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# Recombineering Hunchback identifies two conserved domains required to maintain neuroblast competence and specify early-born neuronal identity

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## SUMMARY

The Hunchback/Ikaros family of zinc-finger transcription factors is essential for specifying the anterior/posterior body axis in insects, the fate of early-born pioneer neurons in *Drosophila*, and for retinal and immune development in mammals. Hunchback/Ikaros proteins can directly activate or repress target gene transcription during early insect development, but their mode of action during neural development is unknown. Here, we use recombineering to generate a series of Hunchback domain deletion variants and assay their function during neurogenesis in the absence of endogenous Hunchback. Previous studies have shown that Hunchback can specify early-born neuronal identity and maintain 'young' neural progenitor (neuroblast) competence. We 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. We identify *pdm2* as a direct target gene that must be repressed to maintain competence, but show that additional genes must also be repressed. We propose that Hunchback maintains early neuroblast competence by silencing a suite of late-expressed genes.

**KEY WORDS:** Hunchback, Ikaros, Competence, Neurogenesis, Temporal identity, Transcription, *Drosophila*

## INTRODUCTION

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 tissue for use in future cell therapy applications. In recent years, substantial progress has been made in the identification of the factors involved in regulating progenitor competence, bringing with it the need to understand the molecular basis of their function.

The identification of genes that regulate neural progenitor competence in 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). Although it is known that Hb and Ik can maintain progenitor competence in the nervous system, the molecular mechanism by which they act remains unknown.

The *Drosophila* embryonic CNS is an excellent system in which to investigate Hb-dependent regulation of neural progenitor competence. Individual neural progenitors (neuroblasts) can be uniquely identified based on their position and pattern of gene expression (Broadus et al., 1995). Each of the 30 neuroblasts in a hemisegment divides to produce an invariant order of neurons and/or glia, the identity of which is determined by the sequential expression of transcription factors: Hb → Kruppel (Kr) → Pdm1 (Nubbin – FlyBase)/Pdm2 (henceforth Pdm) → Castor (Cas) → Grainy head (Grh) (Baumgardt et al., 2009; Bossing et al., 1996; Isshiki et al., 2001; Mairange et al., 2008). Hb is expressed early on 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 to many neuroblast lineages (Cleary and Doe, 2006; Pearson and Doe, 2003). This is achieved by maintaining Hb expression in neuroblasts throughout neurogenesis, or by reintroducing Hb into 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 into 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 Hb regulate gene expression to maintain early neuroblast competence? 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 function in the CNS. In the cellular blastoderm, the Hb protein gradient initiates and establishes the

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spatial expression domains of the gap genes *Kr*, *knirps* (*kni*) and *giant* (*gt*). Rigorous genetic and molecular analyses have shown that Hb acts as 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 the recruitment of Polycomb complex proteins (Kehle et al., 1998). As a result, the multifunctional 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 in maintaining neuroblast competence during nervous system development.

## MATERIALS AND METHODS

### Generation of VP16::Hb chimeric 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. For primer sequences, see Table S1 in the supplementary material. The chimeric gene was verified by sequencing, cloned into the *pUAST* vector (Brand and Perrimon, 1993) and transgenic flies produced (GenetiVision, Houston, TX, USA).

### Generation of tagged Hb deletion proteins

We generated *hb* genes deleted for the six previously described conserved domains (R. Sommer, PhD thesis, University of Munich, 1992) (Tautz et al., 1987), as well as for 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 with 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 (3×FLAG::3×HA) was prepared by PCR amplification using homology primers. For primer sequences, see Table S1 in the supplementary material. 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), and this transgene was used as a UAS control so that each misexpression experiment 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<sup>FB</sup> hb<sup>P1</sup>/TM3 ftz-lacZ* [*hb<sup>FB</sup>* is a null mutant, *hb<sup>P1</sup>* 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<sup>wild-type</sup>/CyO* and *UAS-hb<sup>wild-type</sup>/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*;  
*UAS-hb<sup>ΔAB</sup>/CyO* and *UAS-hb<sup>ΔAB</sup>/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*;  
*UAS-hb<sup>ΔB'</sup>/CyO* and *UAS-hb<sup>ΔB'</sup>/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*;  
*UAS-hb<sup>ΔBBD</sup>/CyO* and *UAS-hb<sup>ΔBBD</sup>/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*;  
*UAS-hb<sup>ΔC</sup>/CyO* and *UAS-hb<sup>ΔC</sup>/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*;  
*UAS-hb<sup>ΔD</sup>/CyO* and *UAS-hb<sup>ΔD</sup>/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*;  
*UAS-hb<sup>ΔE</sup>/CyO* and *UAS-hb<sup>ΔE</sup>/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*;  
*UAS-hb<sup>ΔMZ</sup>/CyO* and *UAS-hb<sup>ΔMZ</sup>/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*; and  
*engrailed-gal4/CyO; hb<sup>FB</sup> hb<sup>P1</sup>/TM3 Ubx-lacZ*.

### Molecular markers and immunostaining

Antibody staining was performed according to standard methods. Polyclonal antiserum 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, USA) for injection into rats. Primary antibodies used were: rat Zfh2, 1:400 (this work; M. Lundell, UT San Antonio, USA); mouse Eve 2B8, 1:10 (Patel et al., 1994); guinea pig Eve, 1:1000, rat Eve, 1:1000 and 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 β-galactosidase, 1:500 (Promega, Madison, WI, USA); rabbit β-galactosidase, 1:1000 (MP Biomedicals, Solon, OH, USA); mouse HA, 1:500 (Roche, Palo Alto, CA, USA); and rabbit HA, 1:1000 (Sigma, St Louis, MO, USA). Secondary antibodies were purchased conjugated to Alexa 488, Rhodamine RedX, Cy5 (Jackson ImmunoResearch, West Grove, PA, USA), biotin (Vector Labs, Burlingame, CA, USA) or alkaline phosphatase (Southern Biotechnology, Birmingham, AL, USA) and used at 1:400.

### Microscopy and statistical analysis

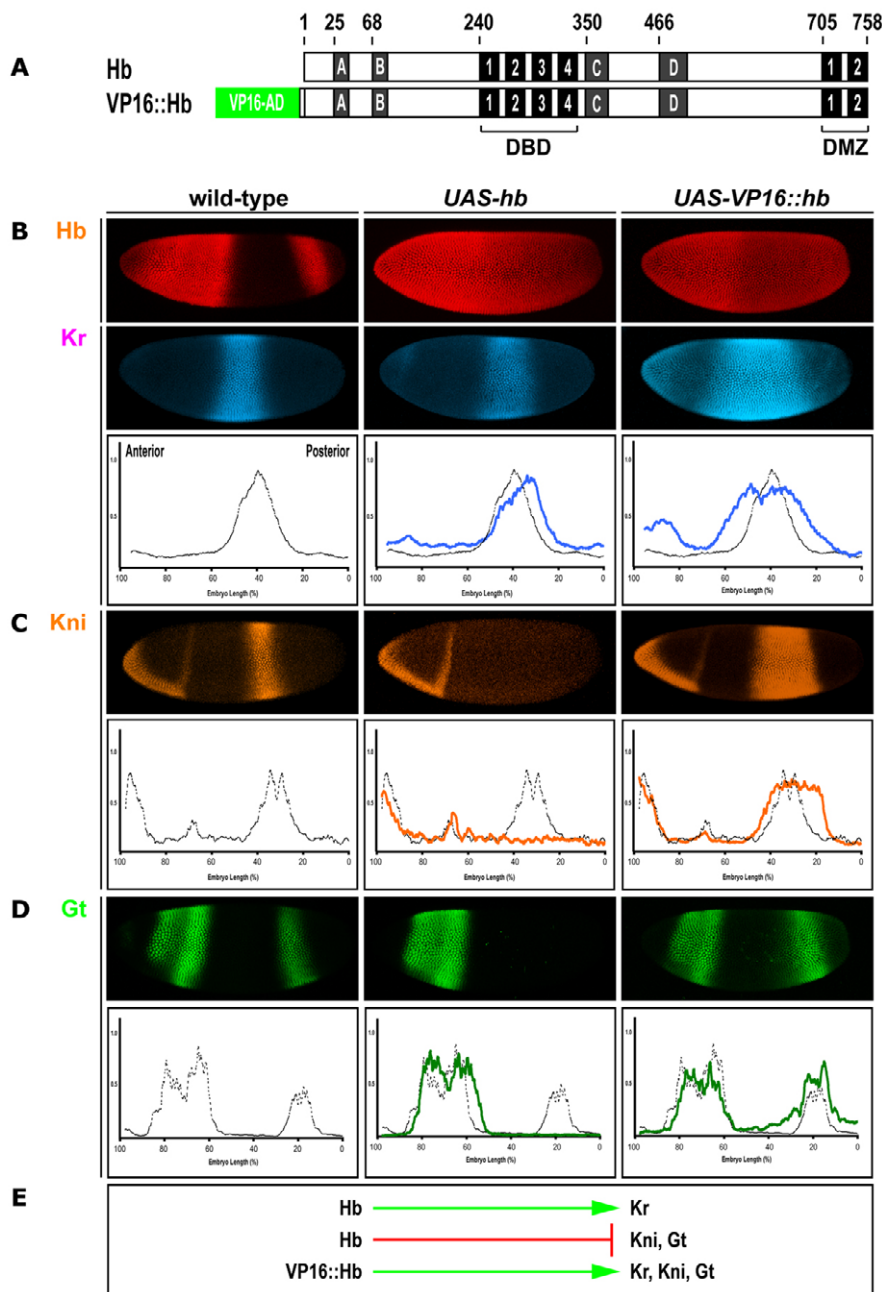
Confocal image stacks were collected using a Leica SP2 or Bio-Rad Radiance 2000 confocal microscope and displayed as two-dimensional projections. Images of histochemical preparations were acquired using a Zeiss AxioCam HRc camera on an Axioplan microscope. Two-tailed *t*-tests were used to determine significance in the differences in cell number.

## RESULTS

### VP16::Hb activates both positively and negatively regulated Hb direct target genes in the early *Drosophila* 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 transactivation 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 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), indicating that VP16::Hb is a potent activator of *Kr* gene expression.



**Fig. 1. VP16::Hb acts as a constitutive transcriptional activator. (A)** Schematic of wild-type Hb protein and the VP16::Hb protein chimera showing the previously characterized conserved domains. DBD, DNA-binding domain; DMZ, dimerization domain.

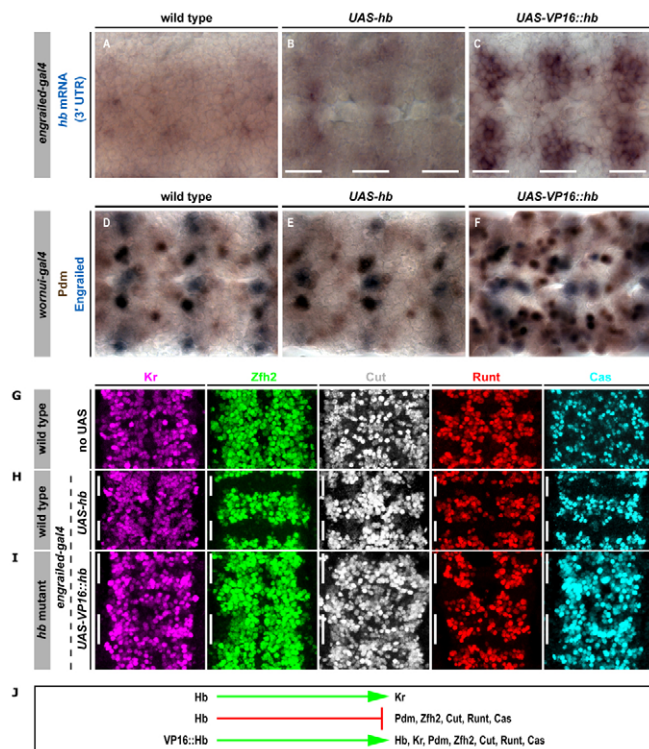
**(B-D)** Expression of gap genes in the *Drosophila* embryonic blastoderm in wild-type, *v32a-gal4 UAS-hb* and *v32a-gal4 UAS-VP16::hb* embryos. **(E)** Summary of gene interactions from B-D.

We further tested the capacity of VP16::Hb as an activator by examining its effect on genes that are normally directly repressed by Hb. Hb expression in the posterior regions of the embryonic blastoderm directly represses the gap genes *kni* and *gt* (Fig. 1C,D) (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,D). 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 targets in the CNS 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 could activate endogenous *hb* transcription via the VP16 activation domain, leading to cells that contain 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 could not induce its own transcription in neuroblasts (Fig. 2A,B),





**Fig. 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 *Drosophila* 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). Each panel shows the neuroblast layer of a stage 15 embryo with anterior to the left. (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 two-dimensional projection of approximately two segments of the ventral nerve cord of a stage 16 embryo. Lines as in B,C. Anterior is up. (J) Summary of gene interactions in the CNS from A–I.

consistent with previous findings (Grosskortenhaus et al., 2005). By contrast, *engrailed-gal4 UAS-VP16::hb* embryos showed strong activation of endogenous *hb* transcription in neuroblasts (Fig. 2C). We conclude that VP16::Hb can activate endogenous *hb* transcription in the CNS.

We next examined whether VP16::Hb can activate *pdm* (*pdm1* and *pdm2*), 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 expressed Pdm (Fig. 2D), and there was 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 upregulation 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 targets in the CNS (Fig. 2G). Hb is known to activate *Kr* and repress *zfh2*, *cut*, *runt* and *cas* (Fig. 2H) (Grosskortenhaus et al.,

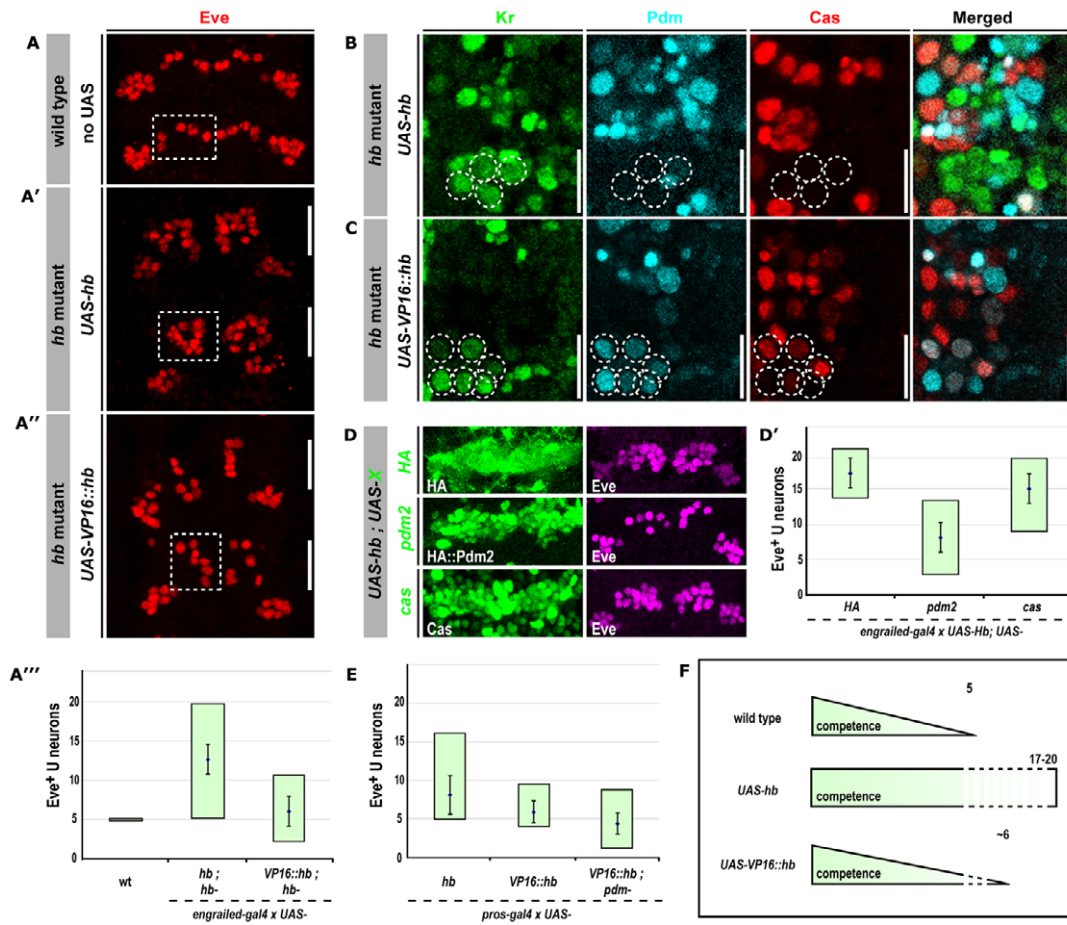
2006; Isshiki et al., 2001; Kambadur et al., 1998), although it is not known whether Hb acts directly or indirectly to regulate the expression of these genes. We overexpressed VP16::Hb in a *hb* mutant background and found that it can activate *Kr* in neurons, similar to wild-type Hb (Fig. 2I). Additionally, 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). 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 might play an essential role in maintaining neuroblast competence, a prediction that we test 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 the 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 ~18–20 *Eve*<sup>+</sup> 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*<sup>+</sup> U motoneurons (Fig. 3A,A'') (Pearson and Doe, 2003; Schmid et al., 1999). Overexpression of wild-type Hb in a *hb* mutant NB7-1 produced 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 generated 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*<sup>+</sup> U neuron production, and suggest that the repression of Hb downstream targets in neuroblasts might 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). By contrast, 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, could lead to the same reduction in ectopic cells as seen in the VP16::Hb-overexpression experiments. In control *engrailed-gal4 UAS-hb UAS-HA* embryos, NB7-1 generated ~17 *Eve*<sup>+</sup> 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 with our control embryos (average of 9, range 3–14, *n*=70; Fig. 3D). *engrailed-gal4 UAS-hb UAS-cas* embryos showed a slight decrease in the number of U neurons generated (average of 15, range 8–20, *n*=118; Fig. 3D). We conclude that Hb normally represses downstream targets, such as *pdm* and *cas*, to maintain neuroblast competence.



**Fig. 3. Hb maintains early neuroblast competence through the repression of multiple target genes. (A-A''')** Quantification of U neurons (within dashed box) specified in various genetic backgrounds. Wild type, average of 5; *engrailed-gal4 UAS-hb*, *hb* mutant, average of 12 ± 2 (s.d.), range 6-18; *engrailed-gal4 UAS-VP16::hb*, *hb* mutant, average of 6 ± 2, range 2-12.  $P \leq 0.001$  for all experiments. **(B,C)** Neuroblast expression profile at early stage 12. One hemisegment is shown. Dashed circles outline neuroblasts, and solid lines indicate the *engrailed-gal4* expression domain. Hb activates *Kr* and represses *pdm* and *cas* in neuroblasts, whereas VP16::Hb activates all three. **(D,D')** Co-misexpression of Hb + Pdm and Hb + Cas results in fewer ectopic U neurons. *UAS-hb UAS-HA* control, average of 17 ± 2, range 13-22; *UAS-hb UAS-pdm2*, average of 9 ± 2, range 3-14; *UAS-hb UAS-cas*, average of 15 ± 2, range 8-20.  $P \leq 0.001$  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*, average of 9 ± 2.5, range 5-16; *pros-gal4 UAS-VP16::hb*, average of 5.6 ± 1, range 4-9; *pros-gal4 UAS-VP16::hb*, *pdm* mutant, average of 4.3 ± 1, range 2-8.  $P \leq 0.001$  for all experiments. **(F)** Summary and comparison of the competence windows generated by Hb and VP16::Hb.

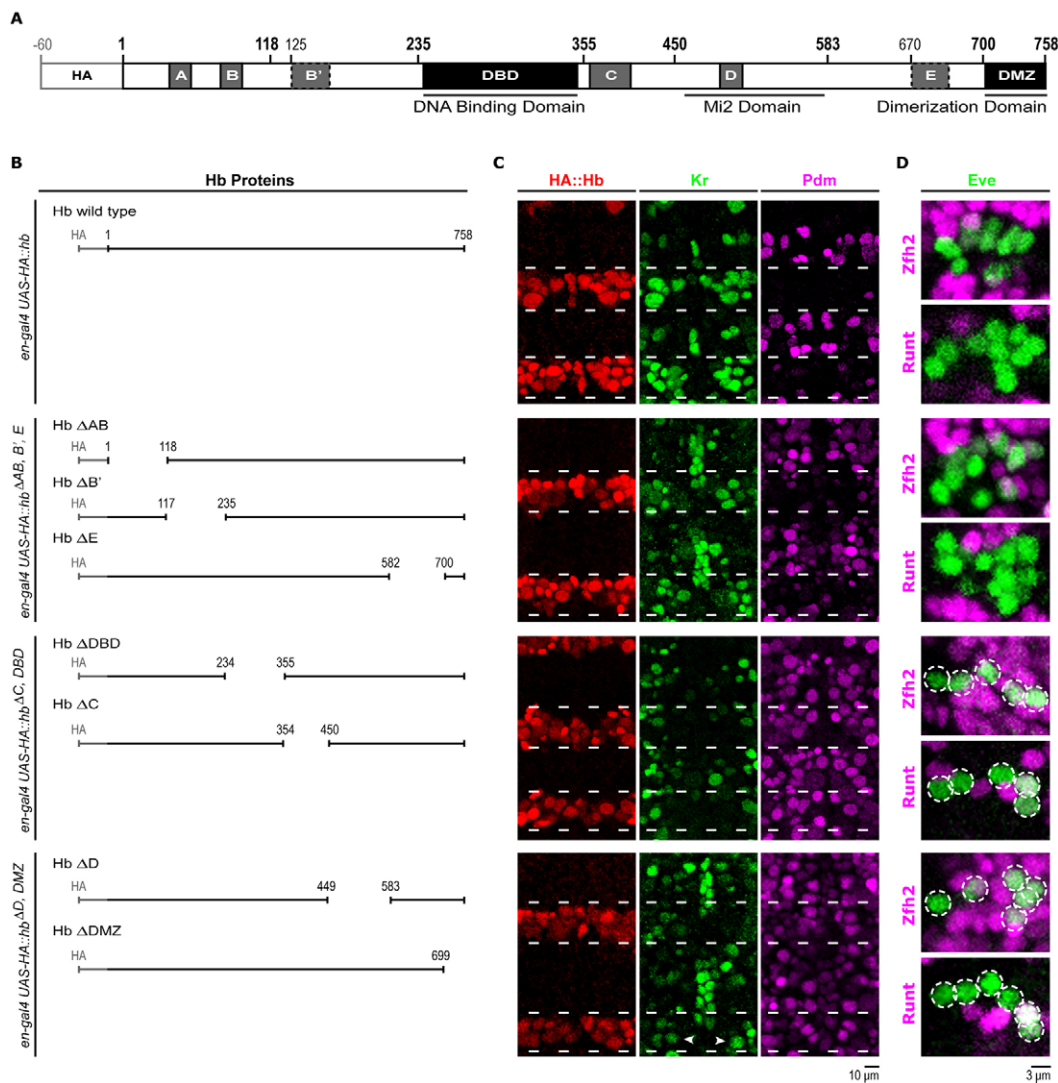
Because Pdm2 is sufficient to block Hb-induced neuroblast competence, we tested whether Pdm2 was also necessary to terminate neuroblast competence. If *pdm2* is the only factor activated by VP16::Hb that limits neuroblast competence, then the overexpression of VP16::Hb in a *pdm* mutant background should extend neuroblast competence and result in the generation of many Eve<sup>+</sup> U neurons. By contrast, if VP16::Hb activates multiple factors, then VP16::Hb might not be able to extend competence or make large numbers of U neurons even in the absence of Pdm. Control *prospero-gal4 UAS-hb* embryos generated an average of nine Eve<sup>+</sup> U neurons per hemisegment (range 5-16,  $n=100$ ; Fig. 3E). By contrast, *prospero-gal4 UAS-VP16::hb* embryos generated on average only 5.6 Eve<sup>+</sup> U neurons (range 4-9,  $n=100$ ,  $P \leq 0.001$ ; Fig. 3E), presumably owing to the transcriptional activation of factors that limit competence. Strikingly, performing the same experiments in a *pdm* mutant embryo (lacking both *pdm1* and *pdm2*) did not increase the number of Eve<sup>+</sup> U neurons (average of 4.3, range 2-8,  $n=90$ ,  $P \leq 0.001$ ; 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 for the maintenance of neuroblast competence, we next sought to identify the Hb protein domain(s) required for transcriptional repression. We generated a series of *hb* transgenes, each deleted for one or more of the eight evolutionarily 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 et al., 1987); and two additional domains (B' and E) that contain short DNA sequences conserved in at least eight sequenced *Drosophila* species (Fig. 4A,B). 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<sup>+</sup>





**Fig. 4. The Hunchback D and DMZ domains are required for transcriptional repression and maintenance of neuroblast competence.** (A) Schematic of *Drosophila* Hb protein showing conserved domains and deletion breakpoints (bold). (B) Deletions grouped by functional phenotype. (C) In each set of panels, which refer to the corresponding deletions in B, 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 $\Delta$ AB, Hb $\Delta$ B' and Hb $\Delta$ E can activate Kr and repress *pdm*; Hb $\Delta$ DBD and Hb $\Delta$ C are non-functional in the CNS; Hb $\Delta$ D and Hb $\Delta$ DMZ can activate Kr, but cannot repress *pdm*. (D) U neurons (encircled) specified by Hb and each deletion construct.

Pdm<sup>+</sup> in the neuroblast layer (Grosskortenhans 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 is required for transcriptional activation or repression. Overexpression of Hb proteins lacking the DBD or the C domain did not activate *Kr* or repress *pdm* (Fig. 4B,C), suggesting that they are non-functional, although these proteins were nuclear localized and exhibited similar stability to wild-type Hb protein in neuroblasts (Fig. 4C). By contrast, overexpression of Hb proteins lacking the D domain (which includes the Mi2 binding sites) failed to repress *pdm*, but still weakly activated *Kr*; an identical result was observed for Hb protein lacking the DMZ domain (Fig. 4B,C). The sparse and intermittent activation of *Kr* might be due to

Pdm repression of *Kr* (Grosskortenhans et al., 2006; Tran and Doe, 2008), which would be expected to counteract Hb-induced *Kr* activation.

We next tested whether the Hb $\Delta$ D and Hb $\Delta$ DMZ proteins fail to repress other known Hb target genes. Whereas wild-type Hb protein efficiently repressed *zfh2*, *cut*, *rumt* and *cas* (Fig. 3C), the Hb $\Delta$ D and Hb $\Delta$ DMZ proteins failed to repress any of these genes (see Fig. S1 in the supplementary material). 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 is not sufficient to extend neuroblast competence, suggesting that this function might require Hb-mediated transcriptional repression. We therefore tested whether the D or DMZ repression domains of Hb are required for extending

**Table 1. Summary of U neuron identity specified by Hb proteins**

Ectopic protein	Genetic background	U neuron identity <sup>*,†</sup>						
		Total <sup>‡</sup>	<i>n</i> <sup>§</sup>	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 <sup>ΔAB</sup>	wt	16	114	8	8	0	0	0
	<i>hb</i> mutant	11.8	65	5	6.8	0	0	0
Hb <sup>ΔB'</sup>	wt	16.2	102	9	7.2	0	0	0
	<i>hb</i> mutant	12.1	62	5	7.1	0	0	0
Hb <sup>ΔDBD</sup>	wt	5	185	1	1	1	1	1
	<i>hb</i> mutant	3	62	0	0	1	1	1
Hb <sup>ΔC</sup>	wt	5	180	1	1	1	1	1
	<i>hb</i> mutant	3	68	0	0	1	1	1
Hb <sup>ΔD</sup>	wt	5.5	242	1	1.25	1.25	0	2
	<i>hb</i> mutant	4	150	0	0	2	0	2
Hb <sup>ΔE</sup>	wt	16	112	8.8	7.2	0	0	0
	<i>hb</i> mutant	13.2	76	6	7.2	0	0	0
Hb <sup>ΔDMZ</sup>	wt	5.3	237	1	1.15	1.15	1	1
	<i>hb</i> mutant	3.6	184	0	0	1.6	1	1

$P \leq 0.001$  for all experiments. wt, wild type.

\*Average number of each cell present per hemisegment based on the markers described in Fig. 5.

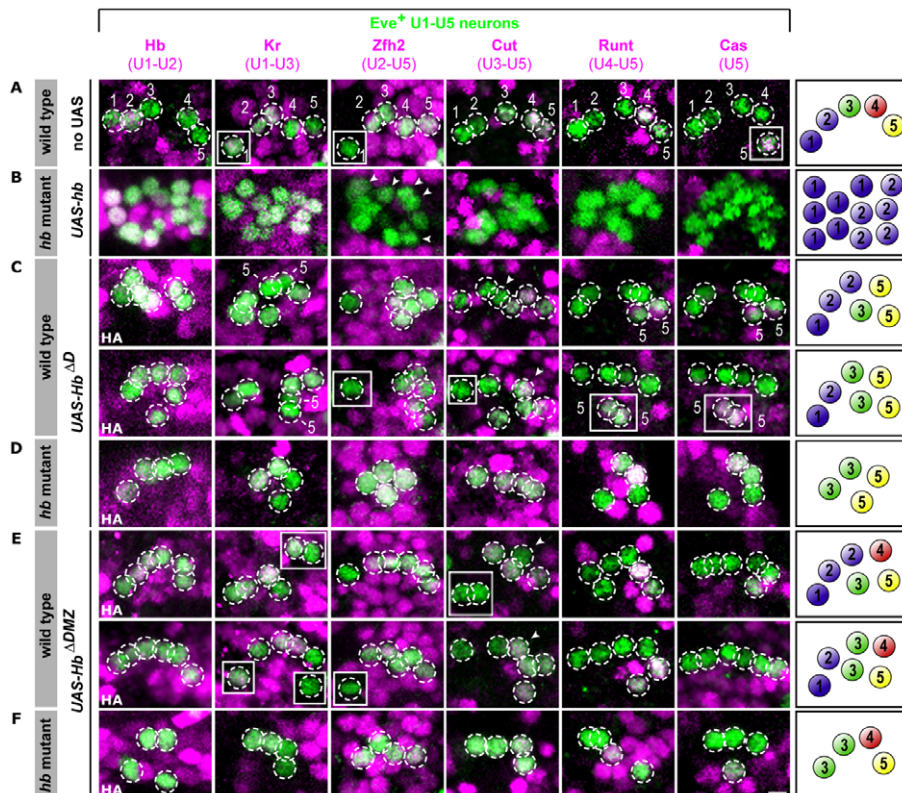
†Cell fate markers: U1, Hb<sup>+</sup> Kr<sup>+</sup> Zfh2<sup>+</sup>; U2, Hb<sup>+</sup> Kr<sup>+</sup> Zfh2<sup>+</sup>; U3, Kr<sup>+</sup> Cut<sup>+</sup>; U4, Runt<sup>+</sup> Cas<sup>-</sup>; U5, Runt<sup>+</sup> Cas<sup>+</sup>.

‡Total number of U neurons.

§Number of hemisegments analyzed.

competence. We assay neuroblast competence by measuring the number of Eve<sup>+</sup> 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 five Eve<sup>+</sup> 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 ~14 Eve<sup>+</sup> 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 ~16 Eve<sup>+</sup> 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 domain neither specified ectopic U neurons nor altered the identity of the existing neurons (Fig. 4D; see Fig. S2 in the supplementary material; Table 1). Interestingly, overexpression of the Hb proteins that lacked



**Fig. 5. The Hb D and DMZ domains are required for the first temporal identity.**

Each panel shows a two-dimensional projection of U neurons from one hemisegment of a stage 16 *Drosophila* embryo; medial is to the left and anterior is to the top. The U1-U5 neurons (as illustrated to the right) can be uniquely identified based on the indicated molecular markers. For quantification of U neuron identity, see Table 1. Scale bar: 3  $\mu$ m. (A) Wild-type embryo. (B) *engrailed-gal4 UAS-hb*, *hb* mutant embryo. Ectopic early-born U1/U2 neurons are specified. Arrowheads indicate weak Zfh2<sup>+</sup> cells. (C) *engrailed-gal4 UAS-hb<sup>ΔD</sup>* embryo. An ectopic U2 or U3 neuron is found (arrowhead) in 50% of hemisegments. All hemisegments contain two Cas<sup>+</sup> U5 neurons. (D) *engrailed-gal4 UAS-hb<sup>ΔD</sup>* in a *hb* mutant embryo. Most hemisegments contain an ectopic U3 neuron but no U1 or U2 neurons are specified. (E) *engrailed-gal4 UAS-hb<sup>ΔDMZ</sup>* embryo. An ectopic U2 or U3 neuron (arrowheads) is found in 25% of hemisegments. All other U neurons differentiate as in wild type. (F) *engrailed-gal4 UAS-hb<sup>ΔDMZ</sup>* in a *hb* mutant embryo. Most hemisegments contain an ectopic U3 neuron and no U1 or U2 neurons are specified.

transcriptional repressor activity, i.e. those lacking the D or DMZ domain, failed to extend neuroblast competence, generating only five or six *Eve*<sup>+</sup> U neurons ( $n > 200$ ; Fig. 4D; Table 1). The identity of the *Eve*<sup>+</sup> U neurons is addressed below, but based simply on the change in the number of *Eve*<sup>+</sup> 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 or *hb* mutant embryos. Wild-type embryos have five U neurons per hemisegment: the first-born U1 neuron is *Hb*<sup>+</sup> *Kr*<sup>+</sup>, the second-born U2 neuron is *Hb*<sup>+</sup> *Kr*<sup>+</sup> *Zfh2*<sup>+</sup>, and the later-born neurons are *Zfh2*<sup>+</sup> *Kr*<sup>+</sup> *Cut*<sup>+</sup> (U3), *Zfh2*<sup>+</sup> *Cut*<sup>+</sup> *Runt*<sup>+</sup> (U4), or *Zfh2*<sup>+</sup> *Cut*<sup>+</sup> *Runt*<sup>+</sup> *Cas*<sup>+</sup> (U5) (Fig. 5A).

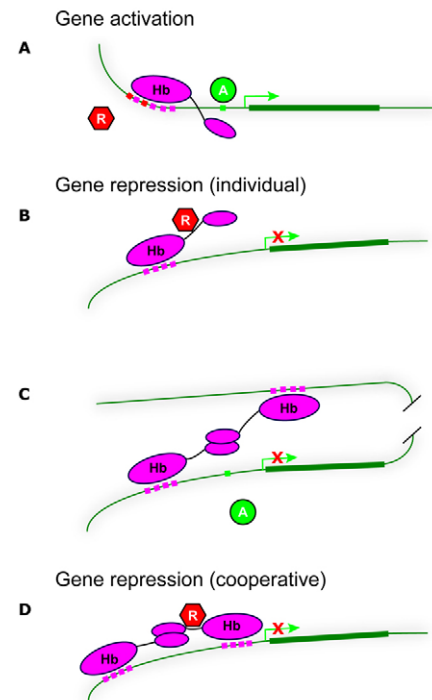
Overexpression of wild-type Hb generated ~14 early-born U1/U2 neurons when endogenous Hb was present (Table 1), and ~12 U1/U2 neurons in a *hb* mutant background (Fig. 5B; Table 1), consistent with previous reports (Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003). Similarly, overexpression of Hb proteins lacking either the A+B domains, the B' domain, or the E domain also generated ~12 *Eve*<sup>+</sup> 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*<sup>ΔD</sup> and *Hb*<sup>ΔDMZ</sup>) generated five to six *Eve*<sup>+</sup> U neurons when endogenous Hb was present (Fig. 5C,E; Table 1). The ectopic neuron was typically the *Kr*<sup>+</sup> U3 neuron, which is consistent with the *Hb*<sup>ΔD</sup> and *Hb*<sup>ΔDMZ</sup> 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 the *Hb*<sup>ΔD</sup> and *Hb*<sup>ΔDMZ</sup> proteins in a *hb* mutant background resulted in the 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*<sup>ΔD</sup> and *Hb*<sup>ΔDMZ</sup> proteins is that *Hb*<sup>ΔD</sup>, but not *Hb*<sup>ΔDMZ</sup>, frequently generated an ectopic U5 neuron (Fig. 5C,D; Table 1; see Discussion). Because both *Hb*<sup>ΔD</sup> and *Hb*<sup>ΔDMZ</sup> 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 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



**Fig. 6. Models for Hb-mediated transcriptional regulation of neuroblast competence in *Drosophila*.**

Proposed molecular interactions underlying Hb function in the CNS. (A) Hb binds to its consensus sequence and recruits co-activators (and/or outcompetes repressors) to promote gene expression. (B) Hb monomers bind to genomic DNA and recruit repressor complexes. (C) Hb monomer binds to the regulatory region of a target gene, which is then repressed by dimerization with a second Hb monomer bound within a heterochromatin domain. (D) Hb dimerization is required for the recruitment of repressor complexes, which might include Mi2. Magenta ovals, Hb; smaller ovals, Hb dimerization domain; magenta squares, Hb binding sites; 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.

dimerization and thus transcriptional repression (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 the D and DMZ domains could act in distinct processes that are both required for transcriptional repression (Fig. 6B,C), or that 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 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*<sup>ΔD</sup> leads to the specification of two U5 neurons at the expense of



the U4 cell identity, whereas overexpression of Hb<sup>ADMZ</sup> results in normal U4 and U5 identities (Fig. 5). Perhaps Hb<sup>ADMZ</sup> retains some ability to repress *cas* expression, allowing the production of the Cas<sup>-</sup> U4 identity. Alternatively, Hb might use the D and DMZ domains to repress different target genes. Currently, we cannot distinguish between these models owing to the limited number of known Hb direct target genes.

Both Hb and the related mammalian protein Iκ 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 systematic deletion series covered the entire protein (see Fig. 4). We can rule out the possibility that the activation domain maps to the D region, as it does in the closely related Iκ protein (Sun et al., 1996), because the Hb<sup>AD</sup> protein has no effect on *Kr* transcriptional activation or the specification of U3 neuronal identity (Fig. 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: (1) Hb activates transcription indirectly by blocking DNA binding of a repressor (Fig. 6A); (2) Hb has multiple activation domains; or (3) the Hb activation domain is tightly linked to an essential domain, such as the DBD. In any case, our VP16::Hb 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 that are involved in extending neuroblast competence? One negatively regulated target is *pdm*, as co-expression of Pdm with wild-type Hb failed 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 function in the CNS will require 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. Such comparative analyses might help to elucidate the complex gene interactions involved in regulating neuroblast competence.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

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