Drosophila Polycomb complexes restrict neuroblast competence to generate motoneurons

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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 (Ishikawa 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 (Bosson 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
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)12:TM3, FRT-2A/Ubac-UAS-2B; (2) UAS-Kr; UAS-Su(z)12/UBac-UAS-2B; (3) ph-d(p201), ph-p202/FM7c; Act-2GFP: 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-Eve[RNai, VDRC stock 39761]; (9) en-GAL4; (10) sca-GAL4; sca-GAL4 (C. Doe, University of Oregon); (11) UAS-Kr; UAS-pha; (12) UAS-ph (G. Cavalli, C.N.R.S., France). Unless otherwise noted, stocks were obtained from the Bloomington Drosophila Stock Center. Su(z)12 heterozygous versus homozygous mutants were identified by staining for &beta;-galactosidase. ph-d(p201), ph-p202 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. Sheehan, Washington University); rabbit VGlut1:400 (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: 1:10 pre-absorbed (A. Brand, University of Cambridge, UK); rat Zfh2: 1:200 (M. Lundeil, University of Texas at San Antonio); and rabbit &beta;-gal: 1:100 (Abcam). Mouse Islet was pre-absorbed and used in combination with the Alexa 488 488 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 wor-GAL4 (Lee et al., 2006) with tubulin-GAL80/hs (temperature-sensitive GAL80) and UAS-Kr, in a genotype we call wor/hs> Kr. At the permissive temperature in wor/hs> 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/hs> 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/hs> 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
PRCs restrict neuroblast competence

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+ 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-d601, 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>Kn; 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>Kn; 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>Kn; 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 (Bosshard 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.

**Polyhomoecic 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 mutations, there is no progressive restriction during the motoneuron phase: competence to respond to
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 therefore provide 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 (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 embryos (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...
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.

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).

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>2XKr) 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

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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/H11003 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. 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.
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
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 mutant (null allele) and ph-d401 (ph-D, ph-P402 is hypomorphic, ph-P402 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-d401, ph-p402 mutants, which was likely to be due to residual PRC 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).

Supporting this idea, we find that once PRC activity alone were responsible for blocking a Kr-specified motoneuron fate 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 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 fate 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.

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 motoneuron versus interneuron has been observed during nervous system development of many insects (Burrows, 1996). Clonal analysis of
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 (Oktava 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, Nvk6 (HGTX – FlyBase), zfh1 and Lim3. All motoneurors that innervate dorsal muscles express Eve (Landgraf et al., 1999), most motoneurons that innervate ventral muscles express some combination of Lim3, Islet, HB9 and Nvk6 (Brohier and Skeath, 2002; Brohier et al., 2004; Cheesman et al., 2004; Oden 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.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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