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reaper is required for neuroblast apoptosis during *Drosophila* development

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SUMMARY

Developmentally regulated apoptosis in *Drosophila* requires the activity of the *reaper* (*rpr*), *grim* and *head involution defective* (*hid*) genes. The expression of these genes is differentially regulated, suggesting that there are distinct requirements for their proapoptotic activity in response to diverse developmental and environmental inputs. To examine this hypothesis, a mutation that removes the *rpr* gene was generated. In flies that lack *rpr* function, most developmental apoptosis was unaffected. However, the central nervous systems of *rpr* null flies were very enlarged. This was due to the inappropriate survival of both larval neurons and neuroblasts. Importantly, neuroblasts rescued from apoptosis remained functional, continuing to proliferate and generating many extra neurons. Males mutant for *rpr* exhibited behavioral defects resulting in sterility. Although both the ecdysone hormone receptor complex and p53 directly regulate *rpr* transcription, *rpr* was found to play a limited role in inducing apoptosis in response to either of these signals.

Key words: *Drosophila melanogaster*, Apoptosis, reaper, p53, Neuroblast

INTRODUCTION

Apoptosis accompanies many developmental processes, from the earliest stages of embryogenesis, to the final sculpting of the mature organism (Jacobson et al., 1997; Vaux and Korsmeyer, 1999). Most physiologic deaths appear to utilize common core effectors, including the caspase proteases. However, less is known about the upstream pathways that initiate programmed cell death. In *Drosophila*, many of the genes that influence developmental decisions have been identified and characterized. Therefore, the study of developmental cell death in flies has the potential to provide important information about how developmental regulators interact with the apoptotic machinery (Bangs and White, 2000).

All developmental apoptosis in the *Drosophila* embryo requires the activity of the *reaper* (*rpr*), *grim* and *head involution defective* (*hid*) genes. These genes are localized within a small genomic region (White et al., 1994). Embryos homozygous for a deletion (*H99*) that removes all three of these genes have virtually no developmental apoptosis, and die as embryos with many extra cells. Each of these genes is sufficient to induce apoptosis in a caspase-dependent manner when overexpressed in insect and mammalian systems (Grether et al., 1995; Hay et al., 1995; Chen et al., 1996; Pronk et al., 1996; White et al., 1996; Evans et al., 1997; Claveria et al., 1998; McCarthy and Dixit, 1998; Haining et al., 1999). At least part of the proapoptotic activity of these genes results from their ability to bind and inactivate the Inhibitor of Apoptosis, or IAP proteins (Vucic et al., 1997; Vucic et al., 1998; Wang et al., 1999; Goyal et al., 2000). IAP proteins can bind to caspases and inhibit their function (Kaiser et al., 1998; Hawkins et al., 1999; Meier et al., 2000). Thus, the combined activity of Rpr, Grim and Hid negatively regulates the amount of IAP-mediated caspase inhibition. The recently identified mammalian SMAC/DIABLO protein acts to potentiate apoptosis through a similar mechanism (Du et al., 2000; Verhagen et al., 2000; Wu et al., 2001). Rpr also binds to the antiapoptotic protein Scythe (Thress et al., 1999). This interaction in turn appears to regulate the molecular chaperone activity of hsp70 (Thress et al., 2001). It is not known if Rpr:Scythe interactions regulate apoptosis in *Drosophila*.

Although *rpr*, *hid* and *grim* may induce apoptosis through similar mechanisms, it is clear that they are not functionally equivalent at the organismal level. The proteins are differentially expressed in dying cells and in response to different signals. Some doomed cells only express a subset of these genes. In both embryonic and adult central nervous systems, *rpr* and *grim* are expressed in a large number of dying cells, while *hid* is only expressed in the midline glia (White et al., 1994; Grether et al., 1995; Chen et al., 1996; Robinow et al., 1997). In contrast, *hid* and *rpr* are expressed in non-neural tissues signaled to die during metamorphosis, while *grim* is not (Jiang et al., 1997). This differential expression reflects the different pathways that regulate these genes. The expression and activity of *hid* are negatively regulated by the Ras/MAPK pathway (Bergmann et al., 1998; Kurada and White, 1998), while *rpr* expression is directly regulated by the p53/DNA damage pathway and the ecdysone receptor (EcR) signaling cascade (Brodsky et al., 2000; Jiang et al., 2000; Ollmann et al., 2000). Many other developmentally important pathways are likely to regulate the tissue-specific expression of these genes.

Are there distinct requirements for these genes in developmental apoptosis? This question is best addressed by the analysis of loss-of-function phenotypes. For example, decreased *hid* function results in extra cells in the developing eye and at the embryonic midline (Zhou et al., 1997; Kurada and White, 1998). These same cells survive when EGF receptor (EGFR) signaling is increased (Dong and Jacobs, 1997; Scholz et al., 1997; Stemerdink and Jacobs, 1997; Miller and Cagan, 1998; Sawamoto et al., 1998). Loss of *hid* function inhibits apoptosis resulting from decreased EGFR signaling (Bergmann et al., 1998; Kurada and White, 1998). Taken together these data indicate that EGFR activity, by regulating *hid* function, regulates cell number in the developing fly.

To dissect the role of *rpr* in apoptosis, we generated a deletion, which removes *rpr* but not *grim* or *hid*. Both developmentally regulated apoptosis as well as DNA damage-induced apoptosis were analyzed in *rpr* null animals. The majority of developmental apoptosis was unaffected by the absence of *rpr*. However, the central nervous systems (CNS) of *rpr* null adults were grossly enlarged. This neural hyperplasia resulted from the failure of some normal nervous system cell death. Among the cells that failed to undergo apoptosis were the neuroblasts of the abdominal neuromeres. When these cells are rescued from apoptosis, they continue to behave like neuroblasts, giving rise to large numbers of neuronal progeny that are incorporated into the adult nervous system. Although both the *Drosophila* p53 homolog, p53, and EcR regulate *rpr* expression, we were surprised to find that *rpr* played only a limited role in apoptosis induced by these regulators.

MATERIALS AND METHODS

Identification of a *rpr*-specific deficiency

Males carrying a ry^+ marked P element (*P(ry$^+$)l(3)02069*) were X-rayed and progeny scored for loss of the marked P element. Out of 120,000 scored flies, 50 showed loss of the P element as judged by the loss of ry^+. One candidate, *XR38*, showed loss of *rpr* DNA in 27% of embryos as assessed by single embryo PCR (see below). Both *grim* and *hid* DNA were present (data not shown). Subsequent in situ hybridization experiments confirmed that *grim* and *hid* expression was normal, while *rpr* was not expressed. GFP-null and GFP-positive embryos (controls) were selected from *XR38/TM3,(Kr-GFP)* (Casso et al., 1999) parents, and whole-mount in situ hybridizations to detect *hid* and *grim* were carried out according to the methods of Grether et al. and Chen et al. (Grether et al., 1995; Chen et al., 1996).

Single embryo PCR

Single embryo PCR was performed as described previously (Franc et al., 1999). Single embryos collected from a cross of *XR38/TM6B* parents were assayed for the presence of *rpr*, *grim* or *hid* DNA. In each reaction, primers for an irrelevant control sequence (*doom*) were included to control for DNA quality. *rpr*-specific primers were: 5′GGCAGTGGCATTCTACATACCCG and 5′CCCGTATTTGTT-AGTTACTCGAATCC. *hid* primers: 5′TCGACGGGCGAGG-ATGAGCGCGAG and 5′GACTGATGTGGCCATGGATGGCAC. *grim* primers: 5′CAACAACAGCAGCAGCAGCGAC and 5′CCGTCGGTTGACGCTGGCTCGAACT. *doom*-specific control primers: 5′AGGGTAAACGGCCACAGAATGT and 5′GATATCGTT-GTAGTTGGCCCG. Out of 48 embryos with a *doom* product, 13 showed no *rpr* PCR product. No loss of *hid* or *grim* DNA was detected. Further mapping by single embryo PCR demonstrated that the *XR38* distal breakpoint was at least 20 kb proximal of *grim* and at least 30 kb distal of *rpr*.

Apoptosis assays

Acridine Orange (AO) staining (Abrams et al., 1993) was performed on the *rpr* null embryos from *XR38/TM3,(Kr-GFP)* (Casso et al., 1999) parents. To test for suppression of *grim* or *hid* killing by the *rpr* mutation, *GMR-grim/+; XR38/H99* was compared to *GMR-grim/+; +/+* and *SM1-GMR-hid/+; XR38/H99* was compared to *SM1-GMR-hid/+; +/+*. Apoptosis of midline glia was assayed in *P(1.0 slit-lacZ); XR38/XR38* embryos according to the method of Zhou et al. (Zhou et al., 1997). X-ray-induced apoptosis was assessed in third instar larval wing discs. *XR38/H99* (the Tb$^+$ progeny of *XR38/TM6B,Tb* X *H99/TM6B,Tb*) and *y w* control larvae were mock treated or irradiated with 4000 rads and subsequently aged for 4 hours at 25°C. Wing discs were dissected and apoptotic cells were visualized by AO (Brodsky et al., 2000). Steroid hormone-induced death was assessed in salivary glands and larval midgut. *XR38/H99* and *y w* white prepupae were collected and aged for 4 hours at 25°C. Midguts were dissected and the presence of gastric caecae was assessed (Jiang et al., 1997). In both *rpr* null and wild-type pupae, the caecae were almost completely histolyzed at this time. To assess salivary gland histolysis, animals were dissected at 16 hours post-puparium formation. Both wild-type and *rpr* mutant salivary glands are undetectable at this time.

BrdU labeling

Larval stainings were done on the Tb$^+$ progeny of *XR38/TM6B,Tb* X *H99/TM6B,Tb*, and on *y w* controls. Larvae were fed continuously on Kankel/White medium (White and Kankel, 1978) containing 0.1 mg/ml of BrdU (Sigma). Central nervous systems from wandering third instar larvae or adults were dissected in calcium-free Ringer's solution and subsequently fixed for 30 minutes in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid), and rehydrated for 2× 20 minutes in PBT (PBS with 0.1% Triton X-100). The nervous systems were then treated with 2 N HCl in PBT for 90 minutes, washed for 2× 10 minutes in PBT and blocked for 2 hours in PBT with 2% BSA and 1% normal goat serum. Mouse anti-BrdU (Roche) was added at 1:50 in PBT with 1% BSA, incubated overnight at 4°C, washed 3× 20 minutes in PBT at room temperature and incubated with FITC-conjugated anti-mouse (Jackson Immunoresearch) 1:200 for 4 hours at room temperature. The tissue was washed 4× 20 minutes in PBT and mounted in Fluoromount-G (Southern Biotech) for confocal microscopy. For double labeling, rat anti-Elav (1:200) (Developmental Studies Hybridoma Bank, University of Iowa) was added with the anti-BrdU, and an anti-rat secondary (Jackson Immunoresearch) was used at 1:200.

Other antibody stainings

Anti-Grainyhead

Stainings were performed on *XR38/H99, NT1B1/+; XR38/NT1B1 H99* (White et al., 1994), *hid^{05014}/H99* (Grether et al., 1995), *dark^{CD4}/dark^{CD4}* (Rodriguez et al., 1999) larvae and *y w* controls, as in Uv et al. (Uv et al., 1997). Staining was detected by confocal microscopy. Grh-expressing cells were counted in *y w, XR38/H99* and *NT1B1/+; XR38/NT1B1 H99* larvae. The number of Grh-expressing cells was counted in A2-A8 on each confocal image, with care not to count the same cell twice. An average of 143±8 Grh-expressing neuroblasts could be counted in the abdominal neuromeres of *XR38/H99* larvae (n=3), while 117±2 Grh-expressing neuroblasts could be counted in the same region of *NT1B1/+; XR38/NT1B1 H99* larvae (n=3). 9.5±3.5 Grh-expressing cells were counted in *y w* control larvae (n=2).

Anti-CCAP and anti-EcRA

Central nervous systems from wild-type, *XR38/TM6B, H99/TM6B* and *XR38/H99* pharate or 2- to 6-day old adults were dissected in PEM (100 mM Pipes, 2 mM EGTA, 1 mM $MgSO_4$). Tissues were

fixed for 20 minutes in 3.7% formaldehyde in PEM, rinsed 2× in PEM, dehydrated in a methanol series, and rinsed 5× in PBT. Nervous systems were incubated overnight at room temperature with 1:1000 anti-CCAP (Ewer and Truman, 1996) or 1:200 anti-EcR-A (Talbot et al., 1993) in PBT and 10% normal goat serum, rinsed 4× in PBT and incubated with biotinylated anti-mouse or anti-rabbit secondary (1:200 in PBT, 10% normal goat serum) for a minimum of 2 hours at room temperature. Nervous systems were rinsed 4× in PBT, incubated with ABC (Vector) and developed in the presence of diaminobenzidine.

Courtship assays

Canton-S, XR38/TM6B, H99/TM6B or *XR38/H99* males were collected within 12 hours of eclosion and aged in individual food vials for 3-5 days. Each male was placed in a courtship chamber with a wet filter paper and an aged Canton S female for observation and videotaping. Courtship indices (CIs) reflect the percentage of time each male directed any courtship step toward his female during a 10 minute observation period. In some cases a male mated before the 10 minute observation period was completed. CIs for these males were based upon percentage of time spent courting until the point of successful copulation.

RESULTS

Reaper is not required for most developmental apoptosis

A specific loss-of-function *rpr* mutation was essential to dissect the role of *rpr* in developmental apoptosis. The isolation of such a mutation has proved challenging; previous attempts to use chemical mutagens to create lethal or visible point mutations in the *H99* region only resulted in the isolation of *hid* alleles (White et al., 1994), prompting us to use an alternative strategy. Males carrying a P element located in the *non-stop* gene, 225 kb proximal to *rpr* (Fig. 1A) (Berkeley *Drosophila* Genome Project) were irradiated and candidate genomic deletions were identified. Loss of *rpr* genomic sequence was assayed by single embryo PCR (Fig. 1B,C). A single *rpr* deletion, *XR38*, was isolated. As assessed by in situ hybridization, homozygous *XR38* embryos showed no *rpr* expression, while no quantitative or qualitative changes in *grim* or *hid* mRNA expression were detected (Fig. 1D-G). The *XR38* deletion is large, removing several genes (Fig. 1A), and *XR38* homozygotes are lethal. However, flies of the genotype *XR38/H99* are likely to be homozygously deleted for the *rpr* gene alone, as the proximal breakpoint of *H99* lies only 15 kb from *rpr* (White et al., 1994), and no other predicted genes lie between *rpr* and this breakpoint (Berkeley *Drosophila* Genome Project). The distal breakpoint of the XR38 deletion lies between *rpr* and *grim* and was found to map more than 30 kb distal to *rpr* and more than 20 kb proximal to *grim*. There are no predicted genes between *rpr* and *grim*.

XR38/H99 flies are viable, and emerge at the expected frequency. They have a shortened lifespan, but do not show any obvious visible defects. Acridine Orange (AO) staining (Fig. 2A,B) did not reveal detectable decreases in the overall levels of embryonic apoptosis. Apoptosis was also assessed in embryos laid by *rpr* null (*XR38/H99*) mothers, to eliminate any potential maternal contribution of Rpr protein or mRNA to embryonic apoptosis. No changes in overall apoptosis were detected in embryos that lacked both maternal and zygotic *rpr* (data not shown).

To more closely examine embryonic apoptosis, the death of

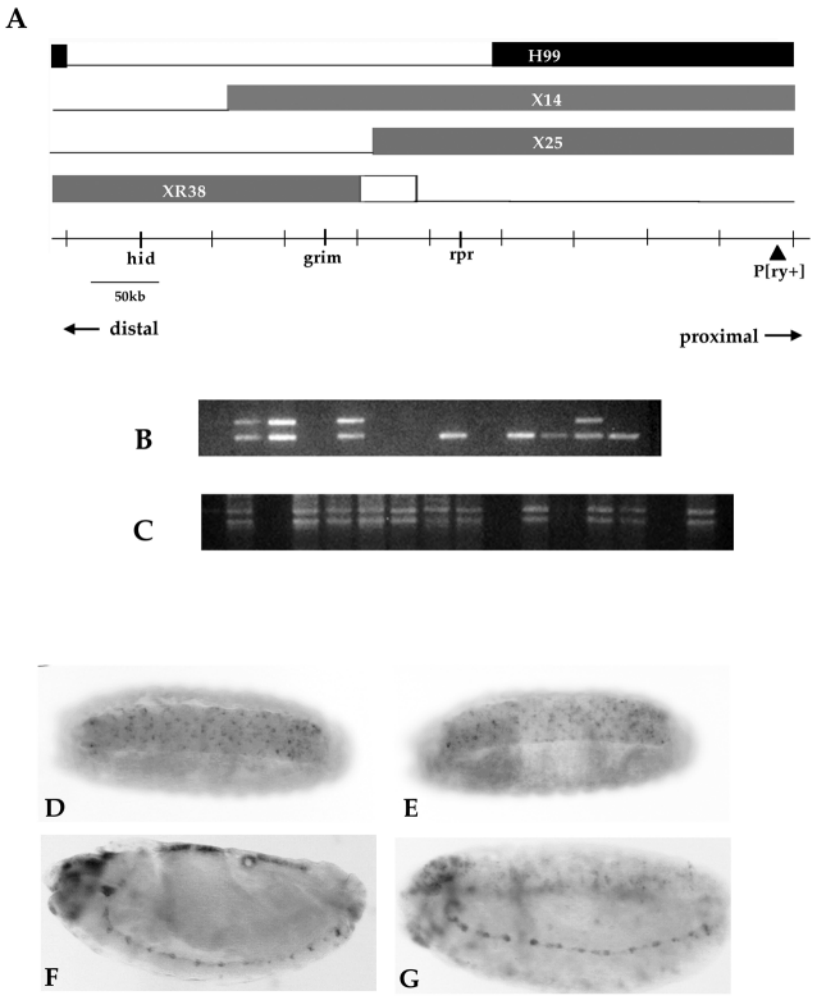

Fig. 1. Generating a *rpr*–specific mutation. (A**)** Previously characterized deletions in the 75C1,2 region include *H99*, which removes *rpr*, *grim* and *hid*, X14, which deletes *hid*, and X25, which deletes *hid* and *grim* (White et al., 1994). Lines represent deleted DNA. Open boxes represent uncertainties in the location of the breakpoints. A deletion that removed *rpr* was generated by irradiating males carrying a marked P element 225 kb proximal to *rpr*. The progeny were scored for the loss of the eye color marker. As scored by single embryo PCR, one candidate line, *XR38*, showed a loss of *rpr* genomic sequences. (B) An example of single embryo PCR on the progeny of *XR38*/TM6B parents. Each lane represents the DNA of a single embryo. Primers from an unrelated gene, *doom*, are used as an internal control (lower band). Lanes with two bands indicate the embryo has both *rpr* (upper band) and *doom*. Lanes with no bands indicate insufficient DNA. Lanes with only the lower band indicate a loss of *rpr* DNA. (C) *grim* genomic sequences are not affected by this deletion. All embryos from *XR38*/+ parents show both *grim* (upper) and *doom* (lower) PCR products. (D-G) In situ hybrizations show that *grim* (D,E) and *hid* (F,G) expression is not detectably altered in *XR38/XR38* embryos (E,G). (D,F) wild-type embryos.

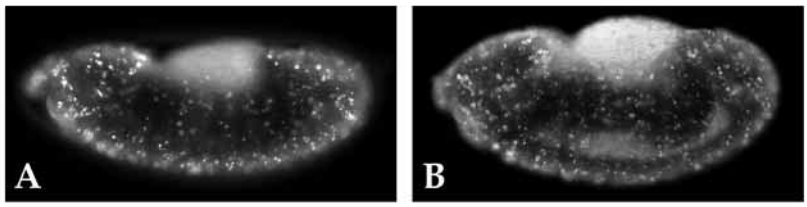

Fig. 2. Most apoptosis is not detectably altered by loss of *rpr*. (A,B) No overall defects were seen in embryonic apoptosis as detected by AO, or by TUNEL (data not shown). (A) Wild-type embryo, (B) *XR38* homozygous embryo.

glial cells at the embryonic midline was assessed. The death of these cells can be easily quantitated *in vivo* using the midline marker P(1.0 slit/lacZ) (Zhou et al., 1995). During embryonic development the number of *lacZ*-expressing midline glia decreases from 9 per segment (stage 12) to 3 (stage 17) due to apoptosis. Previous work has shown that deletion of *hid*, *grim* and *rpr* (*Df(3L)H99*) blocks the death of these cells completely (Zhou et al., 1997). Deletion of *hid* alone doubles the number of surviving cells from 3 to 6 per segment (Zhou et al., 1997), while deletion of *hid* and *grim* (*Df(3L)X25*) increases the number of surviving cells to 8. We found only 3 surviving P(1.0 slit/lacZ)-expressing cells in *XR38/XR38* embryos (data not shown). These data support the idea that *rpr* and *grim* function cooperatively with *hid* to induce apoptosis. The killing activity of *grim* and *rpr* appears to be approximately additive in the midline glia, while *hid* activity is more important. In sum, we could detect no essential role for *rpr* in embryonic apoptosis.

Previous results suggested that *rpr* might be required for the loss of some larval tissues during metamorphosis. Early in pupal development both the larval salivary gland and much of the larval midgut undergo programmed cell death (Jiang et al., 1997). Four hours after puparium formation the midgut has shrunk in size and no longer contains gastric caecae, while salivary gland histolysis occurs during a 30 minute span 14.5 hours after puparium formation. This process is regulated by pulses of the steroid hormone ecdysone (Baehrecke, 2000). *rpr* and *hid* expression immediately precede salivary gland and midgut histolysis. *rpr* is induced directly by the EcR hormone receptor complex, while *hid* is induced as a secondary response to ecdysone during salivary gland histolysis (Jiang et al., 1997). Based on these findings, the loss of *rpr* was predicted to delay or inhibit salivary gland or midgut histolysis. Surprisingly, these deaths were not detectably altered in *rpr* mutant pupae (data not shown). Thus, other apoptotic regulators must compensate for the loss of *rpr* in these hormone-regulated deaths.

The role of *rpr* in DNA damage-induced apoptosis

X-irradiation leads to increased levels of apoptosis in the *Drosophila* embryo and larva. This apoptosis is preceded by a rapid induction of *rpr* expression (Nordstrom et al., 1996). In mammalian systems p53 has been shown to be required for apoptosis induced by DNA damaging agents such as X-irradiation (Lowe et al., 1993). Recently a *Drosophila* homolog of p53 has been identified (*p53*) (Brodsky et al., 2000; Ollmann et al., 2000). p53 induces apoptosis when ectopically expressed, and a dominant negative p53 inhibits apoptosis induced by X-rays. Importantly, p53 was shown to activate a radiation responsive element in the *rpr* promoter (Brodsky et al., 2000). This suggests that p53 induces apoptosis in response to DNA damage by activating *rpr* transcription. Our *rpr*-specific deletion allowed us to test whether *rpr* expression is required for p53 and radiation-induced apoptosis.

When p53 is overexpressed in the wild-type eye using an eye-specific driver (*GMR-p53*), ectopic apoptosis is induced, resulting in small adult eyes (Ollmann et al., 2000) (Fig. 3A).

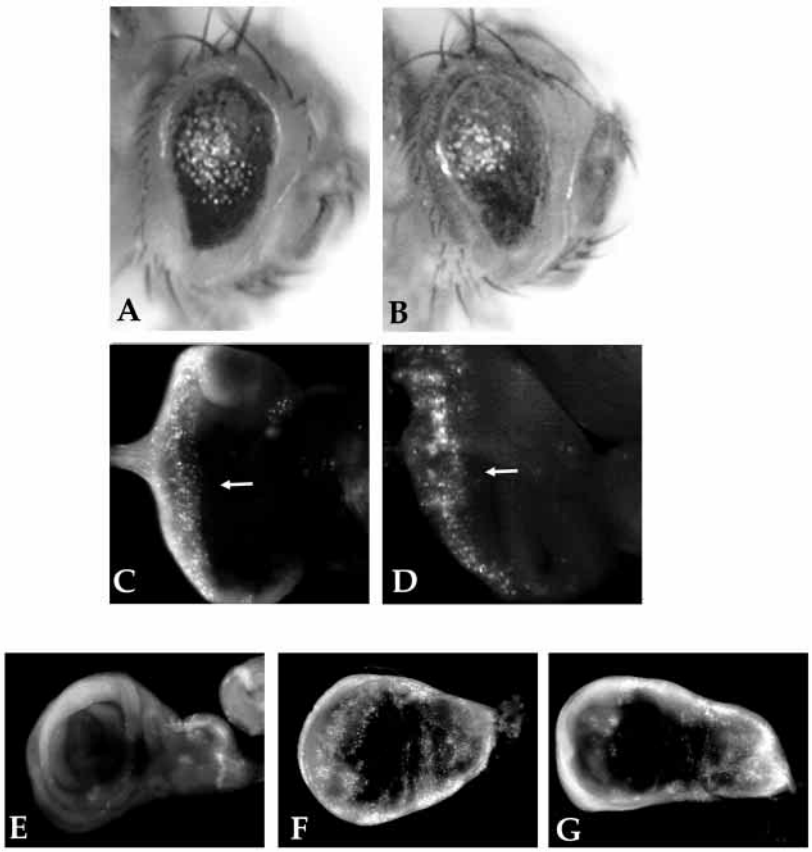

Fig. 3. The role of *rpr* in DNA-damage-induced death. (A,B) The absence of *rpr* does not suppress the ability of p53 to induce apoptosis when overexpressed in the eye. (A,C) GMR-gal4/UAS-p53 (B,D) GMR-gal4/UAS-p53; *XR38*/*H99*. (C,D) AO staining of third instar eye discs detects similar levels of ectopic apoptosis. (E-G) X-ray-induced apoptosis is suppressed in *rpr* nulls. Third instar larvae were irradiated with 4,000 rads and wing discs were dissected 4 hours later and stained with AO to visualize apoptotic cells. Expression of dominant-negative p53 has been shown to block X-ray-induced apoptosis under these conditions (Brodsky et al., 2000; Ollmann et al., 2000) (E) Wild-type unirradiated disc, (F) wild-type irradiated disc, (G) *XR38*/*H99* irradiated disc.

When *GMR-p53* was expressed in *rpr* mutants, the eye size was not modified by the absence of *rpr* (Fig. 3B). As the phenotype of *GMR-p53* might not only result from p53-induced apoptosis, but might reflect additional effects of p53 on eye development, we assayed apoptosis directly in developing eyes, and found that the loss of *rpr* did not suppress or delay this death (Fig. 3C,D). These data indicate that p53 can induce apoptosis in a *rpr*-independent manner.

We directly examined the role of *rpr* in X-ray-induced apoptosis in the developing wing disc. In wild-type larvae, apoptosis is induced in the developing wing within 4 hours of treatment with a high dose of X-rays (Fig. 3F). This death is dependent on the activity of p53 (Brodsky et al., 2000; Ollmann et al., 2000). In the absence of *rpr*, X-ray-induced apoptosis was significantly inhibited, although some ectopic death is clearly visible (Fig. 3G). Thus, *rpr* function is required for high levels of apoptosis in response to DNA damage. The residual apoptosis seen in Fig. 3G indicates that p53 can activate apoptosis through other, *rpr*-independent mechanisms. It is likely that strong overexpression in *GMR-p53* obscures a requirement for *rpr* in p53-induced death in the eye.

rpr is required in the developing nervous system

Although *rpr* mutant flies survive to adulthood, the males are completely sterile. Spermatogenesis appeared normal in *rpr* null males, and large numbers of motile sperm were present in the testes. However, when wild-type females were placed with *rpr* mutant males for several days, no sperm was transferred to the females. Closer analysis revealed that *rpr* mutant males showed almost wild-type amounts of overall courtship behaviors, but only 1 male out of 16 mated with the female. Courtship indices (CIs) were calculated for wild-type, *XR38* and *H99* heterozygous and *XR38/H99* males. The CIs represent the mean amount of time males spent courting individual Canton S females during a given observation period. Wild-type males had a courtship index (CI) of 58±10% (n=13), *rpr* heterozygotes had a CI of 49±9% (n=8) and *rpr* mutant males had a CI of 47±9% (n=16). These numbers are not statistically different by ANOVA analysis ($F_{(2,\ 0.28)}$=0.63, P>0.5). The major block in the mating process appears to be an inability of the males to bend their abdomens sufficiently for copulation.

These defects in adult behavior prompted us to examine the morphology of the adult CNS. Surprisingly, the nervous systems of *rpr* mutant flies were much larger than those of wild-type flies. The thoracic and abdominal ganglia of the ventral nerve cord (VNC) were enlarged (Fig. 4B,D,F), with the most extensive hyperplasia found in the abdominal ganglion.

To determine if this enlargement was due to decreased apoptosis of larval neurons, we looked at the survival of two subsets of neurons. Adult nervous systems were stained with an antibody directed against the crustacean cardioactive peptide (CCAP). Anti-CCAP labels a set of 34-36 neurons in the VNC (Draizen et al., 1999). Six of these neurons are located in the ventral thoracic portion of the VNC, while the remaining neurons are in the abdominal ganglion. Most of these neurons die within 24 hours of eclosion in the wild type, leaving a population of only 4 CCAP-positive cells in the abdominal ganglion (Draizen et al., 1999). Similarly, we observed an average of 3±0.7 CCAP-expressing neurons (n=5) in the abdominal ganglia of 2- to 6-day old wild-type flies (Fig. 4A,C). In 2- to 6-day old *XR38/TM6B* flies we found an average of 5.3±0.9 CCAP-positive neurons in the abdominal ganglion (n=4), a number that is not significantly different from wild type. As previously reported, the 6 ventral thoracic CCAP-expressing cells survive in the absence of 1 copy of *rpr*, *grim* and *hid* (*H99/TM6B*) (Draizen et al., 1999). In *XR38/H99* adults, many more of these cells survived, with 26.6±1.2 CCAP-immunoreactive neurons present 2-6 days after eclosion (n=16) (Fig. 4B,D). These cells include the 6 ventral thoracic cells that survive in *H99/+*, as well as 20 cells in the abdominal ganglion.

To examine the survival of another class of neurons, an antibody to the A isoform of the ecdysone receptor (EcR-A) was used to identify a group of around 300 neurons that normally undergo apoptosis within a day of adult eclosion (Robinow et al., 1993). The death of these cells requires falling

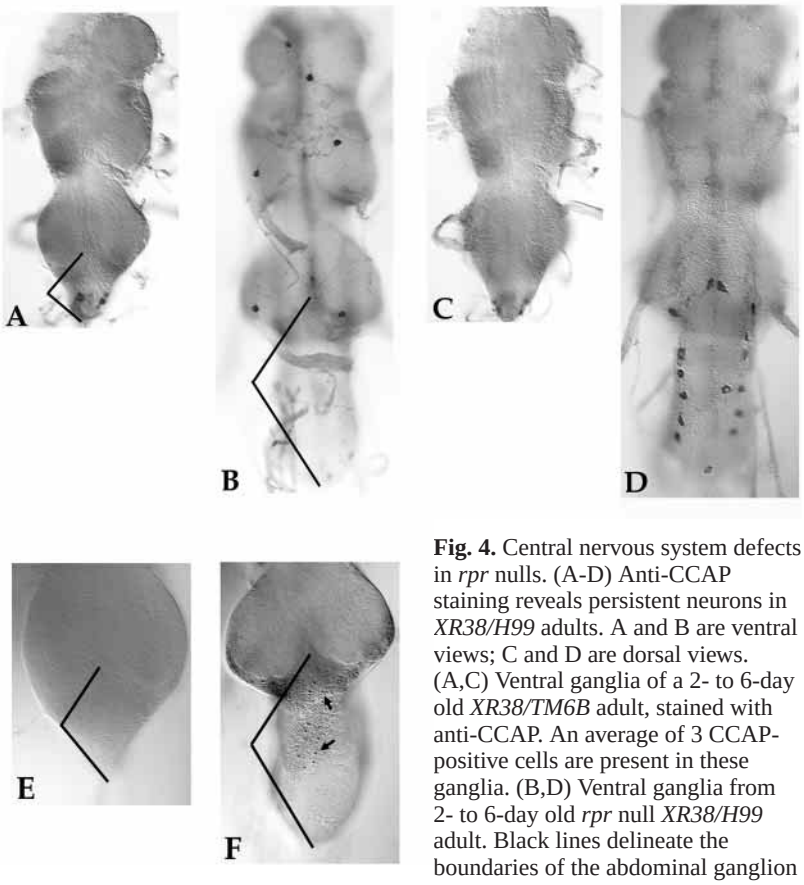

Fig. 4. Central nervous system defects in *rpr* nulls. (A-D) Anti-CCAP staining reveals persistent neurons in *XR38/H99* adults. A and B are ventral views; C and D are dorsal views. (A,C) Ventral ganglia of a 2- to 6-day old *XR38/TM6B* adult, stained with anti-CCAP. An average of 3 CCAP-positive cells are present in these ganglia. (B,D) Ventral ganglia from 2- to 6-day old *rpr* null *XR38/H99* adult. Black lines delineate the boundaries of the abdominal ganglion that are greatly enlarged in *XR38/H99*. About 20 CCAP-positive cells are present in these enlarged abdominal ganglia. (E,F) EcR-A-expressing neurons also persist in *XR38/H99* adults. Ventral view of the T3 thoracic neuromere and abdominal neuromere of a 1- to 2-day old *XR38/TM6B* (E) and *XR38/H99* adult (F). Black lines delineate the boundaries of the abdominal ganglion. All photos are taken at the same magnification.

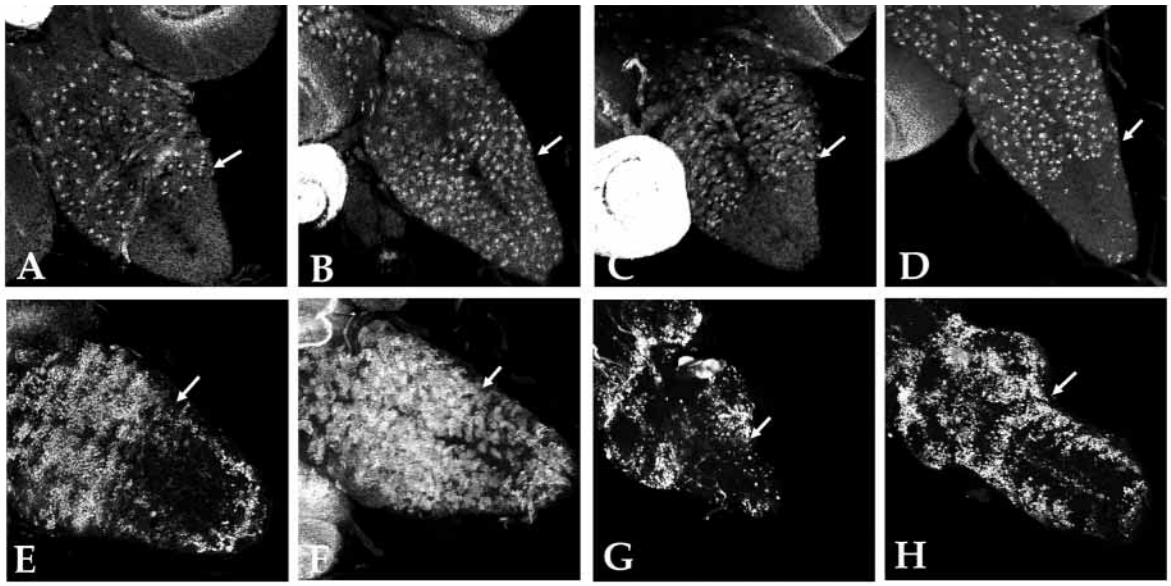

Fig. 5. *rpr* is required to eliminate neuroblasts. (A-D) Anti-Grainyhead staining labels persistent neuroblasts throughout the abdominal neuromeres of *rpr* mutant larvae. (A) Very few neuroblasts are found in the abdominal neuromeres in the VNC of wild-type larvae. The white arrow indicates the boundary between the thoracic and abdominal neuromeres. (B) Many neuroblasts are present in the abdominal neuromeres of *rpr* mutant larvae (*XR38*/*H99*). (C) No ectopically surviving neuroblasts are seen in *hid* mutant larvae, even when one copy of *grim* and *rpr* are also deleted (hid^{05014}/*H99*). (D) Mutation of *dark*, an *Apaf1* homolog, also results in a few ectopically surviving neuroblasts in the abdominal neuromeres. (E-H) BrdU labeling reveals ectopic cell division in the abdominal neuromeres of the ventral nerve cord of *rpr* mutant larvae. (E) CNS from a wild-type third instar larva fed continuously on BrdU-containing food. Very little division is seen in the abdominal neuromeres. *H99*/+ animals also appear wild type (data not shown). (F) In the *rpr* mutant (*XR38*/*H99*), the abdominal region is filled with dividing cells. (G) A few cells labeled with BrdU during larval life are present in the small condensed abdominal neuromeres of wild-type adults. (H) Many cells labeled with BrdU during larval life are found in the enlarged abdominal neuromeres of the *XR38*/*H99* adult.

levels of ecdysone, and is accompanied by increased transcription of *rpr* and *grim* (Robinow et al., 1997). In 1- to 2-day old *XR38/TM6B* adults 2.8±1.4 (*n*=4) EcR-A expressing cells were present (Fig. 4E). In *H99/TM6B* adults 6.9±1.4 (*n*=9) EcRA positive cells were found, while approximately 65±3.8 (*n*=5) of these cells persisted in the *XR38*/*H99* adults (Fig. 4F). These data reveal two populations of neurons that fail to undergo normal programmed cell death in *XR38/H99 rpr* mutants, suggesting that *rpr* is required for at least some post-eclosion neuronal cell death.

The persistence of neurons that should die during or after metamorphosis likely accounts for some of the increased size of the *rpr* null CNS. However, the significant enlargement of the abdominal ganglia of the VNC led us to speculate that this increase might additionally reflect the survival and proliferation of neuroblasts. This speculation was based on patterns of neuroblast apoptosis. In the embryo, neural development begins with the specification of about 50 neuroblasts in each neuromere (repeating segment) of the VNC (Goodman and Doe, 1993). Neuroblast stem cell divisions produce the neurons of the larval CNS, and then the neuroblasts become quiescent. In early larval life, almost all neuroblasts in the brain and the thoracic neuromeres of the VNC reinitiate division, giving rise to the neurons of the adult CNS (Truman and Bate, 1988; Prokop and Technau, 1991). However, very few neuroblasts are reactivated in the abdominal neuromeres (A2-A8) of the larval VNC (Truman and Bate, 1988), as the majority of neuroblasts in the abdominal neuromeres undergo apoptosis by the end of embryogenesis in the wild type (Bray et al., 1989; White et al., 1994).

The apoptosis of the abdominal neuroblasts is prevented in *H99* homozygous embryos (White et al., 1994). However it is not known whether these rescued cells are competent to function as neuroblasts. Can they proliferate? If so, do the progeny of these divisions differentiate as neurons? The embryonic lethal stage of *H99* homozygotes prevented us from assessing whether neuroblasts rescued from death are competent to resume proliferation in the larvae.

Abdominal neuroblast survival in *rpr* null *XR38/H99* larvae was directly assessed by staining for the Grainyhead (Grh) protein (Bray et al., 1989). Grh is expressed in neuroblasts throughout embryogenesis and larval life (Bray et al., 1989; Uv et al., 1997; Prokop et al., 1998). The nervous systems of *XR38/H99* larvae contain many cells expressing Grh in the abdominal neuromeres**,** whereas only a few are present in wild-type nervous systems (Fig. 5A,B).

The death of the abdominal neuroblasts specifically requires *rpr* function. In larvae that completely lack *hid* function, we found no ectopic survival of abdominal neuroblasts (data not shown). Even in larvae that lack *hid* function, and have reduced *rpr* and *grim* function (hid^{05014}/*H99*) (Grether et al., 1995), there was no increase in the survival of these cells (Fig. 5C). Thus, the reduction in *grim* and *hid* in *XR38/H99* is unlikely to contribute to this phenotype. In addition, we were able to partially rescue the *rpr* phenotype with a cosmid transgene containing 30 kb of genomic sequence around *rpr* (NT1B1) (data not shown) (White et al., 1994). The incomplete nature of this rescue may reflect poor *rpr* expression from this transgene in this tissue.

Interestingly, we also found a few ectopic abdominal

neuroblasts in *dark* mutant larvae (Fig. 5D). *dark* is a *Drosophila* homolog of Apaf-1, an important component of the apoptotic effector machinery (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). Apaf-1 and Dark act to facilitate caspase activation in response to the release of cytochrome C from the mitochondria. Mutations in *dark* suppress killing by ectopically expressed *rpr*, suggesting that Dark activates caspases in response to *rpr* expression. The small number of ectopically surviving neuroblasts in *dark* mutants demonstrates that dark is essential for a limited amount of *rpr*-dependent apoptosis in the developing animal.

To determine if these surviving neuroblasts retained their proliferative capacity, larvae were fed on BrdU-containing food. In the larval VNC, virtually the only cycling cells are the neuroblasts and their progeny, the ganglion mother cells (Truman and Bate, 1988). The dividing cells are localized to the ventral surface of the VNC, and the progeny of each neuroblast can be visualized as a cluster of BrdU-positive cells. In wild-type larvae this proliferation is confined mainly to thoracic neuromeres, with the exception of a few divisions in the terminal abdominal neuromere (Truman and Bate, 1988; Taylor and Truman, 1992). In *rpr* mutants, there is a substantial increase in the number of BrdU-labeled cells in the abdominal neuromeres (Fig. 5E,F). These cells are ventrally localized and clustered, similar to the progeny of wild-type neuroblasts. We conclude that neuroblasts rescued from apoptosis in the *rpr* mutant remain competent to proliferate.

The progeny of these rescued neuroblasts are responsible for the enlarged abdominal neuromeres in the adult CNS. Cells that have been BrdU labeled during the larval stages are abundant in the abdominal neuromeres of the *rpr* mutant adult VNC, and much less abundant in the small abdominal neuromeres of the wild type (Fig. 5G,H).

The progeny of these ectopic neuroblast divisions differentiate into neurons. In both wild-type and *rpr* mutant larval VNCs most BrdU-labeled cells expressed Elav, a pan-neural marker (Robinow and White, 1991) (Fig. 6). These newly born neurons are confined mainly to the thoracic neuromeres of the wild-type VNC, but are present throughout the entire VNC in the *rpr* mutant. Taken together, these data indicate that *rpr* function is required to eliminate neuroblasts in the embryo. Neuroblasts that survive this developmental death are competent to divide, giving rise to ectopic neurons in the larval and adult CNS.

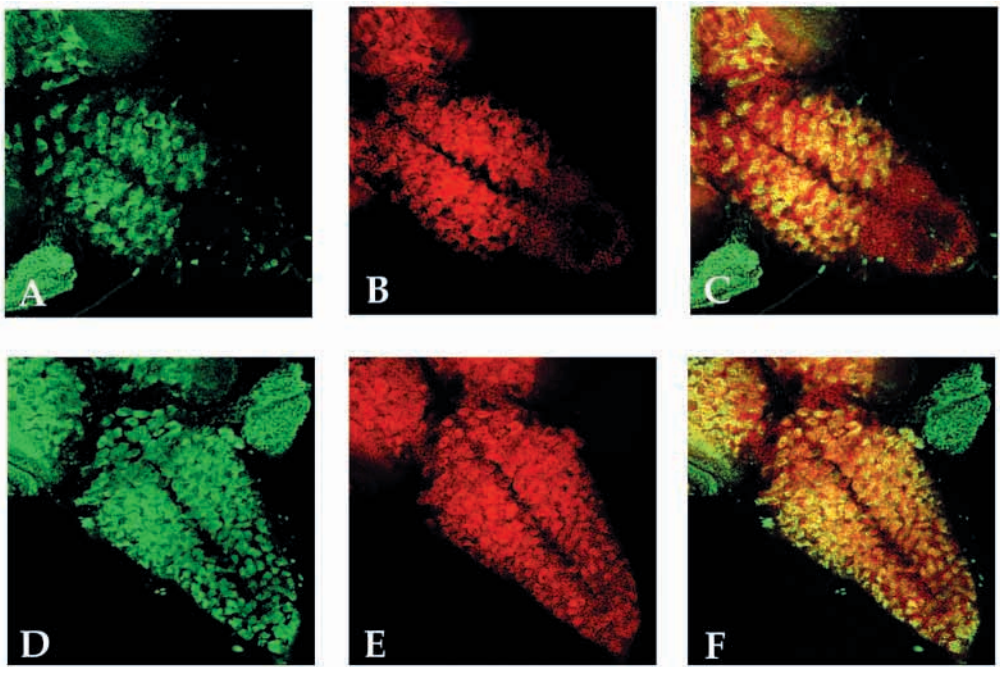

Fig. 6. The progeny of ectopic neuroblast divisions differentiate as neurons. Wild-type (A-C) and *rpr* null (*XR38*/*H99*) (D-F) larvae were fed continuously on BrdU-containing food, and dissected nervous systems were double labeled with anti-BrdU (A,D) and anti-Elav (B,E). The Elav protein is expressed in the nuclei of all neurons. C and F are the merged images of A,B and D,E. Most BrdU-labeled cells in both wild-type and *rpr* null nervous systems label with Elav.

DISCUSSION

The combined activity of the genes *rpr*, *hid* and *grim* is required for the initiation of all apoptotic death in the developing *Drosophila* embryo. Both genetic and molecular data suggest that these proteins act in an additive manner in the cell to induce apoptosis. However, the activities of these apoptotic regulators are governed by diverse developmental and environmental signals. To assess their individual roles in developmental apoptosis we have generated a mutation that removed only *rpr*. The phenotypes of this *rpr* mutant reveal both overlapping and unique functions for this gene. Although *rpr* is expressed in many cells that are fated to die during development, loss of the gene results in very specific defects. In the absence of *rpr*, neuroblasts in the developing CNS survive and proliferate, resulting in neural hyperplasia. Substantial numbers of neurons normally fated to die after eclosion survive in these mutants, suggesting that *rpr* is required for many, but not all, post-eclosion neuronal cell deaths. In contrast, overall levels of apoptosis are not detectably altered in *rpr* mutant embryos, and the ecdysone-induced death of the larval salivary gland and midgut, as well as some DNA damage-induced death can occur normally.

The interactions of *rpr*, *grim* and *hid*

The apparently normal level of death in *rpr* null embryos indicates that *grim* and *hid* functions are sufficient for the majority of embryonic apoptosis. As *grim* is expressed in a very similar pattern to *rpr*, it is likely that the activity of *grim* is able to compensate for the absence of *rpr* in many tissues, with the notable exception of the neuroblasts in the abdominal neuromeres.

In contrast to what is seen in *rpr* mutants, embryos lacking

hid function show a slightly decreased level of overall apoptosis (Grether et al., 1995; Zhou et al., 1997). In the absence of *hid*, *rpr* and *grim* activity are not sufficient to initiate apoptosis in a significant number of cells. These results suggest that *hid* activity is quantitatively or qualitatively different from *rpr* and *grim* activity.

Both in vitro binding studies and genetic data support the model that *rpr*, *grim* and *hid* kill by binding to a *Drosophila* IAP, DIAP1, inactivating its caspase inhibitory activity. In this model, all three proteins are functionally equivalent. The combined activity of the three proteins determines the likelihood that a cell will undergo apoptosis. However there are some findings that suggest functional differences between *rpr*, *hid* and *grim*. Overexpression of *hid* and *rpr* together in the midline glia has been shown to induce apoptosis much more potently than equivalent expression of either protein alone (Zhou et al., 1997). In addition, some caspases appear to be more important for killing by *rpr* than by *hid*, suggesting that there are molecular distinctions between *rpr* and *hid* within the cell (Song et al., 2000). Our preliminary data reveal that deletion of *rpr* and *hid* together has a strong synergistic effect on overall levels of embryonic apoptosis (C. P. and K. W., unpublished). This observation is consistent with distinct mechanisms of action of Rpr and Hid.

The requirement for *rpr* in steroid hormone-induced death shows tissue specificity

The steroid hormone ecdysone regulates programmed cell death at metamorphosis and in the adult central nervous system (Baehrecke, 2000). It is interesting to note that rising levels of ecdysone initiate degeneration in the larval midgut and salivary glands, while falling levels of the hormone are required for the death of the type II neurons in the newly eclosed adult. These different responses to ecdysone may be mediated by different isoforms of the receptor, as the doomed larval midgut and salivary gland cells express primarily the B1 isoform of the receptor, while the doomed neurons express the A isoform (Robinow et al., 1993; Talbot et al., 1993). Although these receptor isoforms share both ligand binding and DNA binding domains, they show functional differences (Bender et al., 1997; Schubiger et al., 1998).

Expression of *rpr* is rapidly induced in the salivary glands after the prepupal pulse of ecdysone. A binding site for the ecdysone receptor complex is present in the *rpr* promoter, which is essential for *rpr* expression in the doomed salivary gland (Jiang et al., 2000). The type II neurons also express *rpr* before they undergo apoptosis (Robinow et al., 1997). Thus *rpr* is a strong candidate to be important in both of these deaths. We found that salivary gland death was not affected in *rpr* mutant pupae, while the death of type II neurons was significantly inhibited. This disparity may be explained by the differences in the other genes expressed in these tissues. In the salivary glands, the induction of *rpr* expression is rapidly followed by *hid* expression (Jiang et al., 2000). In this tissue, as in the embryo, it is likely that *hid* activity compensates for the absence of *rpr*. Expression of the caspase Dronc is also increased in response to ecdysone in these tissues (Dorstyn et al., 1999). High levels of Dronc can induce apoptosis (Quinn et al., 2000) and may contribute to the histolysis of these tissues.

In contrast to the findings in salivary gland and midgut, the ecdysone-regulated death of EcR-A-expressing neurons in the adult nervous system was inhibited in the absence of *rpr*. These cells express *rpr* and *grim* but not *hid* prior to their death (Robinow et al., 1997). This expression pattern may be a common feature of neuronal tissue, as *hid* expression is not detectable in the embryonic central nervous system outside of the midline glia (Grether et al., 1995). In the adult nervous system, *grim* function is apparently not sufficient to induce apoptosis in many of the type II neurons. However, in the embryonic nervous system there is significant apoptosis in the absence of *rpr*. At this stage *grim* activity must be sufficient for most neural apoptosis, with the important exception of the death of the neuroblasts.

p53 requires *rpr* along with other targets to induce apoptosis

In flies, as in mammalian tissues, cells undergo apoptosis in response to DNA damage, and this apoptosis requires the activity of the transcription factor p53 (Ko and Prives, 1996; Brodsky et al., 2000; Ollmann et al., 2000). In flies, the expression of a dominant negative form of p53 largely inhibits X-ray-induced apoptosis (Brodsky et al., 2000; Ollmann et al., 2000). *Drosophila* p53 (p53), can directly bind to a radiation-inducible enhancer in the *rpr* promoter (Brodsky et al., 2000). These data strongly suggest that p53 induces apoptosis in response to DNA damage by activating *rpr* expression. Unexpectedly, no suppression of p53-induced apoptosis was detected in *rpr* null animals. However, X-ray-induced apoptosis is reduced in the absence of *rpr*. These data indicate that *rpr* is an important regulator of apoptosis induced by DNA damage, and that other apoptotic regulators are also involved. When p53 is strongly overexpressed in the eye, these other targets must be sufficient to overcome the absence of *rpr*. The functions of *hid* and/or *grim* are doubtless also involved in DNA damage-induced death, as X-ray-induced apoptosis is very strongly inhibited in *H99* embryos (White et al., 1994).

The survival of neural stem cells in the absence of *rpr*

Two striking phenotypes are found in *rpr* mutants: the adult central nervous system in both males and females is enlarged, especially the abdominal part of the ventral nerve cord, and males are sterile. The hyperplasia of the CNS results in part from the abnormal persistence of some larval neurons in the adult ventral ganglia. More importantly, neuroblasts also survive inappropriately in *rpr* mutants. In the wild-type animal, most of the neuroblasts in the abdominal neuromeres die at the end of embryogenesis, while in the *rpr* mutant many of these neuroblasts survive and proliferate. The progeny of these ectopic neuroblast divisions differentiate into neurons that are integrated into the adult nervous system. Why are the neuroblasts particularly sensitive to the loss of *rpr*? One possibility is that *rpr* is the only apoptosis regulator expressed in these cells. *hid* is not expressed in the embryonic nervous system (Grether et al., 1995). Although widespread expression of *grim* is detected in the embryonic CNS, it is not known if neuroblasts express *grim*. A distinct expression of other apoptotic factors could also account for the specific requirement for *rpr* in neuroblast apoptosis.

Mutations in the *Drosophila* Apaf1 homolog *dark* also result in enlargement of the larval CNS (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). This increased size results at least in part from the survival and proliferation (data not shown) of a

few neuroblasts in the abdominal neuromeres. This implicates *dark* as being required for some *rpr*-dependent apoptosis. It is interesting to note that *dark* mutations, like *rpr* mutations, cause significant male sterility (Rodriguez et al., 1999).

The sterility of *rpr* mutant males appears to be behavioral, as they are unable to copulate, although other courtship behaviors appear normal. The cause of the male copulation defect is unknown, but it is interesting to speculate that the reduction in normal cell death in the abdominal neuromeres is in some way responsible for this behavioral deficit. Indeed, the focus of male copulatory behavior has been mapped to the abdominal nervous system by mosaic mapping techniques (Ferveur and Greenspan, 1998). The presence of additional neurons in the nervous system of *rpr* mutants might interfere with the organization of the appropriate neurons into a functional neural circuit required for copulation. Alternatively, the neural circuit in the CNS might be properly constructed but the presence of additional motorneurons might prevent coordinated movement of the abdomen during copulation.

In *C. elegans*, the majority of developmental apoptosis occurs in the nervous system (Metzstein et al., 1998). In worms that are mutant for the genes *ced-3* or *ced-4*, and thus lacking all apoptosis, there are extra neurons (Ellis and Horvitz, 1986). However, ectopic cell proliferation has not been reported in these mutant animals. Neural hyperplasia is also seen in mice carrying engineered mutations in caspases 3 and 9 and in the Apaf1 caspase activator (Kuida et al., 1996; Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Woo et al., 1998; Yoshida et al., 1998). A detailed analysis of brain development in caspase 3 knockout mice showed a marked increase in proliferating neuroblasts, similar to what is seen in *rpr* mutants (Pompeiano et al., 2000). These mutants provide a graphic example of how normal development can be particularly disrupted when apoptosis of a stem cell population is inhibited, and these cells continue to proliferate. In the future, the *rpr* mutant flies will provide a unique model to explore the fate of ectopic neural stem cells and their progeny in the context of the nervous system.

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REFERENCES

Abrams, J. M., White, K., Fessler, L. I. and Steller, H. (1993). Programmed cell death during *Drosophila* embryogenesis. *Development* **117**, 29-43.

Baehrecke, E. H. (2000). Steroid regulation of programmed cell death during Drosophila development. *Cell Death Differ.* **7**, 1057-1062.

Bangs, P. and White, K. (2000). Regulation and execution of apoptosis during Drosophila development. *Dev. Dyn.* **218**, 68-79.

Bender, M., Imam, F., Talbot, W., Ganetzky, B. and Hogness, D. (1997). Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* **91**, 777-788.

Bergmann, A., Agapite, J., McCall, K. and Steller, H. (1998). The *Drosophila* gene *hid* is a direct molecular target of Ras-dependent survival signaling. *Cell* **95**, 331-341.

Bray, S. J., Burke, B., Brown, N. H. and Hirsh, J. (1989). Embryonic expression pattern of a family of *Drosophila* proteins that interact with a central nervous system regulatory element. *Genes Dev.* **3**, 1130-1145.

Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M. and Abrams, J. M. (2000). *Drosophila* p53 binds a damage response element at the *reaper* locus. *Cell* **101**, 103-113.

Casso, D., Ramirez-Weber, F. A. and Kornberg, T. B. (1999). GFP-tagged balancer chromosomes for Drosophila melanogaster. *Mech. Dev.* **88**, 229-232.

Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A. and Gruss, P. (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* **94**, 727-737.

Chen, P., Nordstrom, W., Gish, B. and Abrams, J. M. (1996). *grim*, a novel cell death gene in *Drosophila*. *Genes Dev.* **10**, 1773-1782.

Claveria, C., Albar, J., Serrano, A., Buesa, J., Barbero, J., Martinez-A, C. and Torres, M. (1998). *Drosophila grim* induces apoptosis in mammalian cells. *EMBO J.* **17**, 7199-7208.

Dong, R. and Jacobs, J. R. (1997). Origin and differentiation of supernumerary midline glia in Drosophila embryos deficient for apoptosis. *Dev. Biol.* **190**, 165-177.

Dorstyn, L., Colussi, P., Quinn, L., Richardson, H. and Kumar, S. (1999). DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl. Acad. Sci. USA* **96**, 4307-4312.

Draizen, T. A., Ewer, J. and Robinow, S. (1999). Genetic and hormonal regulation of the death of peptidergic neurons in the Drosophila central nervous system. *J. Neurobiol.* **38**, 455-465.

Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102**, 33-42.

Ellis, H. and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**, 817-829.

Evans, E., Kuwana, T., Strum, S., Smith, J., Newmeyer, D. and Kornbluth, S. (1997). Reaper-induced apoptosis in a vertebrate system. *EMBO J.* **16**, 7372-7381.

Ewer, J. and Truman, J. W. (1996). Increases in cyclic 3′, 5′-guanosine monophosphate (cGMP) occur at ecdysis in an evolutionarily conserved crustacean cardioactive peptide- immunoreactive insect neuronal network. *J. Comp. Neurol.* **370**, 330-341.

Ferveur, J. F. and Greenspan, R. J. (1998). Courtship behavior of brain mosaics in Drosophila. *J. Neurogenet.* **12**, 205-226.

Franc, N., Heitzler, P., Ezekowitz, R. and White, K. (1999). Requirement for croquemort in phagocytosis of apoptotic cells in *Drosophila*. *Science* **284**, 1994-1998.

Goodman, C. and Doe, C. (1993). Embryonic development of the Drosophila central nervous system. In *The development of Drosophila melanogaster*, vol. 2 (ed. M. Bate and A. Martinez Arias), pp. 1131-1206. New York: Cold Spring Harbor Laboratory Press.

Goyal, L., McCall, K., Agapite, J., Hartwieg, E. and Steller, H. (2000). Induction of apoptosis by *Drosophila reaper*, *hid* and *grim* through inhibition of IAP function. *EMBO J.* **19**, 589-597.

Grether, M. E., Abrams, J. M., Agapite, J., White, K. and Steller, H. (1995). The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev.* **9**, 1694-1708.

Haining, W., Carboy-Newcomb, C., Wei, C. and Steller, H. (1999). The proapoptotic function of *Drosophila* Hid is conserved in mammalian cells. *Proc. Natl. Acad. Sci. USA* **96**, 4936-4941.

Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W. et al. (1998). Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell* **94**, 339-352.

Hawkins, C., Wang, S. and Hay, B. (1999). A cloning method to identify caspases and their regulators in yeast: Identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1. *Proc. Natl. Acad. Sci. USA* **96**, 2885-2890.

Hay, B. A., Wassarman, D. A. and Rubin, G. M. (1995). *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* **83**, 1253-1262.

Jacobson, M. D., Weil, M. and Raff, M. C. (1997). Programmed cell death in animal development. *Cell* **88**, 347-354.

Jiang, C., Baehrecke, E. and Thummel, C. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development* **124**, 4673-4683.

Jiang, C. A., Lamblin, A. F. J., Steller, H. and Thummel, C. S. (2000). A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. *Mol. Cell* **5**, 445-455.

Kaiser, W. J., Vucic, D. and Miller, L. K. (1998). The *Drosophila* inhibitor of apoptosis D-IAP1 suppresses cell death induced by the caspase drICE. *FEBS Letts* **440**, 243-248.

Kanuka, H., Sawamoto, K., Inohara, N., Matsuno, K., Okano, H. and Miura, M. (1999). Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. *Mol. Cell* **4**, 757-769.

Ko, L. J. and Prives, C. (1996). p53: puzzle and paradigm. *Genes Dev.* **10**, 1054-1072.

Kuida, K., Haydar, T., Kuan, C.-Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S.-S., Rakic, P. and Flavell, R. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94**, 325-337.

Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**, 368-372.

Kurada, P. and White, K. (1998). Ras promotes cell survival in *Drosophila* by downregulating *hid* expression. *Cell* **95**, 319-329.

Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A. and Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**, 847-849.

McCarthy, J. and Dixit, V. (1998). Apoptosis induced by *Drosophila* Reaper and Grim in a human system. *J. Biol. Chem.* **273**, 24009-24015.

Meier, P., Silke, J., Leevers, S. J. and Evan, G. I. (2000). The *Drosophila* caspase DRONC is regulated by DIAP1. *EMBO J.* **19**, 598-611.

Metzstein, M., Stanfield, G. and Horvitz, H. (1998). Genetics of programmed cell death in C. elegans: past, present and future. *Trends Genet.* **14**, 410-416.

Miller, D. T. and Cagan, R. L. (1998). Local induction of patterning and programmed cell death in the developing *Drosophila* retina. *Development* **125**, 2327-2335.

Nordstrom, W., Chen, P., Steller, H. and Abrams, J. M. (1996). Activation of the *reaper* gene during ectopic cell killing in *Drosophila*. *Dev. Biol.* **180**, 213-226.

Ollmann, M., Young, L. M., Di Como, C. J., Karim, F., Belvin, M., Robertson, S., Whittaker, K., Demsky, M., Fisher, W. W., Buchman, A. et al. (2000). *Drosophila* p53 is a structural and functional homolog of the tumor suppressor p53. *Cell* **101**, 91-101.

Pompeiano, M., Blaschke, A. J., Flavell, R. A., Srinivasan, A. and Chun, J. (2000). Decreased apoptosis in proliferative and postmitotic regions of the Caspase 3-deficient embryonic central nervous system. *J. Comp. Neurol.* **423**, 1-12.

Prokop, A., Bray, S., Harrison, E. and Technau, G. M. (1998). Homeotic regulation of segment-specific differences in neuroblast numbers and proliferation in the Drosophila central nervous system. *Mech. Dev.* **74**, 99-110.

Prokop, A. and Technau, G. M. (1991). The origin of postembryonic neuroblasts in the ventral nerve cord of *Drosophila melanogaster*. *Development* **111**, 79-88.

Pronk, G. J., Ramer, K., Amiri, P. and Williams, L. T. (1996). Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. *Science* **271**, 808-810.

Quinn, L. M., Dorstyn, L., Mills, K., Colussi, P. A., Chen, P., Coombe, M., Abrams, J., Kumar, S. and Richardson, H. (2000). An essential role for the caspase dronc in developmentally programmed cell death in Drosophila. *J. Biol. Chem.* **275**, 40416-40424.

Robinow, S., Draizen, T. A. and Truman, J. W. (1997). Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the Drosophila CNS. *Dev. Biol.* **190**, 206-213.

Robinow, S., Talbot, W. S., S., H. D. and Truman, J. W. (1993). Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific receptor isoform. *Development* **119**, 1251-1259.

Robinow, S. and White, K. (1991). Characterization and spatial distribution of the ELAV protein during Drosophila melanogaster development. *J. Neurobiol.* **22**, 443-461.

Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X. and Abrams, J. (1999). DARK, a *Drosophila* homolog of Apaf-1/ced-4, functions in an evolutionarily conserved death pathway. *Nature Cell Biol.* **1**, 272-279.

Sawamoto, A., Taguchi, A., Hirota, Y., Yamada, C., Jin, M. and Okano, H. (1998). Argos induces programmed cell death in the developing Drosophila eye by inhibition of the ras pathway. *Cell Death Differ.* **5**, 262-270.

Scholz, H., Sadlowski, E., Klaes, A. and Klambt, C. (1997). Control of midline glia development in the embryonic Drosophila CNS. *Mech. Dev.* **64**, 137-151.

Schubiger, M., Wade, A., Carney, G., Truman, J. and Bender, M. (1998). *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development* **125**, 2053-2062.

Song, Z. W., Guan, B., Bergman, A., Nicholson, D. W., Thornberry, N. A., Peterson, E. P. and Steller, H. (2000). Biochemical and genetic interactions between *Drosophila* caspases and the proapoptotic genes *rpr*, *hid*, and *grim*. *Mol. Cell. Biol.* **20**, 2907-2914.

Stemerdink, C. and Jacobs, J. R. (1997). Argos and Spitz group genes function to regulate midline glial cell number in *Drosophila* embryos. *Development* **124**, 3787-3796.

Talbot, W. S., Swyryd, E. A. and Hogness, D. S. (1993). Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* **73**, 1323-1337.

Taylor, B. J. and Truman, J. W. (1992). Commitment of abdominal neuroblasts in *Drosophila* to a male or female fate is dependent on genes of the sex-determining hierarchy. *Development* **114**, 625-642.

Thress, K., Evans, E. K. and Kornbluth, S. (1999). Reaper-induced dissociation of a Scythe-sequestered cytochrome C-releasing activity. *EMBO J.* **18**, 5486-5493.

Thress, K., Song, J., Morimoto, R. I. and Kornbluth, S. (2001). Reversible inhibition of Hsp70 chaperone function by Scythe and Reaper. *EMBO J.* **20**, 1033-1041.

Truman, J. W. and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster. *Dev. Biol.* **125**, 145-157.

Uv, A. E., Harrison, E. J. and Bray, S. J. (1997). Tissue-specific splicing and functions of the Drosophila transcription factor Grainyhead. *Mol. Cell Biol.* **17**, 6727-6735.

Vaux, D. and Korsmeyer, S. (1999). Cell death in development. *Cell* **96**, 245-254.

Verhagen, A., Ekert, P., Pakusch, M., Silke, J., Connolly, L., Reid, G., Moritz, R., Simpson, R. and Vaux, D. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102**, 43-53.

Vucic, D., Kaiser, W. J., Harvey, A. J. and Miller, L. K. (1997). Inhibition of Reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). *Pcoc. Natl. Acad. Sci. USA* **94**, 10183-10188.

Vucic, D., Kaiser, W. J. and Miller, L. K. (1998). Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by *Drosophila* proteins HID and GRIM. *Mol. Cell. Biol.* **18**, 3300-3309.

Wang, S., Hawkins, C., Yoo, S., Müller, H.-A. and Hay, B. (1999). The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**, 453-463.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in *Drosophila*. *Science* **264**, 677-683.

White, K. and Kankel, D. R. (1978). Patterns of cell division and cell movement in the formation of the imaginal nervous system in Drosophila melanogaster. *Dev. Biol.* **65**, 296-321.

White, K., Tahaoglu, E. and Steller, H. (1996). Cell killing by the *Drosophila* gene *reaper*. *Science* **271**, 805-807.

Woo, M., Hakem, R., Soengas, M. S., Duncan, G. S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S. A. et al. (1998). Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev.* **12**, 806-819.

Wu, J. W., Cocina, A. E., Chai, J., Hay, B. A. and Shi, Y. (2001). Structural analysis of a functional DIAP1 fragment bound to grim and hid peptides. *Mol. Cell* **8**, 95-104.

Yoshida, H., Kong, Y.-Y., Yoshida, R., Elia, A., Hakem, A., Hakem, R., Penninger, J. and Mak, T. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* **94**, 739-750.

Zhou, L., Hashimi, H., Schwartz, L. M. and Nambu, J. R. (1995). Programmed cell death in the Drosophila central nervous system midline. *Curr. Biol.* **5**, 784-790.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L. M., Steller, H. and Nambu, J. R. (1997). Cooperative functions of the reaper and head involution defective genes in the programmed cell death of Drosophila central nervous system midline cells. *Proc. Natl. Acad. Sci. USA* **94**, 5131-5136.

Zhou, L., Song, Z., Tittel, J. and Steller, H. (1999). HAC-1, a *Drosophila* homolog of APAF-1 and CED-4, functions in developmental and radiation-induced apoptosis. *Mol. Cell* **4**, 745-755.