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Coordinated expression of cell death genes regulates neuroblast apoptosis

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SUMMARY

Properly regulated apoptosis in the developing central nervous system is crucial for normal morphogenesis and homeostasis. In *Drosophila*, a subset of neural stem cells, or neuroblasts, undergo apoptosis during embryogenesis. Of the 30 neuroblasts initially present in each abdominal hemisegment of the embryonic ventral nerve cord, only three survive into larval life, and these undergo apoptosis in the larvae. Here, we use loss-of-function analysis to demonstrate that neuroblast apoptosis during embryogenesis requires the coordinated expression of the cell death genes *grim* and *reaper*, and possibly *sickle*. These genes are clustered in a 140 kb region of the third chromosome and show overlapping patterns of expression. We show that expression of *grim*, *reaper* and *sickle* in embryonic neuroblasts is controlled by a common regulatory region located between *reaper* and *grim*. In the absence of *grim* and *reaper*, many neuroblasts survive the embryonic period of cell death and the ventral nerve cord becomes massively hypertrophic. Deletion of *grim* alone blocks the death of neuroblasts in the larvae. The overlapping activity of these multiple cell death genes suggests that the coordinated regulation of their expression provides flexibility in this crucial developmental process.

KEY WORDS: Neuroblast, *Drosophila*, Apoptosis

INTRODUCTION

Programmed cell death is essential for spatial and temporal patterning during development in metazoans, particularly in the central nervous system (CNS), where the majority of cells die during development (Buss et al., 2006). Both mature neurons and dividing progenitors undergo developmentally regulated apoptosis. Mutations in genes important for cell death often result in profound nervous system phenotypes, including increased brain size and defects in morphology (Yuan and Yankner, 2000; De Zio et al., 2005). The appropriate death of proliferating progenitors is particularly crucial to prevent neural hypertrophy (Buss et al., 2006).

We understand relatively little about how particular cells in the CNS are selected to die at a particular time during development. A large number of mature neurons undergo apoptosis in the absence of appropriate targets, and this death is regulated by extrinsic factors, such as the absence of neurotrophins. However, the death of proliferating neural progenitors is likely to be regulated by both extrinsic and intrinsic factors that regulate temporal and spatial identity, as well as interactions with supporting cells (Buss et al., 2006). Examining how neural progenitor cell death is regulated will provide insight into normal neural development. In addition, the viability of these cells might be manipulated to treat disease.

Drosophila neural stem cells or neuroblasts (NBs) provide a well-described, genetically tractable model to examine the regulation of neural stem cell death. In the embryo, NBs are specified and divide to give rise to the neurons and glia of the CNS. At the end of embryogenesis, NBs become quiescent and the vast majority of NBs in the abdominal segments of the developing ventral nerve cord (VNC) undergo apoptosis in late embryogenesis (Fig. 1A, red circle) (Truman and Bate, 1988; White et al., 1994). Of the 30 NBs that are initially present in each hemisegment of the VNC, only three remain in hemisegments A3-A8 at the end of embryogenesis. These occupy characteristic positions and can be identified as the dl, vl and vm NBs in early third instar larvae (Truman and Bate, 1988). During larval life, the dl, vl and vm NBs reinitiate proliferation, giving rise to small neuronal lineages. When NB cell death is inhibited in the embryo, many of the embryonic abdominal NBs survive to become 'ectopic' postembryonic NBs (White et al., 1994; Peterson et al., 2002). These continue to divide and produce neuronal progeny, resulting in a massive enlargement of the abdominal segments of the adult CNS (Peterson et al., 2002). In wild-type larvae, the dl, vl and vm abdominal NBs are eliminated by apoptosis at mid third instar (Fig. 1A, purple circle), which limits the number of neuronal progeny they produce (Bello et al., 2003). Our studies focus on the cell death pathways that select NBs to die in the late embryo and in the larvae.

Developmental apoptosis in *Drosophila* is regulated by the genes *head involution defective* (*hid*; *Wrinkled*, *W* – FlyBase), *grim*, *reaper* (*rpr*) and possibly *sickle* (*skl*), collectively known as the RHG genes (Cashio et al., 2005). These genes are clustered in a 272 kb locus on chromosome III. Developmental apoptosis is completely blocked when *hid*, *grim* and *rpr* are deleted, whereas overexpression of each RHG genes is sufficient to induce caspase-dependent apoptosis (White et al., 1994; Grether et al., 1995; Chen et al., 1996; White et al., 1996; Srinivasula et al., 2002; Wing et al., 2002). A role for *skl* in developmental apoptosis has yet to be

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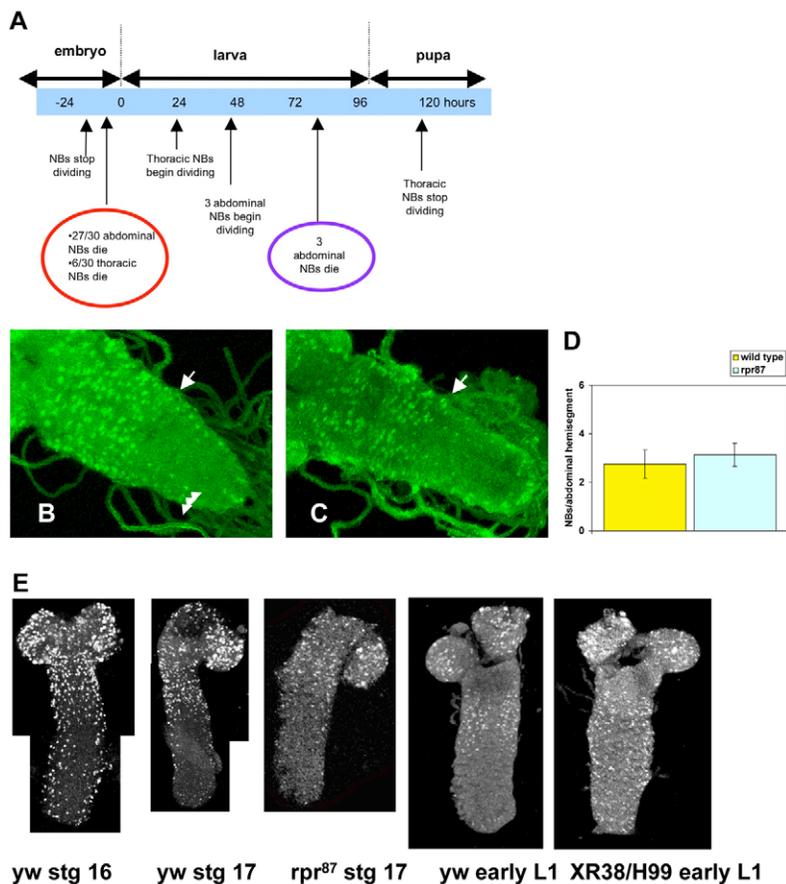


Fig. 1. Deletion of *rpr* alone is not sufficient to inhibit neuroblast (NB) apoptosis. (A) Schematic of NB death during *Drosophila* development, based on data from this study together with published data (Truman and Bate, 1988; White et al., 1996; Bello et al., 2003). Apoptosis occurs at late embryogenesis (red circle) and at the mid third instar stage (purple circle). (B,C) Anti-Dpn staining detects only three abdominal NBs per hemisegment in both wild type (B) and *rpr⁸⁷* (C) early third instar larval CNS. White arrows mark the boundary between 'thoracic' (including A1 and A2) and abdominal segments. Arrowheads mark three NBs in an abdominal segment. (D) Quantitative analysis shows no significant difference in the average number of abdominal NBs between wild type and *rpr⁸⁷* (data shown is the average of four or more animals \pm s.d.). (E) Abdominal NBs (visualized with anti-Dpn) are eliminated from the CNS in late stage 16 embryos in both wild type and *rpr⁸⁷*, whereas they can still be detected after hatching in XR38/H99.

demonstrated. The pro-apoptotic activity of these genes is mediated, at least in part, by the ability to inhibit the anti-apoptotic DIAP1 (Thread – FlyBase) protein (Kornbluth and White, 2005).

Interestingly, the pattern of RHG gene expression largely reflects the pattern of cell death, although *hid* is expressed both in cells that are fated to die and in cells that survive (Bangs and White, 2000). The dynamic pattern of RHG gene expression must reflect complex transcriptional regulation of these genes. We know some of the upstream regulators of RHG gene expression, such as p53, which activates *rpr*, *skl* and *hid* expression in response to DNA damage, and Ecdysone receptor, which induces transcription of *rpr* and *hid* in salivary glands (Brodsky et al., 2000; Jiang et al., 2000; Ollmann et al., 2000; Zhang et al., 2008). The hox protein Deformed directly regulates *rpr* expression in the developing gnathal segments, and Abd-B regulates *rpr* in the posterior segmental boundaries (Lohmann et al., 2002). Expression of *hid* is directly regulated by E2F, modulating sensitivity to DNA damage-induced apoptosis (Moon et al., 2005). Activity of the Ras-MAPK pathway also regulates *hid* transcription (Kurada and White, 1998). During late embryogenesis, *grim*, *rpr* and *skl* are expressed mainly in the central nervous system whereas *hid* is expressed in the midline. The similar expression patterns of *grim*, *rpr* and *skl* at this stage of development suggests that these genes might be regulated in a coordinated manner in the embryonic CNS (Bangs and White, 2000; Srinivasula et al., 2002; Wing et al., 2002).

Previously, we identified a mutant background that results in the ectopic survival of many NBs in the abdominal segments of the larval VNC (Peterson et al., 2002). Overlapping deletions that result in removal of the *rpr* gene, along with ~68 kb of the

surrounding genomic sequence, result in a viable animal with a greatly expanded adult VNC. Although the *rpr* gene is the only known gene in this deletion overlap, we have found that deletion of *rpr* alone does not result in ectopic postembryonic NB survival. Here, we show that genomic sequences between *rpr* and *grim* control expression of *rpr*, *grim* and *skl* in NBs, allowing the coordinated expression of these cell death regulators. We propose that this genomic region serves as an integrator of multiple developmental signals to select this population of cells to undergo apoptosis at the proper time during development.

MATERIALS AND METHODS

Mutations and stocks

Wild-type flies were *yw^{67c23}*, *grim^{A6C}* and *grim^{C15E}* strains were kindly provided in advance of publication by Carrie Brachmann (Wu et al., 2010). The small *rpr⁸⁷* deletion was generated by imprecise P excision (Moon et al., 2008). The following mutants were generated by PiggyBac P-P deletion (Parks et al., 2004): (1) *MM1* removes 32 kb (pp[d11052-f00984]); (2) *MM2* removes 110 kb (pp[d11052-f07860]); (3) *MM3* removes 54 kb (pp[f04656-f00984]) between *rpr* and *grim*. The *grim^{C15E} rpr⁸⁷* (*grim rpr*) recombinant was generated in a two-step recombination using flanking markers. The *skl^{H12}* allele was generated by excising the hobo element from the hybrid *P(wHy)DG39210* element (Huet et al., 2002). *Df(3L)ED224* was obtained from DrosDel, deleting 429 kb on the third chromosome, including *hid*, *grim* and *rpr*. *Df(3L)ED225*, obtained from DrosDel, deletes 435 kb from 5' of *hid* to *non-stop*. *ED225* is a hypomorphic allele of *hid*. *X20* deletes from *hid* through *skl*, with the proximal breakpoint ~30 kb proximal to *skl*. *UAS-grimRNAi* was obtained from the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007) and ubiquitous expression was driven by act5C-gal4 (Ito et al., 1997).

Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) or RNA-protein double labeling was carried out as described by Lecuyer et al. (Lecuyer et al., 2008) with the following modifications: the riboprobe concentration was 1 ng/ μ l, hybridization was done at 57°C and embryos were blocked in PBTB for 30 minutes. In double FISH, the embryos were quenched in 3% H₂O₂ for 30 minutes to completely eliminate residual horseradish peroxidase (HRP) between probes. Riboprobes of biotin-labeled *grim* and digoxigenin (DIG)-labeled *rpr* or *skl* were used in double FISH experiments, and DIG-labeled riboprobes were used to quantify single gene expression. GFP-null embryos were selected from *MM3/TM3*, *Ubi-GFP* parents, and rabbit anti-GFP antibody (Invitrogen) was used to detect GFP protein expression.

Microscopy and neuroblast counting

Fluorescent images were obtained with a Leica TCS 4D or a Nikon A1 SiR scanning confocal microscope. Complete z-series of embryos or larval CNS were taken through the tissue, and z-stack projections were used to quantify NBs, which were identified by their large size, round shape and position. Double staining with an antibody to the neuronal marker ELAV (Robinow and White, 1991) [1:50, Developmental Studies Hybridoma Bank (DSHB)] confirms that these big cells are not neurons and are likely to be NBs. For quantification of gene expression in NBs in the embryo, the CNS of each embryo was divided equally into three sections, and NBs in the posterior third of the VNC were identified and counted. For quantification of early L3 NB number, or late L3 cluster number or cluster size, the NBs in the third to fifth neuromeres of the abdominal VNC were counted and analyzed.

Immunohistochemistry

Neuroblasts were detected with anti-Deadpan (Dpn) staining (Boone and Doe, 2008). Early-mid third instar larvae were collected based on morphology, dissected in PBS (pH 7.4) and the CNS was fixed with 4% formaldehyde for 30 minutes at room temperature. After washing in PBS, the CNS was permeabilized in PBST (PBS with 0.3% Triton X-100) for 30 minutes then blocked with 1% BSA in PBST for 30 minutes. The tissue was incubated with rat anti-Dpn (1:2 or 1:1 in PBST, a gift from C. Doe, University of Oregon) at 4°C overnight. Incubation with donkey anti-rat Alexa 488 secondary antibody (1:200, Invitrogen) was carried out at room temperature for 1.5 hours. To detect newly born neurons, the CNS from late third instar larvae was stained with anti-Neurotactin (Hortsch et al., 1990) (1:50, DSHB) with donkey anti-mouse secondary (1:400, Invitrogen). Anti-phosphohistone H3 antibody (1:400, Millipore) was used to detect mitotic cells.

Quantitative real-time PCR

Embryos of each strain were collected at indicated stages. Total RNA was extracted using RNAqueous-4PCR kit (Ambion) and treated with DNase I to remove genomic DNA. cDNA was synthesized by reverse transcription of total RNA with an Advantage RT-for-PCR Kit (Clontech). Quantitative real-time PCR (qPCR) was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems). Relative levels of specific mRNAs were determined using the SYBR Green I Detection Chemistry System (Applied Biosystems). Quantification was performed using the comparative C_T method as described in the manufacturer's procedures

manual. RP49 (RpL32 – FlyBase) was used for normalization. The following primers were used: *grim*, 5'-TCGGAGTTTGGATGCTGGGATCTT-3' and 5'-AGTCACGTCGTCCTCATCGTTGTT-3'; *rpr*: 5'-AAAGTCCGGCAAATATCGCAAGCC-3' and 5'-TGTTGTG-GCTCTGTGTCCTTGACT-3'; *skl*: 5'-ACGACAACCTGCCAA-GAGTTCAGA-3' and 5'-TATCGACTTGATCGCCACTGGGTT-3'; *RP49*: 5'-CTCATGCAGAACC GCGTTTA-3' and 5'-ACAAATGTG-TATTCCGACCA-3'.

RESULTS

rpr deletion alone does not lead to ectopic NB survival

Previously, we showed that ectopic neuroblasts were present in the abdominal neuromeres of *Df(3L)XR38/Df(3L)H99* (*XR38/H99*) third instar larvae (Peterson et al., 2002). This overlapping combination of deficiencies removes a 70 kb region that extends from ~15 kb upstream of *rpr* to 53 kb downstream, between *rpr* and *grim*. Because the only identified gene that is completely deleted in *XR38/H99* larvae is *rpr* (*hid*, *grim* and *skl* are all heterozygous), the data suggested that *rpr* function is required for NB apoptosis.

To study further the role of *rpr* in the developmental apoptosis of neuroblasts, we generated a small *rpr* deletion (*rpr*⁸⁷) by imprecise P-element excision. This deletion removes ~3 kb, including the entire *rpr* transcription unit (Moon et al., 2008). Surprisingly, *rpr*⁸⁷ homozygotes did not show a significant increase in abdominal NBs as detected by staining with anti-Deadpan (Dpn) in early-mid third instar larvae, or in late embryos (Bier et al., 1992; Boone and Doe, 2008) (Fig. 1). We considered the possibility that homozygous deletion of *rpr* along with loss of one copy of *hid*, *grim* and *skl* might result in ectopic NB survival. However, *rpr*⁸⁷/*Df(3L)X20*, which recapitulates the *hid*^{-/+}, *grim*^{-/+}, *rpr*^{-/-}, *skl*^{-/+} genotype (Table 1), did not result in ectopic postembryonic NBs (data not shown).

We also considered the possibility that other cell death genes could be mutated in *XR38*. We found that the *XR38* chromosome carried a point mutation in *grim* that converts proline 29 to arginine. This proline is conserved in all *Drosophila* species in the melanogaster group, but not in more distantly related *Drosophila* species. When expressed in S2 cells pMT-Grim P29A shows slightly reduced killing when compared with wild type (data not shown). We tested whether this *grim* mutation was significant for NB apoptosis by removing the other copy of *grim*. *Df(3L)X25* deletes *hid* and *grim*, and extends ~30 kb proximal to *grim* (Fig. 2) (White et al., 1994). Larvae of the genotype *XR38/Df(3L)X25* (Table 1) did not contain ectopic abdominal postembryonic NBs (data not shown), indicating that NB cell death occurred normally. These results demonstrated that neither the deletion of *rpr*, nor the *grim* P29A mutation in *XR38* is solely responsible for the

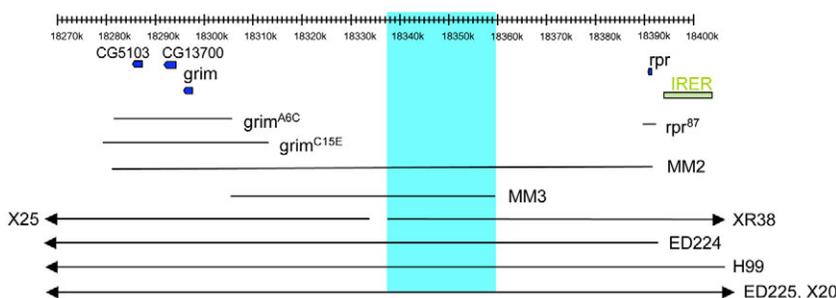


Fig. 2. Genotypes used in this study. Schematic of deletions. Black bars represent deleted regions. The *grim rpr* double mutant is a recombinant between *rpr*⁸⁷ and *grim*^{C15E}. The shaded region is the minimum deletion for neuroblast (NB) survival (the neuroblast regulatory region, NBRR). The IRR-irradiation responsive element, defined by Zhang et al. (Zhang et al., 2008), does not overlap with the NBRR.

Table 1. Table of genotypes used in this study, showing gene dosages for known cell death genes and the neuroblast regulatory region (NBRR)

Genotype	<i>hid</i>	<i>grim</i>	NBRR	<i>rpr</i>	<i>skl</i>	Neuroblasts*
<i>yw</i>	+/+	+/+	+/+	+/+	+/+	wt
<i>XR38/H99</i>	+/-	P29A/+	-/-	-/-	-/-	8.3
<i>rpr⁸⁷/rpr⁸⁷</i>	+/+	+/+	+/+	-/-	+/+	wt
<i>rpr⁸⁷/XR20</i>	+/-	+/-	+/-	-/-	+/-	wt
<i>X25/XR38</i>	-/+	-/P29A [†]	+/-	+/-	+/-	
<i>grim^{A6C}/grim^{A6C}</i>	+/+	-/-	+/+	+/+	+/+	4.0
<i>grim^{A6C}/MM2</i>	+/+	-/-	+/-	+/-	+/+	
<i>skl^{H2}/skl^{H2}</i>	+/+	+/+	+/+	+/+	-/-	
<i>MM3/MM3</i>	+/+	+/+	-/-	+/+	+/+	6.3
<i>MM3/XR38</i>	+/+	+/+	-/-	+/-	+/-	5.6
<i>MM2/XR38</i>	+/+	-/+	-/-	-/-	+/-	8.6
<i>Grim rpr/grim rpr</i>	+/+	-/-	+/+	-/-	+/+	10.4
<i>grim rpr/MM3</i>	+/+	-/+	+/-	-/+	+/+	5.0
<i>MM2/ED224</i>	+/-	-/-	-/-	-/-	+/+	11.8
<i>MM2/ED225</i>	hm/+ [†]	-/-	-/-	-/-	+/-	13.5
<i>X14/H99</i>	-/-	+/-	+/-	+/-	+/+	wt

*Number of neuroblasts present per abdominal hemisegment at early third instar.

[†]Hypomorphic mutation.

wt, ~3 NBs; +/+, wild type; +/-, heterozygous deletion; -/-, homozygous deletion.

XR38/H99 phenotype. Taken together, these results suggest that deletion of the region around *rpr* might be responsible for the ectopic abdominal NB phenotype.

The region between *rpr* and *grim* is important for NB apoptosis

To study the role of the genomic region between *grim* and *rpr* in NB apoptosis, we generated several genomic deletions (Fig. 2). Using FRT-mediated recombination, we generated a deletion that removes only the intergenic region between *rpr* and *grim*, leaving the two transcribed regions intact (*MM3*). In addition we generated a deletion that extends from *grim* through *rpr* (*MM2*). Deletions around *grim* were described previously (Wu et al., 2010). Lastly, a small deletion of *skl* (*skl^{H2}*) was generated by transposable element excision.

Early to mid third instar larvae homozygous for the intergenic *MM3* deletion were assayed for abdominal NB number, using anti-Dpn staining (Bier et al., 1992; Boone and Doe, 2008). We found a significant number of ectopic abdominal NBs in *MM3* homozygous larvae (Fig. 3A), indicating a reduction in embryonic NB cell death (Fig. 1A, red circle). A similar number of ectopic abdominal NBs were found in *MM3/XR38* larvae. These overlapping deficiencies result in homozygous deletion of a 22 kb region between *rpr* and *grim*.

NBs were also visualized using anti-Neurotactin. Neurotactin marks newly born neurons (Hortsch et al., 1990; Bier et al., 1992; Truman et al., 2004). The neuronal progeny of each NB remain clustered around the NB, and can be seen as distinct clusters (Truman and Bate, 1988; Bello et al., 2003). Thus, visualization of Neurotactin-positive NB clusters can be used as a second method to determine whether NBs persist and continue to proliferate. Ectopic Neurotactin-positive clusters were detected in the abdominal neuromeres in *MM3/MM3* and *MM3/XR38* larvae (Fig. 3F). This indicates that the 22 kb intergenic region of overlap between *MM3* and *XR38* must contain a genetic element that is required for abdominal NB apoptosis in the embryo. We call this the NB regulatory region (NBRR).

The NBRR could contain an unidentified transcription unit, either protein coding or a small RNA. Alternatively, this region could contain regulatory elements that control expression of *grim*

or another cell death gene, or multiple cell death genes. No predicted coding genes or non-coding RNAs were identified in this region based on sequence analysis of conserved signatures across the 12 *Drosophila* genomes (Clark et al., 2007; Lin et al., 2007). In addition, RNA deep sequencing analysis by the ModENCODE project (Celniker et al., 2009), as reported on FlyBase, did not detect any regions of significant transcription. Although we cannot rule out a small RNA transcription unit in the region, we investigated whether a transcriptional enhancer for *grim* alone, or for multiple cell death genes, could be found within this region.

grim deletion prolongs NB survival

If *MM3* deletes an enhancer that solely regulates *grim* expression in the NBs, then deletion of *grim* should reproduce the *MM3* NB survival phenotype. Indeed, an increase in abdominal NBs in *grim^{A6C}* homozygous larvae was observed, although to a lesser extent than in the *MM3/MM3* and *MM3/XR38* larvae (Fig. 3A-C). The *grim^{A6C}* deletion removes a 25 kb genomic region including *grim*, *CG5103* and *CG13700*. To exclude the possibility that loss of *CG5103* or *CG13700* contributed to the NB survival phenotype, *grim* was ubiquitously knocked down with *Act5C-Gal4; UAS-grimRNAi*. RNAi knockdown of *grim* led to a similar increase in abdominal NB number (Fig. 3G), indicating that the *grim^{A6C}* phenotype was due to loss of *grim*. In contrast to *grim*, deletion of *skl* or *hid* did not result in persistent abdominal NBs in early L3 (Fig. 3D,E).

We noted that in late L3 *grim* mutant larvae, Dpn-expressing NBs were detected in the locations of the normal dl, vl and vm clusters described by Truman and Bate (Truman and Bate, 1988), in addition to some ectopic NBs (data not shown). This suggests that the *grim* mutant might prolong the lifespan of the dl, vl and vm abdominal NBs that normally die in mid-L3 (purple circle in Fig. 1A). In the absence of cell death at mid-L3, the normal dl, vl and vm abdominal NBs continue to survive and proliferate, resulting in an enlarged cluster of neuroblast progeny (Bello et al., 2003). We investigated whether continued proliferation of abdominal NBs could be detected in *grim^{A6C}* mutant larvae using BrdU, anti-phosphohistone H3 (pH3) and Neurotactin staining. BrdU feeding labels proliferating postembryonic NBs and their progeny (Truman and Bate, 1988; Peterson et al., 2002). Additional BrdU staining was observed in

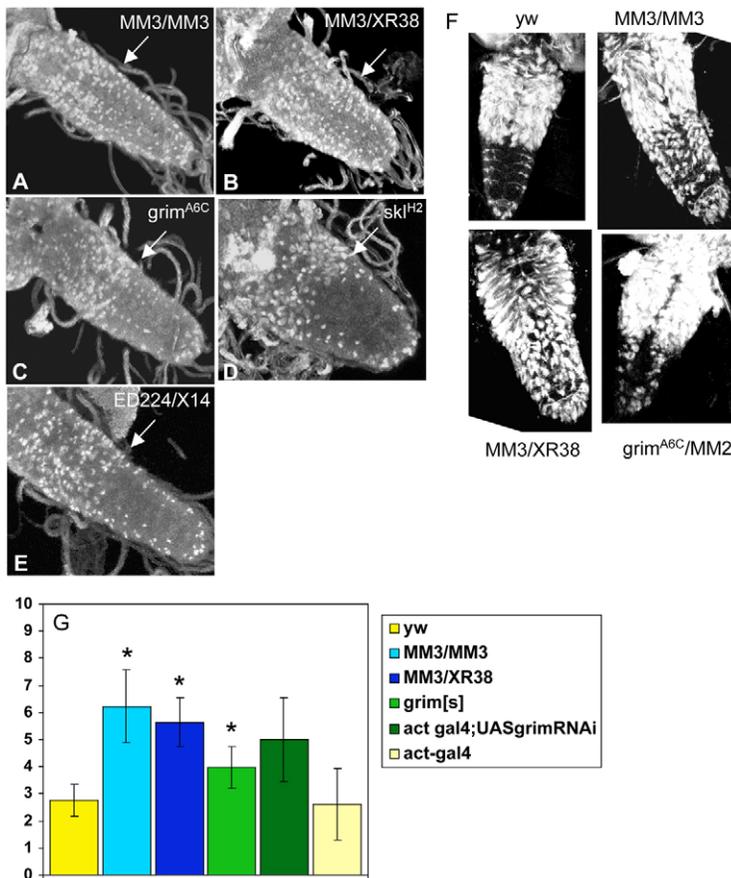


Fig. 3. *grim* and the genomic region between *grim* and *rpr* contribute to the ectopic survival of abdominal neuroblasts (NBs). (A-E) Ectopic abdominal NBs are detected by anti-Dpn in early-mid third instar of *MM3/MM3*, *MM3/XR38*, *grim^{A6C}*, *skl^{H2}* and *X14/ED224* (*hid* null). Deletion of *skl* or *hid* alone does not result in ectopic NBs. Arrows mark the thoracic-abdominal boundary. (F) Clusters of newly born neurons, detected by anti-Neurotactin staining in mid L3, demonstrate that ectopic abdominal NBs continue to divide and produce neurons. (G) Quantification of abdominal NBs detected with anti-Dpn shows that the *MM3* deletion causes a significant increase in the number of abdominal NBs. Deletion of *grim* leads to a smaller increase of abdominal NBs. Data shown is the average of four or more animals \pm s.d., * $P < 0.05$.

grim^{A6C} mutant larvae. By contrast, no ectopic proliferation of abdominal NBs was found in *rpr^{S7}* mutant larvae (data not shown). Staining with anti-pH3 antibody also demonstrated prolonged proliferation in *grim^{A6C}* mutant larvae (Fig. 4A).

Anti-Neurotactin was used to quantify the number of progeny of abdominal NBs. Clusters of Neurotactin-positive cells surround each dividing NB and the number of these cells corresponds well with the NB progeny visualized by BrdU staining [our unpublished data and Bello et al. (Bello et al., 2003)]. Therefore, we examined the number of Neurotactin-positive cells in dorsolateral clusters in *grim^{A6C}* mutant larvae (Fig. 4B,C). The dl cluster is the largest of the normal clusters in the wild type, with about six neurons in late third instar larvae. Deletion of *grim* (*grim^{A6C}/MM2*) resulted in a fourfold increase in Neurotactin-positive cells in clusters in this position. We conclude that *grim* is required for NB death in mid-L3, and that in the absence of *grim*, NBs continue to divide and produce neuronal progeny. Notably, the deletion of both *grim* and *rpr* (*MM2/ED225*) (Fig. 4B,C) or MARCM clones for *H99* (Bello et al., 2003) did not increase cluster size further, suggesting that *grim* is the sole apoptosis gene required to regulate the normal loss of the dl, vl and vm NBs in mid L3. Cluster size in *MM3/MM2* was similar to that observed in the *grim^{A6C}* mutant (Fig. 4C). This suggests that the regulatory sequences deleted in *MM3* control *grim* expression in larval NBs.

***grim*, *sickle* and *reaper* mRNAs colocalize in embryonic NBs**

We considered the possibility that other cell death genes might contribute to abdominal NB loss. It is unlikely that *hid* is involved, as we can not detect expression of *hid* in the embryonic CNS

outside of the midline (Grether et al., 1995). In addition, *hid* mutations did not result in ectopic NB survival (Fig. 3E). However, we cannot rule out the contribution of a low level of *Hid* expression.

We examined the expression of *grim*, *rpr* and *skl* in the embryonic CNS using fluorescent in situ techniques (Lecuyer et al., 2008). Expression of all three genes was almost completely restricted to the CNS from stage 15 (data not shown) (White et al., 1994; Chen et al., 1996; Srinivasula et al., 2002; Wing et al., 2002). Interestingly, *grim* and *skl* mRNAs were almost completely colocalized (Fig. 5A). At stage 15, this included co-expression in many large cells in the abdominal segments of the CNS, identified as NBs by their location, size and shape (Fig. 5A). Double labeling with the neuronal marker anti-Elav confirmed that these cells were not neurons (Fig. 5C,D). Surprisingly, *grim* colocalization with *rpr* was much less extensive at stage 15 (Fig. 5B). Some NB co-expression of *rpr* and *grim* was detected. However, *rpr* was also expressed in many smaller cells in the CNS, most likely to be neurons. Given that the *rpr* gene lies between *grim* and *skl*, this suggests that regulation of gene expression in this region is quite complex.

The *MM3* deletion alters the expression of multiple pro-apoptotic genes in embryonic NBs

To test the idea that the *MM3* deletion removes a common transcriptional regulator of multiple cell death genes in embryonic NBs, we examined overall levels of expression of *grim*, *rpr* and *skl* at late stages of embryogenesis in wild-type and *MM3* homozygous embryos. As abdominal NB loss occurs at mid-stage 16 (Fig. 1D), we examined whole embryo expression by quantitative RT-PCR

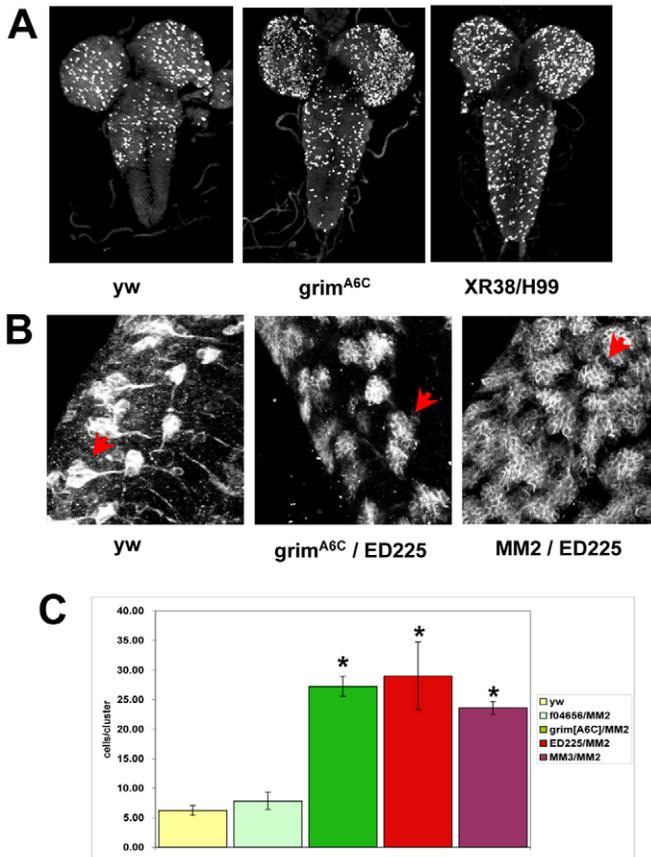


Fig. 4. Loss of *grim* results in continued proliferation of abdominal neuroblasts (NBs) in late third instar larvae. (A) Anti-Phosphohistone staining detects ectopic proliferation in the abdominal neuromeres of the late third instar ventral nerve cord (VNC) in cell death defective mutants. (B) Newly born neurons are labeled with anti-Neurotactin. A cluster in the approximate location of the normal dl cluster is marked with a red arrow. (C) Number of cells in 'dl' clusters. Because neuronal progeny remain closely apposed to the parental NB, the size of a cluster reflects the proliferation of the NB and ganglion mother cells (GMCs). f04656 is one of the P element ends for *grim*^{A6C} (Wu et al., 2010). Data shown is the average of at least five animals \pm s.d., * $P < 0.05$.

(qPCR) at stages 15 and 17. All three genes showed similar temporal patterns of expression, with high expression at stage 15, and a significant drop in expression levels by stage 17 (data not shown). Homozygous *MM3* embryos did not show a significant alteration in overall *grim*, *rpr* or *skl* expression at stages 15 and 17. As NBs represent only a small proportion of the cells expressing *grim*, *rpr* or *skl* at this time, the lack of differences in overall expression suggests that any potential regulatory element deleted in *MM3* might be specific for a small number of cells, including NBs.

We examined NB expression of *grim*, *rpr* and *skl* in *MM3* homozygous embryos using fluorescent in situ analysis. The overall tissue distribution of expression of all three genes was not affected by the deletion of the intergenic region (Fig. 6A,A',B,B',C,C'), in late embryogenesis. To specifically examine NB expression, z-stacks were taken through the ventral nerve cord, and the number of NBs expressing each gene was counted (Fig. 6A'',B'',C''). NBs were recognized by size and location.

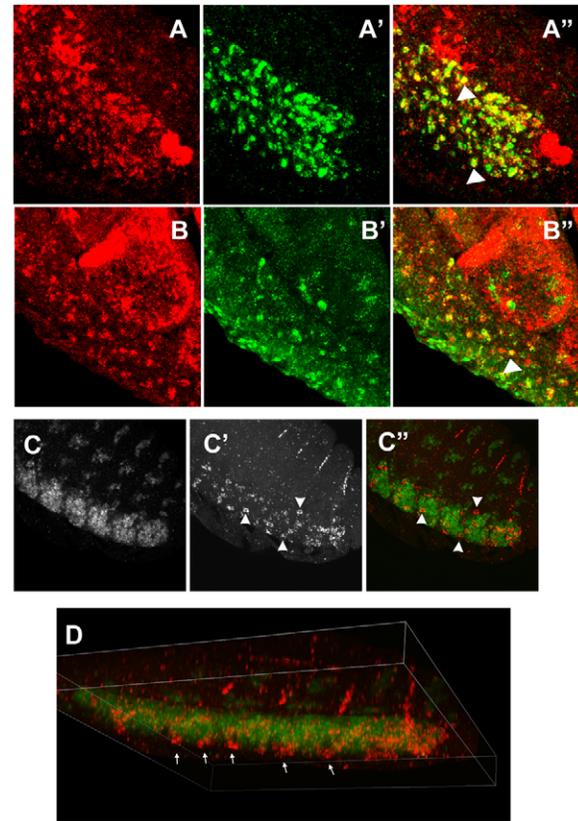


Fig. 5. *grim* mRNA colocalizes with *rpr* and *skl* in the stage 15 embryonic CNS. (A-A'') *grim* (red) and *skl* (green) mRNA colocalize in many cells in the abdominal ventral nerve cord (VNC), including in large neuroblasts (NBs; arrowheads) (B-B'') *grim* (red) and *rpr* (green) mRNA colocalization is much less extensive at this stage. The biotin-avidin detection method used to detect *grim* expression results in some background staining in the embryonic gut. (C-C'') Co-labeling with anti-Elav (green) and *grim* in situ (red), demonstrates that the large cells in the VNC expressing *grim* are not neurons (a few examples are highlighted with arrowheads). (D) 3D reconstruction of the embryo shown in C''. The position of the large *grim*-expressing cells (arrows) in the abdominal VNC demonstrates that they are NBs. Dorsal is up and anterior left in all images.

Analysis of the in situ data showed that the number of abdominal NBs expressing *grim* and *skl* was similar (compare Fig. 6A'' with C''). In wild type, NB expression of *grim* was visible at stage 10, whereas *skl* expression was observed at stage 11. The number of NBs expressing *grim* and *skl* peaked at stage 14. In *MM3* homozygotes, the number of *grim*- and *skl*-expressing NBs at earlier stages was similar to wild type, but the stage 14 peak was blunted. In very late embryogenesis, the number of abdominal NBs expressing *grim* and *skl* in *MM3* was similar to that of wild type.

Expression of *rpr* in abdominal NBs was visible at stage 12. Interestingly, the number of embryonic abdominal NBs expressing *rpr* was about half of the number expressing *grim* at all stages (compare Fig. 6A'' with B''). Similar to *grim* and *skl*, the peak at stage 14 of *rpr*-expressing NBs was essentially missing in *MM3* homozygous embryos. However, the number of *rpr*-expressing NBs in *MM3* stage 14/15 embryos was ~75% of that seen in wild type, whereas the decrease in *grim*-expressing NBs at the same stages was ~50%, and *skl* ~60%. These data indicate that the *MM3*

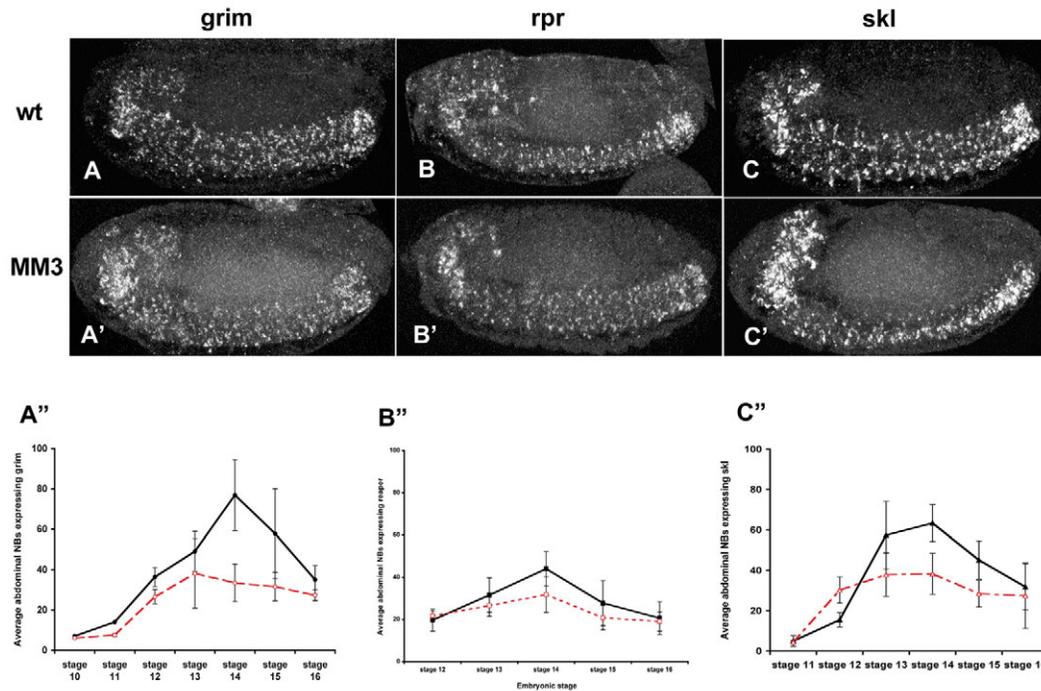


Fig. 6. *MM3* homozygous embryos have fewer NBs expressing *grim*, *rpr* and *skl*. (A-C') Although the pattern of *grim*, *rpr* and *skl* expression, as detected by fluorescent in situ hybridization, does not change in stage 14 *MM3/MM3* embryos, the number of large cells in the ventral nerve cord (VNC) expressing *grim* (A) and *skl* (C) mRNA are substantially decreased (A', C'). The number of cells expressing *rpr* mRNA are more moderately decreased (B, B'). (A''-C'') Quantitative analysis of the average number of abdominal neuroblasts (NBs) expressing *grim* (A''), *rpr* (B'') or *skl* (C'') at embryonic stages as noted. NBs are recognized by size and location. The number of NBs expressing each of these genes peaks at stage 14 in wild type (black line), and this peak is blunted in *MM3/MM3* (red line). Note that the maximum number of NBs expressing *rpr* in wild-type embryos is only ~50% of the maximum expressing *grim* or *skl*. Data shown is the average of at least three animals \pm s.d.

deletion has a larger effect on *grim* and *skl* expression in NBs than it does on *rpr* expression. We conclude that *MM3* deletes a regulatory element that is responsible for the rise in the number of NBs expressing *grim*, *rpr* and *skl* at stage 14, and in the absence of this peak of expression, NBs survive into larval stages.

Deletion of *grim* and *rpr* together results in a large increase in ectopic survival of abdominal NBs

If *grim* and *rpr* act together to regulate NB apoptosis, deletion of the region encompassing both genes should result in a strong NB survival phenotype. In *MM2/ED224*, we observed a fourfold increase in abdominal NBs in early-mid third instar larvae (Fig. 7). The additional deletion of one copy of *skl* in *MM2/ED225* slightly increased the number of NBs. The morphology of the VNC in these mutants was abnormal, with thickened caudal projections that were not seen in wild type. This genotype was semi-lethal; the flies that emerged lived for only one or two days, suggesting that this greatly enlarged CNS is detrimental. However, defects in other tissues cannot be ruled out as the cause of decreased viability.

MM2 deletes the intergenic region as well as both the *grim* and *rpr* coding regions. To confirm that the ectopic NB survival phenotype is due to the loss of both *grim* and *rpr*, we generated a recombinant of the *grim*^{C15E} and *rpr*⁸⁷ deletions. Many ectopic abdominal NBs were seen in *grim*^{C15E} *rpr*⁸⁷ recombinant larvae (*grim rpr*) (Fig. 7). The number of abdominal NBs in *grim rpr* larvae (10.4 NBs/abdominal hemisegment) was comparable to that seen in *MM2/ED224* (11.8 NBs/abdominal hemisegment), but was

much greater than that observed in either *rpr*⁸⁷ or *grim*^{A6C} larva (3-4 NBs/abdominal hemisegment). These data suggest that *grim* and *rpr*, expressed in the embryonic abdominal NBs, act together to induce the apoptosis of these cells.

If the loss of intergenic sequences in *MM3* results in ectopic NB survival due to decreased *rpr* and *grim* expression, then *MM3/grim rpr* should result in ectopic NB survival. Indeed, the number of ectopic NBs in *MM3/grim rpr* (5 NBs/abdominal hemisegment) was comparable to the number in *MM3/MM3* (6.2 NBs/abdominal hemisegment) (compare Fig. 7 with Fig. 3). This demonstrated that loss of the intergenic regulatory sequences on the *MM3* chromosome, coupled with loss of *grim* and *rpr* on the recombinant chromosome, leads to a phenotype that approximates that of two copies of the intergenic deletion (*MM3/MM3*). This finding supports our model of gene regulation in NB cell death.

We observed that the larval CNS of *grim rpr* had an abnormal morphology. Interestingly, homozygous *MM3* larvae did not have this abnormal CNS morphology, and were viable at expected frequencies with a normal lifespan. As *MM3* embryos still had significant NB expression of *grim* and *rpr* (Fig. 4), additional regulatory elements clearly exist for the transcription of these genes and their expression is important for normal CNS development.

When we examined the CNS in one- to two-day-old adults, we saw that the VNC of *grim rpr/MM2* was more than twice as long as wild type (Fig. 7C). The abdominal segments extended far into the abdomen, unlike in the wild type where they extended just to the caudal thorax. The thoracic segments of the CNS were also greatly expanded. When we compared the adult CNS from

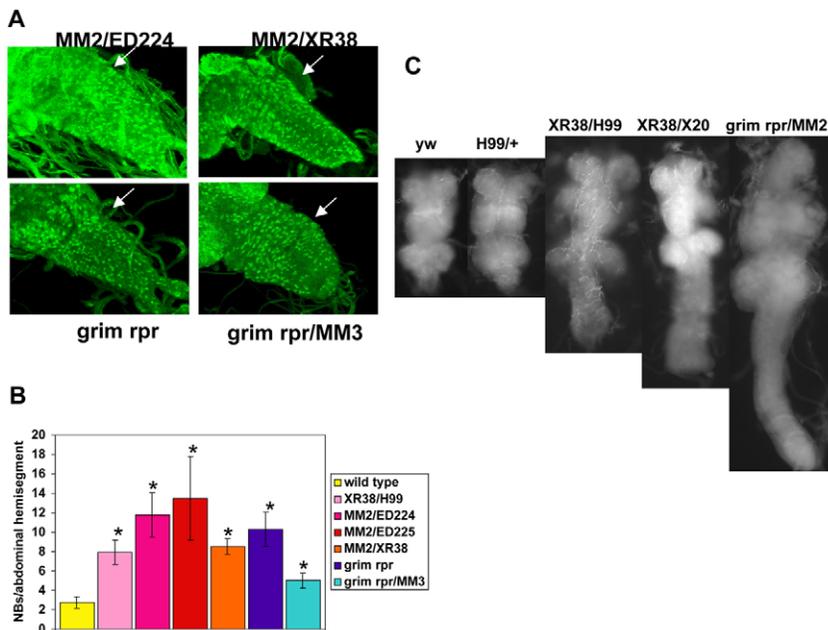


Fig. 7. Both *grim* and *rpr* are required to eliminate abdominal neuroblasts (NBs).

(A) anti-Dpn labeling reveals many ectopic abdominal NBs in *MM2/ED224* early third instar larvae, where the region from *grim* to *rpr* is deleted. Deletion of *grim* and *rpr* without the intergenic regions also allows significant abdominal NB survival (*grim rpr*). *MM2/XR38*, which deletes both copies of *rpr* as well as the intergenic neuroblast regulatory region (NBRR) also blocks NB death, as does deletion of one copy of the NBRR and one copy of *grim* and *rpr* (*grim rpr/MM3*). White arrows indicate the boundary between the thoracic and abdominal neuromeres. (B) Quantitative analysis shows significant increase of abdominal NBs in mutants deleting both *grim* and *rpr*, or deleting *rpr* and the NBRR. Data shown is the average of at least five animals \pm s.d, * $P < 0.01$. (C) Dissected and fixed ventral nerve cords (VNCs) from 1- to 2-day-old adults of the indicated genotypes. Images are taken at the same magnification, and are representative of at least three flies of each genotype.

XR38/H99 to *XR38/X20*, we found that the VNC of *XR38/X20* was more extended. As *XR38/X20* deletes *skl* in addition to *rpr* and the NBRR deleted in *XR38/H99*, this finding suggests that *skl* might contribute to cell death in the VNC.

DISCUSSION

The patterns of developmental cell death are extremely complex and dynamic, and the fate of an individual cell probably represents the integration of multiple signaling pathways. Intrinsic pathways that define the identity and health of a cell, as well as extrinsic growth and survival pathways, are all likely to contribute to the life or death decision. In *Drosophila*, the transcriptional regulation of the RHG genes is an important output of the pathways that regulate cell death. The clustering of these genes to a single large genomic locus suggests that cell death signals can be integrated by regulatory sequences that control the coordinated expression of these genes.

A cell death locus

In this work we show that *rpr*, *grim* and *skl* are co-regulated to eliminate NBs during embryonic development. We identified a genomic region important for this regulation, located between *rpr* and *grim*. Identified genes or transcripts are surprisingly lacking in the 93 kb region between these genes, which is highly conserved between *Drosophila* species (Flybase, 2003). This suggests that crucial cis-regulatory elements might be localized to this region.

We narrowed the genomic region important for NB apoptosis down to 22 kb. The number of NBs expressing *grim* and *skl* is significantly decreased in stage 14/15 embryos homozygous for a deletion of this region. The number of NBs expressing *rpr* is also affected, but to a smaller extent. Interestingly, there is still expression of *grim*, *rpr* and *skl* in NBs from stage 10 through stage 16. However, in the wild type, the number of NBs expressing all three genes increases at stage 14, presaging the loss of these cells by several hours. This increase in NBs expressing *rpr*, *grim* and *skl* is not seen in *MM3* homozygous embryos. As overall *grim*, *rpr* and *skl* expression levels are not significantly altered in the mutants, the deleted regulatory sequences must control *grim*, *rpr* and *skl*

expression in both a temporal- and tissue-specific manner, and baseline NB expression must be regulated by sequences outside of this region.

It is also interesting to note that the *MM3* deletion has a more significant effect on *grim* and *skl* expression than on *rpr* expression. *grim* and *skl* are co-expressed in most cells of the stage 15 embryonic CNS, whereas *rpr* expression is less overlapping. As *grim* and *skl* lie 134 kb apart, on either side of *rpr*, long range enhancer interactions might regulate subsets of genes in this cell death locus. This type of long-range interaction has been implicated previously in the response to irradiation (Zhang et al., 2008). Here again, a single regulatory region regulates multiple cell death genes at a great distance. The radiation responsive element activates *hid* and *rpr*, which are 200 kb apart, without having a major effect on the intervening gene *grim*. It will be important to examine higher order chromatin interactions to understand these long-range enhancer interactions.

The structure of the RHG cell death locus is conserved in other *Drosophila* species, but not obviously in more distantly related insect species, even though IAP inhibitory proteins are found in other species (Flybase, 2003; Zhou et al., 2005). This suggests that a coordinately regulated RHG gene cluster might be particularly important for *Drosophila* development.

Distinct roles of RHG genes in developmental apoptosis

As demonstrated by these studies, the individual roles for the cell death genes in developmental apoptosis are not well understood. Strong genetic and molecular data support a role for *hid* in regulating cell death in the embryonic head and in the developing eye (Grether et al., 1995; Bergmann et al., 1998; Kurada and White, 1998). A role for *grim* in the death of microchaete glial cells has been described recently (Wu et al., 2010). Here, we demonstrate a specific role for *grim* in the elimination of the normal abdominal neuroblasts in the third instar larvae. As described in this work, *rpr* alone is not required for abdominal NB death in the embryo. However, our double mutant analysis demonstrates that *rpr* and *grim* must act together to eliminate these cells.

A possible role for *hid* in NB death cannot be eliminated, although we do not detect *hid* mRNA in the VNC outside of the midline (Grether et al., 1995). A recent study suggests that *rpr* requires *hid* for efficient induction of apoptosis (Sandu et al., 2010). However, loss of *hid* does not result in increased NB survival, as would be expected if *hid* is required for *rpr* activity. Furthermore, loss of one copy of *hid* along with *rpr* and *grim* does not increase NB survival (*MM2/ED224* versus *grim rpr* in Fig. 7B). Knockdown of *rpr*, *grim* and *hid* in NBs with an RHG miRNA (Siegrist et al., 2010) also does not increase the number of surviving NBs over loss of *rpr* and *grim* (6.2 ± 0.7 NB/abdominal hemisegment in *UAS-dcr2; wor-gal4; UAS RHGi* versus 10.4 ± 1.8 NBs/abdominal hemisegment in *grim rpr*). Further studies are needed to examine how *hid* and *grim*, *rpr* and *skl* differentially contribute to developmental apoptosis.

Although *skl* has similar pro-apoptotic activity to *hid*, *grim* and *rpr*, a role for *skl* in developmental apoptosis has not previously been demonstrated, owing to the lack of a *skl* mutation. Here, we show that *skl* deletion alone does not alter NB death. In addition, we found that deletion of one copy of *skl* along with *grim* and *rpr* slightly increase NB survival over deletion of *grim* and *rpr* alone (*MM2/ED225* versus *MM2/ED224*). Deletion of *skl* and *rpr*, along with the NBRR in *XR38/X20* also results in a larger VNC than deletion of *rpr* and the NBRR in *XR38/H88*, again suggesting a role for *skl* in cell death in this tissue. Additional double mutant analysis will be needed to test whether deletion of *skl* along with *grim* or *rpr* supports a role for *skl* in developmental apoptosis. Given the strongly overlapping expression of *grim* and *skl* in the embryonic VNC, we might find that *skl* is redundant with *grim* in the CNS.

Possible advantages of multiple cell death regulators

What are the developmental advantages of multiple cell death regulators? This work demonstrates that multiple RHG genes are activated within a single NB to activate apoptosis. In the salivary gland, *hid* and *rpr*, but not *grim*, are transcribed in the dying tissue, whereas the radiation response appears to involve *hid*, *rpr* and *skl* (Jiang et al., 1997; Zhang et al., 2008). These data suggest that the complex upstream regulation of cell death is likely to impact on different subsets of the RHG genes. If each gene is controlled by different combinations of enhancers this could provide greater flexibility in controlling the activation of cell death. In addition, a requirement for multiple cell death activators could ensure that cells are not killed inappropriately by the accidental activation of a single RHG gene.

The requirement for multiple RHG genes to kill cells might also reflect differences in the pro-apoptotic activities of these genes. For example, the RHG proteins show differential binding preferences to specific domains of the DIAP1 protein, and inhibit DIAP1 anti-apoptotic functions through different mechanisms (Wu et al., 2001; Yoo et al., 2002; Zachariou et al., 2003; Yokokura et al., 2004). Additional activities, such as mitochondrial permeabilization, translational repression or interaction with other proteins such as Bruce or Scythe, also differ between RHG genes (Thress et al., 1998; Vernooij et al., 2002; Thomenius and Kornbluth, 2006; Abdelwahid et al., 2007; Sandu et al., 2010). Furthermore, post-translational regulation of particular RHG proteins might regulate their activity. This has been demonstrated for Hid; phosphorylation by MAPK downregulates the killing activity of Hid (Bergmann et al., 1998). It remains to be demonstrated whether different cell types have differential

sensitivities to particular combinations of cell death genes when expressed at normal levels. This might provide yet another layer of control of the cell death process.

In sum, this work demonstrates that the regulation of NB apoptosis during development involves the coordinated temporal and spatial regulation of multiple cell death genes, controlled by a common regulatory region. By dissecting the RHG gene locus, specific genomic elements that regulate these genes in other developmentally important cell deaths are likely to be identified.

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Competing interests statement

The authors declare no competing financial interests.

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