Drosophila Polycomb complexes restrict neuroblast competence to generate motoneurons

Johnny J. Touma, Frank F. Weckerle and Michael D. Cleary*

SUMMARY

Similar to mammalian neural progenitors, *Drosophila* neuroblasts progressively lose competence to make early-born neurons. In neuroblast 7-1 (NB7-1), Kruppel (Kr) specifies the third-born U3 motoneuron and Kr misexpression induces ectopic U3 cells. However, competence to generate U3 cells is limited to early divisions, when the Eve⁺ U motoneurons are produced, and competence is lost when NB7-1 transitions to making interneurons. We have found that Polycomb repressor complexes (PRCs) are necessary and sufficient to restrict competence in NB7-1. PRC loss of function extends the ability of Kr to induce U3 fates and PRC gain of function causes precocious loss of competence to make motoneurons. PRCs also restrict competence to make HB9⁺ Islet⁺ motoneurons in another neuroblast that undergoes a motoneuron-to-interneuron transition, NB3-1. In contrast to the regulation of motoneuron competence, PRC activity does not affect the production of Eve⁺ interneurons by NB3-3, HB9⁺ Islet⁺ interneurons by NB7-3, or Dbx⁺ interneurons by multiple neuroblasts. These findings support a model in which PRCs establish motoneuron-specific competence windows in neuroblasts that transition from motoneuron to interneuron production.

KEY WORDS: *Drosophila*, Neuroblast, Temporal, Competence, Polycomb, Epigenetic

INTRODUCTION

Cell fate specification requires the coordination of inductive signals and progenitor cell competence to respond to such signals. Temporal regulation of progenitor competence is particularly important during nervous system development (Pearson and Doe, 2004; Hirabayashi and Gotoh, 2010). Mammalian cortical progenitors lose the ability to respond to intrinsic and extrinsic cues over time, so that progenitors at late stages of development are no longer competent to make early-born neurons (Desai and McConnell, 2000). Cortical progenitors also undergo a temporally regulated loss of competence to produce neurons (Miller and Gauthier, 2007). During the early neurogenic phase, Wnt signaling induces expression of the neural fate transcription factor neurogenin 1 (Neurog1). After multiple progenitor divisions, Neurog1 expression ceases and the progenitor switches to making astrocytes. Wnt levels do not change during this transition; instead, the loss of competence is due to Polycomb repressor complexes (PRCs) blocking transcription of *Neurog1* (Hirabayashi et al., 2009). PRCs also regulate the balance between self-renewal and differentiation in the cerebral cortex, with loss of PRC function inducing precocious progenitor differentiation (Pereira et al., 2010). These findings add to a growing list of PRC functions, including the maintenance of pluripotency in mammalian stem cells (Pietersen and van Lohuizen, 2008) and cell cycle regulation in cancer cells (Bracken and Helin, 2009).

PRCs are multi-protein complexes that silence transcription via epigenetic mechanisms (Schwartz and Pirrotta, 2008). There are two main PRC complexes, PRC2 and PRC1, and several modes of silencing by PRCs are known or proposed (Simon and Kingston, 2009). Generally, the complexes work together: PRC2 catalyzes trimethylation of lysine 27 on histone H3, then PRC1 is recruited to H3K27me3 sites and induces changes in chromatin that inhibit transcription. Distinct chromatin-remodeling complexes of the Trithorax group counteract PRCs and favor transcription. Transcription of PRC-regulated genes depends on the balance between the activity of transcriptional activators, Trithorax complexes and PRCs (Zink and Paro, 1995; Simon and Tamkun, 2002). PRC-induced chromatin modifications are maintained following DNA replication and can accumulate over multiple cell divisions (Blomen and Boonstra, 2011). For example, levels of H3K27me3 at the *Neurog1* locus increase with each neural progenitor division, providing a potential mechanism for timing the loss of competence to make neurons (Hirabayashi et al., 2009). In *Drosophila*, PRCs were first identified as regulators of Hox gene expression but have since been shown to control multiple processes, including cell cycle regulation (O'Dor et al., 2006; Martinez et al., 2006), differentiation of germline progenitors (Chen et al., 2005; Narbonne et al., 2004), dendrite remodeling (Parrish et al., 2007) and the diversity of neuronal projection patterns (Wang et al., 2006). Genome-wide mapping of PRC targets in *Drosophila* suggests that PRCs regulate a wide range of developmental programs (Schwartz et al., 2006; Oktaba et al., 2008; Schuettengruber et al., 2009), including potentially regulating cell fate specification during neurogenesis.

During *Drosophila* embryonic neurogenesis, neuroblasts sequentially express a series of transcription factors [Hunchback (Hb), Kruppel (Kr), Pdm (Nubbin – FlyBase) and Castor (Cas)] that specify the temporal identity of progeny (Isshiki et al., 2001). There are 30 neuroblasts in each hemisegment of the ventral nerve cord and clonal analysis has identified the progeny produced by each neuroblast (Bossing et al., 1996; Schmid et al., 1999). Neuroblasts divide asymmetrically, self-renewing and producing a ganglion mother cell (GMC) that typically divides once to produce a pair of differentiated progeny. In the neuroblast 7-1 lineage (NB7-1), the first five neuroblast divisions produce 'U' motoneurons that express the Even-skipped (Eve) transcription factor: U1 is specified by high levels of Hb, U2 is specified by low levels of Hb, U3 is

School of Natural Sciences, University of California Merced, Merced, CA 95343, USA.

*Author for correspondence (mcleary4@ucmerced.edu)

specified by Kr, U4 is specified by Pdm and U5 is specified by Cas (Isshiki et al., 2001; Pearson and Doe, 2003; Grosskortenhaus et al., 2006). GMCs that produce U motoneurons also produce Eve-negative siblings, some of which transiently or permanently express the transcription factor Dbx and develop into interneurons (Lacin et al., 2009). After producing U motoneurons, NB7-1 produces only interneurons and divides five or six more times in abdominal segments of the ventral nerve cord (producing up to 22 neurons) (Bossing et al., 1996). Transcription factors that specify late-born interneuron fates and molecular markers that are specific for NB7-1 interneurons are not known, although as many as four Dbx$^+$ interneurons are produced after the motoneuron portion of the NB7-1 lineage ends (Lacin et al., 2009).

The NB7-1 lineage has proven useful for studying cell fate specification and neuroblast competence. Previous work has shown that misexpression of Hb or Kr alters the fate of later-born cells, but only within a limited competence 'window' (Pearson and Doe, 2003; Cleary and Doe, 2006). Heat shock-induced pulses of Hb or Kr can only alter cell fates within the first five divisions of NB7-1. Competence inhibition increases with sequential neuroblast divisions and is reminiscent of the progressive restriction of competence that occurs in mammalian cortical progenitors (Desai and McConnell, 2000). Constitutive low-level misexpression of Kr induces ectopic U3 fates, but no more than two ectopic U3 cells are ever produced, again corresponding to a five-division window. High-level constitutive misexpression of Kr partially extends competence, suggesting that high levels of Kr can overcome transcriptional repression or other modifications that occur at as yet unknown U3-specifying genes when competence is restricted (Cleary and Doe, 2006).

A similar early competence window has been described in the NB 3-1 lineage, which produces HB9$^+$ Islet$^+$ motoneurons during the first four divisions: high levels of Hb specify the RP1 motoneuron, low levels of Hb specify the RP4 motoneuron, Kr specifies the RP3 motoneuron, and an unknown factor or factors specify the RP5 motoneuron (Tran and Doe, 2008). At the fifth division, NB3-1 switches to producing interneurons. Similar to NB7-1, misexpression of Kr in NB3-1 primarily only converts cell fates during the motoneuron portion of the lineage (Tran and Doe, 2008). By the fifth neuroblast division, when NB3-1 transitions to making interneurons, competence to respond to Kr and produce RP3 motoneurons is lost.

Studies in NB7-1 and NB3-1 have shown that a similar competence window exists in both lineages, yet the mechanism of competence restriction has not previously been identified. In this study, we show that PRCs regulate neuroblast competence. PRCs control competence to produce motoneurons in NB7-1 and NB3-1, whereas interneuron competence in multiple lineages is unaffected by PRC activity. Our findings support a model in which PRCs establish early motoneuron competence windows in neuroblasts that transition from motoneuron to interneuron production.

MATERIALS AND METHODS
Fly stocks and genetics
Trithorax group and Polycomb group mutants used in the initial screen are listed in Table S1. Fly stocks used in other experiments were: (1) *wor-GAL4; Su(z)12³/TM3, Ubx-lacZ*; (2) *UAS-Kr; Su(z)12³/TM3, Ubx-lacZ*; (3) *ph-d⁴⁰¹, ph-p⁶⁰²/FM7c, Act-GFP; hsp70-Kr/CyO*; (4) *wor-GAL4* (C. Doe, University of Oregon); (5) *eve-gal4[+3.5-4.3]*; (6) *UAS-Kr; UAS-HA-UPRT*; (7) *UAS-Kr; UAS-Su(z)12*{RNAi, Vienna *Drosophila* Resource Center (VDRC) stock 42423}; (8) *UAS-Kr; UAS-E(z)*{RNAi, VDRC stock 39761}; (9) *en-GAL4*; (10) *sca-gal4; sca-gal4* (C. Doe, University of

Oregon); (11) *UAS-Kr; UAS-ph*; (12) *UAS-ph* (G. Cavalli, C.N.R.S., France). Unless otherwise noted, stocks were obtained from the Bloomington *Drosophila* Stock Center. *Su(z)12³* heterozygous versus homozygous mutants were identified by staining for β-galactosidase. *ph-d⁴⁰¹, ph-p⁶⁰²* hemizygous mutants were identified by the absence of GFP. For all but the heat-shock experiments, embryos were collected at 29°C. Embryos were staged using standard methods (Campos-Ortega and Hartenstein, 1985). Heat-shock experiments were performed as previously described (Cleary and Doe, 2006), with the exception that embryos were collected for 2 hours and pulses of Kr therefore covered a 2-hour developmental window.

Immunofluorescence and data analysis
Antibody staining was performed according to standard methods (Grosskortenhaus et al., 2005). Primary antibodies, dilutions and sources are: guinea pig Dbx 1:1500 (J. Skeath, Washington University); rabbit VGlut 1:400 (H. Aberle, Max Planck Institute for Developmental Biology, Tübingen, Germany); rabbit HB9 1:1000, rabbit Hb 1:200 (C. Doe, University of Oregon); mouse Islet 1:200, mouse Eve 2B8 1:20 (Developmental Studies Hybridoma Bank, University of Iowa); guinea pig Kr 1:500, guinea pig Hb 1:500, guinea pig Eve 1:500, guinea pig Runt 1:500 (Asian Distribution Center for Segmentation Antibodies); rabbit Cas 1:1000 (W. Odenwald, National Institutes of Health); rabbit Runt 1:500 of 1:10 pre-absorbed (A. Brand, University of Cambridge, UK); rat Zfh2 1:200 (M. Lundell, University of Texas at San Antonio); and rabbit β-gal 1:1000 (Abcam). Mouse Islet was pre-absorbed and used in combination with the Alexa 488 Tyramide Signal Amplification System (Molecular Probes). Species-specific secondary antibodies were conjugated to Alexa 488, Alexa 633 (Molecular Probes) or TRITC (Jackson ImmunoResearch). Images were collected as confocal image stacks on a Nikon C1 confocal microscope, processed in ImageJ (NIH) and shown as two-dimensional projections. Cells that would be obscured from view in projections are shown in the figures as white-boxed insets. Embryos were analyzed at stage 16 or early stage 17 unless otherwise noted, and all analyses were restricted to abdominal hemisegments. Statistical analyses were performed using SigmaPlot (Systat Software, San Jose, CA, USA).

RESULTS
PRC activity is necessary for the restriction of NB7-1 competence
Previous work has shown that constitutive low-level Kr expression in neuroblasts results in limited competence to generate U3 motoneurons and skipping of U4 and U5 fates (Cleary and Doe, 2006). To generate a strain of flies with conditional low-level ectopic Kr expression in neuroblasts, we combined neuroblast-specific *worniu-GAL4* (*wor-GAL4*) (Lee et al., 2006) with *tubulin-GAL80[ts]* (temperature-sensitive GAL80) and *UAS-Kr*, in a genotype we call *wor[ts]>Kr*. At the permissive temperature in *wor[ts]>Kr* embryos, Kr and Pdm are constitutively expressed, as Kr activates Pdm expression (Isshiki et al., 2001; Cleary and Doe, 2006), and Cas is never expressed because Kr inhibits Cas expression (Isshiki et al., 2001; Cleary and Doe, 2006) (data not shown) (summarized in Fig. 1A,B). Embryos that are heterozygous for *wor[ts]>Kr* have limited competence to make ectopic U3 cells: 86% of NB7-1 lineages make only U1, U2 and U3, with no U4 or U5, 5% make U1, U2, U3 and U4, and 9% make U1, U2, U3 and a single ectopic U3 (*n*=66) (Fig. 1B,D). In addition to using Kr as a marker (low expression in U1 and U2 and high expression in U3), cell fates were identified based on expression of Hb (U1, U2), Zfh2 (U2, U3, U4, U5), Runt (U4, U5) and Cas (U5) (Fig. 2A,B). Although the majority of NB7-1 lineages stop producing U motoneurons after three divisions in *wor[ts]>Kr* embryos, NB7-1 maintains normal mitotic activity throughout neurogenesis (supplementary material Fig. S1A,B,D) and there is no increase in apoptotic cells in the developing nervous system, as previously
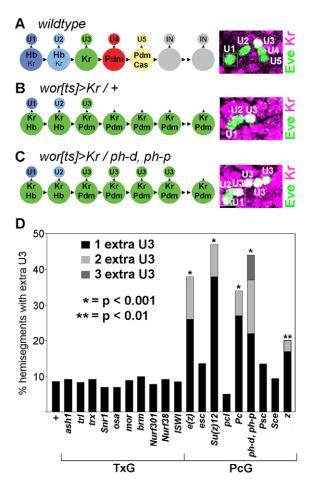
Fig. 1. Mutations in Polycomb group genes extend NB7-1 competence. (**A-C**) The diagrams on the left show cell fates and temporal identity factor expression in the NB7-1 lineage of (A) wild type, (B) *wor[ts]>Kr* crossed to wild type, and (C) *wor[ts]>Kr* crossed to *ph-d, ph-p* mutant. Ganglion mother cells (GMCs) and Eve⁻ siblings are not shown. Images on the right show Eve⁺ U motoneurons for each genotype, with cells identified based on Kr expression and position. In this and all subsequent figures, images are z-series projections, with cells that would otherwise be obscured pasted in a new position and outlined by a white box. Anterior up, midline to the left. (**D**) Results of screen testing for extension of Kr competence in Trithorax group (TxG) and Polycomb group (PcG) mutants. '+' indicates *wor[ts]>Kr* crossed to wild type. A minimum of three embryos and 35 hemisegments were analyzed for each genotype. Statistically significant differences between wild type and mutants were detected using a Kruskal-Wallis test.

described (Cleary and Doe, 2006). These results suggest that when competence to make U motoneurons is lost, NB7-1 continues its progression through interneuron production. These late-born interneurons cannot be identified owing to the lack of specific markers (Cleary and Doe, 2006).

We used *wor[ts]>Kr* to test the role of Trithorax group and Polycomb group genes in the regulation of NB7-1 competence. Loss-of-function mutations in a subset of Polycomb group genes induced statistically significant increases in competence to produce U3 fates, whereas mutations in Trithorax group genes had no effect (Fig. 1C,D). Polycomb group mutants that extend competence encode members of both PRC1 [*Polycomb* (*Pc*), *polyhomeotic-distal, polyhomeotic-proximal* (*ph-d, ph-p*), *zeste* (*z*)] and PRC2 [*Enhancer of zeste* (*E(z)*), *Suppressor of zeste 12*

(*Su(z)12*)]. To determine whether loss of PRC activity alone (without Kr misexpression) affects the NB7-1 lineage, we analyzed homozygous *Su(z)12* mutants and hemizygous *ph-d, ph-p* mutants. The timing and levels of Kr expression in neuroblasts were normal in these mutants, and U motoneuron fates were not altered (data not shown), demonstrating that Polycomb group mutations alone do not alter NB7-1 fates. Although the *ph-d⁵⁰⁴* and *ph-p⁵⁰⁴* mutant alleles are known to cause widespread Eve misexpression in the nervous system (Smouse et al., 1988; Oktaba et al., 2008), the *ph-d⁴⁰¹, ph-p⁶⁰²* alleles used in this study do not have this effect, allowing us to separate the role of Ph in transcriptional regulation at the *eve* locus from the role of Ph in neuroblast competence.

Previous studies have shown that elevated Kr expression in neuroblasts can partially extend competence (Cleary and Doe, 2006). To test the relative effects of Kr versus PRCs in determining competence, we compared embryos expressing high levels of Kr with *Su(z)12* heterozygous and homozygous mutants expressing low levels of Kr (Fig. 2C,D,E). High levels of Kr caused a minor extension of competence compared with *wor[ts]>Kr* embryos, but competence was still lost after the fifth neuroblast division. Su(z)12 levels had a more dramatic effect on competence, extending competence to the seventh division in heterozygous mutants and as far as the eleventh division in homozygous mutants. Analysis of multiple cell fate markers confirmed that the ectopic U3 cells have standard U3 motoneuron characteristics (Fig. 2), including expression of the vesicular glutamate transporter VGlut, which is specific for motoneurons (Mahr and Aberle, 2006). VGlut staining also revealed that the ectopic U3 motoneurons have normal projection patterns out of the central nervous system, fasciculating within the ISN branch of the intersegmental nerve. The ISN branch contains the axons of Eve⁺ motoneurons that innervate dorsal body wall muscles. A slight thickening of the ISN is often detectable in *wor>Kr; Su(z)12⁻/⁻* embryos compared with wild type (particularly at the sites of neuromuscular junction development), reflecting the increased number of U3 motoneurons (supplementary material Fig. S2).

NB7-1 maintains normal mitotic activity in *wor>Kr; Su(z)12⁻/⁻* embryos and ectopic U3 cells are sequentially generated at the expected time and approximate position within the NB7-1 lineage (supplementary material Fig. S1C,D). These results suggest that altered cell cycle timing does not cause the increase in U3 fates and that the ectopic U3 cells are not produced by other neuroblasts. In addition, at early stages when U neurons and their Dbx⁺ siblings are first produced, Dbx⁺ cells are observed adjacent to the ectopic U3 motoneurons in *wor>Kr; Su(z)12⁻/⁻* embryos (supplementary material Fig. S3), arguing against altered sibling fates as the source of ectopic U3 cells. NB7-1 is predicted to divide a maximum of 11 times in abdominal segments, producing 22 neurons (Bossing et al., 1996). Su(z)12 loss-of-function is therefore sufficient to extend competence to the end of the lineage, producing 10.8±1.2 Eve⁺ U motoneurons and the corresponding Eve⁻ siblings.

Polyhomeotic is required for the progressive restriction of NB7-1 competence

To more precisely determine the timing of PRC-dependent competence restriction, we induced pulses of Kr at specific times in the NB7-1 lineage. We have previously shown that, in wild-type embryos, competence is progressively restricted during the first five neuroblast divisions then completely lost at the sixth division (Cleary and Doe, 2006). In *ph* mutants, there is no progressive restriction during the motoneuron phase: competence to respond to
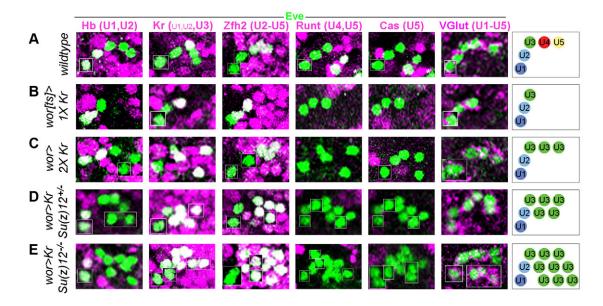
Fig. 2. Su(z)12 levels determine the degree of competence restriction. Representative hemisegments are shown for (**A**) wild type, (**B**) *wor[ts]>Kr* (low-level Kr expression), (**C**) *wor>2×Kr* (high-level Kr expression), (**D**) *wor>1×Kr; Su(z)12+/−* [low-level Kr expression in *Su(z)12* heterozygous mutant], (**E**) *wor>1×Kr; Su(z)12−/−* [low-level Kr expression in *Su(z)12* homozygous mutant]. Cell fate markers are listed in magenta. Boxes to the right show the average result for each genotype. *wor>2×Kr* embryos produced 3.2±0.69 U3 cells per hemisegment (*n*=75), *wor>Kr; Su(z)12+/−* embryos produced 5.1±1.3 U3 cells per hemisegment (*n*=100), and *wor>Kr; Su(z)12−/−* embryos produced 8.8±1.2 U3 cells per hemisegment (*n*=53). *P*<0.001 between all groups based on Kruskal-Wallis test.

Kr is high throughout the first five neuroblast divisions (Fig. 3). Competence also extends into the interneuron phase of the lineage, with ectopic U3 cells produced as late as the ninth division (Fig. 3, supplementary material Fig. S4). Ph is an essential component of PRC1 and is believed to be involved in the formation of higher-order chromatin structures that silence transcription (Simon and Kingston, 2009). These results demonstrate that decreased PRC1 activity significantly delays competence restriction and allows U3 motoneuron production during the interneuron phase of the NB7-1 lineage.
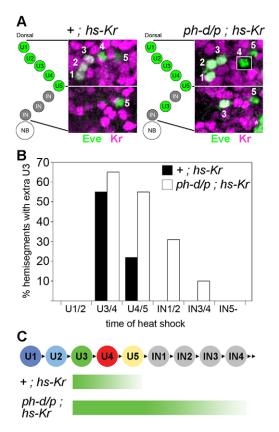
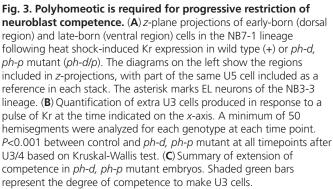## PRCs act within the NB7-1 lineage to restrict competence

Altered competence in PRC mutants could be due to neuroblast lineage non-autonomous effects, such as patterning defects in the neuroectoderm at earlier stages of development. To determine if the relevant PRC activity is neuroblast lineage autonomous, we used cell-specific RNA interference (Dietzl et al., 2007) to decrease expression of Su(z)12 and E(z) in neuroblasts misexpressing Kr (*wor-GAL4 × UAS-Kr; UAS-{RNAi construct}*). *wor-GAL4* drives *GAL4* transcription in neuroblasts and the GAL4 protein is likely to perdure in GMCs and possibly in immature neurons. *wor-GAL4*-driven knockdown of Su(z)12 or E(z) extends competence (Fig. 4B), demonstrating that PRCs act within neuroblasts, and potentially their early progeny, to establish competence states. We also used an *eve-GAL4* driver to knockdown Su(z)12 in U cells misexpressing Kr. Previous work has shown that Kr represses Cas expression in neuroblasts but that ectopic Kr in U5 motoneurons does not repress Cas (Cleary and Doe, 2006). Su(z)12 knockdown in U motoneurons did not alter this regulatory relationship, as Kr-expressing U5 cells continue to express Cas (Fig. 4C). These results suggest that the relevant PRC activity occurs in neuroblasts or GMCs and that cell fate cannot be reprogrammed in mature neurons.

Polyhomeotic gain-of-function precociously restricts NB7-1 competence

To maximally extend competence to generate U3 cells, we used *Engrailed-GAL4* (*en-GAL4*), which has previously been shown to induce high levels of *UAS-Kr* expression throughout the NB7-1 lineage (neuroblast, GMCs and neurons) (Cleary and Doe, 2006). For example, *en-GAL4 × UAS-Kr; UAS-control* embryos [the *UAS-control* transgene is used to normalize UAS copy number (Tran et al., 2010)] produce 2.8±0.79 U3 cells per hemisegment (*n*=54), compared with 1.2±0.41 U3 cells per hemisegment produced in *wor-GAL4 × UAS-Kr; UAS-control* embryos (*n*=96). *En-GAL4* therefore provides an extended competence background in which we can test for conditions that restrict competence. To determine whether elevated PRC activity can induce precocious loss of competence, we used *en-GAL4* to activate expression of a previously described *UAS-ph* transgene (Martinez et al., 2006) combined with *UAS-Kr* [*en-GAL4 × UAS-Kr; UAS-ph* (*en>Kr, ph*)] and compared results with *en-GAL4 × UAS-Kr; UAS-control* (*en>Kr, control*). In *en>Kr, control* embryos, NB7-1 frequently produced ectopic U3 cells: 20% of hemisegments produced three ectopic U3s, 44% produced two ectopic U3s, 31% produced one ectopic U3, and only 5% had no ectopic U3s (*n*=54). Ph gain-of-function (*en>Kr, ph*) caused a significant reduction in competence: 61% of hemisegments produced no ectopic U3s and the remaining 39% produced only one ectopic U3 (*n*=64) (Fig. 5A, supplementary material Fig. S5). We also assayed competence in *en-GAL4 × UAS-ph* embryos (without *UAS-Kr*) and found that U motoneuron fates were unaffected (data not shown). Therefore, PRC restriction of competence in NB7-1 appears to selectively inhibit ectopic U fates.

To further test whether PRC activity inhibits ectopic motoneuron fates, we used a previously described method to delay Pdm and Cas expression (Cleary and Doe, 2006). The *scabrous-GAL4* (*sca-GAL4*) driver is transiently expressed in NB7-1, allowing

Fig. 3. Polyhomeotic is required for progressive restriction of neuroblast competence. (**A**) z-plane projections of early-born (dorsal region) and late-born (ventral region) cells in the NB7-1 lineage following heat shock-induced Kr expression in wild type (+) or ph-d, ph-p mutant (ph-d/p). The diagrams on the left show the regions included in z-projections, with part of the same U5 cell included as a reference in each stack. The asterisk marks EL neurons of the NB3-3 lineage. (**B**) Quantification of extra U3 cells produced in response to a pulse of Kr at the time indicated on the x-axis. A minimum of 50 hemisegments were analyzed for each genotype at each time point. P<0.001 between control and ph-d, ph-p mutant at all timepoints after U3/4 based on Kruskal-Wallis test. (**C**) Summary of extension of competence in ph-d, ph-p mutant embryos. Shaded green bars represent the degree of competence to make U3 cells.

Fig. 4. PRCs act within the NB7-1 lineage to restrict competence. (**A**) Expression patterns of GAL4 lines used in RNAi analysis; GAL4 is transcribed in cells within the corresponding box. (**B**) wor-GAL4-driven RNAi of the Polycomb group (PcG) genes Su(z)12 and E(z). Asterisk marks EL neurons of the NB3-3 lineage. The control genotype is wor-GAL4 × UAS-Kr; UAS-HA-UPRT (with UAS-HA-UPRT controlling for the number of UAS elements, as described in the main text). Control embryos produced 1.2±0.41 U3 cells (n=96), Su(z)12 RNAi embryos produced 3.4±0.89 U3 cells (n=56), and E(z) RNAi embryos produced 3.2±0.91 U3 cells (n=40). P<0.001 between PcG RNAi embryos and control embryos based on Mann-Whitney tests. (**C**) U motoneuron-specific knockdown of Su(z)12. Images show Kr expression in U5 motoneuron and maintenance of Cas expression in the same U5 motoneuron (arrows). Cas expression was never repressed in Kr-positive U5 motoneurons of control embryos (n=8) or Su(z)12 RNAi embryos (n=3).

expression of *UAS-Kr* during early divisions followed by resumption of Pdm and Cas expression when *sca-GAL4* shuts off. *sca-GAL4* was used to drive transient expression of either *UAS-Kr; UAS-control* (*sca>Kr, control*) or *UAS-Kr; UAS-ph* (*sca>Kr, ph*). Similar to the *en-GAL4* results, competence to produce ectopic U3 cells is significantly restricted in *sca>Kr, ph* embryos (Fig. 5B,C). There is also a significant decrease in competence to produce U4 and U5 fates when expression of Pdm and Cas is delayed. *sca>Kr, control* embryos retain competence to make U4 and U5 cells at late divisions, whereas *sca>Kr, ph* embryos have very limited competence to make U4 cells at the fifth division and no competence to make U5 cells at the sixth division (Fig. 5B,C). These results support a model in which PRC activity blocks temporal identity factors from specifying U motoneuron fates at later than normal times. These results also show that the restriction of competence by PRCs is not specific for U3 fates, as Ph gain-of-function also restricts competence to make U4 and U5 motoneurons.
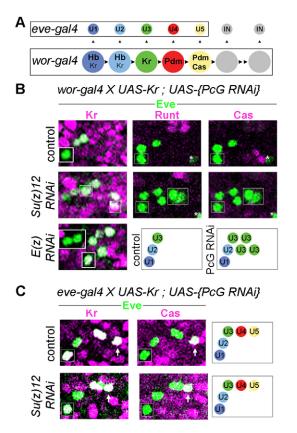
PRC activity does not affect production of Eve⁺ interneurons

To test the role of PRCs in regulating competence in other neuroblast lineages, we analyzed competence in the NB3-3 lineage. In the abdominal ventral nerve cord, NB3-3 produces a series of interneurons, including a cluster of 11 Eve⁺ interneurons (the 'Eve-lateral' or 'EL' interneurons). Kr is expressed at the first division of NB3-3 and specifies an Eve⁻ interneuron, followed by production of the 11 EL interneurons, six of which are at least partially specified by Cas (Tsuji et al., 2008) (Fig. 6A). We used two measures of Kr competence in NB3-3: competence to convert late-born cells to the Eve⁻ fate (identified as a decrease in the number of ELs) and competence to inhibit Cas expression (identified as a decrease in Cas⁺ ELs). Misexpression of high levels of Kr using *wor-GAL4* (*wor>2×Kr*) caused an almost complete loss of EL interneurons: 1.9±0.88 ELs per hemisegment (n=52) and Cas⁺ ELs were never produced (Fig. 6B). This suggests that competence to respond to Kr in NB3-3 is extended far beyond the five-division window observed
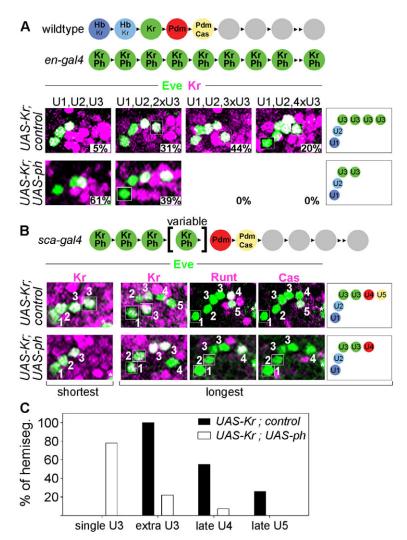
Fig. 5. Polyhomeotic gain-of-function enhances competence restriction in NB7-1. (**A**) Diagram at the top illustrates the timing of ectopic Kr and Ph expression driven by *en-GAL4*. Control embryos (*UAS-Kr; UAS-control*) produced 2.8±0.79 U3 cells (*n*=54), whereas *UAS-Kr; UAS-ph* embryos produced 1.4±0.49 U3 cells (*n*=64); *P*<0.001 based on Mann-Whitney test. Percentages shown in the corner of each image indicate the frequency of hemisegments with the indicated U fates. Summary figures on the right show the highest number of U3 cells observed for either genotype. (**B**) Diagram at the top illustrates the timing of ectopic Kr and Ph expression driven by *sca-GAL4*. Representative hemisegments show the shortest lineage (fewest U neurons) and longest lineage (most U neurons) produced by control and *UAS-ph* embryos. Asterisk marks EL neurons of the NB3-3 lineage. (**C**) Distribution of U fates among *UAS-Kr; UAS-control* embryos (*n*=59) and *UAS-Kr; UAS-ph* embryos (*n*=55). *P*<0.001 between control and *UAS-ph* embryos in each category based on Mann-Whitney tests.

in NB7-1. Low-level Kr misexpression (*wor>Kr*) in NB3-3 caused a less dramatic alteration of cell fates: 6.1±0.83 ELs are formed per hemisegment and 2.7±0.58 are Cas⁺ (*n*=50). Thus, as in NB7-1, Kr competence is dose dependent.

To test if decreased PRC activity affects Kr competence in NB3-3, we assayed EL fates in *Su(z)12* mutant embryos [*wor>Kr; Su(z)12⁻/⁻*]. EL fates were not significantly different from EL fates in *wor>Kr* embryos: 5.9±0.71 ELs are formed per hemisegment and 2.8±0.66 are Cas⁺ (*n*=54) (Fig. 6). We also tested whether Ph gain-of-function affects Kr competence in NB3-3. There was no significant difference between Ph gain-of-function embryos (*sca>Kr, ph*) and controls (*sca>Kr, control*): control embryos produced 7.9±0.65 ELs per hemisegment, with 2.5±0.50 Cas⁺ (*n*=36), whereas Ph gain-of-function embryos produced 8.0±0.68 ELs per hemisegment, with 2.8±0.56 Cas⁺ (*n*=52) (Fig. 6). PRCs therefore differentially regulate two classes of Eve⁺ neurons: competence to produce Eve⁺ motoneurons is restricted by PRC activity, whereas competence to produce Eve⁺ interneurons is unaffected by PRC activity.

PRC activity establishes a motoneuron competence window in NB3-1

To determine whether PRCs regulate the production of other types of motoneurons, we analyzed cell fates in the NB3-1 lineage (Fig. 7A). NB3-1 sequentially produces the RP1, RP4,

RP3 and RP5 motoneurons during its first four divisions and these motoneurons can be identified based on their position and expression of the transcription factors HB9 and Islet (Extra-extra and Tailup – FlyBase) (Tran and Doe, 2008) (Fig. 7A, supplementary material Fig. S6). At the fifth division, NB3-1 switches to producing a series of interneurons (Schmid et al., 1999). Kr specifies the RP3 fate and we found that *wor>2×Kr* embryos produced a limited number of ectopic RP3s (1.8±0.75 per hemisegment; *n*=72) (Fig. 7B, supplementary material Fig. S6), similar to previously described results for this lineage (Tran and Doe, 2008). In *wor>Kr; Su(z)12⁻/⁻* embryos, competence to produce RP3s was significantly extended, with 3.8±1.1 ectopic RP3s per hemisegment (*n*=57) (Fig. 7B, supplementary material Fig. S6). A similar extension of competence was observed when Su(z)12 expression was decreased in neuroblasts using RNAi (data not shown). We also tested whether Ph gain-of-function is sufficient to restrict motoneuron competence in NB3-1. In control embryos (*sca>Kr, control*), ectopic RP3s were produced in 100% of hemisegments (*n*=62), with one ectopic RP3 in 34%, two ectopic RP3s in 58%, and three ectopic RP3s in 8% of hemisegments. NB3-1 was also competent to generate RP5 cells at late divisions, with 29% of hemisegments producing an RP5 at the fifth or sixth division. In Ph gain-of-function embryos (*sca>Kr, ph*), competence to produce RP3 fates was restricted and RP5 cells were never produced: 23% of hemisegments
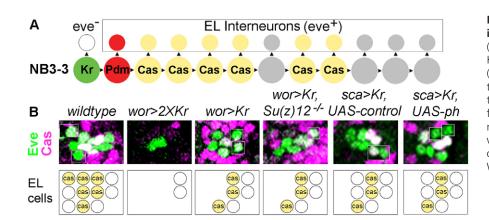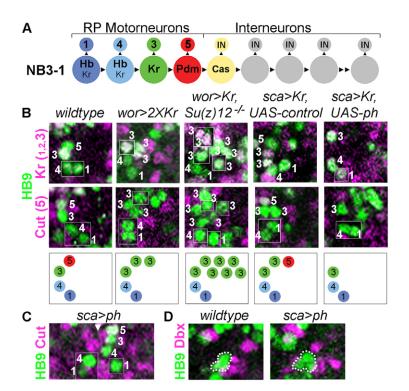
Fig. 6. PRC activity does not affect interneuron production by NB3-3. (**A**) Diagram of the NB3-3 lineage showing the Kr- and Cas-specified interneurons. (**B**) Representative EL interneuron images from the indicated genotypes. Summary figures show the average number of EL neurons and frequency of Cas$^+$ EL neurons (see text for numbers). No statistically significant differences were detected between controls and PRC loss- or gain-of-function embryos based on Kruskal-Wallis test.

produced a single ectopic RP3, 60% produced only the normal RP3 and 17% did not produce any RP3 (n=42) (Fig. 7B, supplementary material Fig. S6).

The absence of any RP3 cell in 17% of NB3-1 lineages suggests that Ph gain-of-function alone is sufficient to inhibit production of the endogenous Kr-specified motoneuron. We tested the ability of Ph gain-of-function to inhibit other motoneuron fates in NB3-1 using *sca-GAL4* × *UAS-ph* and found that the first two motoneuron fates, RP1 and RP4, are always present, whereas RP3 and RP5 are missing in 15% of hemisegments and RP5 is missing in 29% of hemisegments (n=62) (Fig. 7C). To determine if this restriction of HB9$^+$ Islet$^+$ fates was motoneuron specific, we analyzed the production of HB9$^+$ Islet$^+$ interneurons by NB7-3. In the NB7-3 lineage, the first GMC makes the EW1 interneuron and GW motoneuron sibling, the second GMC makes the EW2 interneuron and the sibling apoptoses, and the third GMC directly differentiates into EW3 (Karcavich and Doe, 2005). The three interneurons of the NB7-3 lineage can be identified based on their position and expression of HB9 and Islet. All three EW interneurons were produced in 100% of hemisegments in Ph gain-

of-function embryos (Fig. 7D). As an additional test of interneuron competence, we analyzed the production of Dbx$^+$ interneurons by multiple neuroblasts. Five neuroblast lineages (NB4-2, NB5-2, NB6-1, NB6-2 and NB7-1) produce ~20 Dbx$^+$ interneurons per abdominal hemisegment. The precise birth order of Dbx$^+$ interneurons within individual lineages is not known, and overlap among the Dbx$^+$ populations makes it difficult to assign cells to a specific lineage. However, quantification of the total population of Dbx$^+$ interneurons per hemisegment revealed no difference between controls and Ph gain-of-function embryos (Fig. 7D). The inhibition of HB9$^+$ Islet$^+$ motoneurons and lack of any effect on HB9$^+$ Islet$^+$ interneurons and Dbx$^+$ interneurons further suggests that PRCs specifically regulate motoneuron competence.

DISCUSSION
An epigenetic mechanism of competence restriction in *Drosophila* neuroblasts
We used multiple genetic approaches to investigate the timing and specificity of competence restriction by PRCs in *Drosophila* neuroblasts. Our data show that PRCs establish motoneuron

Fig. 7. PRC activity regulates motoneuron competence in NB3-1, whereas interneuron fates in multiple lineages are unaffected. (**A**) The NB3-1 lineage. (**B**) Summary figures show the average cell fates for each genotype. Kr is expressed at low levels in RP1 and RP4 and at high levels in RP3. Cut is only expressed in RP5. RP motoneurons of the NB3-1 lineage were also identified by staining for Islet (supplementary material Fig. S6). Statistically significant differences (P<0.001) in the mean number of RP3 cells produced were observed between *wor>2×Kr* (2.8±0.74, n=72) and *wor>Kr, Su(z)12$^{-/-}$* (4.8±1.1, n=57) and between *sca>Kr, UAS-control* (2.7±0.59, n=63) and *sca>Kr, UAS-ph* embryos (1.1±0.63, n=42), based on Kruskal-Wallis test. (**C**) Ph gain-of-function inhibits endogenous RP3 and RP5 fates. A single nerve cord segment is shown; arrowhead marks the midline. The NB3-1 lineage on the left is missing RP3 and RP5, whereas the NB3-1 lineage on the right is normal. (**D**) Interneuron production is not affected by Ph gain-of-function. HB9$^+$ EW interneurons (outlined by dotted line) and Dbx$^+$ interneurons are shown for the same hemisegment region of wild-type and *sca>ph* embryos. EW interneurons were also identified by staining for Islet (not shown). There was no difference between wild-type and *sca>ph* embryos in EW production (EW1, 2 and 3 were present in 100% of hemisegments in *sca>ph* embryos; n=62) or in the number of Dbx$^+$ cells per hemisegment [20.0±1.32 in wild type (n=41) and 20.3±1.59 in *sca>ph* (n=48)].

competence windows in two distinct neuroblast lineages, regulating the production of both Eve$^+$ and HB9$^+$ Islet$^+$ motoneurons. This provides a mechanistic explanation for the loss of competence that has been previously described in NB7-1 and NB3-1. Our experiments manipulating the timing of Pdm and Cas expression show that this mechanism is not limited to fate specification by Kr but is involved in establishing a broad motoneuron competence window. Consistent with this model, there appears to be little restriction of competence in a lineage that produces exclusively interneurons (NB3-3) and, correspondingly, PRC activity does not affect the ability of Kr to alter interneuron fates in this lineage. In addition, whereas Ph gain-of-function is sufficient to inhibit production of HB9$^+$ Islet$^+$ motoneurons by NB3-1, the production of HB9$^+$ Islet$^+$ interneurons by NB7-3 and of Dbx$^+$ interneurons by multiple neuroblasts are unaffected.

Our initial screen revealed a requirement for a subset of PRC1 and PRC2 genes in the regulation of competence. Lack of a statistically significant phenotype for other genes might be due to dosage: all embryos are heterozygous for the mutant allele and there is maternal contribution of Polycomb group and Trithorax group transcripts. Our subsequent studies primarily used the $Su(z)12^3$ (null allele) and ph-d^{401}, ph-p^{602} (ph-d^{401} is hypomorphic, ph-p^{602} is null) mutants. $Su(z)12$ is a component of PRC2 and Ph is a component of PRC1, allowing us to assess the roles of each PRC complex. $Su(z)12$ loss-of-function extended competence to the end of the NB7-1 lineage. $Su(z)12$ is an essential co-factor of the E(z) H3K27 methyltransferase and levels of $Su(z)12$ activity correlate with the extent of H3K27 methylation at target genes (Ketel et al., 2005). This suggests that the degree of competence restriction is determined by the levels of H3K27 methylation at genes required for motoneuron production. Progressive restriction of competence was still observed in the ph-d^{401}, ph-p^{602} mutants, which was likely to be due to residual Ph activity. However, competence in these mutants is not completely lost until nearly twice the number of neuroblast divisions have occurred than are normally associated with loss of competence (nine divisions in ph mutants versus five in wild type). We hypothesize that PRC-induced chromatin modifications accumulate over multiple neuroblast divisions and must reach some threshold for inhibiting motoneuron fates, similar to the accumulation of H3K27 trimethylation at the $Neurog1$ locus during competence restriction in mammalian cortical progenitors (Hirabayashi et al., 2009). Without testing additional Polycomb group and Trithorax group genes as homozygous mutants and generating maternal nulls (which in some cases might not survive to the relevant stages of neurogenesis), we cannot precisely identify which Polycomb group proteins are necessary for the restriction of competence. The core components of PRC1 and PRC2 are likely to be ubiquitously and constitutively expressed throughout neurogenesis, and we have confirmed this for Pc and Ph (data not shown). However, cell type-specific PRC complexes and developmentally regulated changes in PRC composition have been described previously (Simon and Kingston, 2009), suggesting that PRC1 or PRC2 co-factors might regulate the timing of competence restriction. It will be interesting to test the role of co-factors that are known or predicted to recruit PRC2 to specific genes, such as the PhoRC complex, Pipsqueak and Grainy head (Schuettengruber et al., 2007).

Temporal regulation of motoneuron versus interneuron production

The sequential generation of motoneurons followed by interneurons has been observed during nervous system development of many insects (Burrows, 1996). Clonal analysis of

Drosophila neuroblasts suggests that motoneurons are always produced first (Schmid et al., 1999), as demonstrated for NB7-1 and NB3-1, although precise birth order data are lacking for most other lineages. In the mammalian spinal cord, motoneurons and interneurons are produced from spatially segregated populations of progenitors that develop along the dorsal-ventral axis of the neural tube. *Drosophila* lacks this spatial segregation of motoneuron-committed or interneuron-committed progenitors. Instead, temporal changes allow single progenitors to produce mixed lineages. PRCs appear to work in parallel to the temporal identity transcription factors by establishing competence windows in which temporal identity factors can specify motoneuron fates. Competence windows might represent a 'quality control' mechanism in which PRCs reinforce the timing of fate specification, similar to the role proposed for miRNAs during *Drosophila* development (Li et al., 2009). Competence windows might also allow temporal identity factors to be 'redeployed' at later divisions. The majority of neuroblasts express Kr and Cas a second time (Cleary and Doe, 2006) and we have confirmed that NB7-1 re-expresses Kr when interneurons are being produced (data not shown). The function of Kr during later neuroblast divisions remains to be determined. If PRC activity alone were responsible for blocking a Kr-specified motoneuron late in the NB7-1 lineage, at least one ectopic U3 might be expected in ph-d, ph-p hemizygous or $Su(z)12$ homozygous mutant embryos. However, we did not detect altered U motoneuron fates in such mutants. There are at least two potential explanations for this result. First, residual PRC activity in these mutants might allow sufficient changes in chromatin states to block endogenous Kr from specifying a motoneuron. This possibility is supported by our data showing a dosage-sensitive relationship between Kr and PRC levels in specifying U3 fates, and the eventual loss of competence in ph mutants subjected to heat shock-induced pulses of Kr. Alternatively, there might be an additional transcription factor (or factors) that specifies interneuron fates in the NB7-1 lineage. This interneuron fate determinant could have a dominant effect, such that even when PRC activity is reduced, interneuron fates (or an Eve$^-$ 'hybrid' fate) prevail. Conversion to an Eve$^+$ motoneuron might therefore only occur in a combined PRC loss-of-function and Kr gain-of-function background.

In both NB7-1 and NB3-1, later-born motoneuron fates are preferentially inhibited in Ph gain-of-function experiments, supporting a link between the number of neuroblast divisions and the restriction of motoneuron competence. The timing of competence restriction might also be regulated by the temporal identity factors themselves. Previous studies of competence in NB7-1 and NB3-1 have shown that constitutive expression of Hb can maintain neuroblasts in a fully competent state (Grosskortenhaus et al., 2006; Tran and Doe, 2008; Tran et al., 2010). In addition, precocious Pdm expression can inhibit Kr expression and block U3 fates in NB7-1 and RP3 fates in NB3-1 (Grosskortenhaus et al., 2006; Tran and Doe, 2008). How Hb or Pdm might interact with Polycomb or Trithorax complexes during the regulation of competence remains to be determined.

What are the PRC-regulated genes that determine motoneuron competence?

In an attempt to identify PRC target genes that affect competence, we analyzed NB7-1 fates in embryos with *wor-GAL4* driving expression of *Kr* in combination with the following candidates: the anterior-posterior patterning Hox genes *Ultrabithorax, abdominal A, Antennapedia* and *Abdominal B*, the nervous system-expressed

Hox gene *BarH1*, the neuroblast fate determinant *gooseberry*, and the cell cycle regulator *Cyclin A*. We did not detect any extension of competence when these PRC targets are coordinately overexpressed with Kr (data not shown). It would be technically very challenging and beyond the scope of this work to identify direct PRC targets in NB7-1 or NB3-1. However, clues are provided by previous studies that identified PRC targets in *Drosophila* embryos (Oktaba et al., 2008; Schuettengruber et al., 2009). One interesting set of PRC targets is a group of genes involved in motoneuron formation or function: *eve*, *islet*, *HB9*, *Nkx6* (*HGTX* – FlyBase), *zfh1* and *Lim3*. All motoneurons that innervate dorsal muscles express Eve (Landgraf et al., 1999), most motoneurons that innervate ventral muscles express some combination of Lim3, Islet, HB9 and Nkx6 (Broihier and Skeath, 2002; Broihier et al., 2004; Cheesman et al., 2004; Odden et al., 2002; Thor and Thomas, 1997; Thor et al., 1999), and all somatic motoneurons express Zfh1 (Layden et al., 2006). None of these genes is sufficient to confer motoneuron fates on their own, and some (*eve*, *HB9*, *islet*) are also expressed in subsets of interneurons. It is possible that PRCs silence the transcription of multiple genes that establish motoneuron fate 'combinatorial codes' (Shirasaki and Pfaff, 2002; Thor and Thomas, 2002). Relevant PRC target genes might be coordinately regulated by the temporal identity transcription factors (as suggested by the ability of high levels of Kr to partially overcome competence restriction) or transcription of these targets might depend on indirect interactions.

Maintenance versus restriction of fate potential by PRCs

In mammalian embryonic stem cells, PRCs maintain pluripotency by inhibiting transcription of developmental pathway genes. These genes contain 'bivalent' histone modifications, with PRC-associated H3K27 methylation and Trithorax-associated H3K4 methylation keeping developmental regulators silenced but poised for activation (Bernstein et al., 2006; Boyer et al., 2006). During differentiation of embryonic stem cells into neural progenitors, neural development genes lose PRC-associated modifications but retain H3K4 methylation, resulting in increased transcription (Bernstein et al., 2006). Although PRC silencing maintains pluripotency in embryonic stem cells, PRCs are likely to have an additional role in restricting fate potential once a progenitor becomes lineage committed. This was recently demonstrated for mouse embryonic endoderm progenitors, which undergo a fate choice for liver or pancreas development. The regulatory elements of liver and pancreas genes have distinct chromatin patterns prior to commitment to either lineage, and EZH2 [an ortholog of *Drosophila* E(z)] promotes liver development by restricting the expression of pancreatic genes (Xu et al., 2011). Similar chromatin 'prepatterns' might exist for motoneuron and interneuron genes in newly formed *Drosophila* neuroblasts, with subsequent PRC activity selectively silencing motoneuron genes in NB7-1 and NB3-1. PRC activity has also been shown to regulate the timing of terminal differentiation in mouse epidermal progenitors (Ezhkova et al., 2009) and the transition from neurogenesis to astrogenesis in mouse cortical progenitors (Hirabayashi et al., 2009). Our identification of a related mechanism in *Drosophila* neuroblasts suggests that temporal restriction of fate potential is a common function of PRCs. *Drosophila* embryonic neuroblasts will provide a useful system for addressing several outstanding questions regarding PRC regulation of fate potential, including how PRCs are recruited to target genes, the composition of the relevant silencing complexes, and how PRC activity is temporally regulated.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.071589/-/DC1

References
Bernstein, B. E., Mikkelsen, T. S., Xie, X., Kamal, M., Huebert, D. J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K. et al. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315-326.

Blomen, V. A. and Boonstra, J. (2011). Stable transmission of reversible modifications: maintenance of epigenetic information through the cell cycle. *Cell. Mol. Life Sci.* **68**, 27-44.

Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. M. (1996). The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev. Biol.* **179**, 41-64.

Boyer, L., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K. et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**, 349-353.

Bracken, A. P. and Helin, K. (2009). Polycomb group proteins: navigators of lineage pathways led astray in cancer. *Nat. Rev. Cancer* **9**, 773-784.

Broihier, H. T. and Skeath, J. B. (2002). Drosophila homeodomain protein dHb9 directs neuronal fate via crossrepressive and cell-nonautonomous mechanisms. *Neuron* **35**, 39-50.

Broihier, H. T., Kuzin, A., Zhu, Y., Odenwald, W. and Skeath, J. B. (2004). Drosophila homeodomain protein Nkx6 coordinates motoneuron subtype identity and axonogenesis. *Development* **131**, 5233-5242.

Burrows, M. (1996). *The Neurobiology of an Insect Brain*. New York: Oxford University Press.

Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of Drosophila melanogaster*. Berlin, Germany: Springer-Verlag.

Cheesman, S. E., Layden, M. J., Von Ohlen, T., Doe, C. Q. and Eisen, J. S. (2004). Zebrafish and fly Nkx6 proteins have similar CNS expression patterns and regulate motoneuron formation. *Development* **131**, 5221-5232.

Chen, X., Hiller, M., Sancak, Y. and Fuller, M. T. (2005). Tissue-specific TAFs counteract Polycomb to turn on terminal differentiation. *Science* **310**, 869-872.

Cleary, M. D. and Doe, C. Q. (2006). Regulation of neuroblast competence: multiple temporal identity factors specify distinct neuronal fates within a single early competence window. *Genes Dev.* **15**, 429-434.

Desai, A. R. and McConnell, S. K. (2000). Progressive restriction in fate potential by neural progenitors during cerebral cortical development. *Development* **127**, 2863-2872.

Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S. et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* **448**, 151-156.

Ezhkova, E., Pasolli, H. A., Parker, J. S., Stokes, N., Su, I. H., Hannon, G., Tarakhovsky, A. and Fuchs, E. (2009). Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell* **136**, 1122-1135.

Grosskortenhaus, R., Robinson, K. J. and Doe, C. Q. (2006). Pdm and Castor specify late-born motor neuron identity in the NB7-1 lineage. *Genes Dev.* **20**, 2618-2627.

Hirabayashi, Y. and Gotoh, Y. (2010). Epigenetic control of neural precursor cell fate during development. *Nat. Rev. Neurosci.* **11**, 377-388.

Hirabayashi, Y., Suzki, N., Tsuboi, M., Endo, T. A., Toyoda, T., Shinga, J., Koseki, H., Vidal, M. and Gotoh, Y. (2009). Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. *Neuron* **63**, 600-613.

Isshiki, T., Pearson, B., Holbrook, S. and Doe, C. Q. (2001). Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* **106**, 511-521.

Karcavich, R. and Doe, C. Q. (2005). Drosophila neuroblast 7-3 cell lineage: a model system for studying programmed cell death, Notch/Numb signaling, and

sequential specification of ganglion mother cell identity. *J. Comp. Neurol.* **481**, 240-251.

Ketel, C. S., Andersen, E. F., Vargas, M. L., Suh, J., Strome, S. and Simon, J. A. (2005). Subunit contributions to histone methyltransferase activities of fly and worm polycomb group complexes. *Mol. Cell. Biol.* **25**, 6857-6868.

Lacin, H., Zhu, Y., Wilson, B. A. and Skeath, J. B. (2009). dbx mediates neuronal specification and differentiation through cross-repressive, lineage-specific interactions with eve and hb9. *Development* **136**, 3257-3266.

Landgraf, M., Roy, S., Prokop, A., VijayRaghavan, K. and Bate, M. (1999). even-skipped determines the dorsal growth of motor axons in Drosophila. *Neuron* **22**, 43-52.

Layden, M. J., Odden, J. P., Schmid, A., Garces, A., Thor, S. and Doe, C. Q. (2006). Zfh1, a somatic motor neuron transcription factor, regulates axon exit from the CNS. *Dev. Biol.* **291**, 253-263.

Lee, C. Y., Robinson, K. J. and Doe, C. Q. (2006). Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. *Nature* **439**, 594-598.

Li, X., Cassidy, J. J., Reinke, C. A., Fischboeck, S. and Carthew, R. W. (2009). A microRNA imparts robustness against environmental fluctuation during development. *Cell* **137**, 273-282.

Mahr, A. and Aberle, H. (2006). The expression pattern of the Drosophila vesicular glutamate transporter: a marker protein for motoneurons and glutamatergic centers in the brain. *Gene Expr. Patterns* **6**, 299-309.

Martinez, A. M., Colomb, S., Déjardin, J., Bantignies, F. and Cavalli, G. (2006). Polycomb group-dependent Cyclin A repression in Drosophila. *Genes Dev.* **20**, 501-513.

Miller, F. D. and Gauthier, A. S. (2007). Timing is everything: making neurons versus glia in the developing cortex. *Neuron* **54**, 357-369.

Narbonne, K., Besse, F., Brissard-Zahraoui, J., Pret, A. M. and Busson, D. (2004). polyhomeotic is required for somatic cell proliferation and differentiation during ovarian follicle formation in Drosophila. *Development* **131**, 1389-1400.

O'Dor, E., Beck, S. A. and Brock, H. W. (2006). Polycomb group mutants exhibit mitotic defects in syncytial cell cycles of Drosophila embryos. *Dev. Biol.* **260**, 312-322.

Odden, J. P., Holbrook, S. and Doe, C. Q. (2002). Drosophila HB9 is expressed in a subset of motoneurons and interneurons, where it regulates gene expression and axon pathfinding. *J. Neurosci.* **22**, 9143-9149.

Oktaba, K., Gutiérrez, L., Gagneur, J., Girardot, C., Sengupta, A. K., Furlong, E. E. and Müller, J. (2008). Dynamic regulation by polycomb group protein complexes controls pattern formation and the cell cycle in Drosophila. *Dev. Cell* **15**, 877-889.

Parrish, J. Z., Emoto, K., Jan, L. Y. and Jan, Y. N. (2007). Polycomb genes interact with the tumor suppressor genes hippo and warts in the maintenance of Drosophila sensory neuron dendrites. *Genes Dev.* **15**, 956-972.

Pearson, B. J. and Doe, C. Q. (2003). Regulation of neuroblast competence in Drosophila. *Nature* **425**, 624-628.

Pearson, B. J. and Doe, C. Q. (2004). Specification of temporal identity in the developing nervous system. *Annu. Rev. Cell Dev. Biol.* **20**, 619-647.

Pereira, J. D., Sansom, S. N., Smith, J., Dobenecker, M. W., Tarakhovsky, A. and Livesey, F. J. (2010). Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. *Proc. Natl. Acad. USA* **107**, 15957-15962.

Pietersen, A. M. and van Lohuizen, M. (2008). Stem cell regulation by polycomb repressors: postponing commitment. *Curr. Opin. Cell Biol.* **20**, 201-207.

Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B. and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. *Cell* **128**, 735-745.

Schuettengruber, B., Ganapathi, M., Leblanc, B., Portoso, M., Jaschek, R., Tolhuis, B., van Lohuizen, M., Tanay, A. and Cavalli, G. (2009). Functional anatomy of polycomb and trithorax chromatin landscapes in Drosophila embryos. *PLoS Biol.* **13**, e13.

Schwartz, Y. B. and Pirrotta, V. (2008). Polycomb complexes and epigenetic states. *Curr. Opin. Cell Biol.* **20**, 266-273.

Schwartz, Y. B., Kahn, T. G., Nix, D. A., Li, X. Y., Bourgon, R., Biggin, M. and Pirrotta, V. (2006). Genome-wide analysis of Polycomb targets in Drosophila melanogaster. *Nat. Genet.* **38**, 700-705.

Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* **25**, 251-281.

Simon, J. A. and Tamkun, J. W. (2002). Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. *Curr. Opin. Genet. Dev.* **12**, 210-218.

Simon, J. A. and Kingston, R. E. (2009). Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat. Rev. Mol. Cell Biol.* **10**, 697-708.

Smouse, D., Goodman, C., Mahowald, A. and Perrimon, N. (1988). polyhomeotic: a gene required for the embryonic development of axon pathways in the central nervous system of Drosophila. *Genes Dev.* **2**, 830-842.

Thor, S. and Thomas, J. B. (1997). The Drosophila islet gene governs axon pathfinding and neurotransmitter identity. *Neuron* **18**, 397-409.

Thor, S. and Thomas, J. B. (2002). Motor neuron specification in worms, flies and mice: conserved and 'lost' mechanisms. *Curr. Opin. Genet. Dev.* **12**, 558-564.

Thor, S., Andersson, S. G., Tomlinson, A. and Thomas, J. B. (1999). A LIM-homeodomain combinatorial code for motor-neuron pathway selection. *Nature* **397**, 76-80.

Tran, K. D. and Doe, C. Q. (2008). Pdm and Castor close successive temporal identity windows in the NB3-1 lineage. *Development* **135**, 3491-3499.

Tran, K. D., Miller, M. R. and Doe, C. Q. (2010). Recombineering Hunchback identifies two conserved domains required to maintain neuroblast competence and specify early-born neuronal identity. *Development* **137**, 1421-1430.

Tsuji, T., Hasegawa, E. and Isshiki, T. (2008). Neuroblast entry into quiescence is regulated intrinsically by the combined action of spatial Hox proteins and temporal identity factors. *Development* **135**, 3859-3869.

Wang, J., Lee, C. H., Lin, S. and Lee, T. (2006). Steroid hormone-dependent transformation of polyhomeotic mutant neurons in the Drosophila brain. *Development* **133**, 1231-1240.

Xu, C. R., Cole, P. A., Meyers, D. J., Kormish, J., Dent, S. and Zaret, K. S. (2011). Chromatin 'prepattern' and histone modifiers in a fate choice for liver and pancreas. *Science* **332**, 963-966.

Zink, D. and Paro, R. (1995). Drosophila Polycomb-group regulated chromatin inhibits the accessibility of a trans-activator to its target DNA. *EMBO J.* **14**, 5660-5671.