Essential role of *grim*-led programmed cell death for the establishment of corazonin-producing peptidergic nervous system during embryogenesis and metamorphosis in *Drosophila melanogaster*

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Summary

In *Drosophila melanogaster*, combinatorial activities of four death genes, *head involution defective* (*hid*), *reaper* (*rpr*), *grim*, and *sickle* (*skl*), have been known to play crucial roles in the developmentally regulated programmed cell death (PCD) of various tissues. However, different expression patterns of the death genes also suggest distinct functions played by each. During early metamorphosis, a great number of larval neurons unfit for adult life style are removed by PCD. Among them are eight pairs of corazonin-expressing larval peptidergic neurons in the ventral nerve cord (vCrz). To reveal death genes responsible for the PCD of vCrz neurons, we examined extant and recently available mutations as well as RNA interference that disrupt functions of single or multiple death genes. We found *grim* as a chief proapoptotic gene and *skl* and *rpr* as minor ones. The function of *grim* is also required for PCD of the mitotic sibling cells of the vCrz neuronal precursors (EW3-sib) during embryonic neurogenesis. An intergenic region between *grim* and *rpr*, which, it has been suggested, may enhance expression of three death genes in embryonic neuroblasts, appears to play a role for the vCrz PCD, but not for the EW3-sib cell death. The death of vCrz neurons and EW3-sib is triggered by ecdysone and the Notch signaling pathway, respectively, suggesting distinct regulatory mechanisms of *grim* expression in a cell- and developmental stage-specific manner.

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Key words: Corazonin, Apoptosis, Metamorphosis, Neuroblast

Introduction

Genetic dissection of programmed cell death (PCD) was first performed in *C. elegans*, in which a defined number of cells (131 out of 1090 cells) were revealed to undergo developmentally controlled death (Ellis and Horvitz, 1986). These studies identified several apoptosis regulators, whose interactions established a molecular paradigm that is more or less conserved in diverse animals (Danial and Korsmeyer, 2004). Extensive genetic and biochemical analyses of the PCD including this species and other genetic model systems such as fruit fly and mouse have identified more components that are responsible for the survival or death of cells (Fraser et al., 1999; Hay et al., 2004; Kornbluth and White, 2005; Steller, 2008; Fuchs and Steller, 2011).

Highly conserved key apoptotic factors are caspases that act as ultimate executioners of PCD (Hay and Guo, 2006). Activation status of the caspases is determined by the balance between proapoptotic and antiapoptotic factors. In mammalian cells, caspases are activated by cytochrome c (intrinsic pathway) or activation of death receptors (extrinsic pathway). In *Drosophila*, BIR-domain containing antiapoptotic protein, inhibitor of apoptosis protein 1 (DIAP1) is a key suppressor of the caspases, thereby promoting cell survival. Paradoxically, however, basal caspase activity is required to cleave and thus activate DIAP1, which in turn inhibits caspase activity levels, resulting in the oscillatory maintenance of the caspase activities below the threshold level (Ditzel et al., 2008). In a cell fated to die, death-promoting factors accumulate and regulate negatively DIAP1, thereby releasing caspases to be activated.

The best-characterized death promoters in *D. melanogaster* are *reaper* (*rpr*), *head involution defective* (*hid*), *grim*, and *sickle* (*skl*) (Grether et al., 1995; White et al., 1996; Chen et al., 1996; Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002). A mammalian homolog of the fly proteins is SMAC/DIAPBLO (Du et al., 2000; Verhagen et al., 2000). The death gene products antagonize DIAP1 through at least two mechanisms: competitive displacement of DIAP1 from its complex with caspases and degradation of DIAP1 (reviewed by Cashio et al., 2005). Three of them, *rpr*, *hid*, and *grim*, collectively have been referred to as *RHG*, are clustered within a small chromosomal region that is

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defined by *Df(3L)H99* (referred to as *H99*) (White et al., 1994). The more recently identified *skl* is located near *rpr* but just outside the *H99*. Embryos homozygous for *H99* are entirely devoid of apoptotic cells, suggesting that most, if not all, aspects of embryonic PCD require the activity of the *RHG* genes. Of interest, it is often found that multiple death genes act in a cooperative or overlapping manner to promote PCD perhaps to prevent inadvertent cell death due to accidental activation of a single death gene (e.g. Sandu et al., 2010). However, spatial and temporal expression patterns of individual death genes are not identical, suggesting distinct *in vivo* functions played by each gene.

PCD, as part of normal animal development, involves sculpturing structures or segmental boundaries during embryonic morphogenesis, or deleting entire structures that are required temporarily for a certain life stage (Fuchs and Steller, 2011). PCD in the central nervous system (CNS) is essential for the establishment of both juvenile and adult CNS (Truman et al., 1994; Ishizuya-Oka et al., 2010). During embryonic CNS development, PCD plays a key role for the removal of superfluously generated neuronal/glial precursors and post-mitotic neurons (Hidalgo and ffrench-Constant, 2003; Yeo and Gautier, 2004). Besides, embryonic neuroblasts (NBs), pioneer neurons, and midline glial cells are known to undergo apoptosis once they have no further functions after the embryonic neural network is established (Sonnenfeld and Jacobs, 1995; Zhou et al., 1997; Miguel-Aliaga et al., 2008; Tan et al., 2011).

PCD of obsolete cells in the juvenile CNS is also a key event to sculpt adult CNS. In insects, neural apoptosis takes place mainly in two distinct developmental periods: the first one during metamorphosis and the second one shortly after adult emergence (Kimura and Truman, 1990; Truman, 1990; Robinow et al., 1993; Awad and Truman, 1997; Draizen et al., 1999; Brodsky et al., 2000; Choi et al., 2006; Tan et al., 2011; Winbush and Weeks, 2011). In addition to the terminally differentiated neurons, postembryonic NBs that continue to produce neuronal precursor cells during larval growth are also removed after they establish lineages of adult-specific neurons (Bello et al., 2003; Tan et al., 2011). Formation of sexually dimorphic CNS is partly due to the sex-specific apoptosis of certain neurons during late pupal development (Kimura et al., 2005). Dysregulation of apoptosis results in structural deformities as well as functional aberration of the CNS, which accentuates the importance of PCD for the CNS development (Kuida et al., 1998; Tan et al., 2011).

Despite the aforementioned studies, it is not well understood the molecular mechanisms of the PCD in a post-embryonic CNS. Peptidergic neurons producing corazonin (Crz) provide a unique opportunity to investigate PCD mechanisms in two different stages of *Drosophila* CNS development. We previously reported that a subset of larval Crz neurons in the ventral nerve cord (for short, vCrz neurons) are eliminated during early prepupal CNS development, and that a pair of dorso-medial Crz neurons in the protocerebrum during late pupal development (Choi et al., 2005; Choi et al., 2006; Lee et al., 2008; Lee et al., 2011). The vCrz neurons are terminally differentiated from their precursor cells (EW3), which are originally derived from the NB7-3 lineage during embryonic development. Mitotic sibling cells of the EW3 (EW3-sib) die of apoptosis in the developing embryos; if they survive, they also differentiate into Crz-producing neurons (Novotny et al., 2002; Lundell et al., 2003; Karcavich and Doe, 2005; this study).

To elucidate cell death mechanisms of the vCrz neurons and EW3-sib cells during metamorphosis and embryogenesis, respectively, we performed comprehensive genetic analyses employing combinations of extant deficiencies deleting multiple death genes, mutations specific to a single death gene and RNA interference. We identified *grim* as a major death gene for the PCD of both vCrz neurons and EW3-sib cells. An intergenic region between *rpr* and *grim* that was recently reported to be important for enhancing expression of *grim*, *rpr*, and *skl* in embryonic NBs (Tan et al., 2011), is also necessary for the vCrz neurons but dispensable for the EW3-sib cell death.

Materials and Methods

Fly strains

Flies were maintained at 25℃ in food vials containing cornmeal, agar, yeast flakes, dextrose and methyl paraben. Canton-S was used as a wild type and *yellow white* (*y w*) or w^{1118} as genetic controls. The following deficiency stocks were used as *trans*-allelic combinations with other mutations: *Df(3L)X14*, *Df(3L)H99*, *Df(3L)X25*, *Df(3L)XR38*, *Df(3L)ED225*, Δ*MM2*, and Δ*MM3*, for short, *X14*, *H99*, *X25*, *XR38*, *ED225*, *MM2* and *MM3*, respectively (for a brief map of the deletion intervals, see Fig. 2) (Peterson et al., 2002; Wu et al., 2010; Tan et al., 2011). $grim^{A6C}$ (Wu et al., 2010) and rpr^{87} (Moon et al., 2008) were used as null alleles. Another putative *grim* mutant ($grim^{MI03811}$) was obtained from the Bloomington Stock Center (stock no. 36978). *UAS* transgenic lines bearing *miRNA*-based short hairpin constructs (*UAS-mirpr*, *UAS-migrim*, *UAS-miskl*, *UAS-miRGH*, and *UAS-mihid*) were generated (Chen et al., 2007). Target sequences for each miRNA construct are: *rpr*, GAAGAAAGATAAACCAACAATG; *hid*, TAAGATATATGCCGATCTAAAC; *grim*, TCATCCTGGTGGAGAGAAAATC; *skl*, TAAAGGCCACCGTTCAAA-TACA.

The UAS lines were crossed to a *Crz-gal4* driver to knockdown cell death genes in the Crz neurons (Choi et al., 2008). For transgenic manipulations in the precursors of vCrz neurons, *engrailed* (*en*)-*gal4*, *eagle (eg)-gal4*, *UAS-*N^{ICD}, and *UAS-p35* were used (Hay et al., 1994; Lundell et al., 2003). *GMR-gal4* (Freeman, 1996), combined each with *GMR-rpr*, *GMR-hid*, or *GMR-grim*, was used to examine specificity of RNA interference in the compound eyes. The following alleles were used for studying maternal effect of caspases, $dronc^{I24}$ and $dron^{51}$ (Chew et al., 2004; Xu et al., 2005): $dark^{CD4}$ and $dark^{82}$ (Rodriguez et al., 1999; Akdemir et al., 2006); $dark^{1}$ and $dark^{2}$ (Mills et al., 2006); $dcp\text{-}1^{Prev1}$, $ice^{\Delta 1}$, and $dcp\text{-}1^{Prev1}$; $ice^{\Delta 1}$ double mutant (Laundrie et al., 2003; Muro et al., 2006); a triple mutation of three initiator caspases, $dredd^{B118}$; $stricа^{4}$; $dronc^{I24}$ (Baum et al., 2007).

Generation of *skl* null mutations

Mobilization of a *P*-element, *P{wHy}DG39210* carrying w^{+} y^{+} markers (Bloomington stock no. 21776), which is located 86-bp upstream of the *skl* locus, was performed. For convenience, we will refer to this allele as skl^{P}. To induce *P*-element excision, skl^{P} homozygous females were crossed to *y w*;; Δ*2-3, Ki* (a genomic source of the *P*-element transposase) (Robertson et al., 1988). The male progeny was then individually crossed to *y w*;; *Ly/TM6C Sb Tb* virgins. From each crossing, two male offspring with yellow body color over the *TM6C* balancer were further crossed to three *y w; Ly/TM6C* virgin females. A total of 280 independent lines was generated and screened for putative *skl*-deletion mutants by PCR using genomic DNA purified from eight homozygous pupae from each excision line. First round of PCR used primers specific to the *skl* open reading frame (ORF) (f2 primer, 2GAGCGACTCAAATATGGCCATTCC; r1 primer, GGAGCCTTAGTTGGTG-CTTAAGTTG). The lines that did not yield PCR product were further analyzed by PCR (skl5p, GTGTGTACGTGACCTTGTCATCGA; r2, TGCACTGGGCCG-ACCACCTACGAG) to detect deletion between 2-kb upstream and 3′ UTR. PCR products shorter than those expected from wild type were sequenced to define deletion breakpoints.

Immunohistochemistry

Newly formed white prepupae were collected and aged on wet filter paper in a Petri dish at 25℃. Whole-mount Crz-immunohistochemistry was performed as described in great detail (Lee et al., 2011). The primary antibodies were detected by TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) at 1:200 dilution. The samples were cleared and mounted in a medium containing 80% glycerol, 0.1 M sodium phosphate buffer (pH 7.4), and 2% n-propyl galate. The fluorescent signals were viewed by Olympus BX61 microscope equipped with CC12 camera and images were obtained by Olympus Microsuite software, analysis 3.1 version (Soft Imaging System).

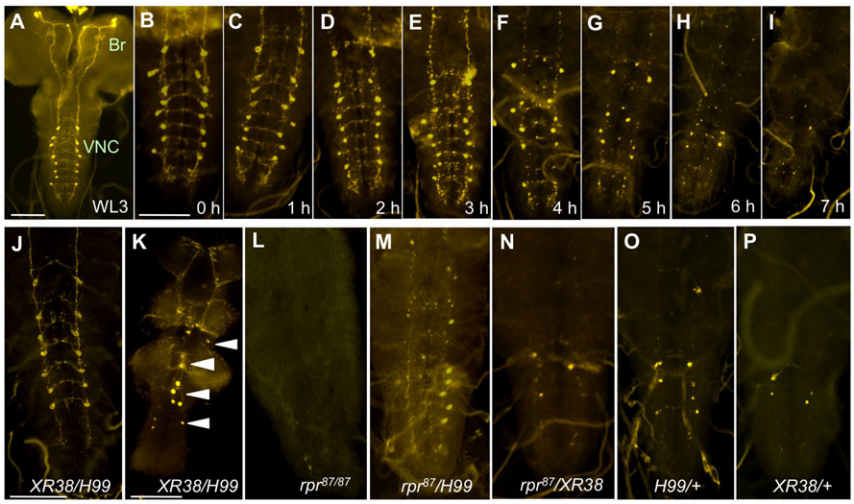


Fig. 1. Time course of vCrz neuronal PCD and its defect in *rpr* mutants. The neurons were detected by Crz-immunohistochemistry. (**A**) Wild-type larval Crz neurons. Two groups of neurons locate in the brain (Br), and another group of 16 vCrz neurons in the ventral nerve cord (VNC). (**B–I**) Progressive elimination of the vCrz neurons in wild type. Removal of vCrz neurons is complete by 7 hours APF but overt apoptotic signs are noticeable around 2–3 hours APF. (**J**,**K**) Survival of the vCrz neurons in *XR38/H99* mutants at 7 hours APF (J) and in a 7-day-old adult (K). White arrowheads indicate surviving vCrz neurons. (**L**) Normal PCD in $rpr^{87/87}$ at 7 hours APF. (**M–P**) Mild PCD defect at 7 hours APF in $rpr^{87}/H99$ and $rpr^{87}/XR38$ is comparable to *H99*/+ and *XR38*/+, respectively. See also Table 1 for quantitative data. Scale bars: 100 µm.

TUNEL assay

Fragmented DNA in the nuclei of dying neurons was detected by using a commercial kit (Deadend Fluorometric TUNEL system, Promega), as described previously (Lee et al., 2011). For double-labeling, the tissues processed for the TUNEL signals were incubated with mouse monoclonal anti-REPO (1/10 dilution) (Developmental Studies Hybridoma Bank) at 4℃ overnight, followed by steps described for Crz-immunohistochemistry.

Results

Dispensable *rpr* and *hid* functions

Previously we showed that vCrz neurons start to display apoptotic signs and caspase activities at 1 hour after puparium formation (APF) and they are removed completely by 6–7 hours APF (Fig. 1A–I) (Lee et al., 2011). In a *trans*-allelic *XR38/H99* combination, which deletes the *rpr* locus (Fig. 2) (Peterson et al., 2002), about 80% of vCrz neurons survived at 7 hours APF and they persisted into adulthood (Fig. 1J,K), suggesting that their death is strongly blocked. The results have led us to assume that *rpr* is essential for developmentally regulated PCD of vCrz neurons. However, in addition to *rpr*, *XR38/H99* removes one copy of each of the three other death genes (Fig. 2). Thus an alternative explanation is that strong PCD defect in *XR38/H99* is a combined effect of the lack of *rpr* and a half dose of three other proapoptotic genes.

To test whether vCrz PCD requires multiple cell death genes, first we attempted to confirm the role of *rpr* only by using an *rpr*-specific deletion mutation (rpr^{87}) (Moon et al., 2008). Surprisingly, all vCrz neurons carried out timely death that was not different from wild-type one (Fig. 1L). However, $rpr^{87}/H99$ and $rpr^{87}/XR38$ mutants displayed mild PCD defects at 7 hours APF, the results that were similar to those observed with heterozygous controls, *H99*/+ and *XR38*/+, respectively (Fig. 1M–P; Table 1). Since the lack of *rpr* did not exacerbate PCD defect in the heterozygous backgrounds for *H99* and *XR38*, *rpr* seemed to be mostly dispensable. This result made us disclaim our previous notion on *rpr*'s essential role for PCD of vCrz neurons.

Next we examined *hid*'s function in hid^{05014} homozygous and $hid^{05014}/X14$ hemizygous mutants. Both mutations did not exert any noticeable effect on normal course of vCrz PCD. Mild PCD defect in $hid^{05014}/H99$ was also comparable to that in *H99*/+ (Table 1). Based on these results, we concluded that PCD deficiency in *XR38/H99* flies is not due to the lack of *rpr* and *hid* but likely due to the reduction of *grim* and/or *skl* functions.

To gain evidence for *skl*'s role, we compared PCD phenotype between $hid^{05014}/H99$ and $hid^{05014}/ED225$. A major difference between the two combinations is that the latter is heterozygous for the *skl* locus. Interestingly $hid^{05014}/ED225$ yielded survival of more vCrz neurons than did $hid^{05014}/H99$ (Table 1). Although the difference between the two genotypes is modest (unpaired *t*-test: *p*-value=0.09), similar levels of difference with greater significance were observed between *H99*/+ and *ED225*/+ (*p*-value=0.001) as well as between $rpr^{87}/H99$ and $rpr^{87}/ED225$ (*p*-value=0.048) (Table 1), supporting a role for *skl* in the PCD of vCrz neurons.

RNA-interference of cell death genes

To investigate the cell-autonomous role of each death gene, we employed RNA interference (RNAi) to knockdown each death

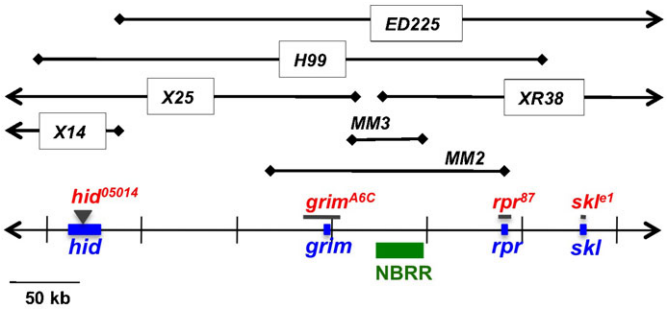


Fig. 2. Physical map of various deficiencies uncovering death genes. Black lines indicate approximately deleted area. Diamond-ends indicate defined breakpoints while arrow-ends signify breakpoints beyond this chromosomal region. A green bar, an overlapping region between *MM3* and *XR38*, designates NBRR site. Mutations specific to each death gene are in red. *P*-element insertion of hid^{05014} allele is shown by a triangle.

Table 1. PCD of vCrz neurons in various dosages of the cell death genes at two developmental time points.

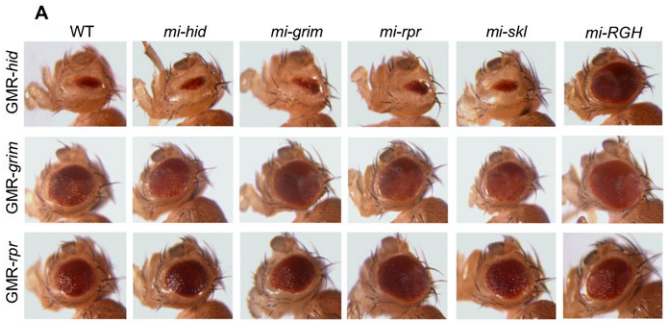
genotype	gene dosage				vCrz neurons	
	hid	*grim*	*rpr*	*skl*	7 h APF	16 h APF
wild type	2	2	2	2	0.0±0.0 (32)	0 (6)
XR38/+	2	2	1	1	2.2±1.6 (13)	0 (3)
H99/+	1	1	1	2	6.7±2.1 (11)	
XR38/X25	1	1	1	1	15.2±1.0 (6)	10.7±1.2 (3)
ED225/+	>1	1	1	1	9.6±1.9 (15)	
rpr						
rpr^{87}/rpr^{87}	2	2	0	2	0.0±0.0 (7)	
$rpr^{87}/XR38$	2	2	0	1	1.7±1.5 (7)	0 (5)
$rpr^{87}/H99$	1	1	0	2	6.3±3.3 (10)	0 (2)
$rpr^{87}/ED225$	>1	1	0	1	8.9±2.3 (11)	
XR38/H99	1	1	0	1	14.3±1.0 (6)	14.3±0.6 (3)
hid						
hid^{05014}	0	2	2	2	0.0±0.0 (7)	
$hid^{05014}/X14$	0	2	2	2	0.0±0.0 (5)	
$hid^{05014}/H99$	0	1	1	2	6.0±2.8 (8)	0 (2)
$hid^{05014}/ED225$	>1	1	1	1	9.0±2.2 (4)	0 (2)
rpr* and *skl						
XR38/ED225	>1	1	0	0	15.3±0.5 (4)	15 (1)
grim						
X25/H99	0	0	1	2	nd	
X25/ED225	1	0	1	1	nd	

Numbers indicate surviving vCrz neurons per VNC (mean±s.d.).
(n): number of specimen examined in each genotype.
nd: not determined due to lethality.

gene within the Crz neurons. For this, we generated transgenic sympUAS lines for *hid*, *grim*, and *rpr*, in which sense and antisense RNAs are produced simultaneously from the flanking UAS promoters, yielding double-stranded RNAs (Giordano et al., 2002). Although these lines were able to rescue small eye phenotypes induced by ectopic expression of each death gene, we found significant cross-interference among them (supplementary material Fig. S1) (see also Y.-J. Choi, Developmental and neurogenetic studies on the peptidergic nerve system in *Drosophila*, PhD thesis, University of Tennessee, 2006).

Since microRNAs (miRNA)-based gene silencing has minimal cross-interference and more efficient knockdown effect (Chen et al., 2007; Haley et al., 2010; Ni et al., 2011), we employed this system. In the compound eyes, *mi-grim* and *mi-rpr* showed a complete rescue of *GMR-grim* and *GMR-rpr*-induced cell death, respectively, and these lines did not display cross-interference (Fig. 3A).

Intriguingly, expression of *mi-grim* directed by a single copy of *Crz-gal4* effectively blocked PCD of all vCrz neurons at 7 hours APF, while *mi-skl* expression did it partially when two copies of the *Crz-gal4* driver were used (Fig. 3B). Consistent with our genetic data, *mi-rpr* and *mi-hid* did not show any anti-PCD effect even with two copies of the *Crz-gal4* transgene (Fig. 3B). Together, these data strongly support *grim* and *skl* as important cell death genes for PCD of vCrz neurons.



B	dose of Crz-gal4		
	1x	2x	
Genotype	7 h APF	7 h APF	16 h APF
mi-hid	0 (5)	0 (7)	-
mi-rpr	0 (10)	0 (9)	-
mi-grim	16.0 ± 0.0 (5)	16.0 ± 0.0 (8)	12.5 ± 2.6 (4)
mi-skl	0 (9)	5.6 ± 2.2 (6)	0 (7)
mi-RGH	16.0 ± 0.0 (9)	16.0 ± 0.0 (3)	16.0 ± 0.0 (3)

Fig. 3. *miRNA*-induced interference of death genes. (**A**) In the compound eyes *mi-rpr* and *mi-grim* rescued eye defect caused by *GMR-rpr* and *GMR-grim* expression, respectively. Expression of *mi-RGH* showed effective rescue against *GMR-hid* and *GMR-grim* expression but moderately against *GMR-rpr* expression. In contrast, *mi-hid* was ineffective in rescuing *hid*-induced cell death, perhaps because of the positional effect that might interfere with *mi-hid* expression. The *miRNA* shows minimal or no cross-interference with non-cognate death genes. (**B**) The numbers of surviving vCrz neurons (mean±s.d.) due to *miRNA* expression at 7 hours and 16 hours APF. Numbers of specimens examined are indicated in parentheses. Expression of *miRNA* was driven by one (1×) or two copies (2×) of the *Crz-gal4* transgene. *mi-grim* and *mi-skl* caused strong and mild PCD defect in a dose dependent manner, respectively. However, *mi-hid* and *mi-rpr* did not interfere with normal PCD. Expression of *mi-RGH* showed stronger PCD defect than did *mi-grim* at 16 hours APF.

A minor role of *skl*

To further define the extent of *skl*'s proapoptotic role, *skl*-null mutations were generated by *P*-element mobilization, as described in Materials and Methods. Out of 280 excision lines, four lines (named skl^{e1-e4}, respectively) were identified to be null alleles lacking the entire ORF and its 5′ flanking region (Fig. 4A). All homozygous mutants are viable and fertile without any noticeable deficit in morphology, development,

and reproduction, suggesting that *skl* functions redundantly with other death gene(s) or is mostly dispensable. The lack of apparent phenotypes is consistent with *skl* mutants independently isolated by another group (Tan et al., 2011).

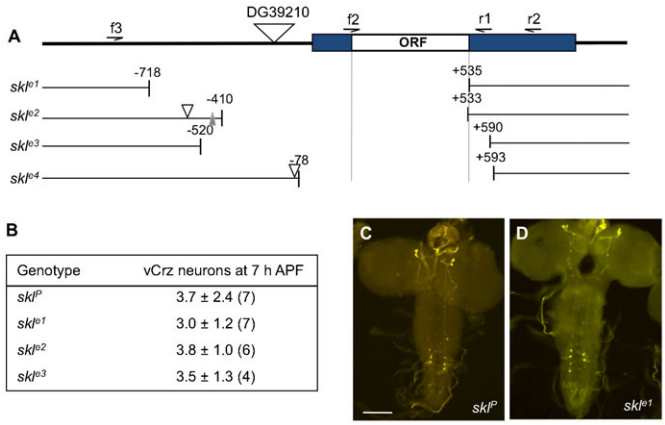
The *skl* homozygous mutants showed mild PCD defect, leaving 3–4 vCrz neurons at 7 hours APF (Fig. 4B–D). Thus, both genetic knockout and transgenic knockdown data confirm that *skl* plays at least a minor role in vCrz neuronal death.

Essential role of *grim*

Since our foregoing data with *mi-grim* expression showed the strongest cell death defect, we attempted to gain supporting evidence for the *grim*'s proapoptotic roles. We employed a recently characterized *grim*-null allele ($grim^{A6C}$), which was generated by FRT-mediated recombination (Wu et al., 2010). Consistent with *mi-grim* data, all of the vCrz neurons survived and appeared normal in the $grim^{A6C/A6C}$ at 7 hours APF ($n=8$) (Fig. 5A). Comparable PCD defect was also observed in various *grim* hemizygous combinations (Fig. 5B–D).

Unexpectedly, we also observed extra Crz-immunoreactive (ir) neurons located laterally to the vCrz neurons (Fig. 5A; Table 3). These ectopic Crz-ir neurons, henceforth referred to as vCrz-sib, are most likely derived from their surviving progenitors (EW3-sib) that normally undergo apoptosis during embryogenesis (Novotny et al., 2002; Lundell et al., 2003). We will address this issue later.

We characterized another putative *grim* mutant stock ($grim^{MI03811}$/*TM3, Sb*) in which a *MiMIC* transposable element disrupts the *grim* open reading frame (Venken et al., 2011). Unlike $grim^{A6C}$ allele, no homozygous adult flies were found in this stock. After we changed the balancer from *TM3* to *TM6B, Tb, Hu*, we found that homozygotes died mostly as third instar larvae. A small fraction of larvae became puparia, but they did not develop any further. However, *trans*-heterozygous $grim^{A6C/MI03811}$ developed into adults as observed with $grim^{A6C}$ homozygotes, suggesting that



Genotype	vCrz neurons at 7 h APF
skl^{P}	3.7 ± 2.4 (7)
skl^{e1}	3.0 ± 1.2 (7)
skl^{e2}	3.8 ± 1.0 (6)
skl^{e3}	3.5 ± 1.3 (4)

Fig. 4. Neuronal PCD in *skl* mutants. (**A**) A diagram showing break points of the four *skl* mutant alleles. Two PCR primers (f2 and r1) were used to screen the excision lines bearing putative deletion of *skl* ORF. Primers (f3 and r2) were used to determine break points. Numbers indicate positions of the break points relative to the transcription start site (+1). White triangles in skl^{e2} and skl^{e4} indicate additional sequences found in these alleles, and a gray one in skl^{e2}, a deletion of three nucleotides. (**B**) Mild PCD defect displayed by *skl* mutant alleles at 7 hours APF. (**C**,**D**) Representative images of surviving vCrz neurons in homozygous skl^{P} and skl^{e1} alleles. Scale bar: 100 µm.

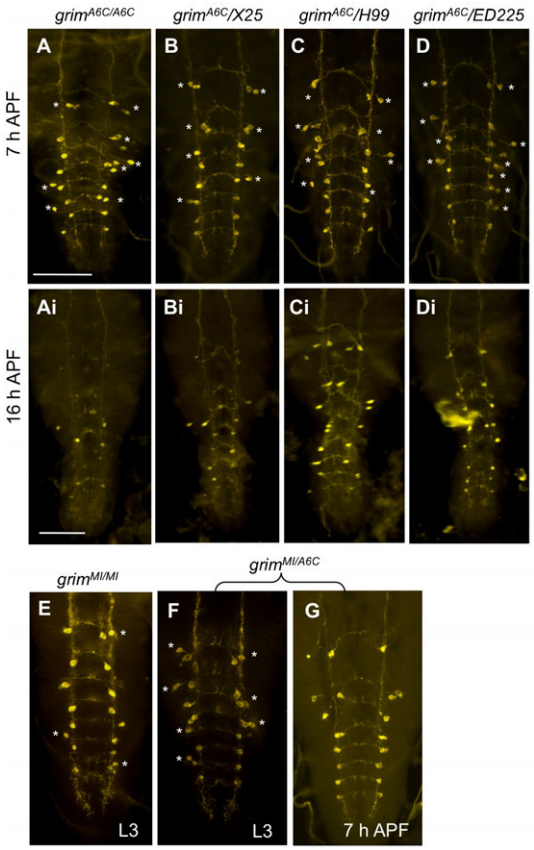


Fig. 5. Essential role played by *grim* for PCD of vCrz neurons. Significant blocking of vCrz neuronal cell death was observed in indicated genotypes at 7 hours (**A–D**) and 16 hours APF (**Ai–Di**). Asterisks indicate ectopic Crz-ir (vCrz-sib) neurons that are derived from their precursor (EW3-sib) cells. (**E**) Crz-ir neurons in $grim^{MI03811}$ homozygous larva. (**F**,**G**) Crz-ir neurons in $grim^{MI03811/A6C}$ *trans*-heterozygous larva (F) and 7-hour-old puparium (G). Scale bars: 100 µm.

the larval lethality of the $grim^{MI03811}$ be associated with a genetic background unrelated to *grim*.

In the larval CNS of $grim^{MI03811}$ homozygotes and $grim^{A6C/MI03811}$, all vCrz neurons and a few ectopic vCrz-sib neurons were detected (Fig. 5E,F; see also Table 3). At 7 hours APF, PCD of vCrz neurons was blocked completely in $grim^{A6C/MI03811}$ mutant (Fig. 5G), as was found in $grim^{A6C}$ mutant. Taking these results together provides a compelling evidence for the *grim* as a principal death inducer in the vCrz neurons during metamorphosis.

grim requires *skl* as a supporting proapoptotic factor

Although vCrz neurons continued to survive in $grim^{A6C}$ homozygous mutants at 7 hours APF (Fig. 5A), we detected only an average of six Crz-ir neurons at 16 hours APF (Fig. 5Ai) and occasionally one or two such neurons in 3-day-old adult CNS ($n=5$) (data not shown). These results from our novel time-course experiment clearly indicate that slow but progressive cell death still takes place even in the absence of *grim* function. Such delayed PCD could be due to activities of other cell death genes. In support of this prediction, $grim^{A6C}/H99$ or $grim^{A6C}/ED225$ showed more pronounced PCD defect at 16 hours APF than did $grim^{A6C/A6C}$ or $grim^{A6C}/X25$ (Fig. 5Bi–Di). Because our foregoing results indicated *skl*'s minor role in the vCrz

neuronal death, we speculate that the delayed death of vCrz neurons in the absence of *grim* is likely caused by *skl*'s activity.

grim is a death inducer of other larval neurons

Since vCrz neurons represent only a subset of larval neurons dying during metamorphosis, we extended our investigation to see if *grim* is required for PCD of other doomed larval neurons. We performed TUNEL assay with wild-type and $grim^{A6C}$ homozygous CNSs dissected from 6–8 hours APF, a period that we previously observed most abundant TUNEL signals (Lee et al., 2011). We detected 308±22 TUNEL-positive cells in the wild-type VNC (n=3) (Fig. 6A,B). Most of them were negative for REPO-immunoreactivity, a pan-glial marker (n=5) (data not shown), implying that these dying cells are neurons. In $grim^{A6C}$ homozygotes, TUNEL signals were reduced to 130±12 cells (n=3) (Fig. 6C,D). These results indicate that *grim* is also important for PCD of many other types of larval neurons during prepupal development.

Mysteries of *XR38/H99*

As shown earlier, *XR38*/*H99* CNS has ~14 vCrz neurons (instead of the expected 16), surviving at 7 hours APF, and all of them continue to survive into adulthood (Table 1; Fig. 1K). Two questions arose from this observation. First, why are 2–3 vCrz neurons undetectable in this genetic background although PCD is strongly compromised? Close examination of the missing vCrz neurons revealed that they are invariably lacking in the 6th abdominal (A6) neuromere (n=25) (Fig. 7C,D) and less frequently in the A5 neuromere (Fig. 7D). A simple explanation for this is that these posterior vCrz neurons undergo PCD in this genetic background during metamorphosis. Surprisingly, however, similarly missing neurons were observed in the 3rd instar larvae (n=16) (Fig. 7E,F) and even in the first instar larvae (n=3) (data not shown) with 100% penetrance. Thus, the lack of A5 and A6 vCrz neurons in early larva-hood is likely due to defective embryonic neurogenesis. The molecular basis for this is unknown.

The second question is 'why does *XR38/H99* show more severe PCD defect than $grim^{A6C}$ homozygous mutation alone does, despite the presence of one copy of wild-type *grim* allele in *XR38*/*H99*?'. Because two main death inducers of vCrz neurons are *grim* and *skl*, PCD phenotype of the *XR38*/*H99* should be comparable to that of flies heterozygous for $grim^{A6C}$ and skl^{e1} ($grim^{A6C}/skl^{e1}$). It wasn't, however, as the PCD took place almost normally in the latter genotypes (Table 2).

Related to the aforementioned question, another puzzling result was made from *X25*/*XR38* combination. This is comparable to those of *ED225*/+ and $rpr^{87}/ED225$ with respect to a dosage of cell death genes (Fig. 2). Surprisingly, however, *X25/XR38* had all 16 vCrz neurons remained at 7 hours APF, while *ED225*/+ or $rpr^{87}/ED225$ did only 9–10 neurons (Fig. 7G; Table 1). Furthermore, the PCD phenotype of *X25/XR38* was very similar to that of $grim^{A6C}/XR38$ (Fig. 7H). From these observations we suspected that the *grim* gene in the *XR38* chromosome is functionally abnormal or subnormal. To address this question, we amplified *XR38-grim* gene ($grim^{XR38}$) from *X25/XR38* genomic DNA by PCR; as such *grim* sequence was derived exclusively from the *XR38* chromosome. As a result, we found two mutations within the *grim* ORF, resulting in the following substitutions: proline 29 to arginine (P29R) and arginine 53 to glutamine (R53Q) (Fig. 7I). P29R was also reported by Tan et al. (Tan et al., 2011), but the latter one was novel. R53Q is in the Gln-rich domain, which was shown to be important for Grim's full proapoptotic function in the cell-based assay (Wu et al., 2010). This result raises the possibility that $grim^{XR38}$ is a hypomorphic allele. This result can also explain

Table 2. PCD of vCrz neurons in *grim* and *NBRR* mutations.

genotype	gene dosage				vCrz neurons	
	grim	*NBRR*	*rpr*	*skl*	7 h APF	16 h APF
grim						
$grim^{A6C}$/+	1	2	2	2	0 (7)	
$grim^{A6C}$/*X14*	1	2	2	2	0 (10)	
$grim^{A6C}/rpr^{87}$	1	2	1	2	0 (11)	
$grim^{A6C}/skl^{e1}$	1	1	2	1	3±2.4 (9)	0 (7)
*$grim^{A6C/A6C}$	0	2	2	2	16±0 (8)	5±0.8 (4)
*$grim^{A6C}$/*X25*	0	2	2	2	16±0 (9)	7.3±1.2 (3)
*$grim^{A6C}$/*H99*	0	1	1	2	16±0 (11)	16±0 (4)
*$grim^{A6C}$/*XR38*	1	1	1	1	15.2±0.8 (9)	8.2±2.8 (5)
*$grim^{A6C}$/*ED225*	0	1	1	1	16±0.0 (12)	16±0 (2)
NBRR						
MM2/+	1	1	1	2	6.2±3.3 (11)	1.3±1.1 (7)
**MM2/*$grim^{A6C}$*	0	0	1	2	16±0.0 (6)	16±0.0 (4)
**MM2/XR38*	1	0	0	1	13.6±2.2 (8)	12.6±2.5 (10)
**MM2/X25*	0	1	1	2	16±0.0 (7)	16 (3)
**MM2/H99*	0	0	0	2	16±0.0 (7)	16 (3)
MM2/MM3	1	0	1	2	13±1.4 (3)	4.5±3.5 (3)
MM3/+	2	1	2	2	0 (9)	
MM3/MM3	2	0	2	2	9.5±0.8 (3)	6 (1)
MM3/XR38	1	0	1	1	5.8±2.9 (5)	0 (5)
MM3/$grim^{A6C}$	1	1	2	2	12.2±2.0 (3)	1.4±0.9 (5)
MM3/X25	1	1	2	2	10.8±1.9 (4)	1.7±1.5 (3)
MM3/H99	1	0	1	2	11.8±1.6 (10)	7.9±3.5 (7)

Numbers indicate surviving vCrz neurons per VNC (mean±s.d.).
(n): number of specimen examined in each genotype.
*Extra Crz-immunoreactive neurons (i.e. vCrz-sib) were detected in these genotypes, but only vCrz neurons were counted.

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Table 3. PCD of EW3-sib cells, as determined by vCrz-sib neurons in the larval CNS.

genotype	EW3-sib cells (n)
$grim^{A6C}$/+	0±0 (15)
$grim^{A6C/A6C}$	10.8±2.0 (10)
$grim^{A6C}$/*X25*	9.8±1.4 (13)
$grim^{A6C}$/*MM3*	2±1.4 (5)
$grim^{A6C}$/*MM2*	9.5±1.8 (8)
$grim^{A6C}$/*XR38*	0.6±0.7 (11)
$grim^{A6C}$/*H99*	9.3±0.6 (3)
$grim^{A6C}$/*ED225*	8.8±1.5
$grim^{MI/MI}$	3.6±1.1 (5)
$grim^{A6C/MI}$	6.8±2.6 (5)
$grim^{L/L}$, $rpr^{87/87}$	11.0±2.9 (11)
$grim^{L/+}$, $rpr^{87/+}$	0.2±0.6 (14)
MM2/+	0±0 (8)
X25/MM2	10±1.3 (8)
MM2/XR38	0.5±0.6 (6)
XR38/H99	0.4±0.8 (18)

Numbers indicate surviving EW3-sib cells per VNC (mean±s.d.). (n): number of specimen examined in each genotype.

why both *X25/XR38* and $grim^{A6C}$/*XR38* displayed stronger PCD defect than did *ED225*/+.

Despite considering hypomorphic $grim^{XR38}$, it is not still fully understood why PCD defect of *XR38/H99* is greater than that of $grim^{A6C}$/*XR38* at 16 hours APF. For instance, nearly 50% of vCrz neurons died in $grim^{A6C}$/*XR38* at 16 hours APF, while all vCrz neurons in *XR38/H99* persisted to adult stage (Tables 1, 2). Such a discrepancy led us to speculate additional genetic element missing in the *XR38/H99* background.

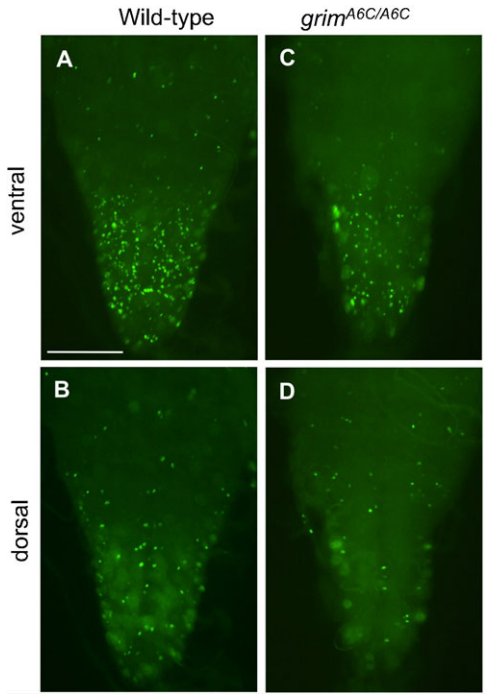


Fig. 6. Reduced TUNEL signals in the *grim* mutant. (**A**,**B**) Abundant TUNEL signals are seen in the abdominal ganglia from both ventral and dorsal sides of wild type at 7 hours APF. (**C**,**D**) Significant reduction of the signals in $grim^{A6C/A6C}$. Scale bar: 100 µm.

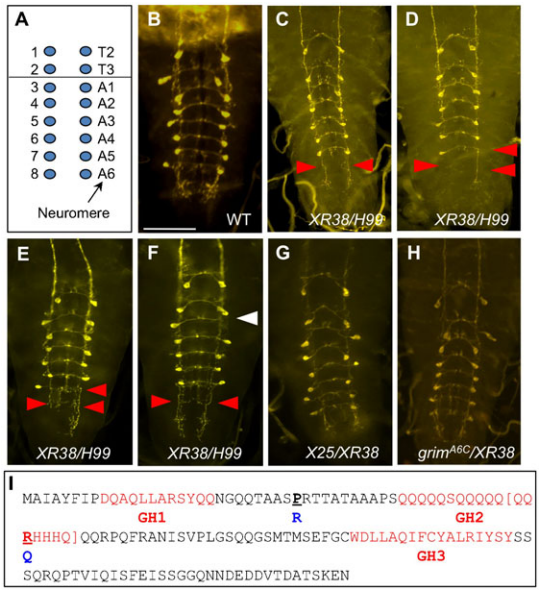


Fig. 7. Absence of vCrz neurons in the 5th–6th abdominal neuromeres (A5 and A6) in *XR38/H99*. (**A**) A diagram illustrating the position of vCrz neurons in each neuromere of a wild-type larval VNC (T, thoracic; A, abdominal). (**B**) Wild-type vCrz neurons. (**C**,**D**) *XR38/H99* prepupal VNC at 7 hours APF lacks 2 or 3 vCrz neurons in the A5 and A6 neuromeres, as indicted by red arrowheads. (**E**,**F**) *XR38/H99* larva lacking the same vCrz neurons as in prepupa. (**G**,**H**) Most of vCrz neurons survived at 7 hours APF in *X25/XR38* and $grim^{A6C}$/*XR38*. (**I**) DNA sequence of the $grim^{XR38}$ allele revealed P29R and R53Q substitutions. Residues in the brackets indicate in-frame deletion found in $grim^{MM3}$ allele. GH stands for Grim Helix (Clavería et al., 2002). Scale bar: 100 µm.

Role of the *neuroblast regulatory region* (*NBRR*) in the PCD of vCrz neurons

Recently an intergenic region between the *rpr* and *grim* loci, termed the *neuroblast regulatory region* (*NBRR*), was proposed to be a remote enhancer for the optimal expression of *rpr*, *grim*, and *skl* in the embryonic NBs (Tan et al., 2011) (Fig. 2). This prompted us to examine whether or not the *NBRR* is important for vCrz neuronal death.

Of note, homozygous *MM3* prepupae lacking the *NBRR* contained 9–10 vCrz neurons detectable at 7 hours APF (Fig. 8A) and 1–2 at 16 hours APF (Fig. 8Ai), while *MM3*/+ showed none (Fig. 8D). This result could support the regulatory role of *NBRR*, as was proposed for the NB death. However, it is more complicated than that because the *MM3* chromosome also carries a *grim* mutation bearing an in-frame deletion of six amino acid residues (52nd–57th) within the Gln-rich domain of *grim* ORF (we confirmed this result with a slight difference, deletion of the 51st–57th residues) (Fig. 7I). The *MM3-grim* (hereafter $grim^{MM3}$) was shown to have a subnormal proapoptotic activity as determined by a cell-based assay (Wu et al., 2010). Thus the hypomorphic property of the $grim^{MM3}$ allele could be a cause of the partial PCD defect seen in *MM3* homozygotes. Consistent with this notion, slightly more vCrz neurons survived in *MM3*/$grim^{A6C}$ and *MM3*/*X25* than in *MM3/MM3* (Fig. 8B,C; Table 2). Therefore, the results from *MM3* alone are not sufficient to support the regulatory role of the *NBRR* for the expression of proapoptotic genes within the vCrz neurons.

We looked into another deficiency, *MM2* carrying a deletion from *grim* to *rpr* locus (Fig. 2). *MM2* heterozygote flies ($grim^{+/-}$,

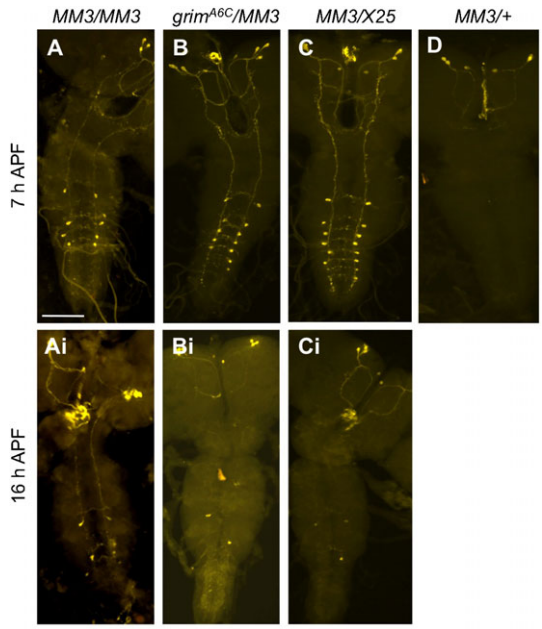


Fig. 8. Role of *NBRR* for PCD of vCrz neurons (A–D). Crz-immunohistochemistry was done to detect surviving vCrz neurons at 7 hours APF (upper panels) and 16 hours APF (lower panels) for the indicated genotypes. Scale bar: 100 µm.

$NBRR^{+/-}$, $rpr^{+/-}$) showed PCD defect similar to those of *MM3/grim^{A6C}* or *MM3/X25* (both $grim^{MM3/-}$, $NBRR^{+/-}$) (Fig. 9A,Ai). In addition, *grim^{A6C}/MM2* and *X25/MM2* genotypes (both $grim^{-/-}$, $NBRR^{+/-}$, $rpr^{+/-}$) produced more severe anti-PCD phenotype particularly at 16 hours APF than did *grim*-null mutation alone (Fig. 9B,C,Bi,Ci). These results could be explained by either synergistic or additive effect by *grim* and *NBRR*, in which the latter might enhance expression of *grim*, *rpr* and *skl* as proposed for the PCD of embryonic NBs (Tan et al., 2011).

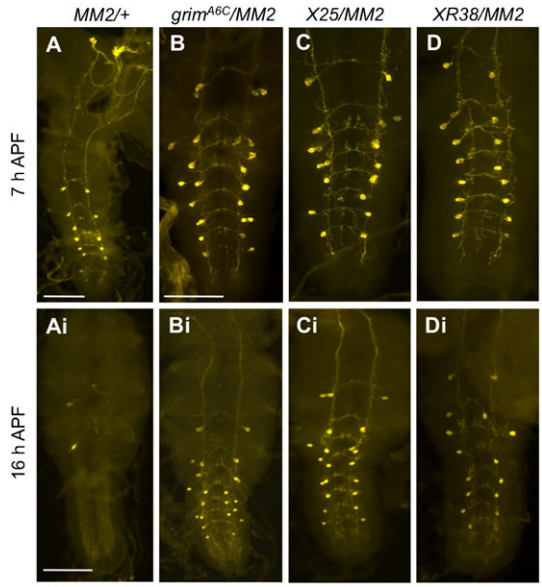
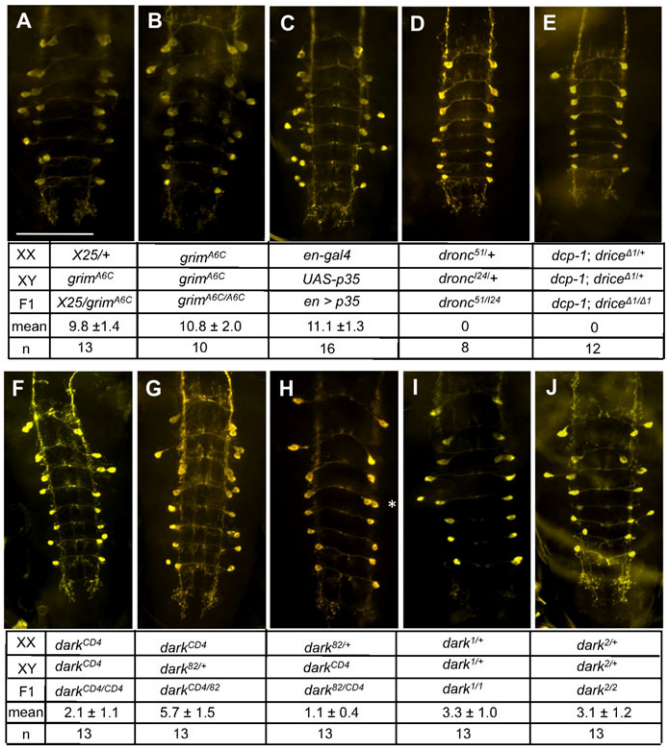


Fig. 9. PCD of vCrz neurons requires both *grim* and *NBRR* (A–Di). Crz-immunohistochemistry was done to detect surviving vCrz neurons at 7 hours APF (upper panels) and 16 hours APF (lower panels) for the indicated genotypes. Scale bars: 100 µm.

Essential role of *grim* for embryonic PCD of the precursors of vCrz-sibling cells

Although a single vCrz neuron exists per hemi-segment from T2 to A6 in the wild-type larval VNC, there are two progenitor cells in the developing embryonic CNS. Third asymmetric division of the NB7-3 gives rise to a GMC-3, which divides once to produce two sister cells, EW3 and EW3-sib; EW3 differentiates terminally into a vCrz neuron while EW3-sib dies of apoptosis (Novotny et al., 2002; Lundell et al., 2003). However, the mechanisms underlying PCD of the EW3-sib are little known.

Since EW3-sib cells, if they survive, also differentiate into Crz-ir neurons (vCrz-sib), we used the larval vCrz-sib neurons as a direct indicator of the surviving EW3-sib cells. To our intrigue, we observed Crz-ir doublets in many hemi-segments of *grim* mutants (Fig. 5) and other *grim*-null genetic combinations (Figs 9, 10). These results suggest that *grim* plays an essential role in the PCD of EW3-sib cells during embryogenesis. To distinguish maternal *versus* zygotic role of *grim*, we compared



XX	*X25/+*	*grim^{A6C}*	*en-gal4*	*dronc$^{51/+}$*	*dcp-1; drice$^{\Delta1/+}$*
XY	*grim^{A6C}*	*grim^{A6C}*	*UAS-p35*	*dronc$^{I24/+}$*	*dcp-1; drice$^{\Delta1/+}$*
F1	*X25/grim^{A6C}*	*grim$^{A6C/A6C}$*	*en > p35*	*dronc$^{51/I24}$*	*dcp-1; drice$^{\Delta1/\Delta1}$*
mean	9.8 ±1.4	10.8 ± 2.0	11.1 ±1.3	0	0
n	13	10	16	8	12

XX	*dark^{CD4}*	*dark^{CD4}*	*dark$^{82/+}$*	*dark$^{1/+}$*	*dark$^{2/+}$*
XY	*dark^{CD4}*	*dark$^{82/+}$*	*dark^{CD4}*	*dark$^{1/+}$*	*dark$^{2/+}$*
F1	*dark$^{CD4/CD4}$*	*dark$^{CD4/82}$*	*dark$^{82/CD4}$*	*dark$^{1/1}$*	*dark$^{2/2}$*
mean	2.1 ± 1.1	5.7 ± 1.5	1.1 ± 0.4	3.3 ± 1.0	3.1 ± 1.2
n	13	13	13	13	13

Fig. 10. Maternal and zygotic effects of *grim*, *dark* and caspases for PCD of EW3-sib cells as determined by the number of ectopic Crz-ir (vCrz-sib) neurons in the larval VNC. (**A**,**B**) *grim*-null larvae derived from *grim* heterozygote (*X25*/+) and homozygote (*grim$^{A6C/A6C}$*) mothers displayed similar number of vCrz-sib neurons. (**C**) Expression of *p35* by *en-gal4* induced survival of 11 EW3-sib cells. (**D**) Maternal provision of a half dose of *dronc* was sufficient for PCD of all EW3-sib cells. (**E**) Both zygotic and maternal loss of *dcp-1* did not show extra vCrz neurons. Zygotic loss of *drice* did not enhance the survival of EW3-sib cells either. (**F–J**) Both maternal and zygotic contributions of *dark* were required for the PCD of EW3-sib cells. An asterisk in (H) indicates a vCrz-sib neuron. XX indicates maternal genotype; XY, paternal genotype; F1, progeny genotype. Numbers of surviving vCrz-sib neurons were given as mean±s.d. for the number of specimen (n). Scale bar: 100 µm.

the numbers of vCrz-sib neurons in the *grim*-null larvae that were derived from either a *X25*/+ or $grim^{A6C/A6C}$ females. Regardless of maternal contribution, mean numbers of surviving cells were about the same, implying an importance of zygotic *grim* expression (Fig. 10A,B). Additional loss or reduction of other death genes besides *grim*, such as in a *grim*, *rpr* double mutation, did not increase EW3-sib cell survival (Table 3). These results strongly suggest that *grim* is the sole proapoptotic factor for the lineage-regulated PCD of EW3-sib.

Maternal role of caspases

engrailed (*en*) is persistently expressed in NB7-3 lineage (Novotny et al., 2002). When P35, a universal inhibitor of caspases, was ectopically expressed by an *en-gal4* driver, we found an average of 11 vCrz-sib neurons in late larval CNSs (Fig. 10C). This result suggests that EW3-sib cells undergo apoptosis in a caspase-dependent manner. Surprisingly, however, we did not find any vCrz-sib neurons in *dronc*-null mutants (Fig. 10D), double mutants of *dcp-1*; *drice*, (Fig. 10E) or triple initiator mutants of *dredd*, *strica*, and *dronc* (n=6) (data not shown). A likely possibility is that maternally provided caspase function is sufficient to induce EW3-sib PCD.

As an alternative approach to identify the role of DRONC for EW3-sib cell death, we investigated DARK, a fly homolog of vertebrate Apaf1 that is required for DRONC activation (Rodriguez et al., 1999; Akdemir et al., 2006). Hypomorphic homozygous $dark^{CD4}$ mutant larvae derived from its homozygous stock had an average of two vCrz-sib neurons (Fig. 10F). When zygotic *dark* expression was reduced further in a $dark^{CD4/82}$ combination that was derived from a crossing between $dark^{CD4/CD4}$ virgins and $dark^{82/+}$ males, significantly more vCrz-sib neurons were found (Fig. 10G). The data support a role for DRONC in the embryonic apoptosis of EW3-sib cells, since DRONC is activated by DARK.

To understand the maternal effect of *dark*, we examined PCD of EW3-sib cells in $dark^{82/CD4}$ larvae derived from $dark^{82/+}$ mother. In this mutant, we detected only one or two vCrz-sib neurons (Fig. 10H), which is in stark contrast to the results of the $dark^{CD4/82}$ larvae from $dark^{CD4/CD4}$ mother (Fig. 10G). The difference between the two cases is the maternal contribution of *dark* products, which is expected to be greater from a $dark^{82/+}$ female than from a $dark^{CD4/CD4}$ one.

Further we examined $dark^{1}$ and $dark^{2}$ alleles, both of which are null alleles due to nonsense mutations (Mills et al., 2006). They produce homozygous larval escapers that die during pupal development, while $dark^{82}$ is a deletion mutation and is homozygous embryonic lethal. Both $dark^{1/1}$ and $dark^{2/2}$ larvae contained consistently more extra vCrz neurons than did $dark^{82/CD4}$ larvae, despite similar maternal contribution of *dark* gene product in all three cases (compare Fig. 10I,J with Fig. 10H). The difference is that $dark^{82/CD4}$ larvae have slightly more zygotic *dark* expression compared to none in $dark^{1/1}$ and $dark^{2/2}$ larvae, because of hypomorphic nature of the $dark^{CD4}$ allele. In summary, the numbers of surviving vCrz-sib cells are inversely proportional to combined amounts of *dark* expression provided both zygotically and maternally, indicating that *dark* gene products from both origins act additively for PCD of EW3-sib cells.

Developmental window for Notch-activated PCD of EW3-sibs

Selective death between EW3 and its sibling cell was shown to require the Notch (N)/Numb signaling pathway. During the mitotic division of the GMC-3, Numb is asymmetrically inherited by one of them, EW3, which differentiates into vCrz neuron, whereas Numb-negative EW3-sib cells undergo apoptosis due to N activation (Lundell et al., 2003). Consistent with this report, expression of a constitutively active form of N (N^{ICD}) by using an *eagle-gal4* (i.e. expression in the EW3 and EW3-sib cells) to bypass Numb's inhibitory activity, resulted in the complete lack of Crz-ir neurons in the larval VNC (Fig. 11A). These observations support the proapoptotic role of N in the EW3-sib cells during embryonic CNS development.

Since the death of EW3-sib cells requires *grim*, N is likely to be an upstream activator of *grim* expression. Interestingly, however, *Crz-gal4* driven N^{ICD} expression (i.e. after differentiation of EW3 into vCrz neurons) did not trigger precocious death of vCrz neurons in larva (Fig. 11B) or during metamorphosis (data not shown). This implies that the N-mediated death of the EW3-sib cells can take place only prior to their terminal differentiation into Crz-ir neurons.

Previously we have shown that ecdysone signaling at the end of larval growth is the developmental cue for the vCrz PCD, as genetic and transgenic disruption of ecdysone receptor (EcR) B1 and B2 isoforms blocks the PCD (Choi et al., 2006). Thus it is reasonable to state that ecdysone signaling sets the course leading to *grim* expression in the vCrz neurons, although the underlying mechanisms are unknown. However, such ecdysone-induced *grim* is unlikely to be the case for EW3-sib cell death, because EW3-sib cells underwent PCD normally in the CNS devoid of EcR functions (Fig. 11C,D). These data suggest that differential upstream regulators are involved in the expression of *grim* in a

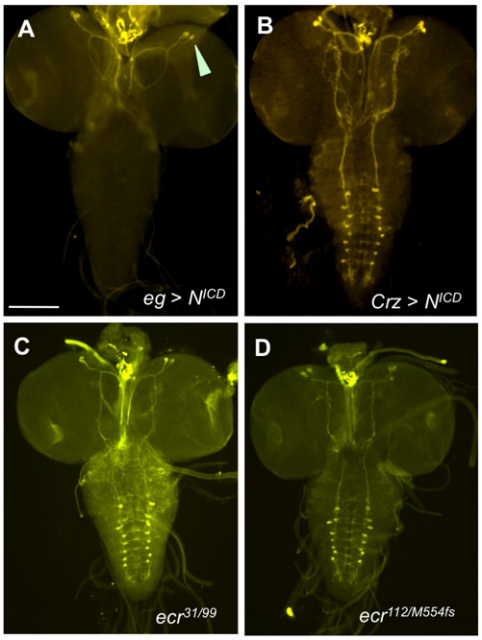


Fig. 11. Effect of Notch and ecdysone signaling on PCD of EW3-sib cells. (**A**) N^{ICD} expression driven by an *eg-gal4* induced killing of both EW3 and EW3-sib cells, as no Crz-ir neurons were present in the VNC (n=8). Note that Crz-ir neurons were normal in the brain (arrowhead). (**B**) Expression of N^{ICD} by a *Crz-gal4* driver did not kill vCrz neurons (n=14). (**C**,**D**) Lack of vCrz-sib neurons in the absence of (C) both EcR-B1 and EcR-B2 isoforms ($ecr^{31/99}$) or (D) EcR-A isoform ($ecr^{112/M554fs}$). These results suggest that the PCD of EW3-sib cells does not involve ecdysone signaling. Scale bar: 100 µm.

manner specific to the cell types and developmental status, as illustrated in Fig. 12.

Discussion

Multiple cell death genes are required to ensure the death of vCrz neurons during metamorphosis

Although major cell death genes found in *Drosophila* have been extensively characterized for their biochemical functions, in cell-based assays, and in transgenic animals, their *in vivo* roles during development have been characterized only in a few cell types. Interestingly, differential requirements of the cell death factors have been found for the PCD of distinct cell types. For instances, synergistic activities of the *rpr* and *grim* are required for the PCD of embryonic neuroblasts (Tan et al., 2011); *rpr* and *hid* for the salivary glands (Jiang et al., 2000); *hid* for the extra inter-ommatidial cells in the developing eye imaginal discs (Yu et al., 2002); *grim* for the microchaete glial cells (Wu et al., 2010) and for the precursor cells of the presumptive sensory neurons and socket cells in the posterior wing margin (Rovani et al., 2012).

Very little is known about the roles of cell death genes in the doomed post-mitotic neurons within the CNS. Based on the observations with *XR38/H99*, we presumed that *rpr* is essential for the PCD of vCrz neurons (Choi et al., 2006). Similar arguments were made for the PCD of RP2 larval motor neurons during metamorphosis (Winbush and Weeks, 2011). Surprisingly, however, our current studies using *rpr*-specific mutations suggest that the survival of vCrz neurons in *XR38/H99* flies is unlikely due to the loss of *rpr* function.

Multiple lines of evidence we presented here clearly support *grim* as the major cell-autonomous proapoptotic factor. Mild PCD defect in the *skl* mutants also suggest that *skl* plays at least a minor role. Although our genetic data do not support *rpr*'s proapoptotic role, we could not completely exclude it for the following reasons. First, *rpr* expression was observed in the doomed vCrz neurons (Choi et al., 2006). Secondly, *mi-RGH* expression completely blocked PCD of vCrz neurons at 16 hours APF, whereas *grim*-null mutation or *mi-grim* did not. *rpr* might play a role with *skl* for PCD of vCrz neurons particularly when *grim* function lacks.

What is the significance of belated function played by *skl* and possibly *rpr*? We propose that when *grim* function is inadvertently disrupted, elimination of unwanted larval neurons is ensured by alternative death triggers. Such a fail-safe mechanism could be important for sculpturing of the adult CNS from its larval predecessor, as accidental survival of larval doomed neurons might interfere with the formation of proper neural circuit during metamorphosis (Buss et al., 2006).

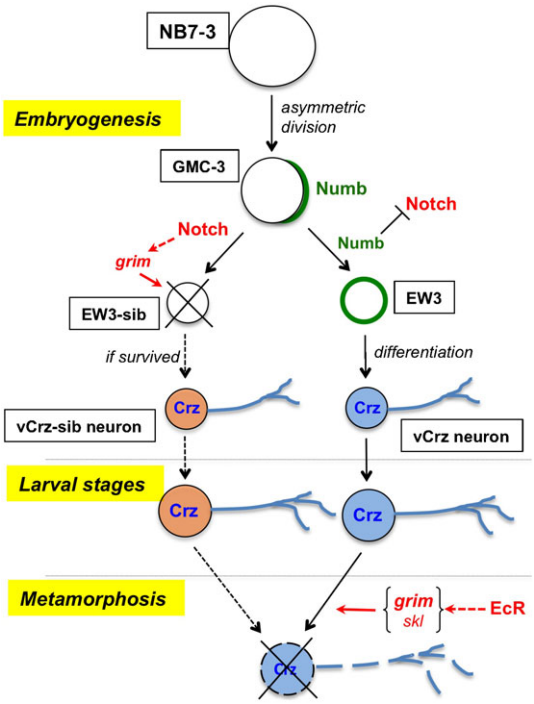


Fig. 12. Schematic illustration showing death genes acting on PCD in the corazonergic lineage during development.

Lineage-regulated PCD of neuronal precursor cells during embryogenesis

Unequal Numb distribution during mitosis determines Notch (N) activities, which direct differential specification of the sister cells in various neuronal lineages (e.g. Spana and Doe, 1996; Tio et al., 2011). In the NB7-3 lineage, daughter cells of the GMC-1 also take distinct fates, one interneuron (EW1) and the other motor neuron (GW), depending on the inheritance of Numb. In contrast, Numb-N determines the 'death fate' of a daughter cell derived from the GMC-2 and GMC-3 (Higashijima et al., 1996; Dittrich et al., 1997; Lundell and Hirsh, 1998; Isshiki et al., 2001; Novotny et al., 2002; Lundell et al., 2003) (see also Fig. 12). Thus Numb-N signaling is important not only for the fate-determination of developing neurons, but also for the regulation of cell numbers generated from a certain neuronal lineage. N-induced apoptosis is also observed in the developing CNS of mammals (Yang et al., 2004), suggesting a conserved role of N for the apoptosis-associated CNS development.

Little is known about the mechanisms underlying N-induced PCD in the nervous system. Because of the transcriptional regulatory functions of the N, it is likely that N signaling involves the expression of proapoptotic genes such as *Bax* and *Noxa* (Yang et al., 2004). Since *grim* is responsible for the PCD of EW3-sib cells, *grim* expression is expected to be a downstream target of the N signaling. However, the upregulation of *grim* expression by the N signaling appears to be developmentally restricted to the neuronal precursor cells, as ectopic expression of an activated N by the *Crz-gal4* did not kill terminally differentiated vCrz neurons. It is likely that only the precursor cells are competent to be responsive to N signal and such competence is lost prior to their final differentiation into functional neurons. It is notable that N-induced apoptosis is also restricted to the neural precursor cells in mammals as well (Yang et al., 2004). In developing *Drosophila* retina, N activates *hid* via antagonizing EGFR-mediated survival signal (Yu et al., 2002). Thus, there seems to be diverse mechanisms underlying N-mediated PCD in different cellular context.

Another interesting finding is that major death factors, *grim*, *dark*, and caspases, responsible for PCD of the EW3-sib cells, are differentially regulated. Zygotic expression of *grim* is essential while maternally provided caspases (Dronc and DrIce) are sufficient to drive PCD. In contrast, *dark* function has to be provided zygotically as well as maternally. These genetic data overall indicate that embryonic cells are preloaded with the death executioners (caspases), which is consistent with a view of 'death by default' (Raff, 1992; Raff et al., 1993).

Differential regulation of *grim* expression

Although *grim* is an essential proapoptotic factor for both vCrz neurons and EW3-sib cells, N signaling is responsible for PCD in EW3-sib cells, while ecdysone signaling is responsible for PCD in vCrz neurons (Fig. 12). These results suggest that *grim* also expression can be regulated by various upstream factors, which determine cell- and stage-specific expression. Such diverse regulatory mechanisms are not unique to *grim*, as *rpr* is also regulated in a complicated manner. Depending on the tissue types, transcription of *rpr* is activated by p53 in response to DNA damage (Brodsky et al., 2000), by ecdysone receptor (EcR) for metamorphosis-associated death of the salivary glands (Jiang et al., 2000), and by a *Hox* gene product, Deformed, for head morphogenesis during embryonic development (Lohmann et al., 2002). These upstream factors have been shown to activate *rpr* through direct binding to distinct 5′ upstream regions. It will be interesting to determine whether 5′ proximal region of the *grim* also contains various *cis* regulatory elements that respond to distinct upstream signals.

In addition to the 5′ upstream region, a remote enhancer *NBRR* is important for the optimal expression of multiple death genes in embryonic NBs (Tan et al., 2011) and *grim* in developing pupa (Wu et al., 2010). Although our genetic data also suggest a functional connection between *NBRR* and *grim* to some extent, further studies are necessary to confirm it in the vCrz neurons. In contrast, *NBRR* is not necessary for the PCD of EW3-sib cells, suggesting that N-induced PCD does not require *NBRR* for *grim* expression, while EcR-mediated PCD does so. Investigations on how *grim* is regulated in response to various death signals will be important to elucidate distinct molecular mechanisms of apoptosis between neuronal precursors and terminally differentiated neurons.

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Competing Interests

The authors have no competing interests to declare.

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Supplementary Material

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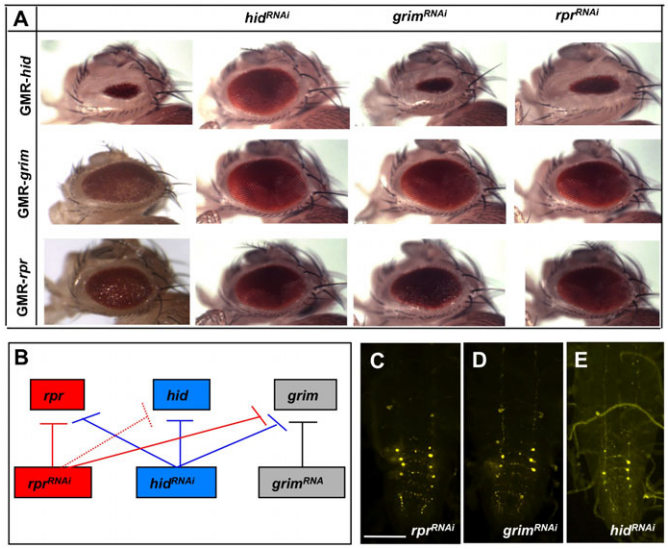

Fig. S1. UAS-double strand RNA (dsRNA) constructs. Specific regions (+55 to +559 for *rpr*; +311 to +1166 for *hid*; +2 to +850 for *grim*. +1 indicates the transcription start site) were amplified by PCR and inserted into pSymp-UAST-w vector at *Xho* I/*EcoR* I sites. The vector allows simultaneous transcription of the insert in both directions in the presence of Gal4, yielding complementary RNA strands. After confirmation of the insert by sequencing, *white* genomic insert was removed by *EcoR* I digestion. The final constructs were injected into *y w* embryos for germline transformation. Results: (**A**) To validate the efficiency and target-specificity of the UAS-RNAi, we tested whether each *RNAi* suppresses rough eye phenotype caused by an ectopic expression of respective cell death gene, as indicated. (**B**) A diagram depicting cross-interference of the RNAi. The eye defect caused by *rpr* overexpression was rescued by hid^{RNAi} and rpr^{RNAi} but not by $grim^{RNAi}$, while *grim*-induced eye phenotype was rescued by all three RNAi. A dotted line indicates a partial rescue effect than solid lines. (**C–E**) Overexpression of RNAi caused mild rescue of vCrz PCD. Scale bar: 100 µm.