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R Sehgal
Z Wang
S Nair
K Kikuno

See next page for additional authors

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Authors
G Lee, R Sehgal, Z Wang, S Nair, K Kikuno, C H. Chen, B Hay, and Jae H. Park
Essential role of grim-led programmed cell death for the establishment of corazonin-producing peptidergic nervous system during embryogenesis and metamorphosis in Drosophila melanogaster

Gyunhee Lee1, Ritika Sehgal1, Zixing Wang2, Sudershana Nair1, Keiko Kikuno1, Chun-Hong Chen3, Bruce Hay3 and Jae H. Park1,2,*

1Neurogenetics Laboratory, Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee, Knoxville, TN 37996, USA
2Graduate Program of Genome Science and Technology, University of Tennessee, Knoxville, TN 37996, USA
3Department of Biology, California Institute of Technology, Pasadena, CA 91125, USA

*Author for correspondence (jhpark@utk.edu; jae.park99@gmail.com)

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...
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 postmitotic 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 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 of a key event to sculpt adult CNS. In insects, neural apoptosis takes place mainly in two different 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; Winburn 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 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; Winburn 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 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; Winburn and Weeks, 2011).

Generation of skl null mutations

Mobilization of a P-element, P[wily]DG39210 carrying w’ y markers (Blooming stock number 21776), which is located 86-bp upstream of the skl locus, was performed. For convenience, we will refer to this allele as sklF. To induce P-element excision, sklF homozygous females were crossed to y w; Df(3R)r5, K(1)Sc (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 Sb Tb virgins. 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) (r1 primer, GGAGCCTTAGTTGGTG-GCAT; r2 primer, GGAGGACATTGAGGAGAAATC; r3 primer, GGAGGACATTGAGGAGAAATC; r4 primer, GGAGGACATTGAGGAGAAATC; r5 primer, GGAGGACATTGAGGAGAAATC). The lines that did not yield PCR product were further analyzed by PCR (skl5p, TGTTGATAGTACCTGTGCATCAGA; r2, TGACCTGACCC-ACCACCTAGG) 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°C. 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).
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<sup>-</sup>) (Moon et al., 2008). Surprisingly, all vCrz neurons carried out timely death that was not different from wild-type one (Fig. 1L). However, rpr<sup>-</sup>/H99 and rpr<sup>-</sup>/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). Mild PCD defect at 7 hours APF in rpr<sup>-</sup>/H99 and rpr<sup>-</sup>/Xr38 is comparable to H99/+ and Xr38/+, respectively. See also Table 1 for quantitative data. Scale bars: 100 μm.

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

| genotype     | gene dosage | 7 h APF | 16 h APF |
|--------------|-------------|---------|----------|
| wild type    | hid 2 | grim 2 | rpr 2 | skl 2 | 0.0±0.0 (32) | 0 (6) |
| XR38/+       | 2 | 2 | 1 | 1 | 6.7±2.1 (11) | 6.7±2.1 (11) |
| H99/+        | 1 | 1 | 1 | 2 | 15.2±1.0 (6) | 10.7±1.2 (3) |
| XR38/X25     | 1 | 1 | 1 | 1 | 9.6±1.9 (15) |
| ED225/+      | >1 | 1 | 1 | 1 | 0.0±0.0 (7) | 0 (5) |
| rpr          | rpr87/+/rpr87 | 2 | 2 | 0 | 2 | 1.7±1.5 (7) | 0 (2) |
| rpr87/+/XR38 | 2 | 2 | 0 | 1 | 6.3±3.3 (10) |
| rpr87/+/H99  | 1 | 1 | 0 | 2 | 8.9±2.3 (11) |
| rpr87/+/ED225| >1 | 1 | 1 | 0 | 14.3±1.0 (6) | 14.3±0.6 (3) |
| hid          | hid05014 | 0 | 2 | 2 | 1 | 3.3 (10) |
| hid05014/X14 | 0 | 2 | 2 | 2 | 0.0±0.0 (5) | 0 (2) |
| hid05014/+/H99| 0 | 1 | 1 | 2 | 6.0±2.8 (8) | 0 (2) |
| hid05014/+/ED225 | >1 | 1 | 1 | 1 | 9.0±2.2 (4) | 0 (2) |
| rpr and skl  | XR38/+/ED225 | 1 | 0 | 1 | 0.0 (7) | 0 (6) |
| grim         | X25/H99 | 0 | 0 | 1 | 2 | 0 (7) |
| 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.

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 skl1–4, respectively) were identified to be null alleles lacking the entire ORF and its 5’ flanking region (Fig. 4A). All homozygous mutants were viable and fertile without any noticeable deficit in morphology, development, 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-sk1 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.

Fig. 3. miRNA4-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 miRNA4 shows minimal or no cross-interference with non-cognate death genes. (B) The numbers of surviving vCrz neurons (mean±s.d.) due to miRNA4 expression at 7 hours and 16 hours APF.

Numbers of specimens examined are indicated in parentheses. Expression of miRNA4 was driven by one (1x) or two copies (2x) of the Crz-gal4 transgene. mi-grim and mi-sk1 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.
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$^{16C}$), 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$^{16C/16C}$ 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$^{M03811}$/TM3, Sb) in which a MIMIC transposable element disrupts the grim open reading frame (Venken et al., 2011). Unlike grim$^{16C}$ allele, no homozygous adult flies were found in this stock. After we changed the balancer from TM3 to TM6B, Tb, Hu, we found that homozygous died mostly as third instar larvae. A small fraction of larvae became puparia, but they did not develop any further. However, trans-heterozygous grim$^{M03811/A6C}$ developed into adults as observed with grim$^{16C}$ homozygotes, suggesting that the larval lethality of the grim$^{M03811}$ be associated with a genetic background unrelated to grim.

In the larval CNS of grim$^{M03811}$ homozygotes and grim$^{16C/M03811}$, 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$^{16C/M03811}$ mutant (Fig. 5G), as was found in grim$^{16C}$ 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$^{16C}$ 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$^{16C}$/H99 or grim$^{16C}$/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 grimA6C 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 grimA6C 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 grimA6C 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 grimA6C and skl (grimA6C/skle1). 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 rpr87/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 rpr87/ED225 did only 9–10 neurons (Fig. 7G; Table 1). Furthermore, the PCD phenotype of X25/XR38 was very similar to that of grimA6C/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 (grimXR38) 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 grimXR38 is a hypomorphic allele. This result can also explain

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

| genotype         | grim dosage | rpr dosage | skl dosage | vCrz neurons at 7 h APF | vCrz neurons at 16 h APF |
|------------------|-------------|------------|------------|-------------------------|-------------------------|
| grimA6C/+        | 1           | 2          | 2          | 2                       | 0 (7)                   |
| grimA6C/X14      | 1           | 2          | 2          | 2                       | 0 (10)                  |
| grimA6C/rpr      | 1           | 2          | 1          | 2                       | 0 (11)                  |
| grimA6C/skle1    | 0           | 2          | 2          | 2                       | 3.2±2.4 (8)             |
| *grimA6C/skle1   | 0           | 2          | 2          | 2                       | 16±0.8 (8)              |
| *grimA6C/X25     | 0           | 2          | 2          | 2                       | 16±0.9 (9)              |
| *grimA6C/XR38    | 0           | 1          | 1          | 2                       | 15.2±0.8 (9)            |
| *grimA6C/ED225   | 0           | 1          | 1          | 1                       | 16±0.0 (12)             |
| NBRR             |             |            |            |                         |                         |
| MM2/+           | 1           | 1          | 1          | 2                       | 6.3±3.3 (11)            |
| *MM2/grimA6C    | 0           | 0          | 1          | 2                       | 16±0.0 (6)              |
| *MM2/XR38       | 1           | 0          | 0          | 1                       | 13.6±2.2 (8)            |
| *MM2/X25        | 0           | 1          | 1          | 2                       | 16±0.0 (7)              |
| *MM2/H99        | 0           | 0          | 0          | 2                       | 16±0.0 (7)              |
| MM2/MM3         | 1           | 0          | 1          | 2                       | 13±1.4 (3)              |
| MM3/+           | 2           | 1          | 2          | 2                       | 10±1.9 (4)              |
| MM3/MM3         | 2           | 0          | 2          | 2                       | 9.5±0.8 (3)             |
| MM3/XR38        | 1           | 0          | 1          | 1                       | 5.8±2.9 (5)             |
| MM3/grimA6C     | 1           | 1          | 2          | 2                       | 12.2±2.0 (3)            |
| MM3/X25         | 1           | 1          | 2          | 2                       | 10.8±1.9 (4)            |
| MM3/H99         | 1           | 0          | 1          | 2                       | 11.8±1.6 (10)           |
Table 3. PCD of EW3-sib cells, as determined by vCrz-sib neurons in the larval CNS.

| genotype          | EW3-sib cells (n) |
|-------------------|-------------------|
| grim\textsuperscript{A6C}/+ | 0±0 (15)          |
| grim\textsuperscript{A6C}A6C | 10.8±2.0 (10)     |
| grim\textsuperscript{A6C}/XR38 | 9.8±1.4 (13)     |
| grim\textsuperscript{A6C}/MM3 | 2±1.4 (5)        |
| grim\textsuperscript{A6C}/XR38 | 9.5±1.8 (8)      |
| grim\textsuperscript{A6C}/MM2 | 0.6±0.7 (11)     |
| grim\textsuperscript{A6C}/XR38 | 9.3±0.6 (3)      |
| grim\textsuperscript{A6C}/ED225 | 8.8±1.5        |
| grim\textsuperscript{A6C}/MM | 3.6±1.1 (5)      |
| grim\textsuperscript{A6C}/MM | 6.8±2.6 (5)      |
| grim\textsuperscript{A6C}/XR38 | 11.0±2.9 (11)    |
| grim\textsuperscript{A6C}/XR38 | 0.2±0.6 (14)     |
| MM2/+            | 0±0 (8)           |
| X25/MM2 | 10±1.3 (8)       |
| MM2/MM | 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\textsuperscript{A6C}/XR38 displayed stronger PCD defect than did ED225/+. Despite considering hypomorphic grim\textsuperscript{XR38}, it is not still fully understood why PCD defect of XR38/H99 is greater than that of grim\textsuperscript{A6C}/XR38 at 16 hours APF. For instance, nearly 50% of vCrz neurons in the larval CNS.

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

Recently an intergenic region between the \textit{rpr} and \textit{grim} loci, termed the \textit{neuroblast regulatory region} (NBRR), was proposed to be a remote enhancer for the optimal expression of \textit{rpr}, \textit{grim}, and \textit{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 it was proposed for the NB death. However, it is more complicated than that because the MM3 chromosome also carries a \textit{grim} mutation bearing an in-frame deletion of six amino acid residues (52nd–57th) within the Gln-rich domain of \textit{grim} ORF (we confirmed this result with a slight difference, deletion of the 51st–57th residues) (Fig. 7I). The MM3\textsuperscript{grim} (hereafter \textit{grim}\textsuperscript{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 \textit{grim}\textsuperscript{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/\textit{grim}\textsuperscript{A6C} and MM3/XR38 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 \textit{grim} to \textit{rpr} locus (Fig. 2). MM2 heterozygote flies (\textit{grim}\textsuperscript{A6C}/X25/MM2),
NBRR$^{+/+}$, rpr$^{+/+}$) showed PCD defect similar to those of MM3/grimA6C or MM3/X25 (both grim$^{MM3/2}$, NBRR$^{+/+}$) (Fig. 9A, Ai). In addition, grim$^{ABC/MM2}$ 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).

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

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$\mu$m.

Fig. 9. PCD of vCrz neurons requires both grim and NBRR (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$\mu$m.
the numbers of vCrz-sib neurons in the grim-null larvae that were derived from either a X2S/+ or grimAG6/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 darkCD4 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 darkCD4/+ combination that was derived from a crossing between darkCD4 virgins and darkCD4+ 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 darkCD4/CD4 larvae derived from darkCD4+ 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 darkCD4/CD4 larvae from darkCD4CD4 mother (Fig. 10G). The difference between the two cases is the maternal contribution of dark products, which is expected to be greater from a darkCD4+ female than from a darkCD4CD4 one.

Further we examined dark1 and dark2 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 dark2 is a deletion mutation and is homozgyous embryonic lethal. Both dark1/1 and dark2/2 larvae contained consistently more extra vCrz neurons than did dark82CD4 larvae, despite similar maternal contribution of dark gene product in all three cases (compare Fig. 10I,J with Fig. 10H). The difference is that dark82CD4 larvae have slightly more zygotic dark expression compared to none in dark1/1 and dark2/2 larvae, because of hypomorphic nature of the darkCD4 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
dICD) 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
dICD 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...
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 interommatidial 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).

**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 (Drone 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).

![Fig. 12. Schematic illustration showing death genes acting on PCD in the corazonergic lineage during development.](image-url)
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 edysone 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 edysone 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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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 XhoI/EcoRI 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 EcoRI 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 hidRNAi and rprRNAi but not by grimRNAi, 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.