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Argos induces programmed cell death in the developing *Drosophila* eye by inhibition of the Ras pathway

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Abstract

We studied the role of Ras signaling in the regulation of cell death during *Drosophila* eye development. Overexpression of Argos, a diffusible inhibitor of the EGF receptor and Ras signaling, caused excessive cell death in developing eyes at pupal stages. The Argos-induced cell death was suppressed by coexpression of the anti-apoptotic genes *p35*, *diap1*, or *diap2* in the eye as well as by the *Df(3L)H99* chromosomal deletion that lacks three apoptosis-inducing genes, *reaper*, *head involution defective* (*hid*) and *grim*. Transient misexpression of the activated Ras1 protein ($Ras1^{V12}$) later in pupal development suppressed the Argos-induced cell death. Thus, Argos-induced cell death seemed to have resulted from the suppression of the anti-apoptotic function of Ras. Conversely, cell death induced by overexpression of Hid was suppressed by gain-of-function mutations of the genes coding for MEK and ERK. These results support the idea that Ras signaling functions in two distinct processes during eye development, first triggering the recruitment of cells and later negatively regulating cell death.

Keywords: Argos; apoptosis; compound eye; *Drosophila*; programmed cell death; Ras

Abbreviations: PCD, programmed cell death; *rl*, *rolled*; *Dsor1*, *Downstream suppressor of raf 1*; PBS, phosphate buffered saline; IAP, Inhibitor of apoptosis protein; DIAP1, *Drosophila* IAP1; DIAP2, *Drosophila* IAP2; *hid*, *head involution defective*; *rpr*, *reaper*; *DER*, *Drosophila EGF receptor*; ICE, interleukin-1β-converting enzyme

Introduction

Programmed cell death (PCD), or apoptosis, is a form of cell death in which supernumerary or harmful cells are removed from an organism. Many lines of evidence indicate that PCD is an active, gene-directed process, the underlying mechanisms of which are highly conserved throughout animal evolution (Steller, 1995; White and Steller, 1995). The genetic cascade underlying PCD has been most intensively characterized in the nematode, *Caenorhabditis elegans* (reviewed by Ellis *et al*, 1991; Hengartner and Horvitz, 1994). In particular, three nematode genes, *ced-9*, *ced-4*, and *ced-3* have been identified as PCD regulators. Of these, *ced-3* and *ced-4* are required for all PCD onset (Ellis and Horvitz, 1986), whereas *ced-9*, in turn, suppresses the PCD induced by *ced-3* and *ced-4* (Hengartner *et al*, 1992). The *ced-9* gene is homologous to the human anti-apoptotic gene *bcl-2* (Tsujimoto *et al*, 1985; Hengartner and Horvitz, 1994). In addition, a mammalian gene *Apaf-1* that shows significant homology with *ced-4* has been identified recently (Zou *et al*, 1997). The *ced-3* gene is homologous to a family of cysteine proteases, called caspases, which includes the interleukin-1β-converting enzyme (ICE) (Yuan *et al*, 1993; Miura *et al*, 1993). It has been shown that the inhibition of caspase activity by the baculovirus p35 protein can prevent PCD in nematodes (Sugimoto *et al*, 1994), insects (Hay *et al*, 1994) and mammals (Rabizadeh *et al*, 1993). Thus, components of the PCD pathway have been highly conserved throughout evolution. Despite good progress in identifying cell death genes, we do not understand much about the molecular mechanisms of PCD at present. The fruit fly, *Drosophila melanogaster*, provides a powerful tool for investigating the mechanisms of PCD using a number of sophisticated genetic strategies.

In *Drosophila*, three genes, *reaper* (*rpr*) (White *et al*, 1994), *head involution defective* (*hid*) (Grether *et al*, 1995), and *grim* (Chen *et al*, 1996) have been identified as playing a central role in the initiation of PCD. Genetic studies have revealed that these three genes are likely to function independently to induce PCD via a caspase pathway (White *et al*, 1996; Grether *et al*, 1995; Chen *et al*, 1996). Among these genes, *rpr* has been best characterized. The transcription of *rpr* is activated by stimuli that cause PCD, such as X-ray irradiation and aberrant development (Nordstorm *et al*, 1996). Thus, the induction of *rpr* expression (and also of *grim* and *hid*, possibly) and the subsequent activation of the caspase family are likely to represent a 'central pathway' for PCD in *Drosophila*. PCD in *Drosophila* is known to be controlled by a number of distinct signals, including extra- and intracellular signals, blockage of cell differentiation, and X-ray induced damage (reviewed by McCall and Steller, 1997). However, the signaling machineries by which the various stimuli activate the central pathway of PCD remain largely unknown.

The *Drosophila* compound eye is a useful system for studying the mechanisms of PCD as well as cell-fate decisions. The compound eye is composed of about 750 repeating units called ommatidia. Each ommatidium contains the same number of cells, including eight

photoreceptor cells (R1-8), four cone cells (lens-secreting cells) and two primary pigment cells. There are also six secondary and three tertiary pigment cells, shared by neighboring ommatidia. Since each ommatidium is composed of exactly the same complement of cells, it is tempting to speculate that there are strict regulatory mechanisms controlling the precise number of each cell type within an ommatidium. An increasing number of such regulatory cues have been shown to be involved in this process: e.g., an inductive event mediated by receptor tyrosine kinases (reviewed by Freeman, 1997) and lateral inhibition accomplished through several mechanisms (reviewed by Sawamoto and Okano, 1996). PCD also plays an important role in regulating the number of cells within each ommatidium.

It is known that massive PCD occurs in the pupal eye, resulting in the elimination of two to three cells per ommatidium (Wolff and Ready, 1991). This event is known to be required as a final step in pattern formation to eliminate supernumerary cells associated with each cluster and to establish the highly ordered lattices of secondary and tertiary pigment cells (Cagan and Ready, 1989; Bonini, 1997). It is generally known that PCD is induced in the *Drosophila* compound eye when normal development, including cell recruitment, is impaired by mutations or events (reviewed by Bonini, 1997). However, much remains to be elucidated about the common regulatory mechanisms required for both cell recruitment and PCD in the developing eye disc. The Ras signaling pathway has been shown to be required for triggering the differentiation of all the cell types in the eye (reviewed by Freeman, 1997). Although Ras has also been implicated in PCD in other systems (reviewed by Pritchard and McMahon, 1997), the role of the Ras pathway in the regulation of PCD during *Drosophila* development is still unclear. In this regard, Freeman (1994) has reported a noteworthy observation that overexpression of Argos, a secreted protein with an EGF-like domain that acts as an inhibitor for the *Drosophila* EGF receptor (DER) and the Ras pathway (Okano *et al*, 1992; Freeman *et al*, 1992; Kretzschmar *et al*, 1992; Schweitzer *et al*, 1995a; Sawamoto *et al*, 1996), causes excessive cell death. This result suggests involvement of Ras signaling in PCD in the developing eye. In the present study, we have characterized the cell death caused by the overexpression of Argos in detail and found a novel function of Ras signaling that prevents PCD in the eyes.

Results

Overexpression of *argos* driven by an eye-specific promoter causes cell death during pupal development

To target *argos* overexpression specifically to the eye, we used the pGMR vector to generate transgenic flies in which *argos* expression was under the control of tandemly-arrayed regulatory elements of the eye-specific transcription factor Glass (Hay *et al*, 1994). The phenotype of the transgenic flies carrying one copy of the *GMR-argos* transgene was similar to that of *HS-argos* (Sawamoto *et al*, 1994; Freeman, 1994; Brunner *et al*, 1994), i.e., a decrease in the numbers of photoreceptor, cone, and pigment cells was observed (see Figure 3). The phenotype appeared to be sensitive to the dosage of the transgene, since flies carrying more copies of the *GMR-argos* transgene showed much stronger phenotypes (data not shown). In this study, we always used flies with one copy of the *GMR-argos* transgene. To determine if overexpressed Argos caused cell death, we stained live discs with acridine orange. In eye discs from wild-type third-instar larvae, dying cells stained with acridine orange were seen both anterior and posterior to the morphogenetic furrow (Figure 1A), as previously described (Wolff and Ready, 1991). The pattern of cell death in the *GMR-argos* larval discs (Figure 1B) was indistinguishable from wild-type (Figure 1A). We also examined photoreceptor differentiation by labeling larval eye discs with anti-Elav antibody, a nuclear marker for all *Drosophila* neurons. No obvious defects were discerned in the *GMR-argos* eyes (data not shown). Approximately 32–35 h after puparium formation (APF), massive PCD occur in the wild-type eyes to eliminate excess cells (Wolff and Ready, 1991; Figure 1C). In the *GMR-argos* eyes, the number of dead cells was

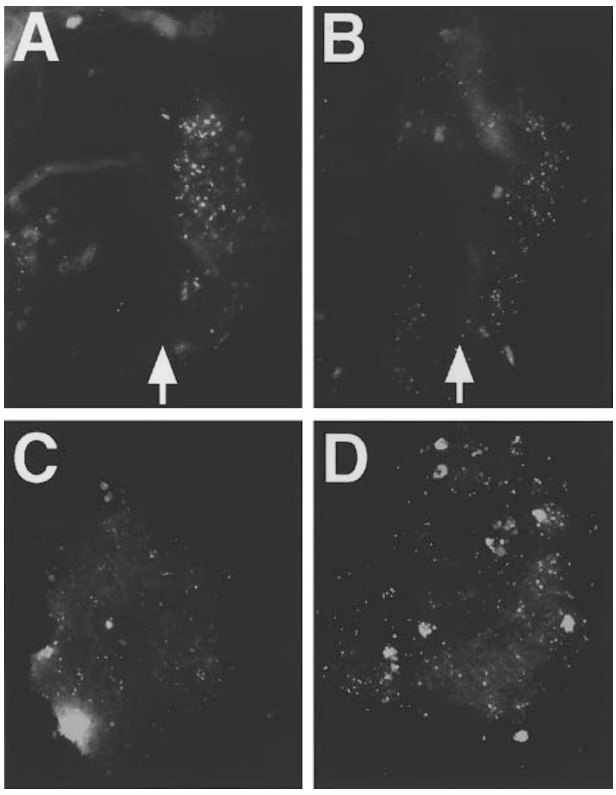

Figure 1 Overexpressed Argos induces excessive cell death in the pupal retina (**A**) Wild-type third instar eye-antennal disc stained with acridine orange. A small number of dying cells are observed within the eye disc. Anterior is to the left. (**B**) Third instar eye-antennal disc from *GMR-argos*/+larva. The level of cell death is indistinguishable from wild-type. Anterior is to the left. (**C**) Wild-type retina at 35 h APF. Excess pigment cells stained with acridine orange are eliminated by apoptosis at this stage. (**D**) Retina from *GMR-argos*/+pupa at 35 h APF. The number of dying cells stained with acridine orange is significantly increased over wild-type. The arrows in (**A**) and (**B**) indicate the position of the morphogenetic furrow

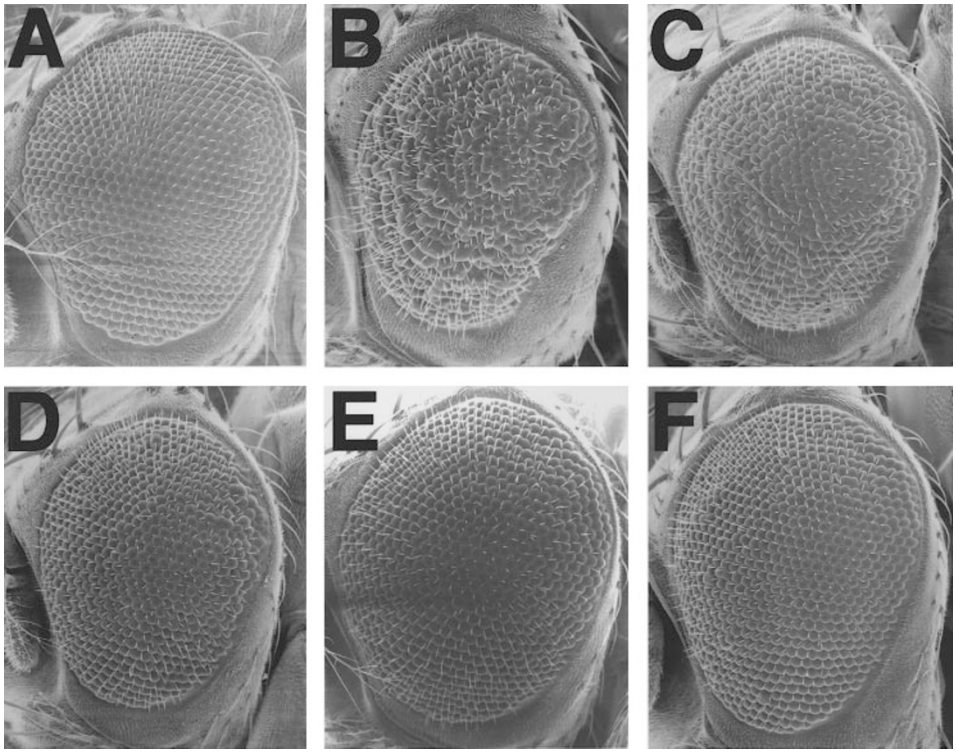

Figure 2 The phenotype of the *GMR-argos* eye is suppressed by co-expression of anti-apoptotic genes and in the *H99* mutation background. (**A**) Wild-type compound eye, composed of a regular array of about 750 ommatidia. (**B**)*GMR-argos*. Note the slight reduction in size and the rough appearance. The phenotype of *GMR-argos* is significantly suppressed by co-expression of the anti-apoptotic genes *p35* (**C**), *diap1* (**D**), and *diap2* (**E**). The *H99* deletion also acts as a dominant suppresser of *GMR-argos* (**F**)

considerably increased in the pupal retina (Figure 1D), as observed previously for *HS-argos* eyes (Freeman, 1994). Thus, one copy of the *GMR-argos* transgene is unlikely perturb the neuronal differentiation and cell death in larval discs, but induces excessive cell death during pupal development.

Cell death in the *GMR-argos* eyes is mediated by caspases and apoptotic activators encoded in the 75C1-2 region

To analyze the mechanisms underlying the excessive cell death in the pupal eyes caused by Argos overexpression, we examined the effects of coexpressing anti-apoptotic factors that act as inhibitors for caspases. Expression of baculovirus p35, which acts as an inhibitor of caspases (Bump *et al*, 1995), is known to prevent PCD in *Drosophila* (Hay *et al*, 1994). Expression of either Diap1 or Diap2, *Drosophila* homologues of the baculovirus inhibitor of apoptosis proteins (IAPs), also prevents PCD in the eye (Hay *et al*, 1995). *GMR-argos* flies were crossed to the files in which *p35*, *diap1*, or *diap2* were expressed specifically in the eyes using the pGMR vector. All of the *GMR-p35* (Hay *et al*, 1994), *GMR-diap1* (Hay *et al*, 1995), and *GMR-diap2* (Hay *et al*, 1995) flies have a mild rough-eye phenotype due to the blockage of normally occurring cell death. *GMR-argos* eyes have a severely rough phenotype and a slightly smaller size (Figure 2B) compared with wild-type (Figure 2A). The *GMR-argos* phenotype was significantly suppressed by the coexpression of any of the anti-apoptotic genes, *p35* (Figure 2C), *diap1* (Figure 2D), and *diap2* (Figure 2E), implying that the overexpression of Argos caused PCD through a common apoptotic pathway that included the activation of caspases.

It is known that the 75C1-2 region of the *Drosophila* genome is essential for all PCD that normally occur during embryogenesis (White *et al*, 1994). The *Df(3L)H99* deletion mutation lacks at least three genes coding for the apoptotic activators, *rpr* (White *et al*, 1994), *hid* (Grether *et al*, 1995), and *grim* (Chen *et al*, 1996) in this 75C1-2 region. To analyze the effects of halving the dosage of genes in this region on the PCD caused by overexpressed Argos in the eyes, we crossed the *GMR-argos* flies to the *Df(3L)H99* mutants. As shown in Figure 2F, the *Df(3L)H99* mutation dominantly suppressed the eye phenotype of the *GMR-argos*. Therefore, gene(s) at 75C1-2 such as *rpr*, *hid*, or *grim* are likely to play essential roles in PCD in the *GMR-argos* eyes.

To determine the cell types that were eliminated by PCD from the *GMR-argos* eyes, sections of adult heads and pupal retinae stained with cobalt sulfide were examined (Figure 3). In wild-type flies, each ommatidium contains eight photoreceptor cells, seven of which can be observed in one tangential section (Figure 3A). Secondary and tertiary pigment cells form highly ordered pigmented lattices surrounding each ommatidium (Figure 3A). The

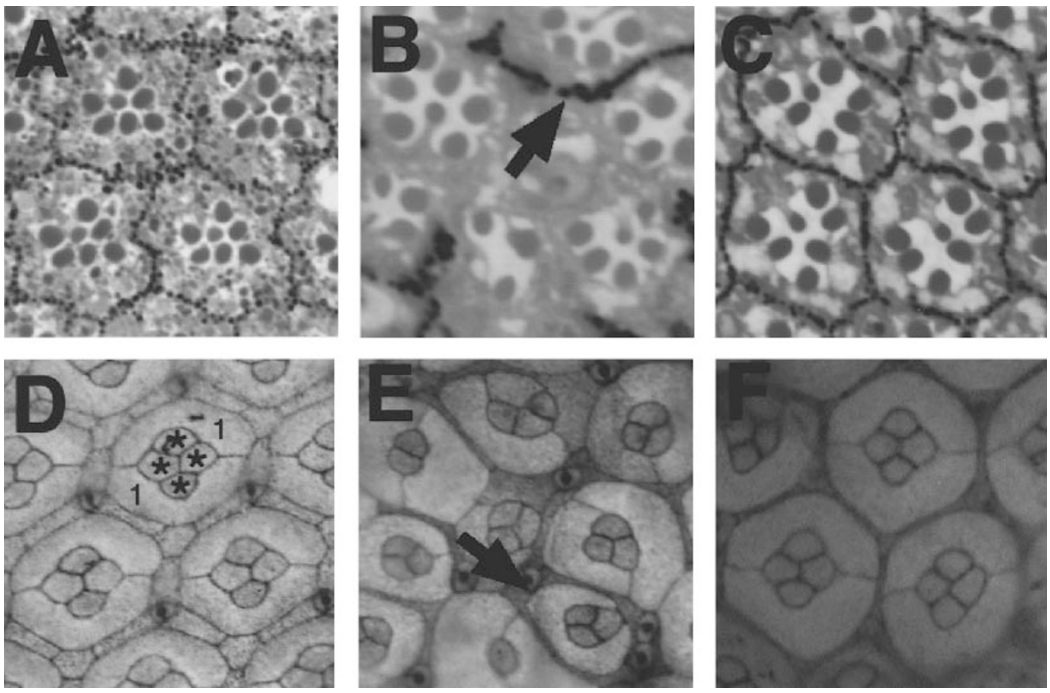

Figure 3 Expression of *diap1* restores all the cell types in the *GMR-argos* eyes. (**A–C**) Sections of adult eyes of wild-type (**A**), *GMR-argos*/+(**B**) and *GMR-diap1*/+; *GMR-argos*/+(**C**) flies. (**A**) In wild-type, each ommatidium contains eight photoreceptor cells, seven of which can be observed in one tangential section. Secondary and tertiary pigment cells form pigmented lattices surrounding each ommatidium. (**B**) The *GMR-argos*/+ eye shows a reduced number of photoreceptor cells and loss of pigmented lattice. The residual pigmented lattices are marked by an arrow. (**C**) Co-expression of *diap1* restores the photoreceptor cells and pigmented lattice in the *GMR-argos* eye. (**D–F**) Cobalt sulfide staining of the wild-type (D), *GMR-argos*/+ (**E**), and *GMR-diap1*/+; *GMR-argos*/+ (**F**) flies. (**D**) In the wild-type eye, the apical surface of each ommatidium displays the outline of four cone cells (indicated by asterisks) and two primary pigment cells (indicated by 1). (**E**) Overexpression of *argos* causes a reduction in the number of cone and primary pigment cells. The arrow indicates an ommatidium that contains three cone cells and one primary pigment cell. (**F**) Normal numbers of both cone and primary pigment cells are seen in the *GMR-diap1*/+; *GMR-argos*/+ommatidia

GMR-argos eye showed a reduced number of photoreceptor cells and loss of pigmented lattices (Figure 3B). This phenotype was almost completely suppressed by the co-expression of *diap1* (Figure 3C). The apical surface of each ommatidium on the wild-type retinae at 40 h APF displays the outline of four cone cells and two primary pigment cells (Figure 3D). Overexpression of *argos* caused a reduction in the number of cone and primary pigment cells (Figure 3E). In contrast, in the *GMR-argos*/*GMR-diap1* eyes, we observed the normal number of cone and primary pigment cells in most of the ommatidia (Figure 3F). Coexpression of either *p35* or *diap2*, or the *Df(3L)H99* mutation, also showed similar suppressive effects on the *GMR-argos* phenotype (data not shown). These results indicate that loss of all the cell types in the *GMR-argos* eyes is caused by the enhanced PCD, mediated – at least in part – through the actions of caspases and the cell-death activators encoded by a gene or genes in the 75C1-2 region.

Ras activation later in pupal development prevents cell death in *GMR-argos* eyes

It is known that PCD is enhanced when cell recruitment is impaired in the developing *Drosophila* eye disc (reviewed by Bonini, 1997). Thus, it is possible that the increased PCD in the *GMR-argos* eyes is the result of impaired cell recruitment due to the inhibition by overexpressed Argos of EGF receptor signaling and subsequent Ras signaling, since EGF receptor signaling is known to be required to trigger the differentiation of all the cell types in the eye (Freeman, 1996). Alternatively, the Ras signaling activity, which is suppressed by the overexpressed Argos, could regulate PCD without affecting the recruitment of retinal cells. To test this possibility, we examined the effect of transiently expressing the constitutively activated Ras1 protein ($Ras1^{V12}$), a downstream effector of the EGF receptor, under the control of the heat-inducible *hsp70* promoter. The wild-type ommatidium is composed of four cone cells, two primary pigment cells, six secondary pigment cells, and three tertiary pigment cells (Figure 4A). Transient expression of $Ras1^{V12}$ by heat shock during early pupal development (0–5.5 h APF) caused an increase in cone cell-like cells (Figure 4B). Heat shocks during late pupal development (20–40 h APF), however, had no effect on cone cell formation, but caused an increase in the number of secondary and tertiary pigment cells (Figure 4C). The time at which over-recruitment of each cell type is induced by expression of the activated Ras1 protein (summarized in Figure 4D) corresponds well with the start of its differentiation (Cagan and Ready, 1989) and the time at which each cell type is sensitive to the inhibition of DER function (Freeman, 1996).

We utilized the cell-type specific effect of transient $Ras1^{V12}$ expression to examine the function of Ras1 in

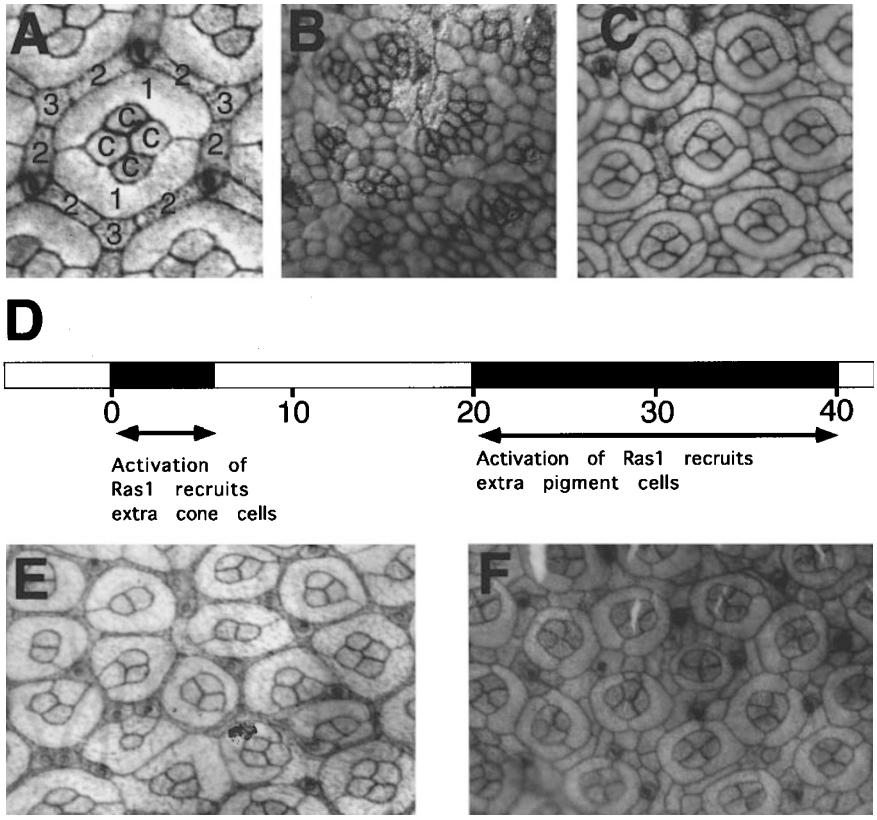

Figure 4 Effects of transient Ras activation on eye development at the pupal stage. Cobalt sulfide staining of retinae at 40 h APF. (**A**) In the wild-type ommatidium, four cone cells (*c*), two primary pigment cells (*1*), six secondary pigment cells (*2*), and three tertiary pigment cells (*3*) are visible. This pattern was not altered by heat-shock at any time during pupal development. (**B – C**) Expression of Ras1^{V12} was induced at 0 – 6 h (**B**) or 20 – 40 h (**C**) with heat shocks and stained with cobalt sulfide at 40 h APF. (**B**) Heat shocks at early pupal stage cause recruitment of extra cone-cell like cells. (**C**) Heat shocks at 20 – 40 h APF cause recruitment of extra pigment cells but do not affect the cone cell number. (**D**) Summary of the effects of Ras1 activation at different times in eye development of wild-type flies. The times are shown as hours after pupation at 25°C. (**E**) *GMR-argos*/+. Note that the number of cone cells is reduced. (**F**) Retina from the *HS-Ras1*^{V12}/+; *GMR-argos*/+fly heat shocked at 20 – 40 h APF. Most of the ommatidia contain normal numbers of cone cells

regulating PCD in the *GMR-argos* eyes. Here, we focused on cone cell death, since we could not distinguish the effect of Ras1^{V12} on the differentiation of pigment cells from its effect on the regulation of PCD (see Discussion). The *HS-Ras1*^{V12}/+; *GMR-argos*/ +eyes showed a slightly weaker phenotype than *GMR-argos* even without heat shock (data not shown), suggesting that Ras1^{V12} expression is constitutively induced at a low level at 25°C. Flies carrying both the *GMR-argos* and *HS-Ras1*^{V12} transgenes were heat-shocked at the late pupal stage (20 – 40 h APF) to exclude the effects of *HS-Ras1*^{V12} on the recruitment of cone cells. The transient expression of *Ras1*^{V12} at 20 – 40 h APF caused a variable but significant reduction in the loss of cone cells in the *GMR-argos* eyes. Heat shocks at 20 – 40 h APF did not cause any visible effect on eye development in the flies without the *HS-Ras1*^{V12} transgene: about half of the ommatidia contained reduced numbers of cone cells in the *GMR-argos* flies (Figure 4E). Heat shocks of the *HS-Ras1*^{V12}/+; *GMR-argos*/+flies at the same stage, however, significantly increased the population of the ommatidia that contained four cone cells (Figure 4F). Therefore, the cone cell death in the *GMR-argos* eyes is likely to result from suppression of the anti-apoptotic effect of the Ras-pathway at 20 – 40 h APF by Argos. The loss of pigment cells by overexpressed Argos was also suppressed by the expression of *Ras1*^{V12} at the late pupal stage (Figure 4F). It is possible that this effect of *Ras1*^{V12} on pigment cells is caused by both overrecruitment of cells and suppression of PCD.

Dsor1 and rolled function to prevent PCD induced by Hid

We next examined the function of the Ras pathway in preventing PCD initiated by stimuli other than Argos. To assess the capacity of other downstream components of the Ras pathway to regulate PCD, we analyzed the effects of hyperactivation of MEK and ERK on ectopic cell death caused by the overexpression of Hid in the eyes. Flies carrying one copy of the *GMR-hid* transgene, where the *hid* gene was expressed specifically in the developing eyes, exhibited near ablation of the adult compound eyes (Grether *et al*, 1995; Figure 5A). We found that the *GMR-hid* phenotype was markedly suppressed by *Dsor1*^{Su1} (Tsuda *et al*, 1993) and *rl*^{Su23} (Sawamoto *et al*, 1996), gain-of-function mutations of the genes encoding MEK and ERK, respectively. The external morphology of the *Dsor1*^{Su1} eye is indistinguishable from wild-type (Lim *et al*, 1997), while the *rl*^{Su23} flies have roughened but

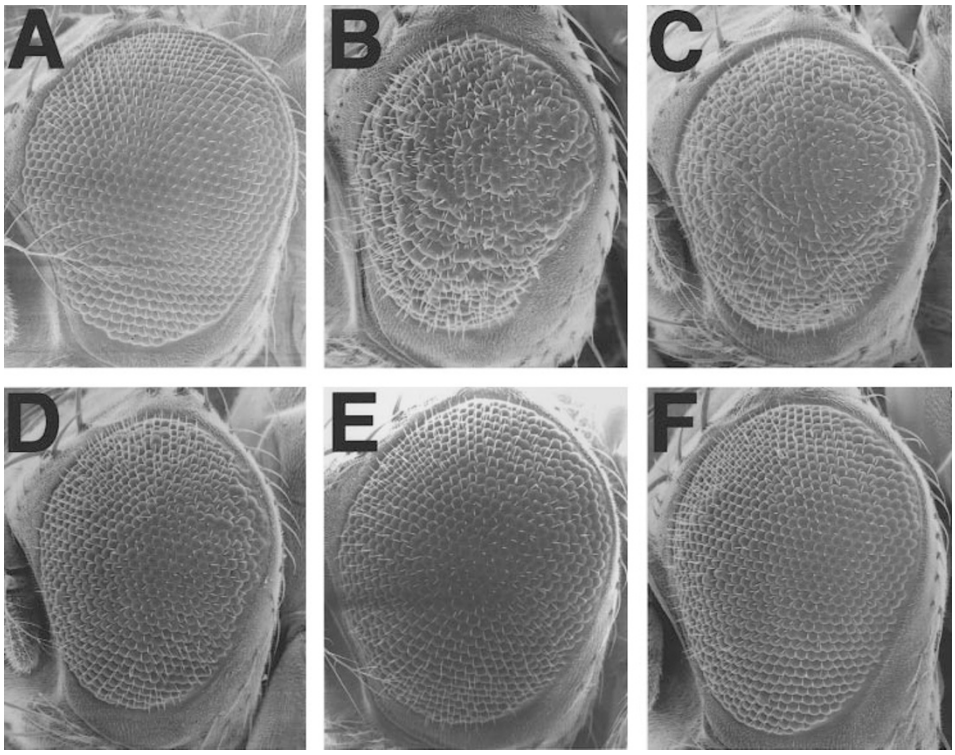

Figure 5 Hyper-activated MEK and MAPK signaling suppress Hid-induced cell death. (**A–C**) Scanning electron micrographs of compound eyes. (**A**) *GMR-Hid*/+. (**B**) *Dsor1*^{Su1}/Y; *GMR-hid*/+. (**C**) *GMR-Hid*/*rl*^{Su23}. Expression of *hid* in the developing retina causes eye ablation (**A**). The gain-of-function mutations of the genes encoding for MEK (**B**) and ERK (**C**) restore the ommatidia. (**D–F**) Acridine orange staining of eye discs from *GMR-Hid*/+(**D**), *Dsor1*^{Su1}/Y; *GMR-hid*/+(**E**), and *GMR-Hid*/*rl*^{Su23} (**F**) larvae. Anterior is to the left. Arrows indicate the position of the morphogenetic furrow. High levels of excessive cell death are present posterior to the morphogenetic furrow in the *GMR-Hid*/+eye discs (**D**). The excessive cell death induced by Hid was significantly suppressed by the mutations, *Dsor1*^{Su1} (**E**) and *rl*^{Su23} (**F**)

normal-sized eyes (Sawamoto *et al*, 1996). The eye tissue removed by Hid was restored in males carrying an X chromosome with the *Dsor1*^{Su1} mutation (Figure 5B), whereas females heterozygous for the *Dsor1*^{Su1} mutation showed less suppression (data not shown). The eye-ablation phenotype of *GMR-hid* was also significantly suppressed by one copy of the *rl*^{Su23} mutation (Figure 5C).

To examine the extent of cell death in larval eye discs, the live discs were labeled with acridine orange. In *GMR-hid* eye discs, increased numbers of dying cells were observed posterior to the morphogenetic furrow (Figure 5D), compared with wild-type (Figure 1A). The Hid-induced cell death in the eye discs was considerably suppressed by either *Dsor1*^{Su1} (Figure 5E) or *rl*^{Su23} (Figure 5F). These results indicate that MEK and ERK act to suppress the ectopic cell death induced by overexpression of Hid.

Discussion

Ras-signaling in the developing *Drosophila* eye is triggered by two receptor tyrosine kinases, Sevenless (Sev) and DER. Sev is required for only the R7 photoreceptor cells (reviewed by Zipursky and Rubin, 1994), while DER is used to induce differentiation of all the cell types (Freeman, 1996). Thus, previous studies have proved that the Ras signaling pathway plays a key role in triggering the differentiation of retinal cells (reviewed by Freeman, 1997). In this study, we examined another role for the Ras-signaling system as a negative regulator of PCD, using a well-characterized model system, the developing *Drosophila* eye.

Interfering genetically with the ability of cells to respond to inductive signals that activate the Ras pathway in the larval eye discs leads to cell death (Rebay and Rubin, 1995; Freeman, 1996). Two transgenic lines, *GMR-yan*^{ACT} (Rebay and Rubin, 1995) and *GMR-DN-DER* (Freeman, 1996) show impaired neuronal differentiation and excessive cell death in larval eye discs. It is possible that the presence of undifferentiated cells that fail to be recruited due to the blockage of Ras signaling may cause PCD immediately in the larval eye discs, since PCD is caused by various developmental defects independent of the Ras pathway (reviewed by McCall and Steller, 1997). Since Argos also acts as an inhibitor of DER (Schweitzer *et al*, 1995b) and Ras signaling (Sawamoto *et al*, 1996), overexpressed Argos could have blocked differentiation by preventing the retinal cells from responding to Spitz, a ligand for DER (Schweitzer *et al*, 1995). However, the pattern of neuronal differentiation and cell death in the *GMR-argos* eye discs appeared nearly normal at the third instar. The differences between the eye phenotypes seen in *GMR-argos* flies at the third instar *versus* adult flies are likely to have resulted from cell death events occurring in the pupal stage. The idea that enhanced PCD in the pupal eye disc is likely to have caused the reduced number of

retinal cells in *GMR-argos* adult flies is supported by the following evidence. First, overexpression of Argos caused excessive cell death in the pupal retina (Freeman, 1994; Figure 1D). Second, the co-expression of the anti-apoptotic gene, *diap1*, suppressed the eye phenotype of the adult *GMR-argos* flies (Figure 3; discussed later). Third, there was a reduced number of retinal cells.

The phenotypes caused by one copy of the *GMR-argos* transgene are different from those of DN-DER and Yan^{ACT}, which are also negative (and possibly stronger) regulators of Ras signaling. DN-DER and Yan^{ACT} caused both suppression of cell recruitment and enhanced PCD in third instar eye discs (Rebay and Rubin, 1995; Freeman, 1996). Flies carrying four copies of the *GMR-argos* transgene showed a small-eye phenotype similar to *GMR-DN-DER* and *GMR-yan*^{ACT} (data not shown). Therefore, it is possible that the inhibitory effect of one copy of the *GMR-argos* transgene on Ras signaling is not strong enough to prevent the onset of cellular differentiation in the larval eye discs, but causes PCD at pupal stages through another mechanism. Thus, the eye of the flies with one copy of the *GMR-argos* transgene provides us a unique opportunity to investigate the role of Ras signaling in the regulation of PCD during pupal development.

During late pupal development, 1500–2000 cells in the wild-type *Drosophila* eye are eliminated by PCD to form the ordered cellular lattices of pigment cells (Cagan and Ready, 1989; Wolff and Ready, 1991). Inhibition of this PCD by ectopically expressed anti-apoptotic genes results in the survival of extra secondary and tertiary pigment cells (Hay *et al*, 1994, 1995). Similar overproduction of the pigment cells can also result from several genetic conditions that activate Ras signaling, e.g., loss-of-function *argos* mutants (Freeman *et al*, 1992), overexpression of the secreted Spitz protein (Freeman, 1996), and overexpression of the constitutively-activated Ras1 protein (this paper). On the other hand, the inhibition of DER signaling by the expression of Argos or dominant-negative DER causes a reduction in the number of pigment cells (Sawamoto *et al*, 1994; Freeman, 1994, 1996). These results suggest the possibility that Ras signaling functions to prevent pigment cell precursors from undergoing normally occurring PCD. Unfortunately, however, the periods for recruitment of the pigment cells by Ras with that for their elimination by PCD overlap, making it difficult to clarify the role of Ras in pigment cell development. Practically, we could not distinguish the effect of the $Ras1^{V12}$ expression on the regulation of PCD from its role in differentiation (Figure 4), so we focused on another cell type, the cone cell. It appears that the loss of cone cells in the *GMR-argos* pupal retina is suppressed by transient expression of the activated Ras1 protein at 20–40 h APF (Figure 4). During this period, Ras signaling acts to recruit only pigment cells but not cone cells (Figure 4). Therefore, the Argos-induced death of cone cells seems to have resulted from the suppression of an anti-apoptotic function of Ras rather than from impaired cell recruitment. It is also possible that unknown factor(s) produced by pigment cells are responsible for the suppression of cone cell death. It is important to note that the *hsp70* promoter is rather leaky. In fact, we observed that the *HS-Ras1*^{V12} transgene could considerably restore the cone cells even without heat shock in the *GMR-argos* eyes. It is most likely that the restoration of cone cells is due to both of the functions of Ras1, i.e., promotion of cone cell recruitment (caused by the leaky expression) in early pupal development and suppression of cell death at a later stage.

Suppression of the *GMR-argos* phenotype by co-expression of p35 or the IAP homologues (Figures 2 and 3) indicates that the loss of each cell type in the *GMR-argos* eye is mainly caused by excessive PCD. Since both p35 and the IAPs act as inhibitors of caspases (Bump *et al*, 1995; Deveralux *et al*, 1997), inhibition of Ras signaling by Argos may cause PCD through the activation of caspases. Two *Drosophila* caspases, DCP-1 (Song *et al*, 1997) and DrICE (Frazer and Evan, 1997) have been identified recently. These two caspases show high homology with mammalian caspases 3 and 6 (CPP32 and MCH2). Both the *Drosophila* proteins have been shown to cleave p35 as a substrate and induce PCD in cultured cells (Song *et al*, 1997; Frazer and Evan, 1997), but its function during eye development remains to be elucidated. Further analyses of the phenotypes of the caspase mutants will be required to clarify the role of Ras signaling in the regulation of caspases.

Several candidate genes that act to bridge between Ras signaling and the activation of the caspases may be located at the 75C1-2 region on the third chromosome, which is known to be essential for the initiation of PCD in *Drosophila*. Molecular analyses of the genomic region has revealed three genes in the *Df(3L)H99* interval: *rpr* (White *et al*, 1994), *hid* (Grether *et al*, 1995), and *grim* (Chen *et al*, 1996). All three genes induce PCD when ectopically expressed, and cell death is blocked by co-expression of p35, indicating that these genes induce PCD through the activation of caspases. We found that the *Df(3L)H99* mutation could dominantly suppress the *GMR-argos* phenotype (Figure 2). Conversely, the eye-ablation phenotype caused by the ectopic expression of *hid* was suppressed by gain-of-function mutations of the genes coding for MEK and ERK, downstream effectors of Ras (Figure 5). These results suggest that inhibition of the Ras pathway by Argos activates caspases via the activation of genes in the 75C1-2 region including *hid*. Alternatively, reduced signaling through the Ras pathway may stimulate unknown mechanisms that act in parallel with the genes in the 75C1-2 region to activate the caspase pathway.

It has been reported that Ras has both pro- and anti-apoptotic influences, depending on cell type and applied stimulus in various mammalian cells. For example, NGF promotes cell survival through the Ras/ERK pathway in PC12 cells (Xia *et al*, 1995). Mice lacking the B-raf protein, a downstream effector of Ras, experience increased apoptosis in their vascular endothelium (Wojnowski *et al*, 1997). In contrast, Ras induces PCD in fibroblasts through the activation of Raf (Kauffman-Zeh *et al*, 1997). Thus, it seems likely that the appropriate biological response to Ras activation and its effect on PCD are influenced by both quantitative and qualitative aspects of signal transduction pathways. The present results show that Ras signaling

functions to inhibit PCD in the developing *Drosophila* eye. However, it is possible that Ras signaling also has a pro-apoptotic function in other *Drosophila* tissues.

Genetic and molecular studies have identified a number of cell-death genes in *Drosophila*, some of which share homology with known genes composing the evolutionally conserved pathway for PCD (McCall and Steller, 1997). However, other components of the conserved PCD pathway such as *ced4* and *ced9/bcl-2* have yet to be elucidated. It is possible that these unidentified genes are involved in the regulation of PCD by the Ras pathway. For example, activation of the Ras pathway may suppress PCD through the transcription of *ced-9/bcl-2* homologs as reported for mammals (Kinoshita *et al*, 1995). Genetic screening for modifiers of the *GMR-argos* phenotype, currently in progress in our laboratory, should identify novel genes involved in the regulation of PCD by the Ras pathway.

Materials and Methods

Drosophila stocks and culture

Flies were raised on a standard cornmeal-glucose-yeast medium at 25°C, except where otherwise noted. Mutant flies used in this study were *GMR-p35* (Hay *et al*, 1994), *GMR-diap1* (Hay *et al*, 1995), *GMR-diap2* (Hay *et al*, 1995), rl^{Su23} (Lim *et al*, 1997), $Dsor1^{Su1}$ (Tsuda *et al*, 1993), *Df(3L)H99* (White *et al*, 1994), and *GMR-hid* (Grether *et al*, 1995). The *HS-Ras1*^{V12} transgenic strain is a gift from N. Perrimon. *Canton-S* or $white^{1118}$ were used as wild-type strains.

Plasmid construction and P-element mediated germline transformation

pGMR-argos was constructed by inserting the 2 kb *Eco*RI fragment of the *argos* cDNA including the entire coding region (Okano *et al*, 1992) into the *Eco*RI site of the pGMR1 vector (Hay *et al*, 1994). The resulting pGMR-argos plasmid was injected into w^{1118}; *Dr/TMS, Sb P*[ry^+, Δ*2-3*] embryos as previously described (Sawamoto *et al*, 1994). Four independent transformant lines showing similar rough-eye phenotypes were obtained. All data presented in this paper are from a single strain.

Histology

Flies were prepared for scanning electron microscopy as described by Kimmel *et al* (1990). Semi-thin sections of adult heads were prepared as described by Sawamoto *et al* (1996). Cobalt sulfide staining and acridine orange staining were performed by methods described in Wolff and Ready (1991). Immunohistochemistry of eye discs was carried out essentially as described (Tomlinson and Ready, 1987), except that discs were fixed in 4% paraformaldehyde in PBS.

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