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Distinct cell killing properties of the *Drosophila reaper, head involution defective*, and *grim* genes

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Abstract

The *Drosophila reaper*, *head involution defective (hid)*, and *grim* genes play key roles in regulating the activation of programmed cell death. Two useful systems for studying the functions of these genes are the embryonic CNS midline and adult eye. In this study we use the Gal4/UAS targeted gene expression system to demonstrate that unlike *reaper* or *hid*, expression of *grim* alone is sufficient to induce ectopic CNS midline cell death. We also show that in both the midline and eye, *grim*-induced cell death is not blocked by the *Drosophila* anti-apoptosis protein Diap2, which does block both *reaper*- and *hid*-induced cell death. *grim* can also function synergistically with *reaper* or *hid* to induce higher levels of midline cell death than observed for any of the genes individually. Finally we analyzed the function of a truncated Reaper-C protein which lacks the NH2-terminal 14 amino acids that are conserved between Reaper, Hid, and Grim. Ectopic expression of Reaper-C revealed cell killing activities distinct from full length Reaper, and indicated that the conserved NH2-terminal domain acts in part to modulate Reaper activity.

Keywords: *Drosophila*; development; Gal4/UAS; Grim; Diap2

Abbreviations: hid, head involution defective; CNS, central nervous system; VUM, ventral unpaired median; IAPs, inhibitor-of-apoptosis proteins; RHG domain, reaper-hid-grim domain

Introduction

The *Drosophila reaper*, *head involution defective (hid)*, and *grim* genes serve as key regulators of cell death activation (reviewed in McCall and Steller, 1997). These genes are all linked at the 75C1,2 region of the third chromosome and mutants that lack all three of these genes, such as Df(3L)H99, exhibit a blockade of essentially all cell death (White *et al*, 1994). *hid*-specific mutants as well as Df(3L)X25, which eliminates *hid* and *grim* but not *reaper*, also exhibit distinct disruptions of normal cell death patterns (Grether *et al*, 1995; Chen *et al*, 1996a; Zhou *et al*, 1997). The expression of *reaper*, *hid*, and *grim* is detected in, but not restricted to, doomed and dying cells, and ectopic expression of each gene can induce some cell types to die (White *et al*, 1994; Grether *et al*, 1995; Chen *et al*, 1996a; Nordstrom *et al*, 1996). These genes all function upstream of one or more caspases (Grether *et al*, 1995; Hay *et al*, 1995; Chen *et al*, 1996a; Pronk *et al*, 1996), as their activities are blocked by p35, a baculovirus caspase inhibitor (Bump *et al*, 1995; Xue and Horvitz, 1995). At present there is limited understanding of how these genes function to regulate the activation of cell death, as the precise biochemical activities of the Reaper, Hid, or Grim proteins are not yet known. Reaper shares some sequence similarity to the 'death domains' present in several vertebrate proteins involved in transducing cell death activation signals (Golstein *et al*, 1995), and Reaper appears to exhibit cytoplasmic localization in dying cells (Vucic *et al*, 1997a). Hid and Grim are both novel proteins whose subcellular localizations are not yet known. Significantly, the NH2-terminal 14 amino acids of Reaper, Hid, and Grim all share sequence similarity, with Reaper and Grim being more closely related to each other than either is to Hid (Grether *et al*, 1995; Chen *et al*, 1996a). This similarity suggests that the NH-2 terminal region may have common functions in all three proteins, and that elucidation of these functions is likely to be crucial for understanding the activities of these key cell death activators.

To begin to decipher the specific functions of *reaper*, *hid*, and *grim*, we initiated a series of *in vivo* functional analyses using the *Drosophila* embryonic central nervous system (CNS) midline as a model system (Zhou *et al*, 1995; 1997). The CNS midline consists of a well characterized set of ~27 cells that can be distinguished using molecular and morphological criteria (see Bossing and Technau 1993; Goodman and Doe, 1993). Among these cells are the midline glia, which are mostly located at the dorsal aspect of the ventral nerve cord, and the (ventral unpaired median) VUM neurons, which are situated ventrally. We and others have shown that the CNS midline exhibits a prominent, lineage-specific pattern of cell death during embryogenesis, as 2/3 of the midline glia are normally eliminated, while few if any of the VUM neurons die (Sonnenfeld and Jacobs, 1995; Zhou *et al*, 1995). Genetic analyses of various 75C1,2 mutants that remove *hid* alone, *hid* and *grim*, or *reaper*, *hid*, and *grim* suggested that the combined functions of all three genes is necessary for the normal pattern of CNS midline cell death (Zhou *et al*, 1997). It was further demonstrated that ectopic expression of *reaper* or *hid* alone could not induce the death of midline cells that normally survive, however, co-expression of both genes resulted in a dramatic loss of midline cells (Zhou *et al*, 1997). These results provided the first indication that these genes can function synergistically, and suggest that they

may act in a combinatorial fashion to regulate cell death in distinct cell types. An important question raised by these results is: what are the distinct cell death-inducing activities that these proteins may carry out?

In this study we use the Gal4/UAS targeted gene expression system (Brand and Perrimon, 1993) to show that *grim* can act in a distinct fashion from *reaper* or *hid*, as targeted expression of *grim* alone was sufficient to induce ectopic midline cell death. We also determined that *grim* cell killing activity is regulated differently than that of *reaper* or *hid*. Thus, the *Drosophila* inhibitor-of-apoptosis protein, Diap2, which blocks both *reaper*- and *hid*-induced cell death (Hay *et al*, 1995; Vucic *et al*, 1997a), fails to block *grim*-induced cell killing both in the midline and adult eye. *grim* can also act co-operatively with *reaper* or *hid* to induce higher levels of midline cell death than observed for expression of the individual genes.

We also analyzed the importance of the conserved NH2-terminal region by generating a truncated Reaper-C protein which lacks amino acids 2–14. The activity of this protein was assayed using the Gal4/UAS system both in the midline and the eye. In the midline, co-expression of Reaper-C with Hid or Grim induced very high levels of cell death, in excess with that observed for similar co-expression studies using wild-type Reaper. In the eye, expression of Reaper-C was able to induce moderate levels of cell death, however, the phenotype was less severe than observed with full length Reaper. Interestingly, unlike the effects of full length Reaper, Reaper-C induced eye cell death was only partially blocked by Diap2. Taken together these data suggest that the conserved NH2-terminal region may serve multiple roles in regulating the activity of Reaper protein in different cell lineages.

Results

grim is a more potent inducer of CNS midline cell death than *reaper* or *hid*

Previous studies indicated that ectopic expression of either *reaper* or *hid* alone is not sufficient to induce the death of midline cells that normally survive (Zhou *et al*, 1997). The more recently identified *grim* gene was not examined in that study, but is of interest because it shares some similarities to *reaper* and *hid*, but can induce cell death during earlier embryonic stages (Chen *et al*, 1996a). In order to analyze the ability of *grim* to induce midline cell death, we generated P[UAS-*grim*] transformant fly strains (see Materials and Methods). Two stains were analyzed, one with a viable second chromosome insertion, P[UAS-*grim*-2], and one with a viable third chromosome insertion, P[UAS-*grim*-3]. These strains were crossed to a P[52A-*gal4*] strain that drives strong expression in the midline glia and VUM neurons from stage 11 onward (Zhou *et al*, 1997). This strain also carried a P[UAS-*lacZ*] chromosome to permit detection of CNS midline cells. The progeny embryos were analyzed via anti-β-gal immunocytochemistry and revealed clear cell killing phenotypes. For P[UAS-*grim*-2], the embryos typically exhibited a loss of all midline glia from 4–5 segments of the ventral nerve cord (Figure 1A and B) and also exhibited a fusion of several commissural axon bundles (Figure 1C and D). This indicated that *grim*-induced killing of midline cells occurred in a rapid fashion during axonogenesis. Thus, unlike *reaper* or *hid*, expression of *grim* alone is sufficient to induce ectopic midline cell death. An even more severe cell death phenotype was observed with P[UAS-*grim*-3], where midline glia were absent in all but 1–2 segments and many of the VUM neurons were also eliminated (Figure 1E). Since previous studies indicated that *grim* cell killing effects are dosage sensitive (Chen *et al*, 1996a), the variability we observe likely reflects different levels of *grim* expression due to positional effects at the different P[UAS-*grim*] chromosomal insertion sites. For all further experiments we utilized the P[UAS-*grim*-2] strain. As expected, the *grim*-induced loss of midline cells was blocked by co-expression of the baculovirus protein p35 (Figure 1F), which inhibits caspase activity (Bump *et al*, 1995; Xue and Horvitz, 1995). Targeted expression of *grim* in a Df(3L)H99 background also resulted in ectopic midline cell death (data not shown), indicating that the functions of the endogenous *reaper*, *hid*, or *grim* genes are not necessary for *grim*-induced midline cell killing.

We then tested whether *grim* can act co-operatively with either *reaper* or *hid*. To address this issue we used P[UAS-*reaper*] and P[UAS-*hid*] strains (Zhou *et al*, 1997), along with P[UAS-*grim*-2], to examine the effects of co-expression of *grim* with *reaper* or *hid*. In embryos expressing both *grim* and *reaper*, nearly all the midline glia and VUM neurons were eliminated (Figure 1G). Similar effects on midline cell death were also detected with *grim* and *hid* co-expression (Figure 1H). In addition, β-galactosidase-expressing cells were also eliminated in other embryonic tissues where P[52A-*gal4*] is expressed, such as the frontal sac as well as somatic and visceral musculature (data not shown). These phenotypes are distinct from the lack of ectopic cell death induced by *reaper* or *hid* alone (Zhou *et al*, 1997), or the moderate effect seen with P[UAS-*grim*-2]. Thus, *grim* can act co-operatively with both *reaper* and *hid* to induce high levels of midline cell death. Taken together with previous findings indicating co-operative interactions between *reaper* and *hid* (Zhou *et al*, 1997), and the overlapping yet distinct expression patterns of these genes (White *et al*, 1994; Grether *et al*, 1995; Chen *et al*, 1996a), these data strongly suggest that *reaper*, *hid*, and *grim* may all act in a combinatorial manner to induce death in cell lineage-specific manner.

grim-induced cell death is not blocked by the anti-apoptotic protein Diap2

The distinct cell killing abilities of *grim* raised the possibility that *grim* activity may be regulated differently than that of *reaper* or *hid*. Although *grim*-induced killing is blocked by the baculovirus p35 protein (Figure 1F; Chen *et al*, 1996a), we were interested in determining whether *grim* may be less sensitive to cellular apoptosis inhibitors. In *Drosophila*, there exist two related inhibitor-of-apoptosis proteins (IAPs), Diap1 and Diap2 (Hay *et al*, 1995), that belong to the IAP family of cell death repressor proteins expressed by viruses and animal cells (e.g. Crook *et al*, 1993; Birnbaum *et al*, 1994; Hay *et al*, 1995; Rothe *et al*, 1995; Liston *et al*, 1996). IAPs generally

contain two or three conserved BIR domains located at the NH2-terminal and center portions of the protein, and most also contain a COOH-terminal RING finger. Ectopic expression of Diap1 or Diap2 can block naturally occurring cell deaths in the developing adult eye, as well as suppress ectopic eye cell death induced by *reaper* or *hid* over-expression (Hay *et al*, 1995). Recent studies have further indicated that the BIR domain of Diaps can directly associate with Reaper protein, and that this interaction results in altered subcellular localization of Reaper (Vucic *et al*, 1997a).

To determine whether Diaps can also block *grim*-induced cell killing, we generated a P[UAS-*diap2*] strain (see Materials and Methods). In embryos where *grim* and *diap2* were co-expressed in developing midline cells, there was a similar loss of midline glia from the nerve cord as detected for expression of *grim* alone (Figure 2A). This

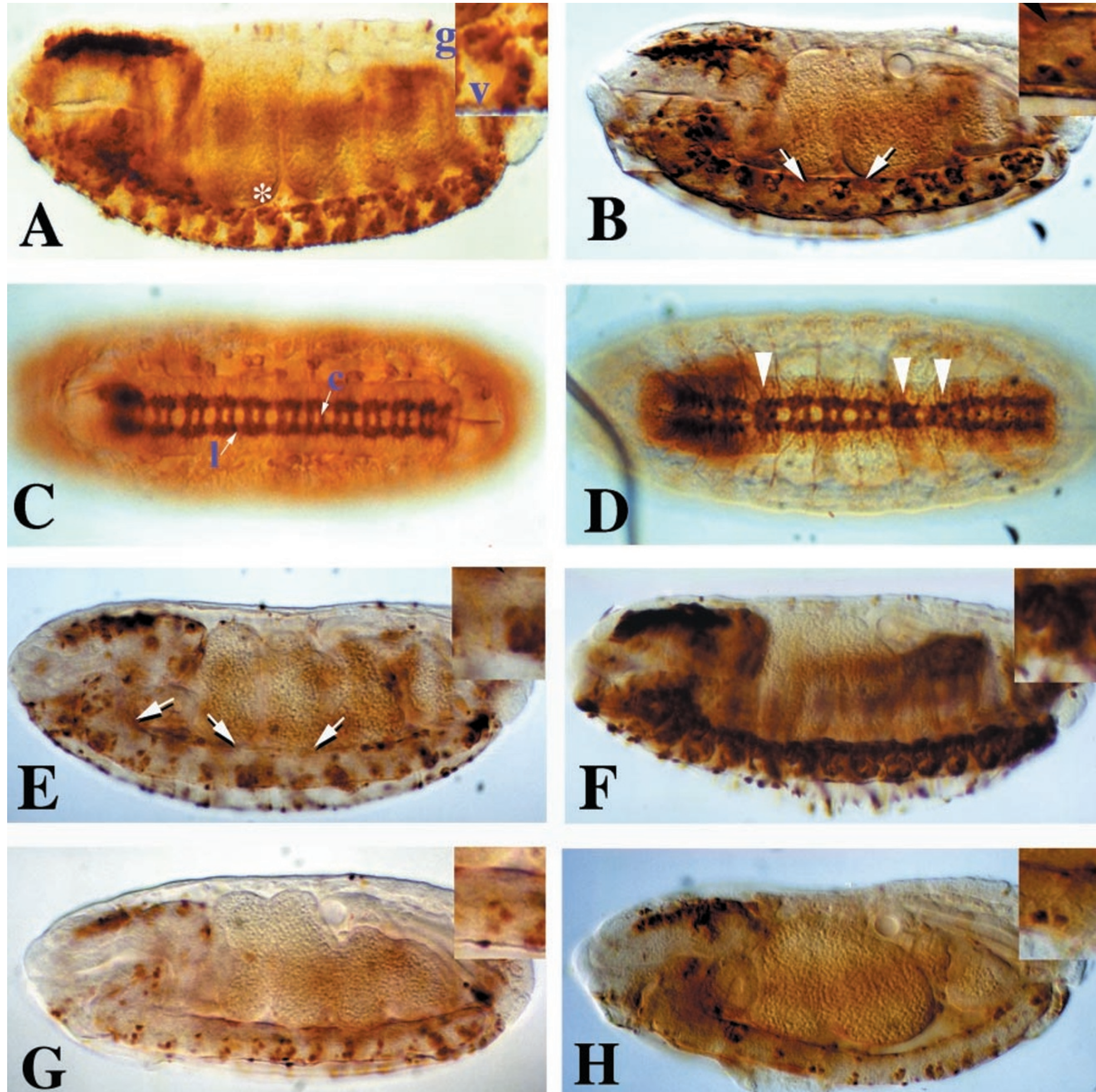

Figure 1 *grim* expression induces ectopic CNS midline cell death. Immunostaining of the following stage 16 embryos using either anti-β-galactosidase serum (**A,B,E,F,G,H**) or Mab BP102 (**C,D**): (**A,C**) P[UAS-*lacZ*]; P[52A-*gal4*]/+ (**B,D**) P[UAS-*lacZ*]; P[52A-*gal4*]/P[UAS-*grim*-2] (**E**) P[UAS-*lacZ*]; P[52A-*gal4*]/+; P[UAS-*grim*-3]/+ (**F**) P[UAS-*lacZ*]; P[52A-*gal4*]/P[UAS-*p35*]; P[UAS-*grim*-3]/+ (**G**) P[UAS-*reaper*]/P[UAS-*lacZ*]; P[52A-*gal4*]/P[UAS-*grim*-2] and (**H**) P[UAS-*lacZ*]/P[UAS-*hid*]; P[52A-*gal4*]/P[UAS-*grim*-2]. (**A**) Note that β-gal expression is normally detected in both midline glia at the dorsal aspect of the nerve cord and the VUM neurons at the ventral region of the nerve cord. Inset shows a higher magnification view of the A2 segment (marked by *) and indicates midline glia (g) and VUM neurons (v). (**C**) The axon scaffold of these embryos exhibits a wild-type organization of longitudinal connectives (1) and commissural axon bundles (c). (**B**) Expression of P[UAS-*grim*-2] typically results in the loss of midline glia from 4–5 segments of the nerve cord (arrows), and (**D**) results in a fused commisure phenotype (arrowheads). (**E**) P[UAS-*grim*-3] induces a more pronounced loss of midline glia (arrows) as well as VUM neurons. (**F**) *grim*-induced ectopic midline cell death is blocked by co-expression of the baculovirus p35 protein. (**G,H**) Co-expression of *grim* and *reaper* (**G**) or *grim* and *hid* (**H**) result in a complete elimination of the midline glia as well as partial elimination of the VUM neurons. Compare this to the moderate midline cell death in (**B**), and absence of midline cell death induced by expression of *reaper* or *hid* alone (Zhou *et al*, 1997). All views are sagittal with anterior to left and dorsal up

result indicated that *diap2* is not capable of blocking *grim*-induced midline cell killing. In contrast, *diap2* was able to block the ectopic midline cell death induced by co-expression of *reaper* and *hid*, as midline glia were present in all segments (Figure 2B; see Zhou *et al*, 1997). The ability of *diap2* to block cell killing induced by *reaper* and *hid*, but not *grim*, is a potentially important regulatory distinction between these three cell death activators.

We extended our analysis on the specificity of *diap2* function by comparing its ability to inhibit *grim*-induced cell death in the developing adult eye. For these experiments we used a P[GMR-*gal4*] strain (see Materials and Methods) to drive Gal4 expression in differentiating photoreceptor cells in the eye imaginal disc (Hay *et al*, 1994). When driven by P[GMR-*gal4*], ectopic expression of *reaper*, *hid*, or *grim* alone, as well as *reaper* and *hid* together, resulted in lethality during late larval and pupal stages. This phenotype differs from previous studies where expression of *reaper*, *hid*, or *grim* were driven in the eye imaginal disc directly by GMR sequences, without the use of the Gal4/UAS system. Thus, P[GMR-*reaper*], P[GMR-*hid*], and P[GMR-*grim*] strains are all viable, but exhibit a dosage sensitive loss of eye cells (White *et al*, 1994, 1996; Grether *et al*, 1995; Hay *et al*, 1995; Chen *et al*, 1996a). Since the eye is not an essential organ, the lethality we observed using the Gal4/UAS system is likely due to expression of *reaper*, *hid*, or *grim* in cells outside of the eye imaginal disc. We examined whether *diap2* could block the lethal effects of P[GMR-*gal4*]-driven expression of these genes. Co-expression of *diap2* was essentially unable to rescue the lethality of ectopic *grim* expression, as less than 1% of pupae eclosed; the few adults that did hatch exhibited a complete elimination of eye tissue (Compare Figure 3A and D to B and E). This effect is likely dosage-sensitive, as high levels of *diap2* expression using the Gal4/UAS system could block the weak eye cell death phenotype of a single copy of a P[GMR-*grim*] chromosome (see Materials and Methods). In contrast, using the Gal4/UAS system *diap2* was able to fully rescue the lethality induced by ectopic expression of *reaper* or *hid* alone (data not shown), as well as co-expression of *reaper* and *hid* together (Figure 3C and F). In addition, 97% of these flies exhibited normal eye morphology while only 3% exhibited a roughened eye phenotype. These results confirm functional specificity for *diap2*, and indicate that *grim* exhibits distinct cell killing properties than *reaper* or *hid* both in the CNS midline and adult eye.

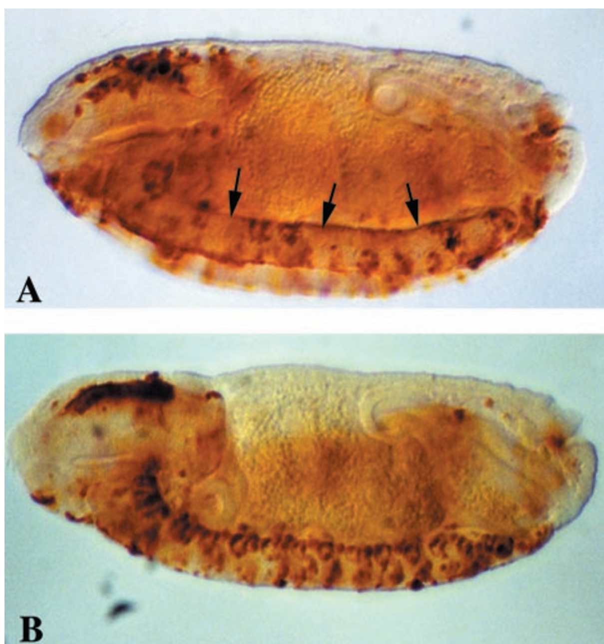

Figure 2 *diap2* does not block *grim*-induced CNS midline cell death. Anti-β-galactosidase staining of the following stage 16 embryos: (**A**) P[UAS-*lacZ*]; P[52A-*gal4*]/P[UAS-*grim*]; P[UAS-*diap2*]/+ and (**B**) P[UAS-*lacZ*]/P[UAS-*reaper*]-P[UAS-*hid*]; P[52A-*gal4*]/+; P[UAS-*diap2*]/+. Note that *diap2* fails to block *grim*-induced midline cell death (**A**), as several segments still lack midline glia (arrows). *diap2* does block midline cell deaths induced by co-expression of *reaper* and *hid*, (**B**), as wild-type numbers of cells are detected. All views are sagittal with anterior to left and dorsal up

Functions of the conserved RHG domain

The Reaper, Hid, and Grim proteins all share a conserved 14 amino acid region at their NH2-termini (Grether *et al*, 1995; Chen *et al*, 1996a), which we propose to refer to as the RHG domain, for Reaper, Hid, and Grim. In particular, the RHG domains of Reaper and Grim are 71% identical, while both are less conserved with that of Hid. This sequence similarity suggests the RHG domain carries out common functions in these three proteins. Previous studies on truncated Reaper proteins in insect tissue culture cells suggested that the RHG domain might be important for normal levels of Reaper activity (Chen *et al*, 1996b; Vucic *et al*, 1997b). Because different cell types, such as the embryonic CNS midline and adult eye, exhibit distinct sensitivity to ectopic Reaper expression, we wished to further address this issue in developing fly tissues. Consequently we generated P[UAS-*reaper*-C] fly strains that express a truncated version of Reaper protein lacking residues 2–14, and compared the cell killing abilities of this Reaper-C protein to wild-type Reaper. Two P[UAS-*reaper*-C] strains were obtained, one with a viable insertion on the X chromosome, P[UAS-*reaper*-C-X], and one with a viable insertion on the third chromosome, P[UAS-*reaper*-C-3]. Both strains exhibited similar activities.

As with wild-type Reaper, targeted midline expression of Reaper-C did not result in any ectopic cell death (Figure 4A). In addition, we did not detect any excess midline glia, indicating that Reaper-C did not act as a dominant negative to block normal midline cell deaths. We then tested whether Reaper-C could function co-operatively with either Hid or Grim. Co-expression of Reaper-C and Grim resulted in a loss of both midline glia and VUM neurons from all segments of the nerve cord (Figure 4B), a level of cell killing much greater than that observed for expression of either Reaper-C or Grim alone. Interestingly, this level of cell death was also more severe than that observed for co-expression of wild-type Reaper and Grim (Figure 1G), suggesting that Reaper-C may possess enhanced midline cell killing activity. Co-expression of Reaper-C with Hid also resulted in a synergistic elimination of midline glia and VUM neurons from most segments (Figure 4C), an effect which

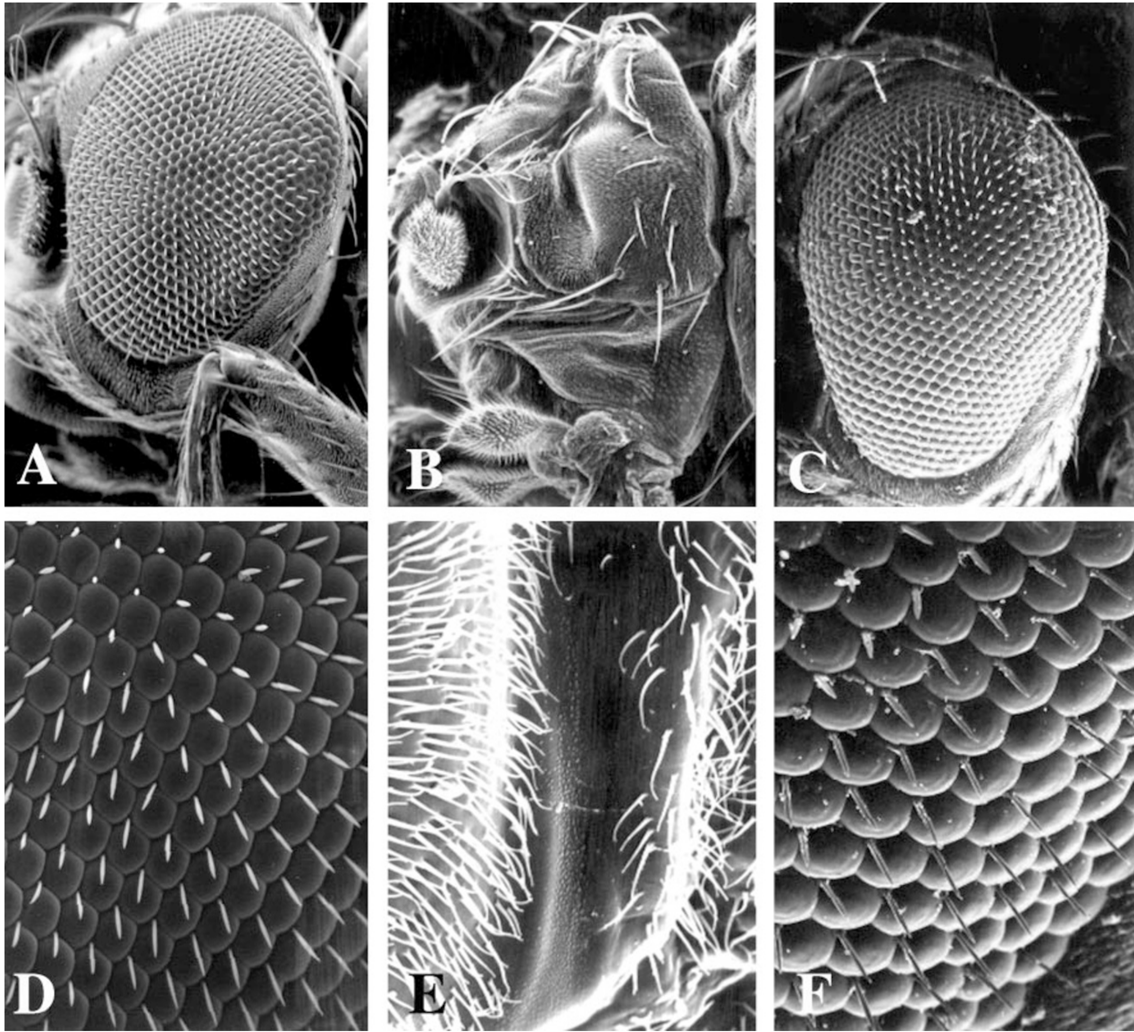

Figure 3 *diap2* specifically fails to block *grim*-induced eye cell death. (**A,D**) Wild-type adult eye. Note ordered array of ommatidia and bristles. (**B,E**) *diap2* failed to rescue the lethality of P[GMR-*gal4*]/P[UAS-*grim*-2] animals, as less than 1% of the P[GMR-*gal4*]/P[UAS-*grim2*];P[UAS-*diap2*]/+ pupae eclosed and these adults exhibited an absence of ommatidia. (**C,F**) *diap2* did rescue the lethality of P[UAS-*reaper*]-P[UAS-*hid*]; P[GMR-*gal4*]/+ animals: 97% of the P[UAS-*reaper*]-P[UAS-*hid*]; P[GMR-*gal4*]/+; P[UAS-*diap2*]/+ adults exhibited a normal eye phenotype and 3% exhibited a roughened eye. All panels are S.E.M. micrographs at magnifications of: (**A,B,C**) ×200, (**D**) ×700, (**E**) ×2000, (**F**) ×1000

differed from the lack of ectopic midline cell death induced by expression of either Reaper-C (Figure 4A) or Hid alone (see Zhou *et al*, 1997). The extent of cell death induced by co-expression of Reaper-C with Hid was also more severe than that observed for co-expression of wild-type Reaper and Hid (see Zhou *et al*, 1997). Taken together, these data indicate that the RHG domain of Reaper is not required for co-operative interactions with Hid or Grim, and suggest that one function of the RHG domain may be to modulate Reaper killing activity.

We also analyzed the cell killing ability of Reaper-C in the adult eye using the P[GMR-*gal4*] strain. Unlike the lethal phenotype observed for wild-type Reaper, flies ectopically expressing Reaper-C were viable, and exhibited a dosage-sensitive elimination of eye tissue. Flies heterozygous for P[GMR-*gal4*] and homozygous for P[UAS-*reaper*-C-X] exhibited a significant reduction of eye tissue, with a loss of ommatidia and disorganization of mechanosensory bristles (Figure 5A, B). In contrast, females heterozygous for P[UAS-*reaper*-C-X] exhibited normal eye morphology (data not shown). Thus, Reaper-C is capable of inducing eye cell death, although the absence of the RHG domain results in less cell killing activity than wild-type Reaper. This result contrasts with the stronger synergistic killing of CNS midline cells by Reaper-C than wild-type Reaper, and implies that differences in the levels of Reaper and Reaper-C proteins are unlikely to be responsible for these contrasting cell death phenotypes. The different effects of Reaper-C in the midline and eye also suggest that there may exist tissue-specific differences in the functions of the RHG domain.

We then examined whether Diap2 was capable of blocking Reaper-C-induced eye cell death. Co-expression of Reaper-C and Diap2 via P[GMR-*gal4*] resulted in only a

partial repression of Reaper-C activity, as 50% of male flies exhibited a wild-type eye while 50% exhibited a roughened eye phenotype that was similar to, but less severe than that of Reaper-C alone (Figure 5C, D). This finding indicates that Diap2 is less effective in blocking the activity of Reaper-C than full length Reaper, suggesting that the cell death inhibitory activities of Diap2 may be mediated, at least in part, through RHG domain.

Discussion

Distinct cell killing properties of *grim-reaper* genes

The *reaper*, *hid*, and *grim* genes encode important cell death activators whose functions are just beginning to be elucidated. Because these genes all function to regulate cell death, share conserved RHG domains, and are linked at 75C1,2, we propose to refer to them as genes of the *grim-reaper* locus. In this study, we show that in contrast to *reaper* and *hid*, ectopic expression of *grim* alone is sufficient to induce the death of CNS midline cells that normally survive. This finding indicates that Grim protein has cell killing capabilities distinct from those of Reaper and Hid. Perhaps Grim is able to access different components of the cell death machinery than Reaper or Hid, or access the same components in a distinct fashion. Another possibility is that Grim's activity may be regulated in a distinct fashion from Reaper or Hid. Consistent with this hypothesis we find that Grim is unique in that cell killing effects are not effectively inhibited by the cellular anti-apoptotic protein Diap2. Thus, while Diap2 can block both Reaper- and Hid-induced cell killing (Hay *et al*, 1995; Vucic *et al*, 1997a; this study), it does not block Grim-induced cell death. Although the mechanisms through which the Diaps block Reaper- and Hid-induced cell deaths are not yet clear, the BIR domains of Iaps can directly associate with Reaper, suggesting potential direct inhibitory interactions (Vucic *et al*, 1997a). Our results suggest this interaction likely requires the conserved RHG domain, as cell killing by a truncated Reaper-C protein was only partially blocked by Diap2. Our results further imply that residues shared between the RHG domains of Reaper and Hid, but which differ from Grim, may be crucial for Diap2 specificity. In this regard there are three such positions, at residues #3, 5, and 6, that are Valine, Phenylalanine, and Tyrosine in Reaper and Hid, and Isoleucine, Tyrosine,and Phenylalanine in Grim (Chen *et al*, 1996a). Previous studies indicated that mutation of the Phenylalanine or Tyrosine residues to Alanine reduced Reaper cell killing activity in insect tissue culture cells (Vucic *et al*, 1997b), and it will also be of interest to examine the effect of mutating these residues on Reaper or Grim killing activity and regulation by Diap2 in developing fly tissues.

Studies of *reaper*, *hid*, and *grim* functions using the embryonic midline as a model system have revealed co-operative interactions between all three of these genes. An important goal will be to define the mechanisms underlying these interactions. One model is that *reaper* and *hid* act together to activate a *diap2*-repressible cell death pathway. In contrast, *grim* can act co-operatively with *reaper* or *hid* but also can function in a distinct *diap2*-independent pathway. This differs somewhat from the actions of *grim-reaper* genes in the adult eye, where each gene is individually capable of inducing cell death and no synergistic actions have been noted (White *et al*, 1994; Grether *et al*, 1995; Hay *et al*, 1995; Chen *et al*, 1996a). This difference suggests there may exist tissue-specific pathways through which these genes activate the cell death machinery. These pathways all appear to ultimately converge on one or more caspases, as cell killing by all three genes, alone or in combination, is blocked by p35 (Grether *et al*, 1995; Hay *et al*, 1995; Chen *et al*, 1996a; Vucic *et al*, 1997; Zhou *et al*, 1997). The ability of *grim-reaper* genes to functionally interact suggests that they may all function in a combinatorial fashion in distinct populations of dying cells. Interestingly, while vertebrate

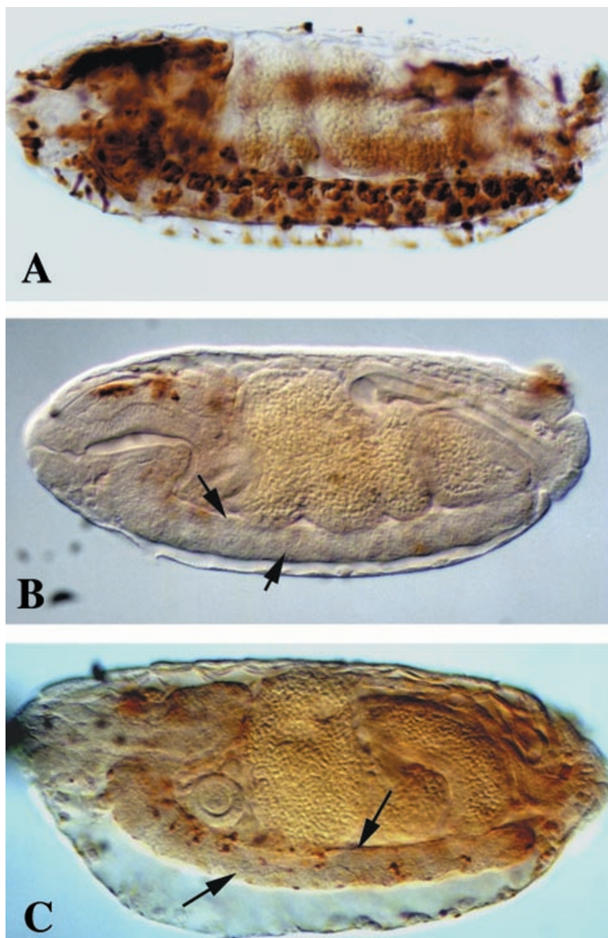

Figure 4 A Reaper-C protein lacking the conserved RHG domain can act co-operatively with Hid and Grim to induce CNS midline cell death. (**A – C**) Anti-β-galactosidase staining of the following stage 16 embryos: (**A**)P[UAS-*lacZ*]; P[52A-*gal4*]/+; P[UAS-*reaper*-C-3]/+ (**B**) P[UAS-*lacZ*]; P[52A-*gal4*]/P[UAS-*grim*-2]; P[UAS-*reaper*-C-3]/+ and (**C**) P[UAS-*lacZ*/P[UAS-*hid*]/; P[52A-*gal4*]/+; P[UAS-*reaper*-C-3]/+. Note that Reaper-C expression alone does not result in any ectopic midline cell death (**A**). Co-expression of Reaper-C with Grim (**B**) or Hid (**C**) results in severe loss of both midline glia and VUM neurons (arrows). This phenotype is more severe than that seen when full length Reaper is expressed with Grim (compare to Figure 1**G**) or Hid (see Zhou *et al*, 1997). All views are sagittal with anterior to left and dorsal up

homologs of *grim-reaper* genes have not yet been identified, like other cell death activators, *reaper*-induced cell killing can result in increased ceramide production (Pronk *et al*, 1996), and can cause release of cytochrome C to induce apoptosis in *Xenopus* egg cell free extracts (Evans *et al*, 1997). These results suggest that *grim-reaper genes* may act through conserved signal transduction pathways.

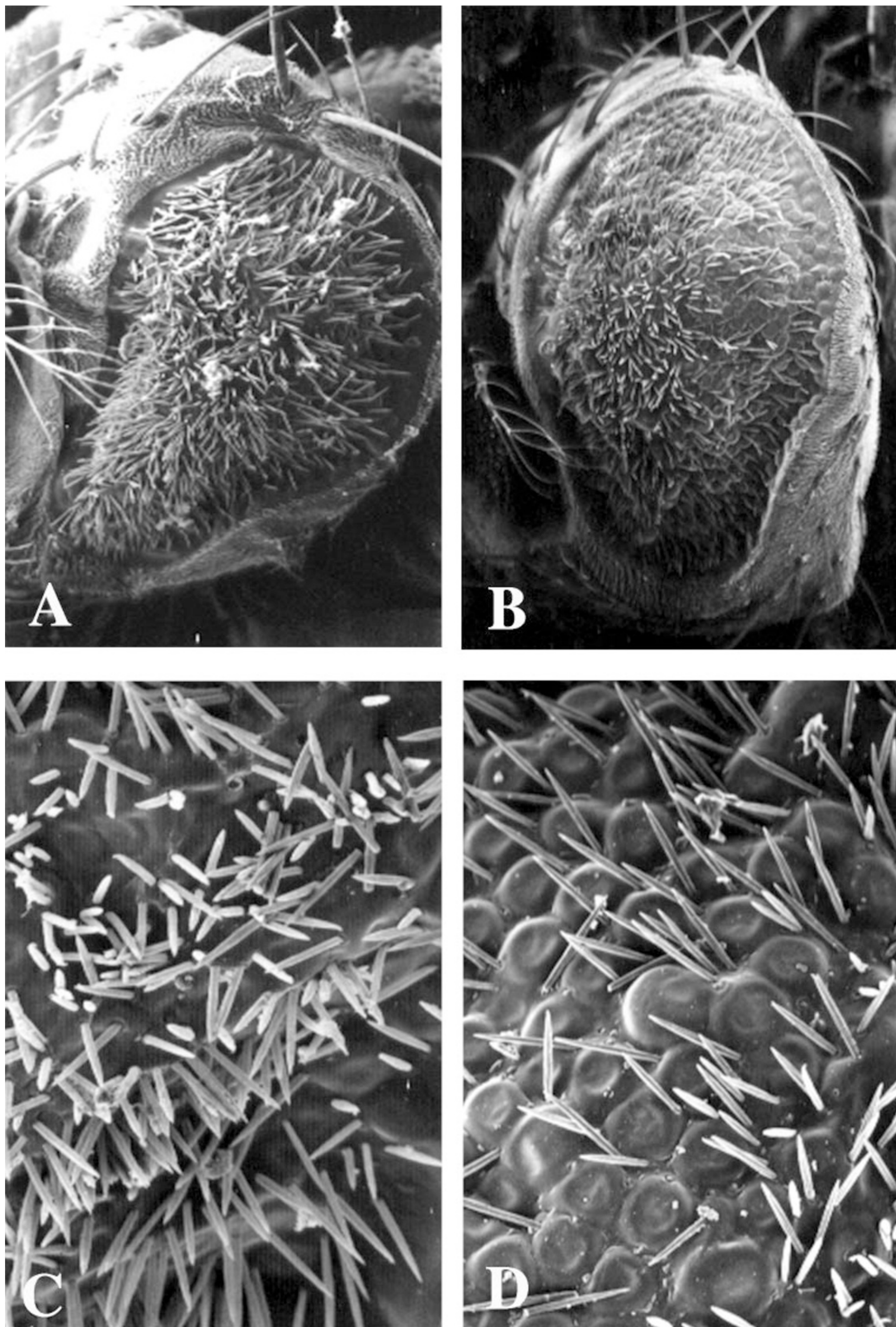

Figure 5 Reaper-C can induce cell death in the adult eye which is not abolished by Diap2. (**A,C**) A male P[UAS-*reaper*-C-X]; P[GMR-*gal4*]/+ fly exhibiting a decrease in eye size (compare to Figure 3**A**). Note also a loss of ommatidia and disorganization of bristles. (**B,D**) Approximately 50% of the male P[UAS-*reaper*-C-X]; P[GMR-*gal4*]/+; P[UAS-*diap2*]/+ flies also exhibited a reduced eye phenotype that was less severe than P[UAS-*reaper*-C-X]; P[GMR-*gal4*]/+ flies

Multiple functions of RHG domain

While the *grim-reaper* genes are crucial for inducing cell death, the biochemical activities of the corresponding proteins are as yet unknown. Reaper does exhibit limited homology to the 'death domain', a protein/protein interaction motif present in a number of signaling proteins that function in cell death pathways (Golstein *et al*, 1995). However, a number of recent studies suggest that Reaper may function differently than the death domains of the vertebrate Fas, FADD, TNFR1 proteins (Chen, 1996b; Kondo *et al*, 1997; Vucic *et al*, 1997b), and mutation of several key residues essential for the functions of vertebrate death domain proteins did not eliminate Reaper activity (Chen *et al*, 1996b; Vucic *et al*, 1997b). One potential clue to the functions of Reaper, Hid and Grim is that they all share a conserved RHG domain at their NH2-termini. By analyzing the activity of a truncated Reaper-C polypeptide lacking this conserved region, we determined that the RHG domain is not required for the co-operative killing of CNS midline cells with Hid or Grim, or the killing of adult eye cells. Thus the COOH-terminal 50 amino acids possess clear cell-killing activity. Although the functions of the Reaper RHG domain are not yet known, it could be important for Reaper stability, as a similarly truncated Reaper polypeptide exhibited low levels of accumulation in tissue culture cells (Vucic *et al*, 1997a). However, this explanation is not sufficient to describe the effects of co-expression of Reaper-C with Hid or Grim in the midline, which led to higher levels of cell death than observed for similar co-expression studies using full length Reaper. Our data support a hypothesis where the RHG domain has multiple functions in regulating Reaper activity. In the CNS midline, the Reaper RHG domain functions largely to restrict Reaper activity, perhaps by association with Diaps. In the adult eye, the RHG domain is required both for full cell killing activity and repression by Diap2. One question that will be of interest to address is whether the respective RHG domains of Reaper, Hid, and Grim confer specific cell killing and regulatory properties.

Developmentally specific killing by *grim-reaper* genes

Ectopic expression of *grim-reaper* genes have been used to induce cell death in several developmental contexts. Our studies on the CNS midline have indicated that cell death is most efficiently induced by co-expression of these genes. In addition to the midline, we have also observed that other embryonic cell types are relatively insensitive to the individual expression of Reaper, Hid, and to a lesser extent, Grim (JPW, LZ, LMS, JRN unpublished observations). These findings are in contrast to the effects of ectopic expression of these genes during postembryonic stages. For example, expression of any one of these genes is sufficient to induce adult eye cell death (White *et al*, 1994; Hay *et al*, 1995; Grether *et al*, 1995; Chen *et al*, 1996a), and ectopic expression of *reaper* or *hid* alone can also effectively induce the death of distinct sets of neurosecretory cells during metamorphosis (McNabb *et al*, 1997; Robinow *et al*, 1997). In addition, there are no apparent synergistic effects of co-expression of these genes in post-embryonic stages, and genetic enhancer/suppressor screens for mutations that influence the eye killing phenotype of *reaper* or *hid* have not revealed interactions between *grim-reaper* genes (Hay *et al*, 1995). These differences suggest there may exist distinct cell killing mechanisms utilized during different developmental stages. Indeed, White *et al*. (1996) have shown that 12–18 h old embryos and late stage pupae exhibit a reduced sensitivity to ectopic *reaper* expression.

It is possible that the differences observed between ectopic cell killing by *grim-reaper* genes in embryonic and post-embryonic stages is that there is simply more time for the corresponding proteins to accumulate and exert their cell killing functions during later stages. For example, genes under control of the cloned GMR sequences are expressed for over 50 h during eye imaginal disc differentiation in third instar larvae (Moses and Rubin, 1991; Hay *et al*, 1994), longer than the entire duration of embryogenesis. However, in our embryonic CNS midline killing experiments the cells are likely exposed to high levels of ectopic *reaper*, *hid*, or *grim* expression for at least 10 h. Both *in vivo* and *in vitro* studies have indicated that for many dying cells, cell death initiation can be detected within 1–2 h after *reaper* expression (White *et al*, 1994; Nordstrom *et al*, 1996; Vucic *et al*, 1997b). These data strongly suggest that timing alone is not sufficient to account for inefficient embryonic cell killing. Instead, they suggest that there may be important differences in the available components of the cell death machinery during different developmental stages. Similar differences may also exist between different cell types, as lineage-specific differences in the sensitivity to expression of *grim-reaper* genes have also been noted, e.g. between the midline glia and VUM neurons. In this regard, as occurs in vertebrates, where expression of multiple caspases and Bcl-2 family members provide lineage-specific regulation of apoptotis (see White, 1996), the three linked and related genes of the *grim-reaper* locus may provide enhanced flexibility in regulating the activation of cell death in distinct developmental and physiological contexts.

In *Drosophila*, embryogenesis is quite rapid and accompanied by a complex series of morphogenic movements, such as germ band extension and retraction, and head involution, that require tight regulation of cell division, differentiation, migration, and death. The pace of embryogenesis necessitates the efficient elimination of unwanted cells, requiring fast-acting mechanisms to mediate cell death. However, the generation of many embryonic stem cells that give rise to large numbers of progeny also requires mechanisms that ensure a very strict control over cell death activation. Thus, aberrant deletion of small numbers of cells in the embryo could have more pronounced effects than a similar cell loss during later developmental stages. This may have led to the establishment of stage- and lineage-specific regulatory mechanisms used to modulate the activity of *grim-reaper* gene products. It is likely that elucidating the mechanisms underlying these differences in cell death activation will ultimately have important implications for understanding and manipulating cell death in a variety of organisms.

Materials and Methods

Fly strains and genetic crosses

P[UAS-*grim*] strains were generated by cloning a full length *grim* cDNA (provided by J Abrams) into the *Eco*RI site of the pUAST vector (Brand and Perrimon, 1993). P element mediated germline transformation into w^{1118} host embryos was performed as originally described by Spradling and Rubin (1982). Two transformant strains were obtained, one with a viable insertion on the second chromosome and the other with a lethal insertion on the third chromosome. A viable third chromosome insertion was subsequently obtained via mobilization of the second chromosome P element insertion using $\Delta 2-3$ as a source of transposase (Robertson *et al*, 1988). A P[UAS-*diap2*] strain was generated using a similar approach. A full length *diap2* cDNA clone (LD04614) was obtained from the Berkeley *Drosophila* Genome Project and digested with *Eco*RI and *Xho*I. This fragment was then cloned into the pUAST vector and microinjected into w^{1118} host embryos. A viable insertion on the third chromosome was obtained and used for all studies described here.

The P[UAS-*reaper*-C] construct was generated by amplifying a DNA fragment encoding amino acids 15–65 of the Reaper protein from a full length *reaper* cDNA clone in the pBluescript vector (Stratagene) using a *reaper*-specific primer: 5′-GCCCGAATTCAT-TAAATTAAATACCGCCATGCGGGAGGCGGAGCAG-3′, and the T3 primer for pBluescript. This fragment was digested with *Eco*RI and cloned into the pUAST vector. P[UAS-*reaper*-C] transformant strains were then generated as described above. Two viable insertions were obtained, one on the X chromosome and one on the third chromosome.

The P[52A-*gal4*] strain (Zhou *et al*, 1997) drives strong CNS midline expression from stage 11 onward (stages defined in Campos-Ortega and Hartenstein, 1997). Expression is also present in several other embryonic tissues, including the frontal sac as well as visceral and somatic muscles. A P[UAS-*lacZ*]; P[52A-*gal4*]/CyO strain was generated using an X chromosome linked P[UAS-*lacZ*] insertion provided by A Brand. Several P[UAS-*p53*] strains were kindly provided by B Hay. For these experiments we utilized a viable 2nd chromosome insertion. The P[GMR-*grim*] strain was provided by Hermann Steller. It contains a viable second chromosome insertion that yields a mild loss of ommatidia as a heterozygote. The P[GMR-*gal4*] strain (a.k.a. *w**; P{w+mC=GAL4-*ninaE*.GMR}12) drives strong expression in and behind and morphogenic furrow in the developing eye imaginal disc; it was generated by M Freeman and obtained from K Matthews at the Indiana Stock Center. X-linked P[UAS-*reaper*], P[UAS-*hid*], and P[UAS-*reaper*]-P[UAS-*hid*] strains were previously described in Zhou *et al*, (1997). All *grim-reaper* gene combinatorial experiments were performed using the viable second chromosome insertion strain, P[UAS-*grim*-2].

Immunocytochemistry and *in situ* hybridization

For immunocytochemical analyses, embryos were collected and fixed using the PEMS/formaldehyde procedure of Patel (1994). A mouse monoclonal anti-β-galactosidase antibody (Promega) was used at 1:1000 to detect lacZ expression, and labeling was identified using a biotinylated horse anti-mouse antibody (Vector Labs) and strepavidin-HRP. The monoclonal antibody BP102 was obtained from the Developmental Studies Hybridoma Bank and used at 1:5 to visualize CNS axon pathways. Labeling was detected using a biotinylated horse anti-mouse (Vector Labs) and strepavidin-HRP. Stained embryos were dehydrated in an ethanol series, cleared in methyl salicylate, and mounted in Permount (Fisher).

Electron microscopy

For EM analyses, adult *Drosophila* tissues were prepared by Dr. Lucy Yin at the University of Massachusetts Central Microscopy Facility. Briefly, tissue samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 3 h at room temperature. After dehydration through a graded ethanol series, they were critical point dried in a Polaron CPD unit. Samples were sputter coated with gold/palladium and examined and photographed using a JEOL 5400 scanning electron microscope.

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