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Polycomb Role in Cell Fate Specification in *Drosophila* Neural Progenitors

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Quantitative and Systems Biology

by

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DEDICATION

In recognition of my families, I dedicate my work to my parents Jamil and Rita Touma, my grandmother Nazilli Alexanian and my beautiful family; my wife Christiane and kids. I am eternally grateful for your love and care.

EPIGRAPH

"Advance, and never halt, for advancing is perfection. Advance and do not fear the thorns in the path, for they draw only corrupt blood."

Gibran Khalil Gibran

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LIST OF ABBREVIATIONS

A-P – anteroposterior
Cas - castor
CNS – central nervous system
D-V - dorsoventral
E(z) - Enhancer of Zeste
E(Z) – Enhancer of zeste
En – engrailed
ESC - Exterior sex com
Eve – even skipped
G.O.F. – gain of function
Grh - Grainyhead
gsb - gooseberry
H1 - histone subunit 1
H2A - histone subunit 2A
H2B - histone subunit 2B
H3 - histone subunit 3
H3K9me3 - trimethylate lysine 9 of histone H3
H4 - histone subunit 4
HAT - histone acetyltransferase
Hb - hunchback
HDACs - histone deacetylase
Kr - Kruppel
L.O.F. – loss-of-function
Mir - miranda
mRNA – messenger RNA
Msh - mesh
NB - neuroblast
NB 3-1 – neuroblast 3-1
NB3-3 - neuroblast 3-3
NB6-4T - Thoracic NB6-4
NB7-1 – neuroblast 7-1
NB7-4 – neuroblast 7-4
Ngn – neurogenin
NSC – neural stem cells
Pc – Polycomb
PCL - Polycomb-like
PH – Polyhomeotic
PHO – Pleihomeotic
PHOL - Pleihomeotic-like
PhoRC – Pleihomeotic repressive complex
PRC – polycomb repressive complex
PRC1 - polycomb repressive complex 1
PRC2 - polycomb repressive complex 2

PRE - Polycomb Response Elements
Pros - Prospero
Psc - Posterior Sex Combs
Psq – Pipsqueak
RNAi – RNA interference
run - runt
SU(Z)12 - Suppressor of zeste-12
Svp - Seven up
TF – transcription factor
UAS - upstream activating sequence
VNC - ventral nerve cord
vnd - ventral nerve cord defective
Wor – wornui
YY1 - Yin-Yang 1
Z - Zeste
zfh2 - Zn finger homeodomain 2

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ABSTRACT OF THE DISSERTATION

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The timing of cell production by progenitor cells is an essential aspect of development. Particularly during neurogenesis, the time at which neurons and glia are produced affects their function and proper integration into neural circuits. In both the mammalian and *Drosophila* central nervous system, neural progenitors progressively lose competence to make early-born cell types, so that "old" progenitors can no longer be induced to make "young" neurons. My dissertation work used *Drosophila* neural progenitors, known as neuroblasts, as a model to investigate the restriction of neural progenitor competence. *Drosophila* neuroblasts sequentially express temporal transcription factors (TTFs) that determine neural and glial cell fate based on birth-order. For example, the second TTF in the series, Kruppel, is necessary and sufficient for all second-born / third-born fates, regardless of cell type or neuroblast lineage. However, neuroblasts lose competence to respond to Kruppel with each division, ultimately completely losing competence to produce Kruppel-specified cell types at late stages of development. I discovered that chromatin remodeling complexes of the Polycomb group are necessary and sufficient for the temporal restriction of neuroblast competence. I found that Polycomb complexes establish distinct competence windows in neuroblasts that transition from early motorneuron production to late interneuron production. This work provides a mechanistic basis for the restriction of neuroblast competence and supports a model in which Polycomb complexes progressively limit the ability of TTFs to activate gene expression programs that induce early-born fates.

Chapter 1: Introduction

Section 1: Temporal Regulation of Neural Cell Fate

1.1 Temporal Identity during Mammalian Neurogenesis

During development, single progenitor cells populate a functional organism with multiple cell types. One prominent example is the central nervous system where a limited number of progenitor cells produce vast numbers of neurons and glia with distinct characteristics. The proper development of the vertebrate and invertebrate central nervous systems depends on spatial patterning of neural progenitor cells (Berry and Rogers, 1965; Reid et al., 1995; Walsh and Reid, 1995; Skeath and Doe, 1996; Rapaport et al., 2001) and temporal control of progeny generation (Skeath and Doe, 1996; Cepko, 1999; Livesey and Cepko, 2001). Spatial patterning has been well studied and involves mechanisms including anterior-posterior regionalization via Hox protein activity and specification of distinct domains via signaling by morphogens such as Sonic Hedgehog and Bone Morphogenetic Proteins (BMPs) (Munoz-Sanjuan et al., 2002; Munoz-Sanjuan and Brivanlou, 2002; De Robertis and Kuroda, 2004; Ciani and Salinas, 2005; Stern, 2006; Levine and Brivanlou, 2007; De Robertis, 2008). In contrast, relatively little is known about the mechanisms that temporally-regulate the generation of the different progeny cell types in the mammalian nervous system (Jacob et al., 2008; Gaspard and Vanderhaeghen, 2011). Here I summarize what is known about temporal-regulation of neuronal fate during development of the mammalian central nervous system.

1.1.1 Cerebral Cortex

The mammalian cerebral cortex is a complex structure in the forebrain region of the central nervous system. Cellular diversification in the cerebral cortex and correct organization of neuronal connections allow the cortex to carry out sophisticated tasks in humans, such as language and reasoning. In all mammals, different areas of the cortex surface are involved in particular functions, such as vision, hearing, language and touch. During the initial stages of neurogenesis, neuroectodermal precursors generate neural progenitors with distinct spatial identities based on position within the developing neural tube. Based on this position and spatial identity, progenitors then generate neurons with unique functions, such as the neurons of the visual cortex versus neurons of the auditory cortex. Within each cortical domain, neurons are organized into six vertical layers named layers 1, 2/3, 4, 5 and 6 (Figure 1). Progeny cells that populate layer 1 are the most superficial whereas those that populate layer 6 are the deepest, located closest to the neural progenitors. Neurons in each layer express the same molecular markers and share unique axonal and dendritic projection patterns (McConnell, 1992; Callaway, 2002; Kubo and Nakajima, 2003). Corticofugal neurons populate the deepest layers 5 and 6 of the cortex and project their axons to the basal ganglia, thalamus, brainstem, and spinal cord (Figure 1). Corticocortical neurons on the other hand are located in layer 2/3 and project axons that connect different areas within the cortex. Input from different regions of the central nervous system, including spinal neurons, reaches the cortex via axons that synapse with neurons that reside in layer 4.

The layered organization of the cerebral cortex is established based on the "birth-order" or temporal identity of neural progenitor progeny. Progeny birth-order of the cerebral cortex was identified using sequential H^3-thymidine incorporation into the mitotic progenitors followed by localization of the radiolabeled post-mitotic progeny within the cortex. This type of approach showed that the cerebral cortex is populated from the deepest layer first to the most superficial layer last, populating the cortical layers in what is known as an "inside-out" order (Berry et al., 1964; Berry and Rogers, 1965; McConnell, 1988). As neural progenitor cells reside in the ventricular zone (VZ), early-born progeny migrate only a short distance away to the deepest layer, layer 6. The next-born progeny cells migrate to layer 5 followed by progeny that migrate to the superficial layer 2/3. An exception to this "inside-out" production of laminar fates is the progeny of layer 1, which are the very first-born progeny and migrate to the outermost layer (Luskin and Shatz, 1985). Progenitor cells in the VZ are multipotent and retroviral lineage studies have shown that individual progenitors can generate neurons of all layers (Walsh and Reid, 1995; Reid et al., 1997). Isochronic and heterochronic transplantation experiments showed that cortical progenitor temporal identity is regulated by an intrinsic mechanism linked to the cell cycle and by extrinsic signals (Frantz and McConnell, 1996). Progenitor cells transplanted in early cell cycle stages were competent to respond to extrinsic host cues that cause neural progeny to migrate to the host-specified layer. In contrast, transplantation of progenitors that had completed at least one full cell cycle resulted in a loss of competence to respond to host cues, with the neural progeny migrating to the donor-specified layer. Further analysis revealed that progenitor cell commitment to produce the neurons of a specific layer occurs in the final G2/M phase of

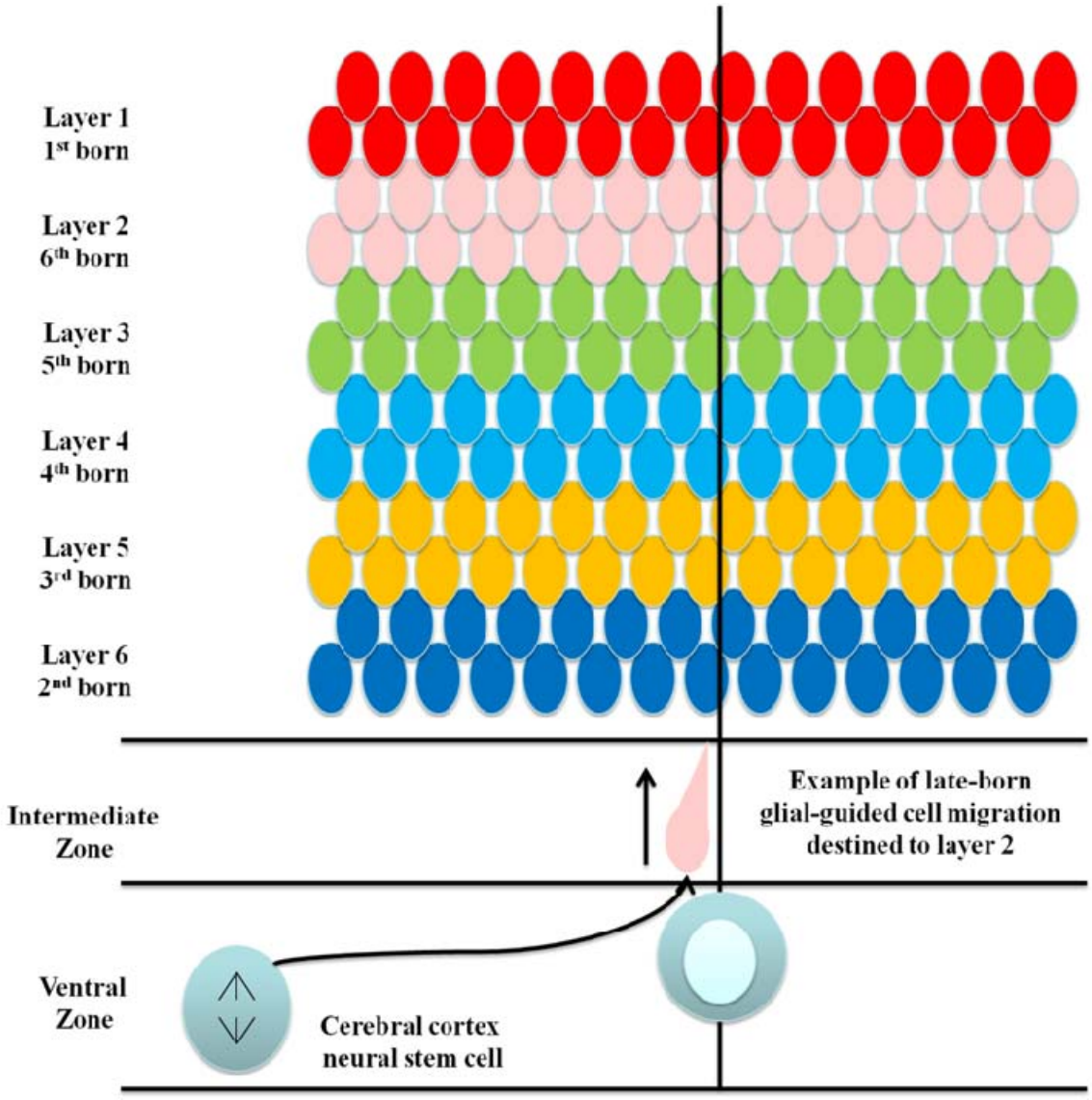

Figure 1: The mammalian cerebral cortex is populated by neural progenitors that reside in the ventral zone. These neural stem cells divide asymmetrically and form progeny cells that will migrate to their specified layers. Neurons of the cerebral cortex are organized into six vertical layers named layers 1, 2, 3, 4, 5 and 6. Progeny cells that populate layer 1 are the most superficial whereas those that populate layer 6 are the deepest. The neuronal progeny in each layer express the same molecular markers and share unique axonal and dendritic projection patterns. Except for the first-born progeny that populate layer 1, early-born progeny populate deepest layers 6 and 5, whereas late-born progeny populate superficial layers 2 and 3.

the cell cycle. Progenitor cells transplanted at early S-phase were competent to respond to extrinsic cues and resulted in progeny cells that populated host-specific layers whereas progenitor cells transplanted at G2/M-phase prior to mitosis, produced donor-specific neurons (McConnell, 1988; McConnell and Kaznowski, 1991). Relatively little is known about the mechanisms that link cell cycle stage and neural progenitor competence. However, these studies have clearly demonstrated a temporally-regulated progression of cell fate specification in the cerebral cortex.

Several transcription factors that appear to regulate temporal identity in the cerebral cortex have been identified. Foxg1 is perhaps the best characterized and has been shown to be both necessary and sufficient to repress early-born layer 1 fates. Foxg1 is expressed after the formation of layer 1 cells and is continuously expressed in the layer 2 through 6 neurons (Hanashima et al., 2004). There are additional transcription factors that are expressed in specific layers in the cortex and are potential temporal identity regulators. Oct6 is a POU-domain containing transcription factor present in layer 5 progeny cells (Frantz et al., 1994a). Otx1 is another factor identified in early progenitor cells that relocates from the cytoplasm to the nucleus of layer 5 and 6 neurons (Frantz et al., 1994b). Late neural progenitor cells were shown to express only low levels of Otx1 in contrast to early progenitor cells that express high levels of Otx1. Otx1 is highly expressed in the deep-layer neurons of layers 5 and 6 in the adult cortex (Frantz et al., 1994b). Otx1 mutant mice loss-of-function experiments showed that Otx1 is required for the development of the entire dorsal telencephalic cortex and it resulted in reduced cortex. In addition, Otx2 have been shown to be involved in the early specification of

rostral neuroectoderm (Simeone et al., 1993; Frantz et al., 1994b). Otx2, Sox2 and Emx2 are expressed in progenitors at early stages of corticogenesis and continue to be expressed in young progeny of layer 6. Otx2 mutant loss-of-function has identified the requirement of Otx2 at earlier stages in the visceral endoderm as shown by the impaired axial mesoendoderm (Frantz et al., 1994b; Leingartner et al., 2003; Bani-Yaghoub et al., 2006). A candidate regulator of late-born cortical fates is Satb2, which is expressed in later progenitor cells producing neurons that populate the superficial layers. Loss-of-function studies have shown that Satb2 is required for proper axon projection patterns in the superficial layer neurons, although it is less clear if Satb2 is required at the time of neuron production to specific the superficial layer fate (Mutch et al., 2009).

An excellent approach to identify candidate genes involved in temporal identity specification has been done by isolating different cortical neurons and analyzing their transcriptome (Arlotta et al., 2005). Fezf2 is one gene identified by this approach, with specific expression in the corticospinal projection neurons of layer 6 (Chen et al., 2005a; Molyneaux et al., 2005). Fezf2 mutant mice were capable of producing progeny that remain in the deepest layer but these neurons failed to form proper axonal projections (Hirata et al., 2004; Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005; Chen et al., 2008). Overexpression of Fezf2 in late progenitors (which normally make superficial layer neurons) results in production of neurons that have deep layer axonal projections, suggesting a temporal-fate transformation (Chen et al., 2005b; Chen et al., 2008).

Much of the current understanding of layer fate specification is based on transcription factor networks that effect layer-specific axon projections. Similar to Fezf2, it is not absolutely clear if these transcription factors are necessary and sufficient within the progenitor to determine temporal-specific fates, or if these transcription factors solely regulate axon projections in the post-mitotic neurons. For example, another gene involved in layer-specific axonal projections is *Ctip2* (Chen et al., 2008). The expression of Ctip2 is downregulated in Fezf2 mutants and phenotypes of neurons with Ctip2 loss-of-function are similar to that of Fezf2 loss-of-function. Ctip2 over-expression in Fezf2 loss-of-function background was capable of salvaging axonal projections of deep layer 5 and 6 neurons. The Sox5 transcription factor is expressed in layer 2/3 neurons and is required to down-regulate Ctip2. Sox5 loss-of-function experiments show that layer 2/3 neurons fate is altered (again based on axon projection patterns) and their timing of delamination from the neural progenitor is delayed, suggesting a temporal identity defect (Kwan et al., 2008; Lai et al., 2008). The final transcription factor known to regulate layer-specific axon projections is Satb2. Satb2 mutant mice ectopically-express Ctip2 in the upper layer progeny and these neurons fail to make proper axon projections (Alcamo et al., 2008; Britanova et al., 2008).

Due to technical limitations (particularly the lack of methods that allow neural progenitor-specific versus post-mitotic neuron-specific loss or miss-expression of these transcription factors), it is difficult to determine the exact stage at which Fezf2, Sox5, and Satb2 might alter cell fates. All of these factors have been shown to regulate Ctip2 expression and Ctip2 is a key regulator of axon projection patterns. Ultimately it will be

necessary to distinguish neuronal fate-specification from axon projection pattern phenotypes to identify transcription factors that are truly necessary and sufficient to determine temporal identity in the cerebral cortex.

Interestingly, cortical progenitor cells at late neurogenesis stages lose their competence to form deep-layer progeny and produce only superficial layer neurons (Walsh and Cepko, 1988). Heterochronic transplantation experiments using the developing cerebral cortex of ferrets as a model demonstrated this restriction of competence. Late-stage progenitor cells from developing ferret cerebral ventricular zone were removed on post-natal day 0 and labeled with H^3-thymidine. These progenitor cells were at the stage of forming neurons of the superficial layer 2/3 (McConnell, 1988; Jackson et al., 1989), and the cells had not yet passed through G2/M, ensuring they were competent to respond to extrinsic cues. Heterochronic transplantation of these "old" progenitor cells into embryonic host brains at early corticogenesis stages revealed that the old / late-stage progenitors had lost competence to respond to extrinsic cues and eventually made only donor-specific neurons. These experiments were repeated with progressively "younger" progenitor cells taken from the donor brain. This analysis revealed that progenitors from earlier stages of corticogenesis retained a higher degree of competence to produce host-specific neurons: progenitors from the very earliest stages of corticogenesis were competent to produce all layer fates, progenitors from intermediate stages of corticogenesis could produce some of the deep-layer fates, and progenitors from the latest stages of corticogenesis could only produce superficial-layer fates (Figure 2). This phenomenon is known as the "progressive restriction" of cortical progenitor fate

potential. The mechanisms responsible for this progressive restriction of competence have yet to be identified. Identification of the complete transcription factor networks that specify temporal layer fates and the associated regulators of progenitor competence is required to completely understand how cortical diversity is generated. For example, while Fezf2 appears to be a key determinant of early-born deep-layer fates, Fezf2 may be insufficient to "reprogram" late-stage progenitors to make deep-layer fates.

1.1.2 Retina

The mammalian retina is formed of seven major cell types. These cells are organized into three different layers. The outer nuclear layer is formed of rod and cone cells, the inner nuclear layer is formed of horizontal, bipolar, amacrine, and Muller cells, and finally the ganglion cell layer is formed solely of ganglion cells (Young, 1985b; Young, 1985a). Birth-dating analysis of the seven different cell types of the retina showed a stereotypic stratification of these progeny. They are grouped into early and late progeny cells. The early differentiating group includes the ganglion, horizontal, cone and amacrine cells. The late differentiating group includes the rod, bipolar and Muller cells last (Young, 1985b; Cepko et al., 1996; Chang and Harris, 1998; Hu and Easter, 1999). Both birth-dating and retroviral analysis showed that retinal progenitor cells are multipotent and capable of forming all seven progeny cell fates.

Ikaros, an ortholog of the *Drosophila* Hunchback transcription factor, has been shown to regulate temporal identity in retinal progenitors (Elliott et al., 2008). Ikaros is

expressed in early progenitors and early-born neurons and Ikaros loss-of-function reduces the ability of retinal progenitors to generate early-born cell types. Ikaros misexpression in late retinal progenitor cells showed that Ikaros was sufficient to alter fate specification and induce production of early retinal neurons, such as horizontal cells. Ikaros appears to at least partially control fate-specification by regulating Prox-1 expression in retinal progenitor cells. Prox-1 is necessary and sufficient for horizontal cell fate specification (Dyer et al., 2003).

As in the cerebral cortex, retinal progenitors undergo a temporally-regulated loss of competence. Heterochronic transplantation and co-culture analyses showed that early retinal progenitor cells are able to form donor-specific fates when cultured in an "old" niche, indicative of high competence in early developmental stages (Morrow et al., 1998a; Morrow et al., 1998b; Belliveau and Cepko, 1999; Rapaport et al., 2001). In contrast, late-stage retinal progenitors co-cultured with "young" niche cells had a limited competence to produce the early ganglion, horizontal, cone and amacrine cells (Ezzeddine et al., 1997). These studies have shown that retinal progenitors undergo a progressive restriction of competence to respond to extrinsic regulatory cues (Kageyama et al., 1997). The process is similar to the progressive restriction of competence that occurs in cortical progenitors, yet the corresponding regulatory mechanisms remain to be determined. Presumably the niche-specific extrinsic cues regulate expression of transcription factors that determine retinal fate, such as Ikaros. Interestingly, the *Drosophila* Ikaros ortholog Hunchback is known to regulate temporal identity during *Drosophila* neurogenesis (described in section 1.2 below). This suggests an evolutionary

conservation of mechanisms that control temporal cell fate-specification in neural progenitors.

1.1.3 Transition from neurogenesis to gliogenesis

In addition to the temporal cell fate transitions described above, mammalian central nervous system progenitors also undergo a temporally-regulated switch from production of neurons to production of glia (Hirabayashi and Gotoh, 2005; Miller and Gauthier, 2007). This neurogenic to gliogenic transition is largely regulated by Wnt signaling (Hirabayashi et al., 2004). Wnt signaling induces transcription of the *neurogenin1 (Ngn1)* and *neurogenin2 (Ngn2)* genes that encode transcription factors necessary for neuronal fates (Israsena et al., 2004). The progenitor switch from neurogenesis to gliogenesis requires downregulation of Ngn1 and Ngn2 expression and the upregulation of expression of glial fate-determining genes (Wen et al., 2009). However, Wnt levels do not change during this transition and the Wnt pathway is still activated in the neural progenitors at the time of transition to gliogenesis.

Analysis of the role of polycomb group complexes (PcGs) showed that these proteins restrict neurogenic competence during the transition from neurogenesis to astrogliogenesis (Hirabayashi et al., 2009). PcG loss-of-function prolonged the neurogenic phase and delayed onset of astrogliogenic phase. PcG complexes are chromatin remodeling factors that generally act to silence transcription of target genes. PcG activity at the Ngn1 locus is necessary and sufficient to block the ability of the β-

catenin / TCF transcription complex (which is activated by the Wnt pathway) to induce transcription of the neurogenin gene. This study demonstrated a role for temporally-regulated chromatin remodeling in restricting neural progenitor competence and inducing a cell fate transition. My dissertation work has tested the role of PcG proteins in regulating temporal identity and competence during *Drosophila* neurogenesis, and I discovered many parallels with PcG function in the regulation of neurogenesis versus gliogenesis in mammals (data presented in chapter 2). In the following sections I describe what is known about temporal identity and competence in the *Drosophila* nervous system followed by a review of PcG complex function.

1.2 Temporal Identity during *Drosophila* Neurogenesis

Drosophila and mammals share several aspects of neurogenesis, including spatial and temporal regulation of cell fate specification (Egger et al., 2008). Studies in the mammalian central nervous system are often hindered by the vast complexity and number of neurons, the lack of progenitor and progeny cell markers, and technical difficulties in tracing the development of single progenitor cell lineages. In contrast, the *Drosophila* central nervous system is a powerful tool to study temporal specification of cell fate during neurogenesis since many relevant transcription factors are known and multiple techniques allow manipulation and analysis of a single progenitor cell and its progeny (Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003; Pearson and Doe, 2004; Grosskortenhaus et al., 2005; Kanai et al., 2005; Cleary and Doe, 2006; Grosskortenhaus et al., 2006; Maurange et al., 2008).

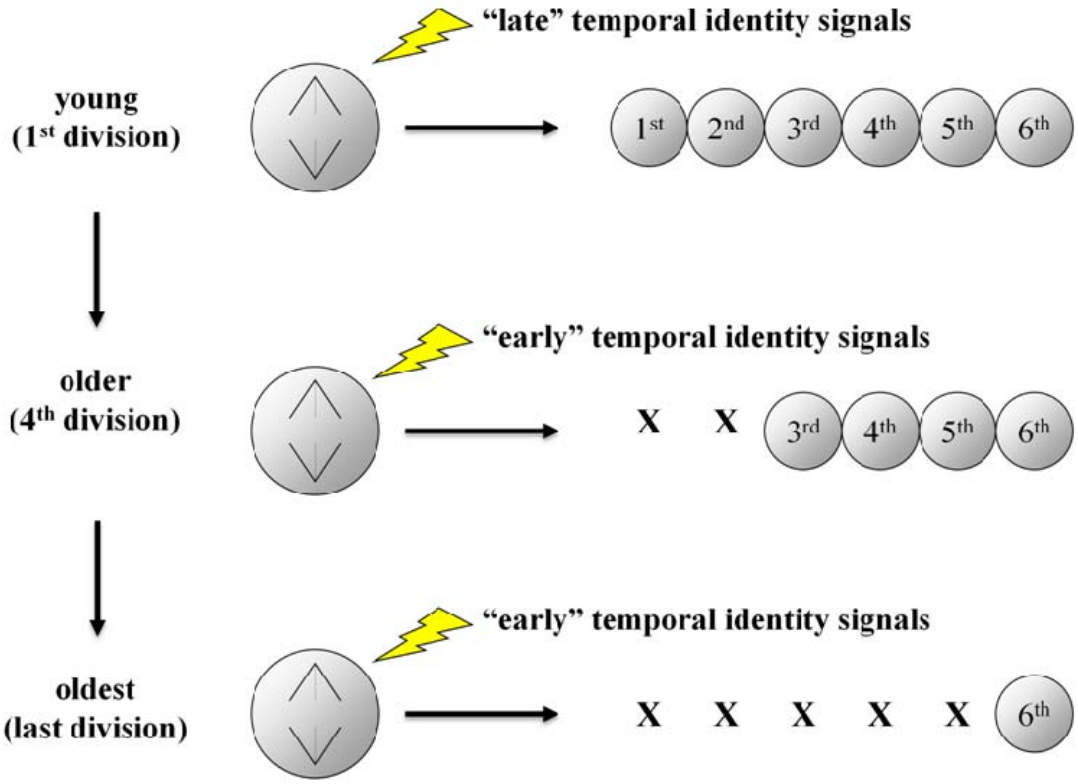

Figure 2: Heterochronic transplantation experiments in the mammalian cerebral cortex showed that competence to make specific layer fates is temporally restricted. Early neural progenitors transplanted to late-development stage embryos were capable of forming all six layers. In contrast, late-development progenitors transplanted into early-development embryos were limited to producing donor-specific "late-born" fates. These data indicate that cues that induce different laminar fates change over time and that the progenitors undergo a restriction of fate potential to respond to these cues.

1.2.1 *Drosophila* Neuroblast Identity

The *Drosophila* central nervous system is populated by neural progenitor cells named neuroblasts (NBs). The central nervous system is formed of the brain and the ventral nerve cord. The *Drosophila* ventral nerve cord (VNC) is segmented along the anterior-posterior axis. During gastrulation, cells from the neuroectoderm are specified to become neuroblasts by a complex set of regulatory cascades that requires both extrinsic signaling molecules and intrinsic genetic program transitions (Campos-Ortega, 1994; Campos-Ortega, 1995; Skeath, 1999). Neuroblasts exit the neuroectoderm through a process known as delamination. They delaminate dorsally and reside in a sub-ectodermal proliferative zone (Hartenstein, 2006). In the proliferation zone, neuroblasts undergo asymmetric divisions that ensure their self-renewal and result in a smaller ganglion mother cell (GMC). GMCs in turn divide once to produce either neurons or glia as seen in Figure 3 (Fuerstenberg et al., 1998). Neurons of the first born GMC are the deepest in the embryo whereas neurons produced at later stages reside close to their respective neuroblast. Spatial patterning and temporal patterning dictate the fate of *Drosophila* neuroblasts (Urbach and Technau, 2003). As a result, progenitor cells can be identified based on their time of delamination from the neuroectoderm and the position they acquire in each hemisegment (Figure 4). For example Neuroblast 3-1 (NB3-1) is located in the third row and first column and Neuroblast 7-1 (NB7-1) is positioned in row 7 and column 1. Each neuroblast has a unique code of gene expression pattern that has helped in identifying different lineages (Figure 4) (Doe, 1992; Broadus and Doe, 1995).

DiI labeling has identified the progeny produced by all thirty neuroblasts in each hemisegment, distinguishing motorneurons, interneurons and glia (Bossing et al., 1996; Schmid et al., 1999). However, these lineage tracing experiments do not reveal the birth-order of neurons within each lineage. Studies of birth order during *Drosophila* embryonic neurogenesis showed that a series of transcription factors are sequentially expressed in neuroblasts during their initial divisions. These transcription factors are known as temporal transcription factors (TTFs) and are expressed in neuroblasts in the following order; Hunchback (Hb) ➔ Kruppel (Kr) ➔ Pdm ➔ Castor (Cas) (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001). As neuroblasts divide asymmetrically, they express each gene transiently, switching to express the next gene in the cascade after each division. In contrast, the daughter GMCs born during each window of TTF expression maintain the expression of that TTF, as do the differentiated neurons and glia produced by that GMC. Therefore, the first to second-born progeny of nearly all neuroblast lineages express Hb, the second to third-born progeny express Kr, the third to fourth born progeny express Pdm, and the fourth to fifth-born progeny express Cas (Figure 5). This temporal pattern of gene expression suggests that there is an internal "clock" that neuroblasts follow irrespective of their position in each hemisegment (Figure 4).

Mutant and misexpression studies have shown that Hunchback is necessary and sufficient for first-born cell fates, whereas Kruppel is necessary and sufficient for second-born cell fates (Isshiki et al., 2001; Cleary and Doe, 2006). This is true in multiple neuroblast lineages and is independent of the cell type produced (Figure 5). Analysis of

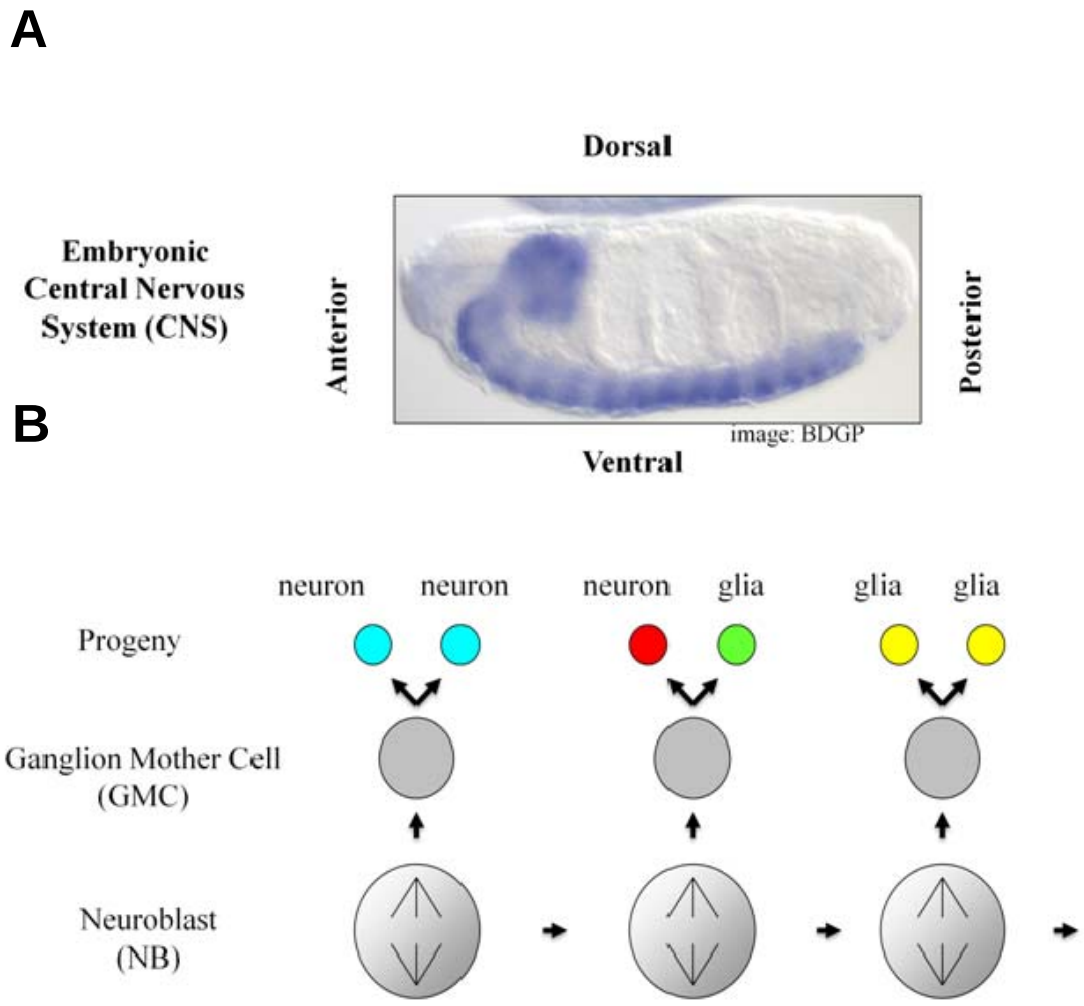

Figure 3: *Drosophila* embryonic central nervous system development. (A) A lateral view of the *Drosophila* embryonic central nervous system, with the brain at the anterior end and the nerve cord along the ventral surface. (B) Neuroblasts divide asymmetrically and form ganglion mother cells that in turn divide once and form neurons, glia or both classes of progeny cell.

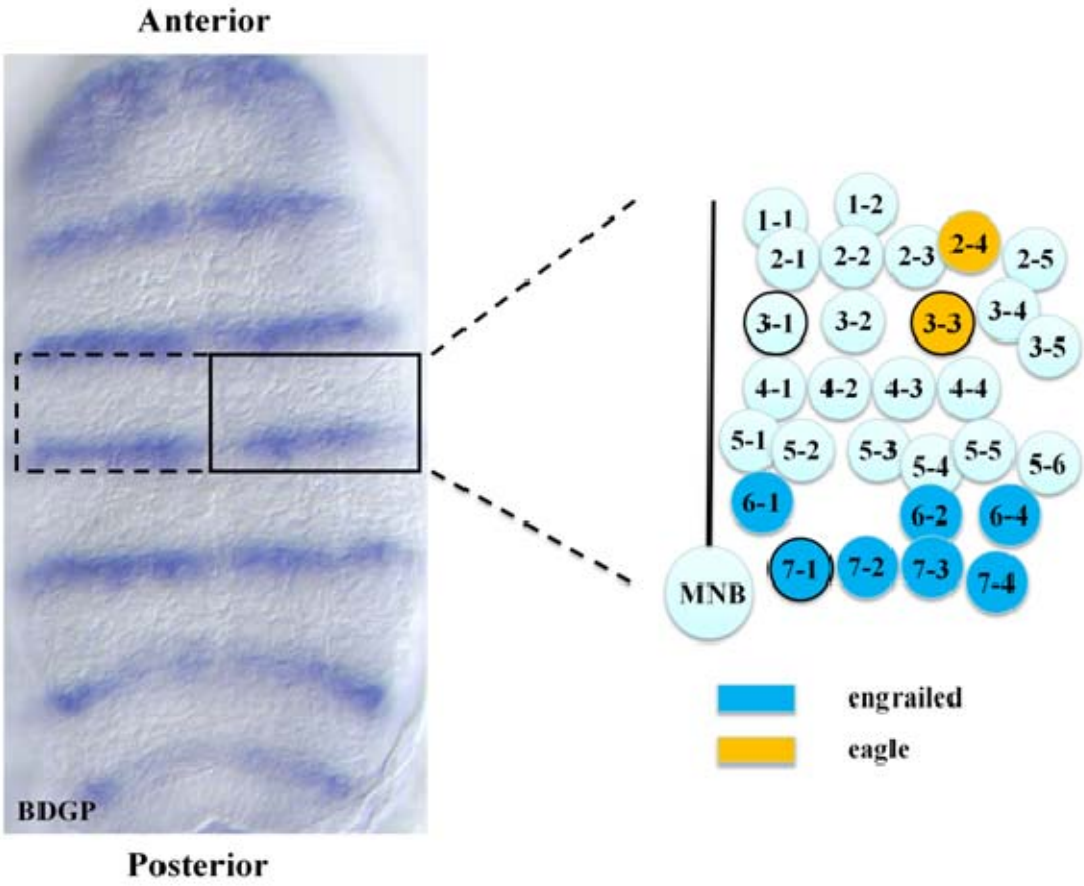

Figure 4: A ventral view of a *Drosophila* embryo on the left. Each segment is divided medially into two hemisegments. Each hemisegment contain 30 neuroblasts with invariant position. Neuroblasts are identified by the transcription factors and other proteins they express. For example, Engrailed is expressed in neuroblasts in row 6 and 7 and Eagle is expressed in neuroblasts 2-4 and 3-3.

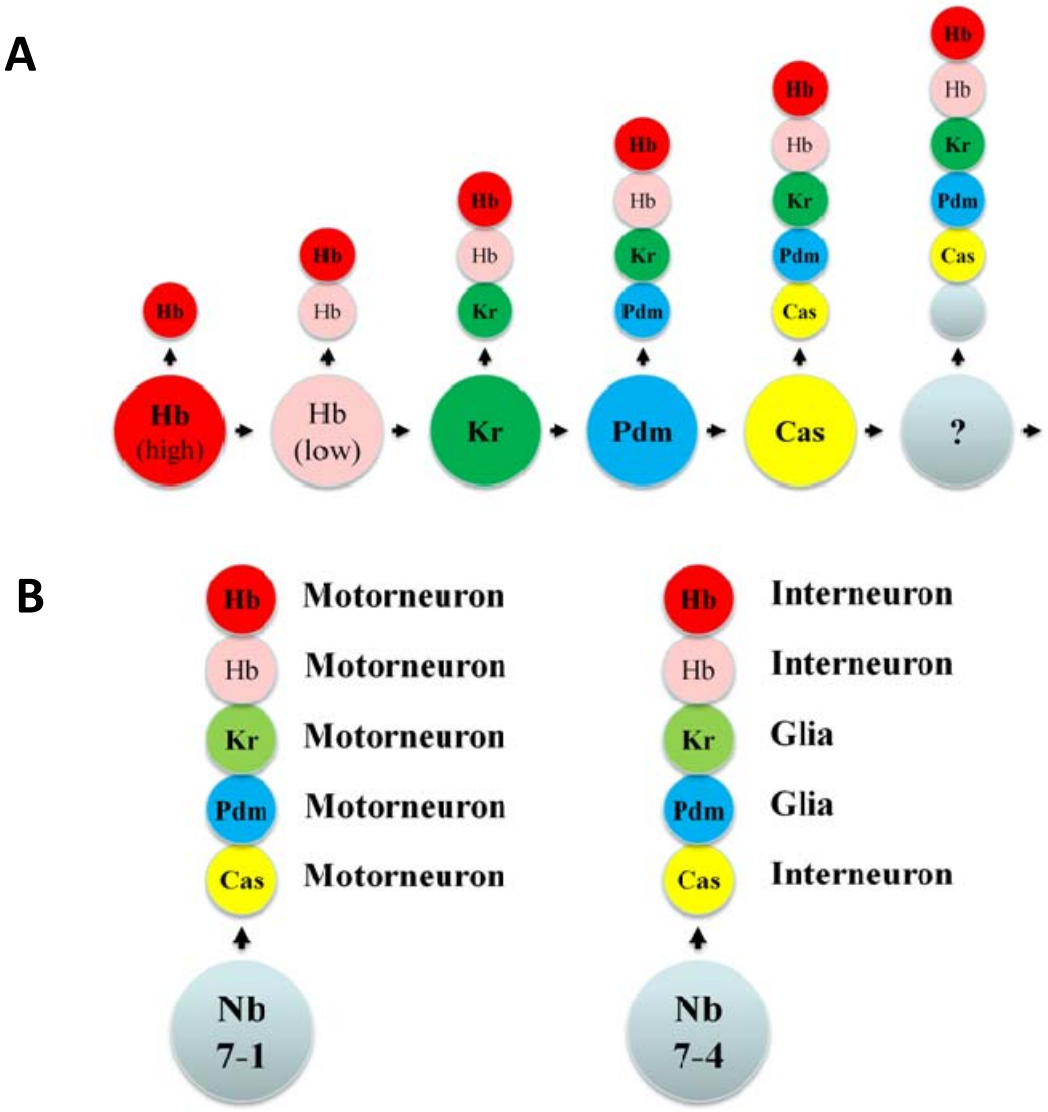

Figure 5: Neuroblast temporal identity. (A) All neuroblasts sequentially express four transcription factors: Hunchback (Hb), Kruppel (Kr), Pdm and Castor (Cas). Neuroblasts express these transcription factors transiently. In contrast, progeny cells formed within a given temporal window constitutively express the corresponding temporal transcription factor. (B) These temporal transcription factors specify the temporal order of progeny formed irrespective of the cell type formed. Hunchback in NB7-1 and NB7-4 are responsible for the formation of the first-born and second-born progeny, however Hb specify motorneuron fate in NB7-1 and interneuron fate in NB7-4.

an early forming neuroblast, NB7-1, in comparison with two late-forming neuroblasts, NB2-4 and NB7-3, showed a remarkable conservation of temporal transcription factors expressed from Hb ➔ Kr ➔ Pdm ➔ Cas (Isshiki et al., 2001). In NB7-1, the first five divisions produce motorneurons (the "U" motorneurons) and the subsequent divisions produce interneurons. U motorneurons can be uniquely identified using immunofluorescent confocal microscopy as seen in Figure 6. Hunchback loss-of-function experiments showed that the U1/U2 motorneurons are lost whereas the rest of the progeny continue to be formed at their correct times. In contrast, driving the expression of ectopic Hb throughout all the NB7-1 divisions showed that all the progeny formed switch their fate to U1/U2 motorneurons (Isshiki et al., 2001). Kruppel loss-of-function has similar effects, the third motorneuron U3 is lost whereas the first born U1/U2 and the later formed U4 and U5 motorneurons are formed. Continuous ectopic expression of Kruppel caused extra U3 cell production and absence of U4 and U5 fates, while the Hb-specified U1/U2 motorneurons were unaffected (Isshiki et al., 2001).

The timing of TTF expression is at least partly determined by regulatory interactions among these transcription factors (Isshiki et al., 2001). Prolonged expression of Hb activates Kr expression and represses Pdm and Cas expression. Kruppel over-expression activates Pdm expression but represses Cas expression. Additional analyses of the regulatory circuit have shown that Pdm over-expression activates Cas expression (Brody and Odenwald, 2000). In summary, each TTF activates transcription of the next TTF gene in the sequence while repressing the TTF gene two steps forward (the "next plus one") in the sequence. This model is summarized in Figure 7.

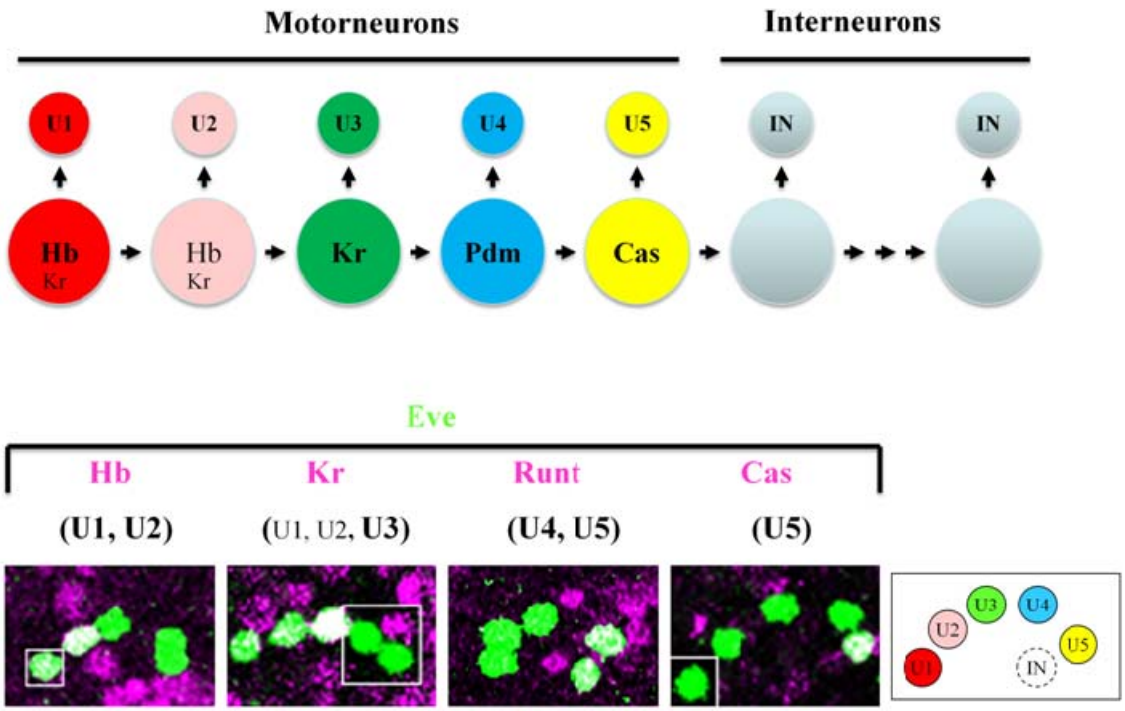

Figure 6: Neuroblast NB 7-1 produces "U" motorneurons that can be identified using immunofluorescent confocal microscopy. U motorneurons of neuroblast NB7-1 can be identified by position and a combination of antibody staining for the Even-skipped (Eve) transcription factor, additional transcription factors like Runt, and the individual TTFs.

Studies on how the temporal gene cascade progression is controlled have identified two stages; a cytokinesis-dependent step responsible for the down-regulation of Hunchback expression by cell division and a cytokinesis-independent step established by feed-forward and feed-back loops between the temporal transcription factors. Downregulation of Hb expression following the first or second NB division has been shown to be a cytokinesis-dependent step. The use of *pebble* mutants allowed the neuroblast to go through cell cycle events but hindered cytokinesis (Cui and Doe, 1995; Weigmann and Lehner, 1995; Prokopenko et al., 2000; Isshiki et al., 2001) and it was found that Hb expression is not downregulated in pebble mutants. In contrast, *string* mutant neuroblasts are G2 arrested and therefore lacking both cytokinesis and cell cycle events (Edgar and O'Farrell, 1989). Interestingly, Hb loss-of-function in *string* mutants allowed for progression through Kr → Pdm → Cas expression independent of the cell cycle, indicating that these transitions are regulated by other mechanisms, potentially the temporal identity factors themselves (Grosskortenhaus et al., 2005).

Multiple regulatory interactions between the TTFs have been identified at the genetic and molecular level. For example, Hb represses Pdm by binding to the cis-regulatory elements within the pdm1/2 enhancer region (Kambadur et al., 1998). Hb loss-of-function causes early Pdm expression in neuroblasts. In addition, Cas loss-of-function causes prolonged Pdm expression (Kambadur et al., 1998; Grosskortenhaus et al., 2006), revealing a negative feedback loop. The intricate control of the temporal gene cascade still requires further investigation, particularly considering the fact that each TTF is not necessary to activate the next TTF in the cascade. For example, Kr is still expressed in

Hb mutants (there is a slight delay in timing of expression) and Pdm is still expressed in Kr mutants (again, with a slight delay in timing) (Isshiki et al., 2001). These results suggest that additional regulators control the temporal transcription factor "clock". One known additional regulator is the orphan nuclear receptor Seven up (Svp). Initially, *svp* mRNA is localized in the nucleus but after the first mitotic division *svp* mRNA gets localized to the cytoplasm, allowing it to be translated. Svp protein then functions as a repressor of Hb transcription, ensuring Hb expression is shut-off after the initial NB divisions (Figure 7).

1.2.2 Restriction of Neuroblast Competence

Progenitor competence can be measured by the ability of a progenitor to respond to factors that specify distinct fates. Similar to the phenomena identified in mammalian cortical progenitors, *Drosophila* neuroblasts progressively lose competence to respond to TTFs, providing an excellent model to study this process.

Initial studies of neuroblast competence tested whether NB7-1 could respond to Hb and produce U1 or U2 motorneurons throughout neurogenesis (Pearson and Doe, 2004). Constitutive expression of high levels of Hb was sufficient to induce U1 fates at essentially all divisions. If ectopic expression of Hb was delayed until after the third division (when Hb has normally shut-off), the neuroblast had limited competence to respond to Hb: high levels of Hb induced U1 cells and low levels induced U2 cells, but not throughout all divisions. To precisely define the time at which competence to

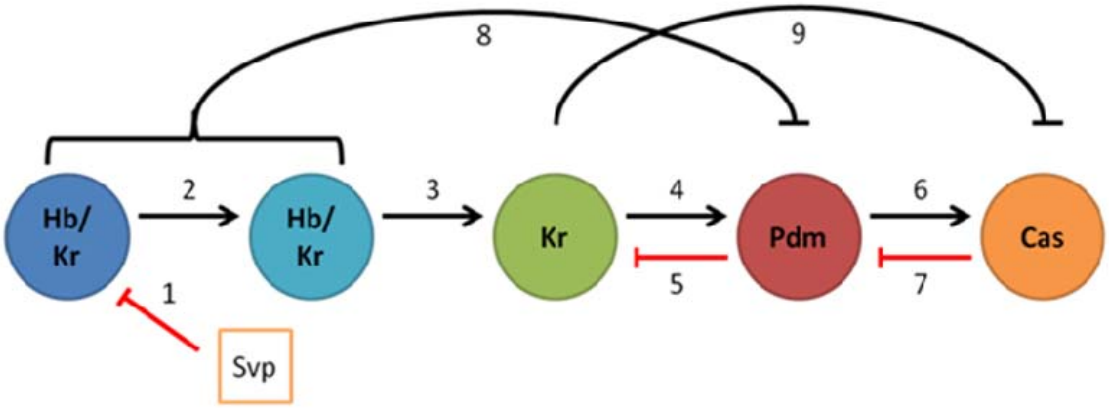

Figure 7: Summary of temporal identity regulation pathways.

1. Svp regulates Hb (Kanai et al. 2005; Mettler et al. 2008).

2. Cytokinesis results in low Hb level in the neuroblast (Grosskortenhaus et al., 2006).

3. Hb gain-of-function results in ectopic Kr expression (Isshiki et al. 2001).

4. Hb loss-of-function results in delayed Pdm expression and Hb gain-of-function leads to precocious Pdm activation (Isshiki et al. 2001).

5. Pdm loss-of-function results in a delayed Kr down regulation and Pdm gain-of-function result in Kr repression (Tran et al. 2008, Grosskortenhaus et al. 2006).

6. Pdm loss-of-function results in delayed Cas expression in NB3-1, but not in NB7-1 (Grosskortenhaus et al., 2006; Tran and Doe, 2008). Pdm loss-of-function results in precocious Cas activation in NB 7-1/NB3-1 (Grosskortenhaus et al., 2006; Tran and Doe, 2008).

7. Cas loss-of-function results in an extended Pdm expression window (Kambadur et al.1998; Isshiki et al. 2001; Grosskortenhaus et al. 2006).

8. Hb represses the expression of Pdm (Isshiki et al. 2001).

9. Kr loss-of-function results in precocious Cas expression (Isshiki et al. 2001).

respond to Hb is lost, ectopic pulses of Hb expression were induced via a heat shock promoter at specific times in the development of the NB7-1 division. These experiments revealed a high degree of competence at the first and second divisions, decreased competence at the third and fourth division (conversion to U1/2 fates was rare), very little competence at the fifth division, and a complete loss of competence after the fifth division. These experiments demonstrated the "progressive" restriction of competence and suggest that after transitioning to interneuron production (at the sixth division), changes occur in the neuroblast that completely block the ability of Hb to induce motorneuron fates (Pearson and Doe, 2003).

Further analysis of the regulation of neuroblast competence showed that multiple temporal identity factors are able to specify distinct neuronal fates within a single early competence window (Cleary and Doe, 2006). This study showed that similar to Hunchback, neuroblasts also lose competence to respond to Kruppel after the fifth division. Neuroblast 7-1 was also shown to lose competence to respond to Pdm and Castor after 5 to 7 divisions. However, the precise timing of loss of competence for Pdm and Cas remains to be determined since the experiments in this study used transient Kr-overexpression to delay Pdm and Cas expression and did not directly assay competence to respond to ectopic Pdm or Cas induced at specific neuroblast divisions. These studies were significant because the discovery of a common "early-fate" competence window supports a model in which a wide-ranging mechanism (such as chromatin remodeling of multiple target genes) regulates competence, as opposed to a TTF-specific mechanism establishing a distinct competence state for each transcription factor.

The importance of neural progenitor competence transitions has been clearly established in both *Drosophila* and mammals, yet relatively little is known about how such transitions are regulated. My thesis work has aimed to identify the mechanistic basis of competence transitions in neuroblasts. In Chapter 2, I present my findings that Polycomb group complexes regulate competence in neuroblasts, similar to the regulation of neurogenic versus gliogenic competence in mammalian neural progenitors. In the following section, I briefly review the molecular basis of Polycomb complex regulation of transcription and I discuss some of the major functions of Polycomb complexes during development.

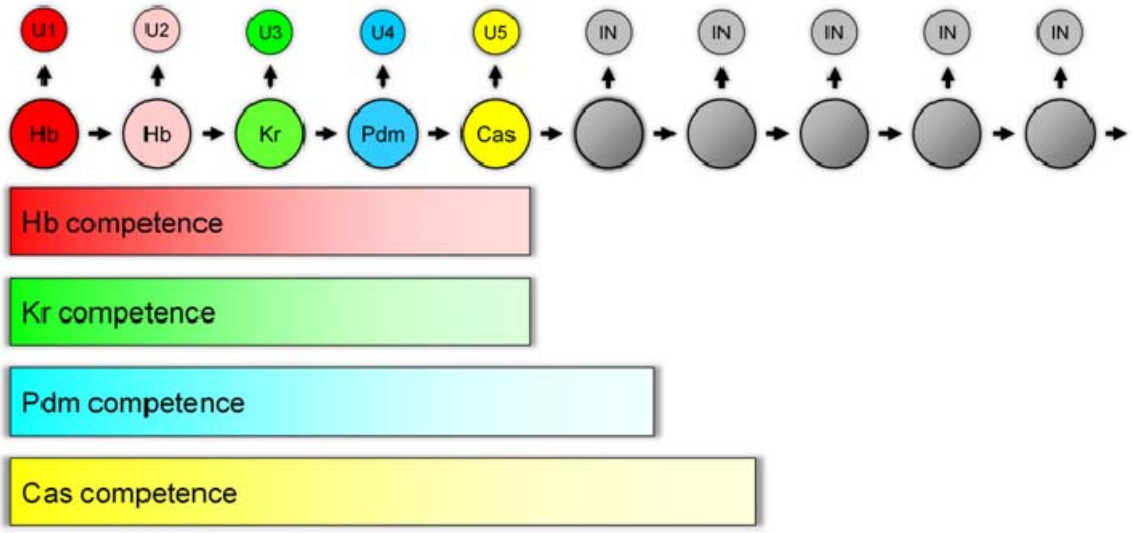

Figure 8: Competence ”windows” of temporal transcription factors in NB 7-1. A single early competence window restricts motorneuron fate potential in NB7-1. Hb and Kruppel have the same competence window limited to 5 divisions. Pdm and Cas have a similar early competence window, potentially extending to the sixth and seventh divisions.

Section 2: Polycomb Repressor Complex Function during Development

2.1. Chromatin Remodeling

Chromatin remodeling is a highly dynamic process that enables genes to be expressed or repressed through the control of transcription factor access to target loci. One group of chromatin remodeling factors catalyzes enzymatic covalent histone modifications. The result of dynamic regulation of the chromatin state is the epigenetic control of many biological mechanisms such as organismal development and stem cell pluripotency and differentiation (Srivastava et al., 2010). Dysfunction of chromatin remodeling proteins has been linked to diseases such as cancer (Pasini et al., 2004; Valk-Lingbeek et al., 2004; Greer and Shi, 2012).

DNA is organized in a highly compacted form within the cell nucleus. DNA wraps around a group of conserved protein subunits known as histones to form the basic subunit of chromatin, the nucleosome. Four histone subunits form the nucleosome octameric complex core. The nucleosome is formed of H2A, H2B, H3 and H4 protein subunits and is wrapped by 146 nucleotide base pairs (Bryan et al., 1979; Simpson and Kunzler, 1979). The H1 histone subunit condenses the nucleosomes, further compacting chromatin (Cole, 1984). Histone subunits of the nucleosome have 25-40 amino acids. The amino-terminal tail region of histones is the target of the enzymatic post-mitotic modifications. Histone covalent modifications can either tighten the histone to DNA interaction and hinder the accessibility of transcription factors or loosen the nucleosome structure and facilitate transcription factor binding to target genes.

One group of enzymes involved in histone modifications are histone acetylases. Histone acetylation is highly dynamic and results in chromatin remodeling. There are two groups of enzymes that regulate histone acetylation. The first group is the histone acetyltransferases (HATs) that act to antagonize the action of the second group known as histone deacetylases (HDACs) (Kuo and Allis, 1998; Ito et al., 2000). Histone acetyltransferases catalyze the transfer of an acetyl group to the lysine side chains. As a result, this acetyl addition leads to a loose conformational change in the histone to DNA interaction and allows for gene expression. On the other hand, HDAC enzymes remove acetyl groups from the lysine residue and this results in chromatin stabilization (Roth et al., 2001; Grozinger and Schreiber, 2002).

A second type of histone remodeling involves histone phosphorylation (Davie and Spencer, 1999). Kinase and phosphatase enzymes carry the addition and removal of phosphate molecules to serine, threonine and tyrosine amino acids in histone tails. Histone kinases catalyze the transfer of a phosphate group from ATP to its target amino acid residue and this addition results in tightening the histone to DNA interaction. On the other hand, histone phosphatases antagonize the effect of histone kinases and allow the expression of the target genes (Hsu et al., 2000). Additional histone modifications also contribute to chromatin remodeling (Jenuwein and Allis, 2001). Deamination, ADP ribosylation, ubiquitylation and SUMOylation are a few other examples of histone modifications. Deamination results in the conversion of arginine to citrulline and decreases the transcriptional expression at target loci. ADP ribosylation adds multiple ADP subunits on glutamate and arginine residues and induces a more relaxed chromatin

state. Ubiquitylation and SUMOylation histone modifications result in a covalent modification of the histone tail. Both ubiquitylation and SUMOylation have been linked to repressed chromatin states (Gill, 2004).

Histone methylation by Polycomb Group proteins is another major chromatin remodeling process (Schwartz and Pirrotta, 2008). My thesis work has focused on the role of Polycomb group complexes in neurogenesis and I review the molecular biology of these complexes and some of the relevant developmental processes affected by Polycomb group complexes in the following sections.

2.2 Polycomb Group Proteins Repressor Activity

Polycomb group proteins function within at least three different complexes that work synergistically. These protein complexes are polycomb repressive complex 2 (PRC2), polycomb repressive complex 1 (PRC1), and PhoRC polycomb proteins.

2.2.1 Polycomb Repressive Complex 2 (PRC2)

PRC2 is a large multi-subunit protein complex composed of four core subunits: Enhancer of zeste (E(z)), Suppressor of zeste 12 (*Su(z)12*), Extra sex combs (Esc) and Nurf55 (Nucleosome remodeling factor 55) (Table 1). E(z) contains a SET (Suvar3-9, Enhancer of zeste, trithorax) protein domain that has histone methyltransferase activity specific to histone 3 Lysine 27 (H3K27) (Cao et al., 2002; Czermin et al., 2002;

Kuzmichev et al., 2002; Muller et al., 2002). These core proteins are required for nucleosome binding and H3K27 methylation by the PRC2 complex (Schwartz and Pirrotta, 2008). In *Drosophila*, this core complex has been purified along with other protein components. One Polycomb Group protein identified is Polycomb-like (Pcl). This protein is required for high levels of H3K27 trimethylation in *vivo* (Nekrasov et al., 2007). Studies in mammals identified three complex proteins, PRC2, PRC3 and PRC4, as homolog complexes to *Drosophila* PRC2 complex. The mammalian PRC2 homologs also share the histone methyltransferase activities. However, these different PRC2 homologs have different characteristics. PRC2, PRC3, and PRC4 contain different EED (Embryonic Ectoderm Development) isoforms (Montgomery et al., 2007). EED is the mammalian homolog of *Drosophila* Esc. Mammalian PRC2 and PRC3 have a preference to catalyze H1 histone protein methylation, favoring stabilization of chromatin structures. While PRC2 and PRC3 appear to function in all cells and tissues, PRC4 has a unique role in undifferentiated embryonic stem cells (ESCs). PRC2 contains the largest EED isoform, Eed1 (Kuzmichev et al., 2005). In contrast, PRC3 contains two smaller isoforms of Eed, Eed3 and 4 (Kuzmichev et al., 2002). Interestingly, PRC4 contains the Eed2 isoform and is only expressed in undifferentiated pluripotent cells (Kuzmichev et al., 2005). The importance of this finding is that subunit diversity may correlate with distinct biological functions and tissue-specific activities, rejecting long-standing hypotheses that PRC2 and related complexes were homogeneous in their expression patterns and function.

2.2.2 Polycomb Repressive Complex 1 (PRC1)

Drosophila PRC1 studies revealed that it contains four core subunits. PRC1 consists of Polycomb (Pc), Polyhomeotic (Ph), Ring and Posterior sex combs (Psc) (Table 1). Pc contains a chromodomain protein that can bind to trimethylated lysine 27 of histone H3 (H3K27me3) code (Cao et al., 2002). Ph contains a zinc finger protein. Ring contains the catalytic subunit for histone H2A ubiquitylation. Finally, Psc contains a RING domain protein that enhances the catalytic activity of Ring (Schwartz and Pirrotta, 2008). As discussed for PRC2, PRC1 purification also revealed many proteins to be incorporated with PRC1 core components. In *Drosophila*, TATA-box-binding protein associated factors (TAFS) (TAFII250, TAFII110, TAFII85, and TAFII62), strongly suggesting that PRC1 directly affects the activity of the RNA polymerase II holoenzyme (Breiling et al., 2001; Saurin et al., 2001). *Drosophila* PRC1 homologs have also been identified in mammals (Levine et al., 2002). In both flies and mammals, PRC1 enzymatic activity occurs via the RING proteins. RING is an E3 ubiquitin ligase that mono-ubiquitylate lysine 119 of histone H2A, thus stabilizing the repression of transcription (Wang et al., 2004). In mammals, the PRC1 core is formed of Ring1A/B, *Drosophila* PSC orthologs (Mel-18, Bmi1, or NSPC1), *Drosophila* Ph orthologs (Phc1, Phc2, or Phc3), and *Drosophila* Pc orthologs (Cbx2, Cbx4, Cbx6, Cbx7, or Cbx8) (Morey and Helin, 2010). The mammalian Pc orthologs, Cbx proteins, have been identified to be involved in stabilizing the interaction of PRC1 at the level of chromatin through direct interaction with the H3K27me3 mark (Bernstein et al., 2006b; Buchwald et al., 2006).

2.2.3 The PhoRC Complex

The third well characterized polycomb complex is the PhoRC complex. The significance of PhoRC is that it can bind directly to DNA (Brown et al., 1998). A closely related protein is the mammalian factor Yin-Yang 1 (YY1). In *Drosophila*, PhoRC complex contains the Pho protein PHO (Table 1) (Kwon and Chung, 2003). PHO is colocalized with PRC1 and PRC2 protein complexes on chromosomes but is not essential for their activities. PHO is also associated with INO80, an ATP-dependent chromatin remodeling complex. This complex is involved in many DNA-dependent processes such as transcription, DNA repair and DNA replication (Conaway and Conaway, 2009). The relationship between INO80 and PcG activity is not yet identified.

2.3 Trithorax-group proteins

Trithorax Group proteins are involved in antagonizing Polycomb Group protein gene silencing (Kennison and Tamkun, 1988). Trithorax group proteins are involved in maintaining active gene expression profiles in *Drosophila*. TrxG and PcG proteins have some complimentary characteristics. TrxG proteins are a heterogeneous group of proteins. There are a vast number of Trithorax group proteins. They can be classified according to their main molecular features. A group of Trithorax proteins contain SET domains. An example of SET domain containing Trithorax proteins in *Drosophila* are Trx and Ash1 and the vertebrate MLL (mixed lineage leukemia) (Yu et al., 1995). Mll is important in human disease, since Mll mutations have been identified in aggressive

human B and T lymphoid tumors and acute myeloid leukemia (AML) (Tenney and Shilatifard, 2005). Another class of TrxG factors is capable of chromatin remodeling using ATP. ATP-dependent chromatin remodeling complexes like SWI/SNF and NURF complexes counteract PcG repression and have been identified to have a role in the suppression of PcG-mediated homeotic transformations (Kennison and Tamkun, 1988).

2.4 Polycomb Complex Recruitment to Target Genes through Polycomb Response Elements

Polycomb and Trithorax group factors target specific elements on the genome (Ringrose and Paro, 2004; Ringrose and Paro, 2007). These DNA elements are called Polycomb Response Elements (PREs). In *Drosophila*, PcG proteins repress their target genes following recruitment to specific PREs (Chan et al., 1994; Bloyer et al., 2003; Muller and Kassis, 2006). There are several DNA-binding proteins that targets PREs. Pleihomeotic (PHO), Pleihomeotic-like (PHOL), GAGA factor (GAF), Pipsqueak (PSQ), Zeste and DSP proteins are capable of targeting binding sites in PREs and affecting chromatin states by recruiting PRC2. Additional proteins that can bind to PREs include CtBP, Grainyhead (GRH) and the Sp1/KLF group proteins. Alternative combinations of different sequence elements in each PRE locus are thought to account for the specificity of PREs and subsequent context-dependent PcG activity. Chromatin immunoprecipitation analysis in vertebrates identified several PcG binding sites (Bracken et al., 2006). These DNA element sites have been identified as both necessary and sufficient for the recruitment of PcG complexes to promoter regions of their target genes.

A mammalian PRE has been shown to be necessary and sufficient for segment specific gene expression in the developing mouse hindbrain (Sing et al., 2009), establishing a pattern similar to the PcG-dependent anterio-posterior segmentation of *Drosophila* embryos.

Drosophila homologs	Mammal homologs	Protein domains
E(z)	EZH1, EZH2	SET
Esc	EED	WD40
Su(z)12	SUZ12	Zinc finger
NURF55	RpAp48, RpAp46	WD40
HP1	HP1	Chromodomain
Psc, Su(z)2	Bmi1, zfp144, mel18	RING finger
Pc	Cbx	Chromodomain
Ph-p, ph-d	HPH1, HPH2, Mph1, Raet8	Zinc finger
Pho	YY1	Zinc finger
Brm	Brg1	DNA helicase
Trx	Mll	DNA methyltransferase, SET

Table 1: Vertebrate homologues of *Drosophila* PcG factors (Gould, 1997; Schumacher and Magnuson, 1997; Satijn and Otte, 1999).

2.5 Polycomb and Trithorax Group Protein Activity in Cell Fate Specification

2.5.1 Maintenance of Pluripotency and Regulation of Differentiation in Mammalian Embryonic Stem Cells

Genome-wide mapping of Polycomb and Trithorax target genes in mammalian embryonic stem cells identified genes encoding regulators of differentiation and cell fate-commitment as targets (Bernstein et al., 2006a). Importantly, this study showed the existence of a "bivalent" chromatin mark on target genes that are involved in key developmental processes. These genes contain both the Polycomb group trimethylated H3K27 mark and the Trithorax group trimethylated H3K4 marks (Barski et al., 2007). This suggests that the bivalent state allows repression of these target genes in the pluripotent stem cell while allowing induction of transcription upon commitment to a specific developmental pathway. For example, stem cell genes that favor self-renewal and proliferation are characterized by decreased H3K27me3 marks and lower levels of targeting by the PcG proteins (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). Loss of PcG function in embryonic stem cells causes developmental defects. For example, Ezh2 loss-of-function mutation in blastocysts resulted in impeded embryonic stem cells growth (O'Carroll et al., 2001). EED loss-of-function caused early differentiation due to the loss of self-renewal capacity (Boyer et al., 2006). Interestingly, genome-wide analyses have shown that there is an overlap between the target genes repressed by PcG proteins and the "Yamanaka" factors, a group of transcription factors (OCT4, SOX2 and NANOG) that are required for stem cell pluripotency (Takahashi and Yamanaka, 2006). Little is known about the interactions between Yamanaka factors and

Polycomb proteins. It is possible that OCT4, SOX2 and NANOG factors establish the transcriptional pattern required for pluripotency, including repression of differentiation genes, and the Polycomb group proteins act to maintain this repression.

2.5.2. Maintenance of Pluripotency and Regulation of Differentiation in *Drosophila*

Genome-wide analysis in *Drosophila* identified Polycomb target genes encoding transcriptional regulators, receptors, cell signaling proteins and morphogens involved in critical pathways during development, findings that are very similar to the PcG targets identified in mammalian stem cells (Schwartz et al., 2006; Oktaba et al., 2008; Schuettengruber et al., 2009). Studies in *Drosophila* and mammals showed that the bivalent histone marks found at developmental regulators are unique to mammals (Schwartz and Pirrotta, 2008).

While PcG-dependent modifications generally associate with repression of transcription across the *Drosophila* genome, there are exceptions in which the H3K27me3 mark is not sufficient for the repression of the target gene, such as in the regulation of *Ubx* expression in the larval wing disc (Kahn et al., 2006; Papp and Muller, 2006). These results show that the H3K27me3 chromatin mark and PcG recruitment does not absolutely determine gene expression, this suggests that additional histone modifications such as acetylation may be important at these genes.

While genome-wide analysis of PcG targets in *Drosophila* suggests a role in development, very little is known about Polycomb regulation of cell fate commitment

within a defined tissue or individual lineage. One well characterized role of PcG complexes in stem cell self-renewal versus differentiation is in the *Drosophila* male germline. During germ cell development, adult stem cells in the testis differentiate into mature sperm cells. This cell-type specific differentiation in the adult stem cells requires the activity of five testis-specific TAFs (tTAFs) encoded by the genes *can, sa, mia, nht*, and *rye* (Hiller et al., 2001; Hiller et al., 2004)(Hiller and Fuller, 2001; Hiller et al., 2004; Cooper and Fuller, 1998).

During *Drosophila* spermatogenesis, Polycomb group proteins are required in the germline stem cell for the repression of differentiation genes and the maintenance of self-renewal (Chen et al., 2011). Upon differentiation, the tTAFs counteract PcG silencing and activate expression of differentiation genes in the developing spermatocytes. tTAFS induce a relocalization of PcG proteins to the nucleolus, apparently decreasing the amount of PcG proteins available to bind and repress target genes (Chen et al., 2005c). Further studies using chromatin immunoprecipitation assays showed that tTAFs bind to promoter regions of differentiation genes and leave a H3K4me3 mark, suggesting that the tTAFs may recruit or stimulate Trithorax-group complexes to additionally counteract PcG-dependent repression. This work is significant because it demonstrates how dynamic regulation of PcG silencing allows differentiation within a defined stem cell lineage. The discovery that tTAFs promote relocalization of PRC1 components to the nucleolus in spermatocytes also implicates sub-nuclear architecture in the regulation of terminal differentiation.

Objectives of Study and Hypotheses Tested

Given the important roles of PcG complexes in multiple developmental processes, particularly stem and progenitor cell differentiation and fate-commitment, we hypothesize that PcG complexes regulate fate-potential during *Drosophila* neurogenesis. Our next chapter presents experimental results that address this hypothesis and reveal a role for PcG complexes in controlling neuroblast competence.

Chapter 2: *Drosophila* Polycomb Complexes Restrict Neuroblast Competence to Generate Motorneurons

2.1. 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 (Martinez and Cavalli, 2006; O'Dor et al., 2006), differentiation of germline progenitors (Narbonne et al., 2004; Chen et al., 2005c), 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' motorneurons 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 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 motorneurons 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 motorneurons, 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 motorneuron 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*$^{+}$ motorneurons during the first four divisions: high levels of Hb specify the RP1 motorneuron, low levels of Hb specify the RP4 motorneuron, Kr specifies the RP3 motorneuron, and an unknown factor or factors specify the RP5 motorneuron (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 motorneuron 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 motorneurons 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 motorneurons 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 motorneuron competence windows in neuroblasts that transition from motorneuron to interneuron production.

2.2. Materials and Methods

2.2.1. Fly Stocks and Genetics

Trithorax group and Polycomb group mutants used in the initial screen are listed in Table 2. Fly stocks used in other experiments were: (1) *wor-GAL4; Su(z)12^{3}/TM3, Ubx-lacZ*; (2) *UAS-Kr; Su(z)12^{3}/TM3, Ubx-lacZ*; (3) *ph-d^{401}, ph-p^{602}/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)12^{3}* heterozygous versus homozygous mutants were identified by staining for β-galactosidase. *ph-d^{401}, ph-p^{602}* 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.

2.2.2 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

Genotype	Allele type	Source
mor^{1}/TM3 ftz-lacZ	Hypomorph	Bloomington 3615
ash1B1/TM3 ftz-lacZ	Hypomorph	Bloomington 5045
Sce1/TM3 ftz-lacZ	Amorph	Bloomington 7364
w; UAS-ISW^{IDN}-HA-6His/TM3 ftz-lacZ	Dominant negative	John Tamkun
Su(z)12^{3}/TM3 ftz-lacZ	Amorph	Bloomington 5068
y', z^{a}/FM7c ftz-lacZ	Hypomorph	Bloomington 1496
Pc^{1}/TM3 ftz-lacZ	Amorph	Bloomington 1728
osa^{0090}/TM3 ftz-lacZ	Hypomorph	Bloomington 11486
Snr1^{01719}/TM3 ftz-lacZ	Unknown	Bloomington 11529
trxj^{14A6}/TM3 ftz-lacZ	Unknown	Bloomington 12137
ln(2R)Pcl^{11}, Pcl^{11}/CyO ftz-lacZ	Amorph	Bloomington 2401
Nurf38^{k16102}/CyO ftz-lacZ	Unknown	Bloomington 12206
E(bx)Nurf$^{301-3}$/TM3 ftz-lacZ	Amorph	Bloomington 9687
esc^{1}/CyO ftz-lacZ	Hypomorph	Bloomington 813
Psc^{h27}/CyO ftz-lacZ	Amorph	Bloomington 5547
brm^{2}/TM3 ftz-lacZ	Amorph	Bloomington 3619
trl^{s2325}/TM3 ftz-lacZ	Unknown	Bloomington 12088
w, ph-d^{401}, ph-p^{602}/FM7c ftz-lacZ	Hypomorph, amorph	Bloomington 5444
E(z)^{731}/TM3 ftz-lacZ	Amorph	Bloomington 24470

Table 2. Fly strains used in screen for enhancers of neuroblast competence in screen for enhancers of neuroblast competence. Females of the mutants listed here were crossed to *wor[ts]>Kr* males.

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).

2.2.3 Kruskal-Wallis Statistical Analysis

Kruskal–Wallis one-way analysis of variance is a non-parametric statistical analysis to test if various variables are from the same distribution. We used Kruskal-Wallis to compare the independent samples of the genetic screen preformed in Figure 9. Our null hypothesis for this genetic screen was that all the various mutations had the same median. In our test, we analyzed the impact of the different loss-of-functions in chromatin remodeling genes on extended competence with respect to Kruppel misexpression background. Our analysis using Kruskal-Wallis test led to significant results with components of Polycomb Group proteins. The main difference between Kruskal–Wallis test and its close related statistical analysis is that it does not assume a normal distribution.

2.3. Results

2.3.1. 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 motorneurons 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 Figure 9 A,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) (Figure 9 B,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) (Figure 10 A,B). Although the majority of NB7-1 lineages stop producing U motorneurons after three divisions in *wor[ts]>Kr* embryos, NB7-1 maintains normal mitotic activity throughout neurogenesis (Figure 11 A,B,D) and there is no increase in apoptotic cells in the developing nervous system, as previously described (Cleary and Doe, 2006). These results suggest that when competence to make U motorneurons 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 (Figure 9 C,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 motorneuron 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 (Doe et al., 1988; Smouse et al., 1988; Oktaba et al., 2008), the $ph\text{-}d^{401}$, $ph\text{-}p^{602}$ 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 (Figure 10 C,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

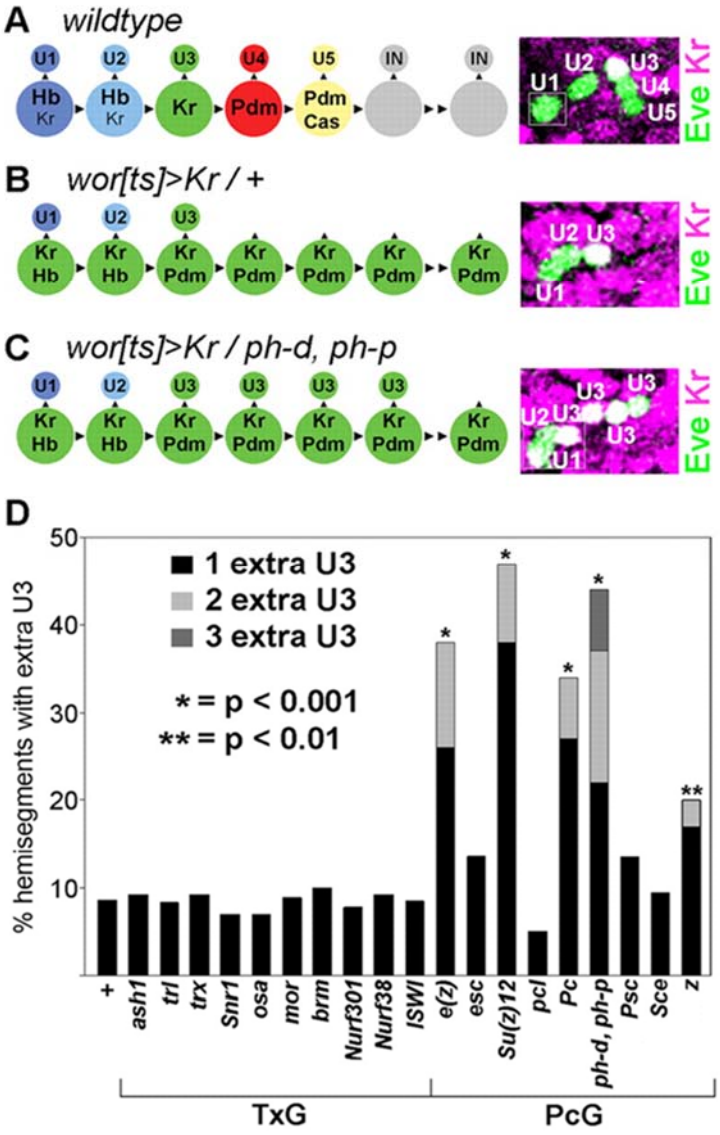

Figure 9: Mutations in Polycomb group genes extend NB7-1 competence. (A-C) The diagrams on the left show cell fates and temporal identity factor expression in the NB7-1 lineage of (A) wild type, (B) wor[ts]>Kr crossed to wild type, and (C) wor[ts]>Kr crossed to ph-d, ph-p mutant. Ganglion mother cells (GMCs) and Eve– siblings are not shown. Images on the right show Eve+ U motorneurons 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 (TrxG) 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.

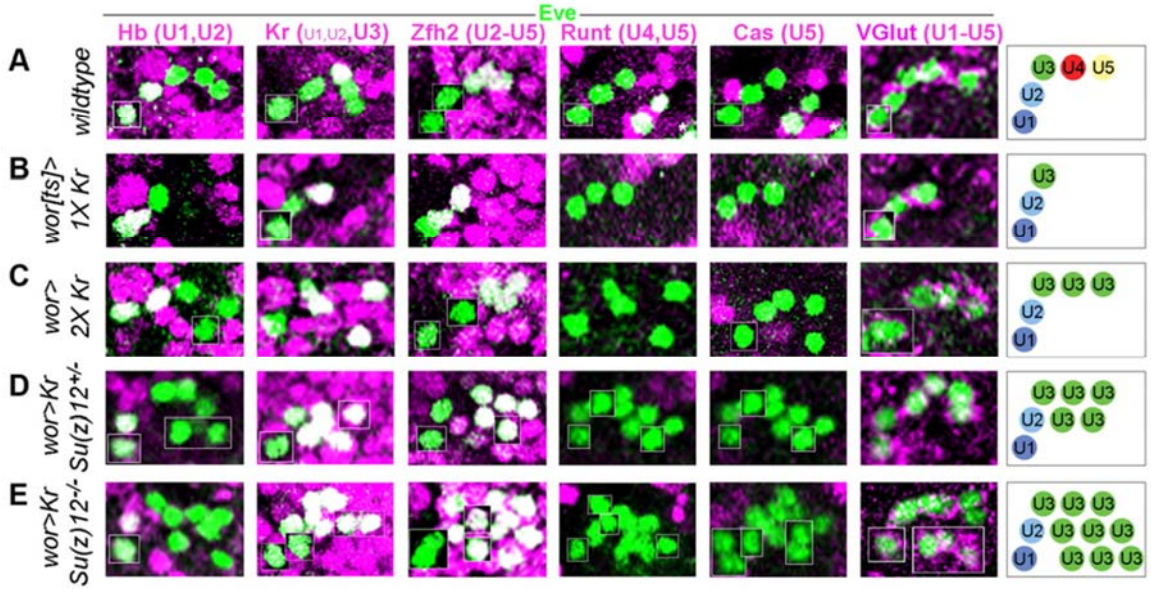

Figure 10: Su(z)12 levels determine the degree of competence restriction. Representative hemisegments are shown for (A) wild type, (B) wor[ts]>Kr (low-level Kr expression), (C) wor>2×Kr (high-level Kr expression), (D) wor>1×Kr; Su(z)12+/– [low-level Kr expression in Su(z)12 heterozygous mutant], (E) 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.

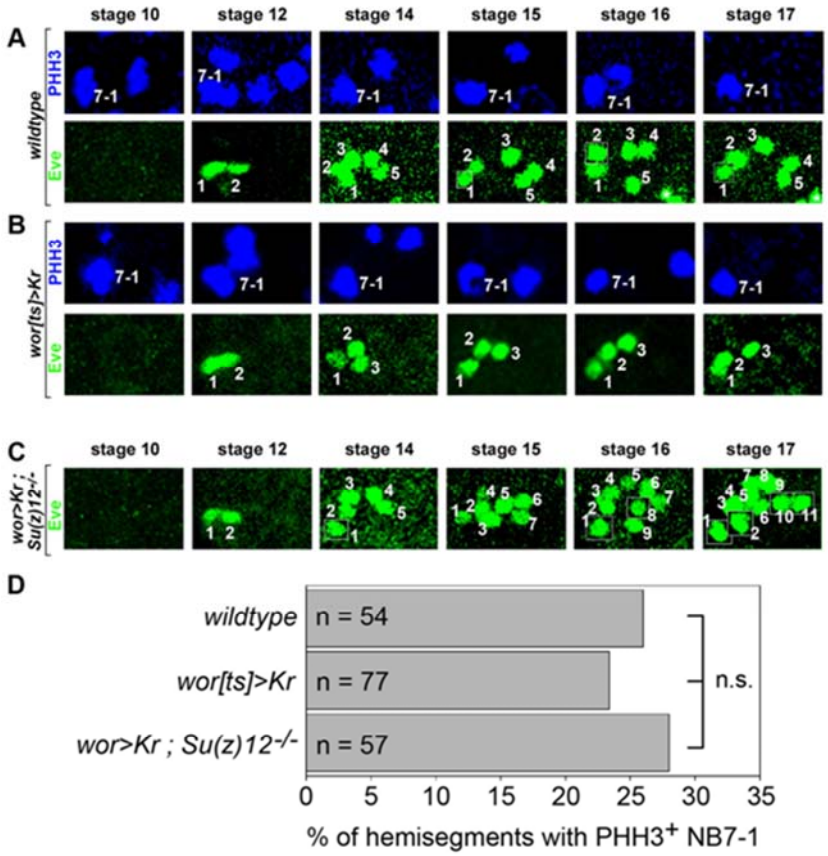

Figure 11: Normal mitotic activity and sequential production of U motorneurons in Kr-misexpressing and *Su(z)12* mutant embryos. (A,B) Phosphorylated Histone H3-positive ($PHH3^+$) neuroblasts (blue) and Eve^+ U motorneurons (green) are shown for the indicated stages of (A) wild-type and (B) *worts>Kr* embryos. (C) Sequential generation of Eve^+ U motorneurons in *wor>Kr; Su(z)12*$^{-/-}$ embryos. Embryos were staged using standard methods (Campos-Ortega and Hartenstein, 1985). The same representative regions of hemisegments are shown in all images. NB7-1 is labeled in the neuroblast layer images, other $PHH3^+$ neuroblasts included in the images vary and are not individually identified. U motorneurons are numbered to indicate both cell fate (U1-U5) and the total number that are present at each stage. Unique U fates were identified as shown in Figure 2 (main text) and all cells after cell number 5 in the *wor>Kr; Su(z)12*$^{-/-}$ embryos are ectopic U3 cells. Asterisk indicates Eve^+ cells of the NB3-3 lineage. (D) Mitotic index of NB7-1 in the indicated genotypes. NB7-1 was identified based on position within the neuroblast layer and expression of Engrailed (not shown) or position relative to the U motorneurons. Data from embryos of stage 14, 15, 16 and early 17 were combined to calculate mitotic index. No significant difference in frequency of $PHH3^+$ NB7-1 was detected between any of the genotypes (n.s., no significant difference), based on Kruskal-Wallis test.

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 motorneuron characteristics (Figure 10), including expression of the vesicular glutamate transporter VGlut, which is specific for motorneurons (Mahr and Aberle, 2006). VGlut staining also revealed that the ectopic U3 motorneurons 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^{+} motorneurons 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 motorneurons (Figure 12).

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 (Figure 11 C,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 motorneurons in *wor>Kr; Su(z)12*$^{-/-}$ embryos (Figure 13), 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 motorneurons and the corresponding Eve^- siblings.

2.3.2 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 motorneuron phase: competence to respond to Kr is high throughout the first five neuroblast divisions (Figure 14). Competence also extends into the interneuron phase of the lineage, with ectopic U3 cells produced as late as the ninth division (Figure 14, Figure 15). 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 motorneuron production during the interneuron phase of the NB7-1 lineage.

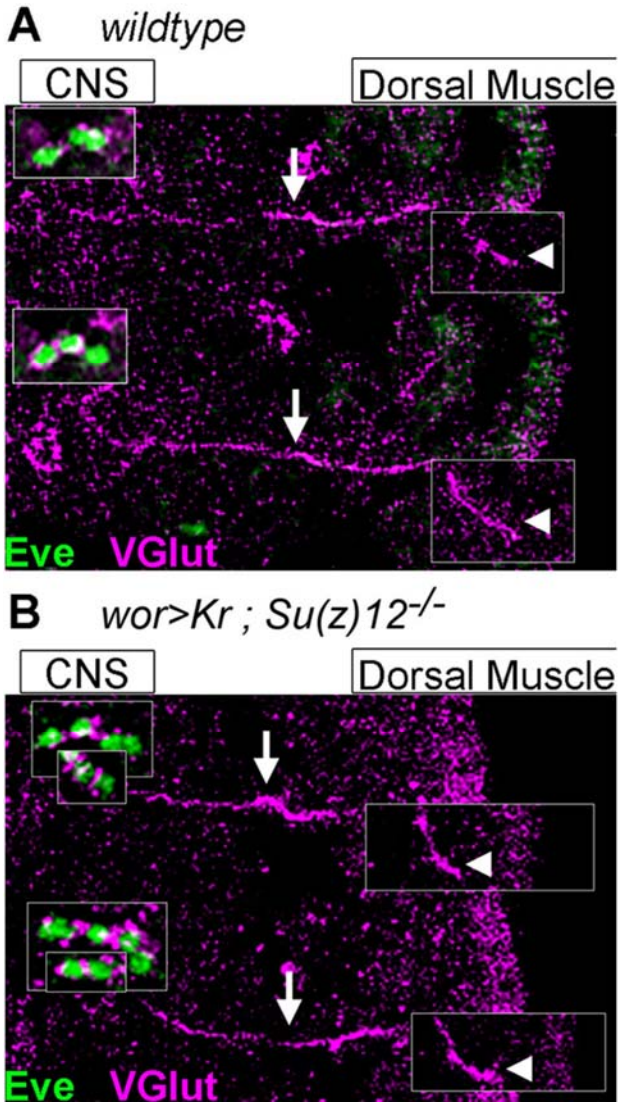

Figure 12: Ectopic U3 motorneurons make normal axon projections. VGlut staining (magenta) shows the fasciculated axons of Eve^{+} motorneurons (green) leaving the central nervous system via the ISN branch of the intersegmental nerve (arrows) and terminating in dorsal body wall muscles (arrowheads). (A) Wild-type embryo showing two segments and U3, U4 and U5 motorneurons. (B) *wor>Kr; Su(z)12*$^{-/-}$ embryos showing two segments and clusters of ectopic U3 motorneurons. No aberrant VGlut projection patterns are observed in the *wor>Kr; Su(z)12*$^{-/-}$ embryos, suggesting that axon pathfinding is not affected. The increased number of U3 motorneurons appears to contribute to a thickening of the nerve, particularly at the site of neuromuscular junction formation. In both A and B, the U motorneurons are pasted into the image in an offset position to allow viewing of the axon projections that initially lie dorsal to the cell bodies and would otherwise be obscured. Corresponding axon terminals at the dorsal body wall muscles are also pasted into the image to allow viewing of regions that would otherwise be obscured.

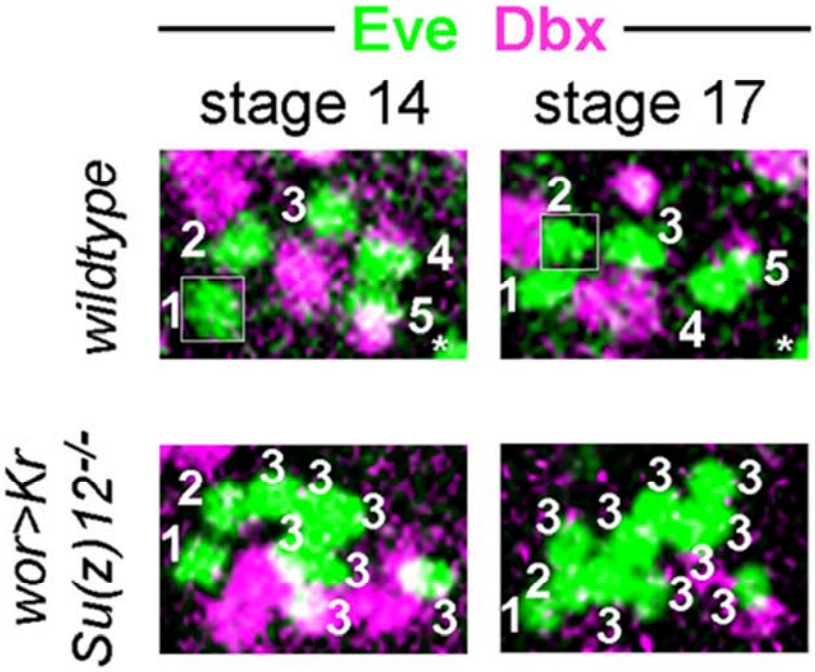

Figure 13: Dbx^{+} interneuron siblings of ectopic U3 motorneurons are produced in*wor>Kr; Su(z)12*$^{-/-}$ embryos. Wild-type and *wor>Kr; Su(z)12*$^{-/-}$ hemisegments are shown for stage 14 and stage 17 embryos. The sibling interneurons of U3 motorneurons express Dbx transiently, with expression fading after stage 14 in wild-type embryos (Lacin et al., 2009). The presumptive Dbx^{+} siblings are observed adjacent (within one or two cell nuclei widths) to U motorneurons in both wild-type and *wor>Kr; Su(z)12*$^{-/-}$ embryos at stage 14. At stage 17, approximately 20 Dbx^{+} neurons are observed per hemisegment in wild-type embryos. At stage 17 in *wor>Kr; Su(z)12*$^{-/-}$ embryos, fewer Dbx^{+} neurons are observed per hemisegment (12.3±1.8, *n*=24), likely due to the loss of late-born interneurons in favor of motorneurons in multiple lineages. As in wild-type embryos, siblings of U3 cells in *wor>Kr; Su(z)12*$^{-/-}$ embryos do not express Dbx at stage 17.

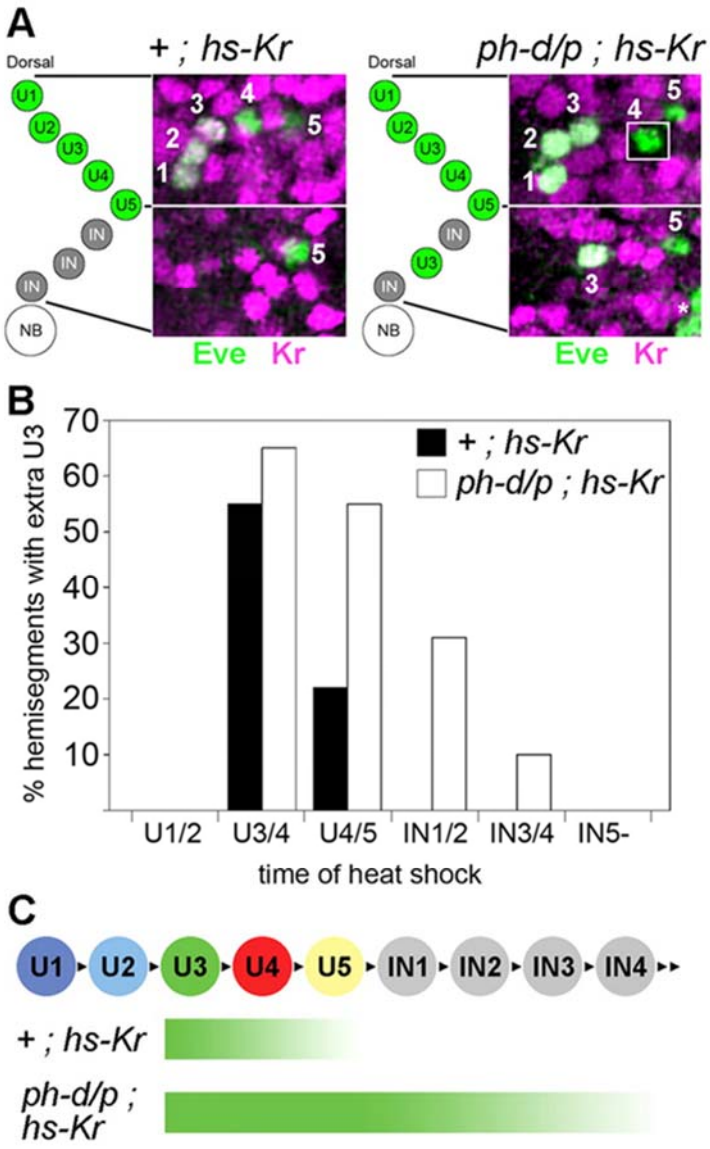

Figure 14: Polyhomeotic is required for progressive restriction of neuroblast competence. (A) z-plane projections of early-born (dorsal region) and late-born (ventral region) cells in the NB7-1 lineage following heat shock-induced Kr expression in wild type (+) or ph-d, ph-p mutant (ph-d/p). The diagrams 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.

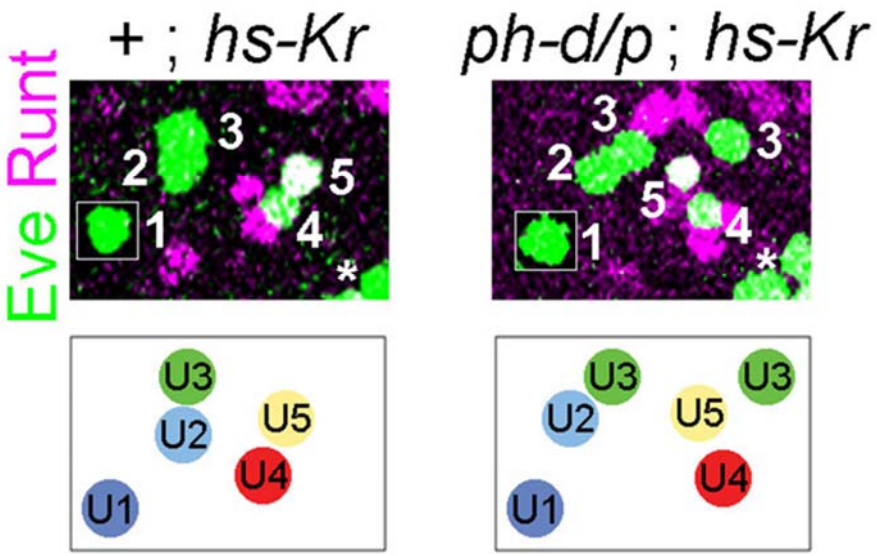

Figure 15: Ectopic cells produced in *polyhomeotic* mutants are U3 cells. Representative hemisegments from stage 17 embryos following heat shock-induced Kr expression at the time of interneuron 1/interneuron 2 formation in the NB7-1 lineage of wild-type (+) or *ph-d, ph-p* mutant (*ph-d*/*p*) embryos (as described for Figure 3 in main text). Cell identity is determined by staining for Eve (green) and Runt (magenta). Runt is expressed in U4 and U5 cells, with higher expression levels in U5 relative to U4. Asterisk indicates Eve^{+} cells of the NB3-3 lineage. Cell identities are summarized in the diagram beneath each hemisegment.

2.3.4 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 (Figure 16 B), 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 motorneurons does not repress Cas (Cleary and Doe, 2006). *Su(z)12* knockdown in U motorneurons did not alter this regulatory relationship, as Kr-expressing U5 cells continue to express Cas (Figure 16 C). These results suggest that the relevant PRC activity occurs in neuroblasts or GMCs and that cell fate cannot be reprogrammed in mature neurons.

2.3.5 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) (Figure 18 A, Figure 17). We also assayed competence in *en-GAL4 × UAS-ph* embryos (without *UAS-Kr*) and found that U motorneuron 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 motorneuron 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 (Figure 18 B,C). There is also a significant decrease in competence to produce U4 and U5 fates when expression of Pdm and Cas is delayed. s*ca>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 (Figure 18 B,C). These results support a model in which PRC activity blocks temporal identity factors from specifying U motorneuron 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 motorneurons.

2.3.6 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) (Figure 19 A). We used two

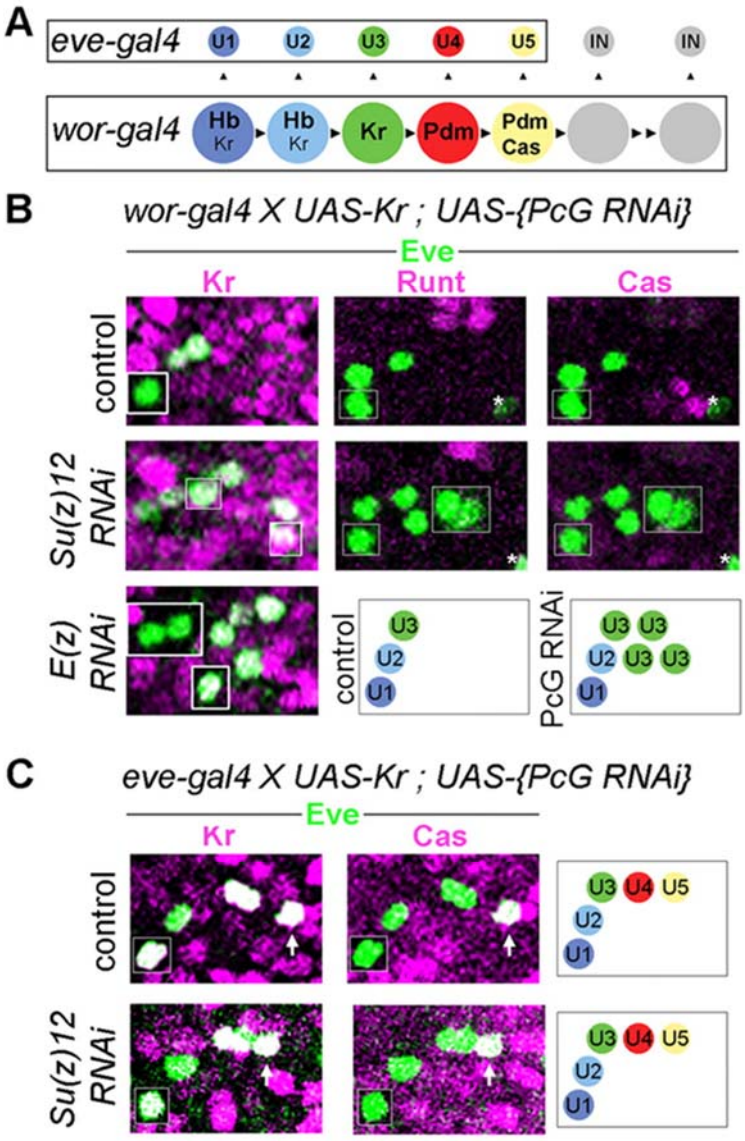

Figure 16: PRCs act within the NB7-1 lineage to restrict competence. (A) Expression patterns of GAL4 lines used in RNAi analysis; GAL4 is transcribed in cells within the corresponding box. (B) wor-GAL4-driven RNAi of the Polycomb group (PcG) genes Su(z)12 and E(z). Asterisk marks EL neurons of the NB3-3 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 motorneuron-specific knockdown of Su(z)12. Images show Kr expression in U5 motorneuron and maintenance of Cas expression in the same U5 motorneuron (arrows). Cas expression was never repressed in Kr-positive U5 motorneurons of control embryos (n=8) or Su(z)12 RNAi embryos (n=3).

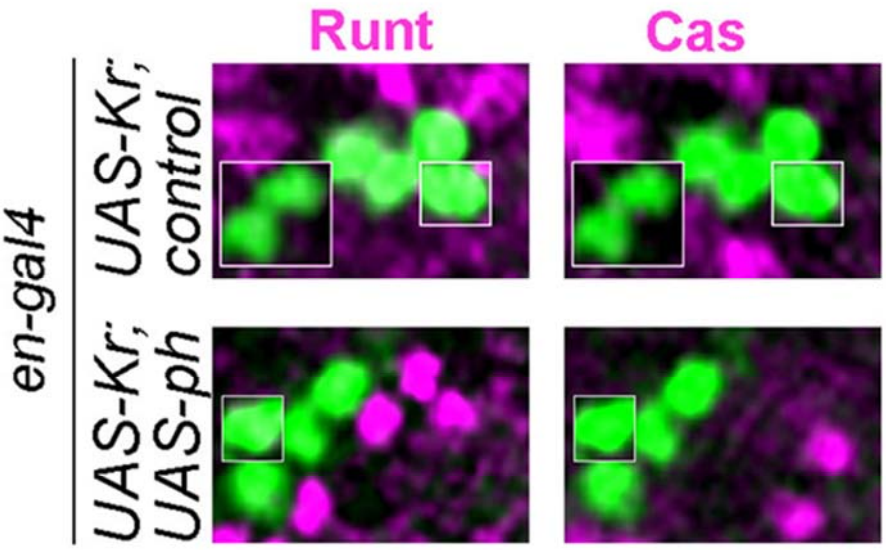

Figure 17: Polyhomeotic gain-of-function restricts U3 fates. Additional confirmation of cell fates in *en-GAL4 × UAS-Kr, UAS-control* embryos and *en-GAL4 × UAS-Kr, UAS-ph* embryos, based on staining for Runt (U4, U5) and Cas (U5). These data complement the Kr stains of the same genotypes shown in Figure 5 of the main text. U4 and U5 cells are never produced in either genotype.

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 (Figure 19 B). This suggests that competence to respond to Kr in NB3-3 is extended far beyond the five-division window observed 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) (Figure 19). 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) (Figure 19). PRCs therefore differentially regulate two classes of Eve^+ neurons: competence to produce Eve^+ motorneurons is restricted by PRC activity, whereas competence to produce Eve^+ interneurons is unaffected by PRC activity.

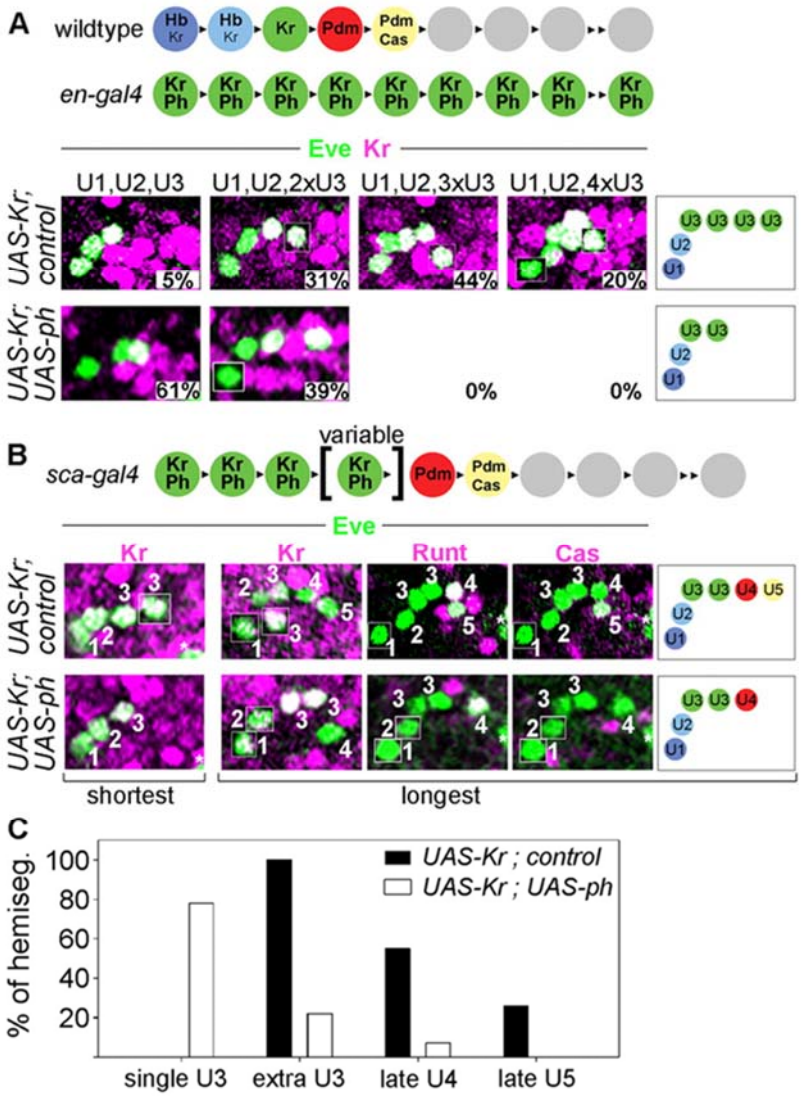

Figure 18: Polyhomeotic gain-of-function enhances competence restriction in NB7-1. (A) Diagram at the top illustrates the timing of ectopic Kr and Ph expression driven by en-GAL4. Control embryos (UAS-Kr; UAS-control) produced 2.8±0.79 U3 cells (n=54), whereas UAS-Kr; UAS-ph embryos produced 1.4±0.49 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.

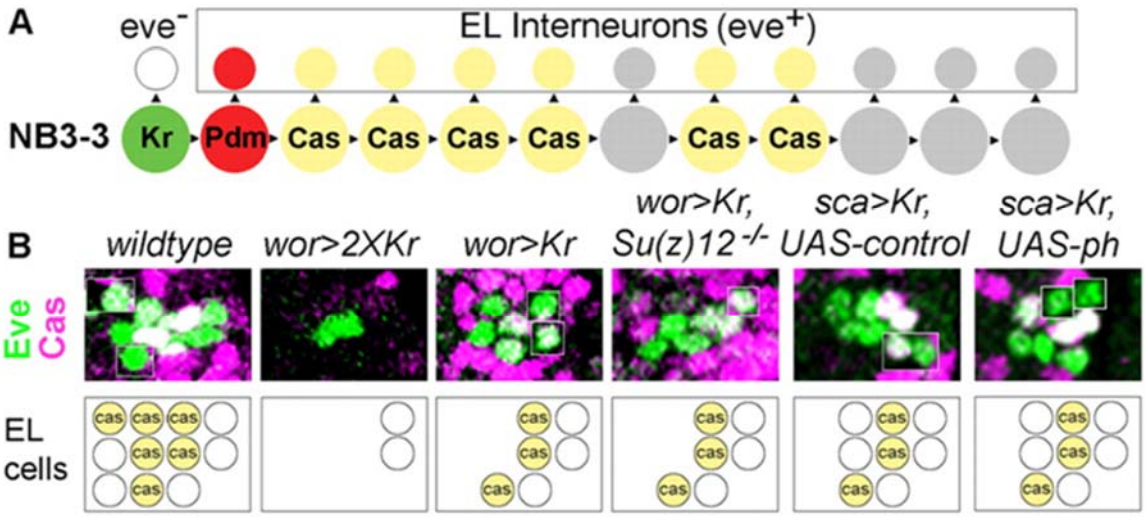

Figure 19: PRC activity does not affect interneuron production by NB3-3. (A) Diagram of the NB3-3 lineage showing the Kr- and Cas-specified interneurons. (B) Representative EL interneuron images from the indicated genotypes. Summary Figures show the average number of EL neurons and frequency of Cas+ 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.

2.3.7 PRC activity establishes a motorneuron competence window in NB3-1

To determine whether PRCs regulate the production of other types of motorneurons, we analyzed cell fates in the NB3-1 lineage (Figure 20 A). NB3-1 sequentially produces the RP1, RP4, RP3 and RP5 motorneurons during its first four divisions and these motorneurons 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) (Figure 20 A, Figure 21). 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) (Figure 20 B, supplementary material Figure 21), 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) (Figure 20 B, Figure 21). 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 motorneuron 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 produced a single

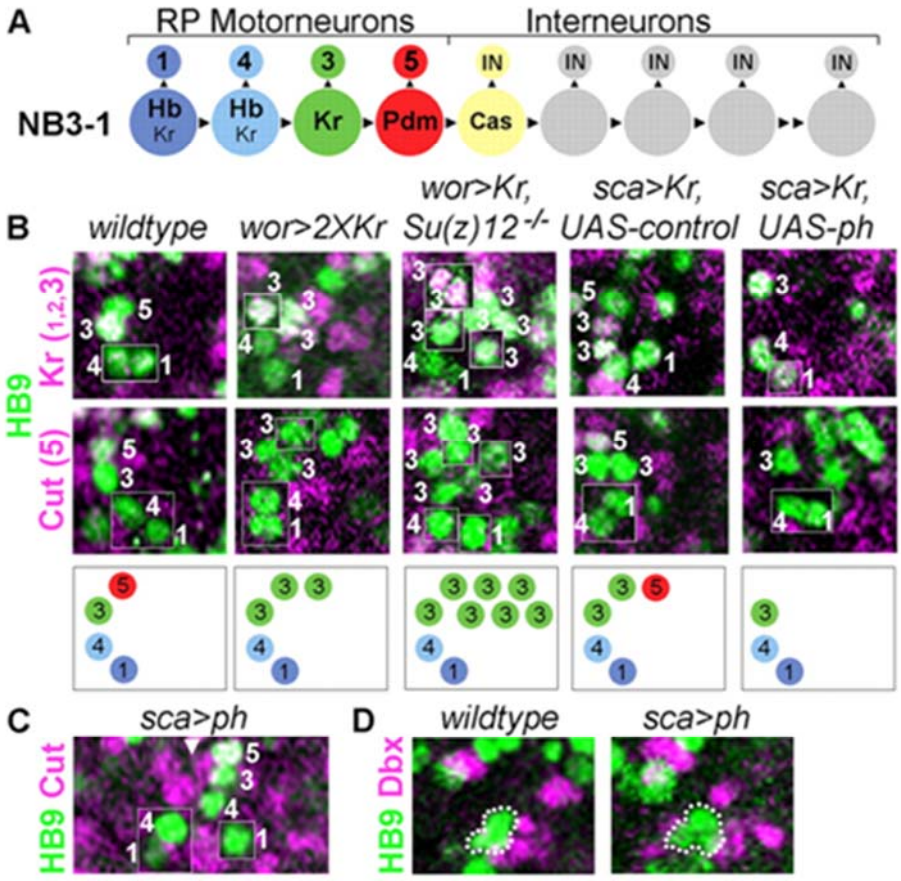

Figure 20: PRC activity regulates motorneuron competence in NB3-1, whereas interneuron fates in multiple lineages are unaffected. (A) The NB3-1 lineage. (B) Summary Figures show the average cell fates for each genotype. Kr is expressed at low levels in RP1 and RP4 and at high levels in RP3. Cut is only expressed in RP5. RP motorneurons of the NB3-1 lineage were also identified by staining for Islet (supplementary material Figure 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)].

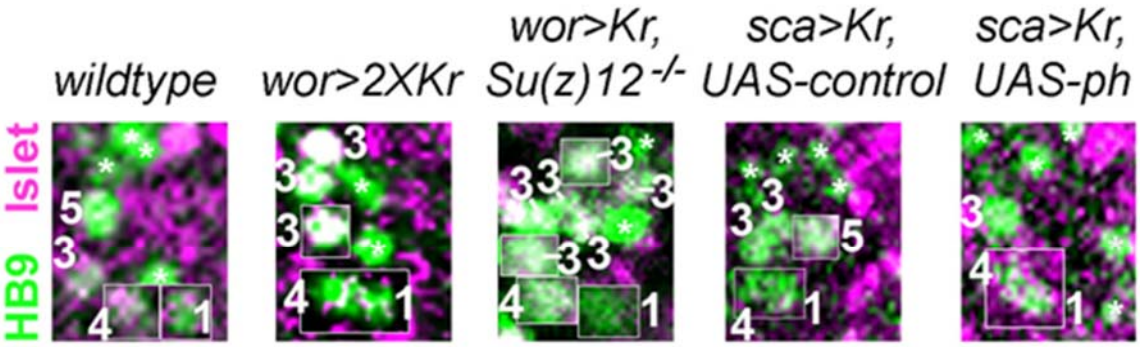

Figure 21. Identification of NB3-1 lineage cells based on combined HB9 and Islet staining. Representative hemisegments for each of the genotypes included in Figure 7 of the main text are shown. Staining for HB9 (green) and Islet (magenta) was used in parallel to staining for Kr and Cut (shown in Figure 7 of main text) to identify cells of the NB3-1 lineage and cell identities within the lineage. The Islet signal is typically weaker in more ventrally located (deep) cells of the embryos, particularly the RP1 and RP4 cells, as previously shown (Tran and Doe, 2008). The presence or absence of Islet signal in $HB9^{+}$ cells and cell position were used to identify cells of the NB3-1 lineage. Cells marked with an asterisk are either $HB9^{+}$ cells that are Islet-negative or double-positive cells that are in the incorrect position to be part of the NB3-1 lineage.

ectopic RP3, 60% produced only the normal RP3 and 17% did not produce any RP3 (n=42) (Figure 20 B, Figure 21).

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 motorneuron. We tested the ability of Ph gain-of-function to inhibit other motorneuron fates in NB3-1 using *sca-GAL4* × *UAS-ph* and found that the first two motorneuron 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) (Figure 20 C). To determine if this restriction of *HB9*$^+$ *Islet*$^+$ fates was motorneuron 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 motorneuron 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 (Figure 20 D). 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

(Figure 21 D). The inhibition of *HB9*$^{+}$ *Islet*$^{+}$ motorneurons and lack of any effect on *HB9*$^{+}$ *Islet*$^{+}$ interneurons and *Dbx*$^{+}$ interneurons further suggests that PRCs specifically regulate motorneuron competence. Figure 22 is a summary of chapter 2 results.

2.4. Discussion

2.4.1. 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 motorneuron competence windows in two distinct neuroblast lineages, regulating the production of both Eve^{+} and *HB9*$^{+}$ *Islet*$^{+}$ motorneurons. 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 motorneuron 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*$^{+}$ motorneurons 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 motorneuron 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 motorneuron 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

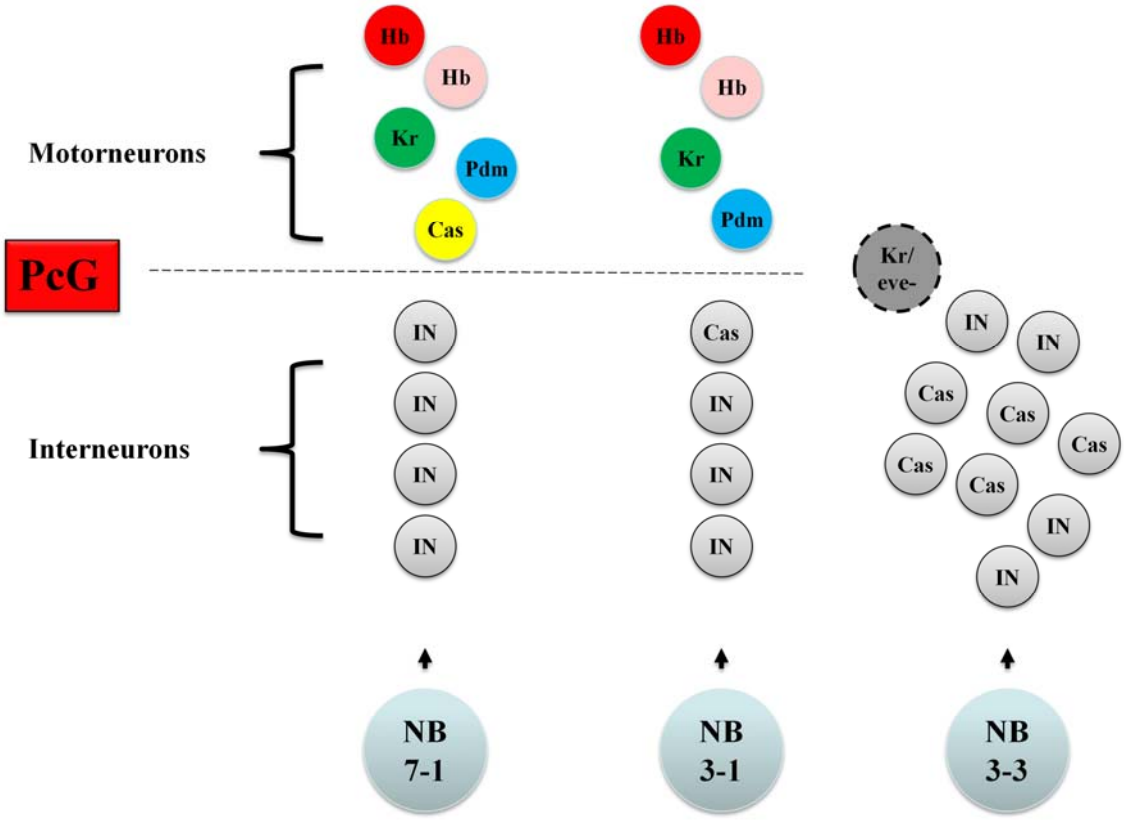

Figure 22: Summary of chapter 2 results show that Polycomb Group proteins repressive activity regulated a motorneuron competence window in NB7-1 and NB3-1. Eve-lateral NB3-3 interneuron progeny is unaffected by Polycomb activity.

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).

2.4.2. Temporal Regulation of Motorneuron versus Interneuron Production

The sequential generation of motorneurons followed by interneurons has been observed during nervous system development of many insects (Burrows, 1996). Clonal analysis of *Drosophila* neuroblasts suggests that motorneurons 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, motorneurons 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 motorneuron-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 motorneuron 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 motorneuron 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 motorneuron 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 motorneuron. 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^{+} motorneuron 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 motorneuron fates are preferentially inhibited in Ph gain-of-function experiments, supporting a link between the number of neuroblast divisions and the restriction of motorneuron 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.

2.4.3 What are the PRC-regulated genes that determine motorneuron 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 motorneuron formation or function: *eve, Islet, HB9, Nkx6* (*HGTX* – FlyBase), *zfh1* and *Lim3*. All motorneurons that innervate dorsal muscles express Eve (Landgraf et al., 1999), most motorneurons that innervate ventral muscles express some combination of Lim3, *Islet*, *HB9* and Nkx6 (Thor and Thomas, 1997; Thor et al., 1999; Broihier and Skeath, 2002; Odden et al., 2002; Broihier et al., 2004; Cheesman et al., 2004), and all somatic motorneurons express Zfh1 (Layden et al., 2006). None of these genes is sufficient to confer motorneuron 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 motorneuron 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.

2.4.4 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 motorneuron and interneuron genes in newly formed *Drosophila* neuroblasts, with subsequent PRC activity selectively silencing motorneuron 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.

Chapter 3: Synthesis and Future Directions

3.1 Remaining Questions Regarding Polycomb Regulation of Competence Windows

As described in the previous chapters, neural progenitors of the mammalian cerebral cortex and the *Drosophila* embryonic nerve cord undergo a progressive loss of competence to make early-born neurons. *Drosophila* neuroblasts are an excellent model for studying this process. The temporal identity factors Hunchback (Hb), Kruppel (Kr), Pdm, and Castor (Cas) are sequentially expressed in embryonic neuroblasts and specify early-born fates. As discussed in chapter 2, I discovered that Polycomb group (PcG) complexes establish motorneuron "competence windows" in neuroblasts. This discovery is significant because it provides a mechanistic basis for the inability of temporal transcription factors to alter cell fates at late stages. However, many questions remain to be answered regarding the role of PcG complexes in establishing competence states. For example, it is not clear whether PcG-regulation of competence is motorneuron-specific (as I have shown for NB7-1 and NB3-1) or if PcG activity affects other cell fate transitions. In addition, the precise PcG components and cofactors relevant for regulation of neuroblast competence remain to be determined. My work has only studied the "core" components of PRC1 and PRC2, but many additional regulators of PcG activity remain to be tested and additional cofactors likely remain to be identified. Understanding the role of different components of the PRC complexes and cofactors in regulating competence, particularly in temporally-restricting fate potential, is pivotal. Finally, the target genes that are regulated by PRC complexes in neuroblasts are a major missing part of my model. I propose in chapter 2 that PRC complexes repress transcription of genes required

for motorneuron development, and that competition between Kr and PRCs determines gene expression patterns. The nature of Kr and PRC interactions (direct or indirect) in neuroblasts, and the neuroblast-specific target genes regulated by PRCs, are significant unknowns. We will address the above questions via experiments designed to test the following hypotheses.

Hypothesis #1: PcG activity regulates multiple cell fate transitions and is a general mechanism to distinguish early versus late cell fates.

To test this hypothesis we aim to identify PcG function in establishing multiple competence states of interneurons and glial cells in neuroblast lineages that have not been previously analyzed for competence transitions. First I propose investigating PcG-regulation of competence during production of Cas-specified interneurons in NB3-3 and Hb and Kr-specified glial fates in NB6-4 and NB7-4. In addition to studying these embryonic neuroblast lineages, I also propose that it would be interesting to investigate PcG effects on larval-born progeny of the thoracic neuroblasts. These neuroblasts are formed during embryonic stages but continue producing progeny during larval neurogenesis and the larval-born progeny are not specified by known TTFs. The larval thoracic neuroblasts therefore provide a model for testing competence to produce embryonic neurons or glia during larval stages.

Hypothesis #2: The timing and magnitude of competence restriction depends on PcG interaction with effector proteins.

Core PcG proteins are constitutively expressed in neuroblasts throughout embryonic neurogenesis, yet competence restriction increases over time. This suggests that PcG effector proteins control the magnitude and timing of competence restriction. PcG effectors include DNA-binding cofactors that recruit PcG complexes to target genes and Trithorax-group complexes that counteract PcG activity. In addition to testing candidate effectors, future work can be enhanced by performing a standard mutagenesis screen to identify modifiers of competence.

Hypothesis #3: PcG activity alters TTF-dependent transcription programs in neuroblasts.

To test this hypothesis, I propose identifying the transcriptional networks in neuroblasts established by the interactions of the TTFs and PcG complexes. Temporal identity factors dictate the cell fate of neuroblast progeny by regulating the expression of an unknown subset of genes. A recently developed technique that allows cell type-specific mRNA purification (known as TU-tagging and described in more detail in section 3.3 below), could be very useful for identifying these target genes (Miller et al., 2009). Neuroblast-specific TU-tagging of mRNA will identify genome-wide changes in transcription profiles that occur in Kr misexpression embryos, PcG loss-of-function embryos, and embryos with combined Kr misexpression and PcG loss-of-function.

While PcG targets genes have been previously identified by chromatin-immunoprecipitation (ChIP) in whole embryos (Schuettengruber and Cavalli, 2009; Schuettengruber et al., 2009), direct PcG targets in neuroblasts are unknown. The results of TU-tagging technique can be analyzed against the known PcG targets from the database established from ChIP in whole embryos to identify promising candidate direct PcG targets. An additional recently developed technique that allows cell type-specific purification of nuclei (known as INTACT (isolation of nuclei tagged in specific cell types)) could be used to confirm target gene binding in neuroblasts by chromatin immunoprecipitation and PCR of candidate genes (described in more detail in section 3.3 below).

3.2 Preliminary Data In Support of Proposed Future Directions

In chapter 2, I presented data showing that Kr-specified interneuron fates are not affected by PcG activity in the NB3-3 lineage. NB3-3 only produces interneurons and our previous work supported the hypothesis that PcG activity only affects motorneuron fates. However, NB3-3 transitions between production of multiple interneuron fates and, importantly, produces Cas-specified interneurons at distinct early versus late times in the lineage. I hypothesized that PcG activity might allow Cas to be "re-used" within the NB3-3 lineage to specify distinct early and late interneuron fates. The progeny of NB3-3 are called EL interneurons (Eve-lateral) (Tsuji and Hasegawa, 2008). Figure 23 A shows the relevant temporal expression pattern of Cas, with an early window of expression generating two Cas+,Kr+ interneurons and a late window of expression generating only

Cas+ interneurons. We found that NB3-3 loses competence to produce early Cas^+,Kr^+ fates even when Cas is continuously over-expressed in the NB (Figure 23 B,C). Crossing constitutive Cas expressing flies to *Su(z)12*^{3} mutants (a null allele of an essential PcG gene) revealed that competence to produce early Cas^+, Kr^+ fates is extended in *Su(z)12*^{3} heterozygous embryos (Figure 23 E). Notably, we also found that *Su(z)12*^{3} homozygous mutant embryos (with no ectopic Cas) occasionally produce extra Cas^+, Kr^+ EL interneurons (Figure 23 D), which we interpret as evidence that loss of PcG function allows Cas to specify early fates during the late competence window. This finding is significant because it supports the model that competence windows allow TTFs to be "re-deployed" within a NB lineage (Cleary and Doe, 2006) and supports my first hypothesis listed above: PcG activity regulates multiple cell fate transitions and is a general mechanism to distinguish early versus late cell fates.

3.3 Future Experimental Approaches

Here I have briefly summarized the types of experiments that could be used to test each of the hypotheses presented at the beginning of this chapter. This experimental plan is presented in a grant proposal format, based on a grant submitted by my advisor and discussions we have had regarding these potential future experiments.

Hypothesis #1: PcG activity regulates multiple cell fate transitions and is a general mechanism to distinguish early versus late cell fates.

My work described in chapter 2 demonstrated that PcG complexes establish motorneuron-specific competence windows in the NB7-1 and NB3-1 lineages (Touma et al., 2012). It remains to be determined if PcG complexes regulate other fate transitions, such as among interneuron subtypes or between gliogenesis and neurogenesis. To test interneuron fate transitions, we will use the NB3-3 lineage. NB3-3 provides a fully traceable lineage for analysis of multiple IN fates, as described in the preliminary results (Figure 23). We are particularly interested in the second round of Cas expression within NB3-3 as this allows us to test the hypothesis that competence windows allow Cas to induce distinct fates in early versus late competence windows. To test glial fates, we will use the abdominal NB7-4 and thoracic NB6-4T lineages. Previous work has shown that Hb specifies the first born glia of the NB6-4T lineage, prior to interneuron production at subsequent divisions (Isshiki et al. 2001). NB7-4 produces Hb-specified interneurons at the first and second division then transitions to glia production at the third division (when the NB expresses Kr), glia production at the fourth division (when the NB expresses Pdm) then back to IN production for subsequent divisions (Schmid et al. 1999; Isshiki et al. 2001). Early specification of glial fates prior to transitioning to interneuron production is reminiscent of the motorneuron to interneuron competence transition we identified in NBs 7-3 and 3-1, suggesting that PcG activity may also inhibit late glial fates. In support of this hypothesis, previous anti-Polycomb ChIP experiments in embryos identified at least two genes known to function in glia specification as Polycomb targets: glial cells missing 1 (gcm1) and reversed polarity (repo) (Schuettengruber and Ganapathi, 2009). PcG regulation of competence in these embryonic NB lineages could easily be tested using the methods described in chapter 2.

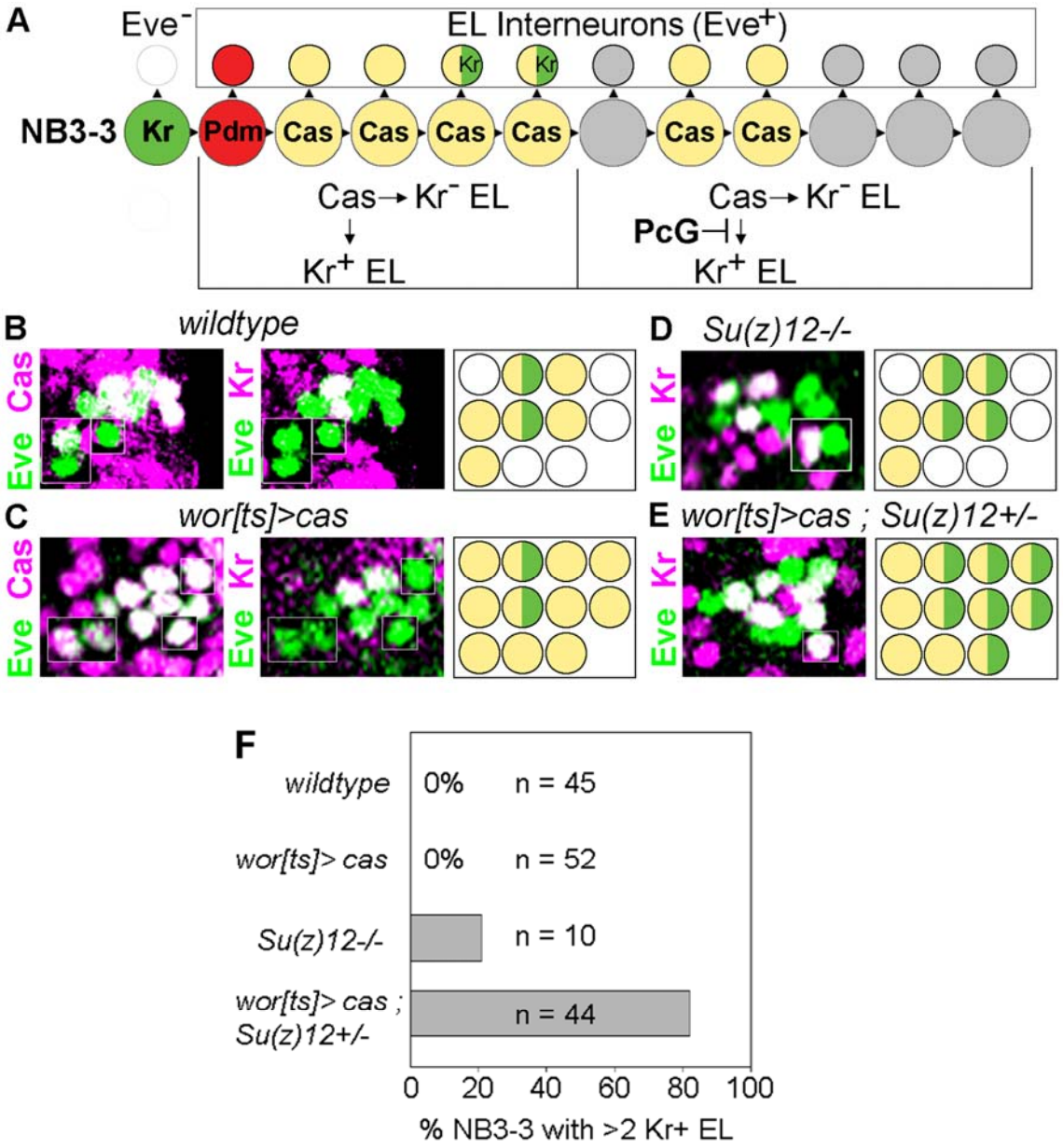

Figure 23: PcG activity regulates cell fate-specification by Cas in NB3-3. (A) NB3-3 lineage and Cas-induced fates within early and late competence windows. The proposed role of PcG activity in blocking late Kr^+ EL fates is shown. (B - E) EL fates in genotypes described in the main text. Cells that would be obscured from view in confocal projections are shown as white-boxed insets. Cas^+ and Cas^+,Kr^+ fates summarized at right. (F) % of NB3-3 lineages with extra Kr^+ ELs. The number of hemisegments scored (single lineage per hemisegment) is listed.

In thoracic segments of the ventral nerve cord, many neuroblasts continue dividing (following a brief quiescent period) and produce large numbers of cells (Truman et al., 2004). These post-embryonic NBs (pNBs) do not express Hb, Kr, or Pdm but do express Cas for multiple early divisions (Maurange et al., 2008). Importantly, larval-born progeny typically have distinct properties from embryonic cells made by the same NB (Truman et al. 2004). For example, NB7-1 makes the five Eve+ U motorneurons during embryonic stages but does not make Eve+ neurons during larval stages (Bahri et al., 2001) and appears to only make interneurons (Truman and Schuppe, 2004). Similarly, NB7-4 does not produce any glia during larval stages, instead making only interneurons (NB6-4 does not produce any larval progeny) (Truman and Schuppe, 2004). In addition to these lineage-specific differences between embryonic and larval progeny, all embryonic and early larval-born neurons express the transcription factor Chinmo while neurons born after ~60 hours of larval development express the transcription factor Broad (and do not express Chinmo) (Maurange and Cheng, 2008). Chinmo and Broad therefore provide useful markers for early versus late neural fates. Interestingly, previous work has shown that NBs undergo a progressive loss of competence for Cas to induce Chinmo+ fates: competence is high in embryos, reduced in early larvae, and completely lost at late stages of larval development (Maurange and Cheng, 2008). This loss of competence as pNBs age is very reminiscent of PcG-mediated competence restriction at embryonic stages. Previous work has shown that PcG loss-of-function in pNBs induces apoptosis due to de-repression of abdominal Hox genes (Bello et al., 2007). However, a simple genetic manipulation (expression of the baculovirus apoptosis inhibitor p35) was sufficient to reverse this affect and allow normal pNB proliferation and lineage

development (Bello et al., 2007). We will take advantage of this technique, combined with PcG loss-of-function and TTF misexpression to address an intriguing question: Is it possible to regenerate embryonic and early larval neurons at late stages of larval neurogenesis?

Hypothesis #2: The timing and magnitude of competence restriction depends on PcG interaction with effector proteins.

Core proteins of PRC1 and PRC2 are constitutively expressed in NBs, yet the magnitude of competence restriction increases over time (Touma et al., 2012). Models of PcG function suggest that multiple effectors could be responsible for such dynamic regulation of PcG activity. Here we use the term "effector" to encompass cofactors that physically associate with PcG proteins and proteins / complexes that act upstream, downstream, or in parallel to PcG complexes. Known effectors include DNA-binding cofactors that recruit PRC2 to "polycomb response elements" (PREs) in target genes and trithorax-group regulators that counteract PcG-mediated silencing. The function of "recruiters" and TrxG proteins in nervous system development is not well understood (Schuettengruber and Cavalli, 2009). One particularly interesting "recruiter" is Grainyhead (Grh) (Blastyak et al., 2006). The Grh expression pattern in embryonic neuroblasts precisely coincides with the strong loss of motorneuron competence that occurs after the fifth division of NB7-1 (Cenci and Gould, 2005). Grh expression is triggered by Cas and continues throughout late embryonic and all larval stages of neurogenesis (Maurange et al. 2008). Grh loss-of-function in pNBs induces apoptosis,

but this effect can be reversed by *UAS-p35* expression (Cenci and Gould 2005). We could easily test the function of known PcG effectors using the methods described in chapter 2 (including genetic mutants and RNAi approaches).

In addition to testing candidate effectors, we could perform a mutagenesis screen to identify modifiers of Kr competence. Genetic screens for modifiers of PcG mutant phenotypes have previously identified PRC1 and PRC2 components and additional regulators of PcG function (Gaytan de Ayala Alonso et al., 2007). The genetic screen in this aim has the potential to identify novel effectors with neurogenesis-specific functions. The mutagenesis approach also has the potential to identify transcription-independent functions of PcG proteins during competence restriction, such as the recent discovery that the PcG protein Posterior sex combs (a PRC1 component that monoubiquitylates histone H2A) also regulates cell cycle progression by ubiquitylating cyclin B and triggering its destruction (Mohd-Sarip et al., 2012).

We will use the previously described *elav-GeneSwitch* line (Osterwalder et al., 2001) (gift of Haig Keshishian) to conditionally mis-express Kr in neuroblasts and neurons. GeneSwitch is a GAL4-progesterone receptor fusion protein that only activates UAS transgene expression in the presence of the drug RU486. I have performed preliminary experiments that confirm that feeding RU486 to *elav-Geneswitch* females (according to published protocols (Osterwalder et al. 2001)) prior to mating with UAS-Kr males produces embryos that mis-express Kr in neuroblasts and produce ectopic U3 motorneurons (data not shown). In the absence of RU486, all NB7-1 lineages are normal. We could recombine UAS-Kr onto chromosome 3, where the *elav-GeneSwitch*

transgene is located. Males carrying isogenized chromosomes homozygous for *elav-GeneSwitch*, UAS-Kr will be treated with 25 mM ethyl methanesulfonate (EMS) and mated according to standard schemes for establishing balanced stocks, in this case using a *TM3, Sb, Ubx-lacZ* balancer. Approximately 3,500 mutant chromosomes will be tested in both RU486-treated and control embryos. 50 embryo stains will be performed per day, using immunohistochemical detection of Eve and beta-gal (to identify homozygous mutants). RU486-negative embryos will be used to identify mutations that alter NB7-1 fates independent of Kr misexpression and these mutations will not be selected for further analysis. We will focus on mutations that allow normal NB7-1 development in the absence of Kr misexpression but cause extension of competence (more U3 cells) or restriction of competence (decreased frequency of extra U3 cells) when Kr is mis-expressed. Mutations will be mapped using standard complementation-based approaches (with available deficiency lines) and BAC transgenics for rescue experiments. Genes identified in this screen will be further characterized by the approaches outlined above (chapter 2, section 2.1): RNA-interference, loss-of-function (including generation of new mutant alleles), gain-of-function, and mRNA / protein expression analysis.

Hypothesis #3: PcG activity alters TTF-dependent transcription programs in neuroblasts.

We hypothesize that PcG complexes alter temporal transcription factor (TTF)-induced transcription programs in neuroblasts. This could be via direct inhibition of TTF activity at coordinately regulated genes, or via indirect effects (such as PcG repression of

genes that are targets of transcription factors induced by the TTF). Competition between Kruppel and PcG complexes is suggested by the fact that high levels of Kr misexpression in neuroblasts can partially overcome PcG-dependent restriction of U3 motorneuron competence (Touma et al., 2012). Competition between transcription factors and PcG complexes has also been demonstrated during differentiation of *Drosophila* male germline stem cells into spermatocytes. Upon commitment to differentiation, testis-specific TATA-binding protein associated factors are expressed and counteract PcG silencing of differentiation genes (Chen et al., 2011). Genetic manipulation of transcription factors and PcG activity has previously been used to identify coordinately regulated genes. For example, microarray analysis was used to identify genes with altered expression profiles in *Drosophila* embryos over-expressing the transcription factor Myc and in embryos with RNAi-mediated knockdown of Polycomb (Goodliffe et al., 2005). This study concluded that up to a third of Myc-regulated genes are potentially silenced by PcG complexes, although the experimental design did not allow identification of direct versus indirect targets of Myc and Polycomb.

Our approach has two major advantages over previously published studies of the interactions of transcription factors and PcG complexes in regulating gene expression. First, we will identify nervous system-specific transcription changes (as opposed to analyzing mRNAs from the entire embryo) using TU-tagging. TU-tagging uses the uracil phosphoribosyltransferase (UPRT) gene of the protozoan parasite Toxoplasma gondii to convert the modified uracil 4-thiouracil (TU) into the nucleotide form, 4-thiouridine-monophosphate, for subsequent incorporation into newly synthesized RNA.

Multicellular eukaryotes lack UPRT activity and thio-containing nucleotides do not naturally occur in eukaryotic mRNAs, thus targeted expression of UPRT allows cell type-specific tagging of mRNAs with TU. Following TU-tagging, RNA from a mixture of cells or a whole organism can be reacted with a thio-specific biotinylation reagent and mRNA from the cells of interest can then be purified using streptavidin-magnetic beads for subsequent analysis by microarrays or RNA-seq.

We will also distinguish direct and indirect PcG and TTF target genes. Candidate direct targets will be identified by comparing TU-tagging data to PcG target genes previously identified by chromatin-immunoprecipitation (ChIP) in *Drosophila* cell lines (Schwartz et al., 2010) and embryos (Schuettengruber and Ganapathi, 2009). These experiments have predicted > 300 PcG targets in the *Drosophila* genome and these data are compiled in a single searchable database: http://purl.oclc.org/NET/polycomb. It is notable that different PcG targets were identified depending on the material analyzed in these previous ChIP studies (for example, there is only ~40% overlap between PcG targets in embryos and S2 cells) (Schuettengruber et al., 2009). This result further supports the theory that PcG complexes regulate gene expression in a tissue / context-dependent manner. Whole embryo ChIP data do not provide cell type-specific information, but the overlap between these data and our TU-tagging data will allow us to identify genes that are good candidates for direct PcG binding in neuroblasts. Candidate direct Kr targets will be identified using the TU-tagging data to identify genes with Kr-dependent changes in transcription and computational methods to identify consensus Kr-binding elements in the enhancer region of these genes.

To validate our target predictions, we will use the recently developed INTACT method to purify nuclei from neuroblasts and perform chromatin immunoprecipitation of Polycomb and Kr to test for binding to candidate genes. Briefly, INTACT uses cell type-specific expression of a nuclear tagging fusion (NTF) protein. The NTF is composed of an outer nuclear pore protein fused to a biotinylation motif that is a substrate for in vivo biotinylation by the E. coli biotin ligase, BirA. BirA is co-expressed with the NTF in cells of interest and biotinylated nuclei can be purified from whole embryo preparations using streptavidin microbeads, based on methodology very similar to the methods we use to purify biotinylated RNA in TU-tagging experiments. INTACT has successfully been applied to the purification of nuclei from mesodermal cells in *Drosophila* embryos and muscle cells in adult *C. elegans* (Steiner et al., 2012). We will use purified neuroblast nuclei for chromatin immunoprecipitation (ChIP) and identification of PcG and Kr target genes, allowing us to answer questions not addressed by traditional mapping of PcG and transcription factor targets in embryos or tissue culture cells.

3.4 Conclusions

It is clear that PcG complexes have the potential to alter transcription factor activity during development (Muller, 1995; Hemberger et al., 2009), yet the effects of PcG activity on establishing cell fate-specific transcription programs during in vivo neurogenesis are not well defined. Our system for studying PcG regulation of neuroblast competence, combined with the innovative technologies described above, has the potential to allow us to identify the transcription networks established by the intersection

of PcG complexes and temporal transcription factors. This will provide a significant advance in our understanding of how PcG complexes establish permissive or restrictive chromatin environments for transcription factors that control neural development. The experiments proposed in this chapter should help establish a blueprint of how PcG complexes regulate neural progenitor competence. This blueprint will be useful for understanding PcG function in many aspects of neurogenesis and will be particularly relevant to the design of regenerative medicine therapies and related techniques. Figure 24 represents our model of Polycomb Group activity to regulate cell fate.

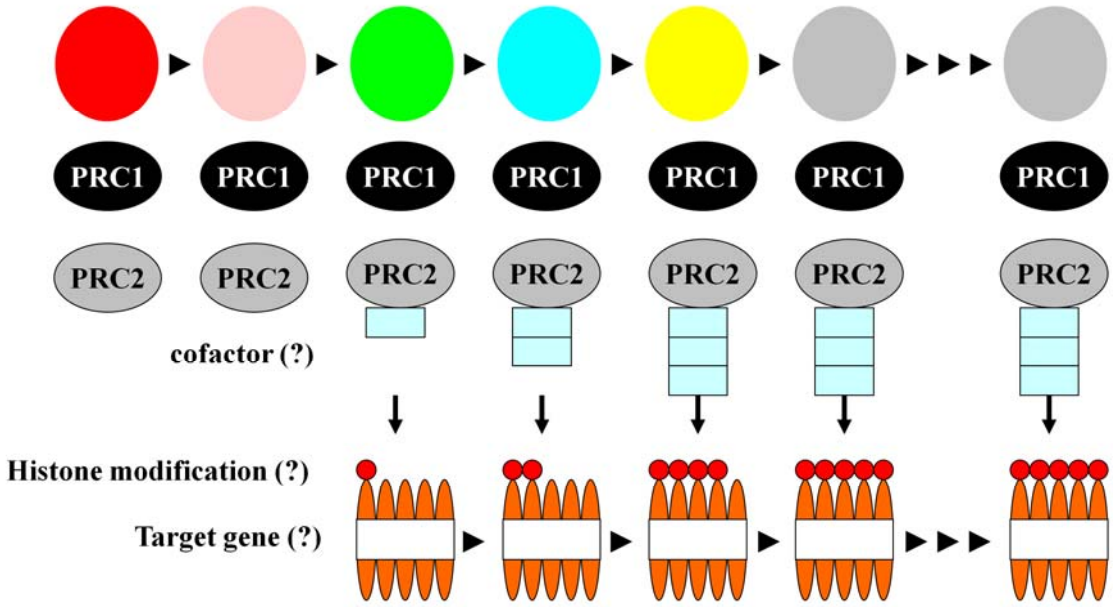

Figure 24: Model of Polycomb Group proteins regulation of cell fate specification. Polycomb repressive complexes PRC1 and PRC2 act to deposit histone modifications on the TTF target genes. We have identified a role of PcG in establishing permissive or restrictive chromatin environments for transcription factors that control neural development in motorneuron cell fate specification.

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