap1^{V153M}$

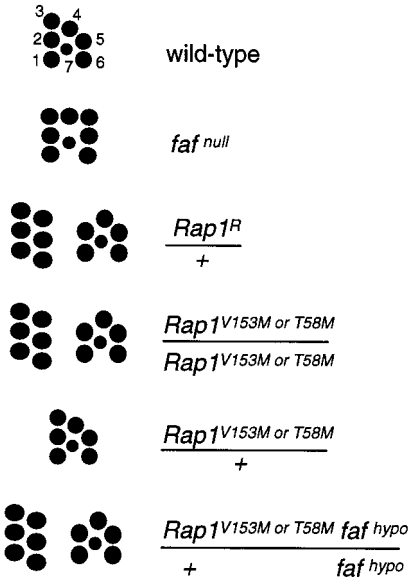

FIG. 3. Summary of mutant eye phenotypes. The numbers refer to R cells, each of which are represented by a solid circle. The *faf* mutant eye phenotype is qualitatively different from the *Rap1* mutant phenotype and hypomorphic *faf* alleles (faf^{hypo}) enhance the mutant phenotype of *Rap1*.

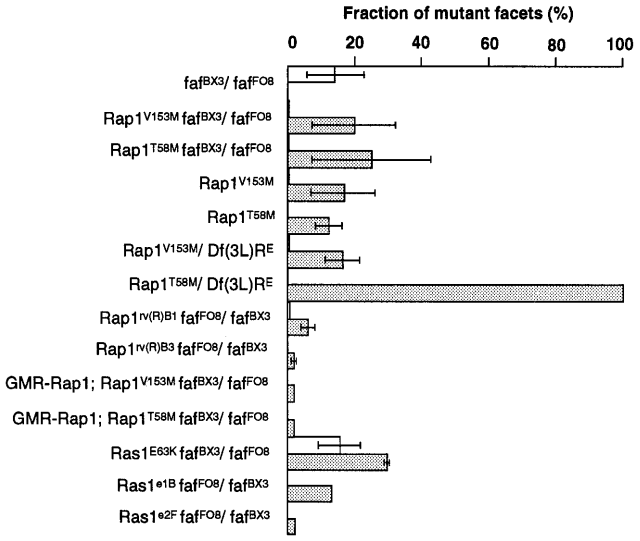

FIG. 4. Quantitation of mutant eye phenotypes. The average fraction of *faf*-type (open bars) and $Rap1^{R}$-type (shaded bars) mutant facets was calculated for each of the genotypes shown. One hundred to 500 facets in two to five eyes were counted for each genotype, except for the genotypes with no error bars where one eye was counted. The error bars represent one standard deviation above and below the mean value obtained for each eye; standard errors are large in the cases where there was significant variation in the penetrance of the phenotype. For reasons that are not understood, the mutant *Rap1* alleles suppress the *faf* phenotype.

Table 1. Analysis of $Rap1^{R}$:$Rap1^{+}$ mosaic facets

R cell	Fraction w^{+} $Rap1^{R}$ observed
R8	0.38
R2	0.42
R5	0.43
R3	0.40
R4	0.38
R1	0.38
R6	0.33
R7	0.19

Clones of homozygous $w^{-}Rap1^{+}$ cells were induced in a $w^{+}Rap1^{R}/w^{-}Rap1^{+}$ background. R-cells in 74 genetically mosaic and phenotypically wild-type facets at the clone borders were scored for the presence (w^{+}) or absence (w^{-}) of pigment granules. In $Rap1^{R}/Rap1^{+}$ eyes, 25% of facets are phenotypically wild type (15), meaning that 25% of each R-cell type in the mosaic facets will be w^{+} or w^{-} randomly. Thus, a particular R-cell in a phenotypically wild-type facet will be $w^{+}Rap1^{R}$ at a frequency of 13%, even if $Rap1^{R}$ produces its mutant phenotype by functioning within that R-cell. Conversely, if $Rap1^{R}$ does not function within a particular R-cell, that R-cell should be $w^{+}Rap1^{R}$ at a frequency of 50%. χ^{2} analysis was used to evaluate whether the observed frequencies for each individual R-cell are significantly different from 0.50 (R8, 2, 5, 3, 4, 1, or 6) or 0.13 (R7). Only the observed frequencies for R6 and R7 are significantly different from 0.50 and 0.13, respectively. Some of the w^{+} cells may have been contained within the twin spot of the $w^{-}Rap1^{+}$ clone and, thus, are homozygous for $w^{+}Rap1^{R}$. This should have little effect on the interpretation of the experiment as $Rap1^{R}$ homozygotes have nearly the identical photoreceptor patterning phenotype to $Rap1^{R+}$ *(15).*

Table 2. Expression patterns of promoter-*faf* constructs

Promoter-*faf* construct*	Expression pattern†
ro-faf	In furrow and R2/5, R3/4
glrs-faf	Posterior edge of furrow and all cells posterior to furrow
sev-faf	M1, M2, R3/4, R1/6, R7, CC
sca-Gal4; *UAS-faf*	Precluster cells at posterior edge of furrow and R8 posterior to furrow
h-Gal4; *UAS-faf*	Stripe of cells anterior to furrow
elav-Gal4; *UAS-faf*	All R-cellss posterior to furrow

*The Gal4-expressing lines are described in *Materials and Methods*.
†See ref. 8 for references to the expressions patterns. Expression patterns using the Gal4/UAS system are delayed several hours (shifted several rows posterior in the eye disc) relative to endogenous protein expression.

faf^{BX3}/faf^{BX4} flies (Fig. 5). The only gene construct that complements the eye defects is *glrs-faf*, which expresses *faf* in all cells posterior to the morphogenetic furrow. Constructs that express *faf* anterior to or within the furrow, or posterior to the furrow but exclusively in cells within the developing photoreceptors all fail to complement (Table 2 and Fig. 5). Three of the transgenes (*glrs-faf*, *ro-faf*, and *sev-faf*) were also tested for their ability to complement the eye defects of $Ras1^{E63K}$ faf^{BX3}/faf^{BX4} mutants. As for the *Rap1* alleles, only *glrs-faf* complements the rough eye phenotype (data not shown). Thus, in its interactions with Rap1 and Ras1, Faf functions posterior to the furrow and in cells outside of the assembling photoreceptors.

In Its Interactions with Rap1 and Ras1, Faf Functions as a Ubp. *P* elements containing a genomic DNA fragment corresponding either to a wild-type (faf^{+}) or a mutant *faf* gene ($faf^{Ser1677}$) were tested for their ability to complement the mutant eye phenotypes of $Rap1^{V153M}$ or $Rap1^{T58M}$ faf^{BX3}/faf^{BX4}. In the $faf^{Ser1677}$ gene, the conserved cysteine residue critical for Ubp enzyme activity is changed to serine (14). This mutation was previously shown to hinder the activity of Faf in a Ubp activity assay and to render the $faf^{Ser1677}$ gene unable to complement faf^{BX4} (9). The *P* element containing the wild-type *faf* gene complements the mutant phenotypes of all three genotypes (Fig. 5). In contrast, the $faf^{Ser1677}$ gene fails to complement any of them (Fig. 5). These results strongly suggest that Faf functions as a Ubp in its Rap1- and Ras1-dependent role.

DISCUSSION

Rap1 and *Ras1* genes bearing specific point mutations display genetic interactions with *faf* mutations. The results of genetic experiments show that in addition to its critical function anterior to the morphogenetic furrow, the Faf deubiquitinating enzyme has a later function in facet assembly posterior to the furrow, evident when the specific Rap1 or Ras1 mutant proteins are expressed. Like its critical function, the later role of Faf is within cells outside the developing facets.

Faf Has a Second Role in Eye Development. The results of two different experiments show that the genetic interactions between *faf* and the specific alleles of *Rap1* and *Ras1* are due to a function of Faf distinct from its essential function. First is the mutant eye phenotype of flies with hypomorphic *faf* mutations when their eye roughness is dominantly enhanced by any of the three mutations described; the eye phenotypes resemble those of *Rap1* or *Ras1* mutations rather than *faf* mutants. Thus, the hypomorphic *faf* background appears to be enhancing the mutant phenotype of the *Rap1* and *Ras1* alleles. Second are the experiments where promoter-*faf* genes were tested for their ability to complement the mutant eye phenotypes of the mutant *Rap1* and *Ras1* alleles in a hypomorphic *faf* background. The results of these experiments show that in its interactions with *Rap1* and *Ras1*, *faf* is functioning later in eye development than in its essential function. The *ro-faf* gene, expressed early in eye development, complements the mutant eye phenotype of *faf* null flies nearly completely, but *glrs-faf*, which is expressed later, complements *faf* nulls weakly (8). In contrast, *ro-faf* has no ability to complement the mutant eye phenotypes of $Rap1^{V153M}$ or $Rap1^{T58M}$ faf^{BX3}/faf^{FO8} or $Ras1^{E63K}$ faf^{BX3}/faf^{FO8} flies, but *glrs-faf* complements extremely well.

Cells Outside the Facet Influence Facet Assembly. As in its critical function anterior to the morphogenetic furrow, the later function of Faf revealed in the background of the mutant Rap1 and Ras1 proteins is in cells outside the facet; expression of *faf* in photoreceptor cells by *sev-faf* and *elav-Gal4*; *UAS-faf* fails to complement the mutant eye phenotype of weak *faf* mutations in a $Rap1^{V153M}$ or $Rap1^{T58M}/+$ or $Ras1^{E63K}/+$ background. Thus, the results presented here suggest that undifferentiated cells surrounding the developing facets play a role in recruiting photoreceptors into the facet. [$Rap1^{R}$ has been shown to interrupt the initial recruitment of photoreceptors rather than their maintenance (15).] This is remarkable, as there is no other evidence that the undifferentiated cells surrounding the facets send any inductive signals.

What Biochemical Pathway Does Faf Regulate Behind the Furrow? There is no signaling pathway known where cells outside the facet affect the development of cells within the facet. Two transcriptional regulators required for proper facet

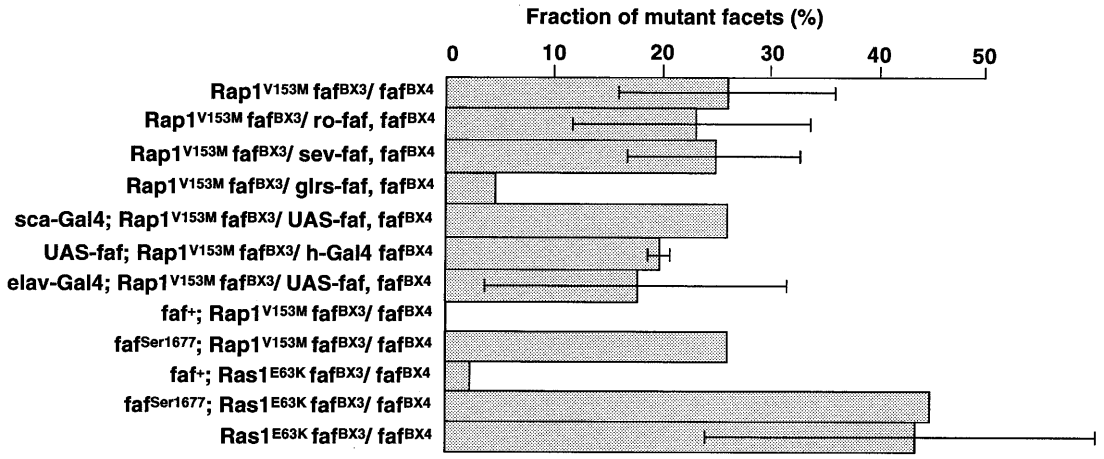

FIG. 5. Complementation of mutant phenotypes by transgenes. The average fraction of mutant facets, indicated by the bars, was calculated for each of the genotypes shown. One hundred to 500 facets in two to five eyes were counted for each genotype, except for the genotypes with no error bars where one eye was counted. (In *glrs-faf*, four eyes were analyzed and the standard error was zero.) The error bars represent one standard deviation above and below the mean value obtained for each eye; standard errors are large in the cases where there was significant variation in the penetrance of the phenotype.

assembly, Jun (25) and Ttk88 (26, 27), are themselves regulated by Ub-dependent proteolysis (28–30). Faf is not likely to be involved directly in the regulation of these proteins because Jun and Ttk88 function autonomously within photoreceptors and cone cells, whereas Faf functions within cells outside the facet.

The Faf–Rap1 and Faf–Ras1 Interactions. Given that the *Rap1* and *Ras1* alleles described are not simple loss-of-function mutations, it is appropriate to ask whether the function of *faf* revealed by these alleles is one that occurs in wild-type cells or only in the presence of these particular proteins. Null alleles of *Rap1* and *Ras1* display weak genetic interactions with *faf* similar qualitatively to the strong interactions observed with the gain-of-function alleles. Thus, it appears that the second function of *faf* is biologically relevant but is much less important in the wild-type fly than in the presence of the specific mutant Rap1 and Ras1 proteins. Also, the interactions described are not specific to the faf^{BX3} and faf^{FO8} alleles; $faf^{BX4}/+$ (faf^{BX4} is a null allele) enhances the $Rap1^{R}/+$ mutant phenotype (data not shown).

The genetic interactions described are unlikely to result from direct physical interaction between Faf and Rap1 or Ras1 proteins. $Rap1^{V153M}$, $Rap1^{T58M}$, and $Ras1^{E63K}$ proteins are likely to function within photoreceptors, because $Rap1^{R}$ functions mainly in R7 and wild-type Ras1 also functions within R cells (12). In contrast, in its interactions with the mutant Rap1 and Ras1 proteins, Faf functions outside the facet. Mosaic data do not, however, exclude the possibility that the mutant Rap1 proteins and Ras1 also function outside the facet.

What Are the Activities of the $Rap1^{V153M}$, $Rap1^{T58M}$, and $Ras1^{E63K}$ Proteins? Rap1 is a GTP-binding protein structurally similar to Ras, but its cellular function is not known, making it difficult to speculate as to how particular amino acid changes might affect Rap1 function. There is evidence that the $Rap1^{R}$ mutation (F157L) increases the proportion of the Rap1 protein in the GTP-bound state (31), suggesting that $Rap1^{R}$ is hyperactive. However, genetic analysis suggests that this may be an oversimplification. As $Rap1^{V153M}$ and $Rap1^{T58M}$ in the fly have phenotypic effects similar to $Rap1^{R}$, their amino acid changes probably have the same final effect on Rap1 function, although not necessarily through the same mechanism. The $Rap1^{V153M}$ amino acid change is located near to the $Rap1^{R}$ mutation, whereas in $Rap1^{T58M}$, the altered amino acid is in the GTP-binding domain. Thus, it is possible that both $Rap1^{V153M}$ and $Rap1^{T53M}$ have altered GDP/GTP binding behavior, similar to, but less severe than, that of $Rap1^{R}$.

Activated (GTP-bound) Ras binds to an effector protein (11). The Ras^{E63K} mutation is located within a 16-amino acid region that may be involved in effector binding because it normally undergoes a conformational change upon Ras activation (32). It has recently been shown that Ras proteins can interact in different ways with different effector molecules (33). Specific mutations in Ras can abolish its ability to interact with subsets of effectors while preserving its ability to activate others. The mutant alleles of *Rap1* and *Ras1* described herein may thus represent alleles that preserve many of the normal functions of these proteins but alter the function of a pathway that is regulated in some manner by a *faf*-dependent signal. These alleles are likely to be useful reagents for the analysis of such a mode of regulation.

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1. Freeman, M. (1997) *Development (Cambridge, U.K.)* **124,** 261–270.
2. Zipursky, S. L. & Rubin, G. M. (1994) *Annu. Rev. Neurosci.* **17,** 373–397.
3. Heberlein, U. & Moses, K. (1995) *Cell* **81,** 987–990.
4. Wolff, T. & Ready, D. F. (1993) in *The Development of Drosophila melanogaster*, eds. Bate, M. & Matinez Arias, A. (Cold Spring Harbor Lab. Press, Plainview, NY), Vol. 2, pp. 1277–1326.
5. Freeman, M. (1996) *Cell* **87,** 651–660.
6. Tio, M. & Moses, K. (1997) *Development (Cambridge, U.K.)* **124,** 343–351.
7. Fischer-Vize, J. A., Rubin, G. M. & Lehmann, R. (1992) *Development (Cambridge, U.K.)* **116,** 985–1000.
8. Huang, Y. & Fischer-Vize, J. A. (1996) *Development (Cambridge, U.K.)* **122,** 3207–3216.
9. Huang, Y., Baker, R. T. & Fischer-Vize, J. A. (1995) *Science* **270,** 1828–1831.
10. Hochstrasser, M. (1995) *Curr. Opin. Cell Biol.* **7,** 215–223.
11. Bollag, G. & McCormick, F. (1991) *Annu. Rev. Cell. Biol.* **7,** 601–632.
12. Simon, M. A., Bowtell, D. L., Dodson, G. S., Laverty, T. R. & Rubin, G. M. (1991) *Cell* **67,** 701–716.
13. Diaz-Benjumea, F. J. & Hafen, E. (1994) *Development (Cambridge, U.K.)* **120,** 569–578.
14. Pizon, V., Chardin, P., Lerosey, I., Olofsson, B. & Tavitian, A. (1988) *Oncogene* **3,** 201–204.
15. Hariharan, I. K., Carthew, R. W. & Rubin, G. M. (1991) *Cell* **67,** 717–722.
16. Hay, B. A., Wolff, T. & Rubin, G. M. (1994) *Development (Cambridge, U.K.)* **120,** 2121–2129.
17. Kramer, S., West, S. R. & Hiromi, Y. (1995) *Development (Cambridge, U.K.)* **121,** 1361–1372.
18. Rebay, I. & Rubin, G. M. (1995) *Cell* **81,** 857–866.
19. Lindsley, D. L. & Zimm, G. G. (1992) *The Genome of Drosophila melanogaster* (Academic, San Diego).
20. Fischer, J. A., Leavell, S. K. & Li, Q. (1997) *Dev. Genet.* **21,** in press.
21. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56,** 779–827.
22. Willumsen, B. M., Papageorge, A. G., Kung, H. F., Bekesi, E., Robins, F., Johnsen, M., Vass, W. C. & Lowy, D. R. (1986) *Mol. Cell. Biol.* **6,** 2646–2650.
23. Sigal, I., Gibbs, J., D'alonzo, J. & Scolnick, E. (1986) *Proc. Natl. Acad. Sci. USA* **13,** 4725–4729.
24. Lev, Z., Kimchie, Z., Hessel, R. & Sogev, O. (1985) *Mol. Cell. Biol.* **5,** 1540–1542.
25. Bohmann, D., Ellis, M. C., Staszewski, L. M. & Mlodzik, M. (1994) *Cell* **78,** 973–986.
26. Xiong, W. C. & Montell, C. (1993) *Genes Dev.* **7,** 1085–1096.
27. Lai, Z. C., Harrison, S. D., Karim, F., Li, Y. & Rubin, G. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5025–5030.
28. Treier, M., Staszewski, L. M. & Bohmann, D. (1994) *Cell* **78,** 787–798.
29. Tang, A. H., Neufeld, T. P., Kwan, E. & Rubin, G. M. (1997) *Cell* **90,** 459–467.
30. Li, S., Li, Y., Carthew, R. & Lai, Z. C. (1997) *Cell* **90,** 469–478.
31. Quilliam, L. A., Zhong, S., Rabun, K. M., Carpenter, J. W., South, T. L., Der, C. J. & Campbell-Burk, S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1272–1276.
32. Milburn, M. V., Tong, L., deVos, A. M., Brunger, A., Yamaizumi, Z., Nishimura, S. & Kim, S.-H. (1990) *Science* **247,** 939–945.
33. Joneson, T., White, M. A., Wigler, M. H. & Bar-Sagi, D. (1996) *Science* **271,** 810–812.