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Mutations in *rugose* promote cell type-specific apoptosis in the *Drosophila* eye

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Abstract

rugose (rg) encodes an A kinase anchor protein and was isolated as a genetic interactor of the Notch and epidermal growth factor receptor (EGFR) pathways during eye development in *Drosophila*. *rg* mutants display a small, rough eye phenotype primarily caused by the loss of cone cells. Here we show that the basis of this phenotype is cell type-specific apoptosis rather than transformation and hence can be rescued by reduction of proapoptotic signals. Moreover, a nearly complete rescue is observed by an increased Notch signal suggesting an antiapoptotic function of Notch in this developmental context. Cone cell loss in *rg* mutants is accompanied by enhanced Jun N-terminal kinase activity and, concomitantly, by a reduction of EGFR signalling activity. Together, these findings support the idea that *rg* plays an important role in the integration of different signals required for the exact regulation of cone cell development and survival.
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Introduction

Programmed cell death (PCD) is an important process for the proper development and homeostasis of multicellular organisms. In *Drosophila*, the induction of apoptosis requires the activity of the closely linked proapoptotic genes *reaper* (*rpr*), *head involution defective* (*hid*) and *grim*, which induce cell death by activating a family of executing proteases known as caspases.[1,2] Cell death is suppressed by the *Drosophila* inhibitor of apoptosis protein I (DIAPI), which directly inhibits caspase activity and promotes their ubiquitination and subsequent degradation.[3] Recent findings suggest that Rpr-mediated DIAP1 inhibition leads to an induction of Jun N-terminal kinase (JNK) signalling, which was shown to induce apoptosis during imaginal disc development in *Drosophila*.[4–6] However, the functional relevance of JNK activation for killing by Reaper is still controversial.[7]

A large number of cells undergo apoptosis during fly embryonic and imaginal development as well as during metamorphosis. One of the best tissues to investigate this process is the eye of the fly, taking into account that it is not an essential structure and easily allows comparative phenotypic analyses. The *Drosophila* eye is composed of approximately 750 ommatidia, each built up of a fixed number of cells. Centrally located are eight photoreceptor cells, covered by four cone cells and surrounded by two primary pigment cells.[8] The ommatidia are separated by interommatidial cells, which differentiate into secondary and tertiary pigment cells and interommatidial bristles. Excess cells are eliminated through apoptosis resulting in the precise hexagonal cellular lattice.[8,9] It was shown that this spatially restricted cell death depends on the balance between epidermal growth factor receptor (EGFR) and Notch receptor signalling. For example, laser ablation-induced cell death during pupal eye development was strikingly blocked by the expression of the activated ras^{V12} allele, a downstream component of the EGFR/mitogen-activated protein kinase (MAPK) pathway.[10] Two independent studies revealed that MAPK signalling counteracts apoptosis by downregulating the function and expression of the proapoptotic factor *hid*.[11,12] Hence, EGFR signalling supports cell survival, whereas activation of the transmembrane receptor Notch in the interommatidial lattice promotes cell death.[9,10,13,14]

Genetic screens identified *rugose* (*rg*) as an interactor of the Notch and EGFR signalling pathway during eye development.[15,16] *rg* encodes a *Drosophila* A kinase anchor protein.[17] Mutants in *rg* are characterized by a rough eye phenotype caused by the loss of cone cells accompanied by further irregularities in the cellular architecture of the pupal retina.[15,16,18]

It was suggested that cone cell loss is based on transformation, albeit the final fate of these cells remained elusive.[16] In this study, we reinvestigated the basis of the observed cone cell loss in *rg* mutant eyes. Our analyses indicate that this phenotype is a result of cell type-specific apoptosis rather than cell fate transformation to 1° pigment cells and occurs largely autonomously in *rg* mutant cells. Accordingly, the mutant phenotype is completely restored by providing survival signals. Moreover, an increase of Notch signalling rescues cone cell death as well, suggesting that Notch provides an anti- rather than proapoptotic signal in this developmental context. Cone cell loss in *rg* mutants is accompanied by an increase of JNK activity, whereas MAPK activity is lowered. This study supports the notion that the major role of *rg* might be the integration of the crosstalk between different signalling pathways to allow precise

regulation of factors involved in cone cell formation and survival.

Results

rg mutation leads to the death of cone cells

rg was isolated as a strong enhancer of the rough eye phenotype caused by the overexpression of the Notch antagonist Hairless (H) in the differentiating eye.[15] The primary cause of the rough appearance of *rg* mutant eyes appears to be a lack of cone cells, which can be visualized by cobalt sulphide stainings of pupal retinae (Figure 1c′).[15,16] In order to follow the fate of the individual cone cells, we used an intermediate allele, rg^{E16}, that
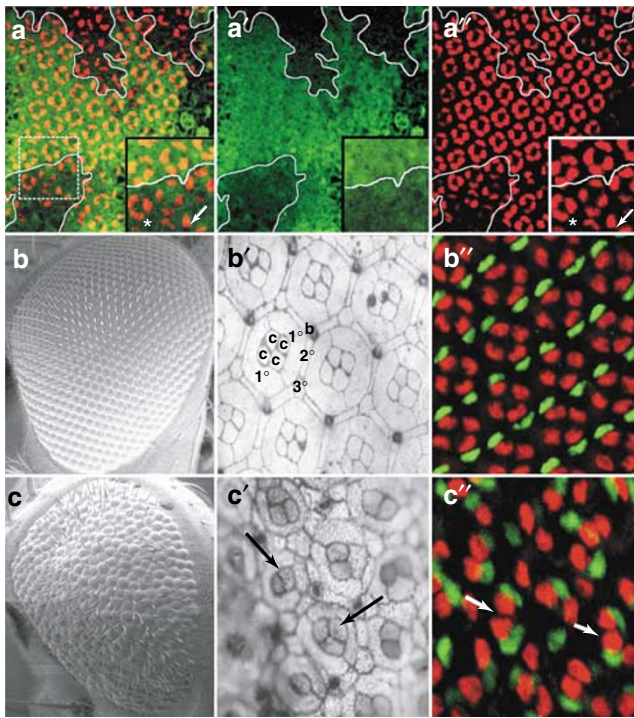

Figure 1 Specific loss of cone cells in rg^{E16} mutant pupal retinae. (**a–a″**) Pupal eye discs with rg^{E16} mutant cell clones are marked by the absence of lacZ, stained with antibodies to β-galactosidase (green, **a, a′**). Cone cells are visualized with anti-Cut staining (red, **a, a″**). The clone borders are outlined. Ommatidia within mutant clones have a reduced number of Cut-positive cone cells. Frequently, only three (asterisk) or even just two (arrow) cone cells remain. Ommatidia close to the clone borders usually have the full complement of four cone cells, implying a positive influence from wild-type cells. Note that Cut levels appear reduced in many mutant cone cell nuclei (see inset). (**b**) Ommatidia of wild-type eyes are ordered regularly (scanning electron microscopy (SEM) picture). (**b′**) Cobalt sulphide staining of 40–48 h old pupae reveals the typical arrangement of four cone cells (**c**) surrounded by two primary pigment cells (1°) and cells of the interommatidial lattice (2°, 3° pigment cells, interommatidial bristle (b)). (**b″**) Antibody staining against Cut (red) and BarH1 (green) was used as marker for cone cell and 1° pigment cell fate, respectively. Note the normal arrangement of four cone cells surrounded by two 1° pigment cells in each ommatidium. (**c–c″**) rg^{E16}/Y mutant eyes. Hemizygous rg^{E16} mutants display a rough eye phenotype (SEM picture (**c**)), caused by the specific loss of cone cells (arrows in **c′**). A loss of Cut-positive cone cells (red) is apparent, whereas the number of BarH1-positive 1° pigment cells (green) is unchanged (**c″**, white open arrows exemplify ommatidia lacking cone cells)

develops a pronounced cone cell loss phenotype little obscured by other retina defects.[15]

Cone cells could be identified because they specifically express Cut, whereas 1° pigment cells strongly accumulate BarH1 protein.[19,20] Using specific antibodies directed against these cell markers allowed to distinguish cone cells from 1° pigment cells in the pupal retinae (Figure 1c″). Early recruitment of cone cells seemed undisturbed in rg^{E16} since Cut staining was indistinguishable from wild type in the larval eye disc and pupal retina of 24 h old pupae (not shown). However, during retina maturation, in about 40 h old pupae ommatidia lacked one to two cone cells on average compared to the four cone cells seen in the wild type. In contrast, 1° pigment cells seemed little affected (Figure 1b–c″). As *rg* encodes a protein kinase A (PKA) anchor protein, one might expect its requirement within the cell of expression. In accordance, *rg* mutant cell clones revealed cell autonomy of the mutant phenotype (Figure 1a–a″). However, we note that ommatidia close to clone borders showed a full complement of four cone cells more frequently than ommatidia within the clone, suggesting a positive input from surrounding wild-type cells. Moreover, Cut expression appeared fainter in some mutant nuclei suggesting an incomplete transformation into a different cell type or fading away of these cells (Figure 1a and a″).

rg mutations result in cell type-specific cell death and not in transformation to 1° pigment cells

Lack of cone cells could be a result of transformation into another cell fate. As no systematic increase of R7 cells is observed, a transformation into the previous cell fate seems very unlikely.[15,16,21] Similarly, the number of 1° pigment cells, which constitute the subsequent cell fate, is also not increased (Figure 1c″). However, it is conceivable that cone cells are fated for 1° pigment cells and replace them. The normal 1° pigment cell might either die as a consequence of wrong signalling input or governed into the next fate, that is, interommatidial lattice cells, eventually forming 2° or 3° pigment cells. This is not without precedence. For example, in the *sevenless* mutant, the presumptive R7 cell is fated for and replaces the normal cone cell.[21] Since the number of lattice cells is reduced by PCD during final stages of retina maturation, such a transformation would not be detectable by an increase of pigment cell numbers. Alternatively, cone cells could simply die in the rg^{E16} mutants maybe due to reduction of survival signals.

In order to distinguish between these possibilities, we increased survival signals in two ways. First, we over-expressed the antiapoptotic factor DIAP1 in cone cell precursors and second, we halved the dose of the proapoptotic genes *hid*, *rpr* and *grim* with a *Df(3L) H99* heterozygous background. We not only observed a complete rescue of the adult eye phenotype (Figure 2a and c) but also of the cone cell loss (Figure 2a′ and c′) without affecting the number of 1° pigment cells (Figure 2a″). Apparently, the missing cone cell(s) specifically die in the rg^{E16} mutant. This can be visualized in a double labelling for Cut and activated Caspase-3, which accumulates in the dying cells (Figure 2e).
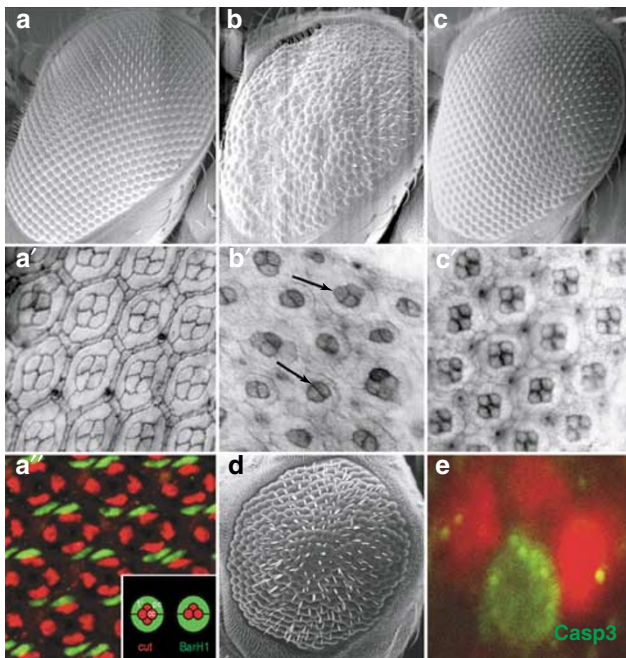
Figure 2 The rg^{E16} mutant eye phenotype involves cone cell-specific cell death rather than cell type transformation. (**a–a''**) rg^{E16}/Y; *Df(3L)H*^{99}/+ flies. The rough eye phenotype and the specific loss of cone cells in rg^{E16} mutants are completely rescued in a heterozygous *Df(3L)H99* background where the gene dose of the proapoptotic genes *rpr*, *hid* and *grim* is reduced by half. The inset in (**a''**) represents a schematic drawing of the cellular make-up of a wild-type (left) *versus* an rg^{E16} mutant (right) pupal ommatidium where cone cells are marked for Cut protein in red and 1° pigment cells are marked for BarH1 protein in green. *rg* mutants are typified by a reduced number of cone cells but normal 1° pigment cell numbers. (**b–b'**) rg^{E16}/Y; *sevGal4*/+ . The rough appearance of *rg* mutant eyes is not influenced by the sev-Gal4 driver line (**b**) nor is the cone cell loss (**b'**) (**c–c'**) rg^{E16}/Y; *sevGal4* > *UAS DIAP1* flies. Overexpression of the antiapoptotic factor DIAP1 in cone cell precursors results in a complete wild-type appearance of the eye (**c**) and the survival of all four cone cells despite an rg^{E16}/Y mutant background (**c'**). Compare the adult eyes from (**b**) and (**c**) and the cobalt sulphide staining from (**b'**) with (**c'**), respectively. The arrow in (**b'**) points to examples of ommatidia lacking one or two cone cells. (**d**) rg^{E16}/Y, *th*^{5}/+ . Accordingly, the eye phenotype of rg^{E16} is enhanced when the gene dose of DIAP1 is halved (compare with Figure 1c). (**e**) rg^{E16} pupal eye disc (48 h after pupariation) stained with α-Cut (red) and α-cleaved Caspase-3 (green). Note that one cell in the ommatidium, which by position seems to be a cone cell albeit lacking the Cut marker, undergoes cell death and consequently accumulates activated Caspase-3

Moreover, reducing the activity of the antiapoptotic factor DIAP1 in a *th*^{5} heterozygous mutant background, which itself is phenotypically wild type, enhances the rough eye phenotype caused by rg^{E16} (Figure 2d, compare with Figure 1c). Protecting cone cells from apoptotic signals in *rg* mutants allows them to survive and to form a normal complement of four (Figure 2a' and c'). Reduction of the proapoptotic gene *rpr* to one copy in the *XR38* mutation results in a slight suppression of the rg^{E16} rough eye phenotype, suggesting an involvement of *rpr* in cone cell death (not shown). The fact that the number of 1° pigment cells is not changed indicates that no cell type transformation occurs (Figure 2a''). Repression of apoptosis by overexpression of DIAPI in cone cells supplies additional survival signals and leads to an inhibition of normal PCD in interommatidial lattice cells also in an *rg* mutant background (Figure 2c'). This does, however, not cause any apparent rough eye phenotype in adults (Figure 2c).

Increase in Notch signalling rescues cone cell death in *rg* mutants

Genetic interactions between rg^{E16} and different components of the Notch signalling pathway were already described.[15,16] It was shown for example that the loss of one copy of the *Notch* ligand *Delta* and therefore a reduction in Notch signalling results in a strong enhancement of the rough eye phenotype caused by rg^{E16}. On the other hand, reducing the gene dose of *H* by half resulted in a rescue of the *rg* rough eye phenotype.[15,16] H acts as antagonist in the Notch signalling pathway by assembling a repressor complex together with Su(H) and corepressors on Notch target genes.[22,23] Hence, lowering H activity should lead to an increase of Notch signalling activity, which might serve as survival signal for cone cells. However, it was shown earlier that Notch promotes PCD in interommatidial lattice cells.[10] Maybe *H* and *rg* mutants interact without involvement of Notch signalling. Indeed, Su(H)-independent activities of H have been described earlier for aspects of wing and bristle development.[24,25]

This reasoning led us to address whether a reduction of *H* to one copy also rescues the cone cell death in rg^{E16} mutants. In fact, pupal retinae of rg^{E16}; *H*^{P8}/+ males were indistinguishable from wild type (Figure 3a and b). Thus, a reduction of *H* gene activity in an rg^{E16} mutant background yields an antiapoptotic signal apparently sufficient to counteract the death stimulus resulting from loss of *rg* activity, thereby repressing cone cell-specific cell death. Apparently, one role of H during retina development is to provide a proapoptotic stimulus in accordance with earlier observations that over-expression of H causes loss of eye or wing tissue.[25,26] We next asked whether this survival signal resulting from reduced H activity involves an increase in N signal. This question is rather difficult to address directly, as overexpression of Notch interferes with multiple aspects of eye development and causes a wide array of phenotypes.[9,27,28] As predicted, our attempts to rescue *rg* mutant phenotype by overexpression of the intracellular domain of N or of Su(H) was uninformative due to vast disorganization of the retina (not shown). We therefore doubled the amount of Notch activity in an rg^{E16} mutant background by including a second Notch gene copy with the *cos479* transgene. As predicted, the adult rough eye phenotype as well as loss of cone cells was rescued by the extra Notch gene dose (Figure 3d and d'). These results are consistent with the idea that a Notch signal supports cell survival in this context.

EGFR signalling is reduced in *rg* mutants

Different genetic screens have revealed numerous interactions between mutants in *rg* and EGFR signalling components. In general, a reduction of EGFR signalling enhances the *rg* rough eye phenotype.[15,16] EGFR has been implicated in the inhibition of proapoptotic signals and the survival of interommatidial lattice cells.[10–12] Thus, mutants in EGFR signalling components might result in a reduction of survival stimuli, thereby enhancing the defects of *rg* mutants. In accordance, mutants in the MAPK *rolled* (*rl*^{1}) have rough eyes and lack cone cells as well.[29] Moreover, heterozygosis for the nuclear effector *pointed* (*pntΔ88/+*) caused a gentle
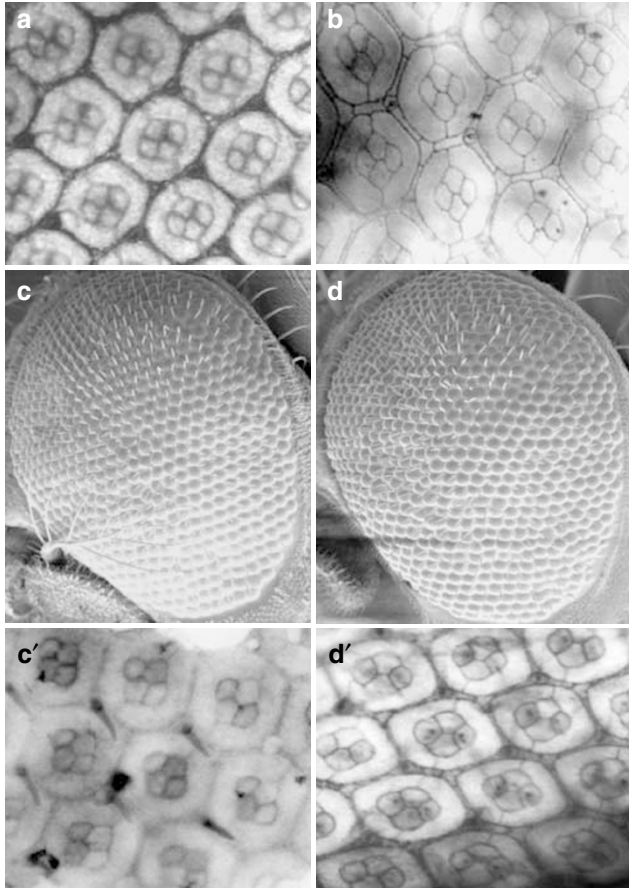
Figure 3 Cell death in rg^{E16} eye discs is influenced by Notch pathway components. (**a**) Pupal eye disc (48 h after pupariation) of $H^{P8}/+$ reveals a wild-type number of cone cells. (**b**) The same is seen in $rg^{E16}/Y; H^{P8}/+$ double mutants. Thus, cone cell loss of rg^{E16} mutants is completely rescued by halving the H gene copy. (**c–c′**) Adult eyes and pupal eye discs of $N^{ts}/Y; cos479/TM3Sb$ males, which have an additional copy of Notch as transgene, appear superficially quite normal (**c**) and have a wild-type assembly of cone cells (**c′**). (**d–d″**) The rg^{E16} eye phenotype is nearly completely rescued by such an additional copy of Notch ($rg^{E16}/Y; cos479/+$). Only a mild roughening of the adult eye remains (**d**), whereas the pupal cone cell equipment is completely restored (**d′**)

enhancement of the rg^{E16} rough eye phenotype (not shown). Conversely, an increase in EGFR activity should result in a rescue. This is indeed observed: halving the gene dose of the transcriptional repressor *anterior open (aop)* rescued the rg^{E16} rough eye phenotype as well as the cone cell loss in pupal retinae to nearly wild type (Figure 4a and a′). As EGFR signal mediates survival of cone cells, does a mutation in *rg* affect the levels of EGFR signalling? Consistent with this hypothesis, we found a significant decrease of the levels of activated MAPK (diP-ERK) in rg^{E16} mutant heads to about 24% of wild-type levels, similar to the levels observed in S^{E10} heterozygotes (Figure 4e and f). This finding was confirmed by antibody staining against diP-ERK in pupal retinae. In agreement with published data,[30,31] a strong, punctate signal was detected within wild-type cone cells (Figure 4b–b″). Although this punctate localization of diP-ERK remained unchanged in rg^{E16} mutants, the intensity of the staining was remarkably lowered (Figure 4c–c″). These results were confirmed in rg^{E16} mutant cell clones, where the signalling
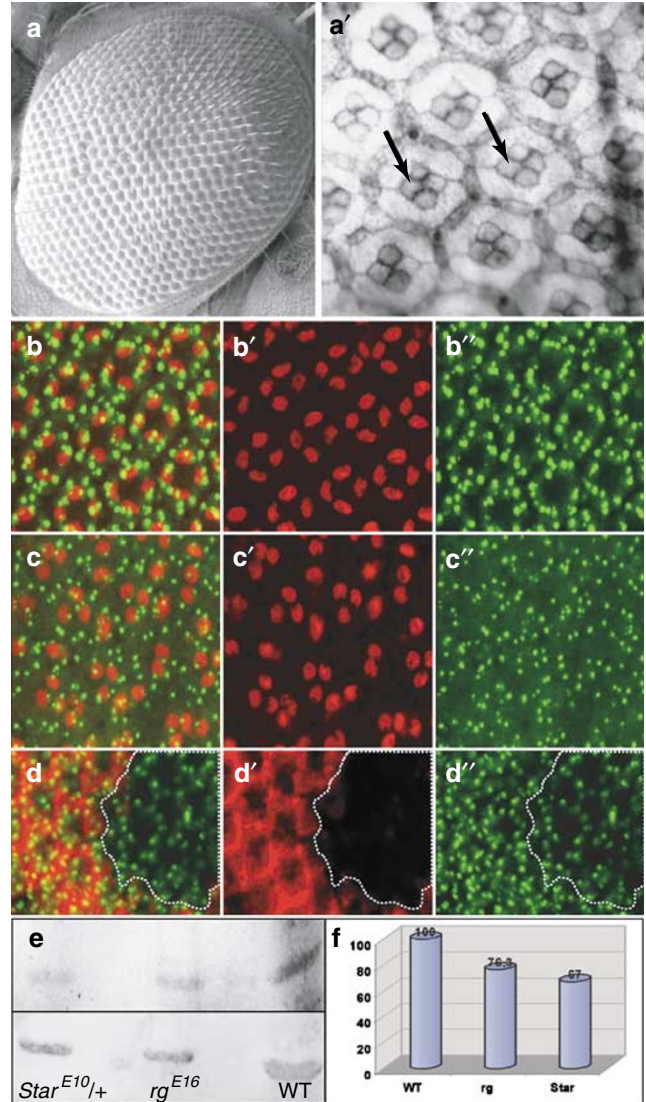

Figure 4 MAPK signalling is reduced in rg^{E16} mutant retinae. Halving the gene dose of *anterior open (aop)*, which acts as a repressor of EGFR target gene activation, suppresses the rg^{E16} rough eye phenotype (**a**; $rg^{E16}/Y; aop/+$) and restores the four cone cells in pupal retinae (**a′**, arrows). Pupal retinae (48 h old) from wild-type (**b–b″**) and rg^{E16} males (**c–c″**) were stained with α-Cut (red) and α-diPERK (green), respectively. Note the dotted staining representing activated MAKP in wild-type cone cells (**b, b″**), which is likewise present, however, at a reduced level in the rg^{E16} mutant (**c, c″**), which also reveals the aforementioned reduction of Cut-positive cells (compare **b–b″** with **c–c″** and **b′** with **c′**). (**b**) and (**c**) are merged images. (**d–d″**) Pupal eye disc with an rg^{E16} mutant cell clone marked by the absence of lacZ, stained with antibodies to β-galactosidase (red, **d, d′**). The clone border is outlined. The signal intensity of the activated MAPK (green in **d, d″**) in cells within the mutant clone is clearly weaker than in their wild-type neighbours. (**e**) Western blot analysis of protein extracts from 20 adult heads each detected a less strong signal of the activated MAPK in rg^{E16} as well as in heterozygous $Star^{E10}$ mutant heads in comparison to the wild type (upper panel). Lower panel: Loading control (α-actin). (**f**) Signals of three independent experiments were quantified by densitometry and normalized to the values of the loading control. This analysis reveals that the levels of activated MAPK are reduced by approximately 24% in the rg^{E16} mutants compared to the wild type. In two attempts, protein extracts of $Star^{E10}/+$ mutant heads were loaded as a positive control showing a reduction of at least 30% of activated MAPK protein

activity of activated MAPK within the mutant area was clearly weaker than in the neighbouring wild-type cells (Figure 4d–d''). Together, these experiments suggest that the PKA anchor protein Rg is required for normal EGFR signalling activity and thereby may regulate levels of survival signals in the developing retina.

JNK signalling is activated in *rg* mutants

Recently, it was shown that the JNK pathway promotes cell death during the development of imaginal discs.[5,6] We thus wondered whether the cell death observed in *rg* mutants is mediated by an increase in JNK signalling. We addressed this question two-fold. First we asked whether reduction in JNK signalling might rescue the *rg* mutant phenotype. Indeed, lowering the gene dosage of the JNKKKK *misshapen* (*msn*) or the JNK *basket* (*bsk*) by one-half significantly rescued the rg^{E16} rough eye phenotype as well as cone cell loss (Figure 5a–b'). Second, we addressed JNK activity directly by analysing the levels of the activated, that is, phosphorylated protein in wild type *versus* the *rg* mutant. Indeed, an increase up to 23% was observed in rg^{E16} mutants compared to wild-type heads, whereas in heterozygous bsk^1, protein levels were reduced by 16% (Figure 5f and g). This observation was further confirmed *in situ*: the activated JNK accumulates in cone cells of the wild-type[32] and much stronger in the remaining cone cells of rg^{E16} mutant retinae as well as in rg^{E16} mutant cell clones when compared to wild-type cells (Figure 5c–e''). We conclude that cone cell loss in *rg* mutants is mediated by an enforced signal of the JNK pathway.

Discussion

In this article, we focused on the analysis of the rough eye phenotype caused by mutations at the *rg* locus. Mutations in *rg* interfere with the correct pattern formation of the retina affecting nearly all retinal cell types to some degree, photoreceptor cells, cone and 2° pigment cells.[15,16,18] In

addition, optic lobe defects were described.[18] The major defect, however, is a loss of cone cells, which is the primary phenotype even in weaker alleles.[15,16] Our work followed in finer detail the fate of these cells. Previously, it was suggested that the cone cells fail to differentiate in the mutant.[16] However, the cone cells, which are born already during late third larval instar, express the Cut marker indistinguishable
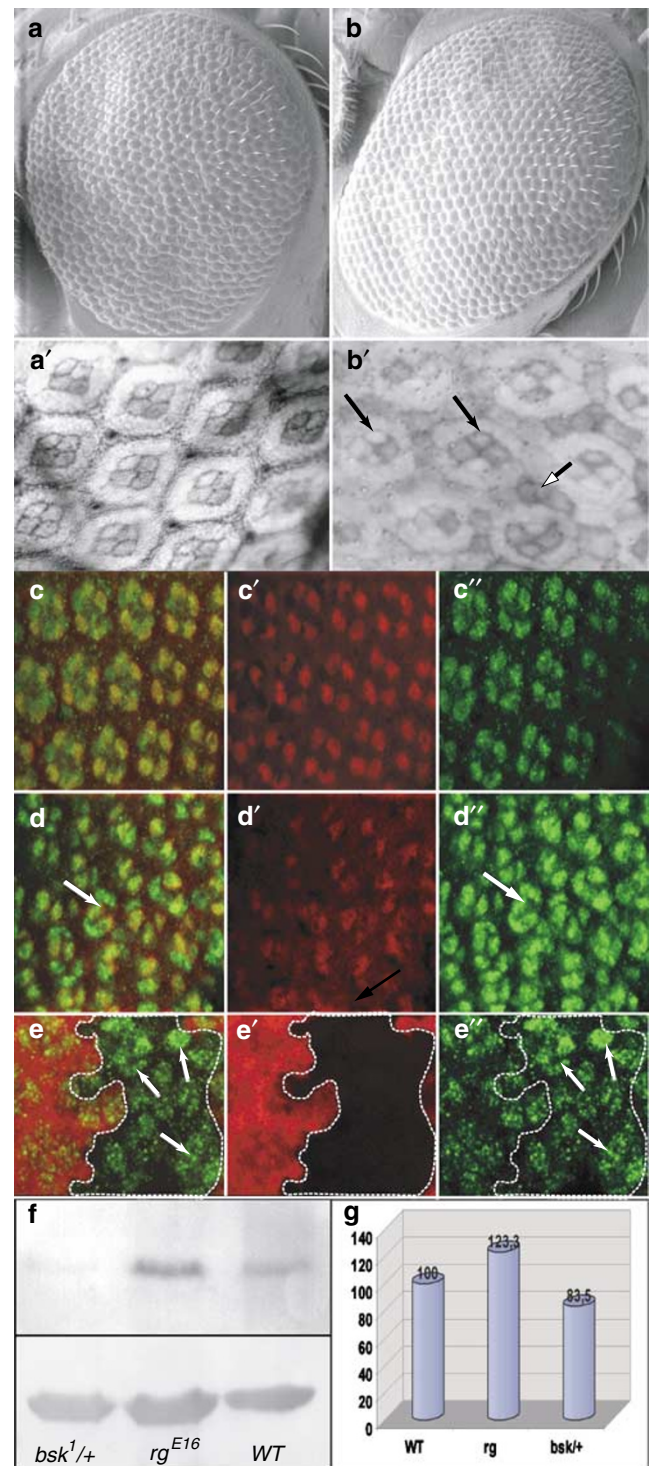
Figure 5 JNK pathway signalling is enforced in rg^{E16} mutant retinae. Reducing the activity of JNK pathway signalling in a heterozygous *msn* (**a–a'**) or *bsk* (**b–b'**) mutant background clearly suppresses the rg^{E16} rough eye phenotype (**a, b**; compare with Figures 2c and 3b) and the typical cone cell loss, visualized by cobalt sulphide stainings of the pupal retinae (**a', b'**, arrows). Moreover, programmed cell death of lattice cells in *bsk* heterozygotes is partly suppressed (**b'**, arrowhead). Activated JNK was detected with specific antibodies (JNK-P, green) in 48 h old wild-type (**c–c''**) and rg^{E16} pupae (**d–d''**). In comparison to the wild type, the mutant showed a stronger signal in the cone cells (α-Cut, red) (compare **c, c''** with **d, d''**). (**c**) and (**d**) are merged images. (**e–e''**) rg^{E16} mutant cell clone (clone border is outlined) marked by the absence of lacZ, stained with antibodies against β-galactosidase (red, **e, e'**). On average, we detected a stronger JNK signal within the mutant cone cells (green, **e, e''**, arrow), which is in line with the results we obtained in the rg^{E16} mutant retinae (**d**). The results were quantified by densitometry of Western blots (**f**). Data were compiled from three independent experiments, and for bsk^1 from two experiments (**g**). Protein extracts from 20 heads each (wild type, WT, rg^{E16} males and $bsk^1/+$ flies) were probed for activated JNK using anti-JNK-P antibodies (upper panel) and anti-actin antibodies (lower panel) for a loading control. (**g**) Quantification by densitometry of three independent experiments reveals an enhancement of activated JNK up to 23% in the rg^{E16} mutants in comparison to the wild type, whereas in the heterozygous bsk^1 mutants a reduction of JNK activity of 16% was observed

from wild type. This suggests that cone cell recruitment is not disturbed by the *rg* mutant. Instead, during mid-pupal stages, cone cells are lost by cell type-specific cell death, explaining the failure to detect these cell types in the pupal retinae.[16] We favour the hypothesis that the AKAP Rg might serve to integrate survival signals during retina development. Presumably, cone cells undergo a specific phase of high sensitivity towards such signals. Thus, a reduction in *rg* activity might cause sporadic to massive loss primarily of cone cells.

Our findings suggest that activation of the JNK pathway contributes to the apoptotic signal in *rg* mutant cone cells. In *Drosophila*, signalling through JNK is required for a variety of processes during embryonic and adult development.[33] Recently, it was observed that the JNK pathway might also function as a kind of 'developmental supervisor' during wing development.[5,6] There it was shown that a deregulation of the positional information maintained by Dpp and Wg signalling results in an induction of the JNK pathway followed by an increased level of apoptosis. Thus, the JNK pathway is obviously activated to maintain correct development when normal signalling is distorted.[5]

Cone cell development also depends on multiple inputs, mainly on the multifunctional Notch and EGFR signals and the transcriptional activator *lozenge*.[34,35] In *rg* mutants, the correct temporal activation level of these signals seems to be impaired, followed by a wrong combinatorial code for cone cell fate development. This could cause increase in JNK signalling and subsequently cone cell-specific cell death. Thus, ablation of cone cells by apoptosis may be a mechanism to proceed with normal retinal development despite impaired normal signalling.

Notch signalling counteracts cone cell death in *rg* mutants

Remarkably, an increased Notch signal, either by doubling the gene dose of *N* or halving the dose of the Notch antagonist *Hairless* (*H*), suppresses the *rg*-specific cone cell death. Notch signalling has previously been associated with both negative and positive effects on apoptosis. A constitutive activated Notch receptor is able to induce cancer and cell transformation probably by impairing apoptosis.[36,37] Moreover, it has been demonstrated that an activated form of Notch is able to inhibit apoptosis mediated by presenilin in *Drosophila*, leading to the speculation that an activated Notch signalling might protect from apoptotic cell death.[38] However, Notch has also been implicated in the induction of apoptosis during different developmental stages in *Drosophila*, for example, during terminal mitotic divisions of the *Drosophila* CNS, PNS and wing development.[39–41] Interestingly, genetic studies revealed that Notch is also able to induce apoptosis during eye development in the pupal retina.[9,42] There it was shown that Notch signalling within the interommatidial lattice cells, which surround the individual ommatidia, promotes cell death, whereas a signal mediated by the activated EGFR promotes cell survival.[10,14] In *rg* mutants, an increase in Notch signalling activity results in effects comparable to those of an enforced EGFR activation, indicating that both pathways

might adopt an antiapoptotic function and therefore behave as 'survival signals' in this developmental context.

How can *rg* influence the activity of the EGFR and other signalling pathways?

A kinase anchoring proteins (AKAPs) contribute to the spatial and temporal PKA signalling specificity by targeting PKA to specific substrates and subcellular compartments.[43] *rg* encodes for one of the two known A kinase anchor proteins in *Drosophila*.[15–17] Reducing the activity of the catalytic subunit of Pka in a *pka-C1^{01272}* heterozygous mutant background results in an enhancement of the *rg* mutant eye phenotype (not shown), supporting the involvement of *rg* in PKA signalling.[44] Although AKAPs are characterized on the basis of their interaction with PKA, they also serve as scaffolding proteins by binding other signalling molecules like kinases and phosphatases as well as components of various other signalling pathways.[45,46] We observe that in *rg* mutants the signalling through the canonical MAP kinase pathway was clearly decreased. This cascade proceeds from the membrane-bound GTP-binding protein Ras, to the sequential activation of at least three Ser-Thr kinases leading to the specific activation of nuclear target genes.[47] Reducing the activity of *rg* might cause mislocalization of activated PKA molecules to the plasma membrane thereby reducing the basal activity of PKA important for the tight regulation of this pathway. Moreover, by interfering with the Ser-Thr kinases itself, *rg* might be important to ensure that signals pass quickly from one kinase to the next following, thus preventing an interplay with functionally unrelated kinases in the same cell.

Interestingly, in addition to their interaction with signal terminators like kinases or phosphatases, AKAPs also assemble signals derived from multiple pathways into multiprotein signalling complexes organizing responses to external and intrinsic signals in different organisms.[45,46,48] One might speculate that *rg* is an important factor for the integration and precise regulation of the different factors known to be involved in cone cell development.

Materials and Methods

Fly work

analysed with a digital scanning microscope from Zeiss and the Orion version 6 software.

Staining procedures

Pupal eye discs shown in this paper (40–48 h after pupal formation) were dissected in PBS and fixed in 4% paraformaldehyde for 20 min. After washing four times with PBT (PBS + 0.1% Tween) and blocking for 1 h with 4% normal goat serum, the retinae were incubated overnight at 4°C with the respective primary antibodies. After several washes in PBT, respective goat secondaries coupled to DTAF or Cy3 were added (1 : 200; Jackson Immuno Research). The following primary antibodies were used: mouse anti-Cut (1 : 20), obtained from the Developmental Studies Hybridoma Bank (University of Iowa, NICHD contract NO1-HD-7-3262), rabbit anti-BarH1,[20] rabbit anti-cleaved Caspase-3 (1 : 200; NEB Cell Signalling Technology), rabbit-anti β-Gal (1 : 5000; Rockland, USA), rabbit anti-active MAPK (1 : 100) and rabbit anti-active JNK-P (1 : 500; both Promega). The specificities of the diPERK and JNKP antibodies were controlled with the help of the Gal4/UAS system: overexpression of the dominant-negative form of EGFR (UAS EGFR[DN])[55] using EqGal4[56] results in a loss of diPERK staining along the dorso-ventral boundary in pupal retinae, whereas the overexpression of the activated JNKK (UAS hep[act])[31] leads to an increase in JNKP staining.

Pictures were taken with a BioRad MRC 1024 confocal system linked to a Zeiss Axioskop using BioRad Laser Sharp 3.1 software. Cobalt sulphide staining was performed as previously described by Wolff and Ready.[8]

Detection of activated kinases

In all, 20 heads each of rg^{E16} hemizygous males, wild-type OregonR1 males and heterozygous $Star^{E10}$ and bsk^1 males were homogenized in 100 μl Em-Lys-buffer (10 mM HEPES pH 7.6, 5 mM EGTA, 5 mM EDTA, 1% Triton X-100, 1 μl protein inhibitor cocktail (Roche)) and boiled for 5 min. After pelleting the debris, 10 μl of each homogenate was separated on 10% PAGE and blotted on nitrocellulose. Membranes were probed with rabbit anti-active JNK-P (1 : 500, Promega, recognizes a 44 kDa band), rabbit anti-active MAPK (1 : 500, Promega, recognizes an 80 kDa band) and rabbit anti-actin (1 : 250, Sigma, recognizes a band at 42 kDa) antibodies as a loading control. The signals were quantified by densitometric scanning of the dry membranes using the wincam software program and normalized with the actin control.

Acknowledgements

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