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Polycomb group genes are required for neural stem cell survival in postembryonic neurogenesis of *Drosophila*

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Genes of the Polycomb group (PcG) are part of a cellular memory system that maintains appropriate inactive states of Hox gene expression in *Drosophila*. Here, we investigate the role of PcG genes in postembryonic development of the *Drosophila* CNS. We use mosaic-based MARCM techniques to analyze the role of these genes in the persistent larval neuroblasts and progeny of the central brain and thoracic ganglia. We find that proliferation in postembryonic neuroblast clones is dramatically reduced in the absence of *Polycomb*, *Sex combs extra*, *Sex combs on midleg*, *Enhancer of zeste* or *Suppressor of zeste 12*. The proliferation defects in these PcG mutants are due to the loss of neuroblasts by apoptosis in the mutant clones. Mutation of PcG genes in postembryonic lineages results in the ectopic expression of posterior Hox genes, and experimentally induced misexpression of posterior Hox genes, which in the wild type causes neuroblast death, mimics the PcG loss-of-function phenotype. Significantly, full restoration of wild-type-like properties in the PcG mutant lineages is achieved by blocking apoptosis in the neuroblast clones. These findings indicate that loss of PcG genes leads to aberrant derepression of posterior Hox gene expression in postembryonic neuroblasts, which causes neuroblast death and termination of proliferation in the mutant clones. Our findings demonstrate that PcG genes are essential for normal neuroblast survival in the postembryonic CNS of *Drosophila*. Moreover, together with data on mammalian PcG genes, they imply that repression of aberrant reactivation of Hox genes may be a general and evolutionarily conserved role for PcG genes in CNS development.

KEY WORDS: Polycomb group, *Drosophila*, Neurogenesis, Hox genes, Neuroblasts

INTRODUCTION

During development, defined genetic programs control proliferation and patterning by regulating tissue-specific gene expression. In many cases, this involves the stable maintenance of an active or repressed state by epigenetic mechanisms. Polycomb group (PcG) genes regulate gene expression by the stable silencing of specific sets of target genes through chromatin modifications that involve the formation of large repressive complexes (for reviews, see Orlando, 2003; Ringrose and Paro, 2004; Simon and Tamkun, 2002). They thus contribute to a cell memory system that prevents changes in cell identity by maintaining the transcription patterns, which are set early in embryogenesis, during subsequent development and in adult life. PcG genes were originally discovered in *Drosophila* as repressors of the homeotic (Hox) genes. Through PcG gene action, spatial patterns of Hox gene expression, which are initially set in early embryogenesis by segmentation genes, are maintained; when the segmentation proteins decay, PcG proteins assume Hox expression control for the remainder of development (Gould, 1997; Kennison, 1995; Pirrotta, 1998; Schumacher and Magnuson, 1997).

In *Drosophila*, approximately 15 PcG proteins are thought to participate in two separate multiprotein complexes. These are the Polycomb repressive complex 1 (PRC1), which contains most of the characterized PcG proteins such as those encoded by *Polycomb* (*Pc*), *Posterior sex combs* (*Psc*), *polyhomeotic* (*ph*) and *Sex combs extra* (*Sce*), and Polycomb repressive complex 2 (PRC2), which contains proteins such as those encoded by *extra sex combs* (*esc*), *Enhancer of zeste* (*E(z)*) and *Suppressor of zeste 12* (*Su(z)12*). PRC2 is thought to be involved in the initiation of gene silencing, whereas PRC1 is implicated in stable maintenance of gene repression. The expression of PcG genes is widespread and nearly ubiquitous throughout fly development, and notably includes expression in the embryonic and larval CNS (e.g. Martin and Adler, 1993; DeCamillis and Brock, 1994; Carrington and Jones, 1996; Bornemann et al., 1998). Homologs of the fly PcG genes have been identified in mammals, and in some cases these gene homologs have also been implicated in developmental Hox gene regulation (Gould, 1997). Indeed, deregulation of Hox gene expression is one of the hallmarks of both *Drosophila* and mammalian PcG phenotypes. However, as general chromatin regulators, PcG genes in *Drosophila* and mammals also control a diverse set of other target genes that include those involved in cell cycle regulation (reviewed by Gould, 1997; Orlando, 2003; Simon, 1995).

Recent work on mouse models indicates that PcG genes are involved in stem cell fate and proliferation control (reviewed by Valk-Lingbeek et al., 2004). Direct evidence for this comes from studies of the murine *Bmi1* gene, which is the homolog of the two closely related fly genes *Psc* and *Suppressor of zeste 2* (*Su(z)2*). The analysis of *Bmi1*-deficient mice, which suffer from progressive loss of neuronal and hematopoietic cells, implicates *Bmi1* in the renewal of multiple stem cell types (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003). In the developing nervous system, *Bmi1* is required for the self-renewal of neural stem cells in the telencephalon and for the proliferation of cerebellar granule cell progenitors (Leung et al., 2004; Molofsky et al., 2003). *Bmi1* has been shown to regulate three Hox genes, *Hoxd8*, *Hoxd9* and *Hoxc9*, in neural stem cells (Molofsky et al., 2003). Since loss of *Bmi1* leads to upregulation of these Hox genes in vitro, it is conceivable that *Bmi1* loss could affect neural stem cells in vivo through aberrant reactivation of Hox gene expression. This possibility has not been addressed experimentally. Although many aspects of PcG gene

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structure and function are conserved from flies to mammals, little is known about the roles of PcG genes in neuronal developmental processes in *Drosophila*. In *ph* mutants, global misrouting of CNS axons and ecdysone-dependent loss of neuronal subtype identity during metamorphosis have been reported (Smouse and Perrimon, 1990; Wang et al., 2006). However, there is currently little evidence for a role of PcG genes in neural stem cell fate or neuronal proliferation in *Drosophila*.

The neuroblasts of *Drosophila* are similar to mammalian neural stem cells in that they self renew and have the potential to generate different types of neurons and glia. Neuroblasts divide repeatedly in an asymmetric mode which is self-renewing and generates smaller ganglion mother cells (GMCs), which usually divide once to produce two postmitotic progeny. *Drosophila* neuroblasts generate the larval CNS during embryonic development. Following a period of quiescence, most neuroblasts resume their asymmetric mode of proliferation and generate the bulk of the adult CNS during postembryonic development (Prokop and Technau, 1991; Truman and Bate, 1988; Truman et al., 1993). Mechanisms involved in asymmetric neuroblast division and neural proliferation during embryogenesis have been characterized in detail (Betschinger and Knoblich, 2004; Chia and Yang, 2002; Jan and Jan, 2001; Pearson and Doe, 2003; Pearson and Doe, 2004; Skeath and Thor, 2003). By contrast, the mechanisms that control the number of cells generated by larval neuroblasts during postembryonic development are not yet well understood (Maurange and Gould, 2005). However, proliferation and self-renewal of larval neuroblasts are currently subjects of intense investigation, given that these processes may have implications for stem cell biology (reviewed by Yu et al., 2006; Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a; Lee et al., 2006b; Bowman et al., 2006. Recently, it has been shown that reactivation of specific Hox genes is involved in terminating neuroblast proliferation during larval stages (Bello et al., 2003). During the last larval instar, a brief pulse of Hox gene expression occurs in abdominal neuroblasts, which activates proapoptotic genes and results in neuroblast death. Thus, the Hox axial patterning system is directly linked to neuronal proliferation and stem cell fate during postembryonic development of the *Drosophila* CNS. Given that PcG genes are involved in maintaining spatial patterns of Hox gene expression, this suggests that PcG genes might also play a role in controlling neuronal proliferation in *Drosophila*.

Here we investigate the role of several PcG genes in postembryonic development of the *Drosophila* CNS. We use mosaic-based MARCM techniques to carry out a clonal analysis of these genes in the persistent larval neuroblasts and their progeny (‘neuroblast clones’). Our findings show that neuroblast clones fail to proliferate normally during larval development in the absence of the PcG genes and that the observed lack of proliferation in the PcG mutants is due to the loss of the neuroblasts through apoptosis. This proliferation defect can be rescued by blocking apoptosis in the neuroblast of mutant clones, indicating that PcG gene action is required to prevent neuroblast death and, thereby, ensure normal neural proliferation. Significantly, ectopic expression of posterior Hox genes, which in wild type leads to neuroblast death, is observed in the PcG mutant clones, as well as in the PcG mutant neuroblasts rescued from apoptosis-block. These results suggest that loss of PcG genes leads to aberrant derepression of posterior Hox gene expression in postembryonic neuroblasts, which causes neuroblast death and termination of proliferation in the mutant clones. Taken together, our findings demonstrate for the first time that PcG genes are essential for normal neuroblast survival and proliferation in postembryonic CNS development of *Drosophila*. Moreover, together with studies on mammalian PcG genes, these studies imply that repression of aberrant reactivation of Hox gene expression may be a general and evolutionarily conserved role for PcG genes in nervous system development.

MATERIALS AND METHODS

Fly strains and genetics

Unless otherwise stated, fly stocks carrying transgenes and recombinant chromosomes were obtained from the Bloomington Stock Center. Recombinant chromosomes carrying null alleles of PcG genes were kindly provided by Jürg Müller: *FRT2A, E(z)^{731}* (Muller et al., 2002); *FRT2A, Su(z)12^{1}*; *FRT42D, Su(z)2$^{1.B8}$*; *FRT2A, Pc^{XT109}*; *FRT82B, Sce^{1}*; *FRT82B, Scm^{D1}* (Beuchle et al., 2001). Additional recombinants were generated for this study: *FRT82B, Scm^{D1}, UAS-p35^{BH2}*; *FRT82B, Sce^{1}, UAS-p35^{BH2}*; *UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}, UAS-p35^{BH1}*. Recombinants not described are found in Bello et al. (Bello et al., 2003). Further details can be found at Flybase: http://flybase.bio.indiana.edu.

Combinations of chromosomes were assembled using standard genetics to generate the following MARCM clones:

wt control (Fig. 1): y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}/tubP-GAL4; FRT82B/FRT82B, tubP-GAL80^{LL3}*.

wt control (Figs 2 and 4): y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}/tubP-GAL4; FRT2A/FRT2A, tubP-GAL80^{LL9}*.

Sce: y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}/tubP-GAL4; FRT82B, Sce^{1}/FRT82B, tubP-GAL80^{LL3}*.

Sce+P35: y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}/tubP-GAL4; FRT82B, Sce^{1}, UAS-p35^{BH2}/FRT82B, tubP-GAL80^{LL3}*.

Scm: y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}/tubP-GAL4; FRT82B, Scm^{D1}/FRT82B, tubP-GAL80^{LL3}*.

Scm+P35: y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}/tubP-GAL4; FRT82B, Sce^{1}, UAS-p35^{BH2}/FRT82B, tubP-GAL80^{LL3}*.

Pc: y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}/tubP-GAL4; FRT2A, Pc^{XT109}/FRT2A, tubP-GAL80^{LL9}*.

Pc+P35: y,w, *hsFLP/+; tubP-GAL4/ UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}, UAS-p35^{BH1}/tubP-GAL4; FRT2A, Pc^{XT109}/FRT2A, tubP-GAL80^{LL9}*.

Ez: y,w, *hsFLP/+; tubP-GAL4, UAS-mCD8::GFP^{LL5}/+; FRT2A, E(z)^{731}/FRT2A, tubP-GAL80^{LL9}*.

Su(z)12: y,w, *hsFLP/+; tubP-GAL4, UAS-mCD8::GFP^{LL5}/+; FRT2A, Su(z)12^{1}/FRT2A, tubP-GAL80^{LL9}*.

Psc-Su(z)^{2}: y,w, *hsFLP/+; FRT42D, Su(z)2$^{1.68}$/FRT42D, tubP-GAL80; tubP-GAL4^{LL7}, UAS-mCD8::GFP^{LL6}/+*.

UAS-abdA: y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}/tubP-GAL4; FRT82B, UAS-abdA^{H3}/FRT82B, tubP-GAL80^{LL3}*.

UAS-abdA+UAS-P35: y,w, *hsFLP/+; UAS-mCD8::GFP^{LL5}, UAS-nlslacZ^{20b}, UAS-p35^{BH1}/tubP-GAL4; FRT82B, UAS-abdA^{H3}/FRT82B, tubP-GAL80^{LL3}*.

For MARCM experiments, embryos of the appropriate genotype were collected on yeasted grape juice agar plates over a 4- to 8-hour time-window and raised at 25°C for 24-28 hours before heat shock treatment. Heat shock induction of FLP in the newly hatched larvae was performed by immersing the plates at 37°C in a water bath for 1 hour. Larvae were then collected and plated at low density on standard cornmeal-yeast-agar medium supplemented with live yeast. GFP-labeled MARCM clones were examined in nervous systems dissected out of wandering third-instar larvae (time of dissection was ~96 hours after heat shock). For the time course experiment shown in Fig. 3, batches of newly hatched larvae were raised in vials on standard medium and heat shocked at 24-hour intervals. Clones were examined on 10-20 nervous systems dissected out at the wandering stage 24, 48 and 72 hours after heat induction.

Immunostaining and antibodies

CNSs were dissected in PBS, fixed in 2% paraformaldehyde in PBL (75 mM lysine-HCl in sodium phosphate buffer, pH 7.4) for 1 hour at room temperature (RT), washed three times for 10 minutes in PBS containing 0.5% Triton X-100 (PBT), blocked for 1 hour at RT in PBT containing 10% normal goat serum, and incubated with primary antibodies in blocking solution overnight at 4°C. Samples were washed three times for 10 minutes each in PBT at RT, and secondary antibodies were applied in blocking solution for 2 hours at RT. After washing three times for 10 minutes each in

PBS, samples were mounted in Vectashield (Vector Labs). The following antibodies were used: rabbit anti-Grh (1:200) (Bello et al., 2006), rabbit anti-PH3 (1:400; Upstate Biotechnology), mouse anti-Mira Mab81 (1:50; gift of P. Overton, VASTox, Abingdon, UK), mouse anti-Pros MaMR1A (1:10; DSHB); rat anti-Elav Mab7E8A10 (1:30; DSHB), rat anti-Abd-A (1:200; gift of J. Casanova, CSIC, Barcelona, Spain), mouse anti-UBX (1:20; gift of R. White, Cambridge University, UK), mouse anti-CycE (Cyclin E2) (1:50; mAb8B10, gift from H. Richardson, Peter MacCallum Cancer Center, East Melbourne, Victoria, Australia), rabbit anti-Caspase-3 (1:75; Cell Signaling Technology). Alexa Fluor-conjugated secondary antibodies (Molecular Probes) were used at 1:200.

All fluorescent images were collected using a Leica TCS SP scanning confocal microscope. Optical sections ranged from 0.5 to 2 μm. Captured images from optical sections were arranged and processed using ImageJ and Adobe Photoshop. All images show projections of multiple focal planes.

RESULTS

Proliferation defects in PcG mutant neuroblast lineages

We investigated the role of six PcG genes in postembryonic development of the *Drosophila* CNS: *Sex combs extra* (*Sce*), *Sex combs on midleg* (*Scm*), *Polycomb* (*Pc*), *Enhancer of zeste* (*E(z)*), *Suppressor of zeste 12* (*Su(z)12*), and the two closely related genes *Posterior sex combs* and *Suppressor of zeste 2* (*Psc-Su(z)2*). Like all other PcG genes, the loss-of-function mutants of these genes are embryonic lethal. In order to study the action of these genes in postembryonic development, we used somatic clonal analysis based on MARCM technology to generate mutant neuroblast clones in a wild-type background. Positively marked somatic clones mutant for PcG genes were generated by inducing random mitotic recombination after larval hatching and examined 4 days later in late third-instar larvae. Wild-type clones were induced in parallel as control. Since heterozygous recessive alleles of PcG genes do not affect development, examination of PcG gene function could be unambiguously assessed in positively labeled MARCM clones.

The extent of ongoing proliferation in the wild-type third larval instar CNS was determined by studying expression of the mitotic marker phosphorylated Histone H3 (PH3), and of the cell cycle marker Cyclin E (Fig. 1A). Highest levels of proliferation were seen in the developing optic lobes where dense zones of mitotically active cells characterized the outer and inner proliferation centers. This proliferation in the optic lobes was not studied further in this report. Clearly delimited from the optic lobes were the scattered proliferative clusters in the developing central brain and thoracic ganglia. Consistent with previous findings, only limited proliferation was seen in the abdominal ganglia (Awad and Truman, 1997). To characterize the proliferating clusters of the wild-type central brain and thoracic ganglia in more detail, we examined expression of the PH3 marker in MARCM neuroblast clones that were positively labeled with nuclear β-galactosidase. Each of these clones was composed of a large neuroblast and its associated progeny. Mitotic activity assayed with the PH3 marker was observed in the neuroblast, in the associated smaller GMC, or in both cell types (Fig. 1B-E). Approximately half of the labeled MARCM clones in late third-instar larvae showed mitotic activity as judged by expression of PH3.

We next examined labeled MARCM clones that were mutant in the PcG genes *Sce*, *Scm*, *Pc*, *E(z)*, *Su(z)12* or *Psc-Su(z)2*. An analysis of the proliferating clusters in the central brain and thoracic ganglia of these PcG mutants revealed a striking phenotype in five of the six cases examined. The overall size of labeled clones was dramatically reduced in *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* mutants as compared with wild-type control clones (Fig. 1F-I). For example, whereas 76% of

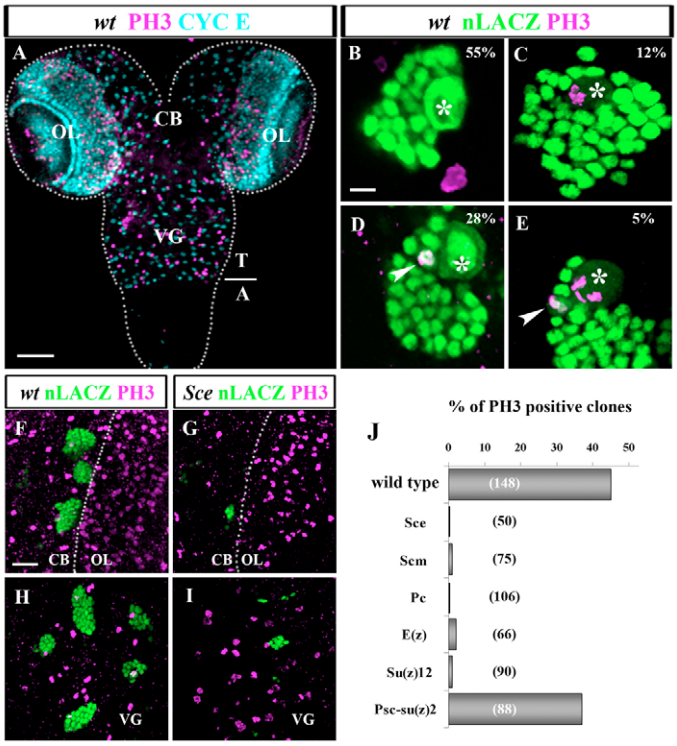

Fig. 1. PcG mutant neuroblast lineages fail to proliferate. Confocal images of late third-instar nervous system immunostained for Cyclin E (CYC E), phosphohistone H3 (PH3) and nuclear β-galactosidase (nLACZ) in positively labeled MARCM clones. All panels show *z* projections of image stacks recorded on the ventral side. (**A**) Ventral view of a whole-mount nervous system showing a large number of mitotic cells in the optic lobes (OL) and in discrete foci evenly distributed in the central brain (CB) and in the thoracic part (T) of the ventral ganglia (VG), but not in the abdominal region (A). (**B-E**) Mitosis in isolated wild-type neuroblast lineages is revealed by PH3 staining in a maximum of two cells: the large neuroblast (asterisk) and/or the closely associated GMC (arrowhead) at the frequency indicated for each panel (*n*=148). In B, mitotic activity is seen outside of the clone. In C, mitotic activity is seen in the neuroblast. In D, mitotic activity is seen in the GMC. In E, mitotic activity is seen in neuroblast and GMC. (**F-I**) Compared with wild-type MARCM neuroblast clones in the central brain (F) or the ventral ganglia (H), *Sce* mutant clones in the same areas (G,I) contain a much smaller number of nuclei and do not stain for PH3. The dotted lines in F,G demarcate optic lobe (OL) and central brain (CB) by the density of mitotic cells. (**J**) The percentage of mitotic *Sce* and other PcG mutant clones is plotted with the number of clones examined indicated in parentheses. For genotypes, see Materials and methods. Scale bars: A, 50 μm; B-E, 5 μm, F-I, 30 μm.

the wild-type clones contained more than 50 nuclei (*n*=25), 81% of *Sce* clones (*n*=75) and 88% of *Pc* clones (*n*=56) contained less than 20 nuclei. This marked reduction in clone size was observed throughout the central brain and thoracic ganglia. In contrast to the other PcG genes studied, mutant clones for *Psc-Su(z)2* in the CNS appeared morphologically normal.

The small size of the labeled clones in the five PcG mutants was associated with a dramatic reduction in mitotic activity as assayed with the marker PH3 (Fig. 1J). Thus, in contrast to wild-type control clones, only 6% of the *E(z)* clones showed PH3 immunoreactivity (*n*=66), and none of the 50 *Sce* clones contained mitotic cells. Mutant clones for *Su(z)12*, *Pc* and *Scm* showed a comparable

reduction in clone size associated with a low mitotic index in the same range. In accordance with their normal overall size, mutant clones for *Psc-Su(z)2* in the CNS had a mitotic index similar to that of wild-type controls. These findings indicate that there is a marked deficit in neural proliferation in the brain and thoracic ganglia of *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* mutants.

Lack of neuronal precursor cells in PcG mutant lineages

To investigate the nature of the proliferation defect in more detail we first studied cellular and molecular features of mutant versus wild-type lineages in MARCM experiments. In the wild type, labeled clones contained one large cell, the neuroblast, that invariably expressed the transcription factor Grainyhead (Grh) (Fig. 2A). By contrast, the markedly smaller labeled clones of the *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* mutants almost never contained Grh-expressing cells of any size (Fig. 2B). This difference in Grh expression as compared with wild type suggests that neuronal precursor cells are lacking in these PcG mutants.

Supporting this notion were differences in the spatial organization of labeled clones in wild type versus mutants. In the wild type, the large Grh-expressing neuroblasts of each clone were located in the outermost cortical layer and their labeled progeny extended in a continuous, columnar fashion from the neuroblast into inner cortical layers (Fig. 2C). As expected for postmitotic neurons, these progeny expressed the neuron-specific label Elav and projected neuronal processes towards the neuropile. By contrast, the small, labeled MARCM cell clones of the *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* mutants were generally located in the inner cortical layers and were rarely associated with a Grh-expressing precursor cell of any size (Fig. 2D). These small clones were composed of cells that expressed Elav and extended neurites, indicating that the cells are postmitotic neurons.

Further evidence for the lack of neural precursors in the mutant clones was provided by studying the expression of Miranda (Mira), which is expressed at the cell cortex in both neuroblasts and GMCs (Ikeshima-Kataoka et al., 1997). As expected, the neuroblasts of labeled MARCM wild-type clones always expressed Mira (Fig. 2E). By contrast, the small clones of *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* mutants were generally Mira-negative and only rarely contained large cells (Fig. 2F,G). Unlike wild-type and *Psc-Su(z)2* mutants clones that always showed Grh- or Mira-expressing cells, the PcG mutants *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* had Grh- or Mira-expressing cells in less than 10% of the clones (Fig. 2G). Taken together with the data on clone size and mitotic activity, these results suggest that the deficit in proliferation observed in *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* mutants is causally related to the absence of neural precursor cells in the brain and thoracic ganglia.

We next investigated if the neuroblasts in PcG mutant clones might be eliminated by programmed cell death. Most of the PcG mutant clones induced after larval hatching and examined 96 hours later at the late third larval instar stage no longer contained neuroblasts. To generate mutant clones that still had neuroblasts, induction was timed to occur 24 hours, 48 hours, and 72 hours before examination at the late third larval instar stage. The percentage of *Pc* mutant clones that contained neuroblasts in each of these conditions is shown in Fig. 3A. Neuroblasts were present in 88% of *Pc* clones examined 24 hours after induction, indicating that significant elimination had not yet occurred during this short time interval. By contrast, neuroblasts were absent in 89% of the mutant clones induced 72 hours before examination, implying that the elimination process had largely taken place during this long time

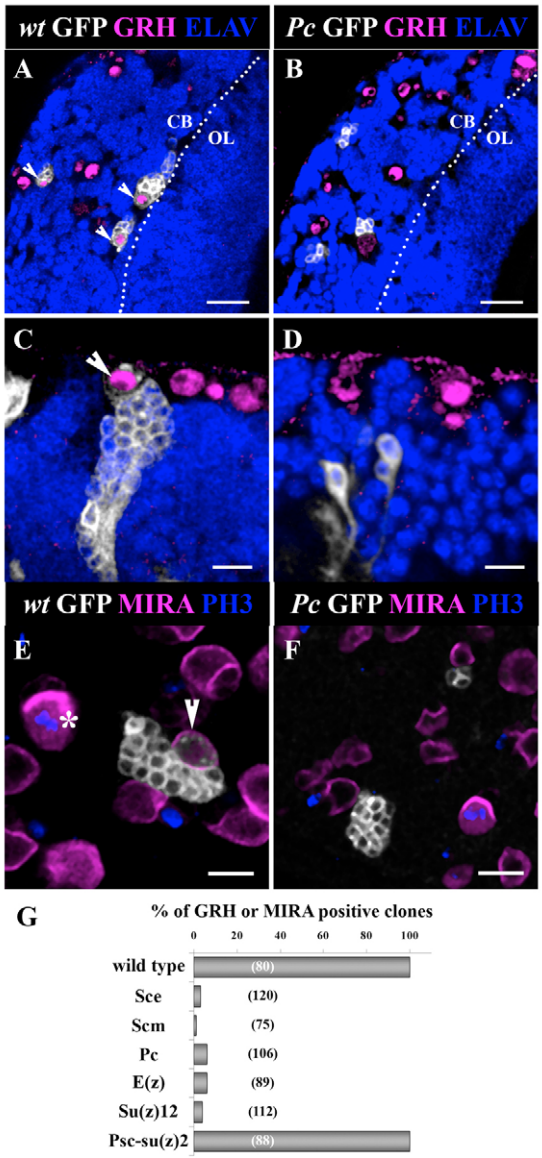

Fig. 2. PcG mutant lineages lack neuronal precursor cells. Confocal images of wild-type control (*wt*) and *Pc* mutant MARCM clones labeled with membrane-tethered GFP (CD8::GFP, white) and immunostained as indicated. (**A-D**) Grh is detectable in the large neuroblasts (arrowheads) of the central brain (CB), but not in the precursor cells of the optic lobes (OL). Elav is expressed in all adult-specific neurons in both areas of the brain hemispheres. A,B are ventral views; C,D are optical cross-sections. Unlike wild-type clones which contain a single neuroblast and a large postmitotic progeny expressing Elav (A,C), the small labeled Pc mutant clones lack Grh-positive nuclei (B,D). Optical cross-sections (C,D) further show that neuronal precursors lie in the outer-most layer (top), and a wild-type neuroblast lineage forms a column spanning the cellular cortex. *Pc* mutant clones comprise a few neurons loosely associated and located away from the neuroblast layer. (**E**,**F**) Mira is evenly detected at the cellular cortex of the large neuroblasts at interphase (PH3-negative, arrowhead). During mitosis, Mira transiently accumulates on one side of the neuroblast forming crescents (PH3-positive, asterisk). The small, labeled mutant *Pc* clones in F contain only postmitotic cells lacking Mira. (**G**) The percentage of wt and PcG mutant clones containing Grh- and/or Mira-positive neuronal precursor cells is plotted with the number of clones examined indicated in parentheses. For genotypes see Materials and methods. Scale bars: A,B, 25 μm; C-F, 10 μm.

interval. However, in mutant clones induced 48 hours before the late third larval instar, neuroblasts were present in approximately half of the cases (59%), suggesting that the neuroblast elimination process might still be ongoing at this intermediate time interval. To investigate this possibility, these clones were immunostained with an antibody against the activated form of the human Caspase-3 protein, shown previously to recognize apoptotic cells of *Drosophila* (Brennecke et al., 2003). Forty-eight percent of the neuroblasts present in *Pc* mutant clones expressed activated Caspase-3 at this stage and were, therefore, undergoing programmed cell death (Fig. 3B). This result supports the notion that neuroblasts in PcG mutant clones are eliminated by apoptosis. Moreover, given the marked proliferative activity of most neuroblasts during the third larval instar, this finding also suggests that loss of PcG gene function can induce neuroblast death even if these precursors are in an actively proliferating state.

Ectopic expression of posterior Hox genes in PcG mutant lineages

The fact that PcG genes are classically involved in maintaining spatial patterns of Hox gene expression prompted us to investigate the expression profile of Hox genes in PcG mutant clones. We focussed on the Hox genes of the Bithorax complex, *Ultrabithorax* (*Ubx*), *abdominal A* (*abd-A*) and *Abdominal B* (*Abd-B*). All three Hox genes are expressed in extensive regionalized and partially overlapping domains in posterior regions of the larval ventral ganglia, and expression anterior to these domains does not occur in the wild type. Accordingly, in the large wild-type MARCM clones, which can be generated in the brain and thoracic ganglia but not in the abdominal ganglia (see above), ectopic expression of the Hox genes anterior to their endogenous expression domains was never observed (Fig. 4A-C). Thus, none of the large, wild-type clones in the larval brain and ventral ganglia were Hox gene-positive, except for those (*Ubx*-expressing) clones that originated in the endogenous expression domain of *Ubx*.

By contrast, in the markedly reduced PcG mutant clones, ectopic expression of Hox genes was regularly observed anterior to their endogenous expression domains. Many of the cells in these PcG mutant clones, which lacked neuroblasts and were much smaller than wild-type clones, showed aberrant expression of the posterior Hox genes (Fig 4D-F). This ectopic Hox gene expression was more prevalent in the PcG mutant clones for *abd-A* and *Abd-B*, in that the number of clones with ectopic Hox gene expression, as well as the number of Hox gene-expressing cells in a given clone, were larger for *abd-A* and *Abd-B* than for *Ubx*. This prevalence of ectopic *abd-A* and *Abd-B* expression was not only seen in the ventral ganglia, but was also characteristic of mutant clones in the central brain (Fig. 4G-I). It should be noted that ectopic Hox gene expression was only seen in *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* mutant clones, and not in *Psc-Su(z)2* mutant clones. These observations imply that the ectopic expression of posterior Hox genes is a cell-autonomous consequence of the loss of specific PcG genes in postembryonic neuroblast lineages.

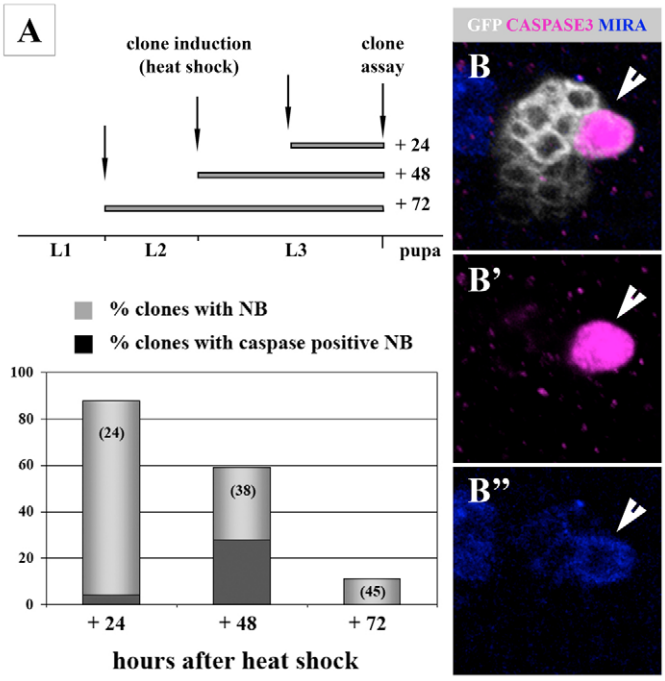

Fig. 3. *Pc* mutant neuroblasts undergo programmed cell death. (**A**) Time course analysis of programmed cell death. Clones were induced at 24 hours, 48 hours and 72 hours before examination in late third-instar larvae (top). The percentage of mutant *Pc* clones containing a neuroblast is plotted for each of the three temporal induction programs (bottom, light gray bars). The number of clones examined is indicated in parentheses. The percentage of clones containing anti-caspase immunoreactive neuroblasts is also plotted (dark gray bar). Caspase activation and disappearance of the progenitor marker was observed predominantly 48 hours after clonal induction. (**B-B"**) Confocal image of a CD8::GFP-labeled *Pc* mutant clone stained for the progenitor marker Mira (blue) showing a dying neuroblast (arrowhead). Programmed cell death is specifically detected in the neuroblast by anti-activated Caspase-3 antibody (magenta).

Targeted misexpression of posterior Hox genes mimics the PcG mutant phenotype

Previous work has shown that experimentally induced misexpression of posterior Hox genes such as *abd-A* in postembryonic neuroblasts causes the death of these precuror cells (Bello et al., 2003). As a consequence of induced neuroblast death, the clonal lineages observed in these experiments were small and consisted of a limited number of postmitotic neurons. The size and cell composition of the lineages obtained in clones that are mutant for the PcG gene *Sce*, are very similar to the size and cell composition of the lineages that result following targeted misexpression of the Hox gene *abd-A* (Fig. 5A-C). In both cases, the clone size is severely reduced as compared with wild type and comprises only small cells; neuroblasts are lacking and clones ectopically express *abd-A*.

The clonal phenotypes observed following misexpression of *abd-A* in postembryonic neuroblasts can be rescued by blocking cell death. Fig. 5D,E shows that blocking cell death in *abd-A* misexpression experiments through co-misexpression of the caspase inhibitor P35 fully restored both clone size and neuroblast survival such that wild-type-like clones were obtained. Thus, rescued lineages consisted of a large neuroblast and a normal number of neuronal progeny that extended in a continuous, columnar fashion from the neuroblast into inner cortical layers. This restoration of wild-type-like lineages occurred in spite of the elevated level of *abd-A* in the neuroblast of the labeled clones. Thus, targeted misexpression of *abd-A* leads to a phenotype that mimics the PcG mutant phenotype, and that can be fully rescued by blocking apoptosis in the neuroblast clones.

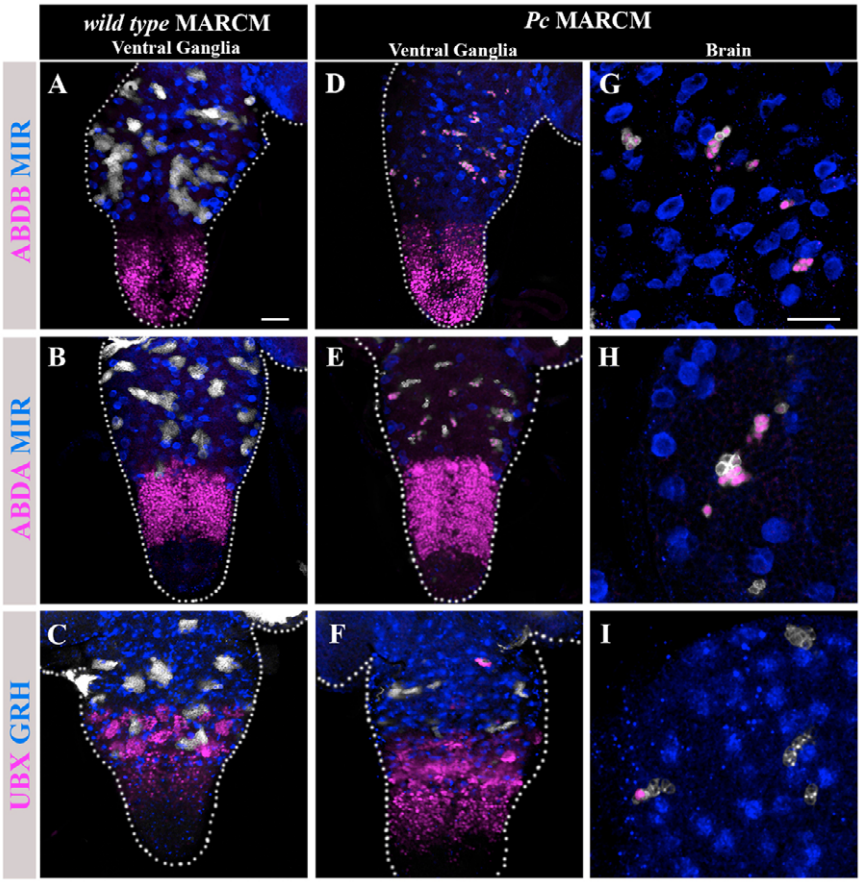

Fig. 4. Abberant expression of homeotic genes in *Pc* mutant clones. Confocal images of wild-type control and *Pc* mutant MARCM clones labeled with membrane-tethered GFP (CD8::GFP, white) and immunostained as indicated. Shown are late third-instar ventral ganglia (A-F, outlined with dots) or close-up views on the central brain (G-I), anterior at the top. (**A-C**) Large wild-type clones are visible in the thorax which contains most of the proliferative neural precursors (labeled for Mira or Grh, blue). Hox genes of the Bithorax complex are expressed in restricted domains along the anteroposterior axis. (**D-F**). Small *Pc* clones show ectopic anterior expression of the homeotic genes (white and magenta). Ectopic expression of UBX (F) is not as widespread as Abd-B (D) or Abd-A (E). (**G-I**) Variability in Hox gene derepression is also evident on the close-up views of isolated small clones in the brain. Scale bars: A-F, 25 μm; G-I, 20 μm.

Restoration of wild-type-like neurogenesis by apoptosis-block in PcG mutant clones

If derepression of Hox genes in PcG mutant lineages restricts lineage size by promoting neuroblast apoptosis in a manner comparable to that caused by the targeted misexpression of Hox genes, then blocking apoptosis should be able to rescue the PcG mutant phenotype. To test this, we expressed P35 in mutant clones for the PcG genes *Pc*, *Sce* and *Scm*. In all three cases we recovered wild-type-like clones characterized by the presence of a large neuroblast and the normal number of neuronal progeny.

An identification of the rescued neuroblasts was based on cellular and molecular features. Thus, in the apoptosis-blocked PcG mutant clones, GFP-labeled lineages contained a single, large cell located at the surface of the CNS. Cells of this type expressed Mira at their cortex and Grh in their nucleus (Fig. 6A-D). Associated with these neuroblasts were assemblies of numerous neuronal cells expressing Elav and the homeodomain-containing protein Prospero (Pros); these neuronal progeny were fully wild-type-like in terms of size and neuron-specific marker expression. Furthermore, we were able to assess the presence of GMCs immediately adjacent to the neuroblast by the lack of Elav, the presence of weak cortical expression of Mira (Fig. 6B), and the coexpression of Grh and Pros in their nuclei (Fig. 6D). As expected, the rescued PcG mutant lineages continued to misexpress posterior Hox genes such as *abd-A* in a number of their cells, including the neuroblast (Fig. 6E-F′).

A quantification of the obtained rescue efficiency is given in Fig. 6G. For all three PcG genes investigated, over 80% of the labeled clones contained neuronal precursor cells based on expression of Mira or Grh. Moreover, approximately half of these clones showed signs of mitotic activity as assayed with PH3 expression; this value corresponds to that seen in wild-type lineages. Taken together, these results demonstrate that apoptosis-block in PcG mutant lineages is sufficient to fully restore wild-type-like properties to CNS neuroblast clones. We therefore posit that PcG mutant lineages are capable of normal neurogenesis if the consequence of homeotic deregulation in neuronal precursors is bypassed through prevention of cell death.

DISCUSSION

Our genetic analysis indicates that the PcG genes *Sce*, *Scm*, *Pc*, *E(z)* and *Su(z)12* are required for postembryonic neurogenesis in the central brain and thoracic ganglia of *Drosophila*. In the absence of any one of these genes, several mutant phenotypes are observed in the third-instar CNS. First, neural proliferation is dramatically reduced and only small numbers of cells are found in neuroblast clones. Second, proliferating postembryonic neuroblasts are absent in most of the mutant clones due to apoptosis. Third, posterior Hox genes are ectopically expressed in the postembryonic neuroblast lineages. We hypothesize that these phenotypes are causally related, in that loss of PcG genes leads to ectopic Hox gene expression in postembryonic neuroblasts resulting in their premature cell death and, thereby, in drastically reduced neuroblast lineage size. Strong support for this hypothesis is provided by the fact that the mutant lineages proliferate normally if apoptosis is blocked. Corollary support for this notion is provided by the fact that *Psc-Su(z)2* mutant clones, which do not show ectopic Hox gene expression, are consistently wild-type-like in size and presence of neuroblast. In the following, we discuss the implications of these findings for our understanding of PcG gene action in *Drosophila* and in mammalian neurogenesis.

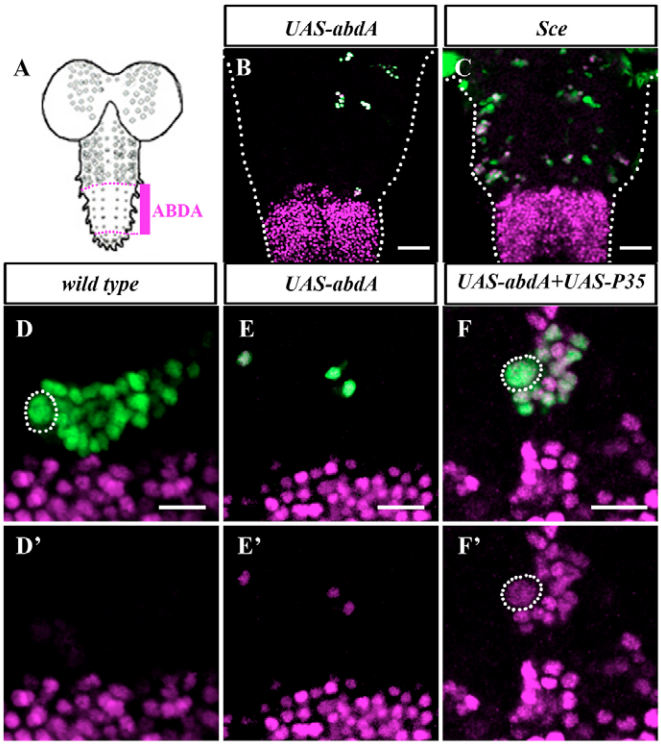

Fig. 5. Targeted misexpression of Abd-A in neuroblast clones mimics the PcG mutant phenotype. (**A**) Schematic of the larval CNS showing the expression domain of Abd-A (see also Fig. 4B,E). (**B-F'**) Confocal images of late third-instar ventral ganglia immunostained for Abd-A (magenta) and the clonal markers CD8::GFP (B, green) or nuclear β-galactosidase (C-F, green). Shown are ventral views at the junction between the endogenous domain of *abdA* expression and the more anterior thoracic region carrying clones. Anterior is at the top. Coexpression of Abd-A and the clonal marker is visible in white (e.g. see Fig. 4B). Close-up views in D-F are shown as split channels for clarity. Targeted misexpression of Abd-A under tubulinGAL4 control in wild-type MARCM clones generates small neuroblast lineages (UAS-abd-A). (B) A large-field view of the ventral ganglia (outlined with dashed lines) shows small clusters of nuclei expressing Abd-A, anterior to the endogenous expression domain in the abdomen. (C) In a similar field, most of the small *Sce* mutant clones also show ectopic expression of Abd-A. (D-F) Close-up views of the thoracic area immediately anterior to the endogenous Abd-A domain and carrying a clone of the genotype indicated. (D'-F') Abd-A expression in the same field without the clonal marker. A wild-type clone in the thorax contains a large progeny around the neuroblast and does not express Abd-A (D,D', neuroblast outlined with dots). Targeted misexpression of Abd-A results in small clones lacking a neuroblast (E,E'). Coexpression of P35 with Abd-A restores to a wild-type-like clone (F,F'), in spite of the elevated level of Abd-A in the neuroblast (dotted circle). Scale bars: B,C, 25 μm; D-F, 10 μm.

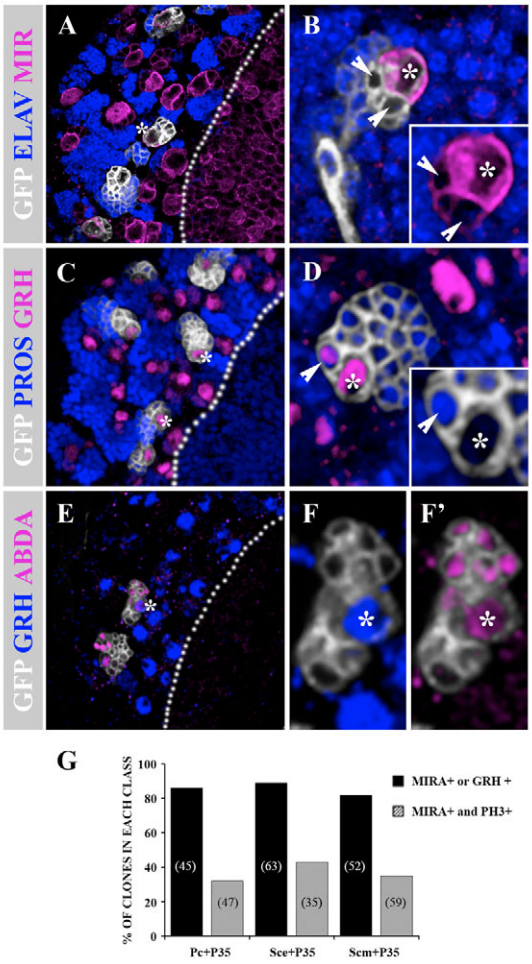

Fig. 6. Blocking apoptosis in PcG mutant clones restores wild-type-like neurogenesis. Confocal images of *Pc*+P35 clones labeled with CD8::GFP in the late third-instar central brain. A,C,E show the region around the junction of the central brain and optic lobe (dotted line); B,D,F,F' show high magnification of isolated clones. (**A-D**) Targeted expression of P35 in *Pc* mutant clones results in clones that contain a single large neuroblast cell (asterisk) expressing Mira (A,B) and Grh (C,D), whereas the adjacent GMCs (arrowheads) express low levels of Mira at the cortex (B and inset) or coexpress Grh and Pros in the nucleus (D shows an isolated clone adjacent to wild-type cells and the inset is a close-up view on the neuroblast and GMC). Non-neural stem cells within the clones express Elav (A,B) and Pros (C,D). (**E-F'**) Neural progenitors survive in spite of the expression of Abd-A and are engaged in mitosis as indicated by PH3 immunoreactivity (G) at a frequency similar to wild-type controls (see Fig. 1J). (**G**) Quantification of the presence of neural progenitors and mitosis indicates a large degree of rescue of the three mutants by P35 (F).

PcG genes are required for postembryonic neuronal proliferation in *Drosophila*

Numerous genes are required for the continued mitotic activity of neuroblasts during postembryonic life (Maurange and Gould, 2005). Our findings provide the first demonstration that PcG genes are essential for neuroblast survival and proliferation in the postembryonic CNS. Previous work on PcG gene action during embryonic neurogenesis has been carried out by Prokop and Technau (Prokop and Technau, 1994). This demonstrated that the derepression of posterior Hox genes in PcG mutants leads to a change in the segmental determination of neuroblasts and their lineage, but not to their mitotic arrest and death. Thus, the effects of PcG gene loss on neurogenesis are context-dependent and differ during embryonic development as compared with postembryonic development. This is underscored in recent work which indicates that the PcG gene *ph* is essential for maintaining neuronal identity and diversity during metamorphosis (Wang et al., 2006).

In postembryonic development of the *Drosophila* CNS, a remarkable link exists between neuroblast survival and Hox gene expression (Bello et al., 2003). In the ventral ganglia, a neuroblast-specific pulse of *abd-A* during the third instar provides the cue for cell death, which limits the number of progeny produced per neuroblast. Our data indicate that this mechanism, which in the wild type relates Hox gene expression to the clone size of neural stem cells, also operates in PcG mutants and is responsible for the PcG mutant phenotypes. Indeed, a general function of PcG genes in postembryonic neurogenesis may be to prevent the premature and widespread operation of this mechanism for temporal regulation of neurogenesis through termination of neuroblast life. It is noteworthy that the Hox gene-dependent activation of apoptosis within the CNS is selective for the neuroblast and does not occur when Hox genes are derepressed in neurons, either during normal development or in misexpression experiments (Bello et al., 2003). This explains why the neurons in PcG mutant clones, which were generated before the induction of neuroblast cell death, continue to survive despite the presence of ectopic Hox gene derepression.

Our data indicate that loss of specific PcG genes in larval neuroblasts leads to ectopic Hox gene expression that is sufficient to cause neuroblast cell death. However, the PcG proteins may also contribute to neuroblast survival by repressing other unidentified target genes which, when derepressed, might result in premature death of postembryonic neuroblasts. Indeed, although deregulation of Hox gene expression is one of the hallmarks of PcG phenotypes in *Drosophila*, a diverse set of other target genes, including genes involved in cell cycle regulation, are controlled by PcG genes (see Gould, 1997; Orlando, 2003; Ringrose and Paro, 2004).

PcG genes act in postembryonic neuronal proliferation in flies and mammals

Interesting parallels to our findings on the role of PcG genes in neural proliferation come from studies of mammalian PcG genes, specifically of the *Bmi1* gene (reviewed by Shakhova et al., 2005; Valk-Lingbeek et al., 2004). *Bmi1* mutant mice develop ataxia, seizures and tremors in early postnatal life, and display a significant reduction in overall brain size, which is particularly severe in the granular and molecular layers of the cerebellum. Strikingly, *Bmi1*-deficent mice become depleted of cerebellar neural stem cells postnataly, indicating an in vivo requirement for *Bmi1* in neural stem cell renewal. *Bmi1* deficiency leads to increased expression of the cell cycle regulators p16Ink-4a and p19Arf (both now known as Cdkn2a – Mouse Genome Informatics), and the neurogenesis defect in the mutant mice can be partially rescued by further deleting p16Ink4a (reviewed by Molofsky et al., 2004). This suggests that one way in which *Bmi1* promotes the maintenance of adult stem cells is by repressing the p16Ink4a pathway. However, it is also likely that Hox gene repression through *Bmi1* is involved in this process, given that loss of *Bmi1* has been shown to cause a deregulation of posterior Hox gene expression in neural stem cells in vitro. Moreover, a direct molecular link between *Bmi1* and Hox gene regulation has recently been discovered in mammalian development, in that the promyelocytic leukemia zinc finger (Plzf; Zbtb16 – Mouse Genome Informatics) protein directly binds *Bmi1* and recruits PcG proteins in the HoxD cluster (Barna et al., 2002).

In *Drosophila*, the homologs of the mammalian *Bmi1* gene are the PcG genes *Psc* and *Su(z)2*. *Psc* and *Su(z)2* encode very similar proteins and are partially redundant in function, but both genes are eliminated in a deletion in the *Psc-Su(z)2* line; see Soto et al. (Soto et al., 1995). Rather surprisingly, mutational loss of *Psc-Su(z)2* does not lead to ectopic Hox gene derepression and, in consequence, does not appear to affect neuronal proliferation in the postembryonic CNS of *Drosophila*. This is in stark contrast to the other five PcG genes investigated, which do play important roles in proliferation control by preventing ectopic Hox gene expression and cell death in postembryonic neuroblasts. The discrepancy between murine *Bmi1* and *Drosophila Psc-Su(z)2* function in neuronal proliferation suggests that although a general role of PcG genes in neuronal proliferation control may be conserved between mammals and flies, conservation of gene action may not always be retained at the level of individual PcG homologs.

In terms of overall development, it is clear that one and the same PcG gene can have very different functions depending on the developmental context in which it acts. For example, as mentioned above, during embryonic neurogenesis the *Drosophila Pc* gene acts in tagmata-specific differentiation of neuroblasts, in contrast to its role in postembryonic neurogenesis (Prokop and Technau, 1994) (this report). Moreover, in postembryonic development of imaginal discs, deletions in the *Drosophila Psc-Su(z)2* genes have been shown to result in cellular hyperproliferation, which contrasts with the lack-of-proliferation phenotype of *Psc-Su(z)2* mutants in postembryonic development of the CNS (Beuchle et al., 2001) (this report). Similarly, in the mouse, the *Bmi1* gene has been implicated in tumor progression in mantle cell lymphoma, colorectal cancer, liver carcinomas and non-small cell lung cancer, in addition to its role in nervous system development. Nevertheless, in all of the *Drosophila* and mammalian phenotypes mentioned, deregulation of Hox gene expression appears to be one of the conserved and thus unifying features of PcG gene functional loss.

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