Undifferentiated cells in the developing *Drosophila* eye influence facet assembly and require the Fat facets ubiquitin-specific protease

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SUMMARY

The *Drosophila* compound eye develops by a complex series of cell interactions where multiple positive and inhibitory cues guide cells in each facet into their positions and fates. The results of many genetic and molecular experiments have led to the view that facet assembly is directed by cells within developing ommatidial preclusters. Here fat facets mutants and the cloned fat facets gene were used to show that, in order to limit the number of photoreceptors in a facet to eight, undifferentiated cells surrounding assembling facets send an inhibitory signal to extraneous cells within the facet preclusters. Generation of the inhibitory signal requires the ubiquitin-specific protease encoded by the fat facets gene and is thus regulated by ubiquitin-dependent proteolysis.

Key words: eye, fat facets, *Drosophila*, ubiquitin, Ubp

INTRODUCTION

Cells in the developing *Drosophila* eye interpret a multitude of positive and negative signals from neighboring cells in order to learn their positions and fates. The compound eye is made up of approximately 800 identical unit eyes, called facets or ommatidia, each of which is composed of 8 photoreceptors (R1-R8) surrounded by a hexagonal lattice of pigment cells and capped by four cone cells (reviewed in Wolff and Ready, 1993). The first event in ommatidial assembly is the singling out of the initial photoreceptor, R8, from a field of competent cells (Jarman et al., 1994) by inhibitory cell interactions involving the Notch signaling pathway and the scabrous gene (Cagan and Ready, 1989; Baker et al., 1990; Mlodzik et al., 1990; Ellis et al., 1994; Parks et al., 1995; Baker and Zitron, 1995; Lee et al., 1996). The R8 cell is then thought to initiate a series of local cell inductions which recruit the appropriate number of photoreceptor cells into each facet (see Fig. 1). First, R8 is thought to signal the adjacent R2 and R5 precursor cells by secreting Spitz protein, a TGFα homolog (Rutledge et al., 1992) likely to be the ligand for the *Drosophila* EGF receptor (DER) which is present on the surfaces of all photoreceptor cell precursors (Tio et al., 1994; Freeman, 1994; Schweitzer et al., 1995a). The R2/5 pair probably secrete Spitz also, and thus contribute to the signaling of the adjacent R3/4 and then R1/6 precursors by R8 (Freeman, 1994; Tio et al., 1994). One of the cellular responses to DER activation is the production of Argos (Golembo et al., 1996), a secreted inhibitor of DER which prevents DER activation in cells other than the photoreceptor precursors adjacent to R8 and R2/5 (Schweitzer et al., 1995b). The best understood induction event is the recruitment of R7, the final photoreceptor cell to join the facet. The Sevenless receptor on the R7 cell surface is activated by its ligand, Bride-of-sevenless, expressed on the surface of R8 (reviewed in Dickson and Hafen, 1993). Thus, once R8 is specified, all positive and negative signals for photoreceptor recruitment appear to emanate from determined cells within the facet.

The fat facets (*faf*) gene product is necessary in order for the so-called ‘mystery cells’ (Tomlinson and Ready, 1987), which are transiently associated with the early 7-cell facet precluster (Fig. 1), to leave the preclusters; the mystery cells become ectopic R3/4-like photoreceptors in *faf* mutant eyes (Fischer-Vize et al., 1992a). Often, *faf* mutant facets also contain ectopic R7-like cells of undetermined origins (Fischer-Vize et al., 1992a; Huang et al., 1995). The *faf* gene encodes a ubiquitin-specific protease (Ubp) (Huang et al., 1995), one member of a large family of enzymes first characterized in yeast (Tobias and Varshavsky, 1991; Baker et al., 1992; Papa and Hochstrasser, 1993; Hochstrasser, 1995). Ubiquitin (Ub) is a highly conserved polypeptide found in all eukaryotes, the major function of which is to target proteins for complete or partial degradation by a multisubunit complex called the proteasome (reviewed in Ciechanover, 1994; Hochstrasser, 1995). Ubps, which cleave Ub-protein bonds, play a variety of roles in Ub-mediated protein degradation (Baker et al., 1992; Papa and Hochstrasser, 1993; Hochstrasser, 1995). Ubiquitin-like (Ub) is a highly conserved polypeptide found in all eukaryotes, the major function of which is to target proteins for complete or partial degradation by a multisubunit complex called the proteasome (reviewed in Ciechanover, 1994; Hochstrasser, 1995). Ubps, which cleave Ub-protein bonds, play a variety of roles in Ub-mediated protein degradation (Baker et al., 1992; Papa and Hochstrasser, 1993; Hochstrasser, 1995). Faf appears to be a regulatory Ubp that removes Ub from perhaps a specific ubiquitinated substrate, thereby preventing the degradation or processing by the proteasome of an unidentified protein (Huang et al., 1995).

The *faf* gene is expressed almost ubiquitously in the larval eye disc, in cells within and outside the facet (Fischer-Vize et al., 1992a). Analysis of facets mosaic for *faf*+ and *faf*− photoreceptor cells suggests that *faf* is not required by the mystery cells nor in any particular photoreceptor cells within the facet (Fischer-Vize et al., 1992a). However, the results of the mosaic analysis are also consistent with a role for *faf* in positively regulating the activity of an inhibitor of photoreceptor fate.
secreted by cells within the facet, like Argos. In addition, the ectopic R7-like cells in faf mutant facets could be cone cell precursors which normally require two different proteins to prevent them from becoming ectopic R7 cells: the DNA-binding protein Yan (Lai and Rubin, 1992; O’Neill et al., 1994; Treier et al., 1995) and Gap1, a negative regulator of Ras activation (Gaul et al., 1992; Rogge et al., 1992). The mosaic experiments do not rule out a role for faf in positively regulating Yan or Gap1 activities within cone cell precursors.

Using hybrid gene constructs to express faf in specific subsets of cells in the developing eye, here it is demonstrated that the faf gene is essential only early in eye development and only outside of the 7-cell precluster and cone cell precursors. Thus, the critical role of faf in eye development is not in the regulation of Argos, Yan or Gap1 activities, nor in the regulation of any other protein that functions within cells of the developing facets. Moreover, the results presented show that undifferentiated cells outside the facet precluster direct ommatidial assembly by sending to the mystery cells and other cells an inhibitory signal, regulated by Faf protein.

MATERIALS AND METHODS

Drosophila strains

All flies were kept on standard food at 25°C unless otherwise noted. Mutant faf alleles are described in Fischer-Vize et al. (1992a).

Gal4 insertions and UAS reporters

All Gal4 insertions and reporters are P[w+]. The Gal4 insertion in elav (Rebay and Rubin, 1995) was obtained from T. Laverty. Two different Gal4 insertions in scabrous (sca) were used; T3 (Kramer et al., 1995) was obtained from Y. Hiromi and scol (M. Mlodzik, unpublished) from M. Mlodzik. Two independent Gal4 insertions in the hairy (h) locus were used; I1J3 (Brand and Perrimon, 1993) was obtained from N. Perrimon and H10 (Y. Hiromi, unpublished) from Y. Hiromi. The UAS-lacZ reporter strain used (Brand and Perrimon, 1993) is a second chromosome insertion obtained from A. Brand that expresses cytoplasmic β-galactosidase and the UAS-nuclear β-galactosidase was a gift of Y. Hiromi.

Suppression of faf by h-Gal4

Surprisingly, both Gal4 insertions in the h locus (I1J3 and H10) behave as partial suppressors of faf in the absence of UAS-faf. Both insertions are known to drive expression of reporter constructs in a wide stripe of cells ahead of the morphogenetic furrow (Ellis et al., 1994; Y. Hiromi, personal communication) and the endogenous Hairy protein is expressed normally in a faf mutant background (see below). Although both I1J3 and H10 cause weak h mutations (Brand and Perrimon, 1993; Y. Hiromi, personal communication), the suppression effect is unlikely to be due to h mutation: three different h mutations, one weak (h1); and two strong (h3 and h3); all obtained from the Bloomington Stock Center), were tested for genetic interactions with faf and none behave as suppressors. Thus the suppression appears to be due to the presence of Gal4 protein in the cells ahead of the furrow. As the h expression pattern ahead of the furrow is the region of strongest endogenous faf expression (Fischer-Vize et al., 1992a) and the experiments described in this paper show that this is where endogenous faf expression is most likely to be critical, it would be expected that faf expression in the h pattern would rescue the faf phenotype, at least partially. However, it was difficult to determine whether or not additional suppression of faf by h-Gal4 occurred in the presence of UAS-faf insertions because the suppression of the faf mutant phenotype by h-Gal4 alone was very strong. Also, it is possible that the presence of Gal4 in these cells somehow changes the cellular physiology such that the addition of Faf protein no longer has any effect. Suppression was not observed with any of the other Gal4 insertion lines used, none of which, in combination with UAS-faf, complement faf mutations even partially. Thus, the observation that h-Gal4 suppresses faf may indicate that the h promoter is indeed expressing Gal4 in the cells in which Faf functions.

Promoter-lacZ reporters

The following strains were used to construct promoter-lacZ reporter genes where the promoters are essentially identical to those in the promotor-faf constructs: ro-lacZ (Heberlein et al., 1994; obtained from U. Heberlein), sev-lacZ (P+[w+], ES3-lacZ);1: R. Carthew, personal communication; obtained from T. Laverty), gglr-lacZ (Brand and Perrimon, 1993) was difficult to determine whether or not additional suppression of faf BX4 insertion that expresses nuclear β-galactosidase and the reporter strain used (Brand and Perrimon, 1993) was obtained from the Bloomington Stock Center), were tested for genetic interactions with faf and none behave as suppressors. Thus the suppression appears to be due to the presence of Gal4 protein in the cells ahead of the furrow. As the h expression pattern ahead of the furrow is the region of strongest endogenous faf expression (Fischer-Vize et al., 1992a) and the experiments described in this paper show that this is where endogenous faf expression is most likely to be critical, it would be expected that faf expression in the h pattern would rescue the faf phenotype, at least partially. However, it was difficult to determine whether or not additional suppression of faf BX4 occurred in the presence of UAS-faf insertions because the suppression of the faf mutant phenotype by h-Gal4 alone was very strong. Also, it is possible that the presence of Gal4 in these cells somehow changes the cellular physiology such that the addition of Faf protein no longer has any effect. Suppression was not observed with any of the other Gal4 insertion lines used, none of which, in combination with UAS-faf, complement faf mutations even partially. Thus, the observation that h-Gal4 suppresses faf may indicate that the h promoter is indeed expressing Gal4 in the cells in which Faf functions.

Mutations tested for modification of faf

The following mutant alleles were tested for dominant enhancement or suppression of the faf mutant phenotype as described in the text. The individuals from whom the flies were obtained or references describing the alleles are indicated in parentheses. Notch pathway and related genes: N5419, Su(H)T4, S(T)115, dx1, manu1115, bis1, vgBG, vgBG (M. Fortini), E(spl)B22 (A. Preiss), zw1M111 (N. Perrimon), sno13 (A. Majumdar), pax1, fts1 [anx (A. Mahowald), sca1A [N. Baker), groBPP2 (Fischer-Vize et al., 1992b), Ras pathway (M. Simon, M. Freeman, T. Laverty): ras11B, drk204, Sos46, Dsor1221, VGl115, raf115, Gap11B, DER signaling (M. Freeman): spi25, spi14.
Plasmid constructions

Vectors designed for expressing any gene in the sevenless (sev) or rough (ro) patterns, under UAS control, or under the control of either ‘long’ or ‘short’ glass (gl) responsive elements were constructed with a unique Asc I site for DNA insertion as described below. The Asc I fragment containing a Myc-tagged faf cDNA and the heat-shock protein 70 (hsp70) expression vector are described in Huang et al. (1995). DNA manipulations were performed using standard procedures (Sambrook et al., 1989).

The short gl-binding site vector (pGLRS-faf) was constructed by changing the KpnI site in Bluescript (Stratagene) to NotI and the BamHI site to AscI to generate pBSKNA. A 150 bp Xbal fragment of pmm27.1 (Riddihough and Ish-Horowicz, 1991) containing the TATA box and transcription start of the hsp27.1 (Riddihough and Ish-Horowicz, 1991) gene (Huang et al., 1995) was cloned into the XbaI site of pBSShsp27 to generate pEGhsp27/BSKNA. An Asc I fragment containing the hsp70 cDNA was cloned into the Asc I site of pBSGsp27/BSKNA. Finally, a NotI fragment containing the short glass-binding site-hs-faf gene was cloned into the NotI site of pCasper3pA, a derivative of Casp3 (Thummel and Pirrotta, 1992; obtained from V. Pirrotta) with an 850 bp SV40 fragment into the NotI site of pSP/HSS (Bowtell et al., 1989; obtained from M. Ellis) containing the long Asc I site of pCasper3pA. The Asc I fragment containing the long glass-binding site hs-faf gene was cloned into the NotI site of pCasper3pA, a derivative of Casp3 (Thummel and Pirrotta, 1992; obtained from V. Pirrotta) with an 850 bp Xbal-EcoRI fragment from pC4Bgal (Thummel et al., 1998) containing SV40 transcription termination sequences cloned into the HpaI site.

The long gl-binding site vector (pGLRL-faf) was constructed by cloning a 250 bp Xbal fragment of pwnpE/KM1/2 (Ellis et al., 1993; obtained from M. Ellis) containing five long gl-binding sites into the Xbal site of pBSGsp27 to generate pEGhsp27/BSKNA. The faf cDNA within an Asc I fragment was then cloned into the Asc I site of pGLRL-faf. The Asc I fragment containing the long glass-binding site hs-faf gene was cloned into the NotI site of pCasper3pA.

The sev vector (pSEV-faf) was constructed by changing the Xbal site of pSP/HSS (Bowtell et al., 1989; obtained from M. Ellis) containing the sev promoter, to AscI to generate pSP/HSS-XA, inserting the 850 bp SV40 fragment into the BamHI site and the faf cDNA within an AscI fragment into the AscI site. The sev-faf gene was then cloned as a NotI fragment into the NotI site of the transformation vector pE5/WS (obtained from M. Ellis), which contains three sev transcriptional enhancers.

The UAS vector (pUAST-XA) was constructed by changing the Xhol site of pUAST (Brand and Perrimon, 1993; obtained from A. Brand) to AscI.

The ro vector (pRO-faf) was constructed by cloning a 2.7 kb NotI fragment of pTHZ50/S’D#24 (Heberlein et al., 1994; obtained from U. Heberlein) containing the ro gene enhancer upstream of a NotI fragment from pshspcDNA, which contains the hs-faf gene (Huang et al., 1995), into the NotI site of Casper3.

P element transformation

Plasmids used for injections were purified on CsCl-EDBr equilibrium density gradients (Sambrook et al., 1989). Injection embryos were co-injected with P element plasmids and helper plasmid according to standard procedures (Spradling, 1986). P element insertions were mapped to a chromosome and stocks established using standard techniques. All transformant lines analyzed were obtained directly from injected embryos except for 15 of the 17 ro-faf lines and 6 of the 7 sev-faf lines which were obtained by mobilization of original P insertions using standard techniques. P element transformants were crossed into a faf^{Bx} background using standard techniques.

Analysis of adult eyes

Scanning electron microscopy (Huang et al., 1995) and fixation, embedding and sectioning of eyes for the light microscope (Tomlinson and Ready, 1987; Fischer-Vize et al., 1992a) were performed as described previously. Eye sections were photographed using a Zeiss Axioscan microscope.

Staining eye discs for β-galactosidase activity

In order to approximate the relative levels of expression of the various promoters, larval eye discs of ro-lacZ, sev-lacZ, glrs-lacZ, glrl-lacZ and sca-Gal4; UAS-lacZ discs were stained histochemically for β-galactosidase activity as described previously (Fischer-Vize et al., 1992a). Staining developed nearly immediately in the glrs-lacZ and sca-Gal4; UAS-lacZ discs. Staining began to develop in discs from all of the other lines after a few minutes.

Staining eye discs with antibodies

Staining with mAbRough (a gift of U. Heberlein) and mAbHairy (a gift of N. Brown) was performed as described in Fischer-Vize et al. (1992a) for mAbRough. mAbGal8 (Promega) and mAbSevenless (42G11 or 36D7; a gift of M. Simon) were used as described in Fischer-Vize et al. (1992a) for mAbGal8, except that the secondary antibody used was HRP-conjugated horse-anti-mouse (Vector) and for staining glrs-lacZ and sev-lacZ discs, Co and Ni were omitted from the developing solution. Instead, after developing, discs were treated with 2% Oso4 in 0.1 M sodium phosphate for 5 minutes on ice, rinsed with 0.1 M sodium phosphate and then dehydrated with ethanol (30/periodial membrane removed/50/100/100). Antibody-stained discs were photographed with a Zeiss Axioscan microscope.

Heat-shock experiments

In order to test for a dominant effect of hs-faf, otherwise wild-type larvae containing two copies of the hs-faf gene were subjected to a wide variety of heat-shock regimens. Embryos were allowed to develop for 4 days (to the early third instar larval stage) and then they were subjected to 30 minute-2 hour heat shocks at 37°C followed by 30 minute-4 hour recovery times 2-5 times daily for 2-5 days.

In order to test how early a heat shock could result in rescue of the faf mutant rough eye phenotype, hs-faf : faf^{Bx}/TM6B flies were transferred to new food vials every 24 hours for 7 days. Eight vials containing animals at different stages of development were heat shocked simultaneously by immersion in a 37°C water bath for 1 hour and then allowed to continue development at 25°C. Thus, flies were heat shocked as embryos (0-24 hours after egg laying (hours AEL)), first instar larvae (24-48 hours AEL), second instar larvae (48-72 hours AEL), early-mid third instar larvae (72-96 hours AEL), mid-late third instar larvae (96-120 hours AEL) and as pupae. No rescue of the faf^{Bx} eye phenotype was ever observed in hs-faf; faf^{Bx} adults that had been heat shocked as embryos or first instar larvae (0-48 hours AEL). Approximately 1/5 of the flies heat shocked at 48-72 hours AEL had ~6-12 rows of wild-type facets in the posterior of their eyes. All flies heat shocked at 72-96 hours AEL showed some rescue and some flies had nearly completely wild-type eyes, except for ~6 rows at the anterior of the eye. Similar results were obtained with flies heat shocked at 96-120 hours AEL, except some eyes with a few rows of anterior or posterior roughness were observed. Pupal heat shocks produced no rescue.

The heat-shock experiments argue that Faf protein is stable for less than 56 hours. There are approximately 32 rows of facets in the eye disc and as each row requires ~2 hours to form, it takes ~64 hours for the furrow to move through all the rows. The furrow starts to move at about 12 hours into L3, or about 84 hours AEL (Tio et al., 1995). Thus, to rescue all but a few rows of the eye, or about 28 rows, Faf needs to be stable for 56 hours. If Faf is stable for 56 hours, a heat shock at 36 hours before the furrow starts to move (48 hours AEL), should result in the rescue of ~10 rows of facets. No rescue was ever seen in flies heat shocked at 48-48 hours AEL. Moreover, only 1/5 of the flies heat shocked at the next time point (48-72 hours AEL)
showed any rescue of the rough eye and the rescue was about 5 rows. Presumably the flies with partially rescued eyes were closer to 72 hours old at the time of heat shock.

**Protein analysis**

Procedures for heat shocking embryos, generating and quantitating embryo protein extracts and Western blotting and developing were exactly as described in Huang et al. (1995). The Coomassie-stained gel was generated using standard procedures (Sambrook et al, 1989).

**RESULTS**

**faf function is essential only outside the facet precluster**

In order to determine which cells in the developing eye require faf expression, hybrid genes that express faf in different groups of cells were generated and tested for their ability to complement faf mutations. Expression of the faf cDNA in different patterns in the eye disc was achieved either by construction of promoter-faf gene fusions or by controlling the expression of a UAS-faf construct with different Gal4 enhancer trap lines (Table 1; Fig. 2). Many P element transformant lines containing each construct were generated and crossed into a faf null mutant (faf^{BX4}) background to test the ability of the construct to rescue the faf mutant eye phenotype to wild-type. It was observed that the three constructs resulting in at least partial rescue of the faf mutant phenotype have two common characteristics, not shared by the non-rescuing constructs: they express faf early in eye development and outside the cells within the developing facets (Table 1; Figs 2, 3).

Four constructs, ro-faf, glrs-faf, sev-faf and sca-Gal4; UAS-faf, are key to the conclusion that faf is required early and outside the cells within the facet. Both constructs that can substitute at least partially for the endogenous faf gene, ro-faf and glrs-faf, are expressed ubiquitously early in eye development before the mystery cells normally leave the preclusters. In contrast, sev-faf and sca-Gal4; UAS-faf, both of which fail to substitute for the endogenous faf gene even partially, also express faf early in eye development but their expression is limited to cells within the facet.

The failure of sev-faf and sca-Gal4; UAS-faf to complement faf mutations is unlikely to be due to low levels of faf expression.

![Diagram](https://example.com/diagram.png)

**Table 1. Complementation of faf^{BX4} by promoter-faf expression constructs in P element transformants**

<table>
<thead>
<tr>
<th>Promoter-faf construct</th>
<th>Expression pattern</th>
<th>Number of transformant lines that rescue faf^{BX4} eye phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-faf</td>
<td>Ubiquitous^1</td>
<td>Partially 4, Nearly completely 14</td>
</tr>
<tr>
<td>ro-faf</td>
<td>In furrow and R2/5, R3/4^2</td>
<td>Partially 3, Nearly completely 14</td>
</tr>
<tr>
<td>glrs-faf</td>
<td>Posterior edge of furrow and all cells posterior to furrow^3</td>
<td>Partially 1, Nearly completely 6</td>
</tr>
<tr>
<td>girl1-faf</td>
<td>All R-cells posterior to furrow^4</td>
<td>Partially 13</td>
</tr>
<tr>
<td>sev-faf</td>
<td>M1, M2, R3/4, R1/6, R7, CC^5</td>
<td>Partially 13</td>
</tr>
<tr>
<td>sca-Gal4; UAS-faf</td>
<td>Precluster cells at posterior edge of furrow and R8 posterior to furrow^6</td>
<td>Partially 7</td>
</tr>
<tr>
<td>elav-Gal4; UAS-faf</td>
<td>All R-cells posterior to furrow^7</td>
<td>Partially 7</td>
</tr>
<tr>
<td>h-Gal; UAS-faf</td>
<td>Stripe of cells anterior to furrow^8</td>
<td>Partially *</td>
</tr>
</tbody>
</table>

All transformant lines were tested with two copies of the P element except that there was only one copy each of the UAS-faf inserts. The structures of the promoter constructs are described in Fig. 2. Representative external eyes and retinas are shown in Figs 3 and 5.

1Pelham, 1982; Lis et al., 1983.
2Kimmel et al., 1990; Heberlein et al., 1994.
3Moses and Rubin, 1991; Ellis et al., 1993.
4Tomlinson et al., 1987; Bowtell et al., 1989.
5Baker et al., 1990; Mlodzik et al., 1990; Two independent sca-Gal4 lines were tested (see Materials and Methods and Fig. 4).
6Bier et al., 1988; Rebay and Rubin, 1995.
7Brown et al., 1991; Ellis et al., 1994; see Materials and Methods.
8*see Materials and Methods.
The role of faf in the fly eye

expression. The eye discs of flies transformed with promoter-
lacZ hybrid genes, in which the promoters are essentially
identical to those driving faf expression, were histochemically
stained for β-galactosidase activity (Materials and Methods).
These experiments revealed that the sca-Gal4; UAS and the
glrs promoter constructs are the strongest, and that the ro and
sev promoter constructs are of similar strength (Materials and
Methods).

In addition, the promoters used are regulated normally in faf
mutant larval eye discs, except for the inevitable differences
due to the developmental defects in faf mutant discs (Fig. 4).

The precise timing of faf expression is not critical

The endogenous faf gene is expressed ahead of and posterior
to the furrow, but not within the furrow (Fischer-Vize et al.,
1992a). Although ro-faf is expressed in a nearly complementary pattern, it can substitute functionally for the endogenous
faf gene (Table 1; Fig. 3). Thus, the precise timing of faf
expression is not crucial to its function.

The essential functions of faf are not temporally
separable

There are several aspects to the faf mutant facet phenotype
(Fischer-Vize et al., 1992a; Huang et al., 1995); most facets
contain one or more extra outer photoreceptor cells, however,
facets often also contain extra Sevenless-independent (J. A. F.-
V., unpublished data) R7-like cells or are missing R7 cells. In
order to determine whether the partial rescue observed with
some ro-faf and all glrs-faf transformant lines was due to
rescue of only one or two of the three mutant facet defects, the
retinas of partially rescued ro-faf; faf BX4 and glrs-faf; faf BX4
eyes were examined. In all cases, partially rescued eyes
contained facets with each of the three types of defects (Fig.
3L and data not shown). Thus, faf does not have critical
functions separable temporally or spatially in these experi-
ments; early expression of faf is sufficient to circumvent the
three kinds of defects in faf mutant eyes.

A short pulse of faf expression is nearly sufficient
for normal eye development

The experiments described above suggest that faf activity
might have a lasting effect because expression of faf in cells
within the furrow (ro-faf) is manifested later by the exclusion
of the mystery cells from the precluster. Flies containing hs-
faq genes in a faf BX4 mutant background were used to investi-
gate how early in eye disc development cells could respond to
Faf activity.

Developing hs-faf; faf BX4 embryos, larvae and pupae were
given a single heat shock at 24 hour intervals after egg laying

Fig. 3. Complementation of faf BX4 by promoter-faf genes. Scanning
electron micrographs of the external eyes (A,E,G,I,K,M) and
tangential sections (B,C,D,F,H,J,L,N) of eyes representative of the
genotypes indicated below are shown. (A-C) wild-type; (D-F)
faq BX4; (G,H) sca-Gal4/ UAS-faf; faf BX4; (I,J) sev-faf; faf BX4; (K,L)
glrs-faf; faf BX4; (M,N) ro-faf; faf BX4. Anterior is to the left in all of
the scanning electron micrographs. C and D are enlargements of
individual facets in B and F, respectively. Numbers in C indicate
photoreceptor cells R1–R7. The bar in N represents ~20 µm in B, F,
H, J, L and M, ~10 µm in C and D and ~140 µm in A, E, G, I, K
and M.
(AEL), allowed to develop into adults and their external eyes examined (for details, see Materials and Methods). The morphogenetic furrow begins to move during the early third instar. Flies heat shocked as embryos or first instar larvae were indistinguishable from non-heat-shocked hs-faf; fafBX4 or fafBX4 flies (Fig. 5A and data not shown). In contrast, flies heat shocked as third instar larvae (72-96 hours AEL) often had nearly completely wild-type eyes, with just a little roughness (~6 rows) at the anterior of the eye, the final facets to complete assembly (Fig. 5C). Later heat shocks resulted in eyes with some roughness at the posterior (Fig. 5B). We interpret these latter eyes as having expressed faf after the furrow had already moved and thus they contain a few rows of facets where the facet preclusters developed without benefit of faf activity.

How can one pulse of faf expression be sufficient for a nearly completely normal eye? These results could be explained in either of two ways: either Faf protein is extremely stable or some or all cells in a third instar larval eye disc are competent to respond stably to Faf activity. The latter interpretation is strongly favored because there are three arguments against Faf protein stability. First, the Faf protein sequence contains several putative PEST sequences (Fischer-Vize et al., 1992a), which are characteristic of short-lived proteins (Rogers et al., 1986). Second, a Faf/LacZ fusion protein containing the first 402 amino acids of Faf protein is unstable as it disappears abruptly in the morphogenetic furrow (Fischer-Vize et al., 1992a). Finally, the results of the heat-shock experiments argue against Faf protein being extremely stable. In order for Faf protein stability to account for rescue of nearly the entire eye with one heat shock, Faf protein would have to be stable for 56 hours (see Materials and Methods). If so, then flies heat shocked at 48 hours AEL should have had ~10 rows of wild-type facets (see Materials and Methods) and rescue was never observed in these flies.

In order to investigate the stability of Faf protein, protein extracts from hs-faf transformant embryos harvested at different times after heat shock were assayed for Faf protein. The Faf protein expressed from the hs-faf gene is tagged with the ‘Myc epitope’ (Materials and
Methods) and thus Faf protein could be detected using antibodies to the Myc epitope in Western blotting experiments. As shown in Fig. 6, large amounts of Faf protein are detected 4 hours after heat shock, the level of Faf decreases dramatically by 8 hours and no Faf protein is detected by 12 hours after heat shock.

Thus, Faf stability cannot account for the ability of one heat shock to rescue nearly the whole faf mutant eye to wild-type. Rather, it appears that undifferentiated cells in the third instar larval eye disc are competent to respond to Faf activity and their response is lasting. Either all cells anterior to the 7-cell precluster stage are competent to respond to Faf, or some cells respond and initiate a self-perpetuating process involving continual communication with more anterior cells as the furrow progresses.

Ectopic faf expression is inconsequential

The ro-faf, glrs-faf and hs-faf constructs were designed to test for an effect of ectopic faf expression in the morphogenetic furrow. Ectopic expression has no detrimental effect on eye development as otherwise wild-type flies transformed with any of the three constructs show no observable eye defects (data not shown). In fact, furrow expression alone can substitute nearly completely for the normal pattern of faf expression (see above).

The faf gene is normally expressed in some tissues other than the eye, but its expression is not ubiquitous (Fischer-Vize et al., 1992a). The hs-faf gene and UAS-faf gene in combination with Gal4 insertions in the scu, elav and h genes all express faf ectopically outside the eye with no obvious effects in a wild-type background.

Interactions between faf and other genes

In order to determine whether faf regulates any known pathway affecting photoreceptor determination in the developing eye, mutations in genes involved in cell cycle regulation and the Notch, Ras and DER signaling pathways, in addition to other genes, were tested for enhancement and suppression of the faf mutant eye phenotype (see Materials and Methods). Loss-of-function mutations in many genes were tested for their ability to dominantly enhance the weak rough eye phenotype of fafBF3 flies; fafBF3 is a very strong mutant allele and fafBF3 is a very weak allele (Fischer-Vize et al., 1992a). The fafBF3/fafBF3 background is known to be highly sensitive to 50% reduction in the levels of other as yet unidentified gene products, as this background was used successfully in a genetic screen for enhancers of faf (J.A.F.-V. and S. Leavell, unpublished data). Suppression was assayed in a fafBF3 homozygous background, which has been shown to be sensitive to suppression by reducing the level of a proteasome component by half (Huang et al., 1995). None of the mutations tested showed significant enhancement or suppression of the faf mutant eye phenotype.

DISCUSSION

It has been demonstrated that the deubiquitinating enzyme encoded by the faf gene is required in a signaling pathway emanating from outside the facet that inhibits inappropriate neural determination of the mystery cells and other cells. The precise timing of faf expression is not critical and ectopic faf expression is not detrimental to facet assembly. Finally, one pulse of faf expression is sufficient for normal eye development, suggesting that some or all cells in early third instar
larval eye discs are competent to respond to faf activity and that the response is lasting.

**In which cells does Faf function?**

The finding that faf works in cells outside the facet preclusters is unusual; the only other gene known to work in these cells is groucho (Fischer-Vize et al., 1992b; see below). The experiments presented here reveal in which cells faf is not absolutely required and offer strong support for one interpretation of a previous analysis of facets mosaic for faf+ and faf− photoreceptors: that is, faf is required in cells other than those within the 7-cell preclusters that emerge from the morphogenetic furrow. The failure of faf expressed in the sev or sca patterns to substitute even partially for the endogenous faf gene demonstrates that faf function is not critical in the mystery cells nor within the other precluster cells that the mystery cells contact (R8 and R3/4).

The experiments presented here do not, however, identify precisely the cells in which faf is essential. Analysis of mosaic facets suggests that to inhibit their misdetermination as photoreceptors, faf works in cells near to the mystery cells (Fischer-Vize et al., 1992a). These cells could be either in the undifferentiated cell pool surrounding the preclusters or cells within the ‘rosette’ structures that emerge from the morphogenetic furrow. One attractive model is that the ‘core cells’ within the rosette (shaded in Fig. 1) send inhibitory signals to the mystery cells, preventing them from determination as neurons. However, the action of Faf may precede the appearance of the core cells behind the furrow, as ro-faf expressed in the furrow is sufficient for normal eye development.

**In what cell communication pathway does Faf function?**

There are several known genes with mutant phenotypes similar to faf in the retina: argos (Freeman et al., 1992; Kretschmar et al., 1992; Okano et al., 1992), yan (Lai and Rubin, 1992; Tei et al., 1992), Gap1 (Gaul et al., 1992; Rogge et al., 1992), Notch (Cagan and Ready, 1989), groucho (Fischer-Vize et al., 1992b), Delta (Park et al., 1995) and strawberry-notch (Coyle-Thompson and Banerjee, 1993). However, the faf gene does not appear to be essential in a common pathway with any of these genes.

The faf gene cannot be critical to the neural inhibition pathway involving Argos because Argos protein is secreted by photoreceptor cells within the facet as a consequence of DER activation (Schweitzer et al., 1995a,b; Golembo et al., 1996); a sev-argos gene can substitute for endogenous argos (Freeman, 1995) while sev-faf is completely ineffective as a replacement for the endogenous faf gene. Similarly, Faf cannot be essential for the regulation of any gene product that functions within the precluster, such as Yan and Gap1. Unlike Faf, both Yan and Gap1 function cell autonomously to repress the development of the mystery and cone cells as R7s (Lai and Rubin, 1992; Gaul et al., 1992; Rogge et al., 1992). sev-faf expresses faf in the mystery cells and cone cells with no effect on the ectopic R7 phenotype of faf mutants. In addition, no significant genetic interactions were detected between faf and yan or Gap1 mutations.

The Notch, Delta, groucho and sno genes are all involved in the Notch signaling pathway and groucho is known to function in cells outside the facet. The failure to detect genetic interactions between faf mutations and mutations in Notch pathway genes suggests that faf is not likely to be a critical regulator of the Notch pathway.

One plausible hypothesis is that faf facilitates the function of a short-range inhibitor of DER, similar to Argos, that emanates from the core cells or other cells outside the 7-cell precluster (see Fig. 1). However, the failure to detect strong genetic interactions between faf and DER pathway genes

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**Fig. 5.** Complementation of faf$^{B4}$ by hs-faf. Scanning electron micrographs of the external eyes of hs-faf; faf$^{B4}$ flies are shown. Anterior is to the left in all panels. Flies contained two copies of the hs-faf gene. (A) No heat shock applied. (B) Flies were heat shocked at 96-120 hours AEL. Note that the eye surface is rough at the very posterior. (C) Flies were heat shocked at 72-96 hours AEL. Note that the eye surface is rough at the very anterior. The bar in C represents ~100 μm in all panels.

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**Fig. 6.** Faf protein stability. (A) Immunoblot of proteins present 4, 8 and 12 hours after heat shock in hs-faf transformant embryos. A protein corresponding to the approximate size of Faf (~300×10$^3$ M$\text{r}$) is detected with anti-Myc epitope antibodies at 4 and 8 hours after heat shock, but not after 12 hours, nor in control (C) embryos that were not heat shocked. Each lane contains 150 μg of total protein. (B) A Coomassie-stained protein gel of the same extracts used in A showing that none of the extracts is degraded. Each lane contains 40 μg of total protein.
suggests that scabrous may regulate an unknown inhibitory pathway for neural determination in the eye.

We are extremely grateful to everyone mentioned in the Materials and Methods section for plasmids, flies and antibodies. We are particularly grateful to Mike Ellis, Marek Mlodzik, Yash Hiromi and Richard Carthew for very generously providing materials and information prior to publication, and to Ulrike Heberlein for fig. 4L.K. Thanks to John Mendenhall at the University of Texas at Austin Cell Research Institute for the scanning electron micrographs, John Loera for fly culture media, Janet Young and Gwen Gage for much help in preparing the figures, Kathleen Mosley and Angelica Cadavid for help with the genetic interaction experiments, Kevin Moses for reading the manuscript and Marek Mlodzik, Nick Baker, Yash Hiromi and everyone in our laboratory for helpful discussions. We also thank one anonymous reviewer whose suggestions improved this paper significantly. This work was supported by a grant to J. A. F.-V. from the NIH (R29HD30880).

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