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Regulation of Temporal Identity Transitions in *Drosophila* Neuroblasts

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Summary

Temporal patterning is an important aspect of embryonic development, but the underlying molecular mechanisms are not well understood. *Drosophila* neuroblasts are an excellent model for studying temporal identity: they sequentially express four genes (*hunchback* → *Krüppel* → *pdm1* → *castor*) whose temporal regulation is essential for generating neuronal diversity. Here we show that *hunchback* → *Krüppel* timing is regulated transcriptionally and requires neuroblast cytokinesis, consistent with asymmetric partitioning of transcriptional regulators during neuroblast division or feedback signaling from the neuroblast progeny. Surprisingly, *Krüppel* → *pdm1* → *castor* timing occurs normally in isolated or G_2-arrested neuroblasts, and thus involves a neuroblast-intrinsic timer. Finally, we find that Hunchback potently regulates the neuroblast temporal identity timer: prolonged Hunchback expression keeps the neuroblast "young" for multiple divisions, and subsequent downregulation allows resumption of *Krüppel* → *pdm1* → *castor* expression and the normal neuroblast lineage. We conclude that two distinct "timers" regulate neuroblast gene expression: a *hunchback* → *Krüppel* timer requiring cytokinesis, and a *Krüppel* → *pdm1* → *castor* timer which is cell cycle independent.

Introduction

Embryogenesis requires both spatial and temporal patterning. Proliferating cells often make a reproducible sequence of cell types over time, either due to exposure to changing extrinsic cues or due to intrinsic changes that occur in the cell (Durand and Raff, 2000; Thummel, 2001). The question of how a cell changes over time to generate an ordered series of cell types is one of the major unanswered questions in developmental biology, and the related question of how a proliferating cell *avoids* changing over time is a key question in stem cell and cancer biology.

The specification of temporal identity—how a proliferative cell changes over time to generate distinct progeny—is particularly important for the development of the central nervous system (CNS). This is due to the vast number of cell types that need to be produced by relatively few progenitor cells during neurogenesis. In the mammalian CNS, individual neural progenitors give rise to an ordered series of cell types in the cerebral cortex, retina, and spinal cord (reviewed in Pearson and Doe, 2004). Gene expression and/or transplantation experiments show that environmental cues change over time and can regulate the type of progeny made by progenitors in the cortex (McConnell and Kaznowski, 1991) and retina (Morrow et al., 1998; Rapaport et al., 2001). However, a cell-intrinsic mechanism for generating the appropriate sequence of cell types in the cortex and retina is supported by in vitro culture experiments (Cayouette et al., 2003; Q. Shen and S. Temple, 2001, Soc. Neurosci., abstract). The relative contribution of intrinsic and extrinsic cues in regulating temporal identity remains controversial.

The *Drosophila* CNS is a good model for investigating the specification of temporal identity. The CNS develops from a bilateral array of 30 neuroblasts, each of which can be individually identified based on position, molecular markers, and its unique and invariant cell lineage (Bossing et al., 1996; Broadus et al., 1995; Schmid et al., 1999; Schmidt et al., 1997). Neuroblasts divide asymmetrically to "bud off" a series of smaller ganglion mother cells (GMCs); each typically generate a pair of neurons (Goodman and Doe, 1993). The neuroblast maintains its position at the superficial (ventral) surface of the CNS, with each new-born GMC pushing older-born GMCs and neurons deeper into the embryo. In this way a histogenic order is built, with early-born neurons located in deep layers and late-born neurons found in more superficial layers (Isshiki et al., 2001; Schmid et al., 1999). These layers can be defined molecularly by the expression of four transcription factors—Hunchback (Hb), Krüppel (Kr), Pdm1, and Castor (Cas)—from deep to superficial (Isshiki et al., 2001; Kambadur et al., 1998). The "laminar" pattern of Hb/Kr/Pdm1/Cas in the mature CNS arises by the sequential expression of each gene in neuroblasts, followed by the maintained expression in GMCs born during each window of neuroblast gene expression (Isshiki et al., 2001). For example, Hb is specifically expressed in early-born GMCs that generate neurons of the deepest layer, whereas Cas is expressed in late-born GMCs that populate a superficial layer of the mature CNS.

The precise timing of Hb → Kr → Pdm1 → Cas expression in the neuroblast is critical for proper CNS development. Extended expression of Hb in the neuroblast results in an excess of early-born neurons at the expense of later-born neurons (Isshiki et al., 2001; Novotny et al., 2001; Pearson and Doe, 2003); the same is true for Kr and the second-born fate (Isshiki et al., 2001). Moreover, inappropriate Hb expression during later portions of a neuroblast lineage can generate ectopic early-born neurons (Pearson and Doe, 2003), so it is vital to keep Hb off following its initial period of expression. Despite the importance of timing gene expression in neuroblasts, remarkably little is known about the mechanisms involved. Misexpression experiments show that Hb and Kr can activate the next gene in the series, raising the possibility of a positive transcriptional cascade; however, *hb* or *Kr* mutants have little effect on the timing of later gene expression (Is-

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shiki et al., 2001). This rules out the simple model of a linear positive transcriptional cascade. Instead, it has been proposed that there is an independent "temporal identity timer" that regulates Hb → Kr → Pdm1 → Cas expression in neuroblasts (Isshiki et al., 2001).

In this paper we investigate the neuroblast temporal identity timer, testing a number of possible mechanisms (Figure 1). We draw three main conclusions. First, the timing of *hb* gene expression is transcriptionally regulated and requires neuroblast cytokinesis. Second, the timing of Kr → Pdm1 → Cas expression occurs normally in G_2-arrested neuroblasts or in vitro isolated neuroblasts, and thus involves an intrinsic timing mechanism. Third, Hb regulates the temporal identity timer: Hb expression keeps the neuroblast "young" for multiple divisions, and release of Hb allows resumption of the normal neuroblast lineage.

Results

Hb Timing Is Regulated at the Transcriptional Level

To determine whether *hb* is regulated transcriptionally or posttranscriptionally in the CNS, we performed double labels for *hb* active transcription (using an intron probe) and Hb protein (using an antibody) (Figure 2). We also performed double labels for *hb* mRNA and protein levels (data not shown). If posttranscriptional regulation plays a major role, we should observe neuroblasts that actively transcribe *hb* but lack Hb protein; if transcriptional regulation is dominant, most neuroblasts should be double positive for active transcription and protein. We observed that the large majority of neuroblasts are double positive for both active transcription and protein (82.5%; Figure 2A), whereas only a small minority have active transcription without detectable Hb protein (2%; Figure 2B). These appear to be newly formed neuroblasts that have just started to express *hb*, although we can't rule out a minor role for posttranscriptional regulation. The remaining neuroblasts show Hb protein without active transcription, and are probably at the end of the *hb* expression window (7%; Figure 2C), or are in mitosis and are transcriptionally inactive as expected for this stage of the cell cycle (8.5%; Figure 2D). Similar results were obtained for *hb* mRNA/Hb protein double labels (data not shown). The tight correlation between active transcription, cytoplasmic mRNA, and nuclear protein indicates that the *hb* mRNA and protein have a short half-life in neuroblasts, because mRNA and protein are rarely detected in transcriptionally inactive cells. Taken together, our data show that *hb* neuroblast expression is primarily regulated at the transcriptional level (Figure 1A), although posttranscriptional regulation may be used to keep mRNA and protein half-life short (see Discussion).

We also assayed *hb* transcription/Hb protein coexpression during neuronal differentiation. We find that early-born GMCs and differentiating neurons maintain active transcription of *hb* (Figures 2E and 2F), including the identified Hb^+ U1 and U2 neurons of the NB7-1 lineage (Figure 2G). Thus, the maintained expression of Hb protein in neurons is not due to a pulse of transcription in the neuroblasts followed by persistence of stable Hb protein in GMC/neuronal progeny, but rather is

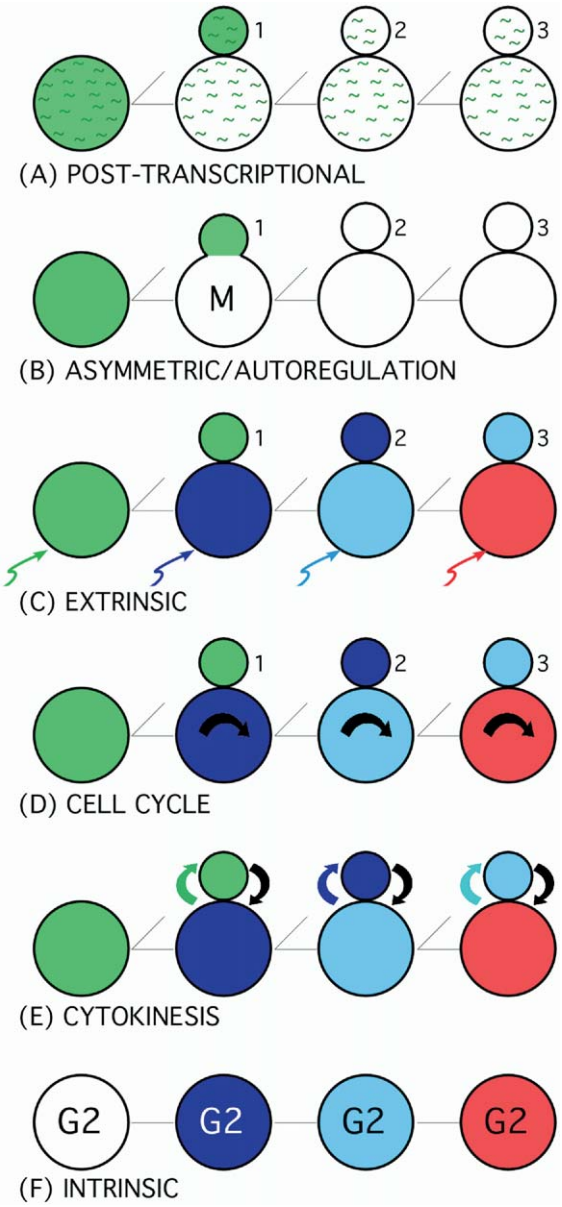

Figure 1. Models for Timing Hb → Kr → Pdm1 → Cas Gene Expression in Neuroblasts

Neuroblasts bud off a series of GMCs over time (small circles, numbers indicate birth order), and sequentially express Hb (green), Kr (blue), Pdm1 (aqua), and Cas (red) proteins.

(A) Posttranscriptional regulation. Downregulation of Hb protein levels is due to posttranscriptional negative regulation; *hb* transcription (green tilde) persists longer than Hb protein.

(B) Asymmetry/autoregulation. Hb protein positively activates its own transcription, and is asymmetrically partitioned out of the neuroblast during mitosis (M).

(C) Extrinsic signals. Cues from outside the neuroblast lineage (wavy arrows) trigger changes in neuroblast gene expression.

(D) Cell cycle counting. Each cell cycle (arrow) changes neuroblast gene expression.

(E) Cytokinesis. GMC formation changes neuroblast gene expression, either by feedback signaling (black arrows) or by partitioning transcriptional activators out of the neuroblast (colored arrows).

(F) Intrinsic timer. Sequential gene expression occurs in G_2-arrested neuroblasts.

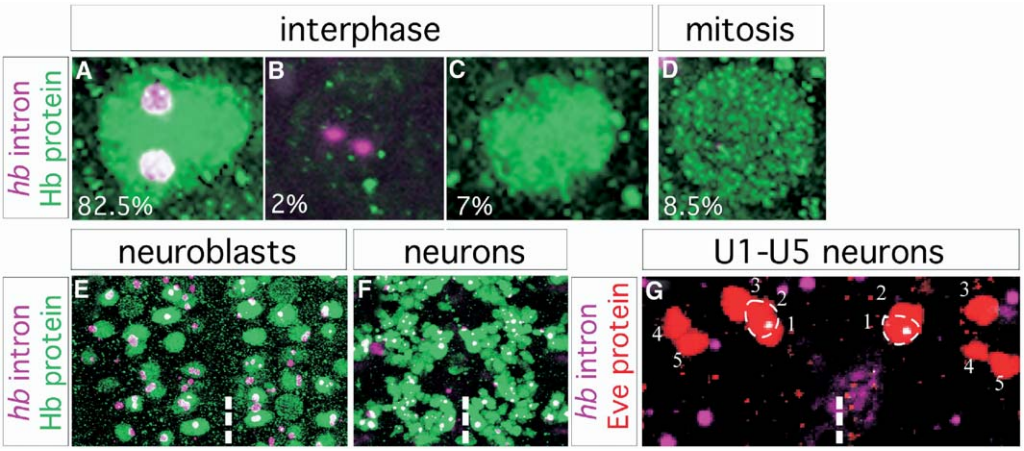

Figure 2. Hb Timing Is Regulated at the Transcriptional Level

(A–D) Neuroblasts double labeled for active *hb* transcription (intron probe, purple) and Hb protein (antibody probe, green). Interphase neuroblasts can be double positive for transcription and protein (A), single positive for transcription (B), or single positive for protein (C). Mitotic neuroblasts (D) are identified by lack of nucleus and show diffuse Hb protein and no active transcription. All neuroblasts were scored in stage 9–10 embryos during the time that Hb protein disappears from neuroblasts; n = 293 neuroblasts.

(E and F) Active transcription of *hb* (purple) and Hb protein (green) are tightly linked in stage 9 neuroblasts (E) and stage 13 neurons (F). Midline indicated by dashed vertical line.

(G) Active *hb* transcription (purple) can be observed in the postmitotic U1/U2 neurons (Eve$^+$, red) at stage 15, but only some U1/U2 neurons show detectable intron probe staining, either due to technical limitations (the neurons are quite small) or due to paused/terminated *hb* transcription. Midline indicated by dashed vertical line. Dotted outlines show U2 (left) and U1 (right).

due to active *hb* transcription in GMCs and mature postmitotic neurons.

Hb Timing Does Not Require Asymmetric Localization of Hb mRNA or Protein

Our finding that *hb* expression is regulated transcriptionally, coupled with the fact that *hb* is known to positively autoregulate its own expression during segmentation (Hulskamp et al., 1994), suggests a simple and elegant "asymmetry/autoregulation" model (Figure 1B), in which asymmetric partitioning of *hb* mRNA or protein into the GMC maintains *hb* transcription in the GMC (due to positive autoregulation) and eliminates it from the neuroblast (due to lack of transcriptional activation). To test this model, we started by assaying *hb* mRNA and Hb protein asymmetric localization in mitotic neuroblasts. Unlike other mRNAs and proteins that we have previously shown to be localized into the GMC (Broadus et al., 1998; Ikeshima-Kataoka et al., 1997; Peng et al., 2000; Spana and Doe, 1995; Spana et al., 1995), we find no evidence that *hb* mRNA or Hb protein is selectively partitioned into the GMC; rather, all neuroblasts showed a uniform distribution of *hb* mRNA or Hb protein throughout mitosis, from prophase to telophase (Figure 3A).

Nevertheless, it remains possible that an Hb cofactor could be partitioned into the GMC, rather than Hb, so we wanted to test this model further by investigating whether Hb positively regulates its own expression within the CNS. We first examined *hb* transcription within the CNS in *hb* mutant embryos, using an *hb* mutant allele that makes a nonfunctional protein. In these mutant embryos, we clearly detect *hb* transcription in neuroblasts (Figure 3D) and GMCs (data not shown), showing that Hb protein is not required for *hb* transcription in the CNS. We next used the Gal4/UAS system (Brand and Perrimon, 1993) to misexpress *hb* within the CNS, and assayed for transcriptional activation of the endogenous *hb* gene using an intron probe. We find that ectopic Hb does not induce transcription of the endogenous *hb* gene within the CNS (Figure 3E). This is in contrast to the epidermis, where a pulse of ectopic Hb is able to induce persistent transcription of the endogenous *hb* gene, presumably by initiating a positive autoregulatory loop (data not shown). We conclude that Hb is not required and is not sufficient to activate its own expression in the CNS. Thus, we can rule out the "asymmetry/autoregulation" model for timing *hb* expression (Figure 1B).

Hb Kr → Pdm1 → Cas Timing Is Independent of Extrinsic Signals

To investigate the role of extrinsic signals in regulating Hb → Kr → Pdm1 → Cas expression (Figure 1C), we isolated neuroblasts, cultured them in vitro, and assayed for Hb → Kr → Pdm1 → Cas expression in these isolated neuroblasts over time. Isolated embryonic neuroblasts divide asymmetrically (Broadus and Doe, 1997) and generate clones of differentiated neuronal progeny which can express Hb, Pdm1, and Cas (Brody and Odenwald, 2000), but gene expression timing in the neuroblast has never been analyzed. We dissociated embryos just after neuroblast formation and cultured the neuroblasts for different times (from 0.5 hr to 5 hr), and then assayed Hb, Kr, and Cas protein distribution within each neuroblast clone (Figure 4). We only scored neuroblast clones that were not in contact with any other cells to avoid the possibility of interclone

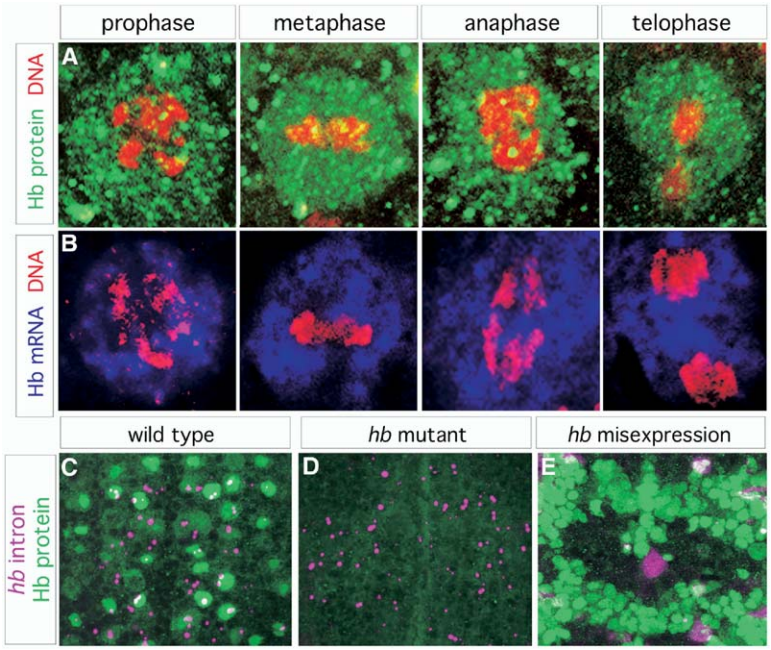

Figure 3. *hb* mRNA/Hb Protein Are Not Asymmetrically Localized and Do Not Positively Regulate *hb* Transcription

(A and B) Hb protein (A, green) and *hb* mRNA (B, blue) are not asymmetrically partitioned into the GMC during neuroblast cell division. Phosphohistone H3 is used to mark the DNA (red). Apical is up in all panels; cell cycle stages indicated at top.

(C–E) Hb is unnecessary and insufficient to activate *hb* transcription in the CNS. (C) In wild-type neuroblasts, Hb protein and *hb* transcription are colocalized. (D) In hb^{FB} mutant neuroblasts, *hb* transcription is still observed (purple) even though there is no functional Hb protein (green). (E) Misexpression of functional Hb protein (green) using the Gal4/UAS system does not activate transcription of the endogenous *hb* gene within the CNS (purple).

signaling (n = 109 clones). We find that neuroblasts shift from Hb^+ to Kr^+ to triple negative (presumptive $Pdm1^+$) to Cas^+ over time in culture (Figure 4A). For example, the highest percentage of Hb^+ neuroblasts is at 0.5 hr, Kr^+ neuroblasts at 2 hr, presumptive $Pdm1^+$ neuroblasts at 4 hr, and Cas^+ neuroblasts at 5 hr (Figure 4A), although there is considerable heterogeneity due to asynchrony of the neuroblasts at the time of embryo dissociation. Perhaps a more convincing measure of the sequential progression of gene expression is that within a single neuroblast clone, the progeny always have the same or an "earlier" transcription factor as found in the neuroblast (Figure 4C). For example, we never observed Hb^+ neuroblasts associated with progeny that were Hb^- Kr^+, Pdm^+, or Cas^+. We conclude that sequential expression of Hb → Kr → Pdm1 → Cas

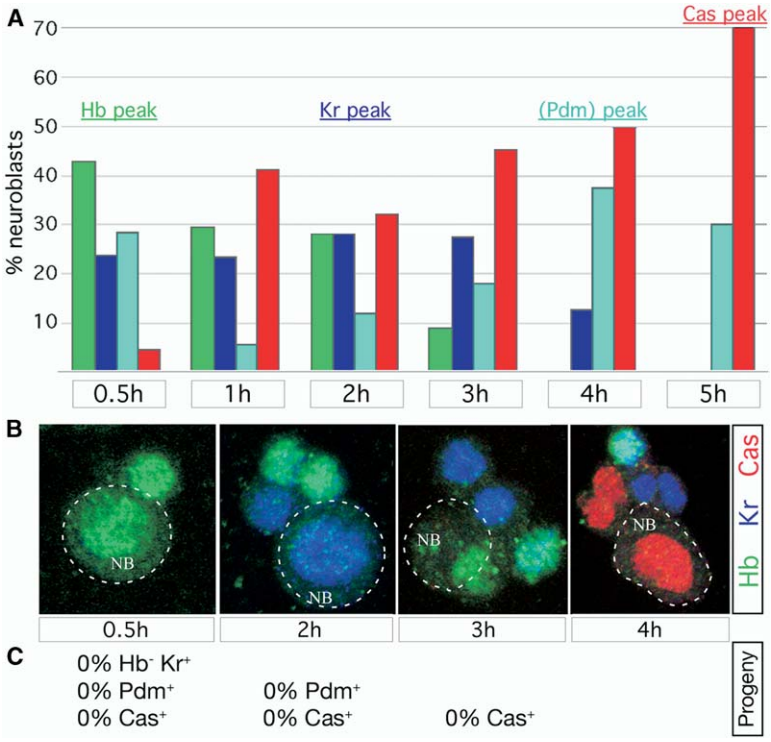

Figure 4. Hb → Kr → Pdm1 → Cas Timing Is Independent of Extrinsic Signals

Neuroblasts are cultured in isolation in vitro, fixed at different time points (0.5 hr–5 hr) and triple labeled for Hb, Kr, and Cas proteins.

(A) Percentage of neuroblasts staining for Hb (green bars), Kr (blue bars), Cas (red bars), or triple negatives (presumptive Pdm1, aqua bars). The Hb^+ neuroblasts also express Kr, as do young neuroblasts in vivo (Isshiki et al., 2001).

(B) Examples of neuroblast clones staining for Hb (green), Kr (blue) and Cas (red) at the indicated time points. The neuroblast (NB, dotted outline) typically moves from Hb^+ to Kr^+ to triple negative (presumptive $Pdm1^+$) to Cas^+ over time in culture. GMCs always express the same or "earlier" transcription factor as the neuroblast.

(C) Percentage of neuroblasts expressing the protein shown in (B) whose progeny are Hb^- Kr^+, triple negative ($Pdm1^+$), or Cas^+. n = 50 for each.

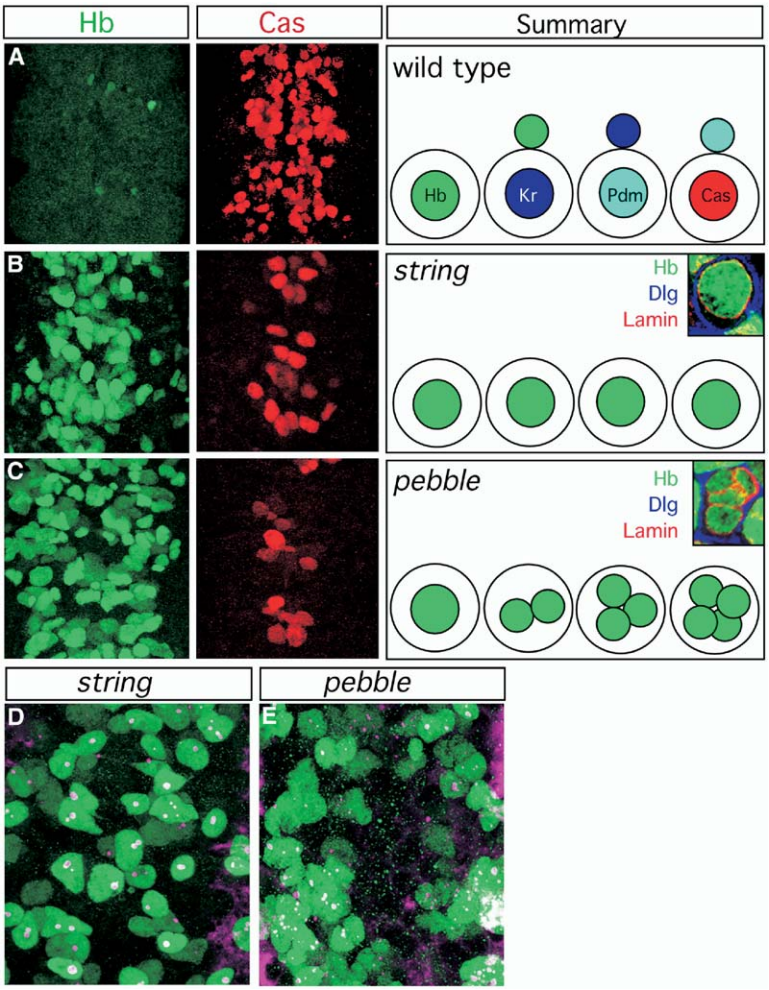

Figure 5. Hb Timing Is Regulated by Neuroblast Cytokinesis and Not Cell Cycle Progression

(A–C) Left panels show a single confocal optical section through the neuroblast layer in two segments of stage 12 embryos stained for Hb (green) and Cas (red). GMCs and neurons are not visible in this focal plane. (A) Wild-type neuroblasts are Hb^{-} Cas^{+}. (B) *string* mutant neuroblasts stay Hb^{+} Cas^{-}. (C) *pebble* mutant neuroblasts stay Hb^{+} Cas^{-}. Right: summary of cell cycle and gene expression for each genotype; the last point in the schematic corresponds to stage 12 shown in the panels to the left. Insets: representative neuroblasts stained for a plasma membrane marker (Discs large, blue), nuclear membrane (lamin, red), and Hb protein (green) showing one nucleus in the *string* mutant neuroblast and four nuclei in the *pebble* mutant neuroblast.

(D and E) *string* mutant and *pebble* mutant neuroblasts fail to downregulate *hb* transcription (intron probe, purple) and Hb protein (green). Ventral view of stage 12 embryos.

in neuroblasts does not require extrinsic signals from outside the lineage (Figure 1C).

Hb Timing Requires Neuroblast Cytokinesis

It has been shown previously that G_2-arrrested neuroblasts maintain a high Hb, low Kr profile similar to newly formed neuroblasts, never making the transition to being Hb^{-} Kr^{+} (Isshiki et al., 2001). However, this experiment does not distinguish between cytokinesis or cell cycle progression for advancing neuroblast gene expression, and it is unknown whether these G_2-arrrested neuroblasts maintain *hb* transcription, or merely show abnormally stable *hb* mRNA or protein. To distinguish between cytokinesis and cell cycle progression for timing Hb → Kr → Pdm1 → Cas expression (Figure 1D versus Figure 1E), we compared *hb* gene expression in *string* mutant neuroblasts, which are G_2 arrested and thus lack both cytokinesis and nuclear cell cycle events (Edgar and O'Farrell, 1989), to *hb* expression in *pebble* mutant neuroblasts, which undergo cell cycle events but do not perform cytokinesis (Prokopenko et al., 1999; Cui and Doe, 1995; Isshiki et al., 2001; Weigmann and Lehner, 1995).

In wild-type embryos at stage 12, neuroblasts have already downregulated Hb and are Cas^{+} (Figure 5A). In *string* mutant embryos at stage 12, neuroblasts remain Hb^{+} Cas^{-} (Figure 5B) (Isshiki et al., 2001); these G_2-arrested neuroblasts continue to actively transcribe *hb* (Figure 5D), which demonstrates that they fail to downregulate *hb* transcription rather than just maintain abnormally stable Hb protein. In *pebble* mutant embryos at stage 12, neuroblasts progress through the cell cycle normally but fail in cytokinesis, resulting in four or more distinct nuclei per cell (Figure 5C, inset). The *pebble* mutant neuroblasts also fail to downregulate Hb protein levels (Figure 5C) and *hb* transcription (Figure 5E). A similar result is observed when cytokinesis is blocked with the microfilament inhibitor Latrunculin B (data not shown). Active *hb* transcription can be detected in all four or more nuclei in the multinucleate neuroblasts (data not shown), so we can rule out the possibility that only one nucleus is transcriptionally active (e.g., nucleus that would have been in the Hb^{+} GMC or neuron). In both *string* and *pebble* mutants, neuroblasts also maintain Kr expression, similar to the initial Hb^{+} Kr^{+} state of newly formed wild-type neuroblasts (Isshiki et al., 2001); importantly, they never become Hb^{-} Kr^{+} or express Pdm or Cas (Figure 5 and data not shown). We draw two major conclusions from these results. First, that repeated cell cycle events such as DNA replication

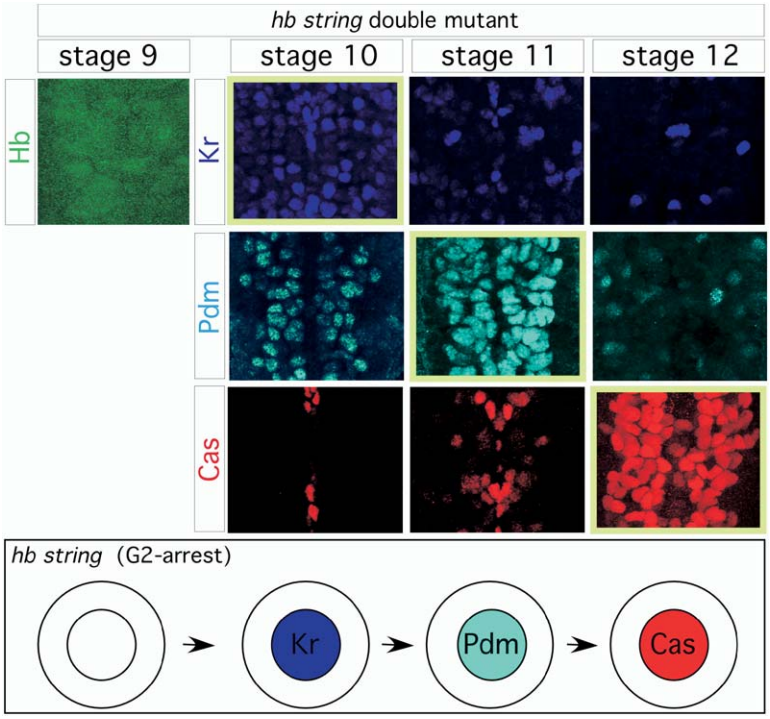

Figure 6. Kr → Pdm1 → Cas Timing Is Regulated by a Cell-Cycle-Independent Mechanism

Single confocal optical section through the neuroblast layer in progressively older embryos shown from left to right (stages labeled at top). Top: ventral view of the neuroblast layer in *hb, string* double mutants stained for Hb, Kr, Pdm1, and Cas (labeled at left). Hb protein is not detected, as expected (image is collected at a higher gain than the rest of the panels to pick up even low levels of protein). The timing of Kr (blue), Pdm1 (aqua), and Cas (red) is in the proper order and at the normal time, despite the fact that all neuroblasts are G_2 arrested. Yellow boxes highlight the stage of peak expression for each transcription factor. Bottom: summary of neuroblast cell cycle and gene expression.

and mitosis are insufficient to advance the Hb → Kr → Pdm1 → Cas temporal identity timer (Figure 1D). Second, that neuroblast cytokinesis is essential for triggering the Hb → Kr transition; this is consistent with feedback signaling from the new-born GMC to the parental neuroblast (Figure 1E, black arrows) or partitioning of an *hb* transcriptional activator into the GMC during asymmetric division (Figure 1E, colored arrows).

Kr → Pdm1 → Cas Timing Is Regulated by a Cell Cycle-Independent Mechanism

To determine whether Kr → Pdm1 → Cas gene expression timing is regulated by cell cycle progression, we needed to eliminate *hb* expression from *string* mutants. This is because prolonged Hb expression will block Kr → Pdm1 → Cas expression (Isshiki et al., 2001; Pearson and Doe, 2003). We made *hb, string* double mutants and scored Kr → Pdm1 → Cas neuroblast expression at different time points. In *hb, string* double mutant embryos, neuroblasts form normally but remain G_2 arrested throughout embryogenesis. If Kr → Pdm1 → Cas timing requires cytokinesis or cell cycle progression, we would expect that *hb, string* double mutant neuroblasts would show persistent Kr expression throughout embryogenesis. We were therefore quite surprised to find that *hb, string* double mutant embryos sequentially express Kr → Pdm1 → Cas with a timing indistinguishable from wild-type neuroblasts, despite the fact that the neuroblasts were G_2 arrested (Figure 6). More specifically, we find that *hb, string* double mutant neuroblasts have high Kr, low Pdm1/Cas at stage 10; high Pdm1, low Kr/Cas at stage 11; and high Cas, low Kr/Pdm1 at stage 12 (Figure 6). This is the same temporal pattern of expression that is observed in wild-type neuroblasts (Isshiki et al., 2001) (data not shown). We conclude that in the absence of Hb, the temporal identity timer can drive Kr → Pdm1 → Cas expression normally even without cell cycle progression. This indicates the presence of a robust neuroblast-intrinsic timing mechanism that is independent of cell cycle progression, asymmetric partitioning of transcription factors, feedback signaling from GMC progeny, and nucleo-cytoplasmic ratio (see Discussion).

Hb Regulates the Temporal Identity Timer

Hb has the ability to prevent sequential Kr → Pdm1 → Cas gene expression in neuroblasts, based on the observation that *string* single mutants have extended Hb and do not sequentially express Kr → Pdm1 → Cas, but *string hb* double mutants show normal Kr → Pdm1 → Cas sequential gene expression. How does Hb prevent Kr → Pdm1 → Cas gene expression? Hb could repress Kr, Pdm1, or Cas expression without blocking advance of the temporal identity timer, or it could "freeze" the temporal identity timer prior to the start of Kr → Pdm1 → Cas gene expression. These two models can be easily distinguished by an Hb "pulse/chase" experiment, in which Hb is maintained for 10–12 neuroblast cell cycles and then turned off, and the temporal identity of the subsequent progeny are scored. If the temporal identity timer is advancing normally, the neuroblast will generate extra early-born neurons at the expense of middle-born neurons; if the temporal identity timer is arrested, the neuroblast will resume with the normal sequence of middle-born neurons.

We performed the Hb "pulse/chase" experiment in the well-characterized NB7-1 lineage (Isshiki et al.,

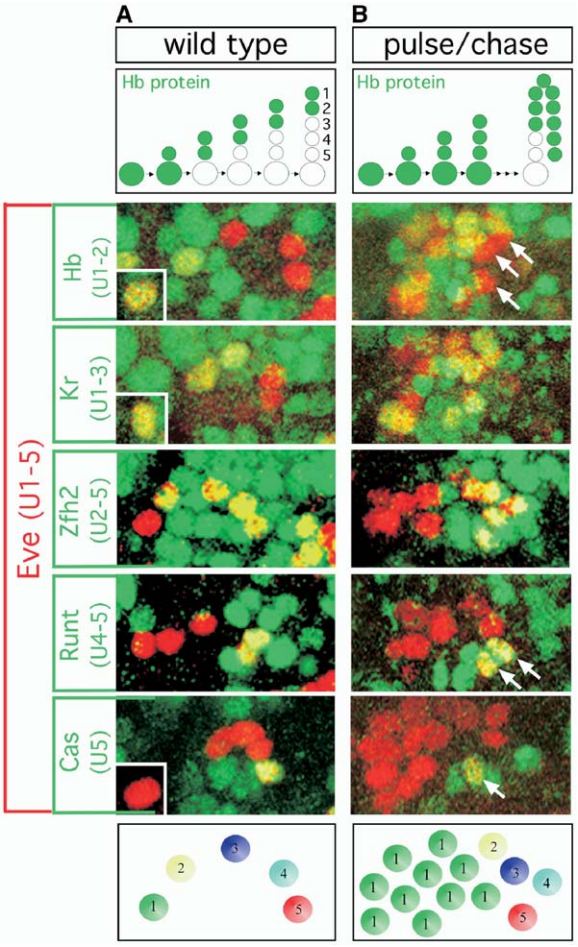

Figure 7. Hb Expression Arrests the Temporal Identity Timer and Uncouples It from Neuroblast Cell Division

The top schematics summarize the timing of Hb protein (green) in the NB7-1 lineage, with the U1–U5 neuron identity indicated by numbers next to their parental GMC (neurons not shown for simplicity); middle panels show the U1–U5 Eve$^+$ neurons (red) stained for the indicated temporal identity markers (green). All panels show one hemisegment of a stage 16 CNS (anterior, up; medial, left). U1 neurons are shown as insets in their approximate spatial position if they are obscured in the projection.

(A) Wild-type. There are five Eve$^+$ U1–U5 neurons; Hb is expressed in the first two GMCs which generate the U1 and U2 neurons.

(B) Hb pulse/chase (*sca-gal4; uas-hb*). Hb is expressed in the first 8–10 GMCs, which all generate Eve$^+$ Hb$^+$ U1 neurons; after decline in Hb expression, the neuroblast resumes its normal lineage to generate the U2–U5 neurons. For example, note the presence of the Runt$^+$ U4 and U5 neurons or the Cas$^+$ U5 neuron.

2001; Pearson and Doe, 2003). This neuroblast generates more than 20 GMCs but only the first five GMCs express the Even-skipped (Eve) transcription factor. Each of the five Eve$^+$ GMCs produces one Eve$^+$ neuron (named U1–U5, based on birth order). In addition, each of the U1–U5 motoneurons can be uniquely identified by marker expression and cell position (Isshiki et al., 2001; Pearson and Doe, 2003). To generate the Hb "pulse/chase" we used *sca-gal4* to misexpress *UAS-hb* in all neuroblasts, which results in extended Hb expression in neuroblasts and delay in Kr → Pdm1 → Cas expression until at least stage 13, at which time the Hb levels gradually decline and disappear (Figures 7A and 7B and data not shown). We assayed U1–U5 neuronal identity in these embryos, and we found the expected pool of 10–12 Hb$^+$ U1 neurons (due to the extended expression of Hb in the neuroblast), but we also detected the normal complement of U2–U5 later-born neurons. The U2–U5 neurons were born later (first detected at stage 16 whereas normally they can be observed at stage 13) and positioned in the most ventral and lateral portion of the Eve$^+$ U neuron cluster, showing that they were the last-born cells of the cluster (Pearson and Doe, 2003). We conclude that Hb expression can arrest the neuroblast temporal identity timer at the earliest stage, independent of the number of cell divisions that occur, and that Hb downregulation is required for advancing the temporal identity timer. Our results, combined with the inability of Hb to induce first-born fates in postmitotic neurons (Pearson and Doe, 2003), raise the possibility that a primary role of Hb is to maintain neuroblasts in a "young" temporal state.

Discussion

We have shown that there are two timing mechanisms that regulate temporal identity transitions in *Drosophila* neuroblasts: a cytokinesis-dependent mechanism that times the Hb → Kr transition, and a cell cycle-independent mechanism that times the Kr → Pdm1 → Cas transitions. We have also shown that Hb can maintain the neuroblast in a temporally "young" state over many cell divisions without losing the potential to resume the lineage upon decline in Hb levels. Our results provide insight into the complexity of temporal patterning mechanisms, as well as provide a foundation for further molecular and biochemical characterization of temporal identity timers in *Drosophila* neuroblasts.

Hb Is Regulated at the Transcriptional Level in Neuroblasts

We have found that *hb* is regulated at the transcriptional level in neuroblasts, based on strong correlation with active transcription (intron probe) and protein levels (antibody probe). In addition, we observed *hb* transcription in GMCs and differentiated neurons, but at this point we cannot determine if the correlation between protein and transcription is as tight as in the neuroblasts. This does not rule out a role for posttranscriptional regulation, however, to ensure a very short half-life of both *hb* mRNA and protein. There are predicted miRNA binding sites in the *hb* 3′UTR (Lin et al., 2003) and protein degradation (PEST) motifs in the Hb protein (data not shown) which may be necessary to restrict Hb protein to the early portion of neuroblast lineages. There is ample precedent for posttranscriptional regulation of *hb* in both *Drosophila* early embryos and *C. elegans*, but only for translational repression. In *Drosophila*, Nanos represses *hb* translation in the early embryo via binding to its 3′UTR (Irish et al., 1989). In *C. elegans*, the *hb* ortholog *hbl-1* regulates temporal identity as part of the heterochronic pathway, and *hbl-1* is a target of microRNA regulation through its 3′UTR (Abrahante et al.,

2003; Lin et al., 2003). We conclude that precise regulation of *hb* transcription, coupled with a short half-life of *hb* mRNA and protein, leads to the observed restriction of Hb protein to the initial cell cycles of neuroblast lineages. Identification of the *hb* cis-regulatory sequences necessary for proper *hb* CNS expression has been initiated (B. Margolis and J. Posakony, personal communication), and it will be interesting to determine the associated factors that positively and negatively regulate *hb* transcription in neuroblasts.

Hb → Kr Timing Requires Neuroblast Cytokinesis

We previously showed that cell cycle-arrested neuroblasts maintain *hb* expression (Isshiki et al., 2001). However, we could not distinguish between a direct role of the cell cycle (e.g., counting S phases) or an indirect role (e.g., generation of a GMC which could signal back to the neuroblast). Here we show that *hb* transcription is maintained in *pebble* mutant neuroblasts, which lack cytokinesis but nevertheless go through repeated cell cycles including DNA replication, nuclear envelope breakdown, chromosome condensation, and spindle assembly. Thus, the timely downregulation of *hb* transcription requires cytokinesis. The requirement for cytokinesis is consistent with two quite different mechanisms: (1) feedback signaling from the GMC to the neuroblast to repress *hb* transcription, and (2) asymmetric partitioning of an *hb* transcriptional activator into the GMC to halt *hb* transcription.

Currently we can't resolve which mechanism is used. We have tested two candidate transcription factors for a role in *hb* regulation, Hb and Prospero. The Hb protein does not positively regulate its own transcription in the CNS, nor are *hb* mRNA or protein partitioned into the GMC during neuroblast cell division. The Prospero transcription factor is known to be partitioned into the GMC during neuroblast division (Hirata et al., 1995; Spana and Doe, 1995), but Prospero protein is cytoplasmic in neuroblasts, and thus unlikely to positively activate *hb* transcription in this cell type. In addition, misexpression of Prospero in neuroblasts is unable to extend the window of *hb* transcription (data not shown) and *prospero* mutants have normal *hb* expression in neuroblasts, although there is reduced Hb protein in GMCs and neurons by stage 13 and beyond (Supplemental Figure S1). Thus, Prospero may have a role in maintaining *hb* transcription in GMCs and neurons, consistent with its nuclear localization in these cell types, but it is not required for timing of *hb* transcription in neuroblasts.

To investigate the role of feedback signaling from the GMC, it would be ideal to do GMC ablations and assay for extended *hb* transcription in the parental neuroblast, but this experiment is technically very demanding, and even short GMC-neuroblast contact might be enough for the signaling to occur. We have tested whether the feedback signal is mediated by the Notch pathway, which is active in all neuroblasts and GMCs examined to date (Skeath and Doe, 1998), and find that blocking the pathway with a *sanpodo* mutant (Skeath and Doe, 1998) has no effect on the timing of *hb* → *Kr* → *pdm1* → *cas* neuroblast expression (data not shown). The identification of *trans*-acting factors that associate with the *hb* cis-regulatory DNA may be the best approach to distinguish between feedback signaling and transcription factor partitioning mechanisms.

Hb → Kr → Pdm1 → Cas Timing Is Normal in Isolated Neuroblasts

Previous work provided strong hints that global extrinsic signals are not required for timing neuroblast temporal identity transitions. First, neuroblast lineages are asynchronous, with later-forming neuroblasts expressing *hb* at the same time adjacent early-forming neuroblasts are expressing *cas*, making it unlikely that global extrinsic signals trigger gene expression transitions (Isshiki et al., 2001). Second, previous in vitro culture experiments reported differentiated neuronal clones containing nonoverlapping populations of Hb^+, $Pdm1^+$, and Cas^+ neurons, consistent with a normal progression of gene expression in the parental neuroblast over time (Brody and Odenwald, 2000), although gene expression timing was not assayed in neuroblasts. Here we confirm and extend these observations. We have shown isolated neuroblasts progress from Hb^+ to Kr^+ to triple negative (presumptive $Pdm1^+$) to Cas^+ over time in culture, and we also never found clones in which the GMCs expressed a later gene than the neuroblast (e.g., Hb^+ or Kr^+ neuroblasts never had $Pdm1^+$ or Cas^+ GMCs). Thus, Hb → Kr → Pdm1 → Cas neuroblast gene expression timing occurs normally in isolated neuroblasts, demonstrating that lineage-extrinsic factors are not required for neuroblast temporal identity transitions. It is possible that extrinsic cues may still override or entrain an intrinsic program, however, which could be tested by heterochronic neuroblast transplants. In summary, in vitro and in vivo data show that timing of temporal identity transitions is regulated by a neuroblast lineage-intrinsic mechanism. For the latter genes in the cascade, it appears that the mechanism is actually intrinsic to the neuroblast itself (see next section).

Kr → Pdm1 → Cas Timing Is Regulated by a Neuroblast-Intrinsic Mechanism

All available data suggest that Kr and Cas timing are regulated at the transcriptional level. *Kr* mRNA and protein are both detected in neuroblasts during embryonic stage 10 and subsequently maintained in a subset of neurons (data not shown). Similarly, *cas* mRNA and protein are both widely detected in neuroblasts only at stage 12, and maintained in a subset of late-born neurons (Cui and Doe, 1992; Mellerick et al., 1992) (data not shown). In the future, it will be important to do mRNA/protein double labels for *Kr*, *pdm1*, and *cas* to determine the extent to which mRNA/protein levels are correlated at the single cell level. Unfortunately, it is not easy to assay for active transcription of *Kr* or *cas* due to the lack of large introns.

Surprisingly, we found that cell cycle-arrested neuroblasts that lack Hb still express Kr → Pdm1 → Cas with the same timing as in wild-type embryos. What mechanism might time Kr → Pdm1 → Cas expression in the absence of cell division? We can rule out extrinsic cues, because isolated neuroblasts still undergo normal Kr → Pdm1 → Cas gene expression timing. We can also rule out a change in nucleo-cytoplasmic ratio, known to time certain early embryonic events (Newport and Kirschner, 1982a, 1982b), because wild-type neuroblasts increase their nucleo-cytoplasmic ratio over time, but G_2-arrested neuroblasts decrease their nucleo-cytoplasmic ratio as they enlarge without dividing.

The most attractive model for Kr → Pdm1 → Cas in G_2-arrested neuroblasts is a cascade of transcriptional regulation between Kr, Pdm1, and Cas. Misexpression studies have shown that each gene can activate expression of the next gene in the series, and repress the "next + 1" gene, which could account for the sequential activation of each gene (Isshiki et al., 2001). If each transcription factor can also repress its activator, similar to the known ability of Cas to negatively regulate *pdm1* expression (Kambadur et al., 1998), it could explain the sequential downregulation of each gene as well. Currently, all misexpression data are consistent with this simple model. However, analysis of *hb* and *Kr* mutants reveals additional complexity. *hb* mutants show relatively normal Kr → Pdm1 → Cas timing, and *Kr* mutants show relatively normal Pdm1 → Cas timing (Isshiki et al., 2001). Thus, there must be at least one unidentified input that can activate *Kr* in the absence of Hb, and *pdm1* in the absence of Kr. Regulation of Hb → Kr → Pdm1 → Cas appears to be primarily at the transcriptional level, and thus identification of the relevant cis-regulatory DNA and associated transcription factors should provide insight into the "timer" mechanism that controls sequential gene expression in neuroblasts.

Hb Regulates the Neuroblast Temporal Identity Timer

Hb seems to have a special role in advancing the temporal identity timer. It is the only factor in the cascade whose downregulation requires cytokinesis, and as long as it is present (either because of cell cycle arrest or misexpression) the timer is unable to advance. Misexpression of Hb beyond its normal expression window leads to generation of extra early cell types and blocks Kr → Pdm1 → Cas progression (Isshiki et al., 2001). However, these experiments do not reveal whether Hb generates these early fates by overriding Kr → Pdm1 → Cas neuronal identity while the temporal timer is advancing or if it arrests progress of the temporal timer. Our results show that continuous expression of Hb blocks the advancement of the temporal identity timer, keeping the neuroblast in a "young" state that is fully capable of resuming its normal cell lineage following downregulation of Hb. The ability of Hb to keep the neuroblast in a "young" multipotent state, despite repeated rounds of cell division, raises the interesting question of how Hb acts at the mechanistic level. Transcriptional targets of Hb in the CNS are so far unknown. A mammalian homolog, Ikaros, is associated with chromatin and remodeling proteins (Sabbattini et al., 2001) and *Drosophila* Hb is thought to regulate chromatin-mediated heritable expression of homeotic genes (Kehle et al., 1998; Farkas et al., 2000). Thus, Hb might modulate chromatin structure in neuroblasts to prevent expression of later temporal identity genes, or to maintain plasticity of gene expression necessary for maintaining the multipotent state of the neuroblast.

Experimental Procedures

Fly Stocks

We used *hb^{FB}*/TM3 *ftz-lacZ, stg^{7M53}*/TM3 *ftz-lacZ, pbl ^{5B}*/TM3 *ftz-lacZ, hb^{FB} stg^{7M53}*/TM3 *ftz-lacZ, $prospero^{17}$/TM3 ftz-lacZ,* and *sca-brous-GAL4* (Figure 7) or *engrailed-GAL4 patched-GAL4* (Figure 3) crossed to *UAS-hb* (Wimmer et al., 2000).

mRNA/Protein Immunolocalization and Drug Treatment

Antibody staining was performed according to standard methods. Primary antibodies, dilutions, and sources are: rabbit anti-β-galactosidase, 1:1000, Cappel; mouse anti-β-galactosidase, 1:500, Promega; mouse anti-Hb, 1:10, Nipam Patel; guinea pig anti-Hb, 1:400, East Asian Distribution Center for Segmentation Antibodies (EADC); guinea pig anti-Kr, EADC, 1:400; mouse anti-Pdm1, 1:10, S. Cohen; rabbit anti-Cas, 1:1000, W. Odenwald; guinea pig anti-Eve, 1:400, EADC; guinea pig anti-Runt, 1: 400, EADC; rat anti-Zfh2, 1:200, M. Lundell; sheep anti-Digoxigenin, 1:1000, Boehringer; and rabbit anti-phospho-Histone3, 1:1000, Upstate. Species-specific secondary antibodies were conjugated to Alexa 488, Rhodamine RedX, Cy5 (Jackson), or Biotin (Vector Labs) and were used at 1:200. The tyramide signal amplification kit (NEN) or ABC Kit (Vectastain) were used in some cases.

mRNA/antibody or intron/antibody double labels were done by sequential RNA hybridization followed by antibody staining. The intron probe was the entire 2.5 kb intron; the cDNA probe was made from EST LD34229. Intron or mRNA detection was done by a modification of the published protocol (Tautz and Pfeifle, 1989), including omission of Proteinase K digestion; subsequent antibody staining was performed using standard methods. A detailed protocol is available upon request. All images were collected as confocal image stacks on a BioRad Radiance confocal microscope, processed in ImageJ (NIH), and shown as two-dimensional projections.

Latrunculin B (Calbiochem) was used at 5 μg/ml for 2 hr on 4- to 5-hr-old wild-type embryos using standard methods (Spana and Doe, 1995).

In Vitro Neuroblast Culture

In vitro neuroblast cultures were prepared and antibody stained as described (Broadus and Doe, 1997) with these changes: we used Chan and Gehring's medium (Chan and Gehring, 1971) with 2% fetal calf serum, used 2 ml of media, changed the media every 30 min, fixed with 4% paraformaldehyde, and subsequently washed with 2% NaN_3 and 0.5% Triton in PBS for 4 min. Neuroblasts were identified by their large size and association with smaller cells in a clone. The neuroblast cell cycle is ~40 min (Campos-Ortega and Hartenstein, 1997), so we can predict the maximum clone size at each time point, and only clones with an equal or fewer cells were quantified to avoid scoring neuroblast clones that were not dissociated at the time of plating.

Supplemental Data

Supplemental data associated with this article can be found at http://www.developmentalcell.com/cgi/content/full/8/2/193/DC1/. The supplemental data set contains one supplemental figure.

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