Mutations Modulating the Argos-Regulated Signaling Pathway in Drosophila Eye Development

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ABSTRACT

Argos is a secreted protein that contains an EGF-like domain and acts as an inhibitor of Drosophila EGF receptor activation. To identify genes that function in the Argos-regulated signaling pathway, we performed a genetic screen for enhancers and suppressors of the eye phenotype caused by the overexpression of *argos*. As a result, new alleles of known genes encoding components of the EGF receptor pathway, such as *Star*, *sprouty*, *bulge*, and *clown*, were isolated. To study the role of *clown* in development, we examined the eye and wing phenotypes of the *clown* mutants in detail. In the eye discs of *clown* mutants, the pattern of neuronal differentiation was impaired, showing a phenotype similar to those caused by a gain-of-function EGF receptor mutation and overexpression of secreted Spitz, an activating ligand for the EGF receptor. There was also an increased number of pigment cells in the *clown* eyes. Epistatic analysis placed *clown* between *argos* and *Ras1*. In addition, we found that *clown* negatively regulated the development of wing veins. These results suggest that the *clown* gene product is important for the Argos-mediated inhibition of EGF receptor activation during the development of various tissues. In addition to the known genes, we identified six mutations of novel genes. Genetic characterization of these mutants suggested that they have distinct roles in cell differentiation and/or survival regulated by the EGF receptor pathway.

THE epidermal growth factor (EGF) receptor plays important roles in cell proliferation, differentiation, and survival. Activation of the receptor and its downstream signals must be tightly regulated for cells to grow and function normally. In fruit flies, the Drosophila EGF receptor (DER) is activated by multiple ligands, including Spitz, Gurken, and Vein (Perrimon and Perkins 1997). Binding of these ligands to DER triggers the activation of the Ras1/MAPK pathway. Argos, a secreted protein with an EGF-like domain, has a structure similar to these activating ligands (Freeman *et al.* 1992; Kretzschmar *et al.* 1992; Okano *et al.* 1992; Freeman 1994); however, it inhibits DER activation (Schweitzer *et al.* 1995; Sawamoto *et al.* 1996a). Loss-of-function *argos* mutants show increased numbers of cells in various tissues where cellular differentiation is triggered by DER activation (reviewed by Schweitzer and Shilo 1997). On the other hand, forced expression of *argos* inhibits cell differentiation.

The developing Drosophila compound eye is a very useful model system for studying the function and regulatory mechanisms of the EGF receptor signaling pathway in animal development. The compound eye is composed of ~800 units called ommatidia. Each ommatidium consists of 8 photoreceptor cells, 4 cone cells and 11 pigment cells. The differentiation and survival of these cells are dependent on signaling through the Ras1/MAPK pathway, which is triggered by the interaction of DER with secreted Spitz (Freeman 1996; Dominguez *et al.* 1998; Miller and Cagan 1998; Sawamoto *et al.* 1998). Regulation of DER activation by Argos is required for normal ommatidial development (reviewed by Sawamoto and Okano 1996). Loss of Argos function results in the formation of extra ommatidial cells due to excessive differentiation and decreased cell death (Freeman *et al.* 1992; Kretzschmar *et al.* 1992; Okano *et al.* 1992; Brunner *et al.* 1994; Freeman 1994). Overexpression of Argos inhibits cellular differentiation and induces programmed cell death, resulting in a decreased number of retina cells (Brunner *et al.* 1994; Freeman 1994; Sawamoto *et al.* 1994, 1998). The DER/Ras1/MAPK pathway promotes cell survival by downregulating the expression and function of *head involution defective* (*hid*), a cell death regulator (Bergmann *et al.* 1998; Kurada and White 1998; Sawamoto *et al.* 1998). In spite of these results from genetic and biochemical experiments, the precise mechanisms by which Argos regulates the DER/Ras1/MAPK pathway, which may involve unidentified genes, remain unknown.

One approach for further elucidating the mechanisms of Argos' action is to identify components that interact

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with *argos* genetically. Screens for modifiers affecting the eye phenotype caused by mutations of a gene have been used successfully to identify genes that function in a common signaling pathway (for example, Hay *et al.* 1995; Dickson *et al.* 1996; Karim *et al.* 1996; Ma *et al.* 1996; Verheyen *et al.* 1996; Neufeld *et al.* 1998). To identify genes that function in the Argos-regulated signaling pathway, we carried out a genetic screen for enhancers and suppressors of the eye phenotype induced by eye-specific overexpression of Argos. In this article, we describe the mutants isolated from this screen, which include those for both novel and known genes.

MATERIALS AND METHODS

Drosophila stocks: *Canton-S* or w^{1118} were used as wild-type strains. The *CyO, GMR-argos* and *TM3, GMR-argos* chromosomes were generated by transposition of the *GMR-argos* transgene (Sawamoto *et al.* 1998) onto *CyO* and *TM3* balancer chromosomes using a transposase expressed from the P[ry^+, $\Delta 2$-*3*] transgene (Robertson *et al.* 1988). *sev-Ras1*^{N17} (Karim *et al.* 1996), *sev-Ras1*^{V12} (Fortini *et al.* 1992; Karim *et al.* 1996), *GMR-hid* (Grether *et al.* 1995), *GMR-rpr* (White *et al.* 1996), *hs-argos* (Sawamoto *et al.* 1994), *Raf* ^{HM7} (Melnick *et al.* 1993), *Dsor1*^{Su1} (Tsuda *et al.* 1993), *Sos*^{JC2} (Rogge *et al.* 1991), *rl*^{Su23} (Lim *et al.* 1997), *clown*^{4a1} (Wemmer and Klämbt 1995), *bulge*^{6d7} (Wemmer and Klämbt 1995), *sprouty*$^{\delta 5}$ (Hacohen *et al.* 1998), and S^{218} (Kolodkin *et al.* 1994) were previously described. Fly stocks with multiple recessive markers and the deficiency kits for second and third chromosomes were obtained from the Bloomington Stock Center.

Plasmid construction and *P*-element-mediated germline transformation: p*UAS-argos* was constructed by inserting the 2-kb *Eco*RI fragment of the *argos* cDNA, which includes the entire coding region (Okano *et al.* 1992), into the *Eco*RI site of the p*UAST* vector (Brand and Perrimon 1993). The resulting p*UAS-argos* plasmid was injected into w^{1118} ; *Dr*/*TMS*, *Sb* P[ry^+, $\Delta 2$-*3*] embryos as previously described (Sawamoto *et al.* 1994). Several independent transformant lines with similar phenotypes were obtained. All data presented in this article are from a single strain, *14-1*.

Genetics: Fly cultures and crosses were performed according to standard procedures at 25°, except where otherwise noted. For heat-shock experiments, second instar larvae were collected in a vial containing medium and repeatedly heat-shocked at 36° for 1 hr with a 5-hr interval at 25°, using a temperature-programmable incubator.

Male w^{1118} flies were subjected to mutagenesis with EMS. Two- to five-day-old males were starved for 6 hr at 25° and then fed 5–25 mm EMS in a 10% sucrose solution overnight as described previously (Lewis and Bacher 1968). The males were mated to *CyO, GMR-argos*/*Tft* virgin females. Approximately 140,000 EMS-treated F_1 progenies were examined under a dissecting microscope for an enhancement or suppression of the rough-eye phenotype of *CyO, GMR-argos.* Putative enhancers and suppressors were backcrossed to the w^{1118}; *CyO, GMR-argos*/*Tft* stock to test for chromosomal linkage and balanced over *CyO, GMR-argos* or *TM3, GMR-argos.*

Complementation tests were performed based on lethality or by having a visible phenotype in *trans*-heterozygotes. As a result, three complementation groups were identified on the second and third chromosomes. Other mutations were presumed to be single hits. One of the three complementation groups was homozygous viable with apparent phenotype and the other two groups were homozygous lethal.

Mutations of enhancers and suppressors were mapped meiotically, using the markers *b pr c px sp* and *ru h th st cu sr e ca* for the second and third chromosomes, respectively. After their map positions were determined, mutant flies were crossed with deficiency stocks in the relevant regions.

Histology: For scanning electron microscopy, flies were prepared as described by Kimmel *et al.* (1990). Semithin sections of adult heads were prepared as described by Sawamoto *et al.* (1994). The anti-ELAV antibody was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. Immunohistochemistry of eye discs was carried out essentially as described by Tomlinson and Ready (1987), except that discs were fixed in 4% paraformaldehyde in PBS. Cobalt sulfide staining and acridine orange staining were performed by methods described in Wolff and Ready (1991).

RESULTS AND DISCUSSION

Screen for dominant modifiers of *GMR-argos*: To target the expression of Argos to developing eyes, we previously generated *GMR-argos* transgenic flies using the p*GMR* vector (Sawamoto *et al.* 1998). The p*GMR* vector contains a multimer cluster of binding sites for the Zn-finger protein Glass and provides eye-specific expression by Glass-dependent promotor activity in all the cells posterior to the morphogenetic furrow in the eye imaginal discs (Hay *et al.* 1994). The adult compound eyes of the flies carrying one copy of the *GMR-argos* transgene showed a mild rough morphology (Figure 1B) compared with the wild-type eye (Figure 1A). Some photoreceptor cells and pigmented lattices were often lost in the *GMR-argos*/+ eyes (Figure 1E). The effects of *argos* overexpression were dose dependent: flies with two copies of the transgene had smaller eyes with a more irregular array of ommatidia containing fewer cells (Figure 1, C and F) than flies carrying one copy (Figure 1, B and

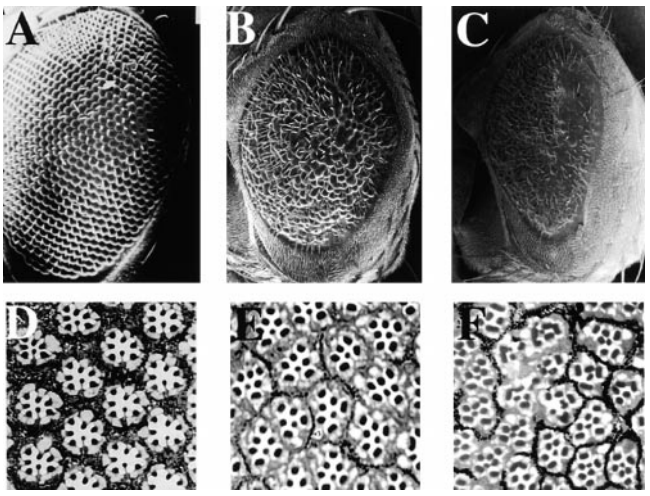

Figure 1.—Phenotype of *GMR-argos.* (A–C) Scanning electron micrographs of the adult compound eyes of wild type (A), *GMR-argos*/+ (B), and *GMR-argos*/*GMR-argos* (C). (D–F) Tangential sections of the adult compound eyes of wild type (D), *GMR-argos*/+ (E), and *GMR-argos*/*GMR-argos* (F).

E). Thus, the phenotype of *GMR-argos*/+ eyes is sensitive to the amount of Argos protein and also possibly to other molecules that have functions related to Argos. In addition, the phenotype of the *GMR-argos* flies is stable and not altered by wild-type chromosomes or balancers (data not shown). Therefore, we used the phenotype caused by one copy of the *GMR-argos* transgene to screen for enhancers and suppressors. As a pilot test, we crossed *GMR-argos* flies to a collection of flies with mapped deficiencies and scored for modification of the rough-eye phenotype. As a result, we found that the *GMR-argos* phenotype was modified by a number of deficiencies corresponding to regions in which known components of the DER/Ras1/MAPK pathway and genes implicated in cell death signaling are located (data not shown). These results suggest that a twofold reduction in the dose of a gene that functions in the Argos-regulated pathway should alter the signaling strength and modify the rough-eye phenotype of *GMR-argos*. For example, loss-of-function mutations in one copy of a gene that acts positively on the DER/Ras1/MAPK pathway should reduce signaling and enhance the *GMR-argos* phenotype. Conversely, loss-of-function mutations in one copy of a gene that acts negatively on the pathway should increase signaling and suppress the *GMR-argos* phenotype. In addition, gain-of-function mutations in such genes may also be isolated in this screen. Since homozygotes for mutations in many of the genes required for the DER/Ras1/MAPK pathway are expected to be lethal, the ability to detect mutations as heterozygotes is critical for the efficient isolation of mutations.

Females carrying a *GMR-argos* construct on the *CyO* balancer were mated to w^{1118} males that had been subjected to mutagenesis with EMS. As a parental line for screening, flies with the *GMR-argos* transgene inserted into *CyO* were used to prevent recombination between the new mutations and the transgenes. F_1 progenies carrying *CyO* were observed under the dissecting microscope and their eye morphology, size, and color were scored for modification of the *GMR-argos* phenotype. To isolate dominant modifiers of *GMR-argos*, we screened $\sim$140,000 F_1 progeny. Enhancer mutations were identified as those resulting in an increased roughness and smaller size of eyes (Figure 2). Suppressor mutations were identified by the occurrence of reduced ommatidial fusions and the reappearance of straight ommatidial rows (Figure 3). In this screen, we recovered three enhancers and 10 suppressors (Tables 1 and 2). First, the chromosomal linkage of these mutations was determined. Each mutation was located on either the second or third chromosomes. The mutations were then balanced over *CyO* or *TM3* chromosomes carrying the *GMR-argos* transgene. To determine allelism, complementation tests were performed among all the mutations on a given chromosome. Failure to complement was scored either by lethality or by a mutant eye phenotype in adult *trans*-heterozygotes. The enhancers fell into one complementation group consisting of two alleles and a mutant of a single allele (Table 1), and the suppressors fell into two complementation groups and six mutants of single alleles (Table 2). Examples of the modified phenotypes are shown in Figures 2 and 3. *EF2-1*enhanced the *GMR-argos* phenotype, resulting in a smaller eye and loss of photoreceptor and pigment cells (Figure 2, B and E). *EM3-1* acted as a weak enhancer of *GMR-argos* due to a reduction in retina cells

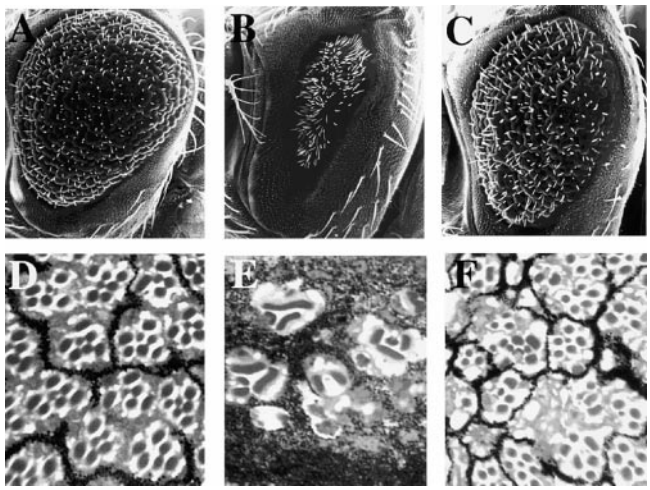

Figure 2.—Enhancement of the *GMR-argos* phenotype by the enhancer mutations. (A–C) Scanning electron micrographs of the adult compound eyes of *GMR-argos*/+ (A), *EF2-1*/*GMR-argos* (B), and *GMR-argos*/+; *EM3-1*/+ (C). (D–F) Tangential sections of the adult compound eyes of *GMR-argos*/+ (D), *EF2-1*/*GMR-argos* (E), and *GMR-argos*/+; *EM3-1*/+ (F).

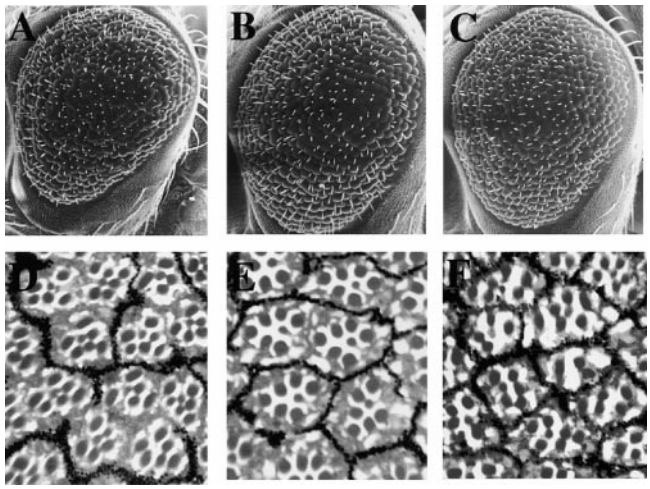

Figure 3.—Suppression of the *GMR-argos* phenotype by the suppressor mutations. (A–C) Scanning electron micrographs of the adult compound eyes of *GMR-argos*/+ (A), *GMR-argos*/+; *SF3-2*/+ (B), and *GMR-argos*/+; *SM3-2*/+ (C). (D–F) Tangential sections of the adult compound eyes of *GMR-argos*/+ (D), *GMR-argos*/+; *SF3-2*/+ (E), and *GMR-argos*/+; *SM3-2*/+ (F).

TABLE 1

Genetic interactions with enhancers of *GMR-argos*

Chromosome	Mutant	Allelic gene	Map position	*Cyo, GMR-argos*	*Cyo, sev-Ras1*^{V12}	*Cyo, sev-Ras1*^{N17}	*HS-argos* eye	*HS-argos* wing	*GMR-rpr*	*GMR-hid*	*sev-argos*
Second	*EF2-1*	*Star*	21E2	↑↑↑	↓↓↓	↑↑↑	↑↑↑	↑↑	↑	—	↑↑
	EM2-4	*Star*	21E2	↑↑↑	↓↓↓	↑↑↑	↑↑↑	↑↑	↑	—	↑↑
Third	*EM3-1*		72D-73A	↑↑	—	↑	↑↑	—	ND	—	↑

sev-argos, sev-gal4/CyO; UAS-argos/TM6; ↓, suppresses; ↑, enhances; —, little or no effect; ND, not determined because these are lethal.

and the fusion of lenses (Figure 2, C and F). On the other hand, *SF3-2* resulted in considerable recovery of the *GMR-argos* phenotype (Figure 3B). The decrease in number of photoreceptor cells in *GMR-argos* was rescued by this mutation to a nearly wild-type appearance, although the pigment cell phenotype was not recovered (Figure 3E). In contrast with *SF3-2*, another single-hit suppressor, *SM3-2* suppressed the *GMR-argos* phenotype by restoring the phenotype of pigment cells but not that of photoreceptor cells (Figure 3, C and F).

Classification and characterization of modifiers by genetic tests: Modifiers isolated in screens based on eye phenotypes often contain mutations of genes that do not have functions related to the mutation used as the background for the screen. Therefore, some of the mutants isolated in our screen may not have been specifically involved in the cellular differentiation and/or survival processes regulated by Argos. Moreover, the modifiers may have included a variety of genes, since the roles of the EGF receptor pathway are pleiotropic. To classify and characterize modifiers, we carried out four genetic tests to examine the effects of modifier mutations on (1) the eye phenotype caused by *argos* overexpression using promoters other than *GMR*, (2) the eye phenotype caused by mutant *Ras1* overexpression (*sev-Ras1*^{N17} and *sev-Ras1*^{V12}), (3) the cell death induced by overexpression of *hid* and *rpr*, and (4) the wing vein phenotype caused by *argos* overexpression. These results are summarized in Tables 1 and 2.

1. Interactions with sev-argos and hs-argos: Since Argos expression was induced under the control of the Glass binding sites in the *GMR-argos* eyes, mutations in genes including *glass* that regulate the expression of *argos* from this construct might modify the eye phenotype by increasing or decreasing the expression level of the *GMR-argos* transgene. For example, 11 alleles of *glass* mutations were isolated in a screen for modifiers of *GMR-sina* (Neufeld *et al.* 1998), although any *glass* mutations were not isolated in our screen. To examine the effect of the modifier mutations isolated in this study on the phenotype caused by *argos* overexpression induced using other promoters, we crossed the modifier mutants to the flies carrying both *sev-GAL4* and *UAS-argos* transgenes. The *sev-GAL4*/+ ; *UAS-argos*/+ flies showed a rough-eye phenotype similar to *GMR-argos* (data not shown). All of the mutants showed modifying effects on the *sev-GAL4*/+ ; *UAS-argos*/+ phenotype that were similar to those on *GMR-argos* (Tables 1 and 2). In addition, we also crossed all the modifier mutants to *hs-argos* transgenic flies (Sawamoto *et al.* 1994), where *argos* expression is induced under the control of the *hsp70* promoter. The eye phenotype of the *hs-argos* flies was also modified by all of the modifier mutations (Tables 1 and 2). These results imply that the effects of these mutations on the *GMR-argos* phenotype were not dependent on the change in transcriptional activity regulated by Glass.

2. Interactions with Ras1 mutation alleles: Some of the

modifiers isolated in our screen were expected to be mutants of genes encoding components of the Ras pathway. To examine whether the modifier mutations affect the phenotype caused by increased or decreased Ras1 activity, we crossed them to *sev-Ras1*^{V12} and *sev-Ras1*^{N17} lines. The *sev-Ras1*^{N17} transgenic fly expresses a dominant-negative *Ras1* allele under the control of the *sev*-enhancer/promoter and produces a rough-eye phenotype caused by the absence of the R7 cell in ~25% of the ommatidia and a lack of outer photoreceptor cells (Figure 4, A and D; Karim *et al.* 1996). All the enhancer mutations showed similar enhancing effects on *sev-Ras1*^{N17} (Table 1) and the suppressors, except *SM3-5* and *SM3-9*, rescued the phenotype of *sev-Ras1*^{N17} (Table 2). Examples of these effects are shown in Figure 4. The two strong enhancers of *GMR-argos*, *EF2-1* (Figure 4, B and E) and *EM2-4* (data not shown) enhanced the rough-eye phenotype of *sev-Ras1*^{N17}, so that the number of photoreceptor cells was decreased in >90% of the ommatidia. The two suppressors of *GMR-argos*, *SM3-8* (Figure 4, C and F) and *SM3-6* (data not shown), rescued the loss of R7 in almost all the ommatidia. In contrast to *sev-Ras1*^{N17}, the *sev-Ras1*^{V12} transgenic fly expresses a constitutively activated Ras1 protein and shows a rough-eye phenotype caused by excess R7 cells and destruction of the regular ommatidial array (Figure 4, G and J; Karim *et al.* 1996; Karim and Rubin 1998). It has been shown that the *sev-Ras1*^{V12} phenotype is not affected by mutations of genes that function upsteam of *Ras1* (Karim *et al.* 1996). On the other hand, the *sev-Ras1*^{V12} phenotype was suppressed by mutants identified as enhancers of *GMR-argos*, *EF2-1* (Figure 4, H and K) and *EM2-4* (data not shown), and enhanced by two suppressors of *GMR-argos*, *SM3-6* (Figure 4, I and L) and *SM3-8* (data not shown), suggesting that these genes may function downstream of or in parallel to *Ras1*.

3. Interactions with GMR-hid and GMR-rpr: Each of the three apoptotic activators, *rpr* (White *et al.* 1994), *head involution defective* (*hid*; Grether *et al.* 1995), and *grim* (Chen *et al.* 1996), can induce programmed cell death when overexpressed. It is likely that these factors induce cell death through the activation of Dapaf-1/DARK, a recently identified protein homologous to CED-4 and Apaf-1 (Kanuka *et al.* 1999; Rodriguez *et al.* 1999), and multiple caspases (Fraster and Evan 1997; Inohara *et al.* 1997; Song *et al.* 1997; Chen *et al.* 1998; Dorstyn *et al.* 1999). The DER/Ras1/MAPK pathway promotes cell survival by regulating the expression and function of *hid* (Bergmann *et al.* 1998; Kurada and White 1998; Sawamoto *et al.* 1998). Since the decreased number of cells in the *GMR-argos* eyes is caused by excessive cell death (Sawamoto *et al.* 1998), we expected to isolate mutations of genes involved in the regulation of cell-death signaling in this screen. To examine the effects of the modifier mutations on cell death, we crossed them to *GMR-rpr* and *GMR-hid* (Grether *et al.* 1995; Chen *et al.* 1996; White *et al.* 1996). The eye discs

TABLE 2

Genetic interactions with suppressors of *GMR-argos*

Chromosome	Mutant	Allelic gene	Map position	*Cyo, GMR-argos*	*Cyo, sev-Ras1*^{V12}	*Cyo, sev-Ras1*^{N17}	*HS-argos* eye	*HS-argos* wing	*GMR-rpr*	*GMR-hid*	*sev-argos*
Second	*SM2-1*		29C-30C	→	—	→	→	—	—	—	→
Third	*SM3-5*	*sprouty*	63D11-2	→	—	—	⇉	⇉	—	—	→
	SM3-9	*sprouty*	63D11-2	→	—	—	⇉	⇉	—	—	→
	SF3-2	*clown*	68C-D	⇉	—	→	⇉	⇉	⇉	→	→
	SF3-3	*clown*	68C-D	⇉	—	→	⇉	⇉	⇉	→	→
	SF3-1	*bulge*	72E4-5	→	—	⇉	⇉	⇉	—	—	⇉
	SM3-3		68A-69A	→	—	→	⇉	—	—	—	→
	SM3-2		ND	→	—	→	⇉	—	→	—	→
	SM3-6		ND	→	⇈	⇉	→	—	—	—	→
	SM3-8		ND	→	⇈	⇉	⇉	⇉	—	—	⇉

sev-argos, sev-gal4/CyO; UAS-agros/TM6; ↓, suppresses; ↑, enhances; —, little or no effect; ND, not determined.

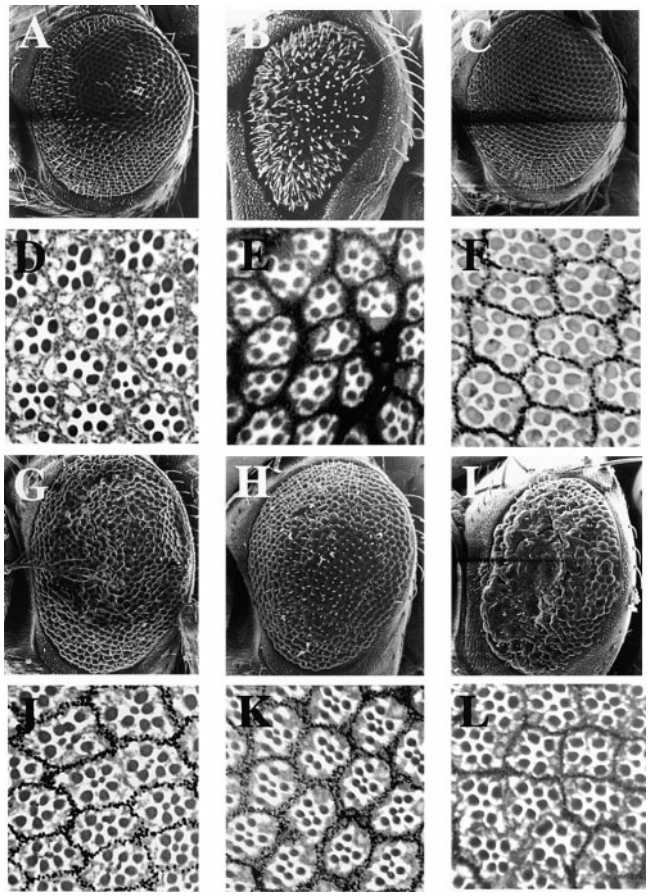

Figure 4.—Effects of enhancer and suppressor mutations on the phenotype induced by constitutively active and dominant-negative *Ras1* mutations. (A–C) Scanning electron micrographs of the adult compound eyes of *sev-Ras1*^{N17}/+ (A), *sev-Ras1*^{N17}/*EF2-1* (B), and *sev-Ras1*^{N17}/+ ; *SM3-8*/+ (C). (D–F) Tangential sections of the adult compound eyes of *sev-Ras1*^{N17}/+ (D), *sev-Ras1*^{N17}/*EF2-1* (E), and *sev-Ras1*^{N17}/+ ; *SM3-8*/+ (F). (G–I) Scanning electron micrographs of the adult compound eyes of *sev-Ras1*^{V12}/+ (G), *sev-Ras1*^{V12}/*EF2-1* (H), and *Ras1*^{V12}/+; *SM3-6*/+ (I). (J–L) Tangential sections of the adult compound eyes of *sev-Ras1*^{V12}/+ (J), *sev-Ras1*^{V12}/*EF2-1* (K), and *Ras1*^{V12}/+ ; *SM3-6*/+ (L).

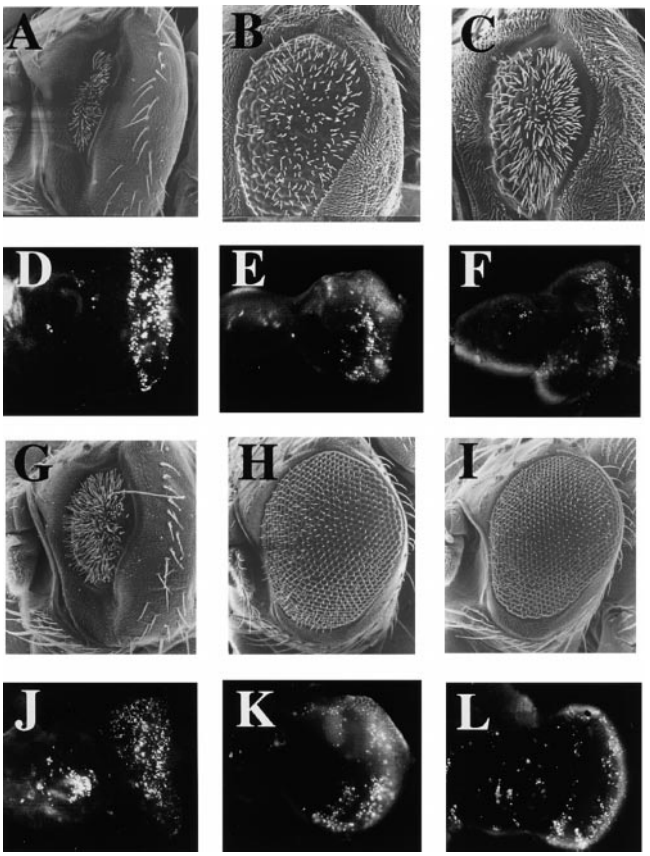

Figure 5.—Effects of the modifier mutations on the excessive cell death caused by overexpression of *hid* and *rpr*. (A–C) Scanning electron micrographs of the adult compound eyes of *GMR-hid*/+ (A), *GMR-hid*/*SF3-2* (B), and *GMR-hid*/*SM3-9* (C). (D–F) Acridine orange staining of eye imaginal discs from third instar larvae of *GMR-hid*/+ (D), *GMR-hid*/*SF3-2* (E), and *GMR-hid*/*SM3-9* (F). (G–I) Scanning electron micrographs of the adult compound eyes of *GMR-rpr*/+ (G), *GMR-hid*/*SF3-2* (H), and *GMR-hid*/*SM3-2* (I). (J–L) Acridine orange staining of eye imaginal discs from third instar larvae of *GMR-rpr*/+ (J), *GMR-hid*/*SF3-2* (K), and *GMR-hid*/*SM3-2* (L). Anterior at left; dorsal at top.

of both *GMR-hid* and *GMR-rpr*, which had a small-eye phenotype (Figure 5, A and G), showed increased numbers of dying cells posterior to the morphogenetic furrow (Figure 5, D and J). *SF3-2* considerably suppressed the small-eye phenotype and cell death in the eye discs induced by both *GMR-hid* and *GMR-rpr* (Figure 5, B and E and H and K). The *hid*-induced cell death was markedly suppressed by three of the suppressors, including *SM3-9* (Figure 5, C and F; Table 2). The *rpr*-induced cell death was enhanced by *EF2-1* and *EM2-4* (data not shown) and suppressed by three of the suppressors, including *SM3-2* (Figure 5, I and L; Table 2). These observations suggest that these modifiers, which affect the programmed cell death induced by *hid* and/or *rpr*, are mutations of genes involved in cell death.

4. Effects on wing vein development: In addition to eye development, the DER/Ras1/MAPK pathway also plays important roles in several other developmental processes (for review, Schweitzer and Shilo 1997). The development of the wing vein is another well-studied model system for investigating the function of the EGF receptor pathway in cellular differentiation. Overexpression of Argos under control of the *hsp70* promotor results in loss of the wing veins (Figure 6B; Sawamoto *et al.* 1994) due to inhibition of the Ras signaling pathway (Sawamoto *et al.* 1996a). We examined whether the dominant enhancers and suppressors of *GMR-argos* could modify the wing vein loss induced by *argos* overexpression. An enhancer mutation, *EF2-1*, dominantly enhanced the loss of the L2 and L3 veins in the *hs-argos* wings (Figure 6C). Moreover, heterozygotes for four of the suppressor mutations, including *SF3-1* (Figure 6D; Table 2), could completely restore this vein phenotype. These results suggest that these modifiers, which altered

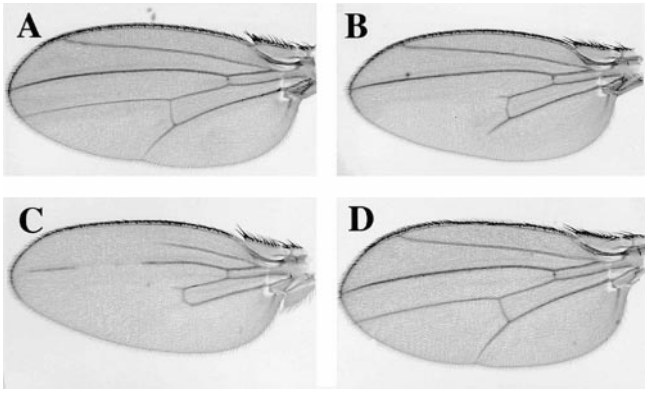

Figure 6.—Effects of the modifier mutations on the wing vein phenotype caused by overexpression of *argos*. Photomicrographs of adult wings from wildtype (A), *hs-argos*/+; *hs-argos*/+ (B), *hs-argos*/*EF2-1*; *hs-argos*/+ (C), and *hs-argos*/+; *hs-argos*/*SF3-1*(D).

the wing vein phenotype of *hs-argos*, are mutations of genes regulating cell differentiation, not only in the eye, but also in other tissues, including the wings.

Mutations of known genes: Each mutation was mapped by meiotic recombination using several recessive markers and deficiency stocks (see materials and methods). After the map positions were determined, each mutant was crossed to a number of mutant flies with defects in known genes located near the mutations to determine the allelism. All three complementation groups were found to be mutations of known genes that had been previously implicated in the DER/Ras1/MAPK pathway.

EF2-1 and *EM2-4* were allelic to *Star*(*S*). We have reported previously that *S* enhanced the *argos* overexpression phenotype and suppressed the loss-of-function *argos* phenotype in various tissues (Sawamoto *et al.* 1996a,b), suggesting that *argos* and *S* function in a common pathway. In fact, *S* interacts with components of the EGF receptor signaling pathway (Kolodokin *et al.* 1994). Moreover, S is involved in the processing of Spitz, an activating ligand for DER (Pickup and Banerjee 1999). *SM3-5* and *SM3-9* failed to complement *sprouty* (*spry*) mutations. *spry* is a regulator for EGF receptor signaling in Drosophila tracheal development (Hacohen *et al.* 1998). Recently, *spry* mutants were also isolated as regulators of EGF receptor signaling in a genetic screen similar to ours (Casci *et al.* 1999). Spry binds to Drk and Gap1 and acts as an intracellular inhibitor of Ras signaling (Casci *et al.* 1999; Kramer *et al.* 1999). *SF3-2* and *SF3-3* were found to be new alleles of the gene *clown* (Wemmer and Klämbt 1995). One allele of the *clown* gene, $clown^{4a1}$ has been identified as a suppressor of a gain-of-function mutation of the *bulge* gene (Wemmer and Klämbt 1995). Among the remaining single-hit mutations, *SF3-1* failed to complement the loss-of-function allele of *bulge* (Wemmer and Klämbt 1995). Loss-of-function mutants of both *bulge* and *clown* suppress the eye phenotype caused by *argos* overexpression (Wemmer and Klämbt 1995), suggesting that they have an antagonistic effect on EGF receptor signaling similar to that of *argos*. Thus, all the mutations of known genes isolated in this work are likely to function in DER/Ras1/MAPK pathway, indicating that our screen was highly specific for isolating genes in this pathway.

Karim *et al.* (1996) performed a nearly saturated screen for modifiers of $sev\text{-}Ras1^{V12}$ to identify genes that function downstream of *Ras1* and isolated numerous modifier mutations. Interestingly, mutants of the four known genes (*S*, *spry*, *clown*, and *bulge*) identified in our screen have not been isolated as modifiers of $sev\text{-}Ras1^{V12}$. Since we used a phenotype induced by overexpression of Argos, a diffusible protein that functions upstream of *Ras1*, our screen could have detected mutations of the genes both downstream and upstream of *Ras1*. For example, *spry*, identified in this screen, is known to act upstream of *Ras1* (Casci *et al.* 1999).

Phenotypic analysis of the *clown* mutants: We examined phenotype of the *clown* mutants in more detail, since two alleles isolated in our screen are adult viable. *clown* has been implicated in *argos* functioning (Wemmer and Klämbt 1995); however, its precise roles in eye development have remained largely unknown. To better understand *clown* function, we analyzed the phenotypes of the two new alleles of *clown*, $clown^{SF3\text{-}2}$ and $clown^{SF3\text{-}3}$, isolated as suppressors of *GMR-argos* in our present screen. Homozygotes for the $clown^{SF3\text{-}2}$ and $clown^{SF3\text{-}3}$ mutations are viable and have extremely rough eyes (Figure 7A) showing a characteristic "white and red" appearance due to loss of pigments (data not shown) similar to that of $clown^{4a1}$ (Wemmer and Klämbt 1995). The phenotype of $clown^{SF3\text{-}2}$ was more severe than those of $clown^{4a1}$ and $clown^{SF3\text{-}3}$. Flies carrying $clown^{SF3\text{-}2}$ *in trans* to a deficiency lacking the 68C-D region, where *clown* has been mapped, showed a phenotype indistinguishable from the $clown^{SF3\text{-}2}$ homozygotes (data not shown). Therefore, it is likely that $clown^{SF3\text{-}2}$ is an amorphic allele, and $clown^{4a1}$ and $clown^{SF3\text{-}3}$ are hypomorphic alleles.

To study the function of *clown* in eye development, we analyzed the phenotype of the null allele $clown^{SF3\text{-}2}$ in detail. Sections through the adult compound eyes revealed that the normal structure of the ommatidia was disrupted almost completely (Figure 7B). To analyze the development of cone and pigment cells, we stained pupal retinas with cobalt sulfide. The wild-type ommatidium is composed of four cone cells, two primary pigment cells, six secondary pigment cells, and three tertiary pigment cells (Figure 7C). In the $clown^{SF3\text{-}2}$ mutant, the number of secondary and tertiary pigment cells was increased (Figure 7D). The increase in pigment cells may be caused by the inappropriate differentiation of excess cells and/or by impaired cell death. Cone cells in the $clown^{SF3\text{-}2}$ mutant were deformed and their number and arrangement in the ommatidia were irregular (Fig-

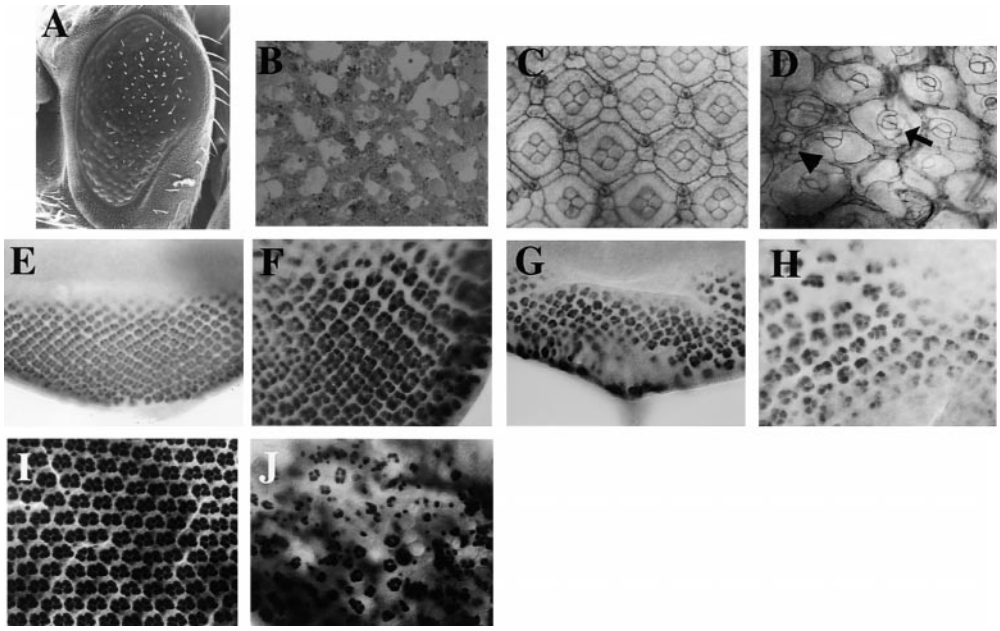

Figure 7.—Analysis of the eye phenotype of the *clown* homozygous mutant. (A and B) Scanning electron micrograph (A) and tangential section (B) of the adult compound eye of a *clown*$^{SF3\text{-}2}$ homozygous mutant. (C and D) Cobalt sulfide staining of developing retinas from wild-type (C) and *clown*$^{SF3\text{-}2}$/*clown*$^{SF3\text{-}2}$ (D) pupae at 40 hr after puparium formation (APF). Arrowhead shows increased numbers of pigment cells and arrow shows an ommatidium with extra cone cells. (E–H) Anti-ELAV staining of eye discs from the wild-type (E and F) and *clown*$^{SF3\text{-}2}$/*clown*$^{SF3\text{-}2}$ (G and H) third instar larvae. F and H are higher magnification views. (I and J) Anti-ELAV staining of developing retinas from wild-type (I) and *clown*$^{SF3\text{-}2}$/*clown*$^{SF3\text{-}2}$ (J) pupae at 40 hr APF.

ure 7D). These defects are similar to the phenotype of *clown*^{4a1} (Wemmer and Klämbt 1995).

We then analyzed the differentiation of photoreceptor cells in the *clown*$^{SF3\text{-}2}$ mutant. Eye imaginal discs from third instar larvae were stained with an antibody against a neuronal marker ELAV. In the wild-type eye discs, a regular succession of neuronal differentiation was observed (Figure 7, E and F). Posterior to the morphogenetic furrow, relatively normal five-cell preclusters developed in the *clown*$^{SF3\text{-}2}$ mutant (Figure 7, G and H). However, the adjacent clusters lacked ELAV-positive cells, resulting in the formation of a region with no photoreceptor cells. At the posterior region of the eye discs, clusters with variable numbers of ELAV-positive cells were observed. Anti-ELAV staining of pupal retinas showed that photoreceptor cells degenerated in the *clown*$^{SF3\text{-}2}$ eyes during pupal development (Figure 7, I and J). The precise mechanisms by which the *clown* mutation causes such a complex phenotype are unclear. Interestingly, however, this phenotype is similar to that of *Ellipse*, a gain-of-function *DER* mutation (Baker and Rubin 1989, 1992), and the phenotype caused by the overexpression of secreted Spitz (Freeman 1996). Therefore, it is possible that the loss of *clown* function resulted in a hyperactivation of the EGF receptor signaling in the photoreceptor cell precursors, causing this phenotype. This hypothesis is supported by our observation that *clown* mutations suppressed the *GMR-argos* photoreceptor cell phenotype (Figure 3E).

Adult flies homozygous for the putative null allele *clown*$^{SF3\text{-}2}$ are viable and fertile. Except for the phenotype affecting the compound eyes, they do not show any visible morphological defects on the body surface. Therefore, *clown* is unlikely to be required for the development of other tissues in which cellular differentiation is triggered by EGF receptor signaling. To examine whether *clown* is involved in the development of other organs, we examined its genetic interaction with components of the DER/Ras1/MAPK pathway in wing vein development. Inhibition of DER activation by overexpression of *argos* under the control of the *hsp70* promoter results in partial loss of wing veins (Sawamoto *et al.* 1994; Figure 8A). Flies homozygous for *clown*$^{SF3\text{-}2}$ have normal wing vein patterns (data not shown). However, the wing vein phenotype induced by *argos* overexpression was significantly suppressed by halving the dose of the *clown* gene (Figure 8B). We then examined the effect of the *clown*$^{SF3\text{-}2}$ mutation on the phenotypes caused by hyperactivity of the DER/Ras1/MAPK signaling. The flies carrying gain-of-function mutations for both *Sos* and *Dsor1* had wings with extra veins (Figure 8C). In addition, the wings of the *rl*^{Su23} mutants showed a similar extra vein phenotype (Figure 8E). These phenotypes were considerably enhanced by halving the dose of the *clown* gene (Figure 8, D and F). These results indicate that the *clown* gene product has a redundant function in the regulation of wing vein development possibly through antagonizing the DER/Ras1/MAPK pathway.

To determine the epistasis between *argos* and *clown*, the effect of *argos* overexpression was examined in flies homozygous for the *clown*$^{SF3\text{-}2}$ mutation. The eyes of *GMR-argos*/+ ; *clown*$^{SF3\text{-}2}$/*clown*$^{SF3\text{-}2}$ flies showed a phenotype indistinguishable from that of *clown*$^{SF3\text{-}2}$/*clown*$^{SF3\text{-}2}$ (data not shown). This observation indicates that *clown* functions downstream of or in parallel to *argos*. Since

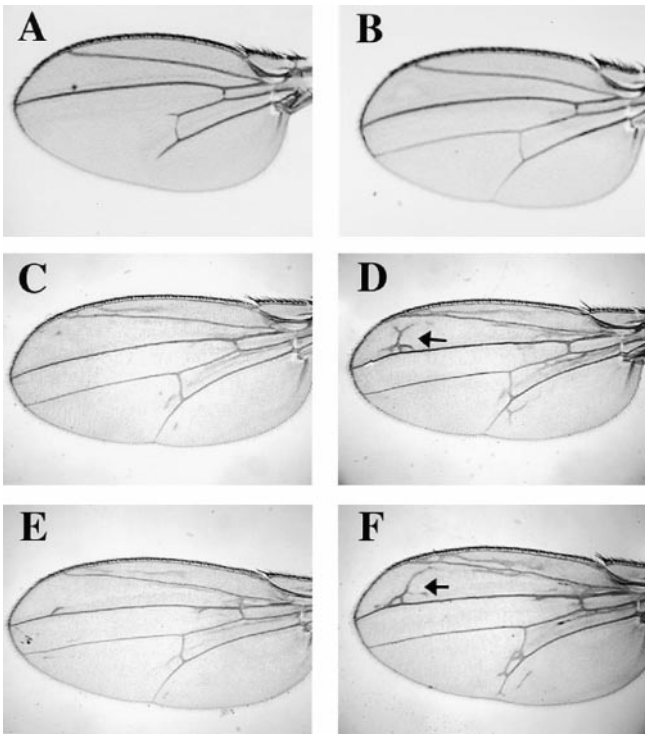

Figure 8.—Genetic interaction of *clown* with *argos*, *Dsor1*, *Sos*, and *rolled* in wing vein development. Photomicrographs of adult wings are shown. (A) *hs-argos*/+; *hs-argos*/+. (B) *hs-argos*/+; *hs-argos*/*clown*$^{SF3\text{-}2}$. (C) *Dsor1*^{Su1}/Y; *Sos*^{JC2}/+. (D) *Dsor1*^{Su1}/Y; *Sos*^{JC2}/+; *clown*$^{SF3\text{-}2}$/+. (E) *rl*^{Su23}/+. (F) *rl*^{Su23}/+; *clown*$^{SF3\text{-}2}$/+. Anterior at top. *clown*$^{SF3\text{-}2}$ enhances formation of extra veins in *Dsor1*^{Su1}/Y; *Sos*^{JC2}/+ and *rl*^{Su23}/+. The additional ectopic veins between L2 and L3 are marked by arrows.

clown mutations suppressed the dominant-negative Ras1 allele but did not affect the phenotype caused by a constitutively activated Ras1 (Table 2), the *clown* gene product may play an important role in the Argos-mediated inhibition of the EGF receptor activation upstream of Ras1.

Mutations of novel genes: Results from complementation tests revealed that the other six modifiers were mutations of novel genes. Since the results from characterizing the mutants of known genes showed a high specificity of this screen for identifying genes involved in a common pathway with *argos*, we expect that these novel genes play similar important roles. *SM3-6* and *SM3-8* enhanced *Ras1*^{V12} and suppressed *Ras1*^{N17}, suggesting that they are mutations of novel genes that function downstream of *Ras1* as negative regulators. *SM3-2* may be involved in the cell-death signaling pathway regulated by *rpr*, since it suppressed cell death induced by *rpr* but not by *hid*. As shown in Figure 3F, suppression of the *GMR-argos* phenotype by *SM3-2* was observed only in pigment cell death. Therefore, *SM3-2* may function in pigment cell precursors that are known to undergo programmed cell death in normal eye development. *EM3-1*, *SM2-1*, and *SM3-3* altered the *Ras1*^{N17}-induced defects due to photoreceptor differentiation but did not affect cell death induced by *rpr* and *hid*, or the wing vein phenotype caused by *argos* overexpression. It is possible that these three genes are involved in cellular differentiation during ommatidial development. Further characterization of these mutants and molecular cloning of the genes would unequivocally clarify their functions in cellular differentiation and/or survival regulated by the DER/Ras1/MAPK pathway.

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