

TRANSCRIPTIONAL REGULATION OF EARLY PROGENITOR COMPETENCE IN
THE *DROSOPHILA* CENTRAL NERVOUS SYSTEM

by

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Neurogenesis in *Drosophila* and mammals requires the precise integration of spatial and temporal cues. In *Drosophila*, embryonic neural progenitors, called neuroblasts, sequentially express the transcription factors Hunchback, Kruppel, Pdm1/Pdm2 (Pdm) and Castor as they divide to generate a stereotyped sequence of neuronal and glial progeny. Hunchback is necessary and sufficient to specify the first-born cell identity in many neuroblast lineages. Additionally, Hunchback is able to maintain an early-competence state in which early-born cells are generated. Furthermore, the Hunchback mammalian ortholog, Ikaros, possesses a similar ability to specify early-born cells in the vertebrate nervous system. However, the mechanisms underlying the function of Hunchback/Ikaros are unknown.

Pdm and Castor are expressed later in many neuroblasts and can specify late-born neuronal cell identities in a model neuroblast lineage, NB7-1. Previous work studying their function in the NB7-1 lineage showed that Pdm and Castor act as repressors of *Kruppel* gene expression and inhibit the generation of the Kruppel-dependent cell identity. It is not known if the functions of Pdm and Castor are conserved across multiple neuroblast lineages during neurogenesis or whether these factors impart any restrictions on the ability of a factor like Hunchback to maintain early competence.

To investigate the transcriptional mechanisms regulating early neuroblast competence in *Drosophila*, I have focused my dissertation research on two aims. The first is to examine the function of Pdm and Castor across multiple neuroblast lineages to characterize their potential roles as competence restricting factors; the second is to determine how Hunchback maintains early neuroblast competence and specifies early-born cell identities (e.g. as a transcriptional activator, repressor, or both). My work demonstrates that Pdm and Castor control the timing of *Kruppel* gene expression, and possibly the timing of other genes, in neuroblasts. Furthermore, I have shown that Hunchback acts as a transcriptional repressor of multiple target genes, including *pdm* and *castor*, to maintain early neuroblast competence. Because Hunchback must repress at least one additional unknown factor that can restrict neuroblast competence, I have piloted a screen to identify and characterize novel Hunchback target genes in the nervous system.

This dissertation includes previously published and unpublished co-authored materials.

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CHAPTER I

INTRODUCTION TO THE *DROSOPHILA* CENTRAL NERVOUS SYSTEM AS A MODEL FOR TEMPORAL REGULATION OF NEURAL PROGENITORS

*“The brain is a wonderful organ. It starts working the moment
you get up in the morning and does not stop
until you get into the office.”*

-- Robert Frost

INTRODUCTION

Background on temporal regulation of vertebrate neural stem cells during nervous system development

Normal development of the central nervous system (CNS) depends on both the spatial patterning of progenitor domains, as well as the tempo at which individual progenitors generate distinct subtypes of neurons and glia (Berry and Rogers, 1965; Cepko, 1999; Doe and Skeath, 1996; Harris, 1997; Livesey and Cepko, 2001; Rapaport et al., 2001; Reid et al., 1997; Walsh and Reid, 1995). In the mammalian cerebral cortex,

individual neural stem cells generate progeny capable of populating all laminar layers (Reid et al., 1997; Walsh and Reid, 1995). Birth-dating studies revealed that each layer is occupied by neurons of similar birth-order, so that early-born neurons occupy the deepest layers and late-born neurons occupy the most superficial layers (McConnell, 1995). It has been shown that the transcription factor *Foxg1* is required at a precise moment during neural stem cell proliferation to repress the first-born cell fates and allow progenitors to specify later-born cells in the lineage (Hanashima et al., 2007; Hanashima et al., 2004). These findings highlight the importance of temporal cues during the development and diversification of the mammalian nervous system.

Background on nervous system development in *Drosophila*

Neural stem cells in the *Drosophila* embryonic nerve cord, called neuroblasts, delaminate from the epithelium to the interior of the embryo marking the start of neural differentiation. Individual neuroblasts can be identified based on the time at which they are formed, their position within each hemisegment (for example, NB7-1 is positioned in the seventh row, first column of the neuroblast array), and their pattern of gene expression (Broadus et al., 1995; Doe, 1992). In addition, each neuroblast generates a unique and invariant cell lineage (Bossing et al., 1996; Karcavich and Doe, 2005; Lundell and Hirsh, 1998; Pearson and Doe, 2003; Schmid et al., 1999; Schmidt et al., 1997) resulting from a series of asymmetric cell divisions where neuroblasts “bud-off” ganglion mother cells (GMCs) that typically undergo an additional division to produce two post-mitotic neurons. These neurons can differentiate as motoneurons, interneurons, or glia;

and all three cell types can arise from a single parent neuroblast. The unique cell lineage that is specified by each individual neuroblast depends on the identity of that neuroblast; and that identity is conferred by positional information and spatial gene expression (Broadus et al., 1995; Doe, 1992). While the spatial patterning cues that give rise to individual progenitor identity has been well characterized, less is known about the regulation of these progenitors over time.

In this dissertation, I discuss the mechanisms that promote the production of diverse cell identities by single progenitors over time. I will show that unique temporal identities are a product of well orchestrated interactions between temporal identity factors and timing factors; and that these timely interactions are critical to the normal development of the *Drosophila* central nervous system.

Background on temporal regulation of *Drosophila* neural progenitors

Each of the 30 neuroblasts in a hemisegment divides to produce an invariant order of neurons and/or glia whose identity is determined by the sequential expression of the transcription factors: Hunchback (Hb) → Kruppel (Kr) → Pdm1 (Nubbin, Flybase)/Pdm2 (henceforth Pdm) → Castor (Cas) → Grainyhead (Grh) (Baumgardt et al., 2009; Isshiki et al., 2001; Mairange et al., 2008). In this manner, neurons resulting from first-born GMC division are pushed to deep positions in the CNS and express molecular markers for early-born cells, while later-born neurons occupy more ventral positions and express late-born fate markers (Isshiki et al., 2001). The early- and late-born markers are excellent candidates for genes that specify cell identity based on birth-order, also called

temporal identity genes (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003).

Two transcription factors are known to have critical roles in specifying temporal identity: Hunchback (Hb) and Krüppel (Kr). Hb is an Ikaros-type zinc finger protein expressed in newly formed neuroblasts and in their early-born GMCs and neuronal progeny; it is necessary and sufficient to specify the first temporal identity in multiple neuroblast lineages (Cleary and Doe, 2006; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002; Pearson and Doe, 2003). Note that we define the "first" temporal identity as the neuronal fates specified during the window of Hb expression; this can be just one GMC and its sibling neurons as in the NB7-3 lineage, or two GMCs and their neuronal progeny as in the NB7-1 lineage. In the latter case, high Hb specifies the first GMC/U1 neuron fate, and low Hb specifies the second GMC/U2 fate (reviewed in Pearson and Doe, 2004). Kr is a zinc finger protein that is detected at low levels together with Hb, and at high levels in neuroblasts and their progeny immediately following Hb down-regulation; it is necessary and sufficient to specify the second temporal identity in both the NB7-1 and NB7-3 lineages (Isshiki et al., 2001). We define the second temporal identity to be that following the Hb-dependent first temporal identity; this can be the second-born or the third-born GMC in a lineage.

It is unknown what factors specify the temporal identity that comes after Krüppel. In NB7-1, Pdm and Castor specify the U4 neuronal identity, and Castor alone specifies the U5 neuronal identity. It is not known whether Pdm and Castor are specifying successive temporal identities following the Krüppel-dependent second temporal identity,

or whether they have a lineage specific role of specifying the U4 and U5 neurons in NB7-1. Furthermore, to classify Pdm and Castor as bonafide temporal identity factors, they must specify the same temporal identity in multiple neuroblast lineages. In Chapter II, I present and discuss results from my investigation of Pdm and Castor function in a neuroblast lineage where the temporal identity of progeny neurons have never been characterized, NB3-1. My work shows that Pdm and Castor act as timing factors that regulate the gene expression window of factors that precede them in many neuroblast lineages.

Background on the regulation of early neuroblast competence in *Drosophila*

Small pools of multipotent neural progenitors give rise to a large number of neurons and glia to allow proper assembly of a functional nervous system (Cepko, 1999; Doe and Skeath, 1996; Rapaport et al., 2001; Walsh and Reid, 1995). However, as progenitors change over time to accommodate the production of different tissues, they also undergo a progressive restriction and lose their competence to produce the full assortment of cell types (Desai and McConnell, 2000; Rapaport et al., 2001). The ability to maintain progenitors in their early competent states yields the capacity to generate any desired tissues for use in future cell therapy applications. In recent years, substantial progress has been made in the identification of new factors involved in regulating progenitor competence, bringing with it the need to understand the molecular basis underlying their function.

The identification of genes regulating neural progenitor competence in both vertebrates and insects has provided an entry point for investigating the molecular mechanism of progenitor competence (Elliott et al., 2008; Hanashima et al., 2004; Isshiki et al., 2001; Novotny et al., 2002). The zinc-finger transcription factor Ikaros (Ik) is both necessary and sufficient to specify early-progenitor competence, leading to the production of early-born cell types in the mouse retina (Elliott et al., 2008). This function of Ikaros mimics that of its *Drosophila* ortholog, the zinc-finger transcription factor Hunchback (Hb), which has also been shown to promote early progenitor competence during *Drosophila* neurogenesis (Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003).

Hb is expressed early in many neuroblasts and is required for the specification of the first-born cell identity, or first-temporal identity, in those lineages. In addition, Hb can also confer the early-competence state where early-born progeny are specified in many neuroblast lineages. Interestingly, the ability of Hb to specify and extend the early-competence window declines over time (Cleary and Doe, 2006; Pearson and Doe, 2003). When Hb is reintroduced to NB7-1 at progressively later time points, its ability to specify ectopic U1/U2 neurons is greatly reduced. Eventually, NB7-1 is unable to respond to Hb to specify early-born cells after the fifth neuroblast division. It is possible that some unknown factor or factors expressed later in the neuroblast lineage may act to advance neuroblast programming beyond the early-competence state where the neuroblast normally specifies early-born cell identities. Two likely candidates that may function to restrict early neuroblast competence are Pdm and Castor, as they are expressed later in

many neuroblast lineages; and in NB7-1, they are expressed around the time when the neuroblast loses the ability to respond to Hb. In NB7-1, the loss of Castor leads to the specification of ectopic U5 neurons and extends the window of motoneurons production. This provides some evidence that Castor is required to properly advance NB7-1 from a motoneurons producing state to an interneuron producing state. In Chapter II, I characterize the roles of Pdm and Castor in the NB3-1 lineage and provide more evidence suggesting that Pdm and Castor can impose restrictions on the ability of a neuroblast to specify early-born cell identities.

While it is known that Hb can maintain progenitor competence in the nervous system, the molecular mechanisms underlying its function remains unknown. Hunchback is a transcription factor previously characterized to have both activator and repressor activities in the early fly embryo (Kraut and Levine 1991; Schulz and Tautz 1994; Papatsenko and Levine 2008). During the first few hours of development, very high levels of Hb repress Kr, while low levels activate Kr. Hb can also repress the posterior gap genes *Knirps* (*Kni*) and *Giant* (*Gt*), at low concentrations. While the mechanisms of Hb-mediated gene regulation in the early embryo have been well characterized, the mechanisms underlying Hb function in the CNS have yet to be described. In Chapter III, I investigate the mechanisms of Hb-mediated gene regulation in the nervous system, and address the following questions: First, how does Hb regulate gene expression to maintain early-neuroblast competence? And second, why does this ability decline over time? I present and discuss the results of my investigation, and show that Hb must repress multiple late-expressing genes in order to maintain early neuroblast competence. One

such target is the previously discussed gene, Pdm. My work suggests that factors such as Pdm can advance neuroblast timing beyond the early-competence window, and that Hb must normally act as a repressor of these factors to create a unique window of opportunity for neuroblasts to specify early-born cells.

For results presented in the proceeding chapters, Khoa D. Tran and Chris Q. Doe designed research; Khoa D. Tran performed research; Khoa D. Tran and Chris Q. Doe analyzed data. Michael R. Miller provided new reagents for the work presented in Chapter III.

Bridge to Chapter II

In the following chapter, I present the findings from my investigation of the roles of Pdm and Castor in specifying neuronal identity during early neurogenesis in the *Drosophila* embryo. Because Pdm and Castor have only been assayed in one lineage, it is unknown whether their function is restricted to only the NB7-1 lineage, or whether they function more broadly as late temporal identity genes in all neuroblast lineages. To investigate the roles of Pdm and Castor outside of the NB7-1 lineage, I identify the neuronal birth-order and molecular markers within the NB3-1 cell lineage, and then use this lineage to assay Pdm and Castor function. I show that Hunchback and Kruppel specify first and second temporal identities in the NB3-1 lineage, similar to their roles in other neuroblast lineages; surprisingly, Pdm does not specify the third temporal identity, but rather acts as a timing factor to close the second temporal identity window. Similarly, Castor closes the third temporal identity window. This work lead us to conclude that

Hunchback/Kruppel specify the first/second temporal identity, an unknown factor specifies the third temporal identity, and Pdm/Castor are timing factors that close the second/third temporal identity windows in the NB3-1 lineage. These results provide a new neuroblast lineage for investigating temporal identity; and reveal the importance of Pdm and Castor as timing factors that regulate precise temporal identity windows during which unique cell identities are specified. Furthermore, these results suggest that late-onset genes such as Pdm and Castor may also have a role in regulating the early-competence window in many neuroblasts.

CHAPTER II

PDM AND CASTOR ARE TIMING ELEMENTS THAT CLOSE SUCESSIVE TEMPORAL IDENTITY WINDOWS IN THE NEUROBLAST 3-1 LINEAGE

“The only reason for time is so that everything doesn't happen at once.”

-- Albert Einstein

Khoa D. Tran and Chris Q. Doe designed research; Khoa D. Tran performed research; Khoa D. Tran and Chris Q. Doe analyzed the data and wrote the manuscript published below.

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INTRODUCTION

While previous studies have identified temporal identity factors that specify the first and the second temporal identities, it remains unknown what factor or factors specify the cell identities following Kruppel. The best candidate for a multi-lineage third

temporal identity factor is Pdm (a pair of co-expressed, redundantly-functioning POU domain proteins, Pdm1/Nubbin and Pdm2). Pdm is expressed immediately after Kr in many neuroblasts, and is known to specify the third temporal identity (U4 neuron) within the NB7-1 lineage (Grosskortenhaus et al., 2006). However, the analysis of just one neuroblast lineage does not resolve whether Pdm has a specific function in specifying U4 motoneuron identity, or a more general function as a multi-lineage third temporal identity factor. This is a crucial distinction, because many transcription factors are likely to regulate different neuronal subtype specification without having anything to do with temporal patterning. In fact, Pdm is also required for specification of the first-born progeny in the NB4-2 lineage (Yang et al., 1993; Yeo et al., 1995), raising some doubt about its role as a multi-lineage temporal identity gene.

The best candidate for a multi-lineage fourth temporal identity factor is the zinc finger protein Castor (Cas), which is detected in neuroblasts just as Pdm fades away, and together with Pdm specifies the fourth temporal identity (U5 neuron) in the NB7-1 lineage (Grosskortenhaus et al., 2006; Isshiki et al., 2001). As with Pdm, it is impossible to know whether Cas has a specific role in specifying U5 identity or a general role as a fourth temporal identity gene without analyzing its function in additional neuroblast lineages. This has been difficult, because most neuroblast lineages have not been characterized past the first or second cell division, and few molecular markers are known for late-born neurons. For example, NB7-3 generates neurons with well-characterized molecular markers (Isshiki et al., 2001; Lundell et al., 1996; Lundell and Hirsh, 1998; Novotny et al., 2002), but it only divides three times and never expresses Cas; in contrast,

NB2-4 divides many times and expresses Pdm and Cas (Isshiki et al., 2001) but there are no molecular markers available to identify late-born neurons in this lineage. Thus, to test the role of Pdm and Cas as multi-lineage late temporal identity factors, and to test any new candidate late-born temporal identity factors, it is necessary to characterize a new neuroblast lineage for both birth-order lineage data as well as neuronal molecular markers.

Here we trace the birth-order of the first four divisions in the NB3-1 lineage and develop molecular markers to distinguish early-born and late-born neuronal identity, allowing us to use this lineage to assay late temporal identity gene expression and function. We find that Hb and Kr specify early temporal identity in this lineage, extending their role as multi-lineage temporal identity factors to a different spatial domain of the CNS. Surprisingly, we find that Pdm is not required to specify the third temporal identity, but rather that Pdm is required to repress Kr and thus close the second temporal identity window. Similarly, we find that Cas is required to close the third temporal identity window in this lineage. We conclude that Hb and Kr are multi-lineage temporal identity factors, while Pdm and Cas are timing factors that close successive temporal identity windows in the NB3-1 lineage.

MATERIAL AND METHODS

Fly stocks

We used the following fly stocks to analyze wild-type and mutant phenotypes at 23°C: *hb^{P1}, hb^{FB} / TM3 hb-lacZ* to remove Hb CNS expression (Hulskamp et al., 1994; Isshiki et al., 2001); *Kr¹, Kr^{CD} / CyO hb-lacZ* to remove Kr CNS expression (Isshiki et al., 2001; Romani et al., 1996); *Df(2L)ED773* which removes both *pdm1* and *pdm2* (Grosskortenhaus et al., 2006), *cas²⁴ / TM3 ftz-lacZ* (formerly called *ming²⁴* (Cui and Doe, 1992)), *red e spdo^{ZZ27} / TM3* and *numb² pr cn Bc / CyO ftz-lacZ* (Skeath and Doe, 1998); *cas-lacZ* (Cui and Doe, 1992); *svp^{e22}/svp^{z4}* (Miller et al., 2008; Mlodzik et al., 1990). Unless otherwise noted, for misexpression experiments we crossed *insc-gal4* (*1407-gal4*, Bloomington Stock Center) on chromosome II to *UAS-hb* on chromosomes II and III (Wimmer et al., 2000), *UAS-Kr* on chromosomes II and III (Hoch and Jackle, 1998), *UAS-HA:pdm2* on chromosomes II and III (Grosskortenhaus et al., 2006), and *UAS-cas* on chromosomes II and III (W. Odenwald, NIH, Washington, DC) at 29°C. Recombinant clones were generated using flies with the following genotype: *y w hs-FLP / + ; X-15-33 / X-15-29* (courtesy of Allan C. Spradling, Carnegie Inst., Washington DC).

Molecular markers and immunostaining

Antibody staining was performed according to standard methods. Primary antibodies, dilutions, and sources: rabbit HB9, 1:1000 (Odden et al., 2002); guinea pig HB9, 1:500 and rat Islet, 1:500 (Broihier and Skeath, 2002); mouse Islet, 1:200, mouse

FasII, 1:100, mouse FasIII, 1:5 (Developmental Studies Hybridoma Bank, Iowa); rabbit Hb, 1:200 (this work); guinea pig Kr, 1:500 (East Asian Distribution Center for Segmentation Antibodies); rat Pdm2, 1:10 (Grosskortenhaus et al., 2006); rabbit Cas, 1:1000 (Kambadur et al., 1998); rat Zfh2, 1:400 (M. Lundell, UT San Antonio); mouse En 4D9, 1:5 (Patel et al., 1989); mouse Late Bloomer, 1:4 (C. Goodman, UC Berkeley); mouse Beta-galactosidase, 1:500 (Promega, Madison, WI). Secondary antibodies were purchased conjugated to Alexa 488, Rhodamine RedX, or Cy5 (Jackson, West Grove, PA), biotin (Vector, Burlingame, CA), or alkaline phosphatase (Southern Biotechnology, Birmingham, AL) and used at 1:400. Confocal image stacks were collected using a Leica SP2 confocal microscope, processed using ImageJ (NIH), and shown as two-dimensional projections. Histochemical preparations were acquired using a Zeiss Axioplan.

Identification of NB3-1 and the RP motor neurons

We staged embryos using standard methods (Campos-Ortega and Hartenstein, 1985), and identified NB3-1 using spatial and morphological features along with the expression of Engrailed (marking neuroblast in rows six and seven). RP1, RP4, RP3, and RP5 neurons were identified as Isl⁺ HB9⁺ neurons in the dorsal-medial region of each hemisegment. The only other nearby Isl⁺ HB9⁺ neurons are the more posterior/lateral EW neurons from the NB7-3 lineage, which can be distinguished from the RP neurons by their expression of Engrailed (Isshiki et al., 2001; Lundell et al., 1996). RP1 and RP4 are often shown as insets as they appear directly ventral to RP3 and are often obstructed from view.

RESULTS

Temporal identity gene expression and neuronal birth-order in the NB3-1 lineage

To identify a new lineage ideal for temporal identity analysis, we focused on one that was outside neuroblast row 7 (where the previously characterized NB7-1 and NB7-3 reside), and contained several well-characterized neurons. We chose NB3-1 by virtue of its position within the anterior region of the segment, far from the posterior row 7, and because it was known to generate the well-characterized RP1, RP4, RP3, and RP5 motor neurons (Bossing et al., 1996; Landgraf et al., 1997; Schmid et al., 1999). We first characterized the expression of the known and candidate temporal identity genes Hb, Kr, Pdm, and Cas in NB3-1 as it begins its cell lineage. We detect Hb and Kr expression in the newly formed NB3-1 at stage 10; Kr alone during early stage 11; Pdm alone at mid stage 11; and Cas expression from late stage 11 into stage 12 (Fig. 1A, B). We conclude that NB3-1 sequentially expressed Hb/Kr→ Kr→ Pdm→ Cas during the initial phase of its cell lineage, and that this lineage is appropriate for investigating the role of all four genes in specifying temporal identity.

Next, we characterized the birth-order of the RP neurons and determined which known or candidate temporal identity gene was expressed at the time of their birth. We use both molecular markers and cell body position to identify and distinguish the RP neurons (Fig. 2A,B; see methods for details). Motor neuron backfills show that RP1/4 are the most dorsal, RP3 is intermediate, and RP5 is most ventral in position within the CNS (Landgraf et al., 1997; Schmid et al., 1999). Early-born neurons occupy deeper layers

(Isshiki et al., 2001), consistent with a birth-order of $RP1/RP4 \rightarrow RP3 \rightarrow RP5$. Here we used molecular markers to identify the NB3-1 derived RP neurons, and assayed their Hb, Kr, Pdm, and Cas expression profile. We find that $RP1/4$ are $Hb^+ Kr^+$, $RP3$ is $Hb^- Kr^+$, and $RP5$ is $Hb^- Kr^-$ (Fig. 2A). This precisely matches the sequence of gene expression within NB3-1 as it goes through the early portion of its lineage (Fig. 1). We conclude that $RP1/4$ are born during the early $Hb^+ Kr^+$ neuroblast expression window, $RP3$ is born during the $Hb^- Kr^+$ neuroblast expression window, and $RP5$ is generated after Hb and Kr expression is lost from the neuroblast.

The $RP1$ and $RP4$ neurons express the same molecular markers (Fig. 2A) and have identical axon projections (Landgraf et al., 1997), raising the possibility that they are sibling neurons. To determine whether $RP1$ and $RP4$ are sibling neurons, we used *sanpodo* and *numb* mutants to equalize sibling cell fate (Skeath and Doe, 1998). We found that *sanpodo* mutants typically generate a pair of $RP1$ neurons and a pair of $RP4$ neurons, and *numb* mutants show the opposite phenotype (Fig. 2B; Table 1). These results show that the $RP1$ and $RP4$ neurons are not siblings; but rather that they each have a non-RP sibling that assumes the RP fate in *sanpodo* mutants. Furthermore, we have generated clones by mitotic recombination that label the entire NB3-1 lineage except $RP1$ ($n=4$; Fig. 2C); as all four neurons are definitively produced by NB3-1 based on DiI labeling (Bossing et al., 1996; Schmid et al., 1999), this proves that $RP1$ is derived from the first-born GMC in the lineage. We conclude that NB3-1 sequentially generates the $RP1 \rightarrow RP4 \rightarrow RP3 \rightarrow RP5$ neurons (and their non-RP siblings), followed by a pool of local interneurons (Fig. 2D).

Hunchback specifies the first temporal identity in the NB3-1 lineage

Hb is known to specify the first temporal identity in two closely positioned lineages, NB7-1 and NB7-3, located within the *Engrailed*⁺ posterior region of the neuromere (Broadus et al., 1995). Here we test whether Hb has a similar function in the NB3-1 lineage, which is located in the anterior region of the neuromere. We used *hb* mutants that were rescued for Hb segmentation expression (Isshiki et al., 2001), and found a loss of the early-born RP1 and RP4 neurons; the later-born RP3 and RP5 neurons were unaffected (Fig. 3A, Table 1). Thus, Hb is required for the specification and/or survival of the early-born RP1 and RP4 neurons. To determine if Hb is sufficient to induce the first-born RP1/RP4 temporal identity, we used *insc-gal4 UAS-hb* to prolong expression of Hb in NB3-1 beyond its normal expression window. We observed as many as nine RP neurons per lineage, and all appeared to take the early-born RP1/RP4 identity based on molecular markers (Fig. 3B-C, Table 1). Consistent with the increase in RP1/RP4 motor neurons, we observe a thickening of *FasII*⁺ and *FasIII*⁺ motor axon fascicles exiting the CNS and entering the ventral longitudinal muscle fields (Fig. S1). Late-born RP3 and RP5 neurons expressing *Zfh2* or *Cut* were never detected. We conclude that Hb is necessary and sufficient to specify early-born RP1/RP4 temporal identity within the NB3-1 lineage, paralleling to its role in specifying the first temporal identity in the NB7-1 and NB7-3 lineages (Isshiki et al., 2001; Novotny et al., 2002).

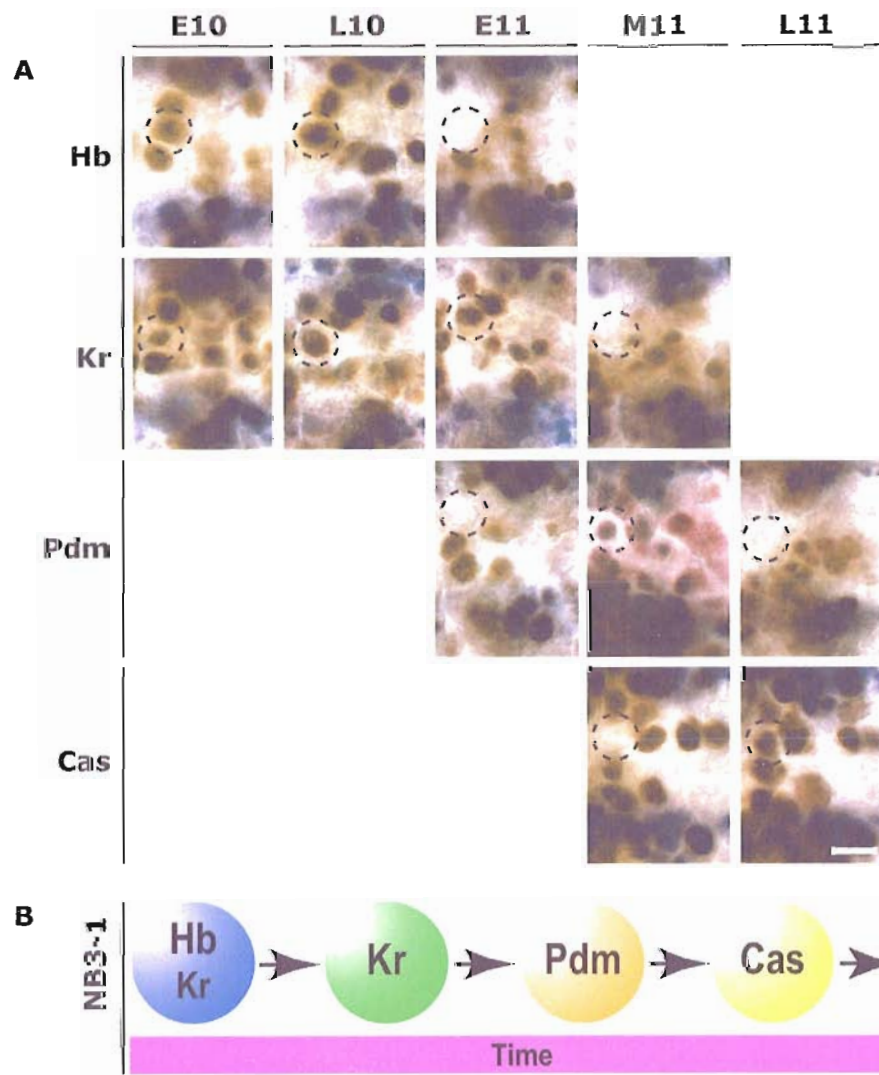


Figure 1. Temporal identity gene expression in NB3-1.

(A) NB3-1 (*black circle*) sequentially expresses Hb, Kr, Pdm, and Cas. A portion of one hemisegment is shown, with Engrailed marking the most posterior NB rows 6/7 (blue); midline, left; anterior, up. Embryonic staging from Campos-Ortega and Hartenstein (1985): early stage 10 (E10) when NB3-1 forms; late stage 10 (L10); early stage 11 (E11); mid stage 11 (M11); late stage 11 (L11). Scale bar is 10 μ m.

(B) Summary of gene expression in NB3-1.

Figure 2. Molecular markers and cell position can be used to identify each RP motor neuron in the NB3-1 lineage.

- (A) RP1 and RP4 are Hb^+ , Kr^+ , Cut^- , $Zfh2^-$ and are often shown in insets as they are usually obstructed in the projection ($n > 100$). RP3 is Hb^- , Kr^+ , Cut^- , $Zfh2^+$ ($n > 100$). RP5 is Hb^- , Kr^- , Cut^+ , $Zfh2^+$ ($n > 100$). A single representative hemisegment of a stage-16 CNS is shown as a maximum intensity projection; midline, left; anterior, up; phenotype summary, top panel; scale bar, 3 μm .
- (B) Top row: RP motor neurons are $Islet^+$ $HB9^+$ (*white outline*). RP1 and RP4 occupy the deepest layer; RP1 is more dorsal and expresses HB9 at higher levels than RP4 after stage 15 ($n > 100$). RP3 is directly ventral to RP1 and RP4 ($n > 100$). RP5 is ventral and anterior to RP3 ($n > 100$). NB7-3 derived EW interneurons (*white arrowheads*); midline, dashed vertical line; ventral views of two segments are shown from deep (left) to superficial (right) focal planes. Bottom row: Each RP neuron has a non-RP neuron sibling, based the duplication of each RP neuron in *sanpodo* mutants. Eight $Islet^+$ $HB9^+$ Late Bloomer⁺ RP motor neurons are observed in each hemisegment; quantified in Table 1. Midline, between each pair of panels.
- (C) Recombination-induced activation of the lacZ gene in NB3-1 labeled RP4, RP3, RP5 and the late-born interneurons but not RP1, showing that RP1 is the first-born neuron in the lineage. HB9 is green, beta-gal is magenta, and RP neurons and NB3-1 are outlined and labeled. One hemisegment of a stage-16 CNS is shown as a maximum intensity projection; midline, left; anterior, up. A summary of the clone is to the right. Scale bar is 3 μm .
- (D) Schematic of NB3-1 gene expression and cell lineage. The vertical dashed line represents the transition between RP neuron and subsequent interneuron specification. Nb, sibling cell fate specified by Numb; N, sibling cell fate requires Sanpodo and active Notch signaling.

Table 1 Summary of phenotypes in the NB3-1 lineage

Genotype*	Protein gain or loss (+/-)	RP neuron identity [‡]						Conclusion
		Total # of RP (n)	RP1	RP4	RP3	RP5		
wild-type		4	(100)	1	1	1	1	wild type
<i>spdo</i> mutant	- Sanpodo	8.0	(70)	2	2	2	2	Spdo specifies RP sibling fates
<i>numb</i> mutant	- Numb	1.0	(88)	1 [†]		0	0	Numb specifies RP fates
<i>hb</i> mutant	- Hb (CNS)	2	(163)	0	0	1	1	Hb is required for RP1/RP4 identity
2x <i>UAS-hb</i>	+ Hb	6.9	(198)	6.9 [‡]		0	0	Hb sufficient for RP1/4 identity
<i>Kr</i> mutant	- Kr (CNS)	2.7	(152)	1	1	0	0.7	Kr is required for RP3 identity
2x <i>UAS-Kr</i>	+ Kr	4.3	(147)	1	1	2.3	0	Kr is sufficient for RP3 identity
<i>pdm</i> mutant	- Pdm1/2	5.1	(172)	1	1	1.8	1.3	Pdm closes the RP3 temporal window
2x <i>UAS-pdm2</i>	+ Pdm2	2.9	(142)	1	1	0.6	0.3	Pdm represses RP3/RP5 identity
<i>cas</i> mutant	- Cas	5.6	(183)	1	1	1	2.6	Castor closes the RP5 temporal window
2x <i>UAS-cas</i>	+ Cas	2.8	(156)	1	1	0.8	0	Cas represses RP5 identity

$P < 0.01$ for all experiments.

* All genotypes are described in Materials and Methods.

|| Average number of each cell type present per hemi-segment based on markers described in Figure 1.

‡ We currently lack the markers to distinguish between RP1 and RP4 in functional analysis.

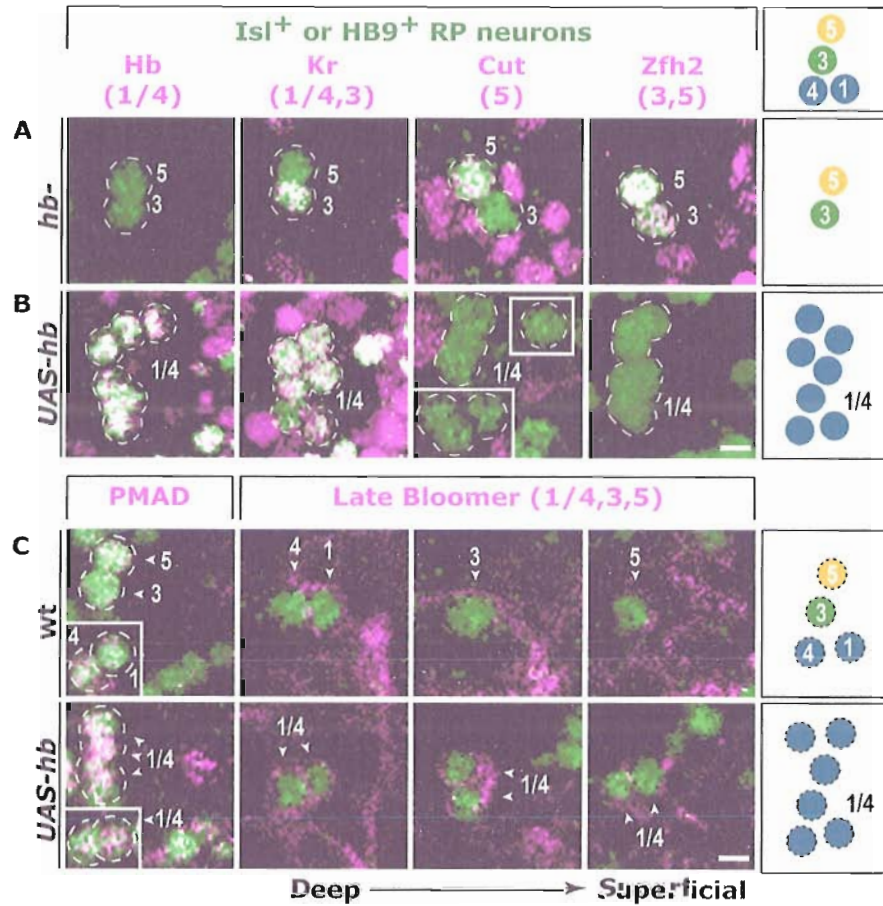


Figure 3. Hb is necessary and sufficient to specify the first temporal identity.

(A) *hb* CNS mutants lack the RP1/RP4 neurons, but have normal RP3 and RP5 neurons; quantified in Table 1. Wild type has RP1, RP4, RP3, and RP5 neurons (Fig. 2).

(B) *hb* misexpression (*insc-gal4 UAS-hb*) generates ectopic RP1/4 neurons based on molecular markers; quantified in Table 1.

(C) Top row: Wild type RP motor neurons express the pan-motor neuron markers pMAD and Late Bloomer. Bottom row: *hb* misexpression (*insc-gal4 UAS-hb*) generates ectopic RP1/4 neurons that are pMAD and Late Bloomer double positive (100%, n = 48). For all panels, a single representative hemisegment of a stage-16 CNS is shown as a maximum intensity projection; midline, left; anterior, up; phenotype summary shown in right panel, with Late Bloomer expression indicated by dashed circles. Scale bar is 3 μ m.

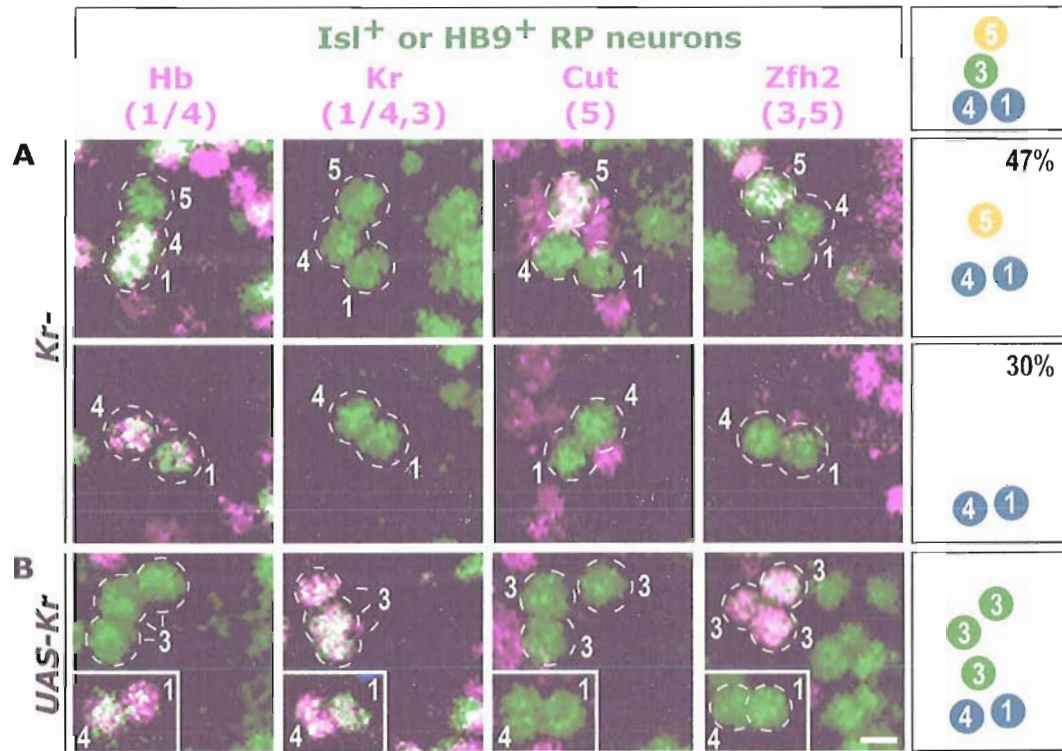


Figure 4. Kr is necessary and sufficient to specify the second temporal identity.

(A) In *Kr* CNS mutant embryos, RP3 is missing in the majority of hemisegments examined (both rows), and RP5 is occasionally missing (second row); RP1/RP4 are normal; quantified in Table 1. Wild type has RP1, RP4, RP3, and RP5 neurons (Fig. 2).

(B) *Kr* misexpression (*insc-gal4 UAS-Kr*) generates ectopic of RP3 neurons, RP5 is usually absent, and RP1/RP4 are normal; quantified in Table 1. For all panels, a single representative hemisegment of a stage-16 CNS is shown as a maximum intensity projection; midline, left; anterior, up; phenotype summary, right.

Scale bar is 3 μ m.

Kruppel specifies the second temporal identity in the NB3-1 lineage

Kr is known to specify the second temporal identity in the NB7-1 and NB7-3 lineages (i.e. the fate of the GMC born immediately after Hb downregulation) (Cleary and Doe, 2006; Isshiki et al., 2001). Here we test whether Kr has a similar function in the NB3-1 lineage. We used *Kr* mutants that were rescued for early segmentation expression (Isshiki et al., 2001), and found a loss of the RP3 neuron; the early-born RP1/RP4 neurons and the late-born RP5 neuron were mostly unaffected (Fig. 4A, Table 1). Thus, Kr is required for the specification and/or survival of the RP3 neuron; this is the RP neuron born during the Kr neuroblast expression window. To determine if Kr is sufficient to induce the RP3 identity, we used *insc-gal4 UAS-Kr* to prolong expression of Kr in NB3-1 for the entire length of its cell lineage. We observed a maximum of three RP3 neurons per lineage (Fig. 4B, Table 1). We saw no deleterious effect on the specification of RP1 and RP4, but the Cut⁺ RP5 neuron was typically missing (Fig. 4B). We conclude that Kr is necessary to specify the second temporal identity in the NB3-1 lineage (RP3) -- within a competence window -- similar to its role in specifying the second temporal identity in the NB7-1 and NB7-3 lineages (Isshiki et al., 2001).

Pdm is required to close the second temporal identity window, but not for specifying the third temporal identity in the NB3-1 lineage

Pdm expression follows Hb and Kr in most neuroblasts, and thus is an excellent candidate for specifying the third temporal identity. Indeed, Pdm is necessary and sufficient to specify the third temporal identity (U4 neuron) within the NB7-1 lineage

(Grosskortenhause et al., 2006). To determine if Pdm is a multi-lineage temporal identity gene, we assay its loss of function and misexpression phenotype in the NB3-1 lineage. We assayed embryos homozygous for the deficiency *Df(2L)ED773* (which eliminates both *pdm1* and *pdm2*; henceforth called *pdm* mutant embryos). In *pdm* mutant embryos, we observed normal timing of Hb expression in NB3-1 and other neuroblasts (data not shown), a modest extension of Kr expression, and a similar delay in Cas expression (Fig. 5A). Consistent with this change in neuroblast gene expression, *pdm* mutant embryos show normal specification of the early-born Hb⁺ RP1 and RP4 neurons, possess extra Kr⁺ RP3 neurons, followed by an apparently normal Cut⁺ late-born RP5 (Fig. 5C, Table 1). We conclude that Pdm is not required to specify the third temporal identity (the Cut⁺ RP5 neuron), but it is required to limit Kr expression in the neuroblast and thus close the second temporal identity window after the birth of just one Kr⁺ RP3 neuron.

We next determined if continuous expression of Pdm in NB3-1 was sufficient to induce ectopic RP5 neurons (i.e. extend the third temporal identity window). We used *insc-gal4 UAS-pdm2* to generate continuous Pdm expression in neuroblasts, and observed normal timing of Hb expression in NB3-1 and other neuroblasts (data not shown), but premature loss of Kr expression and precocious Cas expression (Fig. 5B). Consistent with this change in neuroblast gene expression, we observe normal specification of the early-born Hb⁺ RP1 and RP4 neurons, but lack Kr⁺ RP3 neurons; there is also a loss of the Cut⁺ late-born RP5 neuron (Fig. 5D, Table 1). We conclude that Pdm is not sufficient to specify the third temporal identity (RP5), but rather it acts as a timer element to define the window of Kr expression, and thus the length of the second temporal identity

window. The precocious expression of Cas in these Pdm misexpression embryos may result in the precocious formation of Cas⁺ interneurons at the expense of the RP5 neuron (see below).

Castor is required to close the third temporal identity window in the NB3-1 lineage

Cas is expressed in NB3-1 following Hb, Kr, and Pdm, but it is not detected in any of the post-mitotic RP1-RP5 motor neurons (Figs. 1A, 2A). In addition, we examined flies carrying the *cas-lacZ* reporter transgene (Cui and Doe, 1992) and found no residual β -galactosidase expression in any NB3-1 derived RP neurons (data not shown). This suggests that Cas expression is initiated after NB3-1 has made its fourth GMC, at the time it shifts to producing local interneurons (Fig. 2D). Thus, we can test whether Cas is important for closing the third (RP5) temporal identity window, but due to the lack of interneuronal markers we are unable to assay for a Cas function in specifying the fourth (interneuron) temporal identity.

To test whether Cas is required to close the third temporal identity window, we assayed *cas* null mutant embryos (Cui and Doe, 1992). We find that *cas* mutants have normal Hb and Kr expression in neuroblasts (data not shown) but prolonged Pdm expression (Fig. 6A), consistent with previous work showing that Cas is required to repress *pdm* (Grosskortenhaus et al., 2006; Kambadur et al., 1998). At the neuronal level, we find that *cas* mutants have normal early-born RP1, RP4, and RP3 neurons but possess ectopic RP5 neurons (Fig. 6B, Table 1), consistent with a prolonged third temporal identity window. The ectopic RP5 neurons are not specified by the persistent Pdm

protein, because *pdm* mutants still form apparently normal RP5 neurons (Fig. 5), and *pdm cas* double mutants still form Cut⁺ RP5 neurons (data not shown). Interestingly, *cas* mutants have a few RP-like (Islet⁺ HB9⁺) neurons that lack expression of the motor neuron marker Late Bloomer and thus may have a mixed interneuron/RP motor neuron identity (Fig. S2). We next examined *insc-gal4 UAS-cas* embryos which have continuous expression of Cas in NB3-1. We find that RP5 is often missing, but the early-born RP1, RP4 and RP3 are normal (Fig. 6C, Table 1). We conclude that the precocious expression of Cas is sufficient to close the third temporal identity window, in which RP5 is specified. Taken together, our results suggest that Cas is necessary and sufficient to close the third temporal identity window in the NB3-1 lineage.

DISCUSSION

We have characterized the neuronal birth-order of the first four motor neurons within the NB3-1 lineage, described the temporal identity gene expression pattern within NB3-1 and its motor neuronal progeny, and performed functional analysis of all four known and candidate temporal identity genes. Our results confirm and extend previous conclusions that Hb and Kr are multi-lineage temporal identity genes, and reveal novel aspects regarding the role of Pdm during the specification of temporal identity. We find that both Pdm and Cas play essential roles as part of the neuroblast gene expression timer: Pdm closing the second temporal identity window, and Cas closing the third temporal identity window.

Hunchback and Kruppel are multi-lineage temporal identity factors

We have shown that Hb and Kr are necessary and sufficient to specify the first and second temporal identities in the NB3-1 lineage. We can now conclude that Hb and Kr function as temporal identity factors in many spatial domains of the CNS – anterior-medial (NB3-1), posterior-medial (NB7-1), posterior-lateral regions (NB7-3) – showing that temporal identity and spatial identity are independent with regards to Hb and Kr. Furthermore, Hb and Kr maintain similar functions in neuroblasts that form at distinct times during embryogenesis – early (NB7-1), middle (NB3-1), and late (NB7-3) – thus confirming that temporal identity is a lineage-autonomous event that is not coordinated by embryo-wide timing events (Brody and Odenwald, 2000; Grosskortenhaus et al., 2005). Overall, our data strongly support the conclusion that Hb and Kr are multi-lineage temporal identity genes.

Our data also provide insight into neuroblast competence. When we misexpressed Hb in the NB3-1 lineage, we can generate up to nine RP motor neurons; if each has a non-RP sibling, it would be near the expected number of cells for the entire lineage (Schmid et al., 1999). Thus, Hb seems capable of maintaining at least three very different neuroblast lineages (NB3-1, NB7-1, and NB7-3) in a "young" state for their entire lineage. In contrast, misexpression of Kr produces only a few RP3 motor neurons before NB3-1 proceeds to make the later-born neurons. The inability of Kr to maintain a second temporal identity state may be due to the initiation of progressive restriction in neuroblast competence in NB3-1, as occurs in NB7-1 (Cleary and Doe, 2006; Pearson and Doe, 2003).

Pdm closes the second temporal identity window in the NB3-1 lineage

Our findings show that Pdm is not required to specify the third temporal identity in the NB3-1 lineage, but rather that Pdm is a timer element that represses Kr expression and closes the second temporal identity window. Loss of Pdm allows for a transient extension of the Kr expression window leading to a few ectopic Kr-specified RP3 followed by a Cut⁺ RP5. We hypothesize that the production of the RP5 cell is possible because Kr is not permanently maintained in the neuroblast. In contrast, permanent expression of Kr in NB3-1 (*insc-gal4 UAS-Kr*) also leads to extra RP3 neurons but does not allow production of a Cut⁺ RP5, perhaps due to the continuous expression of Kr. Pdm is not the first transcription factor known to act as a timing element. The orphan nuclear hormone receptor Seven-up (Svp) is required for repressing Hb to close the first temporal identity window in the NB7-1 and NB7-3 lineages (Kanai et al., 2005; Mettler et al., 2006), and in the NB3-1 lineage (data not shown). It should be noted that Svp represses Hb expression in all neuroblasts tested to date, whereas Pdm represses Kr expression in some but not all neuroblasts.

Pdm does not act as a timer element in all neuroblast lineages. For example, *pdm* mutants do not show extended Kr expression in the NB7-1 or NB7-3 lineages, based on the lack of ectopic Kr⁺ neurons in these lineages (Grosskortenhaus et al., 2006)(Fig. S3). These results suggest that the spatial identity of a neuroblast can alter its response to timing factors such as Pdm. While this is counter to the simple model that spatial and temporal factors are independent and act combinatorially to specify birth-order identity within each lineage (Pearson and Doe, 2004), it is consistent with the finding that spatial

identity occurs at the time of neuroblast formation (Chu-LaGraff and Doe, 1993; Prokop and Technau, 1994; Skeath et al., 1995), prior to the expression of temporal factors. Taken together, these data suggest that spatial cues allow individual neuroblasts to respond differently to a temporal identity factor expressed at a similar time in all lineages.

The prior expression of early temporal identity factors is also likely to alter the response of a neuroblast to later temporal identity factors. Previous work has shown that misexpression of later temporal factors such as *Kr*, *Pdm*, or *Cas* has no detectable effect on the fate of first-born Hb^+ neurons in the NB7-1 lineage (Cleary and Doe, 2006; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Pearson and Doe, 2003). Consistent with these results, we find that in the NB3-1 lineage, *Pdm* misexpression cannot repress *Kr* or activate *Cas* during the early Hb^+ expression window (Fig. 5B). Just as prior spatial patterning cues may alter the response to a later temporal identity factor, so to may prior temporal identity factor expression alter the response of a neuroblast to later temporal factors. The mechanism by which spatial and temporal factors confer heritable changes to neuroblasts remains a mystery. One entry into this mechanism could be the investigation of how *Hb* blocks *Pdm* from repressing *Kr* gene expression.

If *Pdm* does not specify temporal identity in the NB3-1 lineage, what is the third temporal identity factor in this lineage? It has recently been reported that the *SoxB* family member *Dichaete* is expressed immediately prior to *Cas* in many embryonic neuroblast lineages (Maurange et al., 2008); however *Dichaete* is only transiently expressed in

Figure 5. Pdm closes the second temporal identity window in the NB3-1 lineage.

(A-B) Neuroblast expression of Kr and Cas in *pdm* mutant and Pdm misexpression embryos. One hemisegment shown; Kr or Cas, brown; the positional marker Engrailed, blue. NB3-1 is outlined in black. Scale bar is 10 μ m. (A) In *pdm* mutants, Kr expression persists through late stage 11 (normally off by mid stage 11; see Fig. 1A) and Cas expression is delayed until mid stage 12. (B) In *pdm* misexpression embryos (*insc-gal4 UAS-pdm2*), Kr expression is lost prematurely and Cas is expressed precociously (compare to Fig. 1A).

(C-D) RP neuron specification in *pdm* mutant and Pdm misexpression embryos. One hemisegment of a stage 16 CNS is shown as a maximum intensity projection; midline, left; anterior, up; phenotype summary, right; scale bar, 3 μ m. (C) In *pdm* mutant embryos there are 2-3 ectopic RP3 neurons; RP1, RP4, and RP5 neurons are usually normal; quantified in Table 1. (D) *pdm* misexpression (*insc-gal4 UAS-pdm2*) results in the frequent loss of the RP5 neuron (both rows) and occasional loss of the RP3 neuron (second row); RP1/RP4 are normal; quantified in Table 1.

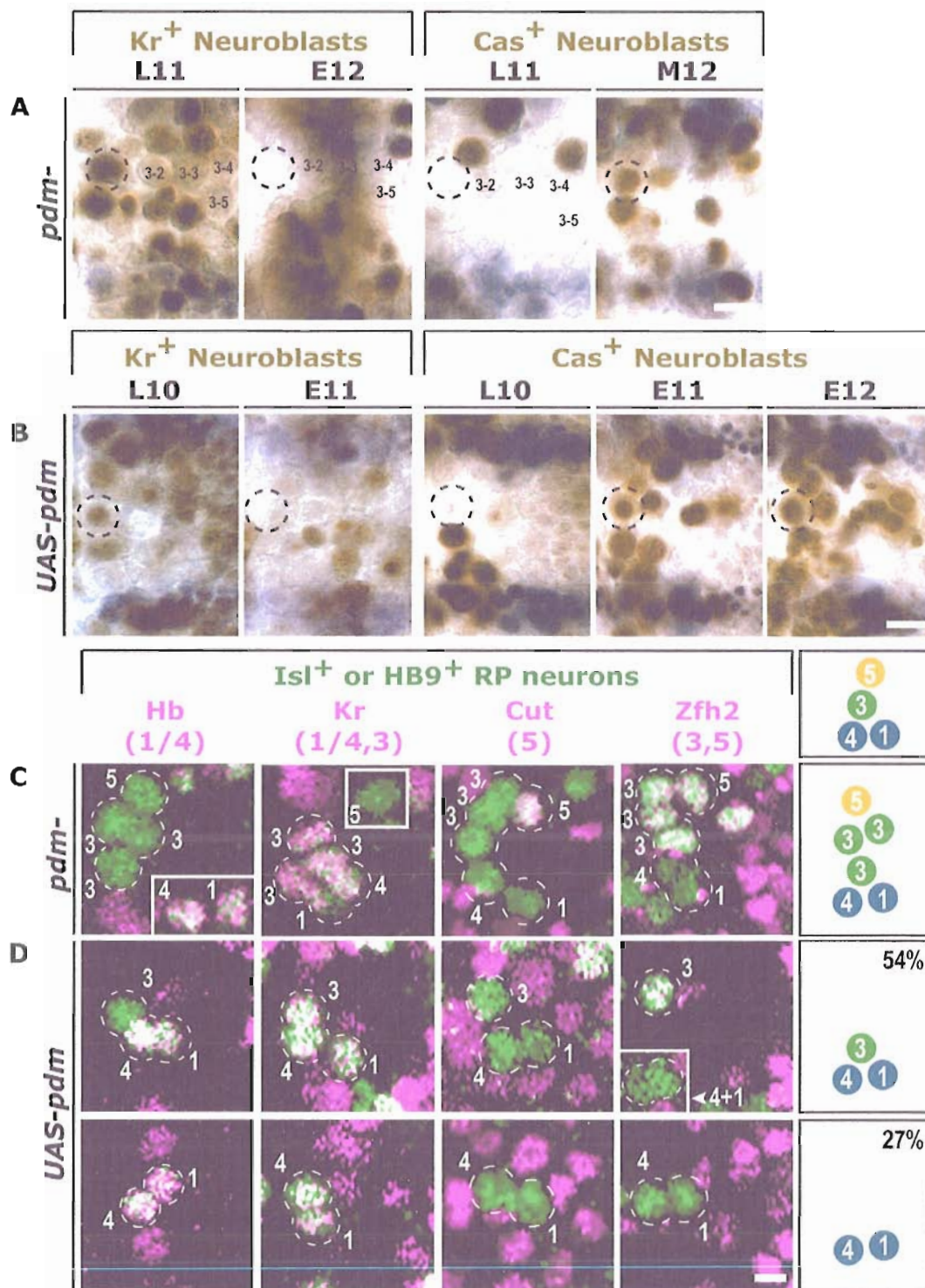


Figure 5

Figure 6. Cas closes the third temporal identity window in the NB3-1 lineage.

- (A) *cas* mutants have persistent Pdm expression in NB3-1 until at least mid stage 12 (M12); in wild type Pdm is gone from NB3-1 by late stage 11 (Fig 1A). At mid stage 16, neuroblasts in the medial column no longer expressed Pdm (*white arrowheads*); one of these neuroblasts is likely to be NB3-1. Scale bar, 10 μ m.
- (B) In *cas* mutants, there are up to four ectopic Cut⁺ RP5 neurons; RP1, RP4 and RP3 are normal; quantified in Table 1. Wild type has RP1, RP4, RP3, and RP5 neurons (Fig. 2).
- (C) *cas* misexpression (*insc-gal4 UAS-cas*) results in frequent loss of RP5 and occasional loss of RP3; RP1, RP4 are normal; quantified in Table 1. For all panels, a single representative hemisegment of a stage-16 CNS is shown as a maximum intensity projection; midline, left; anterior, up; phenotype summary, right.

Scale bar is 3 μ m in *B* and *C*.

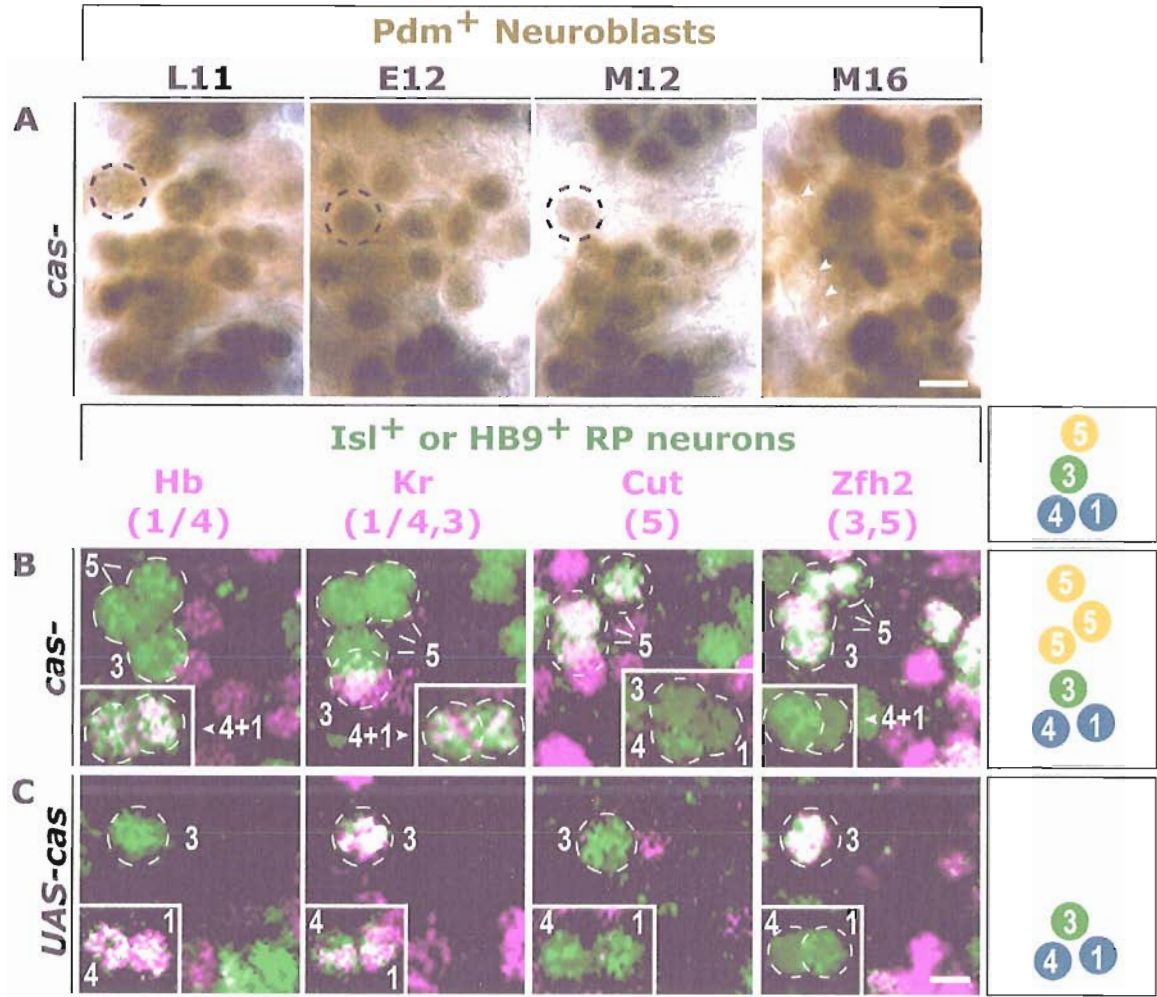


Figure 6

medial column neuroblasts such as NB3-1 at their time of formation (Zhao and Skeath, 2002) and thus does not have the proper timing for a third temporal identity factor in this lineage. Alternatively, the absence of Hb, Kr, and Cas may specify the third temporal identity, with Pdm acting solely as a timing factor to establish a gap between Kr and Cas expression. Another possibility is that a currently unknown factor specifies the third temporal identity in the NB3-1 lineage. Finally, Pdm may specify aspects of RP5 identity that we are not able to detect with our limited number of markers; unfortunately due to severe morphological defects in late-stage *pdm* mutant embryos, we have been unable to assay the RP5 axon projection to its target muscle, which is a sensitive read-out of proper neuronal identity.

Castor closes the third temporal identity window

Cas is expressed right after Pdm in most neuroblasts, and at the time NB3-1 is generating its fourth temporal identity (interneurons). We find that *cas* mutants have an extended window of Pdm neuroblast expression and production of ectopic RP5 neurons. Thus, Cas is required to close the third (RP5) temporal identity window. In addition, we find that precocious expression of Cas can prematurely close the third temporal identity window and repress the specification of RP5. We observed comparable phenotypes in the NB7-1 lineage where loss of Cas leads to ectopic U4 formation and gain of Cas results in the repression of that identity (Grosskortenhaus et al., 2006). Based on these observations, we predict that Cas functions in multiple neuroblast lineages to close the third temporal identity window. Does Cas specify the fourth temporal identity? We

cannot answer this question in the NB3-1 lineage due to lack of interneuron markers, but Cas does specify the fourth temporal identity (together with Pdm) in the NB7-1 lineage (Grosskortenhaus et al., 2006). In the future, the role of Cas in the NB3-1 lineage could be examined by making CD8::GFP-marked *cas* mutant clones and assaying neuronal identity by axon projections, or by developing molecular markers for interneurons within the lineage.

Temporal identity genes, timing factors, and neuronal cell type specification

We propose that there are two classes of genes that regulate neuroblast temporal identity. One class of genes encodes temporal identity factors that are necessary and sufficient to directly specify a particular temporal identity in multiple neuroblast lineages (Isshiki et al., 2001); Hb and Kr are good examples. A second class of genes encodes timing factors that establish the timing of temporal identity gene expression, but do not directly specify temporal identity (Fig. 7). Timing factors, however, may indirectly influence the specification of temporal identities as seen in NB3-1 where *pdm* is required to restrict the specification of RP3 and properly advance the neuroblast to the Cas-positive state (Fig. 5). Previously only one timing factor had been identified, Seven-up, which down-regulates Hb protein levels, and along with cytokinesis, closes the first temporal identity window to facilitate the Hb→Kr transition (Grosskortenhaus et al., 2005; Kanai et al., 2005). The Kr→Pdm→Cas transitions are independent of cell cycle progression (Grosskortenhaus et al., 2005). Here we show that Pdm closes the second temporal identity window by repressing Kr expression and activating Cas in NB3-1.

Taken together, our observations suggest that Kr and Pdm are involved in a negative-feedback loop: where Kr activates Pdm which in turns represses Kr and activates Cas to advance neuroblast timing independent of cell cycle progression. Through its role as a regulator of Kr and Cas timing, Pdm can restrict the production of neuronal cell types and advance the NB3-1 lineage.

Bridge to Chapter III

In Chapter III, I present the results from my investigation of the molecular mechanisms underlying Hunchback function to maintain early neuroblast competence and specify early neuronal-identity. Hunchback/Ikaros proteins can directly activate or repress target gene transcription during early insect development, but their mode of action in during neural development is unknown. Previous studies show that Hunchback can specify early-born neuronal identity and maintain "young" neural progenitor (neuroblast) competence. In this chapter, I use recombineering to generate a series of Hunchback domain deletion variants and assay their function during neurogenesis, in the absence of endogenous Hunchback. I identify two conserved domains required for Hunchback-mediated transcriptional repression, and show that transcriptional repression is necessary and sufficient to induce early-born neuronal identity and maintain neuroblast competence. I identify one direct target gene that must be repressed to maintain competence, *pdm-2*, but show that additional genes must also be repressed. Base on these findings, we propose that Hunchback maintains early neuroblast competence by silencing a suite of late-expressed genes.

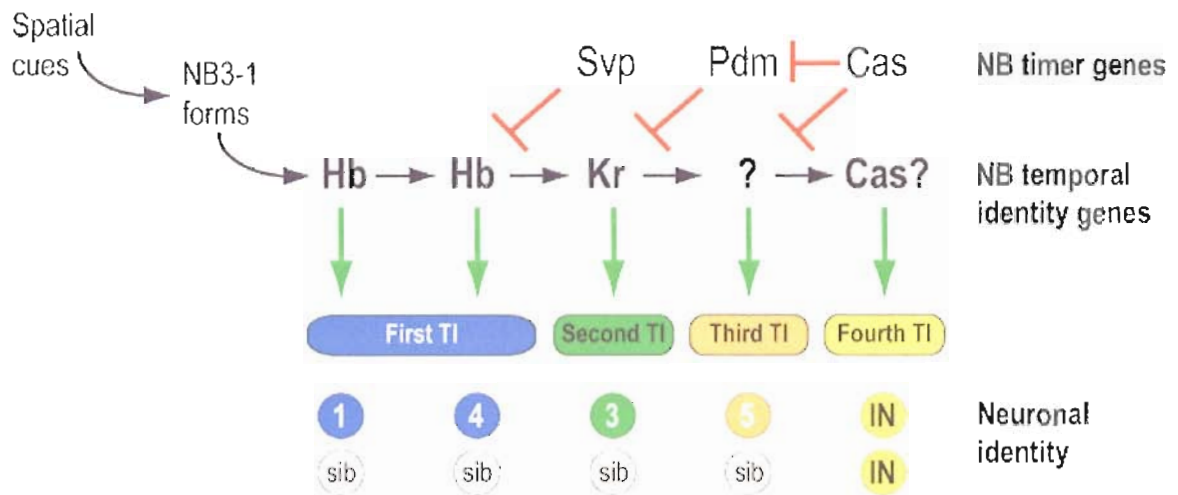


Figure 7. Hb and Kr specify early temporal identity, whereas Svp, Pdm, and Cas act as timer elements within the NB3-1 lineage.

Left: Spatial cues specify the NB3-1 identity and formation, which allows the neuroblast to respond in a potentially unique way to subsequently expressed timer genes and temporal identity genes.

Top row: Timer elements include Seven-up (Svp), Pdm, and Cas; these factors close successive temporal identity windows. Timer genes indirectly control cell fate through regulation of temporal identity genes.

Middle row: Temporal identity genes include Hb and Kr, which specify first and second temporal identities respectively in this and other lineages.

Bottom row: Neuronal identity within the lineage. GMC-1 makes RP1/sibling neurons; GMC-2 makes RP4/sibling neurons; GMC-3 makes RP3/sibling neurons; GMC-4 makes RP5/sibling neurons; GMC-5 makes interneurons.

CHAPTER III

HUNCHBACK REPRESSES MULTIPLE DOWN-STREAM FACTORS TO MAINTAIN NEUROBLAST COMPETENCE AND SPECIFY EARLY-BORN NEURONAL IDENTITY

“Age is an issue of mind over matter. If you don’t mind, it doesn’t matter.”

-- Mark Twain

Khoa D. Tran and Chris Q. Doe designed the research; Khoa D. Tran performed research; Michael R. Miller contributed new reagents; Khoa D. Tran and Chris Q. Doe analyzed the data and wrote the manuscript published below.

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INTRODUCTION

Hb is expressed early in many neuroblasts and is required for the specification of the first-born cell identity, or first-temporal identity, in those lineages (Isshiki et al., 2001; Novotny et al., 2002). In addition, Hb can also confer the early competent state in

many neuroblast lineages (Cleary and Doe, 2006; Pearson and Doe, 2003). This is achieved by maintaining Hb expression in neuroblasts throughout neurogenesis, or by re-introducing Hb in neuroblasts after its normal expression window. The ectopic Hb expression results in the specification of extra early-born progeny.

Interestingly, the ability of Hb to specify and extend the early-competence window declines over time (Cleary and Doe, 2006; Pearson and Doe, 2003). When Hb is reintroduced to NB7-1 at progressively later time points, its ability to specify ectopic U1/U2 neurons is greatly reduced. Eventually, Hb is unable to specify early-born cells after the fifth neuroblast division. This raises two interesting questions regarding neuroblast competence. First, how does the Hb regulate gene expression to maintain early-neuroblast competence? And second, why does this ability decline over time?

Hb regulates gene expression via multiple, well-characterized modes during the formation of the *Drosophila* body plan; however, little is known about its modes of operation in the CNS. In the cellular blastoderm, the Hb protein gradient initiates and establishes the spatial expression domains of the gap genes *Kruppel* (*Kr*), *knirps* (*kni*) and *giant* (*gt*). Rigorous genetic and molecular analyses have shown that Hb acts a concentration dependent transcriptional activator and repressor of gene expression during embryonic segmentation (Berman et al., 2002; Hoch et al., 1991; Pankratz et al., 1992; Rivera-Pomar et al., 1995; Schulz and Tautz, 1994; Struhl et al., 1992). Furthermore, Hb can induce permanent repression of target genes through its interaction with Mi2 and recruitment of Polycomb complex proteins (Kehle et al., 1998). As a result, the multi-functional Hb protein is a potent regulator of gene expression in the early embryo. Here,

we extend the analysis of Hb-mediated gene regulation to include its role of maintaining neuroblast competence during nervous system development.

MATERIALS AND METHODS

Generation of VP16::Hb chimera protein

We generated the VP16::Hb chimera by PCR amplifying the *VP16* activation domain (Lai and Lee, 2006) using primers with a 3'-tail that contained *hb* 5' sequence, and ligating to PCR amplified full length *hb* coding sequence. Primer sequences are available upon request. The chimeric gene was verified by sequencing, cloned into the *pUAST* vector (Brand and Perrimon, 1993), and transgenic flies were made (GenetiVision, Houston, Texas).

Generation of tagged Hb deletion proteins

We generated *hb* genes deleted for the six previously described conserved domains (Tautz, 1987), as well as two additional domains (B' and E) that we identified as conserved in at least eight sequenced *Drosophila* species using EvoPrinter (Odenwald et al., 2005). Each *hb* deletion construct (except the D domain deletion) was generated using recombineering by targeted insertion and replacement of the *galK* expression cassette (Warming et al., 2005). *GalK* targeting cassettes were prepared by PCR amplification of the *galK* expression cassette using primers containing homology to *hb*. To insert *galK*, SW102 cells containing BAC clone BACR01F13 were electroporated with the

appropriate targeting cassette and plated on minimal medium with galactose and chloramphenicol. To replace *galK*, SW102 cells were electroporated with the appropriate replacement cassette and plated on minimal medium with glycerol, 2-deoxy-galactose, and chloramphenicol. The replacement cassette for epitope tagging (3xFLAG::3xHA) was prepared by PCR amplification using homology primers. Primer sequences are available upon request. The D domain deletion was generated by two-step PCR. Each construct was sequenced to confirm that Hb was modified correctly. Deletions were cloned into a *pUAST(attB)* vector (Bischof et al., 2007) and sent to GenetiVision for injections into flies carrying the *attP40* docking site on Chromosome 2 (Markstein et al., 2008). In addition to the deletions, we also generated flies carrying the same epitope tagged wild type Hb in the *attP40* locus as a standard control. The fly stocks generated are described below.

Fly stocks

The following pre-existing fly stocks were used: *yw* (wild type); *v32a-gal4* for ubiquitous embryonic expression (Siegrist and Doe, 2005); *UAS-hb* (Wimmer et al., 2000); *engrailed-gal4* for expression in the posterior compartment of each segment (Harrison et al., 1995; Isshiki et al., 2001; Pearson and Doe, 2003); *worniu-gal4* for expression in neuroblasts (Albertson et al., 2004); *UAS-HA* is *UAS-HA::UPRT* (Miller et al., 2009), this transgene was used as a UAS control so that each misexpression experiments had two UAS transgenes; it does not change the number of U neurons when expressed alone.

The following fly stocks were generated in this work:

UAS-VP16::hb on Chromosomes 2 and 3

UAS-VP16::hb; hb^{FB} hb^{P1}/TM3 ftz-lacZ hb^{FB} hb^{P1}/TM3 ftz-lacZ [*hb^{FB}* is a null-mutant, *hb^{P1}* is a segmentation rescue construct (Hulskamp et al., 1994; Isshiki et al., 2001)]

UAS-VP16::hb; Df(2L)ED773/CyO ftz-lacZ [*Df(2L)ED773* removes both *pdm1* and *pdm2*, (Grosskortenhaus et al., 2006)]

UAS-hb^{wild-type}/CyO and *UAS-hb^{wild-type}/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ*

UAS-hb^{ΔAB}/CyO and *UAS-hb^{ΔAB}/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ*

UAS-hb^{ΔB'}/CyO and *UAS-hb^{ΔB'}/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ*

UAS-hb^{ΔDBD}/CyO and *UAS-hb^{ΔDBD}/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ*

UAS-hb^{ΔC}/CyO and *UAS-hb^{ΔC}/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ*

UAS-hb^{ΔD}/CyO and *UAS-hb^{ΔD}/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ*

UAS-hb^{ΔE}/CyO and *UAS-hb^{ΔE}/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ*

UAS-hb^{ΔDMZ}/CyO and *UAS-hb^{ΔDMZ}/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ*

engrailed-Gal4/CyO; hb^{FB} hb^{P1}/TM3 Ubx-lacZ

Molecular markers and immunostaining

Antibody staining was performed according to standard methods. Polyclonal antisera against Zfh2 was made by expressing a GST-fusion protein containing amino acids 1641-2149 of the Zfh2 protein (pZFH-2c, (Lai et al., 1991)), and purified proteins were sent to Alpha Diagnostic International (San Antonio, TX) for injections into rats. Primary antibodies, dilutions, and sources: rat Zfh2, 1:400 (this work; M. Lundell, UT

San Antonio); mouse Eve 2B8, 1:10 (Patel et al., 1994); guinea pig Eve, 1:1000, rat Eve, 1:000, guinea pig Kr, 1:800 (Kosman et al., 1998); rabbit Hb, 1:200 (Tran and Doe, 2008); rabbit Kr, 1:500 (Gaul et al., 1987); rat Pdm2, 1:10 (Grosskortenhaus et al., 2006); rabbit Cas, 1:2000 (Kambadur et al., 1998); mouse En 4D9, 1:5 (Patel et al., 1989); mouse Beta-galactosidase, 1:500 (Promega, Madison, WI); rabbit Beta-galactosidase, 1:1000 (MP Biomedicals, Solon, OH); mouse HA, 1:500 (Roche, Palo Alto, CA); rabbit HA, 1:1000 (Sigma, St. Louis, MO). Secondary antibodies were purchased conjugated to Alexa 488, Rhodamine RedX, Cy5 (Jackson, West Grove, PA), biotin (Vector, Burlingame, CA), or alkaline phosphatase (Southern Biotechnology, Birmingham, AL) and used at 1:400.

Microscopy and statistical analysis

Confocal image stacks were collected using Leica SP2 or Biorad Radiance 2000 confocal microscopes and shown as two-dimensional projections. Histochemical preparations were acquired using a Zeiss AxioCam HRc camera on an Axioplan microscope. Two-tailed T tests were used to determine the significance in the differences in cell number.

RESULTS

VP16::Hb activates both positively- and negatively-regulated Hb direct target genes in the early embryo

To address the molecular mechanism underlying Hb maintenance of neuroblast competence, we began by examining whether Hb achieves this role through the transcriptional activation or repression of target genes. Because no transcriptional activation or repression domains have been identified within Hb, we began by converting Hb to act solely as a transcriptional activator by fusing the VP16 trans-activation domain (Triezenberg et al., 1988) to the N-terminus of full-length Hb (Fig. 1A). We then tested whether VP16::Hb could induce competence or first-born temporal identity.

Before assaying the effects of VP16::Hb in the CNS, we first examined the ability of VP16::Hb to recognize and activate known Hb direct target genes in the early embryo. In the embryonic blastoderm, Hb is a direct activator of the gap gene *Kr* (Hoch et al., 1991). Expression of Hb throughout the embryo using a heat-shock promoter can activate *Kr* and expand the *Kr* central gap domain to the posterior of the embryo (Hoch et al., 1991; Hulskamp et al., 1990; Struhl et al., 1992). We expressed wild type Hb throughout the embryo using the Gal4/UAS gene expression system (Brand and Perrimon, 1993) with a maternal Gal4 driver (*v32a-gal4 UAS-hb*) and were able to reproduce this expansion of the *Kr* central gap domain (Fig. 1B). Next, we examined *v32a-gal4 UAS-VP16::hb* embryos and found a dramatic increase in *Kr* expression

throughout the embryo (Fig. 1B). The activation of *Kr* throughout the embryo indicates that VP16::Hb is a potent activator of *Kr* gene expression.

We further tested the capacity of VP16::Hb as an activator by examining its effects on genes that are normally directly repressed by Hb. Hb expression in the posterior regions of the embryonic blastoderm directly represses the gap genes *knirps* (*kni*) and *giant* (*gt*) (Fig. 1C, 1D) (Berman et al., 2002; Berman et al., 2004; Pankratz et al., 1992; Pelegri and Lehmann, 1994). However, VP16::Hb overexpression activated both *kni* and *gt*, expanding their domains anteriorly and posteriorly into the domains of their respective repressors (Fig. 1C, 1D). Based on these observations, we conclude that VP16::Hb is also sufficient to activate genes that are normally repressed by Hb (Fig. 1E). The strong activation and expansion of the Kr, Kni, and Gt gap domains indicates that VP16::Hb acts as a potent transcriptional activator that overcomes or eliminates the normal transcriptional repression function of Hb. Taken together, we conclude that VP16::Hb has the capacity to recognize and activate Hb target genes whether they are normally activated or repressed by Hb in the early embryo.

VP16::Hb activates all known Hb-regulated genes in the CNS

Because VP16::Hb acts as a strong activator of Hb direct target genes in the early embryo, we next examined the expression of Hb CNS-targets in response to VP16::Hb expression. Previous studies suggest that Hb regulates its own transcription in the blastoderm, but not in the CNS (Grosskortenhaus et al., 2005; Treisman and Desplan, 1989). However, it is possible that the VP16::Hb chimera will activate endogenous Hb

Figure 1. VP16::Hb acts as a constitutive transcriptional activator.

- (A) Schematic of wild type Hb protein and the VP16::Hb protein chimera with previously characterized conserved domains. DBD, DNA-binding domain; DMZ, dimerization domain.
- (B-D) Expression of gap genes in the *Drosophila* embryonic blastoderm in wild type, *v32a-gal4 UAS-hb*, and *v32a-gal4 UAS-VP16::hb* embryos.
- (E) Summary of gene interactions.

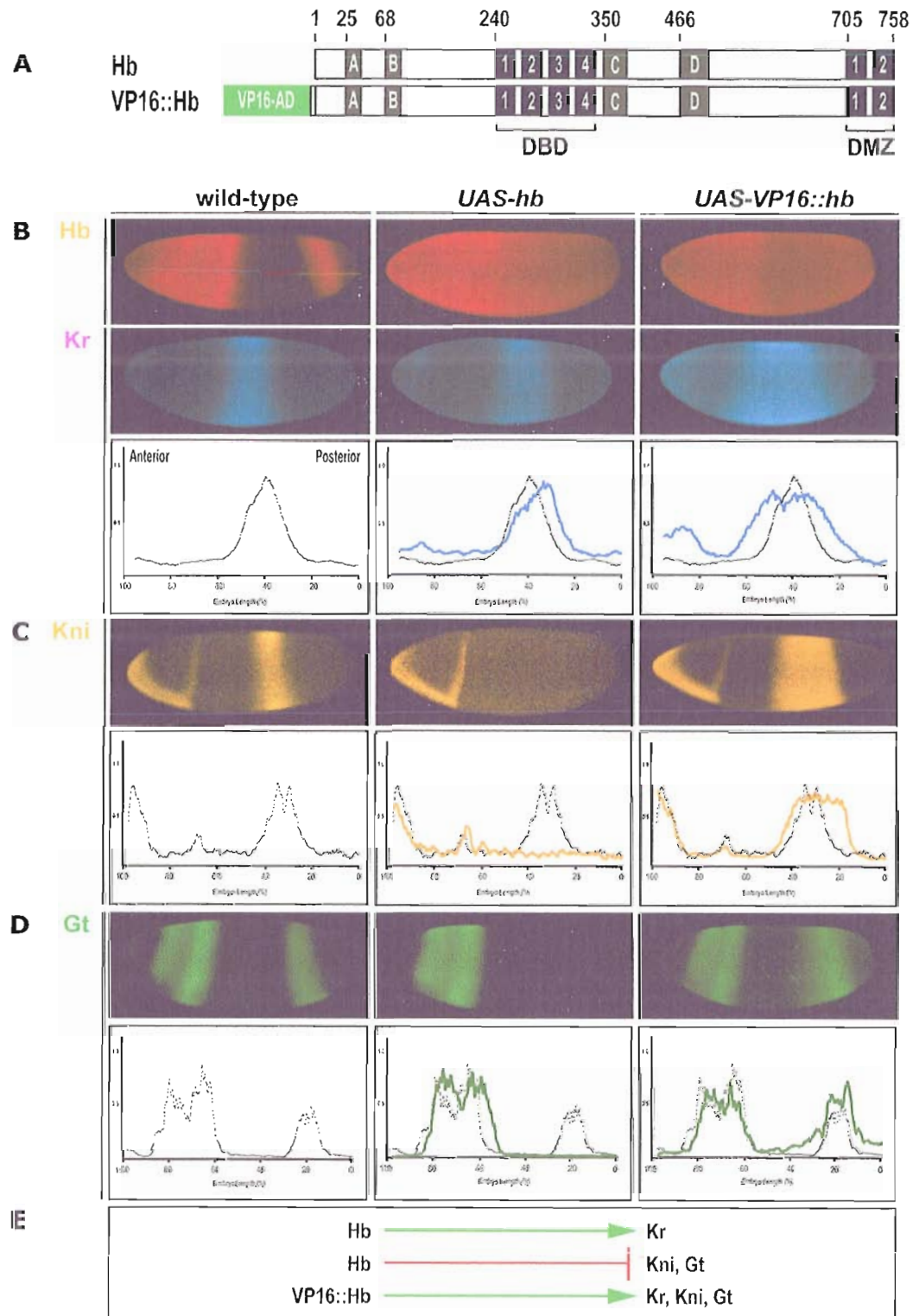


Figure 1

transcription via the VP16 activation domain, leading to cells containing both a transcription activating Hb protein (VP16::Hb) and a potential transcription repressing Hb protein (endogenous Hb). To test whether VP16::Hb can activate endogenous Hb transcription, we performed in-situ hybridizations against the 3' UTR of endogenous *hb* mRNA. In *engrailed-gal4 UAS-hb* embryos, we found that Hb cannot induce its own transcription in neuroblasts (Fig. 2A, B), consistent with previous findings (Grosskortenhause et al., 2005). In contrast, *engrailed-gal4 UAS-VP16::hb* embryos show strong activation of endogenous Hb transcription in neuroblasts (Fig. 2C). Based on these observations, we conclude that VP16::Hb can activate endogenous *hb* transcription in the CNS.

We next examined whether VP16::Hb can activate *pdm*, a gene normally repressed by Hb through well-characterized Hb-binding sites in its CNS enhancer element (Kambadur et al., 1998). In stage 15 embryos, only a few neuroblasts express Pdm (Fig. 2D), and we found minimal change in Pdm following the expression of wild type Hb protein (Fig. 2E), probably because most neuroblasts can no longer respond to Hb at this stage (Cleary and Doe, 2006). However, the overexpression of VP16::Hb in neuroblasts resulted in the up-regulation of Pdm (Fig. 2F). We conclude that VP16::Hb can activate the direct target *pdm* in the CNS.

Having shown that VP16::Hb can activate Hb direct target genes, we extended our analyses of VP16::Hb activity to all other known Hb CNS target genes (Fig. 2G). Hb is known to activate *Kr*, and repress *zfh2*, *cut*, *runt*, and *cas* (Fig. 2H) (Grosskortenhause et al., 2006; Isshiki et al., 2001; Kambadur et al., 1998), although it is not known whether

Hb acts directly or indirectly to regulate the expression of these genes. We overexpressed VP16::Hb in a *hb* mutant background and found that it can activate *Kr* in neurons similar to wild type Hb (Fig. 2I). Additionally, we found that VP16::Hb expression also results in the activation of the normally repressed target genes *zfh2*, *cut*, *runt*, and *cas* in neurons (Fig. 2I). Based on these observations, we conclude that VP16::Hb can activate all known Hb CNS targets, whether they are normally activated or repressed by Hb. Because Hb normally acts as a transcriptional repressor of most CNS targets (Fig. 2J), we suggest that this repression may play an essential role in maintaining neuroblast competence, and we test this prediction below.

Overexpression of VP16::Hb in the CNS reveals that Hb maintains neuroblast competence by transcriptional repression of multiple target genes

Because VP16::Hb is a potent transcriptional activator with little ability to repress gene expression, we can use it to test whether Hb-mediated transcriptional activation of target genes is sufficient for maintaining neuroblast competence. Overexpression of wild type Hb can extend neuroblast competence and production of early-born neuronal cell types (Isshiki et al., 2001; Novotny et al., 2002; Tran and Doe, 2008). In NB7-1, Hb misexpression produces approximately 18-20 Eve⁺ U motoneurons. If Hb maintains early-competence by acting solely as a transcriptional activator, then VP16::Hb should mimic Hb function and specify the same or more U neurons. In wild-type embryos, NB7-1 generates five Eve⁺ U motoneurons (Fig. 3A, A'') (Pearson and Doe, 2003; Schmid et al., 1999). Overexpression of wild type Hb in a *hb* mutant NB7-1 gives an

average of 12 U neurons (range=6-18, n=100; Fig. 3A'). However, the overexpression of VP16::Hb in a *hb* mutant NB7-1 only generates an average of six U neurons (range=2-12, n=88; Fig. 3A''). We conclude that the constitutive activator VP16::Hb is not as good as wild type Hb at maintaining the early-competence window necessary for Eve⁺ U neuron production; and suggest that the repression of Hb downstream targets in neuroblasts may be essential to maintain early-competence.

Because Hb normally represses downstream targets such as *pdm* and *cas*, and VP16::Hb can activate these targets, we tested whether the repression of these downstream genes is required to maintain neuroblast competence. First, we examined *engrailed-gal4 UAS-hb, hb* mutant embryos at early stage 12 and found that neuroblasts in the *engrailed-gal4* domain expressed *Kr* but not *pdm* and *cas* (Fig. 3B) (Isshiki et al., 2001). In contrast, we found that the majority of neuroblasts in the *engrailed-gal4* domain of *engrailed-gal4 UAS-VP16::hb, hb* mutant embryos expressed *Kr, pdm*, and *cas* (Fig. 3C). We conclude that VP16::Hb can ectopically induce *pdm* and *cas* expression in neuroblasts.

Next, we tested whether the co-expression of wild type Hb plus Pdm or Hb plus Cas can lead to the same reduction in ectopic cells as seen in VP16::Hb overexpression experiments. In control *engrailed-gal4 UAS-hb UAS-HA* embryos, NB7-1 generated about 17 Eve⁺ U-neurons (range=13-22, n=87; Fig. 3D). We next examined *engrailed-gal4 UAS-hb UAS-pdm2* embryos for the total number of U neurons generated and found a large decrease in the number of ectopic U neurons compared to our control embryos (average=9, range=3-14, n=70; Fig. 3D). *engrailed-gal4 UAS-hb UAS-cas* embryos

showed a slight decrease in U neurons generated (average=15, range=8-20, n=118; Fig. 3D). Taken together, we conclude that Hb normally represses down-stream targets such as *pdm* and *cas* to maintain neuroblast competence.

Because *Pdm2* is sufficient to block Hb-induced neuroblast competence, we tested if *Pdm2* was also necessary to terminate neuroblast competence. If *pdm2* is the only factor activated by VP16::Hb that limits neuroblast competence, then the overexpression of VP16::Hb in a *pdm* mutant background should have extended neuroblast competence and result in the generation of many Eve⁺ U neurons. On the other hand, if VP16::Hb activates multiple factors, then VP16::Hb may not be able to extend competence and make large numbers of U neurons even in the absence of *Pdm*. Control *prospero-gal4 UAS-hb* embryos can generate an average of nine Eve⁺ U neurons per hemisegment (range=5-16, n=100; Fig. 3E). In contrast, *prospero-gal4 UAS-VP16::hb* embryos generated only 5.6 Eve⁺ U neurons (range=4-9, n=100, p<<.001; Fig. 3E), presumably due to the transcriptional activation of factors limiting competence. Strikingly, performing the same experiments in a *pdm* mutant embryo (lacking both *pdm1* and *pdm2* genes) did not increase the number of Eve⁺ U neurons (average=4.3, range=2-8, n=90, p<<.001; Fig. 3E). We conclude that Hb must normally repress multiple factors, in addition to *pdm1/pdm2*, to maintain early neuroblast competence.

Identification of two domains required for Hb transcriptional repression

Because Hb repression of target genes is essential to the maintenance of neuroblast competence, we next sought to identify the Hb protein domain(s) required for

Figure 2. VP16::Hb activates Hb direct and indirect targets in the CNS.

- (A-C) VP16::Hb activates endogenous *hb*. In-situ hybridization against endogenous *hb* mRNA. Each panel shows the neuroblast layer of a stage 12 embryo with the anterior to the left. Lines in B and C indicate the *engrailed-gal4* expression domain.
- (D-F) VP16::Hb activates the direct target, *pdm*. Histochemical detection of Pdm (brown) and the segment boundary marker Engrailed (purple) proteins. Each panel shows the neuroblast layer of a stage 15 embryo with anterior to the left. *wornui-gal4* drives gene expression in all neuroblasts.
- (G-I) Expression of *Kr*, *zfh2*, *cut*, *runt*, and *cas* in wild-type, *engrailed-gal4 UAS-hb*, and *engrailed-gal4 UAS-VP16::hb*, *hb* mutant embryos. Each panel shows a 2D-projection of approximately two segments of the ventral nerve cord of a stage 16 embryo. Anterior is up. Lines in B and C indicate the *engrailed-gal4* expression domain.
- (J) Summary of gene interactions in the CNS.

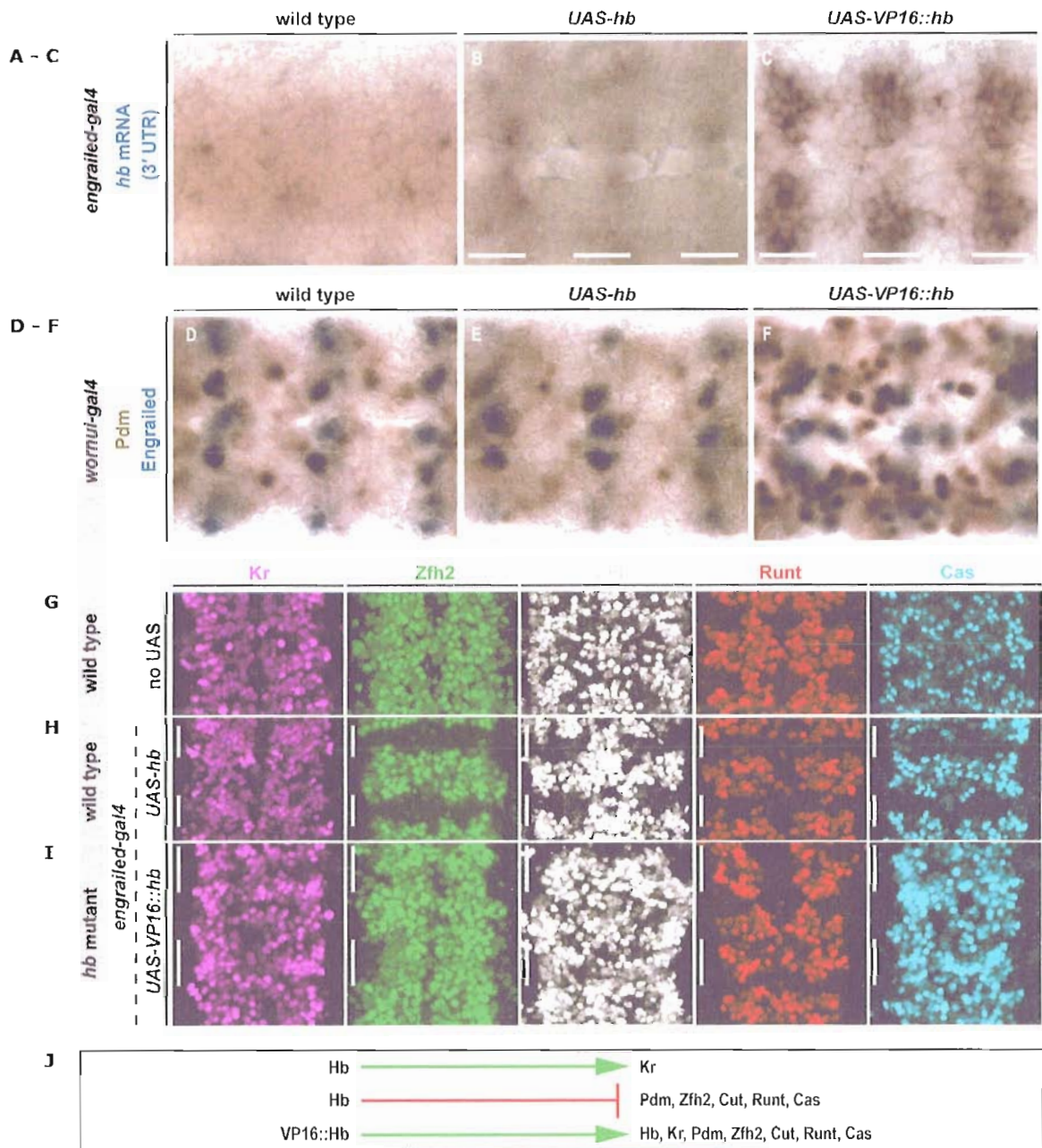


Figure 2

transcriptional repression. We generated a series *hb* transgenes, each deleted for one or more evolutionarily-conserved domains (Fig. 4A, B). Hb has eight conserved domains: the first group of zinc-finger domains bind DNA (DNA-binding domain; DBD), the second group of zinc-finger domains allow for Hb dimerization (dimerization domain; DMZ), four previously identified conserved domains A, B, C, and D (Tautz, 1987), and two additional domains containing short DNA sequences conserved in at least eight sequenced *Drosophila* species (B' and E; Fig. 4A). We deleted each of these domains in a series of UAS-HA::Hb transgenes, which we placed in the same *attP* site on chromosome 2 so that the results of each transgene could be directly compared.

We tested each protein for its ability to activate the direct target *Kr* (Hoch et al., 1991) or repress the direct target *pdm* (Kambadur et al., 1998) within the CNS. Wild type embryos at stage 11 are $Kr^- Pdm^+$ in the neuroblast layer (Grosskortenhaus et al., 2005; Isshiki et al., 2001; Tran and Doe, 2008). Overexpression of the full length wild type Hb protein resulted in the activation of *Kr* and repression of *pdm* (Fig. 4B, C), consistent with previous findings (Isshiki et al., 2001; Kambadur et al., 1998). Similarly, overexpression of Hb proteins lacking either the A+B domains, the B' domain, or the E domain also activated *Kr* and repressed *pdm* (Fig. 4B, C), showing that none of these domains are required for transcriptional activation or repression. Overexpression of Hb proteins lacking the DBD or the C domain cannot activate *Kr* or repress *pdm* (Fig. 4B, C), suggesting that they are non-functional, although they are nuclear localized and have similar stability to wild type Hb protein in neuroblasts (Fig. 4C). In contrast, overexpression of Hb proteins lacking the D domain (which includes the Mi2-binding

Figure 3. Hb maintains early neuroblast competence through the repression of multiple target genes.

(A-A'') Quantification of U neurons (dashed box) specified in various genetic backgrounds. Wild-type, avg=5; *engrailed-gal4 UAS-hb hb* mutant, avg=12 $\pm$ 2 (standard deviation), range=6-18; *engrailed-gal4 UAS-VP16::hb hb* mutant, avg=6 $\pm$ 2, range=2-12. $p < .001$ for all experiments.

(B-C) Neuroblast expression profile at early stage 12. One hemisegment is shown. Dashed circles outline neuroblasts, solid lines indicate *engrailed-gal4* expression domain. Scale bar equals 10 μ m. Hb activates *Kr* and represses *pdm* and *cas* in neuroblasts, whereas VP16::Hb activates all three factors.

(D-D') Co-misexpression of Hb + Pdm and Hb + Cas results in less ectopic U neurons. *UAS-hb UAS-HA* control, avg=17 $\pm$ 2, range=13-22; *UAS-hb UAS-pdm2*, avg=9 $\pm$ 2, range=3-14; *UAS-hb UAS-cas*, avg=15 $\pm$ 2, range=8-20. $p < .001$ for all experiments.

(E) Overexpression of VP16::Hb in a *pdm* mutant embryo does not result in the recovery of ectopic U neurons. *pros-gal4 UAS-hb*, avg=9 $\pm$ 2.5, range=5-16; *pros-gal4 UAS-VP16::hb*, 5.6 $\pm$ 1, range=4-9; *pros-gal4 UAS-VP16::hb pdm* mutant, avg=4.3 $\pm$ 1, range=2-8. $p < .001$ for all experiments.

(F) Summary and comparison of the competence window generated by Hb and VP16::Hb.

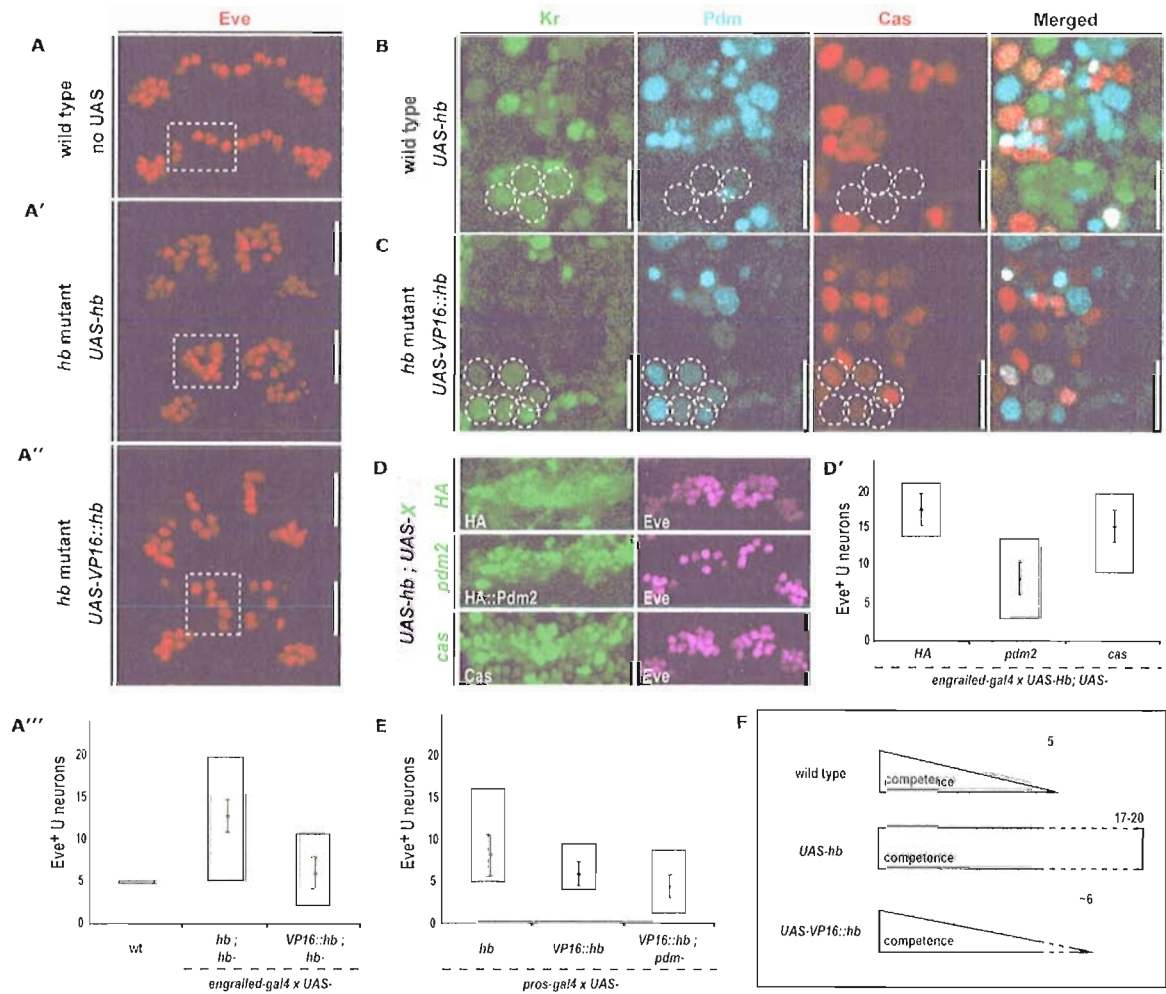


Figure 3

sites) fails to repress *pdm*, but still weakly activates *Kr*; an identical result was observed for Hb protein lacking the DMZ domain (Fig. 4B, C). The sparse and intermittent activation of *Kr* may be due to Pdm repression of *Kr* (Grosskortenhaus et al., 2006; Tran and Doe, 2008), which would be expected to counteract Hb-induced *Kr* activation. We next tested whether the Hb^{ΔD} and Hb^{ΔDMZ} proteins fail to repress other known Hb target genes. Whereas wild type Hb protein can efficiently repress *zfh2*, *cut*, *runt*, and *cas* (Fig. 3C), the Hb^{ΔD} and Hb^{ΔDMZ} proteins failed to repress all of these genes (Fig. S1). We conclude that both the D and DMZ domains are required for Hb-mediated transcriptional repression.

Hb repression domains are required for maintenance of neuroblast competence

We showed above that overexpression of the constitutive transcriptional activator VP16::Hb was not sufficient to extend neuroblast competence, suggesting that this function may require Hb-mediated transcriptional repression. Here we test whether the D or DMZ Hb repression domains are required for extending competence. We assay neuroblast competence by measuring the number of Eve⁺ U neurons that can be induced by overexpression of Hb within the NB7-1 lineage using the *engrailed-gal4* driver (Isshiki et al., 2001; Pearson and Doe, 2003). Wild type embryos have 5 Eve⁺ U neurons per hemisegment (Fig. 3A)(Isshiki et al., 2001), whereas overexpression of wild type Hb can extend neuroblast competence to allow the formation of approximately 14 Eve⁺ U neurons (n=100; Fig. 4D; Table 1). Similarly, overexpression of Hb proteins lacking either the A+B domains, the B' domain, or the E domain generated approximately 16

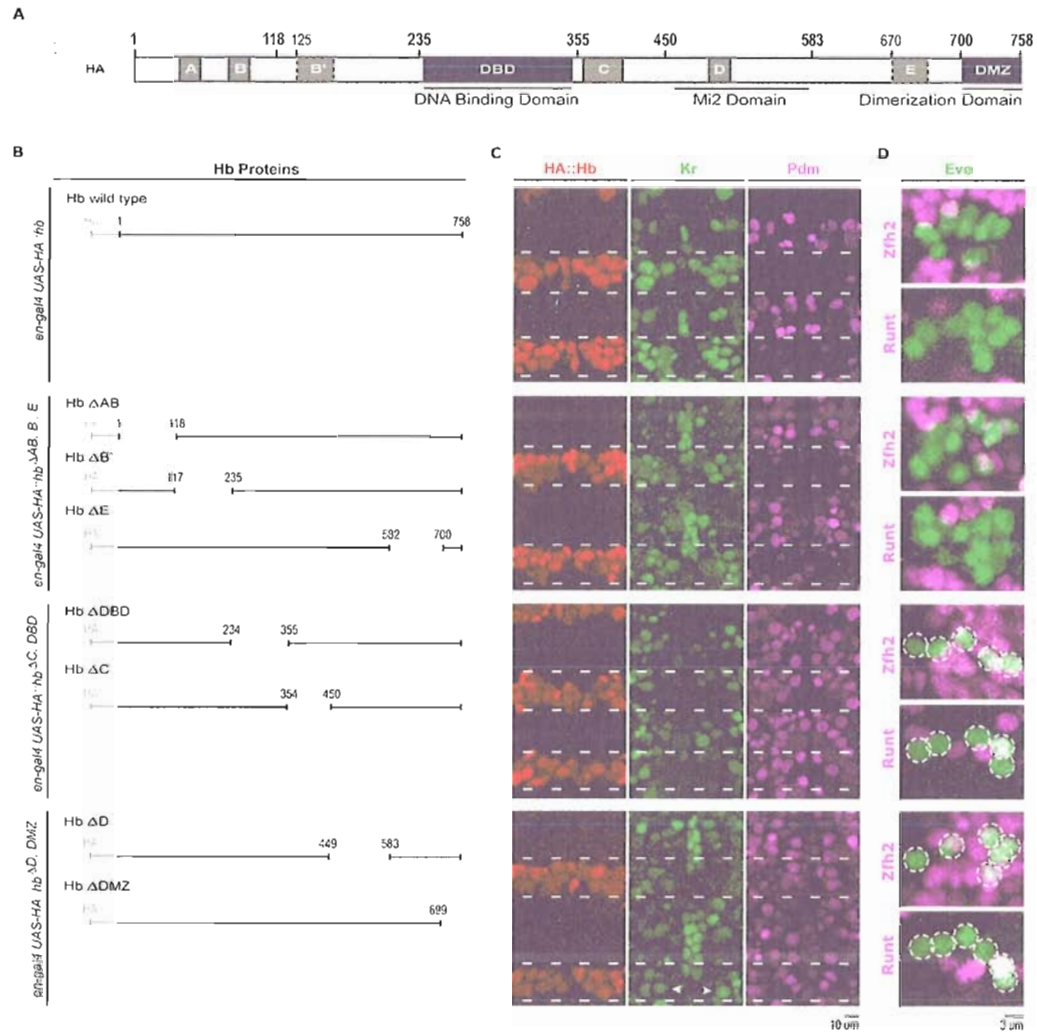


Figure 4. The Hunchback D and DMZ domains are required for transcriptional repression and maintenance of neuroblast competence.

(A) Schematic of Hb protein showing conserved domains and deletion breakpoints (bold).

(B) Deletions grouped by functional phenotype.

(C) In each panel set, two segments of a stage 11 embryo are shown with neuroblasts stained for Kr and Pdm proteins. Dashed lines indicate *engrailed-gal4* expression domains. Wild type Hb, Hb ΔAB , Hb $\Delta B'$, and Hb ΔE can activate *Kr* and repress *pdm*; Hb ΔDBD and Hb ΔC are non-functional in the CNS; Hb ΔD and Hb ΔDMZ can activate *Kr*, but cannot repress *pdm*.

(D) U neurons specified by Hb and each deletion construct.

Eve⁺ U neurons (n>100 each; Fig. 4D; Table 1), showing that none of these domains is required for Hb-mediated extension of neuroblast competence. As expected, overexpression of the non-functional Hb proteins lacking the DBD or the C domains cannot specify ectopic U neurons or alter the identity of the existing neurons (Fig. 4D, S2; Table 1). Interestingly, overexpression of Hb proteins that lack transcriptional repressor activity, i.e. those lacking the D or DMZ domain, both fail to extend neuroblast competence, generating only 5-6 Eve⁺ U neurons (n>200, Fig. 4D; Table 1). The identity of the Eve⁺ U neurons will be addressed in the next section, but based simply on the change in the number of Eve⁺ U neurons, we conclude that Hb-mediated transcriptional repression using the D and DMZ domains is required to extend neuroblast competence.

Hb repression domains are required for the specification of first-born neuronal identity

In addition to its role in regulating neuroblast competence, Hb has an essential role in the specification of early-born neuronal identity in multiple neuroblast lineages (Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003; Tran and Doe, 2008). To determine whether Hb transcriptional repression is required to specify early-born neuronal identity, we expressed Hb domain deletion proteins in the NB7-1 lineage, either in wild type embryos or *hb* mutant embryos. Wild type embryos have five U neurons per hemisegment: the first-born U1 neuron is Hb⁺ Kr⁺, the second-born U2 neuron is Hb⁺ Kr⁺ Zfh2⁺, and the later-born neurons are Zfh2⁺ Kr⁺ Cut⁺ (U3), Zfh2⁺ Cut⁺ Runt⁺ (U4) or Zfh2⁺ Cut⁺ Runt⁺ Cas⁺ (U5) (Fig. 5A).

Overexpression of wild type Hb generated approximately 14 early-born U1/U2 neurons when endogenous Hb is present (Table 1), and approximately 12 U1/U2 neurons in a *hb* mutant background (Fig. 5B; Table 1), consistent with previous reports (Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003). Similarly, overexpression of Hb proteins lacking either the A+B domains, the B' domain, or the E domain also generated about 12 Eve⁺ U1/U2 neurons in a *hb* mutant background (Table 1), showing that none of these domains is required for Hb-mediated specification of early-born neuronal identity.

Overexpression of the Hb deletion proteins that lack transcriptional repression activity (Hb^{ΔD} and Hb^{ΔDMZ}) have 5-6 Eve⁺ U neurons when endogenous Hb is present (Fig. 5C, E; Table 1). The ectopic neuron is typically the Kr⁺ U3 neuron, which is consistent with the Hb^{ΔD} and Hb^{ΔDMZ} proteins having the ability to transcriptionally activate *Kr* (Fig. 5C) and thus specify U3 identity (Cleary and Doe, 2006; Isshiki et al., 2001). Consistent with this result, overexpression of Hb^{ΔD} and Hb^{ΔDMZ} deletion proteins in a *hb* mutant background results in loss of the Hb-dependent early-born U1/U2 neurons, while still generating an ectopic U3 neuron (Fig. 5D, F). The only difference we have observed between the Hb^{ΔD} and Hb^{ΔDMZ} proteins is that the Hb^{ΔD}, but not Hb^{ΔDMZ}, frequently generated an ectopic U5 neuron (Fig. 5C, D; Table 1; see *Discussion*). Because both the Hb^{ΔD} and Hb^{ΔDMZ} proteins retain the ability to activate *Kr* expression, we conclude that Hb-mediated transcriptional repression through the D and DMZ domains is required for the specification of first-born neuronal identity.

DISCUSSION

We have shown that Hb acts as an activator and repressor of gene expression in the CNS, but only its transcriptional repressor function is essential for maintaining neuroblast competence and specifying early-born neuronal identity. We have identified two repression domains within the Hb protein: the Mi2-binding D domain and the DMZ dimerization domain.

How do the D and DMZ domains repress gene expression? It is interesting to note that the D and DMZ domains are not dedicated repression domains such as the one found in Engrailed (Han and Manley, 1993; Jaynes and O'Farrell, 1991). Instead, both are known to mediate protein-protein interactions. The DMZ allows Hb dimerization, leading to the proposal that high Hb levels promote dimerization and thus transcriptional repression ability (Papatsenko and Levine, 2008). For example, at cellular blastoderm stages, high levels of Hb in the anterior of the embryo are required to repress *Kr*, whereas low Hb levels activate *Kr* (Hulskamp et al., 1990; Schulz and Tautz, 1994; Struhl et al., 1992), and mutations in the DMZ lead to an anterior expansion of the *Kr* expression domain (Hulskamp et al., 1994). Yet it remains unknown how Hb dimerization leads to gene repression. The D domain is also involved in protein-protein interactions. The region of Hb containing the D domain is known to bind the chromatin regulator Mi2, and this interaction promotes epigenetic silencing of the Hb target gene *Ubx* during early embryonic patterning (Kehle et al., 1998). Our results suggest that D and DMZ domains

Table 1 Summary of U neuron identity specified by Hb proteins

Ectopic protein	Genetic Background	U- neuron identity *						
		Total [‡]	n	U1	U2	U3	U4	U5
None	wt	5	100	1	1	1	1	1
	<i>hb</i> mutant	3	100	0	0	1	1	1
Hb (wild type)	wt	14	116	8	6	0	0	0
	<i>hb</i> mutant	11.5	77	5.5	6	0	0	0
Hb ^{ΔAB}	wt	16	114	8	8	0	0	0
	<i>hb</i> mutant	11.8	65	5	6.8	0	0	0
Hb ^{ΔB}	wt	16.2	102	9	7.2	0	0	0
	<i>hb</i> mutant	12.1	62	5	7.1	0	0	0
Hb ^{ΔBBD}	wt	5	185	1	1	1	1	1
	<i>hb</i> mutant	3	62	0	0	1	1	1
Hb ^{ΔC}	wt	5	180	1	1	1	1	1
	<i>hb</i> mutant	3	68	0	0	1	1	1
Hb ^{ΔD}	wt	5.5	242	1	1.25	1.25	0	2
	<i>hb</i> mutant	4	150	0	0	2	0	2
Hb ^{ΔE}	wt	16	112	8.8	7.2	0	0	0
	<i>hb</i> mutant	13.2	76	6	7.2	0	0	0
Hb ^{ΔDMZ}	wt	5.3	237	1	1.15	1.15	1	1
	<i>hb</i> mutant	3.6	184	0	0	1.6	1	1

$P < 0.001$ for all experiments.

^{||} Average number of each cell present per hemisegment based on markers described in Fig. 5.

* Cell fate markers: U1, Hb⁺ Kr⁺ Zfh2⁻; U2, Hb⁺ Kr⁺ Zfh2⁺; U3, Kr⁺ Cut⁺; U4, Runt⁺ Cas⁻; U5, Runt⁺ Cas⁺.

[‡] Total number of U neurons.

n = Total number of hemisegments analyzed.

Figure 5. The Hb D and DMZ domains are required for the first-temporal identity.

Each panel shows a 2D-projection of U neurons from one hemisegment of a stage 16 embryo; medial is to the left and anterior is on top. Quantification U neuron identity can be found in Table 1. Scale bar equals 3 μ m.

- (A) Wild type embryo. The U1-U5 neurons can be uniquely identified based on the indicated molecular markers.
- (B) *engrailed-gal4 UAS-hb* embryo. Ectopic early-born U1/U2 neurons are specified. Arrowheads indicate weak $Zfh2^+$ cells.
- (C) *engrailed-gal4 UAS-hb^{AD}* embryo. An ectopic U2 or U3 neuron can be found (arrowhead) in 50% of hemisegments. All hemisegments contain two Cas^+ U5 neurons.
- (D) *engrailed-gal4 UAS-hb^{AD}* in a *hb* mutant embryo. Most hemisegments contain an ectopic U3 neuron, and no U1 or U2 neurons are specified.
- (E) *engrailed-gal4 UAS-hb^{ADMZ}* embryo. An ectopic U2 or U3 neuron can be found (arrowheads) in 25% of hemisegments. All other U neurons differentiate as in wild-type.
- (F) *engrailed-gal4 UAS-hb^{ADMZ}* in a *hb* mutant embryo. Most hemisegments contain an ectopic U3 neuron, and no U1 or U2 neurons are specified.

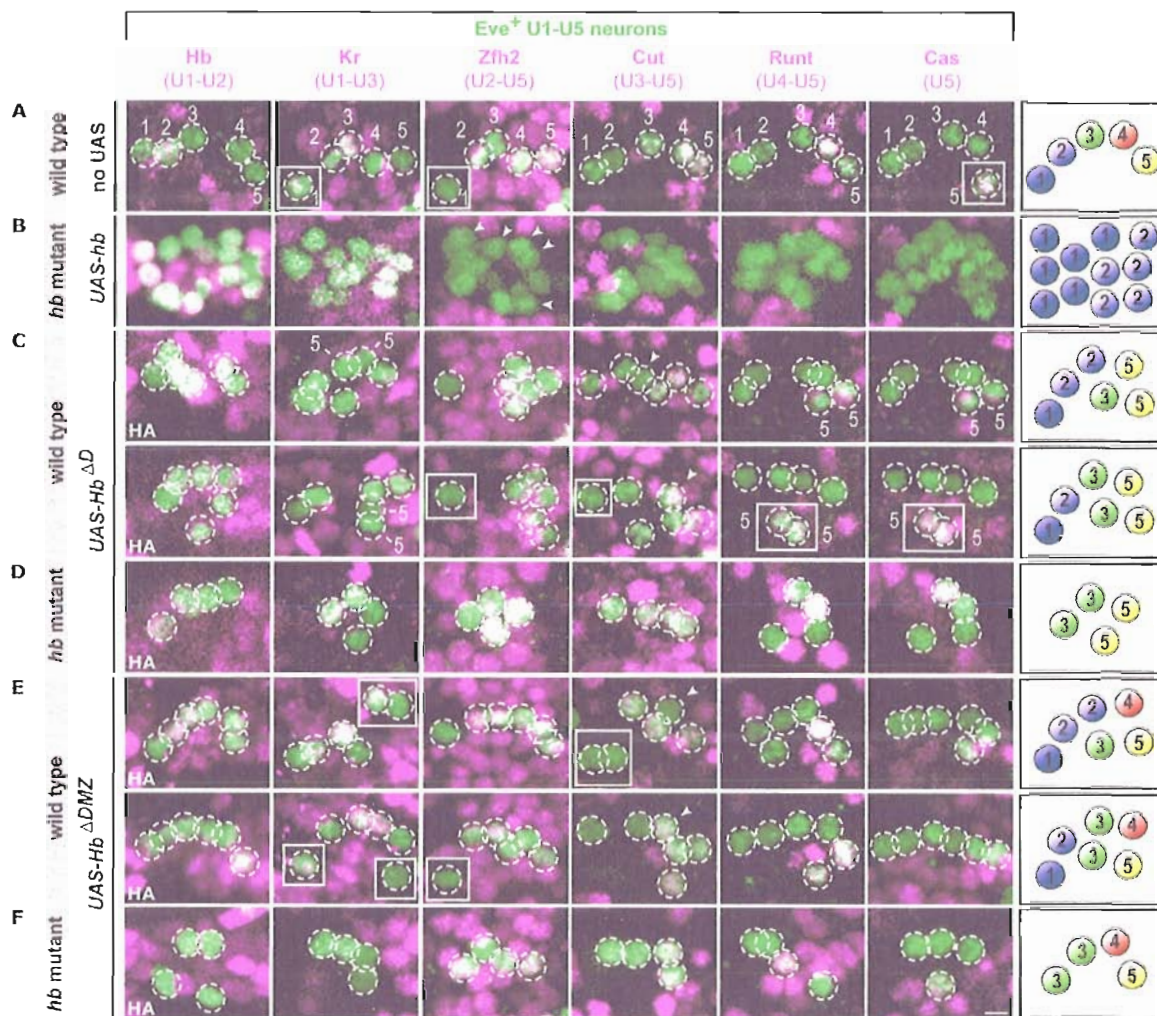


Figure 5

could act in different processes that are both required for transcriptional repression (Fig. 6B, C), or they could act in a common pathway such as dimerization-dependent recruitment of Mi2 and/or other repressor proteins to the D domain (Fig. 6D).

Hb proteins lacking the D or the DMZ domain have very similar phenotypes in the CNS (this study). Although both the D and DMZ domains appear to be required for Hb-mediated transcriptional repression, they do not have identical functions.

Overexpression of Hb^{ΔD} leads to the specification of two U5 neurons at the expense of the U4 cell identity, whereas overexpression of Hb^{ΔDMZ} results in normal U4 and U5 identities (Fig. 5). Perhaps Hb^{ΔDMZ} retains some ability to repress *cas* expression, allowing the production of the Cas⁻ U4 identity. Alternatively, Hb may use the D and DMZ domains to repress different target genes. Currently we can't distinguish these models due to the limited number of known Hb direct target genes.

Both Hb and the related mammalian protein Ikaros have major roles as transcriptional repressors, but are also weak transcriptional activators. How does Hb activate gene expression within the CNS? We were unable to identify a discrete activation domain despite the fact that our deletion series covered the entire protein. We can rule out the possibility that the activation domain maps to the D region, similar to its location in the closely related Ikaros protein (Sun et al., 1996), because Hb^{ΔD} protein has no effect on *Kr* transcriptional activation or the specification of U3 neuronal identity (Figure 4). We can also rule out the presence of a single activation domain within the A, B, B', E, or DMZ domains for the same reason. Mechanisms for Hb-mediated transcriptional activation consistent with our data are: Hb activates transcription

indirectly by blocking DNA binding of a repressor (Fig. 6A), Hb has multiple activation domains, or the Hb activation domain is tightly linked to an essential domain like the DBD. In any case, our Hb:VP16 experiments together with our repression domain deletion experiments show that Hb-mediated transcriptional repression -- not transcriptional activation -- is essential for maintaining neuroblast competence and specifying early-born neuronal identity.

What are the Hb-repressed target genes involved in extending neuroblast competence? At least one negatively regulated target is *pdm*, because we find that co-expression of Pdm with wild type Hb fails to extend neuroblast competence. However, overexpression of VP16::Hb in a *pdm* mutant background (lacking both *pdm1* and *pdm2*) was incapable of extending neuroblast competence, showing that Hb must repress multiple genes to extend competence. In the future, further characterization of Hb CNS function will require a genomic analyses, such as chromatin immunoprecipitation to identify Hb binding sites within the genome or TU-tagging (Miller et al., 2009) experiments to identify all the genes regulated by Hb within the CNS. This type of comparative analyses may help elucidate the complex gene interactions involved in regulating neuroblast competence.

Bridge to Chapter IV

In Chapter III, I have shown that Hb act as a transcriptional repressor of multiple targets to maintain early neuroblast competence and specify the first temporal identity. To better understand the mechanism by which Hb functions, I have piloted a screen to

Figure 6. Models for Hb mediated transcriptional regulation of neuroblast competence.

(A-D) Proposed molecular interactions underlying Hb CNS function. Magenta ovals, Hunchback; smaller ovals, Hb dimerization domain; magenta squares, Hb binding sights; dark-green line, genomic DNA; green arrow, transcription start site; dark-green boxes, gene; A, activator; green square, activator binding sites; R, repressor; red squares, repressor binding sites.

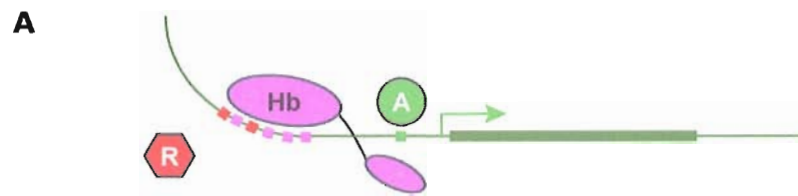
(A) Hb binds to its consensus sequence and recruits co-activators (and/or out compete repressors) to promote gene expression.

(B) Hb monomers bind to genomic DNA and recruit repressor complexes.

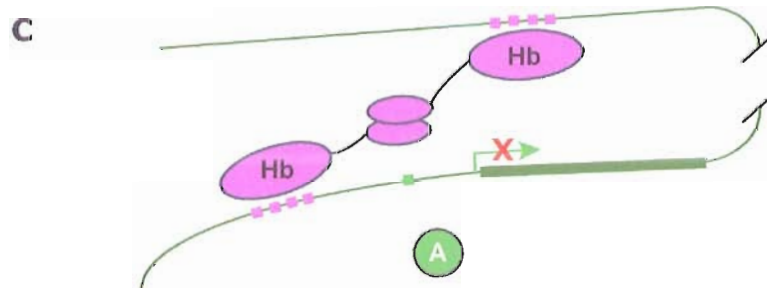
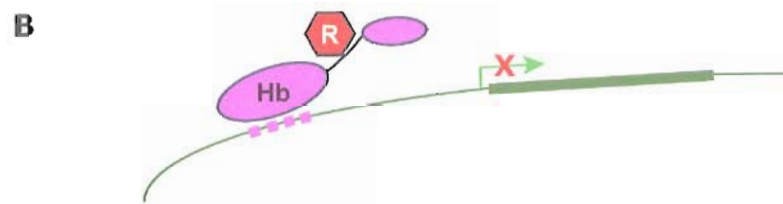
(C) Hb monomer bind to the regulatory region of a target gene, which is repressed by Hb dimerization with a Hb monomer bound in a heterochromatin domain.

(D) Hb dimerization is required for the recruitment of repressor complexes which may include Mi2 and other repressor proteins.

Gene activation



Gene repression (individual)



Gene repression (cooperative)

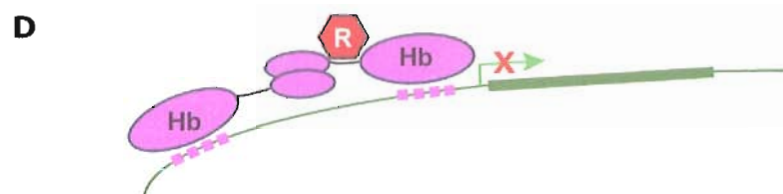


Figure 6

identify Hb target genes that may: 1) function to restrict the early-competence window, or 2) specify late-born temporal identities. I have performed a microarray analysis to compare the gene expression profiles of wild-type and Hb misexpression embryos during early neurogenesis. Gene transcripts that appear less abundant in Hb misexpression embryos are potentially interesting as they may be Hb targets that must be kept off in order to maintain early-competence. Furthermore, genes that appear to be repressed similar to *pdm* and *castor* can potentially be factors that specify late-born neuronal identities. The initial arrays have generated a list of approximately 170 candidate genes. In chapter IV, I will discuss the rationale, methods, and my preliminary results.

CHAPTER IV

IDENTIFICATION OF NOVEL HUNCHBACK TARGET GENES THAT ARE CANDIDATES FOR RESTRICTING EARLY NEUROBLAST COMPETENCE AND SPECIFY LATE-BORN NEURONAL IDENTITIES

“If Edison had a needle to find in a haystack, he would proceed at once with the diligence of the bee to examine straw after straw until he found the object of his search. I was a sorry witness of such doings, knowing that a little theory and calculation would have saved him ninety per cent of his labor.”

-- Nikola Tesla

Khoa D. Tran and Chris Q. Doe designed the research; Khoa D. Tran performed research; Khoa D. Tran and Chris Q. Doe analyzed the data.

INTRODUCTION

The mechanisms governing stem-cell multipotentiality and the ability to specify the correct cells at the right time and place is an active area of research in both developmental and stem-cell biology. Yet, little is known about the cell-autonomous

mechanisms regulating a progenitor's decision to produce different cells over time. The ability to manipulate progenitors and to coerce them to produce specific cells when needed may one day prove useful in therapeutic treatments for tissue repair after a life altering event such as a stroke, or damage due to neurodegenerative diseases.

The *Drosophila* C2H2 zinc-finger transcription factor Hunchback (Hb) is an essential regulator of anterior-posterior patterning and neurogenesis (Isshiki et al., 2001; Pearson and Doe, 2004; Schulz and Tautz, 1994; Struhl et al., 1992; Tran et al., 2010). During early *Drosophila* development, Hb transcriptional regulation of HOX genes establishes the anterior-posterior axis of the organism. During neurogenesis, Hb is expressed early in *Drosophila* neuroblasts and is necessary and sufficient to specify the “young” neuroblast state in which first-born progeny are produced (Isshiki et al., 2001). Loss of Hb in the CNS results in embryos lacking many first-born neurons; and prolonged Hb expression produces a supernumerary of cells that differentiate with first-born identity (Isshiki et al., 2001; Novotny et al., 2002).

Previous studies have shown that Hb must act as a transcriptional repressor of multiple target genes to maintain early neuroblast competence and specify early born cells (Tran et al., 2010). Misexpression of a Hb chimera protein (VP16::Hb) that act as a strong transcriptional activator revealed that Hb transcriptional repressor activity is necessary for its function to maintain neuroblast competence. One gene that must be repressed is the Hb-direct target, *pdm*. Co-misexpression of Hb and Pdm results in the repression of the Hb misexpression phenotype, generating less ectopic U neurons than the misexpression of Hb alone (Isshiki et al., 2001; Novotny et al., 2002; Tran et al., 2010).

However, *Pdm* is not the only factor that must normally be repressed by Hunchback, as the misexpression of VP16::Hb in a *pdm* mutant did not result in the recovery of ectopic U neurons. Therefore, Hb must be repressing at least one other factor to maintain neuroblast competence.

To identify novel Hb target genes that may act as competence restricting factors, or factors that may specify late-born neuronal identity, I have piloted a screen to compare the gene expression profile of wild-type embryos and Hb misexpression using microarray analysis. Additionally, I have compared the gene expression profile of wild-type embryos to those that overexpress different Hb-deletions discussed in the previous chapter. Using a gene cluster analysis, I have identified approximately 175 candidate Hb-target genes that behave similar to *pdm* across multiple Hb misexpression genotypes. In the following months, I will conduct follow-up studies from this dataset. Below, I describe my progress and future directions.

MATERIALS AND METHODS

Fly stocks

Wild-type (*yw*) embryos were collected at 23°C and fixed using standard fixation methods as previously described (Grosskortenhaus et al., 2005; Tran and Doe, 2008). For misexpression experiments, flies carrying *insc-gal4* (*1407-gal4*, Bloomington Stock Center) on chromosome II were crossed to flies carrying *UAS-hb* on chromosomes II and

III (Wimmer et al., 2000), *UAS-Hb*^{ΔDBD}, *UAS-Hb*^{ΔC}, *UAS-Hb*^{ΔD}, and *UAS-Hb*^{ΔDMZ} on chromosome II at 25°C (Tran et al., 2010).

Microarray experiments

Stage 11-12 embryos were collected and total RNA were extracted according to standard methods. RNA was processed using the Quick-Amp Labeling Kit from Agilent Technologies and hybridized to a *D. melanogaster* oligonucleotide microarray representing all 14,141 genes from the Flybase release 5.4 genome according to Agilent's protocol (Agilent Technologies, Santa Clara, CA). Microarray slides were scanned using an Axon GenePix 4000B scanner and fluorescent ratios for each microarray element were recovered and normalized using GenePix Pro 6.0. For arrays comparing wild-type embryos to wild-type Hb misexpression embryos, we quantified and averaged the change in transcript levels of each gene over four biological replicates. Experiments comparing wild-type embryos to various Hb-deletions misexpression embryos were performed in triplicates.

In-situ probe production and in-situ hybridization

In-situ probes were prepared and in-situs were performed according to the Berkeley Drosophila Genome Project (BDGP) protocols. In summary, probe templates were obtained by PCR from cDNA clones or a cDNA library, transcribed and labeled with Digoxigenin (DIG) (Roche, Pleasanton, CA), and detected using alkaline-phosphatase conjugated sheep anti-DIG antibodies (Roche, Pleasanton, CA). In-situ

images were captured using a Zeiss AxioCam HRc camera on an Axioplan microscope. Images were processed in Adobe Photoshop and figures were prepared using Adobe Illustrator (Adobe Systems, San Jose, CA).

RESULTS

Transcriptional profiling and cluster analysis of multiple Hb misexpression genotypes identify potential Hunchback target genes

To identify potential Hb targets that may act to restrict early neuroblast competence or specify late-born temporal identities, we have performed microarray experiments to compare the transcriptional profile of wild-type embryos to those of various Hb misexpression genotypes. In addition to wild-type Hb, misexpression experiments were conducted with Hb-deletion proteins that lack the repression domains (Hb^{ΔD} and Hb^{ΔDMZ}) and Hb-deletion proteins that appear to be non-functional in the nervous system (Hb^{ΔDBD} and Hb^{ΔC}) (Tran et al., 2010). To filter our microarray data and minimize background changes in the transcription profile of experimental embryos, we employed a clustering algorithm to identify genes with similar expression profiles across all misexpression genotypes.

Because *pdm* is a Hb target that must be repressed to maintain neuroblast competence, and because *pdm* can specify late-born cell identity in at least one neuroblast lineage, we focus our attention on genes that behave similarly to *pdm* in microarray experiments across multiple genotypes. We have identified approximately 175 candidate

genes that cluster closely with *pdm*, in that they are repressed by wild-type Hb but not repressed by Hb-deletions lacking the repression domains (Fig. 1, Table S1). One known Hb CNS target, the gene *castor*, also clustered closely to *pdm*, suggesting that our array experiments can identify genes normally repressed by Hb in neuroblasts (Fig. 1) (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Tran et al., 2010).

To further filter our array results, we searched the BDGP embryonic gene expression database to look for expression of our candidate genes in the nervous system. This search identified 26 genes with expression in the nervous system, 30 genes that are not expressed in the nervous system, 75 genes with no expression data, and 44 genes that require further analysis due to insufficient data in the database (Fig. S1, Table S1). Our in-silico search reduced the list of candidate Hb target to 145 genes, 83 of which are conserved in vertebrates.

DISCUSSION

We have identified 145 candidate Hb CNS target genes that cluster closely to known Hb-targets such as *pdm* and *castor*. Further characterization is required to determine whether these genes are expressed in neuroblasts at the right stages. In the future, whole mount in-situ hybridization for these candidate genes in wild-type and Hb-misexpression embryos will confirm whether they are repressed by Hb during neurogenesis.

At this time, we are currently examining the 145 candidate genes from a relatively small cluster containing *pdm* and *castor* (Fig. 1). Factors that are identified as Hb targets will be functionally examined for their roles during neurogenesis. This includes the potential role for specifying unique temporal identities in multiple neuroblast lineages, as well as the potential role for factors that restrict neuroblast competence to specify early-born cell types.

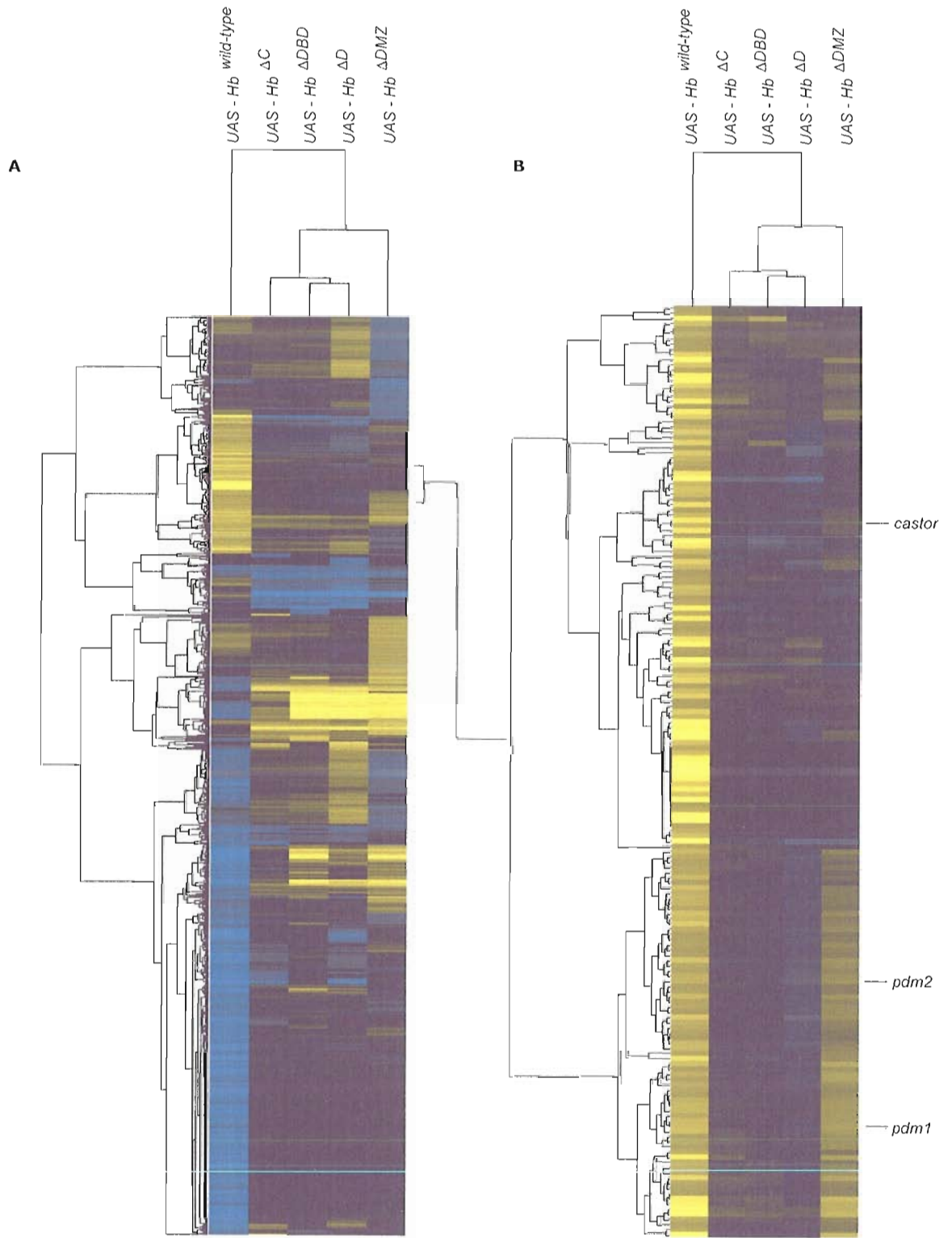
Although our “core” cluster of potential Hb-target genes contains only 170 candidate genes, this analysis can be expanded to include an additional 230 genes in adjacent clusters. We will examine this second tier of candidate genes after completing our initial investigation of the core cluster. And, while we have limited our initial efforts to genes that are repressed by Hb, this data set can also be used to identify factors that are activated by Hb. But, that will be another story for another time and person.

Figure 1. Cluster analysis identifies potential Hb CNS targets.

Microarray experiments and cluster analysis comparing the transcriptional profiles of wild-type and multiple Hb-misexpression phenotypes. Each column represents an average over multiple biological replicates (*UAS-hb^{wild-type}*, four; all others, three). Blue represents up-regulated genes, and yellow represents down-regulation genes.

(A) Cluster of all genes that are differentially expressed. Only genes that are present in all biological replicates are included in this analysis.

(B) Cluster containing genes that behave similarly to known Hb CNS targets across all genotypes.

**Figure 1**

CHAPTER V

CONCLUSIONS

My dissertation work has focused on the temporal regulation of *Drosophila* neuroblasts with an emphasis on the molecular mechanisms and genetic interactions underlying the specification of neuronal cell identities over developmental time. I, and others, have shown that temporally regulated genes encode for factors that specify temporal identities as well as factors that regulate the timing of gene expression in neuroblasts. In this text, I have highlighted the importance of precise cross-regulation between the temporal identity factors Hb and Kr, and timing factors Pdm and Cas; and how they must function in perfect unison to promote the proper specification of neuronal identities at the correct time, and in the correct place.

In Chapter II, I discussed the findings from my investigation of the functions of Pdm and Cas in a new neuroblast lineage. While previous studies conducted in the NB7-1 lineage shows that Pdm and Cas specifies late-born cell identities (Grosskortenhaus et al., 2006), my studies in the NB3-1 lineage revealed that they act to regulate the timing of the temporal identity windows under which late-born cell identities are specified. Since my initial characterization of Pdm and Cas as regulator of temporal identity windows in

NB3-1, others have identified important roles for Cas as the initiator of important gene regulation cascades required for the specification of late-born cell identities in an additional neuroblast lineage, NB5-6 (Baumgardt et al., 2009; Karlsson et al., 2010). These findings underscore the need to investigate the role of temporally regulated factors in multiple lineages in order to gain a more comprehensive understanding of temporal regulation during neural development. In the future, as more tools are developed to aid in the analysis of single neuroblast lineages, it will be possible to identify and compare the multi-faceted roles of factors such as Pdm and Cas in many neuroblasts.

In Chapter III, I discussed in detail the findings from my investigation of how Hb maintains early neuroblast competence. It has been well documented, by myself and others, that Hb is necessary and sufficient to maintain early neuroblast competence and specify early-born cells in many neuroblast lineages (Isshiki et al., 2001; Novotny et al., 2002; Tran and Doe, 2008). However, the mechanisms underlying Hb function are unknown; and this understanding is crucial for helping to identify novel factors involved in specifying the early-competence state. Therefore, I conducted a molecular dissection of Hb functions during early neurogenesis. I found that although Hb is capable of activating and repressing gene expression in the embryonic nervous system, it is the transcriptional repression of multiple target genes that is necessary to maintain early-competence and specify early-born neuronal identities. Further, I found that this repression is mediated through two well conserved protein-protein interaction domains, suggesting that Hb may regulate gene expression by recruiting one or more co-factors. In

the future, it will be important to identify these binding partners and characterize how they regulate gene expression together with Hb.

Another question that stems from the work I described in Chapter III is “What are the factors that must be repressed in order to maintain early neuroblast competence?” Thus, in Chapter IV I describe my initial attempt to identify Hb CNS targets that must be repressed to maintain early neuroblast competence. I have identified a list of candidate genes and will proceed to characterize their function during nervous system development upon confirmation that they are indeed targets of Hb.

CLOSING REMARKS

Recent studies have also shown that the Hb ortholog, Ikaros, is necessary and sufficient to specify early neural progenitor competence and early-born cells in the vertebrate retina. The orthologs of *Kruppel* and *pdm* are also present in vertebrates and have important roles in stem-cell biology. The Kruppel-like factor 4 and Oct4 (POU domain protein family which includes Pdm) are sufficient to create induced pluripotent stem cells from fully differentiated cells in mouse models (Kim et al., 2009; Kim et al., 2008). Thus, a fundamental understanding of how different states of neural progenitor competence in *Drosophila* is specified and maintained may have further reaching implications than merely that of a conceptual understanding of how progenitors can stay “young”.

APPENDIX A**SUPPLEMENTAL MATERIALS FOR CHAPTER II**

Figure S1. Misexpression of Hb generates ectopic RP1/RP4 motor neurons and a thicker motor nerve root.

- (A) Wild type embryo stained for HB9 and Fasciclin III (FasIII). RP motor neurons express FasIII (*white chevron*) and exit the CNS via the ISNa nerve root (*white arrowhead*). Dashed vertical lines show the edge of CNS in A and B.
- (B) Misexpression of Hb (*worniu-gal4 UAS-hb*) results in more RP motor neurons and a thicker ISNa fascicle (*white arrowhead*). Scale bar is 3 μm in A and B.
- (C) Wild type RP motor neurons project to the indicated lateral muscles (muscle names in white, RP axons labeled in yellow text). Nerve roots, white arrowheads. Image is one dissected hemi-embryo shown in mirror image, with muscles shown on the right side.
- (D) Misexpression of Hb (*worniu-gal4 UAS-hb*) shown with the same orientation and labeling as in C. Nerve roots are thicker (white arrowheads) and RP motor neurons reach their normal muscle field (yellow labels). Scale bar is 10 μm in C and D.

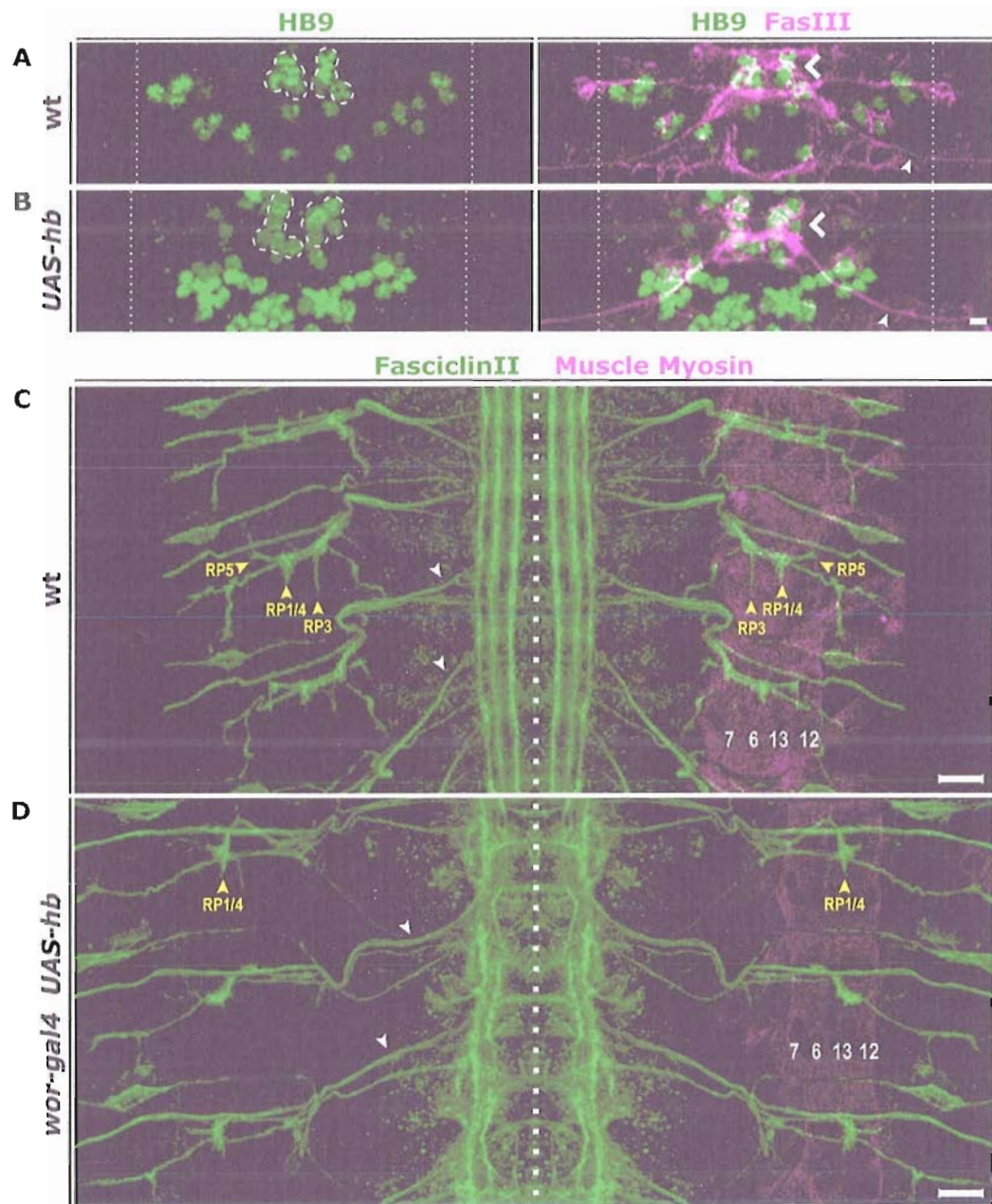


Figure S1

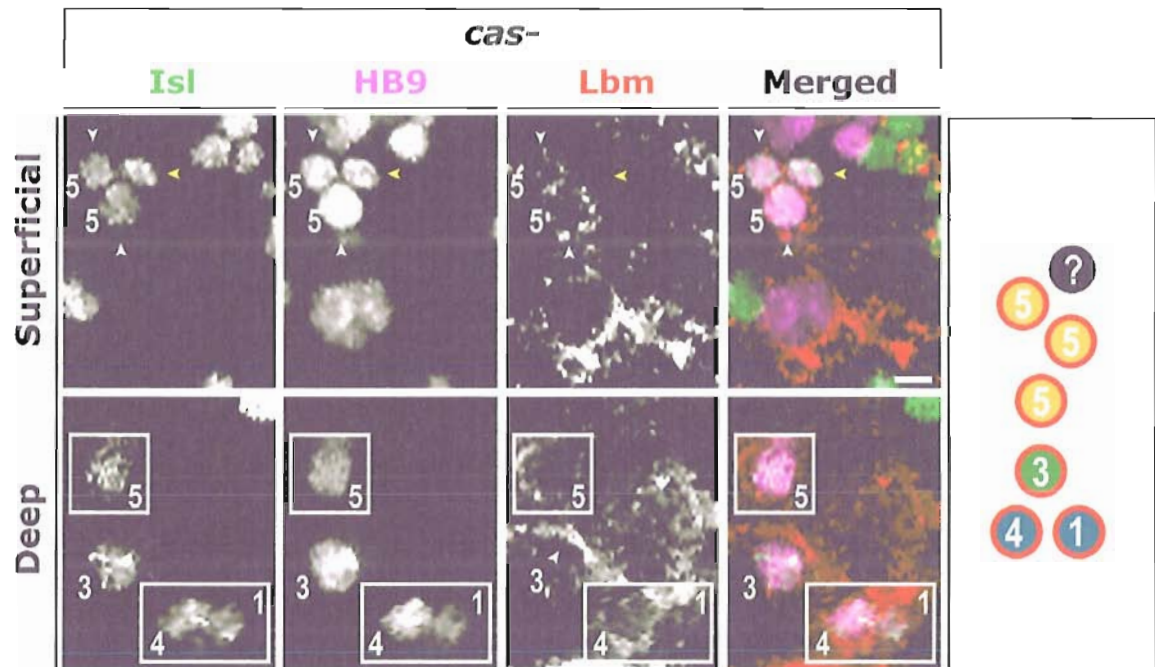


Figure S2. Ectopic Isl^+ $HB9^+$ cells that are superficial to RP5 in *cas* mutant embryos do not express the motor neuron marker, Late Bloomer.

Islet, green; HB9, magenta; Late Bloomer, red. RP neurons, white arrowheads; ectopic Isl^+ $HB9^+$ cells (yellow arrowhead; 1-2 per hemisegment, 90%, $n=183$). For all panels, a single representative hemisegment of a stage-16 CNS is shown as a maximum intensity projection; midline, left; anterior, up; phenotype summary, right (Late Bloomer, red outlines); scale bar, 3 μ m.

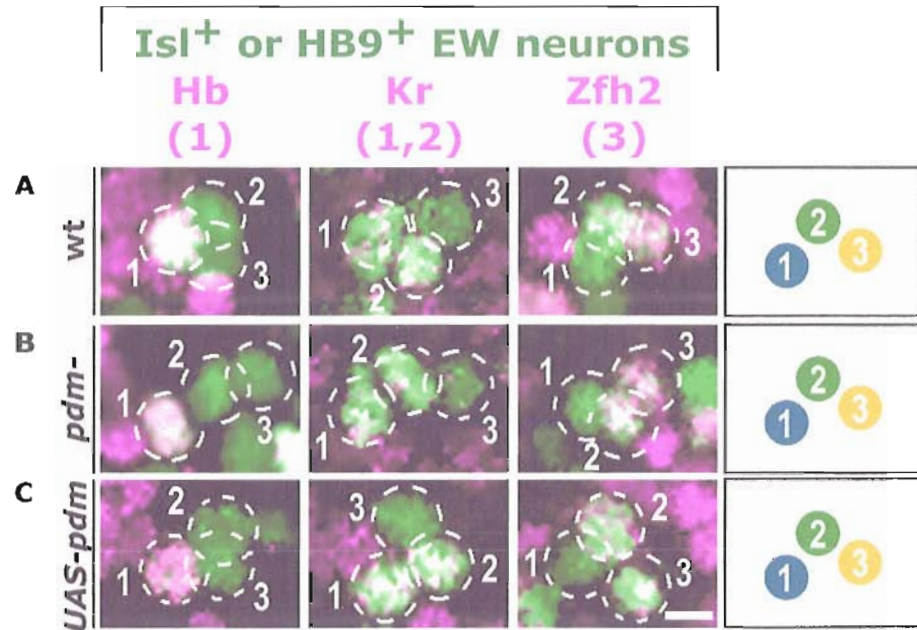


Figure S3. NB7-3 progeny are unaffected in *pdm* mutants and after Pdm misexpression.

(A-C) Interneurons of the NB7-3 lineage. Molecular markers shown at top; genotypes shown to left; schematic of neuronal identity shown to right. The first GMC makes the Hb⁺Kr⁺ EW1 interneuron (and the GW motoneuron, not shown), the second GMC makes the Hb⁻Kr⁺ EW2 interneuron, and the third GMC makes the Zfh2⁺ EW3 interneuron (Isshiki et al., 2001; Novotny et al., 2002). Pdm is expressed in the EW3 interneuron (Isshiki et al., 2001) and possibly transiently in other neurons in the lineage (Novotny et al., 2002). Anterior, up, midline, left. Scale bar is 3 μ m.

(A) Wild type (wt) embryos have EW1-EW3 neurons.

(B) *pdm* mutant embryos have normal EW neuron fates.

(C) Pdm misexpression embryos (*insc-gal4 UAS-pdm2*) have normal EW neuron fates.

APPENDIX B**SUPPLEMENTAL MATERIALS FOR CHAPTER III**

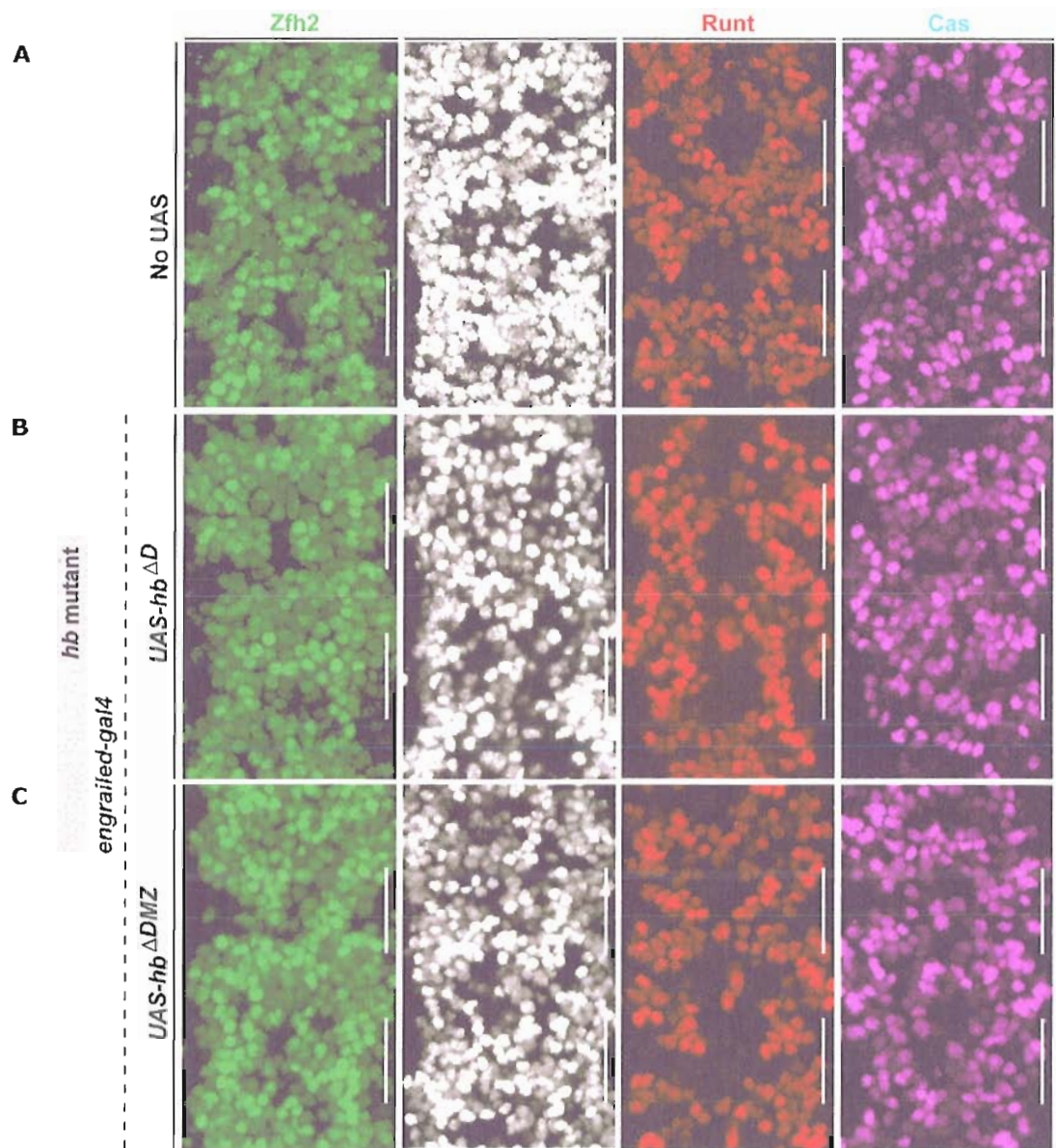


Figure S1: The D and DMZ domains are required for Hb repression of CNS targets.

(A-C) The overexpression of Hb^{ΔD} and Hb^{ΔDMZ} fail to repress Hb CNS targets normally repressed by Hb.

(A) Expression of Zfh2, Cut, Runt, and Cas in *hb* mutants.

(B-C) There is no noticeable change in the expression of Hb CNS target genes.

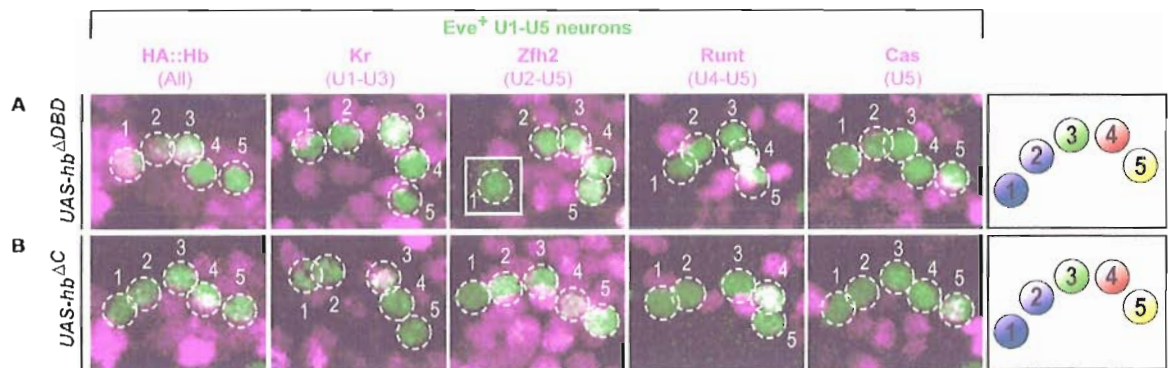


Figure S2: Overexpression of Hb^{ΔD} and Hb^{ΔDMZ} does not alter U neuron identities.

Each panel shows a 2D-projection of U neurons from one hemisegment of a stage 16 embryo; medial is to the left and anterior is on top. Scale bar equals 3 μm.

(A-B) U1-U5 neurons differentiate normally.

APPENDIX C

SUPPLEMENTAL MATERIALS FOR CHAPTER IV

Distribution of expression data from BDGP expression database

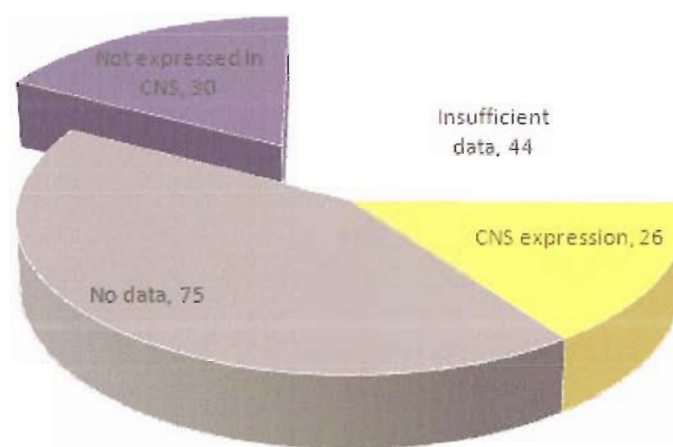


Figure S1. Distribution of candidate genes based on established expression database.

The application of an in-silico filter identifies 26 genes with known CNS expression and removes 30 genes with no CNS expression.

Table S1 Candidate Hb-target gene cluster

	Gene Name	Fold Change	Known Expression Pattern	Vertebrate Homolog
1	Klp61F	-1.4	CNS Brain	Yes
2	l(1)sc	-1.6	CNS Brain Gut Strip	Yes
3	ena	-1.4	CNS	Yes
4	asp	-1.4	CNS Brain	Yes
5	Mcm7	-1.5	CNS Brain	Yes
6	dnk	-1.5	CNS Brain Gut	Yes
7	Ect3	-4.5	CNS	Yes
8	hb	-1.5	CNS	Yes
9	cas	-2.0	CNS Brain	Yes
10	Chrac-16	-2.0	CNS Brain	Yes
11	pdm2	-1.4	CNS Brain Gut	Yes
12	Nub	-2.7	CNS Brain	Yes
13	CG11398	-1.5	maternal	Yes
14	CG11403	-1.4	maternal	Yes
15	CG12863	-1.6	maternal	Yes
16	CG13277	-2.6	maternal	Yes
17	CG13398	-1.4	maternal	Yes
18	CG15747	-2.0	maternal	Yes
19	CG17219	-1.6	maternal	Yes
20	CG17470	-1.8	maternal	Yes
21	CG31120	-1.4	maternal	Yes
22	CG40191	-1.5	maternal	Yes
23	CG5857	-1.5	maternal	Yes
24	CG5969	-1.5	maternal	Yes
25	CG5987	-1.6	maternal	Yes
26	CG6071	-2.1	maternal	Yes
27	CG7597	-1.4	maternal	Yes
28	CG8441	-1.4	maternal	Yes
29	CG9723	-1.7	maternal	Yes
30	fh	-1.4	maternal	Yes
31	RpL17	-2.3	maternal	Yes
32	RpL37a	-1.5	maternal	Yes
33	scpr-C	-1.5	maternal	Yes
34	Sse	-1.5	maternal	Yes
35	mit(1)15	-1.4	Muscle Brain	Yes
36	CG11327	-2.0	No expression	Yes
37	CG11436	-1.6	No expression	Yes

	Gene Name	Fold Change	Known Expression Pattern	Vertebrate Homolog
38	CG4680	-1.4	No expression	Yes
39	CG7102	-1.4	No expression	Yes
40	Clk	-1.7	No expression	Yes
41	Rala	-4.0	No expression	Yes
42	Rh7	-1.7	No expression	Yes
43	pxb	-2.1	Stripes	Yes
44	Aats-gln	-1.5	no data	Yes
45	ac	-1.7	CNS, 4 neuroblasts	Yes
46	amos	-1.4	PNS, sensory neurons	Yes
47	ato	-1.5	PNS, sensory neurons	Yes
48	baf	-1.5	no data	Yes
49	BobA	-2.0	no data	Yes
50	CG10469	-1.4	no data	Yes
51	CG10581	-1.4	no data	Yes
52	CG10859	-2.1	no data	Yes
53	CG11839	-1.4	no data	Yes
54	CG12269	-2.3	no data	Yes
55	CG12325	-1.8	no data	Yes
56	CG13083	-2.1	no data	Yes
57	CG1311	-1.5	no data	Yes
58	CG13361	-27.2	no data	Yes
59	CG13427	-1.4	no data	Yes
60	CG13454	-1.8	no data	Yes
61	CG13465	-2.0	no data	Yes
62	CG13794	-12.1	no data	Yes
63	CG14057	-1.4	no data	Yes
64	CG14966	-1.4	no data	Yes
65	CG15456	-1.5	no data	Yes
66	CG15816	-1.6	no data	Yes
67	CG17118	-1.7	no data	Yes
68	CG17233	-1.6	no data	Yes
69	CG17264	-1.6	no data	Yes
70	CG18190	-1.5	no data	Yes
71	CG1946	-2.0	no data	Yes
72	CG2453	-2.0	no data	Yes
73	CG30272	-1.8	no data	Yes
74	CG3071	-1.7	no data	Yes
75	CG31012	-1.4	no data	Yes

	Gene Name	Fold Change	Known Expression Pattern	Vertebrate Homolog
76	CG31606	-21.1	no data	Yes
77	CG32150	-1.7	no data	Yes
78	CG32182	-1.4	no data	Yes
79	CG32572	-27.2	maternal	Yes
80	CG32581	-2.8	no data	Yes
81	CG33791	-1.7	no data	Yes
82	CG3781	-1.4	no data	Yes
83	CG4645	-2.6	no data	Yes
84	Esp	-1.4	Ectoderm	Yes
85	HLHm5	-1.7	Ectoderm	Yes
86	SAK	-1.7	Ectoderm	Yes
87	Toll-6	-1.5	Ectoderm	Yes
88	slp2	-1.4	Ectoderm Stripes	Yes
89	CG6910	-2.2	fat body	Yes
90	Dhpr	-1.6	fat body	Yes
91	boi	-1.5	Gut	Yes
92	bwa	-1.5	Gut	Yes
93	CG18600	-1.6	Gut	Yes
94	CG6512	-1.4	Gut	Yes
95	CG9279	-4.2	Gut	Yes
96	tst	-1.4	Gut	Yes
97	CG9281	-1.4	Gut Muscles	Yes
98	Doc1	-1.5	Guts Muscles	Yes
99	Doc2	-1.4	Guts Muscles	Yes
100	Doc3	-1.4	Guts Muscles	Yes
101	CG15628	-1.4	Gut	Yes
102	CG30431	-1.5	Gut, Yolk Sac	Yes
103	CG8436	-1.4	muscle	Yes
104	bin	-1.4	muscle	Yes
105	CG12022	-1.7	Ubi	Yes
106	CG8191	-1.5	Ubi	Yes
107	CG11407	-1.7	Yolk nuclei	Yes
109	CG3085	-1.7	not interesting	Yes
110	CG3597	-2.1	not interesting	Yes
111	CG30043	-2.1	not interesting	Yes
112	Sry-alpha	-1.7	not interesting	Yes
113	CG7768	-1.4	CNS	Not identified
114	CG14937	-1.5	CNS Brain	Not identified

	Gene Name	Fold Change	Known Expression Pattern	Vertebrate Homolog
115	CG17321	-1.5	CNS Brain	Not identified
116	Sr-CII	-1.7	CNS Ectoderm	Not identified
117	CG12236	-1.4	CNS (faint)	Not identified
118	ial	-1.5	CNS Brain	Not identified
119	mod(mdg4)	-1.5	CNS Brain	Not identified
120	Nrt	-1.5	CNS Brain	Not identified
121	BEAF-32	-1.4	CNS Brain Gut	Not identified
122	Mes2	-1.4	CNS Brain Gut	Not identified
123	CG3036	-1.5	CNS	Not identified
124	msb1l	-1.5	CNS Ubi	Not identified
125	mira	-1.4	CNS	Not identified
126	Ote	-1.4	CNS Brain	Not identified
127	CG4676	-1.5	no data	Not identified
128	CG5167	-1.5	no data	Not identified
129	Reck	-1.4	no data	Not identified
130	CG5602	-1.4	no data	Not identified
131	Npc2f	-1.4	no data	Not identified
132	lr68a	-1.4	no data	Not identified
133	CG6475	-1.8	no data	Not identified
134	CG6520	-1.4	no data	Not identified
135	CG6729	-1.6	no data	Not identified
136	CG7763	-3.0	no data	Not identified
137	CG7912	-12.9	no data	Not identified
138	CG8783	-1.5	no data	Not identified
139	CG9270	-1.7	no data	Not identified
140	cmet	-1.4	no data	Not identified
141	CS-2	-1.4	no data	Not identified
142	DNApol-epsilon	-1.4	no data	Not identified
143	Dsp1	-1.4	no data	Not identified
144	Fancd2	-1.4	no data	Not identified
145	fz	-1.4	no data	Not identified
146	Grip84	-1.5	no data	Not identified
147	gsb	-1.4	Subset of neuroblasts	Not identified
148	HLHm7	-1.6	no data	Not identified
149	Hsp70Bc	-1.5	no data	Not identified
150	ind	-1.4	no data	Not identified

	Gene Name	Fold Change	Known Expression Pattern	Vertebrate Homolog
151	Kap3	-5.6	no data	Not identified
152	kek5	-1.6	no data	Not identified
153	Klp54D	-10.5	no data	Not identified
154	l(2)05714	-1.4	Ectoderm, maybe CNS	Not identified
155	HipHop	-1.5	no data	Not identified
156	malpha	-1.4	no data	Not identified
157	MED7	-1.4	faint ubi	Not identified
158	MRP	-1.7	no data	Not identified
159	mus205	-1.4	no data	Not identified
160	nmdyn-D7	-1.4	no data	Not identified
161	Notum	-1.4	no data	Not identified
162	pad	-1.4	no data	Not identified
163	Pen	-1.4	no data	Not identified
164	polo	-1.5	no data	Not identified
165	Ppt1	-1.4	no data	Not identified
166	RfC3	-1.5	no data	Not identified
167	RpL37A	-1.4	maternal	Not identified
168	sc	-1.4	Ectoderm, not CNS	Not identified
169	sca	-1.5	no data	Not identified
170	sens	-1.4	Ectoderm, not CNS	Not identified
171	Six4	-1.6	no data	Not identified
172	spn-E	-1.9	no data	Not identified
173	sqz	-1.4	CNS Brain	Not identified
174	wnd	-2.0	no data	Not identified
175	Cyt-b5-r	-2.2	Gut	Not identified

Gene expression data were collected from the Berkeley Drosophila Genome Project database (as of June 1, 2010).

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