

Developmentally Regulated Subnuclear Genome Reorganization Restricts Neural Progenitor Competence in *Drosophila*

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SUMMARY

Stem and/or progenitor cells often generate distinct cell types in a stereotyped birth order and over time lose competence to specify earlier-born fates by unknown mechanisms. In *Drosophila*, the Hunchback transcription factor acts in neural progenitors (neuroblasts) to specify early-born neurons, in part by indirectly inducing the neuronal transcription of its target genes, including the *hunchback* gene. We used in vivo immuno-DNA FISH and found that the *hunchback* gene moves to the neuroblast nuclear periphery, a repressive subnuclear compartment, precisely when competence to specify early-born fate is lost and several hours and cell divisions after termination of its transcription. *hunchback* movement to the lamina correlated with downregulation of the neuroblast nuclear protein, Distal antenna (Dan). Either prolonging Dan expression or disrupting lamina interfered with *hunchback* repositioning and extended neuroblast competence. We propose that neuroblasts undergo a developmentally regulated subnuclear genome reorganization to permanently silence Hunchback target genes that results in loss of progenitor competence.

INTRODUCTION

Stem and progenitor cells typically produce different cell types in a stereotyped birth order during development. Over time, progenitors can lose competence to generate earlier-born cell fates. How stem and/or progenitor competence is lost, and how it can be experimentally regained, is a fundamental question that has important implications for understanding development and for using stem cells therapeutically. Progressive loss of competence has been well characterized in mammalian and insect neural progenitors, in which single progenitors make an ordered series of distinct neural progeny (reviewed in Cepko et al., 1996; Kwan et al., 2012; Pearson and Doe, 2004; Sousa-Nunes et al., 2010). For example, mammalian neural progenitors have an early competence window to generate neuronal subtypes and a later competence window to generate glia

(reviewed in McConnell, 1989; Okano and Temple, 2009). Yet virtually nothing is known about how competence is regulated in any mammalian or insect progenitor cell lineage.

Drosophila embryonic neural progenitors (neuroblasts) provide an excellent model system for investigating progenitor competence. Neuroblasts sequentially express the transcription factors Hunchback (Hb) > Krüppel (Kr) > Pdm1/2 > Castor > Grainyhead and generate specific neuronal or glial cell types during each window of gene expression (Baumgardt et al., 2009; Benito-Sipos et al., 2011; Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998; Kao et al., 2012; Karlsson et al., 2010; Mettler et al., 2006; Touma et al., 2012; Tran and Doe, 2008) (Figure 1A). The best characterized of these factors is Hb, which is a zinc finger transcription factor homolog of mammalian Ikaros (Elliott et al., 2008; Kim et al., 1999). *hb* transcription is activated by an unknown mechanism in newly formed neuroblasts and is repressed one to two divisions (~1 hr) later by the repressor Seven-up (Svp) (Kanai et al., 2005; Kohwi et al., 2011; Mettler et al., 2006) (Figure 1A). Neurons born from the Hb-expressing neuroblasts maintain *hb* transcription via a neuron-specific *cis*-regulatory element (Hirono et al., 2012).

Hb is necessary and sufficient to specify early-born neuronal identity in multiple neuroblast lineages (Cleary and Doe, 2006; Isshiki et al., 2001; Kanai et al., 2005; Novotny et al., 2002; Tran and Doe, 2008). This has been best studied in neuroblast 7-1 (NB7-1). NB7-1 produces the Even-skipped (Eve)-positive U1-U5 motoneurons during its first five cell divisions and then ~20 Eve-negative interneurons during its subsequent divisions (Figure 1A) (Bossing et al., 1996; Pearson and Doe, 2003; Schmid et al., 1999). *hb* mutants lack early-born U1/U2 neurons, and prolonged *hb* expression in NB7-1 can generate ectopic U1/U2 neurons (Isshiki et al., 2001; Novotny et al., 2002). Importantly, NB7-1 loses competence to make U1/U2 neurons in response to ectopic Hb after its fifth division (Cleary and Doe, 2006; Pearson and Doe, 2003). We refer to this five-division interval as the neuroblast “early competence window.” Furthermore, ectopic Hb is unable to induce U1/U2 identity when misexpressed specifically in postmitotic neurons (Pearson and Doe, 2003). Thus, Hb acts in the neuroblast to specify early-born U1/U2 neuronal identity, and NB7-1 loses competence to respond to Hb after its fifth cell division.

Here we investigate the molecular mechanism by which neuroblast competence to specify Hb+ early-born neuronal identity is lost over time. We find that the *hb* genomic locus moves from

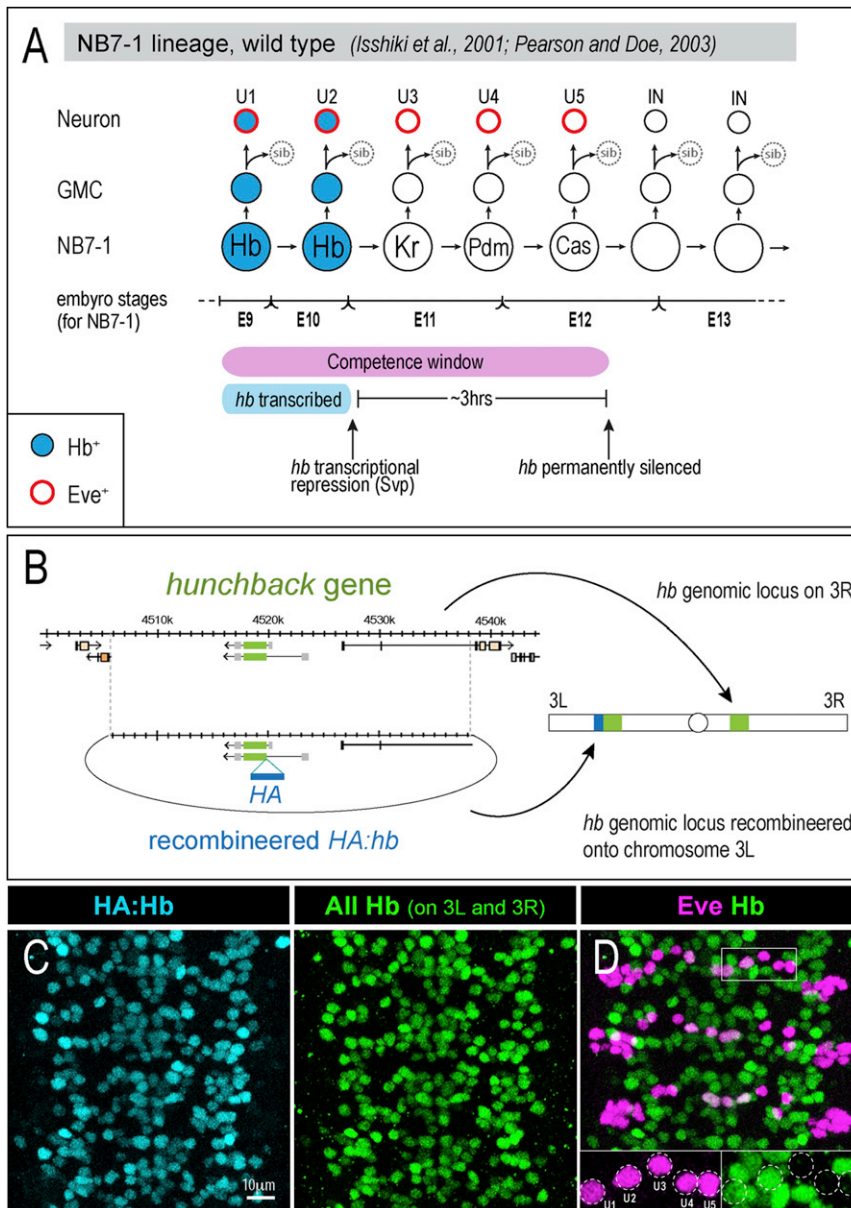


Figure 1. Temporal Identity within the NB7-1 Lineage and Defining the Early Competence Window

(A) Wild-type NB7-1 cell lineage. Hb expression is shown in blue; subsequent temporal identity gene expression is indicated for NB7-1 but not shown in the progeny. *hb* transcription is terminated after the neuroblast second division by the transcriptional repressor Seven-up (Svp). The *hb* gene becomes permanently silenced after the neuroblast fifth division (Pearson and Doe, 2003). Embryonic stage transitions (bottom line) refer to NB7-1 only. (B) We used recombining (Venken et al., 2006) to make a 32 kb genomic construct with an HA-epitope in frame with *hb* coding sequences. The Φ C31 system (Bischof et al., 2007; Groth et al., 2004) was used for site-specific integration into an *attP* site on the left arm of chromosome 3. (C) Immunostaining for HA (blue, to detect the HA:Hb protein) shows the same pattern as staining for both HA:Hb and endogenous Hb (green). (D) Embryos containing both the endogenous *hb* gene and the recombined *HA:hb* gene have wild-type numbers of Eve⁺ Hb⁺ U1/U2 neurons (see insets).

window. We further show that the Pip-squeak domain protein, Distal antenna (Dan), is downregulated in neuroblasts concurrent with *hb* movement to the nuclear periphery; prolonging Dan expression in neuroblasts prevents *hb* gene repositioning to the lamina and extends neuroblast competence.

RESULTS

Recombined HA-Tagged *hb* Mimics Endogenous *hb* Expression and Is a Specific Marker for U1/U2 Neuronal Identity

Previously, heat shock-induced pulses of ectopic Hb expression were used to reveal that NB7-1 is competent to specify Hb+ U1/U2 neurons during its first five divisions, referred to as the “early competence window” (pink bar, Figure 1A; Pearson and Doe, 2003). In contrast, using the Gal4/UAS system to continuously express ectopic Hb in NB7-1 (*engal4 UAS-hb*) resulted in the generation of over 20 extra neurons with U1/U2-like identity (Isshiki et al., 2001; Pearson and Doe, 2003). This result suggested that Hb could extend the neuroblast early competence window. However, the extra U1/U2-like neurons were only assayed in the embryo, when some Gal4/UAS-induced Hb protein may still have been present. Therefore, it is unknown whether the ectopic U1/U2 neurons are able to express endogenously transcribed *hb*, a key molecular characteristic of early-born identity, or whether they are able to maintain Hb expression into larval stages after ectopic Hb protein has declined. Since Hb functions as both the inducer of early-born identity (in

the nuclear interior to the nuclear periphery, a transcriptionally repressive subnuclear compartment, nearly synchronously within the entire neuroblast population. Strikingly, the timing of *hb* gene repositioning to the nuclear periphery correlates with the end of the NB7-1 early competence window, which occurs three divisions (~3 hr) after *hb* transcription has ceased. Depletion of Lamin, an intermediate filament component of the nuclear envelope, reduces *hb* positioning at the nuclear periphery and extends neuroblast competence. Therefore, the *hb* gene undergoes two distinct levels of transcriptional regulation in NB7-1: *hb* transcription is terminated after the second cell division and then, after the fifth division, the *hb* locus becomes repositioned to the nuclear periphery, where it becomes permanently silenced and ends the neuroblast early competence

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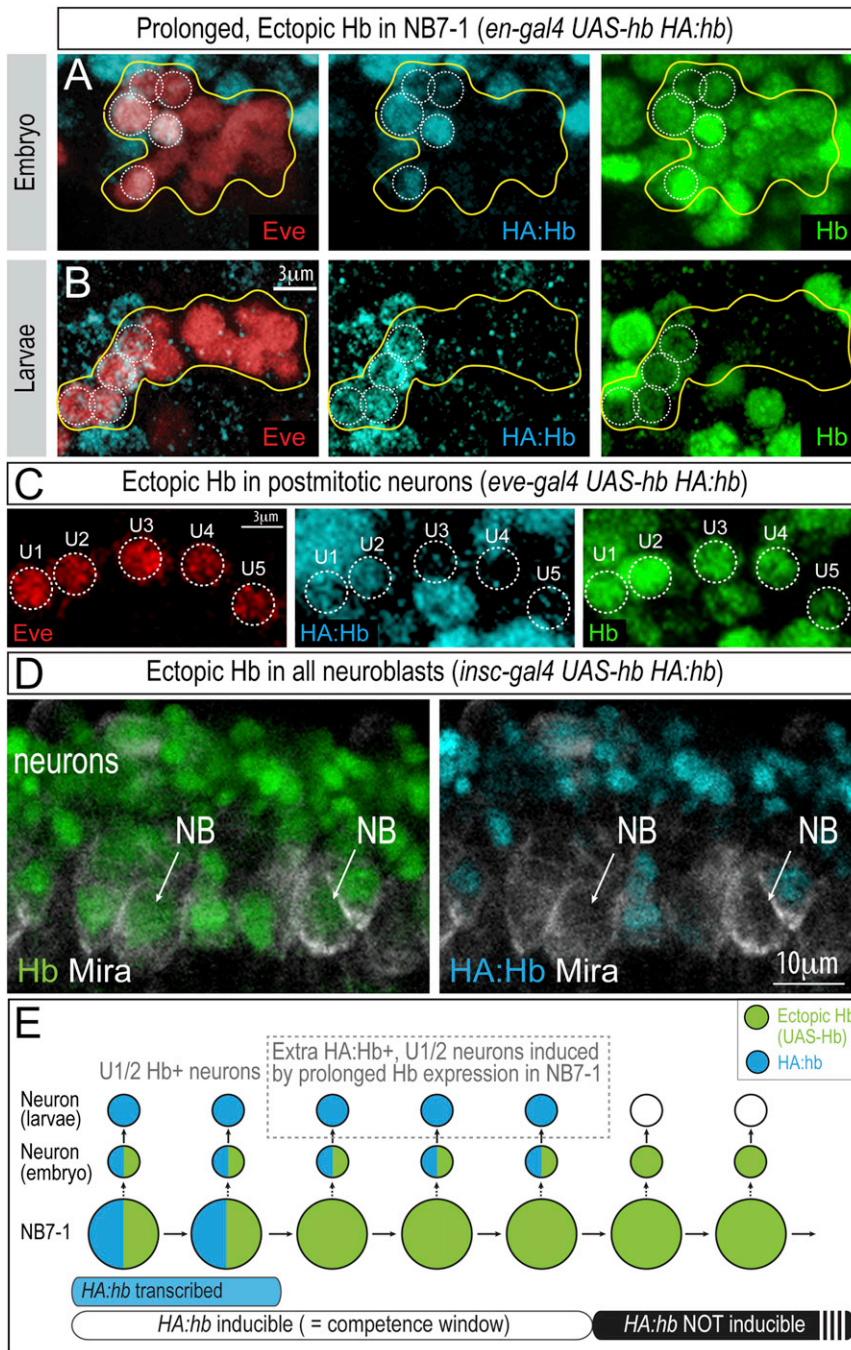


Figure 2. Hb Does Not Directly Activate *hb* Transcription but Is Required within the Neuroblast for Subsequent Expression of HA:Hb in Neuronal Progeny Born during the Competence Window

(A) Prolonged Hb expression in NB7-1 results in all Eve+ neurons being Hb+, but only ~5 Eve+ neurons are HA:Hb+. Stage 14 embryo shown is (*en-gal4 UAS-hb HA:hb*).

(B) Only ~5 Eve+ neurons are HA:Hb+ (blue) in larvae (*en-gal4 UAS-hb HA:hb*).

(C) Ectopic Hb protein in postmitotic neurons (green) does not induce HA:Hb expression (blue). Only early-born U1/U2 neurons express HA:Hb. Ventral view, stage 16 (*eve-gal4 UAS-hb HA:hb*).

(D) Ectopic Hb protein in neuroblasts (green; NBs, arrows) does not induce expression of HA:Hb expression (blue) within neuroblasts. Lateral view, stage 11 (*insc-gal4 UAS-hb HA:hb*). Also see Figure S1.

(E) Schematic summary. Blue indicates endogenous HA:Hb, whereas green indicates Hb protein generated from *UAS-hb* ectopic overexpression. Blue bar at the bottom indicates active *hb* transcription in the neuroblast; white bar indicates the NB7-1 early competence window, when ectopic Hb in the neuroblast can induce HA:Hb expression in neuronal progeny.

grated into the genome in addition to the existing endogenous *hb* gene (Figure 1D). Furthermore, *HA:hb* rescued an *hb* null mutant to viability (data not shown). Thus, immunostaining for HA in the *HA:hb* genotype can be used as a proxy for endogenous *hb* transcription and is a specific, positive marker for early-born U1/U2 neuronal identity. We can now ectopically express untagged Hb protein and use HA staining as a molecular readout for endogenous *hb* gene expression and U1/U2 neuronal identity.

U1/U2 Identity Can Be Induced by Hb Only during the Neuroblast Competence Window

Using our HA:Hb reporter, we revisited the question of whether Hb can extend the NB7-1 early competence window.

We ectopically expressed untagged Hb

protein throughout the NB7-1 lineage (*en-gal4 UAS-hb HA:hb*) and assayed for the number of U1/U2 neurons generated (Figure 2). We stained for Eve (to identify all U neurons), HA:Hb (to detect authentic U1/U2 neurons), and Hb (to detect both endogenous Hb and the ectopically expressed Hb). Consistent with our previous results, we found all Eve+ U neurons were also Hb+ in the embryo (Figure 2A, green; Pearson and Doe, 2003). Importantly, however, only 5.4 ± 0.4 neurons were HA:Hb+ and thus authentic U1/U2 neurons (Figure 2A, blue). The HA:Hb+ U1/U2 neurons were all clustered at the dorsal side of the CNS (data

the neuroblast) as well as the responder of cell fate (in the neuronal progeny), we wanted to develop a definitive molecular marker for U1/U2 neuronal identity and to assay neuronal identity in larval stages. The only known positive marker for U1/U2 identity is endogenous *hb* expression. Hence, we generated a hemagglutinin (HA)-tagged recombinered (Venken et al., 2006) genomic construct (*HA:hb*) that contains 32 kb genomic DNA from the *hb* locus (Figure 1B). Immunostaining for HA showed that HA:Hb precisely mimicked the endogenous *hb* expression pattern (Figure 1C) and did not generate extra U1/U2 neurons when inte-

not shown), indicating that they were the earliest-born neurons in the lineage (Pearson and Doe, 2003). Consistent with their bona fide U1/U2 neuronal identity, these were the only neurons that maintained endogenous Hb expression into larval stages (Figure 2B), which is well after the *en-gal4* driver turns off and ectopic Hb protein is no longer present. We conclude that Hb can specify U1/U2 neuronal identity but only during the NB7-1 five-division competence window.

Having shown that ectopic Hb can induce U1/U2 neuronal identity only during the early neuroblast competence window, we next asked whether ectopic Hb acts in neuroblasts or neurons to induce endogenous Hb expression. We observed that ectopic Hb expression specifically in postmitotic U1–U5 neurons (*eve-gal4 UAS-hb HA:hb*) was unable to induce HA:Hb expression (Figure 2C), consistent with previous observations (Pearson and Doe, 2003), suggesting that Hb must act in the neuroblast to specify U1/U2 identity. Interestingly, ectopic expression of Hb protein in neuroblasts did not directly induce HA:Hb expression within the neuroblasts themselves (Figure 2D), but rather indirectly induced subsequent HA:Hb expression in the neurons derived from the neuroblast (Figure 2A; summarized in Figure 2E). Similarly, Gal4/UAS-induced expression of Hb in neuroblasts was not able to directly activate endogenous *hb* transcription (Figure S1 available online) (Grosskortenhaus et al., 2005). We conclude that ectopic Hb protein in the neuroblast can induce expression of *HA:hb* and *hb* in its neuronal progeny, but importantly this occurs only during the early competence window. Thus, the *hb* gene transitions through three states during the NB7-1 lineage: during the first two divisions the *hb* gene is actively transcribed in the neuroblast (Figure 2E, blue bar), during the next three divisions the *hb* gene is not transcribed but can be induced in the postmitotic neurons by ectopic Hb protein (Figure 2E, white bar), and after the fifth division the *hb* gene becomes permanently silenced and is refractory to induction by ectopic Hb protein (Figure 2E, black bar).

The *hb* Gene Shows a Near-Synchronous Movement to the Nuclear Lamina within the Entire Neuroblast Population

The above data led us to hypothesize that the *hb* locus must undergo a transition within the neuroblast after the fifth division that renders it nonresponsive to Hb protein, thus ending the neuroblast early competence window. A strength of the *Drosophila* neuroblast system is that one can image neuroblasts in vivo as they progress through their cell lineages. Therefore, we tracked the subnuclear position of the *hb* genomic locus within the population of wild-type neuroblasts at different developmental stages in vivo. We used immunostaining to detect neuroblasts and DNA fluorescence in situ hybridization (DNA FISH; Bantignies et al., 2011) to detect the *hb* genomic locus within these cells in intact embryos. Our goal was to determine whether the *hb* gene becomes repositioned into a repressive subnuclear domain—e.g., DAPI-intense foci or the nuclear lamina (Zhao et al., 2009)—concurrent with loss of neuroblast competence.

We generated a fluorescently labeled DNA probe spanning 10 kb surrounding the *hb* coding region (this sequence is also present in the 32 kb *HA:hb* genomic transgene). After probe hybridization, we immunostained embryos with antibodies to

the panneuroblast marker Worniu and the nuclear envelope protein Lamin (Figures 3A–3C) or stained with DAPI (data not shown). We could detect up to two *hb* gene foci per neuroblast nucleus at all stages of development examined (average 1.35; $n = 926$ foci). Duplication of the *hb* locus (*HA:hb* on chromosome 3L, endogenous *hb* on chromosome 3R) increased the number to up to four foci per neuroblast (average 2.12; $n = 823$ foci), confirming the specificity of the DNA FISH probe. We did not observe an obvious relationship between *hb* gene position and DAPI-intense foci at any embryonic stage. In contrast, we observed a striking movement of the *hb* gene from the nuclear interior to the nuclear lamina nearly simultaneously within the entire neuroblast population at stage 12 (blue bar, Figure 3D). Quantifying the position of the *hb* gene at various distances from the nuclear lamina at progressively later embryonic stages (9–15) revealed a gradual movement from the nuclear interior toward the nuclear lamina between stages 9–11 (white bar, Figure 3D). We conclude that *hb* gradually moves closer to the nuclear periphery as the neuroblasts age, with a final, near-synchronous localization to the nuclear envelope at stage 12.

We performed several additional experiments to better understand *hb* subnuclear repositioning in neuroblasts. Our *hb* FISH probe detected both the endogenous *hb* gene and the *HA:hb* 32 kb recombiner gene, and in embryos containing both of these genes, we observed the same repositioning of *hb* gene FISH signals to the nuclear periphery at stage 12 (Figure 3E); thus, the subnuclear positioning of *hb* and *HA:hb* are similarly regulated. This result shows that the *cis*-regulatory DNA sequence for repositioning to the nuclear lamina must lie within the 32 kb span of the *HA:hb* genomic construct, and such a lamina-targeting mechanism can act on distinct chromosomal regions (*hb* on chromosome 3R; *HA:hb* on chromosome 3L). In contrast, the *worniu* and *muscleblind* genes (continuously or never expressed in all neuroblasts, respectively) showed no change in neuroblast subnuclear position over the same interval (Figure 3F). Similarly, the *hb* gene did not undergo repositioning in epithelial cells, which do not express *hb* (Figure 3G). These results show that gene repositioning is gene specific (*hb* and *HA:hb*) and cell type specific (neuroblasts). We never detected more than ~70% of any loci positioned to within 0.4 μm of the lamina (Figures 3D–3G); this may be the maximum percentage possible due to the dynamics of chromosome movements within the nucleus (Chubb et al., 2002; Heun et al., 2001; Marshall et al., 1997; Peric-Hupkes and van Steensel, 2010). We conclude that *hb* and *HA:hb* genes show a developmentally coordinated repositioning to the nuclear periphery within the entire neuroblast population at stage 12. The timing of this event was unexpected, because gene targeting to the lamina is typically associated with transcriptional repression (Guelen et al., 2008; Meister et al., 2010; Peric-Hupkes et al., 2010; Pickersgill et al., 2006; Shevelyov et al., 2009; Towbin et al., 2010; Williams et al., 2006), and termination of *hb* transcription occurs much earlier at stage 10 (see Figure 1A).

Repositioning of the *hb* Locus to the Nuclear Lamina Correlates Tightly with the End of the NB7-1 Competence Window

To determine how closely the timing of *hb* gene repositioning to the nuclear lamina correlates with the end of the neuroblast early

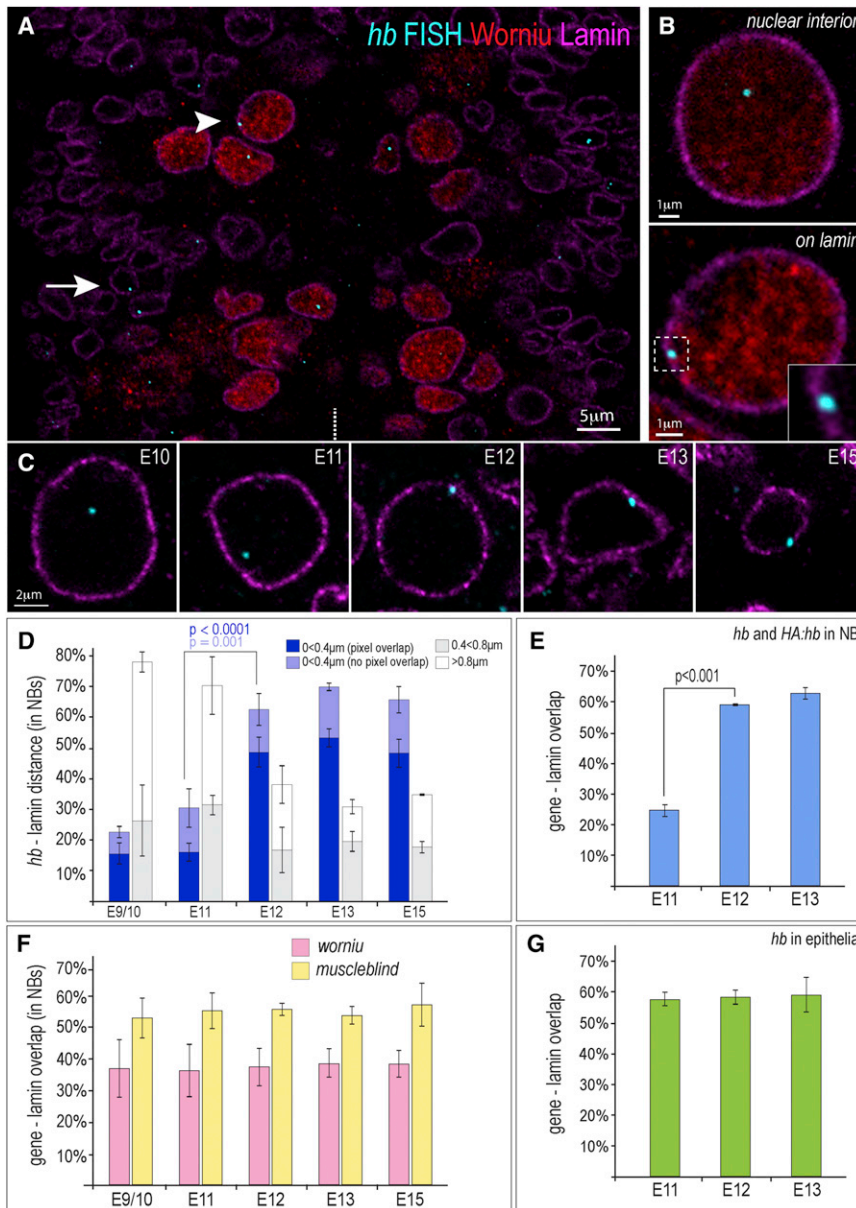


Figure 3. Coordinated Movement of the *hb* Genomic Locus in the Neuroblast Population to the Nuclear Lamina at Stage 12

(A) A single optical plane of a stage 12 embryo (ventral view) hybridized with DNA probe against *hb* genomic locus (blue) and immunostained for the Worniu neuroblast marker (red) and Lamin nuclear envelope marker (magenta). Arrowhead, neuroblast; arrow, epithelial cell; white dotted line, midline.

(B) Representative high-magnification images of a single optical plane through a neuroblast showing the *hb* gene positioned in the neuroblast nuclear interior (top) or at the nuclear periphery (bottom). Inset shows the enlarged region indicated by white dotted box.

(C) Representative images of single neuroblasts indicating position of the *hb* gene (blue) within the nucleus at all analyzed stages (Worniu not shown).

(D) Percentage of *hb* loci positioned at the indicated distances from nuclear Lamin within neuroblasts at the indicated embryonic stages. E9/10 = 5 embryos (225 *hb* alleles), E11 = 3(246), E12 = 5(395), E13 = 3(285), and E15 = 3(227). Dark blue bar indicates pixel overlap between the *hb* FISH signal and the lamin signal (p value < 0.0001 for stages 11 and 12). Light blue indicates the *hb* FISH signal was detected within 0.4 μm of lamin, but no pixels overlapped (p value = 0.001 for all signals detected < 0.4 μm from lamin for stages 11 and 12).

(E) Percentage of *hb* loci with Lamin-FISH pixel overlap in *HA:hb* embryos. Both *hb* and *HA:hb* genes are detected with the FISH probe, yet a similar shift of the loci to the nuclear periphery is observed as that of *hb* alone (see D), indicating that *HA:hb* locus subnuclear position is regulated the same as the *hb* locus. Numbers scored: E11 = 3 embryos (277 alleles), E12 = 3(274), and E13 = 3(272).

(F) Percentage *worniu* (pink) or *muscleblind* (yellow) loci with overlapping pixels with the lamin signal. Numbers scored for *worniu*, *muscleblind*, respectively, are the following: E9/10 = 5 embryos (259 *worniu* alleles), 3 embryos (145 *muscleblind* alleles); E11 = 4(155), 4(168); E12 = 5(272), 3(129); E13 = 4(215), 3(156); and E15 = 4(143), 3(135).

(G) Percentage of *hb* alleles with overlapping pixels with the lamin signal in epithelial cells (green). Numbers scored are the following: E11 = 3(210), E12 = 3(232), E13 = 3(221). Error bars represent SD.

competence window, we focused on NB7-1, for which the length of the competence window has been well defined (Cleary and Doe, 2006; Pearson and Doe, 2003). One major advantage of the *Drosophila* neuroblast model system is that neuroblasts can be individually identified by their stereotyped position within the neuroblast array at early embryonic stages (Doe, 1992). This allowed us to track the position of the *hb* genomic locus not just among the neuroblast population, but also at the resolution of a single, identified neuroblast. Neuroblast positions become less stereotyped after stage 11, however, making it difficult to identify NB7-1 when its competence window ends at late stage 12 (Pearson and Doe, 2003). Therefore, we used the split-gal4

system (Luan et al., 2006) to generate a tool for marking NB7-1 throughout neurogenesis. Briefly, we used recombineering to make a transgene that expresses the Gal4 DNA-binding domain under the control of a *gooseberry* enhancer and a second transgene that expresses the Gal4 VP16 transactivation domain under the control of an *achaete* enhancer (Figure 4A). *gooseberry* and *achaete* are expressed in distinct subsets of neuroblasts, but only NB7-1 expresses both genes. Thus, combining the two transgenes resulted in the formation of a functional Gal4 protein in NB7-1 (Figures 4A–4C). Using the NB7-1 split-gal4 line, we were able to label and unequivocally identify NB7-1 at all embryonic stages. Combined with immuno-DNA FISH, we

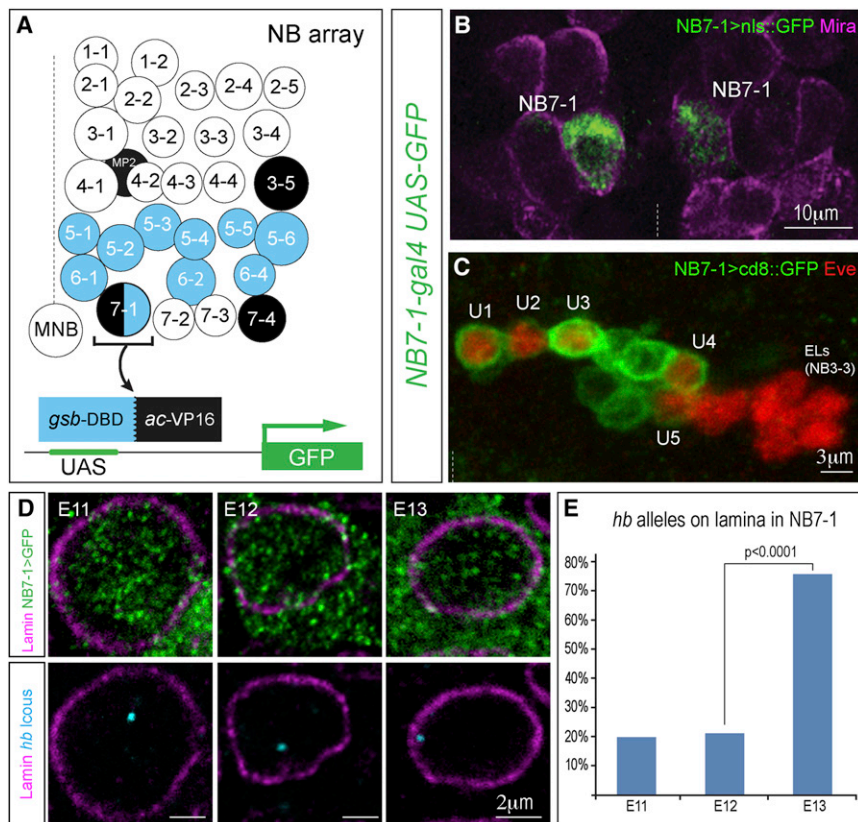


Figure 4. Movement of *hb* to the Nuclear Lamina Is Concurrent with Loss of Competence in NB7-1

(A) Schematic of all neuroblasts present in each hemisegment, with *gooseberry* (blue) or *achaete* (black) expression indicated; only NB7-1 expresses both. Infrequently, NB6-1 is also labeled, but these can be identified by position and were excluded from the *hb* FISH quantifications.

(B) The NB7-1 split-gal4 driver was used to drive nuclear-localized GFP expression (green) and costained for the neuroblast marker Miranda (magenta) to show expression in the bilateral NB7-1 at stage 11.

(C) The split-gal4 driver marks the neuronal progeny of NB7-1 in one hemisegment at stage 16. All five U motoneurons born from NB7-1 marked by Eve expression (red) are labeled by *cd8::GFP* (green), but not the Eve+ EL (Eve Lateral) neurons derived from NB3-3.

(D) NB7-1 split-gal4 driver was used to drive GFP expression (green) to identify NB7-1 at different stages. Note that GFP protein fills the cytoplasm and can be detected outside of the nuclear membrane border (magenta). DNA FISH was used to detect *hb* subnuclear gene position (blue) in NB7-1.

(E) Percentage *hb* loci with overlapping pixels with the lamin signal in NB7-1. Hemisegments scored (number of embryos examined): E11 = 45(9), E12 = 42(8), and E13 = 53(8). Increased *hb* positioning at the nuclear periphery is observed by stage 13, precisely when NB7-1 is no longer competent to specify early-born fate. p value is from chi-square test. Midline is denoted in (B) and (C) with dashed line.

were able to track the position of the *hb* gene specifically in NB7-1 throughout neurogenesis. We found that in NB7-1, the *hb* gene was initially localized to the nuclear interior but became repositioned to the nuclear periphery by stage 13 (Figures 4D and 4E). This is slightly later than the timing of *hb* repositioning to the periphery in the entire neuroblast population (see above), but it precisely correlates with the loss of NB7-1 competence by stage 13 (Pearson and Doe, 2003).

Depletion of Lamin Decreases *hb* Positioning at the Nuclear Periphery, Reduces *hb* Silencing, and Extends Neuroblast Competence

Our data show that at the end of the neuroblast early competence window, the *hb* gene becomes repositioned to the nuclear lamina, a nuclear compartment that many recent studies have shown is a highly repressive domain (reviewed in Peric-Hupkes and van Steensel, 2010; Rajapakse and Groudine, 2011; Shevelyov and Nurminsky, 2012). To determine whether *hb* positioning at the nuclear periphery is required for permanent *hb* gene silencing and termination of the neuroblast early competence window, we used a *lamin^{sz18}* null mutation, or expression of a *lamin* RNAi transgene, to reduce Lamin levels in neuroblasts. We first assayed homozygous *lamin^{sz18}* null mutants. Due to perdurance of maternal gene products, Lamin protein levels are high in early embryos and gradually decline beginning at stage 12

(Figure S2). Whereas wild-type neuroblasts have uniform Lamin staining around the nuclear envelope at stages 12 and 13 (Figure 5A), *lamin^{sz18}* mutants at the same stages show gaps in Lamin staining at the nuclear envelope (Figure 5B, arrowheads). In these *lamin^{sz18}* mutant neuroblasts, we observed a decrease in *hb* positioning at the nuclear periphery (Figure 5B; quantified in Figure 5C). Next, we tested whether the reduction of *hb* positioning at the nuclear periphery affects *hb* silencing and/or the length of the competence window. We asked whether extra Hb+ U1/U2 neurons could be produced in response to brief ectopic Hb expression by transiently expressing a pulse of ectopic Hb protein in NB7-1 late in its competence window, in either wild-type or *lamin^{sz18}* mutant embryos. We observed very few ectopic Hb+ U1/U2 neurons in wild-type, as expected (Figure 5D, black bars). In contrast, *lamin^{sz18}* mutants given the same Hb pulse showed a significant increase in the percentage of NB7-1 lineages with ectopic Hb+ U1/U2 neurons (Figure 5D, blue bars). We conclude that lowering Lamin protein levels reduces *hb* gene silencing, thereby allowing more Hb+ U1/U2 neurons to be generated within the early competence window.

We next determined whether reduced Lamin levels could extend neuroblast competence. We used a *UAS-lamin^{RNAi}* transgene to reduce Lamin levels and probed for the length of the early competence window by continuously expressing ectopic Hb in NB7-1 and assaying the number of HA:Hb+ U1/U2

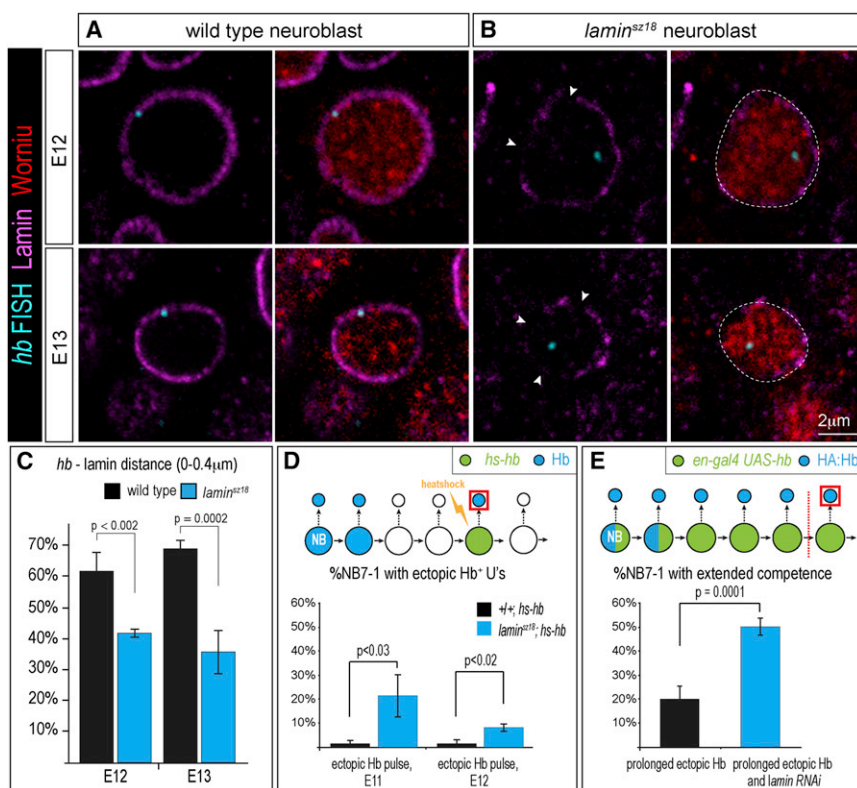


Figure 5. Decrease in Lamin Levels Reduces *hb* Silencing and Extends Neuroblast Competence

(A and B) Wild-type neuroblasts have continuous Lamin at the nuclear envelope (A), but *lamin^{sz18}* mutants have breaks in Lamin staining at the nuclear envelope (B, arrowheads). Also see Figure S2.

(C) *lamin^{sz18}* mutants show reduced *hb* positioning at the nuclear periphery. Number of embryos scored (number of hemisegments examined): wild-type, E12 = 5(395), E13 = 3(285); *lamin^{sz18}* mutants, E12 = 3(191), E13 = 5(285). Error bars represent SD.

(D) Percentage of hemisegments with extra Hb+ U1/U2 neurons at stage 16. Either wild-type (black bar) or *lamin^{sz18}* mutants (blue bar) received a transient pulse of ectopic Hb at the indicated embryonic stage (E11, E12). Number of embryos scored (number of hemisegments examined): wild-type, E11 = 5(72), E12 = 4(56); *lamin^{sz18}* mutants, E11 = 3(47), E12 = 4(72). Error bars represent SE.

(E) Percentage of hemisegments that have >5 HA:Hb+ neurons in embryos with prolonged ectopic Hb expression either with *lamin* RNAi, blue bar, $n = 11$ embryos (188 hemisegments) or without *lamin* RNAi, black bar, $n = 11$ embryos (194 hemisegments). Above each graph is a schematic of the experiment. Error bars represent SE. Also see Figure S3.

neurons generated. Prolonged expression of ectopic Hb in wild-type neuroblasts (*en-gal4 UAS-hb HA:hb*) gave >5 HA:Hb+ neurons in 20.3% \pm 5.1% (SE) of the lineages (Figure 5E, black bar); in contrast, overexpression of Hb and the *lamin* RNAi transgene (*en-gal4 UAS-hb UAS-lamin^{RNAi}*) more than doubled the number of lineages with >5 HA:Hb+ neurons to 50.3% \pm 3.6% (SE) ($p = 0.0001$; Figure 5E, blue bar). Theoretically, reduced Lamin levels could result in a derepression of Hb, but we did not observe Hb derepression in the *lamin^{sz18}* mutant neuroblasts at stage 12 (Figure S3). We conclude that *hb* positioning at the nuclear periphery is required for efficient *hb* gene silencing and the loss of neuroblast competence.

The Distal Antenna Protein Regulates *hb* Subnuclear Positioning and Neuroblast Competence

We previously showed that Dan, a Pipsqueak-motif nuclear protein, is detected in all neuroblasts from stages 9 to 11 and is downregulated nearly simultaneously in all neuroblasts during stage 12 (Kohwi et al., 2011; Figure S4), with one of the final neuroblasts to downregulate Dan being NB7-1 at late stage 12 (Figure 6A). Thus, downregulation of Dan is tightly correlated with *hb* gene movement to the nuclear lamina both within the whole neuroblast population and within NB7-1. Dan is part of a protein superfamily defined by an N-terminal Pipsqueak DNA-binding domain, which includes the CENP-B centromere protein and Pogo transposase (Siegmond and Lehmann, 2002). CENP-B and Pogo form oligomers to bring distal DNA elements together in nuclear space through their DNA-binding domains and then perform catalytic functions using their

C-terminal domains (Siegmond and Lehmann, 2002). Dan contains a single Pipsqueak domain and can form oligomers in vitro (Curtiss et al., 2007) but lacks known C-terminal catalytic domains (Siegmond and Lehmann, 2002).

The tight temporal correlation of Dan downregulation and *hb* repositioning to the lamina—plus the role of other Dan protein superfamily members in genome organization—led us to hypothesize that Dan downregulation may be required for movement of the *hb* gene to the nuclear lamina. Indeed, prolonged Dan expression in all neuroblasts (*inscutable-gal4 UAS-dan*) blocked movement of the *hb* gene to the nuclear lamina, and the proportion of *hb* loci at the nuclear periphery remained low from stage 9/10 through stage 15 (Figure 6B, pink bars). In contrast, prolonged expression of ectopic Hb in the neuroblast had no effect on the timing of *hb* gene repositioning to the nuclear lamina (Figure 6C, green bars), consistent with our conclusion that Hb specifies early-born neuronal identity but has no effect on the competence window. We conclude that Dan has the ability to regulate subnuclear gene organization in neuroblasts.

Prolonged Dan expression in neuroblasts prevented the *hb* genomic locus from repositioning to the nuclear lamina. If *hb* gene movement to the nuclear lamina terminates neuroblast competence, as we propose, then prolonged Dan expression should extend neuroblast competence. Wild-type neuroblasts have an approximately five-division competence window, and neuroblasts with prolonged ectopic Hb expression (*en-gal4 UAS-hb HA:hb*) cannot generate more than approximately five HA:Hb+ U1/U2 neurons (Figure 6D; quantified in Figure 6G).

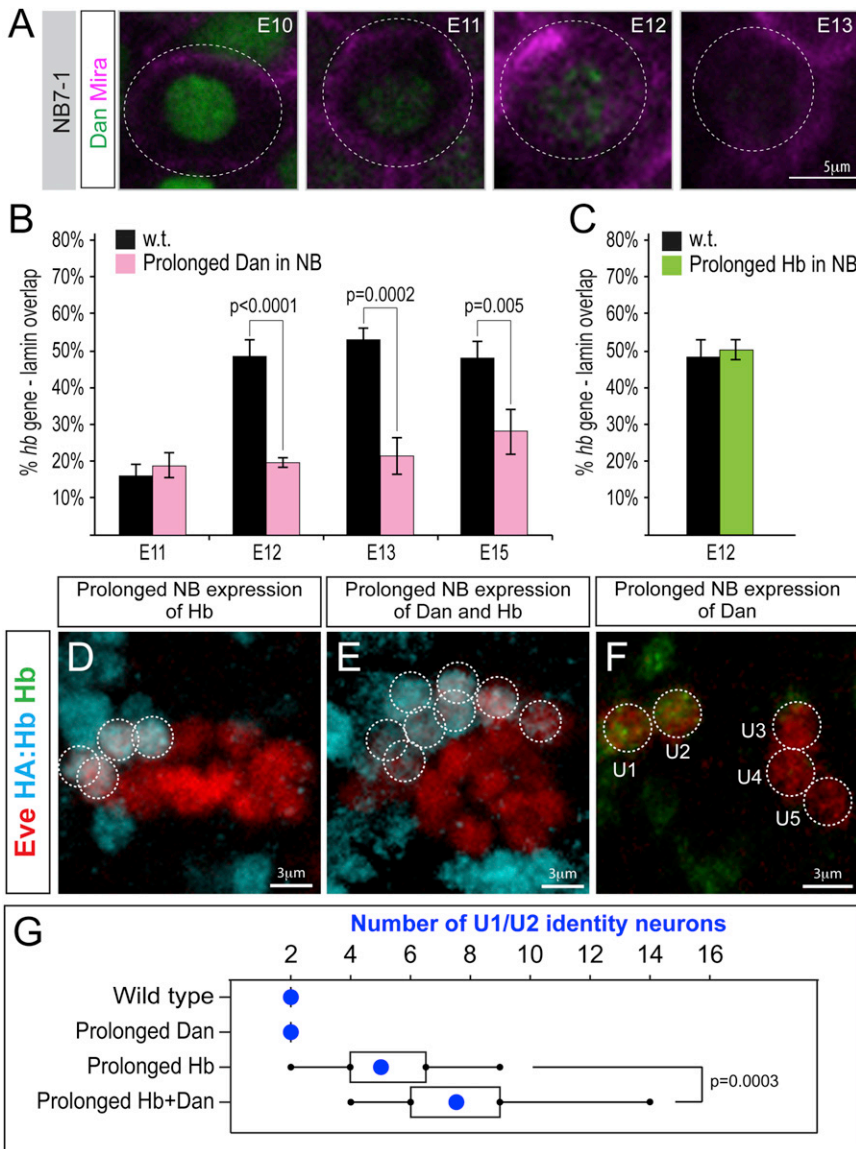


Figure 6. Prolonging Dan Expression Blocks *hb* Gene Movement to the Nuclear Lamina and Extends Neuroblast Competence

(A) In NB7-1, Dan nuclear protein (green) is detectable until late stage 12 (magenta, neuroblast marker Miranda; engrailed positional marker is not shown). Also see Figure S4.

(B) Percentage of *hb* loci with overlapping pixels with the lamin signal in wild-type (black) or neuroblasts with prolonged Dan expression (*insc-gal4/UAS-dan*; pink). Number of embryos (hemisegments) scored for embryos with prolonged Dan expression: E11 = 4 embryos (240 alleles); E12 = 4(289); E13 = 3(390); E15 = 3(311).

(C) Percentage of *hb* loci with Lamin-FISH pixel overlap at stage 12 in wild-type (black) or neuroblasts with prolonged Hb expression (*insc-gal4/UAS-hb*; green). Four embryos (112 hemisegments) scored for embryos with prolonged Hb expression.

(D–F) Embryos overexpressing Hb, Hb plus Dan, or Dan alone stained for the U neuron marker Eve (red) and HA:Hb (blue; D and E) or Hb (green; F). Also see Figure S5.

(G) Box/whisker plot showing median numbers (box) and spread (whiskers) of early-born identity neurons in (D–F). Error bars represent SD.

of Dan expression (Figure S3, bottom). We conclude that prolonged expression of Dan, but not Hb, can maintain *hb* in the nuclear interior and extend neuroblast competence.

If Dan is sufficient to extend competence and maintain *hb* in the nuclear interior, is it necessary for these events? We assayed a mutant (*dan danr*) lacking both Dan and its close relative, Dan-related (Danr), and observed no change in the length of the competence window or in the timing of *hb* subnuclear position (Figure S5), providing additional support

When we prolonged expression of Dan together with Hb (*en-gal4 UAS-hb UAS-dan HA:hb*), we observed an increase to approximately eight HA:Hb+ U1/U2 neurons (Figure 6E; quantified in Figure 6G), significantly more than with misexpression of Hb alone. Thus, prolonged expression of Dan extended NB7-1 competence to respond to ectopic Hb. In contrast, prolonged expression of Dan alone in neuroblasts (*en-gal4 UAS-dan*) did not increase the number of neurons with Hb+ early-born identity (Figure 6F; quantified in Figure 6G). This shows that prolonged expression of Dan in neuroblasts does not change the timing of *hb* transcription or directly specify U1/U2 neuronal identity. In light of these results, we note that it is theoretically possible that the reduction in *hb* gene silencing observed in our *lamin* mutant analysis could have been due to prolonged expression of Dan in the neuroblast, rather than through reduction in *hb* gene positioning at the nuclear periphery. However, *lamin*^{sz18} mutant neuroblasts showed no change in the levels or timing

for the tight relationship between *hb* subnuclear position and competence, and raising the possibility that Dan may function redundantly to regulate competence (see Discussion). Taken together, our data highlight the independent regulation of neuronal fate specification and progenitor competence: Hb potently induces early-born neuronal identity but has no effect on the competence window, whereas Dan can extend competence but cannot induce early-born cell fates.

DISCUSSION

We find that the *Drosophila* embryo undergoes a reorganization of genome architecture that is gene, cell type, and developmental stage specific. As neuroblasts age, the *hb* genomic locus becomes repositioned to the nuclear periphery, which marks the end of the neuroblast competence window to specify early-born cell fates. Why can ectopic Hb not induce early-born fates after

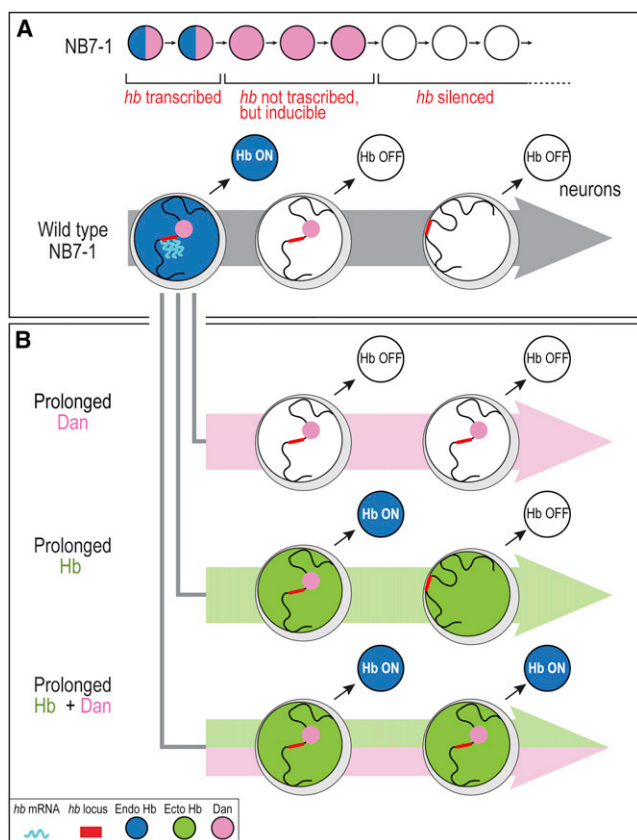


Figure 7. Neuroblast Temporal Identity and Neuroblast Competence Are Independently Regulated

(A) Neuroblast competence model.

(B) Hb and Dan independently regulate neuroblast temporal identity and competence, respectively. Note that ectopic Hb protein cannot induce endogenous Hb expression within the neuroblast. Rather, endogenous Hb is induced in the neurons born during the early competence window.

the close of the competence window? We propose that *hb* is just one of many genes that move to the nuclear lamina at the end of the early competence window—that a genome-wide reorganization shifts the neuroblasts into a state in which Hb is unable to regulate the same targets it could during the competence window. In support of this model, we have shown that misexpression of Hb in the NB5-6 lineage has no effect on the activation of late-born cell fate factors (data not shown), consistent with a new genome organization that is refractory to Hb-induced early-born neuronal identity.

Our data lead us to propose that neural progenitors undergo a developmentally regulated reorganization of genome architecture as they age, potentially changing the palette of genes available to specify cell fate in aging progenitors. We propose the following three-step model for neuroblast competence. (1) In the newly formed NB7-1, the *hb* gene is in the nuclear interior and is transcriptionally active; this is the time when early-born Hb+ U1/U2 neurons are generated (Figure 7A, left). (2) After the second division of NB7-1, the transcriptional repressor Svp terminates *hb* transcription (Kanai et al., 2005; Mettler et al., 2006); however, the *hb* gene remains accessible in the nuclear

interior where ectopic Hb protein can indirectly induce *hb* neuronal transcription to generate extra U1/U2 neurons (Figure 7A, middle). (3) After the fifth division of NB7-1, Dan protein is downregulated, resulting in the movement of *hb* (and probably many other Hb target genes) to the nuclear lamina; at this point, ectopic Hb in the neuroblast can no longer induce transcription of *hb*, and the competence window is closed (Figure 7A, right).

Our results are consistent with growing evidence from multiple organisms that repositioning of a gene to the nuclear lamina can cause transcriptional repression (Guelen et al., 2008; Peric-Hupkes and van Steensel, 2010; Pickersgill et al., 2006). For example, forced tethering of reporter genes to Lamin can repress reporter expression (Finlan et al., 2008; Meister et al., 2010; Reddy et al., 2008; Shevelyov et al., 2009; Williams et al., 2006), and Lamin depletion can derepress silent genes (Shevelyov et al., 2009; Towbin et al., 2010). An important difference in our work, however, is that *hb* movement to the lamina occurs ~3 hr after termination of *hb* transcription, when *hb* undergoes an additional level of repression to become permanently silenced. Does lamina targeting induce permanent *hb* gene silencing or vice versa? Depletion of the nuclear envelope protein Lamin displaces *hb* away from the lamina, decreases *hb* silencing, and increases neuroblast competence; this strongly suggests that lamina targeting is an early and essential step in permanent *hb* gene silencing and the loss of neuroblast competence. However, we cannot rule out the possibility that *hb* positioning at the nuclear periphery might maintain, rather than establish, the permanently silenced state.

Our results show that neuroblast cell fate specification and neuroblast competence are independently regulated temporal programs. Prolonged expression of Dan can extend the competence window but cannot induce neuronal identity (Figure 7B, top). Conversely, Hb can specify early-born neuronal identity but cannot extend the competence window (Figure 7B, middle). Importantly, coexpression of Dan and Hb act synergistically: Dan extends the neuroblast competence window, and Hb “fills” this window with U1/U2 neurons, thereby specifying more early-born identity neurons than prolonging Hb alone (Figure 7B, bottom).

The mechanism and function of Dan is poorly understood. Why might Dan be sufficient, but not necessary, to promote neuroblast competence? One common finding is that *dan* and *dan* related (*danr*) genes show weak double mutant phenotypes, but strong misexpression phenotypes, in all tissues examined. For example, *dan danr* double mutants can live to adulthood with weak antennal and eye defects (Curtiss et al., 2007; Emerald et al., 2003) and show no change in the neuroblast competence window (this work). These findings are consistent with a redundant protein or pathway that can compensate for loss of Dan/Danr. A second model arises from comparing the *dan danr* mutant and Dan overexpression phenotypes. *dan danr* double mutants have a slight delay *hb* transcription termination at stage 10 (distinct from permanent *hb* silencing at stage 12) (Kohwi et al., 2011) but no effect on *hb* gene movement to the nuclear periphery (this work). Conversely, prolonged Dan expression has no effect on *hb* transcription but blocks *hb* gene movement and extends the competence window. Thus, during the competence window, Dan may promote a genome organization that

allows timely access of the Svp transcriptional repressor to the *hb* locus, whereas after the competence window, Dan must be eliminated to allow a new genome organization to form. This model emphasizes the role of Dan in maintaining a genome organization in which *hb* and Hb target genes can be efficiently regulated. A third, not mutually exclusive, model is that Dan binds DNA via its Pipsqueak domain to competitively inhibit other Pipsqueak-like factors from recruiting *hb* and other loci to the nuclear lamina. Indeed, the founding member of the Pipsqueak-motif family, Pipsqueak, is a GAGA-binding factor (Huang et al., 2002), and recent work has shown an enrichment for GAGA motifs in Lamin-associated DNA sequences (Zullo et al., 2012). Consistent with this model, Dan protein is dispersed throughout the nucleoplasm (Figure 6A), where it could associate with *hb* and other loci.

What is the normal function of a competence window? Competence windows could provide both flexibility and limitations on the production of neural diversity. They could serve as a substrate for natural selection by allowing variation in neuronal subtype numbers through fluctuations in the length over which a progenitor is exposed to a temporal identity cue. Conversely, a competence window could also prevent stochastic fluctuations in the expression of a temporal identity cue from generating a neuronal subtype at a completely inappropriate time (e.g., an early-born fate at the end of a progenitor lineage), thereby limiting potentially deleterious effects. Another potential function of competence windows is that successive competence windows may allow the same cell fate determinant to generate different cell types. Indeed, during spinal cord development, Olig2 first promotes neurogenesis and later induces oligodendrogenesis (reviewed in Rowitch, 2004), and retinal progenitors are thought to progress through multiple competence states during which they can specify only limited cell fates (Livesey and Cepko, 2001; Okano and Temple, 2009). In support of this model, we previously showed that Dan has two waves of neuroblast expression, one at stages 9–late 12 and a second at stages 13–16 (Kohwi et al., 2011). Bimodal Dan expression may produce two competence windows in which the same temporal identity factors can access different genomic targets to generate additional neuronal diversity. For example, during the first Dan expression window, Hb, Kr, Pdm, Castor, and Svp specify U1–U5 motoneuron identities in the NB7-1 lineage (Isshiki et al., 2001; Kanai et al., 2005; Kohwi et al., 2011; Mettler et al., 2006; Novotny et al., 2002); during the second Dan expression windows, Kr, Castor, and Svp are re-expressed and a different population of neurons is produced (Benito-Sipos et al., 2011; Isshiki et al., 2001; Kanai et al., 2005; Kohwi et al., 2011).

Mammalian Ikaros, an Hb homolog, is expressed in young progenitors in which it specifies early-born retinal ganglion cell (RGC) identity (Elliott et al., 2008). As with Hb, re-expression of Ikaros in older progenitors in vivo cannot induce specification of the early-born RGCs, although Ikaros misexpression in late retinal progenitors cultured in vitro can activate RGC-specific genes (Elliott et al., 2008). Perhaps in vitro cultured progenitors are lacking an extrinsic cue that closes the competence window, allowing Ikaros to generate more early-born neurons. Interestingly, Dan downregulation could also be regulated by an extrinsic cue, because Dan downregulation occurs nearly

simultaneously in the entire neuroblast population, despite each neuroblast being at a different stage of its cell lineage.

Mammalian retinal and cortical progenitor cells change competence over time, generating an ordered series of distinct neural cells (Cepko et al., 1996; Livesey and Cepko, 2001; McConnell, 1989). In the future, it would be interesting to determine whether the genes expressed in early-born cortical or retinal cell types (Kwan et al., 2012; Leone et al., 2008; Trimarchi et al., 2007) are repositioned to the nuclear lamina in mammalian neural progenitors as competence to specify that these cells are lost over time. Determining the mechanisms underlying loss of competence and identifying the molecular players in this process would have important implications for understanding normal brain development, adult tissue homeostasis, and tissue repair.

EXPERIMENTAL PROCEDURES

Fly Stocks

We used recombineering (Venken et al., 2009) to make *HA:hb* transgenes containing three HA tags in frame with the *hb* coding sequence and ~32 kb of genomic DNA, and Φ C31-mediated integration was targeted to *attP* sites on the second or third chromosome (Bischof et al., 2007; Groth et al., 2004). We also used recombineering to make the *gooseberry-DBD*; *achaete-VP16* split-gal4 stocks, which were inserted into *attP* sites on the second and third chromosomes. We used the following previously reported fly stocks: *UAS-lamin^{RNAi}* (TRIP consortium), *engrailed-gal4* (Harrison et al., 1995; Isshiki et al., 2001; Pearson and Doe, 2003), *inscutable-gal4* (*1407-gal4*, Bloomington Stock Center), *UAS-hb* chromosomes II and III (Wimmer et al., 2000), *hsp70-hb* chromosome III (Struhl et al., 1992), *UAS-dan*, and *dan dan^{ex56}* (Emerald et al., 2003).

Immunostaining, RNA In Situ Hybridization, and Immuno-DNA FISH

Standard methods were used for antibody staining (Carney et al., 2012; Kohwi et al., 2011); larvae overexpressing Hb do not hatch and were manually devitelinized. The following primary antibodies were used: Dan rat 1:400 (gift from Jennifer Curtiss, New Mexico State University, NM, USA), Eve mouse 3C10-c 1:50 (Developmental Studies Hybridoma Bank [DHSB]), Eve guinea pig 1:500 (gift from John Reinitz, Asian Distribution Center for Segmentation Antibodies, Mishima, Japan), green fluorescent protein (GFP) chicken 1:500 (Aves Labs), HA mouse 1:500 (Roche), Lamin rabbit R836 1:2,000 (gift from Paul Fisher), Lamin mouse AD95-c 1:100 (DSHB), Hb rabbit 1:200 (Doe laboratory), Miranda rat 1:500 (Doe laboratory), and Wormiur rat 1:1 (Doe laboratory). Fluorescent secondary antibodies were from Jackson Laboratory Immunoresearch or Invitrogen. Standard methods were used for RNA in situ hybridization (Groszkortenhaus et al., 2005).

We followed the immuno-DNA FISH protocol described in Bantignies et al. (2011). We used the DNA FISH Tag kit (Invitrogen, F32948) to generate DNA probes. Primers used to generate DNA FISH probes are listed in Table S1. DNA FISH embryos were imaged on Zeiss 700 confocal with a 0.4 μ m step size. Pinholes were adjusted to equalize the resolution of all wavelengths in the x, y, and z axes. To measure gene position within the nucleus, we selected the optical section with the strongest FISH signal and measured the shortest distance from the center of the FISH signal to the nuclear lamina using ImageJ. FISH foci that were positioned within 0.4 μ m of the lamin fluorescent signal were further separately scored as either Lamin-FISH “pixel overlapping” or “no pixel overlapping.” Neuroblast FISH signals at stages 11 and 12 (the transition stages when *hb* gene movement to the lamina occurs) were initially scored by M.K., then scored blindly (with regard to stage) by a second person. The results were statistically indistinguishable.

Heat Shock Hb Overexpression

Standard methods were used for heat shock Hb experiments (Pearson and Doe, 2003; Cleary and Doe, 2006) using fly stocks with a single *hs-hb* insertion on chromosome 3. The pulse of ectopic Hb is delivered at stage 11 or 12, but

the NB7-1 progeny neurons are scored at stage 16. Some +/-; *hs-hb* and *lam-in^{sz18}*; *hs-hb* embryos were fixed and stained just prior to heat shock to ensure that the embryonic stages were synchronized between the genotypes.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2012.11.049>.

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