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The deubiquitination enzyme Fat facets negatively regulates RTK/Ras/MAPK signalling during *Drosophila* eye development

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Abstract

The *Drosophila fat facets* (*faf*) gene encodes a deubiquitination enzyme with a putative function in proteasomal protein degradation. Mutants lacking zygotic *faf* function develop to adulthood, but have rough eyes caused by the presence of one to two ectopic outer photoreceptors per ommatidium. Here we show that *faf* interacts genetically with the receptor tyrosine kinase (RTK)/Ras pathway, which induces photoreceptor differentiation in the developing eye. The results indicate that RTK/Ras signalling is increased in *faf* mutants, causing normally non-neuronal cells to adopt photoreceptor fate. Consistently, the protein level of at least one component of the Ras signal transduction pathway, the transcription factor D-Jun, is elevated in *faf* eye discs at the time when the ectopic photoreceptors are induced. We propose that defective ubiquitin-dependent proteolysis leads to increased and prolonged D-Jun expression, which together with other factors contributes to the induction of ectopic photoreceptors in *faf* mutants. These studies demonstrate the relevance of ubiquitin-dependent protein degradation in the regulation of RTK/Ras signal transduction in an intact organism. © 1997 Elsevier Science Ireland Ltd.

Keywords: fat facets; RTK signal transduction; Eye development; Ubiquitin-dependent proteolysis

1. Introduction

Intracellular signal transduction pathways convey information from the cell surface to the nucleus and enable the cell to respond to its environment by changes in gene expression. Among the best understood examples are pathways that are triggered when receptor tyrosine kinases (RTK) bind specific extracellular ligands (Perrimon, 1994). The resulting increase in RTK activity can initiate signalling through downstream components, such as small GTPases and mitogen activated protein kinase (MAPK) cascades. Activated MAP kinases may then phosphorylate pre-existing transcription factors, which in turn causes activation or repression of target genes in a signal-regulated manner (reviewed by Treisman, 1996).

RTK-triggered signal transduction can be positively and negatively regulated at different steps. The activity of components of the pathway is commonly modulated by phosphorylation/dephosphorylation (reviewed by Hunter, 1995). In addition, several other mechanisms for negative regulation exist; ligand-bound receptors can be inactivated by internalization and degradation (Sibley et al., 1987). Small GTPases such as Ras are negatively regulated by GTPase activating proteins (GAPs) (reviewed by McCormick, 1992). The prevalence of such negative regulation illustrates the importance of mechanisms that limit the strength and duration of RTK signalling.

A well-established experimental system to study the regulation of RTK/Ras/MAPK signalling in vivo is photoreceptor determination in the developing *Drosophila* eye (reviewed by Zipursky and Rubin, 1994). In the larval eye imaginal disc photoreceptor precursors are recruited to ommatidial preclusters and induced to differentiate by cell–cell interactions mediated by an RTK/Ras/MAPK pathway. Two RTKs have been implicated in this process. The *Drosophila* EGF receptor (DER) directs the formation of all photoreceptors (Xu and Rubin, 1993; Freeman, 1996), whereas the Sevenless receptor (Sev) has a more specialized function and is only required for induction of the R7 cell, a photoreceptor neuron sensitive to light in the UV range. The two receptors act via the same downstream factors (Brunner et al., 1994a; Diaz-Benjumea and Hafen, 1994), which

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include Ras1, Raf, the MAPK Rolled and transcription factors such as Pointed (Brunner et al., 1994b; O'Neill et al., 1994), Yan (Lai and Rubin, 1992) and *Drosophila* Jun (D-Jun) (Bohmann et al., 1994). DER and Sev can even functionally substitute for each other (Freeman, 1996). Thus, it has been suggested that for induction of R7 cell differentiation both RTKs must act by sequentially stimulating the same pathway (Freeman, 1996).

Gain-of-function alleles of *sev*, *Ras1*, *D-raf* and other Ras pathway components can cause the differentiation of supernumerary photoreceptors (Basler et al., 1991; Dickson et al., 1992; Fortini et al., 1992). A similar ectopic photoreceptor-phenotype was observed in animals carrying mutations in the *fat facets* (*faf*) gene (Fischer-Vize et al., 1992). As opposed to the other genes mentioned above, however, *faf* does not encode a classical signalling component, but a deubiquitination enzyme that can cleave the covalent bond between ubiquitin and an attached protein (Huang et al., 1995). Deubiquitination is involved in ubiquitin-dependent protein degradation, which is increasingly recognized as an important mechanism for regulation of cellular functions. A number of key regulatory decisions depend upon ubiquitin-dependent proteolysis; degradation of cyclins and mitotic inhibitors regulate the timing of the cell cycle (reviewed by Peter and Herskowitz, 1994; Murray, 1995) and proteolysis of p53 and IκB-α control cell differentiation and gene transcription (reviewed by Isaksson et al., 1996; Pahl and Baeuerle, 1996).

Ubiquitin is a small peptide of 76 amino acids which can be covalently bound to an internal lysine of a target protein in the form of one or more multimeric chains, a process that is mediated by a complex and highly selective enzymatic machinery (reviewed by Hochstrasser, 1995). Such multi-ubiquitination marks the substrate protein for degradation by the 26S proteasome (reviewed by Peters, 1994; Rubin and Finley, 1995).

The role of deubiquitination enzymes in this process has been best characterized in *Saccharomyces cerevisiae* where 15 genes have been identified (reviewed by Hochstrasser, 1995). The deubiquitination enzymes can broadly be divided into two groups, one promoting and the other inhibiting ubiquitin-dependent proteolysis. Sequence alignments indicate that Faf may be functionally homologous to Doa4, which promotes efficient ubiquitin-dependent degradation (Papa and Hochstrasser, 1993). Doa4 is associated with the 26S proteasome and appears to be required for removing the ubiquitin tail from substrate proteins (Hochstrasser, 1995). Lack of *Doa4* function generally impairs ubiquitin-dependent degradation, since both natural and artificial substrates are degraded less efficiently. The resulting accumulation of several endogenous proteins in *Doa4* mutants is thought to be the cause of the pleiotropic phenotype, characterized by slow growth, radiation sensitivity and defects in initiation of DNA replication (Singer et al., 1996).

The homology between DOA4 and Faf suggests that the *faf* phenotype might also be caused by stabilization and accumulation of proteins, which are normally ubiquitin-dependently degraded. We asked whether such proteins might affect photoreceptor differentiation in *faf* mutants through the DER/Ras/MAPK pathway, which induces photoreceptor cell fates in wildtype animals. Our genetic data indicate that the activity of the pathway is increased in *faf* animals. Consistently, the protein level of a nuclear component of the DER/Ras/MAPK pathway, D-Jun, is increased in the mutant and may contribute to the phenotype. Thus, it appears that negative regulation of D-Jun, and probably other factors, by ubiquitin-dependent degradation is required for proper control of RTK signalling and eye development.

2. Results

2.1. Genetic interactions between DER/Ras/MAPK responsive transcription factors and faf

To investigate whether the ectopic photoreceptors observed in homozygous *faf* mutants differentiate in a Ras-independent manner, or whether neuronal differentiation still requires DER/Ras/MAPK signals, we examined if loss-of-function mutations affecting the pathway genetically interacted with *faf*. To monitor modifications of the *faf* phenotype we quantitated the number of ectopic outer photoreceptors in different genetic backgrounds.

The allele faf^{BX4}, which we have used throughout this study, is homozygous viable and behaves genetically as a null mutation. Molecularly, faf^{BX4} was found to contain a small inversion, which should abolish the enzymatic activity of the Faf protein (Fischer-Vize et al., 1992). A typical homozygous faf^{BX4} ommatidium has seven to eight outer photoreceptors with large light-dense rhabdomeres, one to two more than the six invariably found in wildtype ommatidia. In addition, a second ectopic R7 photoreceptor can occasionally be observed in *faf* mutant ommatidia (Fischer-Vize et al., 1992). On average only 14% of the ommatidia show the wildtype complement of six outer photoreceptors, but they are not arranged in the trapezoidal pattern typical of wildtype ommatidia (see figure legends for details on calculations).

The first mutation tested for an interaction with *faf* was the ETS-domain transcription factor *pointed* (*pnt*), which is a nuclear target of the DER/Ras/MAPK pathway (Brunner et al., 1994b; O'Neill et al., 1994). The hypomorphic allele pnt^{1277} is homozygous viable with no defect in eye development (Scholz et al., 1993). Fig. 1C shows a section through a pnt^{1277}, faf^{BX4}/pnt^{1277}, faf^{BX4} double mutant eye. The extra-photoreceptor phenotype observed in faf^{BX4}/faf^{BX4} animals is clearly suppressed; many more ommatidia have six outer photoreceptors in a trapezoidal arrangement characteristic of wildtype ommatidia (60% as opposed to 14%) (Fig. 1B,D, examples indicated by black arrows).

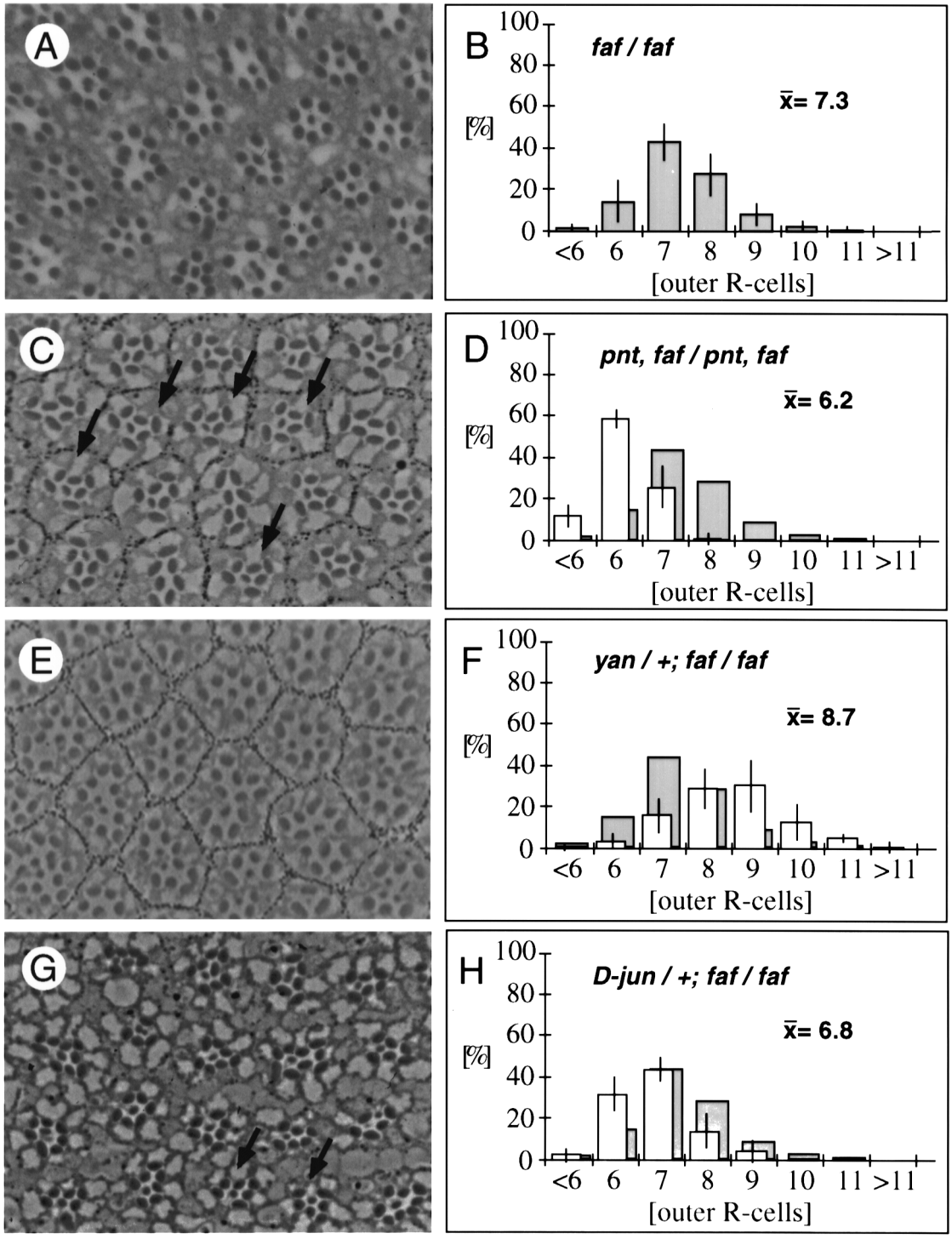

Fig. 1. Genetic interactions between *faf* and loss-of-function mutations in nuclear components of the RTK/Ras/MAPK pathway. (A,C,E,G) Tangential sections through adult retina. Differences in pigmentation between genotypes are due to the presence or absence of a *white*$^{+}$ gene and do not affect the number or arrangement of photoreceptors. (B,D,F,H) Histograms presenting statistical analyses of sections. The number of outer photoreceptors in single ommatidia was counted. The percentage of ommatidia with a specific number of outer photoreceptors was calculated separately for each eye. The average value from at least three independent eyes was calculated and plotted against the number of outer photoreceptors. Error bars indicate ± SD. (A,B) faf^{BX4}/faf^{BX4}. (A) Note the supernumerary photoreceptors of the R1–6 subclass with large dark rhabdomeres. (C,D) pnt^{1277}, faf^{BX4}/pnt^{1277}, faf^{BX4}. (C) Black arrows indicate ommatidia with six outer photoreceptors with their rhabdomeres arranged in the trapezoidal pattern characteristic of wildtype ommatidia. (D) pnt^{1277}, faf^{BX4}/pnt^{1277}, faf^{BX4} (white bars). Fifty-five percent of the ommatidia have six outer R-cells. For side by side comparison, the histogram for faf^{BX4}/faf^{BX4} (B) is shown behind in grey (D,F,H). (E,F) $yan^{1}/+$; faf^{BX4}/faf^{BX4}. (E) Note the increased number of R1–6-like rhabdomeres and ectopic R7-like cells. (G,H) $D\text{-}jun^{2}/+$; faf^{BX4}/faf^{BX4}. (G) Ommatidia with six outer R-cells are indicated by black arrows.

The average number of outer R-cells is reduced from 7.3 to 6.2.

The *pointed* null mutation $pnt^{\Delta 88}$ is homozygous lethal (Scholz et al., 1993). Removing one copy of *pnt* with the $pnt^{\Delta 88}$ mutation dominantly suppresses the *faf* phenotype (data not shown).

Thus, the differentiation of ectopic photoreceptors induced by mutant *faf* alleles appears sensitive to a reduction in Pointed activity and indicates that the mutation does not obviate the requirement for Pnt observed under wildtype conditions.

Yan is a negatively acting Ets-family transcription factor which antagonizes Pnt in the Ras-dependent induction of photoreceptor cell fate (Brunner et al., 1994b; O'Neill et al., 1994). Heterozygosity of the recessive hypomorphic mutant allele yan^{1} does not cause any phenotypic abnormalities in an otherwise wildtype eye, but dominantly enhances the number of photoreceptors in various genetic backgrounds in which the DER/Ras/MAPK signalling is increased (Lai and Rubin, 1992; Rebay and Rubin, 1995). yan^{1}/+ in combination with faf^{BX4}/faf^{BX4} strongly enhances the *faf* phenotype (Fig. 1E,F). About 50% of the ommatidia have more than eight R1–6-like photoreceptors, in marked contrast to less than 10% in faf^{BX4}/faf^{BX4} (Fig. 1B,F). The average number of outer R-cells increased from 7.3 to 8.7. In addition, R7 differentiation which is under negative control by Yan (Lai and Rubin, 1992), is enhanced in the yan^{1}/+; faf^{BX4}/faf^{BX4} double mutant (data not shown). The finding that a loss-of-function mutation in *yan* can dominantly enhance the faf^{BX4}/faf^{BX4} phenotype supports the idea that inductive neurogenic signalling is hyperactivated in *faf* mutants.

Previous studies have identified D-Jun as a nuclear target of the DER/Ras/MAPK pathway (Bohmann et al., 1994; Peverali et al., 1996; Kockel et al., 1997a). Moreover, D-Jun co-operates with Pnt and Yan in photoreceptor determination (Treier et al., 1995). We tested whether reducing D-Jun activity would also suppress the faf^{BX4}/faf^{BX4} phenotype using a recently identified *D-jun* allele (Kockel et al., 1997a). In sections of eyes with the genotype $D\text{-}jun^{2}$/+; faf^{BX4}/faf^{BX4} the average percentage of ommatidia with the wildtype complement of six R1–6-like cells is 32%, up from 16% in faf^{BX4}/faf^{BX4} (Fig. 1B,H). Furthermore, a significant number of the $D\text{-}jun^{2}$/+; faf^{BX4}/faf^{BX4} ommatidia display a wildtype configuration of photoreceptors (Fig. 1G, examples indicated by black arrows). The average number of ommatidia has decreased from 7.3 in faf^{BX4}/faf^{BX4} to 6.8 in $D\text{-}jun^{2}$/+; faf^{BX4}/faf^{BX4} (Fig. 1B,H).

The above experiments show that a mild reduction in the activity of Ras-responsive transcription factors which does not lead to phenotypic abnormalities in a wildtype background can nevertheless influence the extra-photoreceptor phenotype of *faf* mutants significantly. This indicates that the ectopic photoreceptor differentiation observed in strains where *faf* function is missing is regulated by these factors. To investigate whether they are still under the control of the Ras/Raf signalling pathway in a *faf* mutant context, we next examined genetic interactions between *faf* and genes encoding upstream components of the Ras pathway.

2.2. Mutations in upstream components of the DER/Ras/MAPK pathway also modify the faf phenotype

14-3-3 proteins are mediators of signal-dependent activation of Raf and have been placed between Ras1 and D-Raf based on genetic experiments (Chang and Rubin, 1997; Kockel et al., 1997b; Roberts et al., 1997). D-14-3-3ζ is a positive regulator of photoreceptor differentiation (Kockel et al., 1997b). A presumed null allele, $D\text{-}14\text{-}3\text{-}3\zeta^{E16}$, is homozygous lethal, but under heterozygous conditions causes no mutant eye phenotype in a wildtype background (Kockel et al., 1997b). In a section of an eye with the genotype $D\text{-}14\text{-}3\text{-}3\zeta^{E16}$/+; faf^{BX4}/faf^{BX4}, however, on average 40% of the ommatidia have six outer photoreceptors, a more than two-fold increase compared to the faf^{BX4}/faf^{BX4} genotype (Figs. 1B and 2B). Furthermore, the appearance of the ommatidia is more organized in the case of the double mutant combination than in eyes mutant only for *faf*.

We also tested a hypomorphic mutation in *D-raf* called $D\text{-}raf^{HM\text{-}7}$ (Melnick et al., 1993) for interaction with faf^{BX4}/faf^{BX4}. The $D\text{-}raf^{HM\text{-}7}$ mutation does not affect the coding sequence but strongly reduces the D-Raf protein level when hemizygous. In $D\text{-}raf^{HM\text{-}7}/Y$ males the average number of outer R-cells is reduced to 5.5. When this genotype is combined with *faf* ($D\text{-}raf^{HM\text{-}7}/Y$; faf^{BX4}/faf^{BX4}), the resulting eye phenotype is indistinguishable from $D\text{-}raf^{HM\text{-}7}/Y$ alone. Taken together these experiments indicate that the *faf* mutant phenotype is sensitive to reduction in *D-14-3-3ζ* gene dosage and cannot bypass the requirement for *D-Raf*.

2.3. R7 cells are induced independently of sevenless in faf mutant background

As mentioned above, in *faf* mutant eyes a small number of ommatidia (about 10%) have ectopic R7-like cells. To investigate whether the induction of these ectopic R7-like cells requires *sev*, we analyzed photoreceptor differentiation in various *sev* and *faf* mutant combinations. As expected, in tangential sections of sev^{d2}/sev^{d2} (Gerresheim, 1988) as well as sev^{d2}/sev^{d2}; faf^{BX4}/+ eyes R7 cells are absent (Fig. 3A). However, R7-like cells can form in the absence of *sev* function in a sev^{d2}/sev^{d2}; faf^{BX4}/faf^{BX4} double mutant (Fig. 3B). Surprisingly, 60% of the ommatidia have an R7 cell in this genotype (Fig. 3D). Apparently, in the sev^{d2}/sev^{d2}; faf^{BX4}/faf^{BX4} double mutant R7 precursors frequently differentiate into R7 cells. Thus, *faf* appears to alleviate the requirement for *sev* in the R7 precursor. This result is interesting in light of previously suggested models for the sequential requirement of both DER and Sev signalling for R7 differentiation (Freeman, 1996). It is conceivable that DER activity is increased and/or prolonged in *faf* mutants and that this

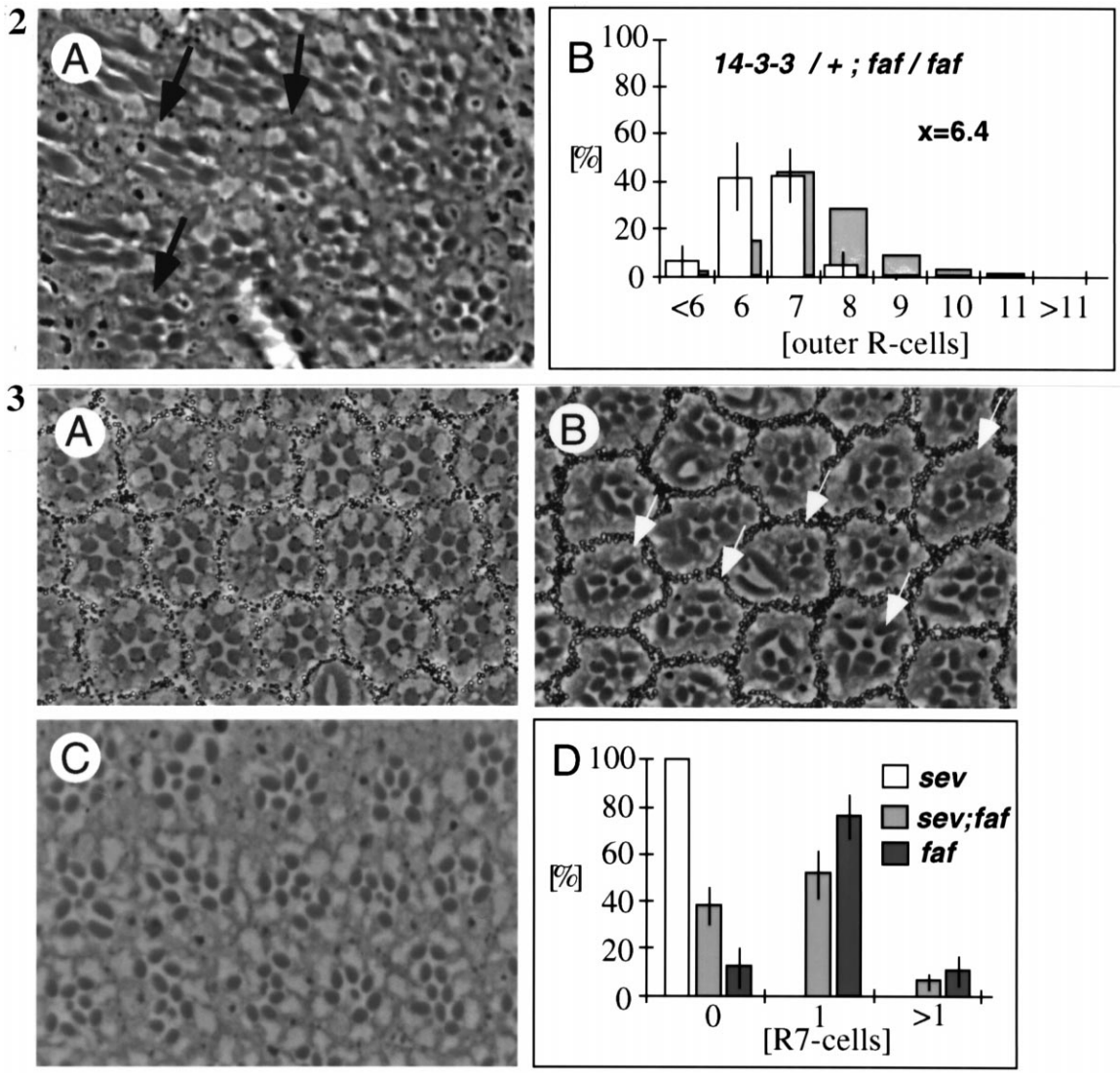

Fig. 2. Genetic interactions between *faf* and a loss-of-function mutation of a non-nuclear component in the RTK/Ras/MAP pathway. A tangential section through an adult *D-14-3-3ζ*^{E16}/+; *faf*^{BX4}/*faf*^{BX4} retina is shown in (A). Ommatidia with six outer photoreceptors arranged in the trapezoidal pattern characteristic of wildtype eyes are indicated by black arrows. A histogram shows the average fraction of ommatidia ± SD plotted against the number of outer R-cells in *D-14-3-3ζ*^{E16}/+; *faf*^{BX4}/*faf*^{BX4} (B). For side by side comparison, the histogram for *faf*^{BX4}/*faf*^{BX4} (Fig. 1B) is shown behind in grey.

Fig. 3. R7-like cells form independently of *sev* in *faf* mutant eyes. (A,B,C) Tangential sections of adult retina. A histogram shows the average percentage of ommatidia plotted against the number of R7-like cells and error bars indicate ± SD (D). (A) *sev*^{d2}/*sev*^{d2}; *faf*^{BX4}/+. Note the absence of R7 cells. (B) *sev*^{d2}/*sev*^{d2}; *faf*^{BX4}/*faf*^{BX4}. Ommatidia with an R7 cell are indicated by white arrows. (C) *faf*^{BX4}/*faf*^{BX4}. (D) *sev*^{d2}/*sev*^{d2}; *faf*^{BX4}/+ (white bars), all ommatidia have no R7 cell. *sev*^{d2}/*sev*^{d2}; *faf*^{BX4}/*faf*^{BX4} (light grey bars). Note that 60% of ommatidia have one R7 cell and about 10% have more than one R7 cell. *faf*^{BX4}/*faf*^{BX4} (dark grey bars). About 80% of ommatidia have one R7 cell and about 10% have more than one R7 cell.

effect obviates the need for a second RTK signal which normally would be delivered by Sev.

Since reducing the activity of all the components tested so far (except for *sev*) can modulate the *faf* phenotype, it appears that the DER/Ras/MAPK pathway is required for induction of the ectopic photoreceptors in the *faf* mutant.

2.4. Expression of D-Jun protein is elevated and prolonged in faf mutant eye discs

The Faf homologue in yeast, DOA4, is required for efficient ubiquitin-dependent protein degradation, suggesting a similar function for Faf. Thus, a plausible explanation for the *faf* mutant phenotype would be inefficient proteolysis and increased function of one or several positively acting components of the DER/Ras/MAPK pathway. For several reasons the transcription factor D-Jun could be such a protein. First, it is a nuclear target of the pathway (Bohmann et al., 1994; Peverali et al., 1996) and is involved in induction of outer photoreceptors (Bohmann et al., 1994; Treier et al., 1995; Kockel et al., 1997a). Second, D-Jun is a normally short-lived protein, as seen by its transient expression pattern in a wildtype eye disc (Fig. 4, left panel; Bohmann et al., 1994). Third, c-Jun has been shown to be ubiquitin-

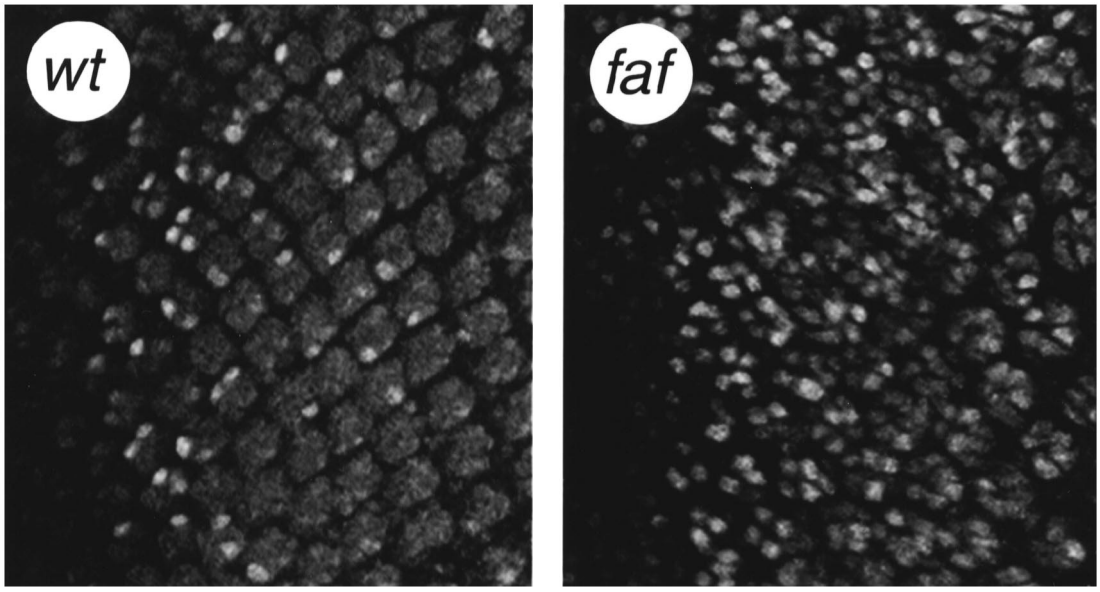

Fig. 4. D-Jun expression in wildtype and *faf*$^{-}$ eye discs. Posterior is to the right. Left panel, wildtype (*wt*) eye disc immunostained with antibodies against D-Jun. Right panel, *faf*$^{-}$ (*faf*) eye disc immunostained in parallel. Note that in the *faf*$^{-}$ disc clusters of cells continue to express D-Jun protein at high levels in the posterior part of the disc. Importantly, the D-Jun expression profile across the disc differs qualitatively as-well as quantitatively between wildtype and *faf* mutants, which rules out artefacts due to variable staining conditions.

dependently degraded in mammalian cells (Treier et al., 1994). Fourth, expression of a constitutively active mutant of c-Jun in the eye induces the differentiation of ectopic photoreceptors of both the R1–6 and the R7 class (Treier et al., 1995). This demonstrates that increased Jun activity can result in a phenotype similar to the one observed in *faf* mutants.

During ommatidial development, elevated levels of Jun are expressed very transiently as photoreceptor precursors undergo neuronal determination. This results in a temporally well-defined pattern of Jun expression posterior to the morphogenetic furrow (Fig. 4, left panel; Bohmann et al., 1994). We compared the Jun expression pattern in wildtype and *faf* imaginal discs by immunostaining (Fig. 4). In *faf* mutant eye imaginal discs the onset of D-Jun expression remains unchanged as compared to the wildtype. This D-Jun expression, however, reaches higher levels and is much more protracted than in control flies. The immunostaining indicates that D-Jun protein persists in clusters of cells in the posterior part of the *faf* disc, where it is only detectable at basal levels in the wildtype. This expression pattern in *faf* eye discs is consistent with the stabilization of D-Jun protein, presumably due to deficient ubiquitin-dependent degradation.

3. Discussion

3.1. Ubiquitin-dependent protein degradation and signal transduction

Our data indicate that the extra-photoreceptor phenotype observed in *faf* mutants is the consequence of increased signalling through the RTK/Ras/MAPK pathway which relays a neurogenic signal during *Drosophila* eye development and induces photoreceptor differentiation. The identification of the *faf* gene product as a deubiquitination enzyme suggested that the phenotype might originate from defective degradation and the resulting increase in activity of one or more components of this pathway. The prolonged and increased expression of D-Jun in *faf* eye discs is consistent with defects in ubiquitin-dependent degradation of this transcription factor. Formally, we cannot rule out that the extended expression of D-Jun in the developing eye is due to a transcriptional mechanism (*D-jun* mRNA could not be detected in a specific pattern in wildtype or *faf* discs, presumably due to low levels of expression; data not shown). Based on the nature of the mutation and the fact that c-Jun is a known target for ubiquitination (Treier et al., 1994), we consider it likely that the augmented expression of D-Jun is due to defective degradation.

A contribution of defective Jun degradation to the ectopic photoreceptor differentiation observed in *faf* eyes is supported by the finding that phenotype is suppressed by reducing *D-Jun* gene dosage (see model in Fig. 5). However, stabilization of D-Jun is not likely to be the only cause for the *faf* phenotype, as elevated levels of Jun per se do not elicit a gain-of-function effect as shown by transgenic expression of Jun in a wildtype background (Bohmann et al., 1994; Treier et al., 1995). Nevertheless, in combination with even small disturbances in the Ras pathway, as in the presence of the mildly activated *tor*^{Y9} allele of *sev*, which in a *sev*$^{-}$ background appears largely normal, D-Jun overexpression causes marked differentiation of extra photoreceptors (Bohmann et al., 1994). We conclude that the levels of D-Jun must be reasonably well controlled during eye devel-

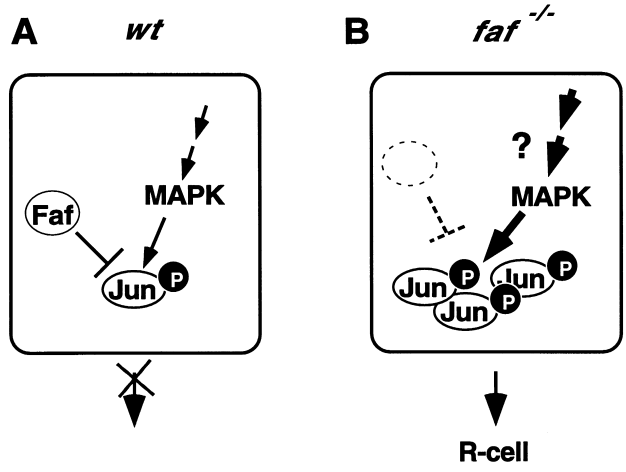

Fig. 5. A model for induction of ectopic photoreceptors by the *fat facets* mutation. (A) In a wildtype imaginal disc cell Faf facilitates the ubiquitin-dependent degradation of D-Jun and other factors. The cell does not adopt photoreceptor cell fate. (B) In a *faf* mutant cell the D-Jun protein level is elevated presumably due to deficient ubiquitin-dependent degradation. D-Jun and possibly other factors induce the mystery cell to adopt an R1–6-like cell fate. Jun requires phosphorylation triggered by the RTK/Ras pathway. Signalling by components upstream of D-Jun may also be increased in *faf* mutants.

opment. Otherwise fluctuations in the upstream signalling cascade might lead to aberrant developmental decisions. We consider it likely that such 'upstream disturbances' occur in *faf* mutants.

Due to the pleiotropic effects expected from a deficient ubiquitin system, it is conceivable that other components of the pathway might be activated in *faf* mutants and could lead to increased phosphorylation of the elevated levels of D-Jun. Indeed, as pointed out above, the observation that the Sevenless signal becomes largely obsolete for R7 induction in a *faf* background suggests that the signalling through the DER pathway is increased in the mutant. The molecular basis for such an effect is not clear at present. It could be mediated by the stabilization of signalling proteins such as Raf, Spitz or DER itself. It appears reasonable that such effects in conjunction with the increased levels of Jun would cause the *faf* phenotype.

Increased signalling due to reduced degradation of signalling components may also affect photoreceptor differentiation cell non-autonomously. This idea is consistent with the clonal analysis of the *faf* mutant phenotype (Fischer-Vize et al., 1992), which suggests, in addition to autonomous effects of the *faf* mutation, a mild and short ranging non-autonomous function for *faf*. Indeed, recent results are consistent with the idea that activation of the DER/Ras/MAPK pathway can have non-autonomous effects on neighbouring cells. Clonal analysis shows, for example, that Spitz expression is only required in R8, R2 and R5, but has non-autonomous effects on the development of the ommatidium (Freeman, 1994; Tio et al., 1994).

In contrast to our conclusions, a previous publication states that *faf* does not interact genetically in a significant way with members of the DER/Ras/MAPK pathway (Huang and Fischer-Vize, 1996). However, the effects we report here would not have been reliably detected without cytological sections and statistical evaluation, which was not included in that report.

Huang et al. (1995) have shown a genetic interaction between *faf* and an antimorphic temperature-sensitive mutation in a proteasome subunit. Based on the suppression of the *faf* phenotype by this mutation it has been proposed that Faf may inhibit ubiquitin-dependent degradation. So far, this is the only interaction with a proteasome subunit that has been reported. Since it is not known if this component influences proteasome function positively or negatively, it makes the interpretation of the experiment difficult. On the other hand, mutations in Doa4, a yeast protein homologous to Faf, led to stabilization and increased expression of proteins normally degraded by the proteasome (Papa and Hochstrasser, 1993). Taken together with the increased expression of D-Jun protein in *faf* mutant eye discs, our data support the hypothesis that Faf, in analogy to Doa4, promotes ubiquitin-dependent proteolysis by the 26S proteasome.

3.2. How general is the regulation of RTK/Ras signalling by the ubiquitin system?

One may ask why a null mutation in a component of the ubiquitin-dependent protein degradation system like faf^{BX4} has only a comparatively mild eye phenotype, especially if as suggested by this study, Ras and D-Jun, factors of rather pleiotropic function throughout development (Perrimon, 1994; Kockel et al., 1997a) are affected by this lesion. It is clear that *faf* has functions other than inhibiting photoreceptor differentiation. Maternal *faf* function is required for early embryogenesis (Fischer-Vize et al., 1992). The maternal contribution of *faf* rescues the early lethality and maybe other phenotypes. Furthermore, homozygous faf^{BX4} animals show a reduced viability and suffer from other defects such as problems in mitosis (A. Isaksson, unpublished data). The defect in the eye may be particularly apparent because eye development is a very dynamic process that requires a sequence of precisely timed events. It is possible that even mild effects on the timing of signals, as they might be caused by deficient ubiquitin-dependent degradation of signalling proteins, can have visible phenotypic consequences. Therefore, the observations presented here may provide a starting point for studying the relevance of the timing and kinetics of signal transduction.

4. Experimental procedures

4.1. Drosophila strains

All crosses were performed on standard fly food at 25°C. The alleles used were described earlier, i.e. faf^{BX4} (Fischer-

Vize et al., 1992), *D-14-3-3ζ*^{E16} (Kockel et al., 1997b), *D-raf*$^{HM-7}$ (Melnick et al., 1993), *yan*^{1} (Lai and Rubin, 1992), *pnt*^{1277}, *pnt*$^{\Delta 88}$ (Scholz et al., 1993), *D-jun*^{2} (Kockel et al., 1997a) and *sev*^{d2} (Gerresheim, 1988).

4.2. Histology

Tangential sections of adult retinae were performed as described in Tomlinson and Ready (1987) and viewed with phase contrast. Immunostaining of third instar eye disc was carried out as in Bohmann et al. (1994) except fluorescence detection was used. The affinity purified anti-D-Jun antibody is described in Bohmann et al. (1994) and was used at a dilution of 1:10 000 in 0.4% saponin. A goat anti-rabbit secondary antibody coupled with Biotin (Jackson Laboratories) was used at a dilution of 1:50. The discs were incubated in secondary antibody solution for 1 h and washed three times in 0.1 M phosphate buffer (pH 7.2) (0.4% saponin and 10% normal serum). A streptavidin coupled FITC fluorophor (Jackson Laboratories) at a dilution of 1:1250 in phosphate buffer was allowed to bind to the secondary antibody for 45 min. After three washes in phosphate buffer the discs were mounted in moviol containing DABCO and imaged on the EMBL CCM confocal microscope.

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