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Neuroblast entry into quiescence is regulated intrinsically by the combined action of spatial Hox proteins and temporal identity factors

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Neural stem cell quiescence is an important feature in invertebrate and mammalian central nervous system development, yet little is known about the mechanisms regulating entry into quiescence, maintenance of cell fate during quiescence, and exit from quiescence. *Drosophila* neural stem cells (called neuroblasts) provide an excellent model system for investigating these issues. *Drosophila* neuroblasts enter quiescence at the end of embryogenesis and resume proliferation during larval stages; however, no single neuroblast lineage has been traced from embryo into larval stages. Here, we establish a model neuroblast lineage, NB3-3, which allows us to reproducibly observe lineage development from neuroblast formation in the embryo, through quiescence, to the resumption of proliferation in larval stages. Using this new model lineage, we show a continuous sequence of temporal changes in the neuroblast, defined by known and novel temporal identity factors, running from embryonic through larval stages, and that quiescence suspends but does not alter the order of neuroblast temporal gene expression. We further show that neuroblast entry into quiescence is regulated intrinsically by two independent controls: spatial control by the Hox proteins Antp and Abd-A, and temporal control by previously identified temporal transcription factors and the transcription co-factor Nab.

KEY WORDS: *Drosophila*, Nab, Neuroblast, Quiescence, Temporal

INTRODUCTION

In the mammalian central nervous system (CNS), quiescent adult neural stem cells exist in both spinal cord and brain (Gould, 2007; Zhao et al., 2008). Quiescent adult neural stem cells in the mouse forebrain can generate multiple cell types over time, in response to niche-derived sonic hedgehog activity (Ahn and Joyner, 2005). Although such extrinsic controls of stem cell proliferation have been reported, the mechanisms by which neural stem cells/progenitors enter and exit quiescence remain largely unknown.

During development of *Drosophila* CNS, neural stem cells (neuroblasts; NBs) proliferate in the embryo to generate the neurons that drive larval behaviors. Once embryogenesis is completed, most abdominal NBs are eliminated through programmed cell death (Abrams et al., 1993; Peterson et al., 2002; White et al., 1994), whereas most of the cephalic and thoracic NBs enter mitotic quiescence at the embryo-larval transition. After a long period of quiescence in the larval stage, the quiescent NBs that transformed into larval types receive extrinsic mitogenic signals, such as Hedgehog, in a niche-dependent fashion, and resume cell division to produce the huge number of neurons needed to control the highly developed cephalic and thoracic segments of the adult fly (Barrett et al., 2008; Britton and Edgar, 1998; Datta, 1995; Ebens et al., 1993; Park et al., 2003; Prokop and Technau, 1991; Truman and Bate, 1988).

One advantage of studying neural stem cell quiescence in *Drosophila* is the ability to trace identified NBs and their lineages. NBs divide asymmetrically to bud off a series of daughter cells (ganglion mother cells; GMCs), each of which typically makes two postmitotic neurons. Each *Drosophila* hemisegment contains a set of ~30 NBs that can be individually identified and named according to their position within the hemisegment (Broadus et al., 1995; Doe, 1992). Each NB has a unique cell lineage (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997). NBs with identical positions in a segment, but located in different segments, share many gene expression and developmental features but generate slightly different segment-specific neuronal clones. For example, abdominal NB3-3 (NB3-3A) generates 11 Even-skipped (Eve)-positive neurons, whereas the thoracic NB3-3 (NB3-3T) generates just six Eve-positive neurons, yet the axon projections of these interneurons are virtually indistinguishable (Schmid et al., 1999; Schmidt et al., 1997).

A single NB can produce diverse neuronal cell types in an invariant order by changing its property over time. In the earliest stages of lineage development, NBs sequentially express a series of transcription factors: Hunchback (Hb), Seven-up (Svp), Krüppel (Kr), Pdm1/Pdm2 (Pdm) and Castor (Cas), which control the temporal change of NBs and temporal cell fate specification of their progeny (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kambadur et al., 1998; Kanai et al., 2005; Novotny et al., 2002; Pearson and Doe, 2003). As these transcription factors mutually regulate each other's expression in a network of feed-forward loops, the temporal change of NBs proceeds in a cell-intrinsic manner (Grosskortenhaus et al., 2005).

To ensure that a sufficient variety of neurons are generated before quiescence, NB entry into quiescence must be temporally regulated. However, the timing of entry into quiescence and whether it is controlled extrinsically or intrinsically in NBs is currently unknown. How NBs re-adopt the appropriate cell lineage upon re-entry to the cell cycle after quiescence is also unclear. Such issues have been difficult to address because markers and tools for analyzing the later stages of embryonic NB lineages have been lacking.

In this study, we first searched for transcription factors that are expressed temporally at the later stages of most, if not all, NB lineages, and characterized the model NB lineage NB3-3. This new

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model lineage allowed us to observe reproducibly the entire process of lineage development from embryogenesis to larval stages at the level of single cell divisions. We report that the sequence of temporal changes in NBs, defined by the switching of temporal transcription factor expression, runs continuously from embryonic to larval stages, and that quiescence suspends – but does not disrupt – the switching of temporal gene expression. Moreover, we report that NB entry into quiescence is regulated intrinsically and cooperatively by two independent controls: spatial control by Hox genes and temporal control by known temporal transcription factors in conjunction with the transcription co-factor Nab.

MATERIALS AND METHODS

Fly stocks

Flies were raised at 25°C or as otherwise noted. Fly stocks used are w^{1118}, $cas^{20\text{-}1}$, *eagle-GAL4*$^{27\text{-}1}$, *MZ360-eagle-GAL4* (Dittrich et al., 1997), *worniu-GAL4* (Lee et al., 2006), *pdm*^{ED773} and UAS-*pdm* (Grosskortenhaus et al., 2006), *nab*^{SH143} and *UAS-nab* (Terriente Felix et al., 2007), *nab*^{G26} (Clements et al., 2003), *sqz*^{ie} (Allan et al., 2003), *sqz*^{02102}, *Antp*^{25}, *UAS-abd-A*, *UAS-mCD8GFP*, *UAS-LacZ* and *scabrous-GAL4* (Bloomington Drosophila Stock Center). The *cas*$^{20\text{-}1}$ null mutant line was generated by imprecise P-element excision from the 1530 line (Cui and Doe, 1992). *UAS-sqz* was generated by inserting the cDNA fragment from RE47124 (BDGP) into pUAST. *eagle-GAL4*$^{27\text{-}1}$ was generated by inserting an *eagle* enhancer 2.0 kb fragment into pGAWB. To visualize NB3-3 and NB2-4 lineage clones, we crossed either *eagle-GAL4*$^{27\text{-}1}$ or *MZ360-eagle-GAL4* to *UAS-mCD8GFP* or *UAS-LacZ*. To misexpress *UAS-nab*, *UAS-pdm*, *UAS-sqz* or *UAS-abd-A*, we crossed *worniu-GAL4* or *scabrous-GAL4* to the *UAS* lines at 29°C.

Antibodies and immunostaining

Antibody staining for embryos or larval CNS was performed according to standard protocols. Primary antibodies used were mouse and rat anti-BrdU (1:200, BD Biosciences, Abcam), rabbit and mouse anti-GFP (1:500, Invitrogen, 1:200, Roche), mouse anti-b-galactosidase (1:300, Promega), guinea pig anti-Kr, guinea pig anti-Castor, guinea pig and rat anti-DmLin29, rat anti-Seven-up, guinea pig anti-Nab, rabbit anti-Even-skipped, guinea pig anti-Miranda (1:500), rat anti-Squeeze (1:750), rat anti-β-galactosidase (1:1000), rabbit and mouse anti-Miranda, (1:2000, 1:50) (Matsuzaki et al., 1998; Ohshiro et al., 2000), rabbit anti-Grainyhead (1:300) (Bello et al., 2006), rabbit anti-Nab (1:500) (Terriente Felix et al., 2007), rat anti-Pdm2 (1:10) (Grosskortenhaus et al., 2006), rabbit anti-Eagle (1:1000) (Karcavich and Doe, 2005), rabbit anti-Castor (1:2000) (Kambadur et al., 1998) and mouse anti-Even-skipped monoclonal 2B8 (1:50, DSHB). Detailed information about primary antibodies can be supplied upon request. Images were obtained using Zeiss LSM510 META or LSM5 LIVE confocal microscopes. Images and counted data were from T3 or A2-A6 segments unless otherwise noted.

Microarray analysis

We isolated the CNS from *cas*$^{20\text{-}1}$ homozygous and heterozygous embryos 12.5-13.5 hours after egg laying (AEL) (stages late 15 to early 16) in PBS. We used *Drosophila* Genome 2.0 Array (Affymetrix). Total RNA was extracted from the CNS and purified. Experiments were performed using 1 μg total RNA following the Affymetrix manual.

BrdU labeling

A 1-hour collection of embryos was dechorionated, permeabilized in octane for 4 minutes, and incubated in BrdU solution (BrdU 1 mg/ml in Schneider's medium) for 20 minutes. Immediately after labeling, embryos were fixed. As for larvae, a 5-hour collection of newly hatched larvae was transferred to BrdU-containing medium (BrdU 0.1 mg/ml), and grown for the specified time. To prepare 0 to 6 hour after larval hatching (ALH) larvae, each isolated CNS was incubated in BrdU-containing Schneider's medium (BrdU 1 mg/ml) for 20 minutes.

In vitro NB culture

In vitro NB culture was performed according to Grosskortenhaus et al. (Grosskortenhaus et al., 2005), except that NB cultures were made from 5 to 7 hour AEL embryos.

RESULTS

Thoracic NB3-3 enters quiescence and changes shape, whereas abdominal NB3-3 continues to proliferate

To investigate when and how NBs enter quiescence, we first examined the timing of entry into quiescence using a model NB, NB3-3. We chose NB3-3 because we could use anti-Eagle antibody or an *eagle-GAL4* transgene to identify NB3-3 and its progeny throughout embryonic and larval stages (Dittrich et al., 1997; Higashijima et al., 1996). To examine when NB3-3 stops entering a new S phase, we observed the incorporation of BrdU into NB3-3 during transient labeling (Fig. 1). When we labeled embryos at stages late 13 to 14 (10-11 hours AEL), 76% of thoracic NB3-3 (NB3-3T) and 92% of abdominal NB3-3 (NB3-3A) incorporated BrdU (Fig. 1A-C). Labeling just 1 hour later [stages late 14 to 15 (11-12 hours AEL)] resulted in a significant reduction of BrdU incorporation in NB3-3T cells, and after 12-hour AEL (stage 15) we rarely observed BrdU-positive NB3-3T (Fig. 1A,C). Thus, NB3-3T enters quiescence at stages late 14 to 15. By contrast, more than 70% of NB3-3A incorporated BrdU even at stage 16 (Fig. 1B,C), showing that NB3-3A proliferates. Despite the different number of GMCs made in each lineage, both lineages generate neurons with similar axonal projections (Schmid et al., 1999; Schmidt et al., 1997).

We also found that, upon entering quiescence, NB3-3T underwent marked changes in shape. We used Miranda, a cargo-binding protein involved in NB asymmetric cell division (Ikeshima-Kataoka et al., 1997), as a marker for visualizing NB shape. Whereas mitotic NBs were round, NB3-3T began to extend a thin protrusion around the time it entered quiescence (stages late 14 to 15) (Fig. 1A). This non-spherical shape persisted until at least stage 16, well after NB3-3T entered quiescence (Fig. 1A). At stage 16, many such elongated Miranda-positive cells were detected in thoracic, but not abdominal, segments. These cells never incorporated BrdU (data not shown). These observations strongly suggest that the elongated Miranda-positive cells are indeed quiescent NBs. Individual NBs underwent this shape change in a stereotypical order, rather than simultaneously at a specific time of development (Fig. 1E). Furthermore, isolated NBs cultured in vitro often exhibited quiescent NB-like features (see Fig. S1 in the supplementary material).

Next, we evaluated the timing of NB3-3T exit from quiescence in larvae by continuously feeding larvae with BrdU after hatching. NB3-3T could first be labeled with BrdU at late first-instar to early second-instar stages (20-25 hours ALH) and assumed a round shape at this point (Fig. 1D). We conclude that NB3-3T exits quiescence by late first-instar to early second-instar.

These observations revealed the period of quiescence in the model NB (NB3-3T), and that quiescent NBs have distinct morphological features that may reflect their unique cell physiology. Furthermore, the fact that NBs change shape asynchronously and in vitro suggests that an intrinsic property in each NB controls the timing of quiescence.

Relationship of sequential expression of temporal transcription factors and the timing of quiescence in NB3-3T and NB3-3A

NBs sequentially express a series of temporal identity factors that intrinsically regulate sequential neuronal identity (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kanai et al., 2005). To determine whether any correlation exists between sequential expression of these factors and quiescence, we analyzed the timing of expression of the known temporal transcription factors Hb, Kr, Pdm, Cas, Svp and Grainyhead (Grh) in NB3-3T (Almeida and Bray, 2005; Cenci

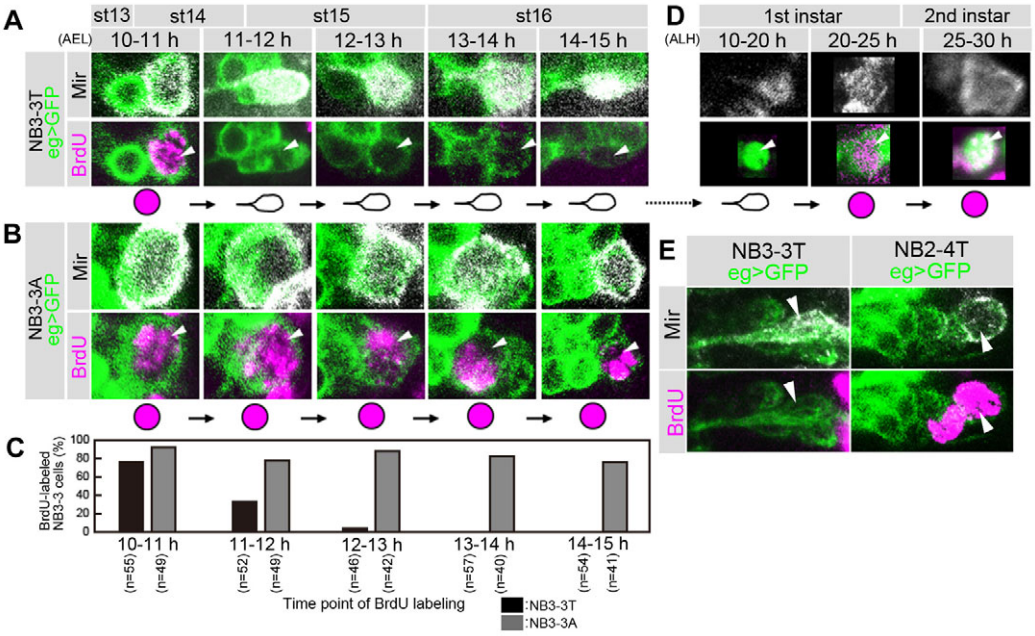

Fig. 1. Quiescence of NB3-3T and proliferation of NB3-3. NB3-3 (arrowhead) and its progeny were visualized by *eagle-GAL4>UAS-mCD8GFP* (*eg*>GFP; green) or *eagle-GAL4>UAS-LacZ* (*eg*>LacZ; green). *Drosophila* NBs were labeled using anti-Miranda (Mir; white). (**A-C**) Embryos were pulse-labeled with BrdU (magenta) at the indicated time (AEL, after egg laying). (A) Thoracic NB3-3 (NB3-3T) and (B) abdominal NB3-3 (NB3-3A). (C) BrdU-positive NB3-3 cells were counted. n, number of NB3-3 scored. (**D**) Wild-type larvae were continuously labeled with BrdU (magenta) from hatching until the indicated time (ALH, after larval hatching). (**E**) NB3-3T (left) and NB2-4T (right) from the same hemisegment of embryo pulse-labeled with BrdU at stage 15. When NB3-3T became elongated, its neighboring NB2-4T remained round and incorporated BrdU (magenta). The shape and BrdU incorporation of NB3-3 are represented diagrammatically beneath A,B,D.

and Gould, 2005; Maurange et al., 2008) (Fig. 2). NB3-3T was not Hb positive at birth but sequentially expressed Kr, Pdm, Cas, Grh and Grh/Cas (Fig. 2A,F). Two distinct windows of Cas expression have previously been noted (Cleary and Doe, 2006) but never analyzed in an identified NB. In NB3-3T, the second Cas expression phase began at stage 15, when NB3-3T started to change shape (i.e. enter quiescence), and Cas levels were maintained in the quiescent NB (Fig. 2A,F). Within the NB3-3T lineage, the quiescent NB was the only Cas-positive cell at late first-instar to early second-instar (20-25 hours ALH) (Fig. 2C,D); at this point the NB had increased Cas levels, returned to a round shape, began to incorporate BrdU and exited quiescence (Fig. 2C,D; Fig. 1D). By early- to mid-second instar (30-35 hours ALH), Cas expression decreased in NB3-3T, and the NB expressed the temporal factor Seven-up (Svp), which transiently overlapped with the Cas expression window (Fig. 2D). Similar to NB3-3T, most thoracic NBs showed transient late Svp expression in larval stages but not in embryos (see Fig. S2 in the supplementary material).

Next, we analyzed the NB3-3A lineage to determine whether it exhibited the same timing of temporal identity gene expression, despite its lack of a window of quiescence. We found that NB3-3T and NB3-3A expressed the temporal identity genes in the same order but that the sequence was faster in NB3-3A than in NB3-3T (Fig. 2A,B,F). In particular, the switch from the second phase of Cas expression to the phase of Svp expression in NB3-3A occurred in the embryo, but it occurred in the larva in NB3-3T (Fig. 2B,D). The switch to Svp expression in the late embryo was shared in many abdominal NBs (see Fig. S2 in the supplementary material).

We conclude that the model NB3-3 lineage allows us to track the sequence of temporal identity gene expression in abdominal segments (where it occurred rapidly and was completed during embryogenesis) and in thoracic segments (where it is interrupted by a period of quiescence and completed only in larval stages) (Fig. 2E,F). The fact that a period of quiescence delays but does not disrupt the sequence of temporal identity factor switching suggests that the NB can 'remember' its temporal identity during quiescence.

Hox proteins regulate segment-specific entry into NB quiescence

In embryos, NB3-3T becomes quiescent, whereas NB3-3A continuously proliferates. Among the Hox proteins, Antennapedia (Antp) and Abdominal-A (Abd-A) are expressed in NBs in thoracic T1-T3 segments and in abdominal A1-A7 segments, respectively (Carroll et al., 1986; Carroll et al., 1988; Hirth et al., 1998; Prokop et al., 1998). We therefore investigated whether these two Hox proteins are involved in the regulation of quiescence in NB3-3A and NB3-3T (Fig. 3).

In *Antp* mutant embryos, NB3-3T incorporated BrdU until late in embryogenesis, and maintained a round shape (Fig. 3J). Thus, Antp is required for NB3-3T to enter quiescence during mid-embryogenesis. Moreover, *Antp* mutants occasionally showed precocious Svp expression in NB3-3T during embryogenesis (13%, *n*=23) (Fig. 3A,C,G). Corresponding to the change in mitotic behavior, *Antp* mutant NB3-3T generated an increased number of GMCs and neurons. Wild-type NB3-3T in the T3 segment typically produced seven GMCs that generated six Eve-positive lateral interneurons (EL neurons) (Fig. 2F; Fig. 3B), whereas *Antp* mutants generated as many as nine EL neurons (average of 7.8 cells, *n*=41) (Fig. 3D). This is an increase in number but is still fewer than the number of EL neurons produced by NB3-3A: at least 12 GMCs generated 11 EL neurons (Fig. 2F, Fig. 3H; see Fig. S3 in the supplementary material) (see also sections below).

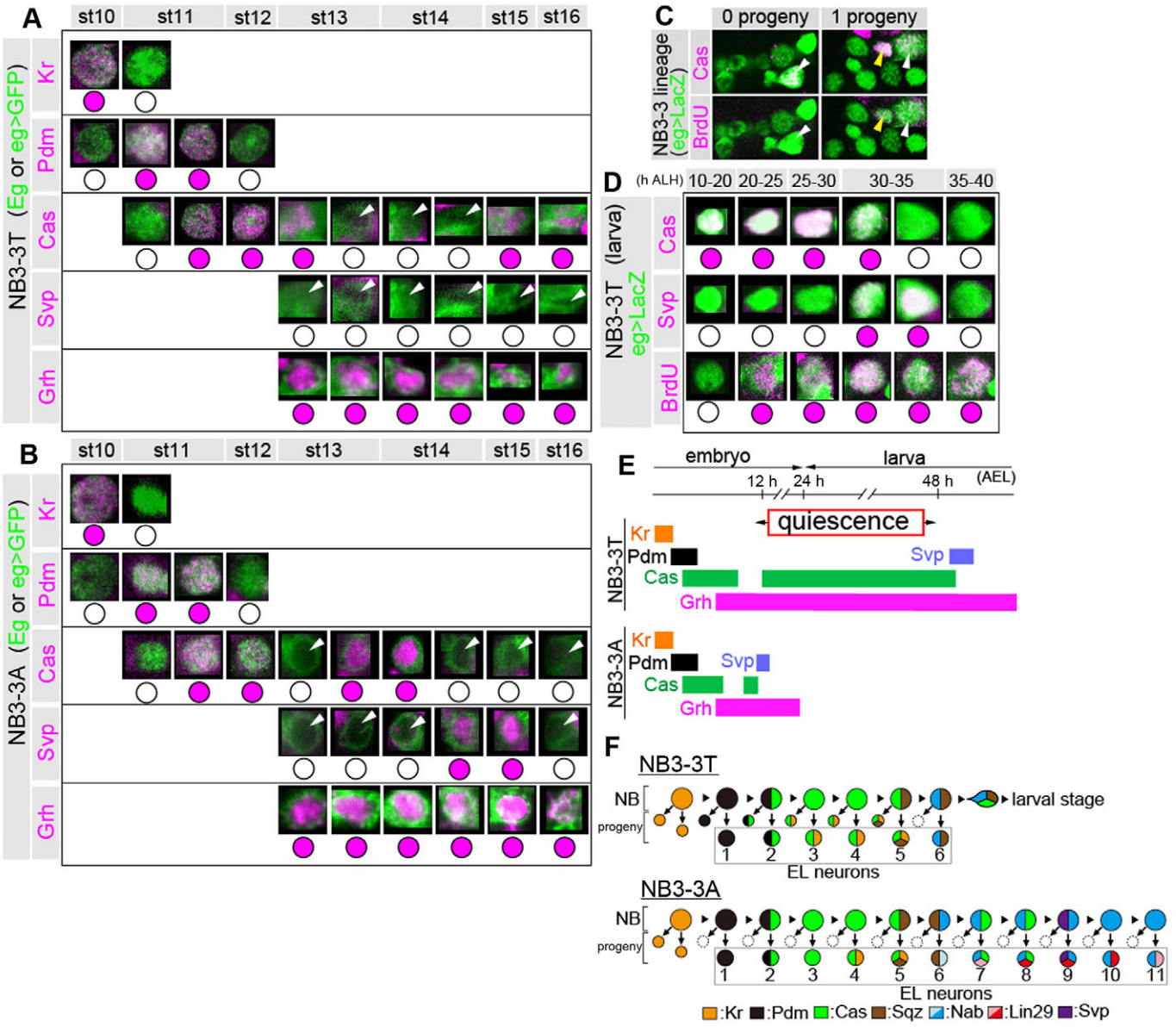

Fig. 2. Sequential expression of temporal transcription factors in NB3-3T and NB3-3A. The *Drosophila* NB3-3T and NB3-3A lineages were visualized using Eg, *eg*>GFP or *eg*>LacZ (green). White arrowheads indicate NB3-3. (**A**,**B**) Kr, Pdm, Cas, Svp and Grh expression (magenta) in NB3-3T (A) and NB3-3A (B) during embryogenesis. (**C**) NB3-3T lineage from wild-type larva at 20-25 hours ALH (0 progeny, left) and 25-30 hours ALH (1 progeny, right) continuously labeled with BrdU from hatching. Cas expression and BrdU incorporation are shown in magenta. Yellow arrowhead indicates the first NB3-3T larval progeny. (**D**) Cas and Svp expression and BrdU incorporation (magenta) in larval NB3-3T at the indicated time (ALH). (**E**) Schematic of the quiescence period in NB3-3T and expression of the temporal transcription factors in NB3-3T (upper) and NB3-3A (lower). (**F**) Schematic model of NB3-3 lineage development. Expression patterns of the temporal factors are shown by the indicated color. EL neurons are enclosed by gray rectangles. The late Cas expression was downregulated without Svp function (data not shown).

We further characterized the NB3-3 lineage using a marker: the protein encoded by the gene *CG2052*. This protein is a Krüppel-type zinc-finger protein related to *Caenorhabditis elegans* LIN-29, which we named DmLin29. DmLin29 was expressed in most or all late-born neurons (data not shown). We thus consider DmLin29 as a marker for late-born neurons. In the NB3-3T lineage, DmLin29 was not expressed in any of the six EL neurons but was expressed after quiescence in larval stages (Fig. 2F, Fig. 3B,I; see Fig. S4 in the supplementary material). Not surprisingly, *Antp* mutants that had a delay in NB3-3T quiescence showed up to three DmLin29-positive EL neurons (average of 2.3 cells, n=18), born after the first six DmLin29-negative EL neurons (Fig. 3D). This was similar to the NB3-3A lineage, which does not enter quiescence at mid-embryogenesis and generated five DmLin29-positive late-born EL neurons during embryogenesis (Fig. 2F, Fig. 3H,I; see Fig. S3 in the supplementary material). We can thus conclude that in *Antp* mutants NB3-3T fails to enter quiescence during mid-embryogenesis, leading to the precocious expression of Svp and DmLin29 during embryogenesis.

When the abdominal Hox gene *abd-A* was continuously expressed in all NBs beginning at about stage 11, NB3-3T exhibited the same phenotype as loss of *Antp*: a failure to enter quiescence at

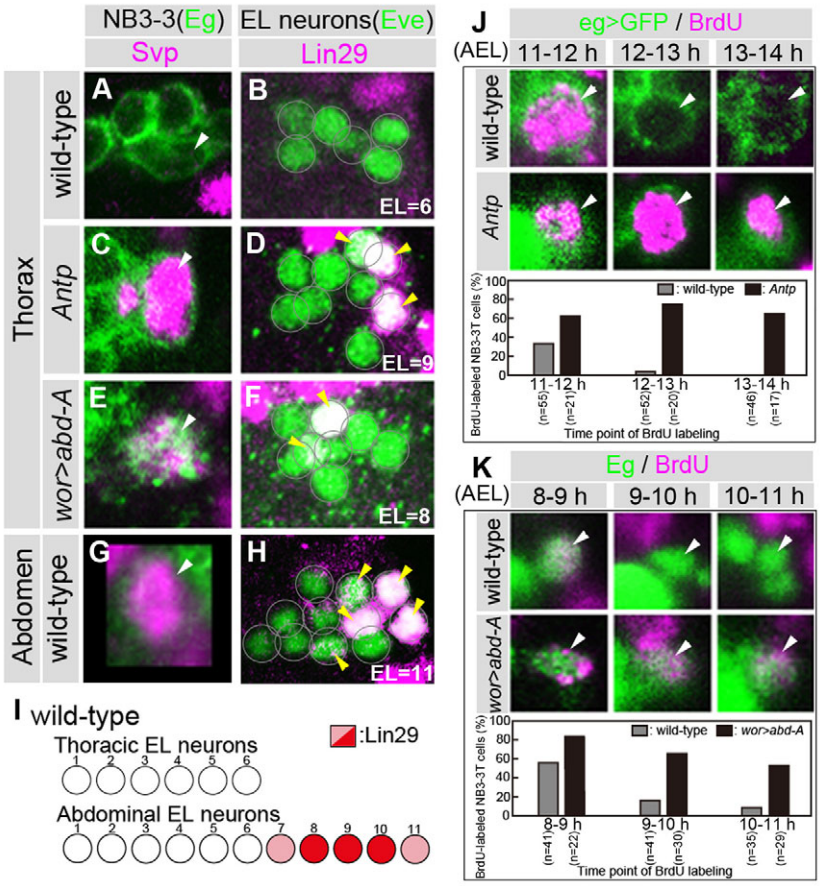

Fig. 3. Hox genes spatially regulate NB quiescence. *Drosophila* embryos were labeled for markers indicated at the top. (**A-H**) NB3-3T (A,C,E; stage 16) and thoracic EL neurons (B,D,F; stage 17) of wild-type (A,B), *Antp* (C,D) and *wor>abd-A* (E,F) and NB3-3A (G, stage 15), and abdominal EL neurons (H, stage 17) of wild type. White arrowhead, NB3-3; yellow arrowhead, DmLin29-positive EL neuron. (**I**) Schematics of DmLin29 expression in EL neurons. The seventh to 11th late-born abdominal EL neurons are DmLin29 positive, although the expression in seventh- and 11th-born EL neurons is hard to detect at stage 17 owing to the decay and late expression of DmLin29. (**J**,**K**) BrdU incorporation in wild-type *Antp* (J) and *wor>abd-A* (K) NB3-3T (arrowhead) pulse-labeled at the indicated times. In K, embryos were grown at 29°C. n, number of NB3-3T scored.

the appropriate time and an extension of its embryonic cell lineage (compare Fig. 3E,F,K with Fig. 3C,D,J). The conversion of the lineage pattern was confirmed by examining expression of temporal identity factors (see Fig. S5 in the supplementary material). We conclude that Hox proteins Antp and Abd-A spatially control NB entry into quiescence.

Pdm inhibits NB quiescence

Because NB3-3T enters quiescence at a specific time in its lineage, quiescence must be controlled temporally during the sequential switching of transcription factors. A series of four temporal identity factors – Hb, Kr, Pdm and Cas – is known to regulate temporal identity within several NB lineages (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Novotny et al., 2002). Thus, we tested whether any of these factors also regulate the timing of entry into quiescence (Fig. 4). In *pdm* mutants, many thoracic NBs prematurely changed to an elongated cell shape and appeared to enter quiescence (Fig. 4C,G). By contrast, in *cas* mutants most thoracic NBs retained the round shape characteristic of proliferating NBs (Fig. 4B,F). When we assayed the model NB3-3 lineage, we found that *pdm* mutants showed precocious quiescence of NB3-3T and generated fewer thoracic EL neurons (average of 4.2 cells, *n*=20) (Fig. 4K,K′,W,Z). *pdm* mutant NB3-3T lost its ability to incorporate BrdU 3 hours earlier than in the wild type (Fig. 4Y, compare with wild type in Fig. 1C). By contrast, *cas* mutants showed prolonged proliferation of NB3-3T and generated extra thoracic EL neurons (average of 6.8 cells, *n*=15) (Fig. 4J,J′,V,Z). We conclude that Pdm delays quiescence in NBs, whereas Cas induces quiescence.

How do Pdm and Cas oppositely regulate quiescence? *cas* mutants show prolonged Pdm expression in NBs (Grosskortenhaus et al., 2006), suggesting that the *cas* mutant phenotype (delayed quiescence) may simply be due to prolonged Pdm expression. To test this hypothesis, we assayed *pdm cas* double mutants. In *pdm cas* double mutants, we found that most NBs showed precocious quiescence similar to *pdm* mutants (Fig. 4D,H,L,L′,S,T,X). Furthermore, forced expression of Pdm delayed quiescence, regardless of Cas (see Fig. S6 in the supplementary material). Pdm thus acts downstream of Cas to inhibit NB quiescence. Because Pdm expression normally disappeared 3-4 hours before NB3-3T entered quiescence, Pdm probably controls expression of other genes to regulate the timing.

nab is a Pdm-regulated gene required for triggering NB quiescence

To identify unknown Pdm-regulated factors involved in triggering NB quiescence, we performed microarray analyses to compare wild-type embryos with *cas* mutant embryos, in which entry into quiescence is inhibited due to prolonged Pdm. We selected genes that show significant differences in expression at the time when most NBs enter quiescence. Genes that are upregulated in *cas* mutant (Pdm overexpression) embryos might be involved in inhibiting quiescence, and conversely downregulated genes are good candidates for promoting quiescence. Among the candidate genes that showed severe reductions at the transcript level in *cas* mutant embryos was *nab*, which encodes a transcriptional cofactor of the NGFI-A-binding protein family (Clements et al., 2003; Terriente Felix et al., 2007). Although it has previously been reported that Nab

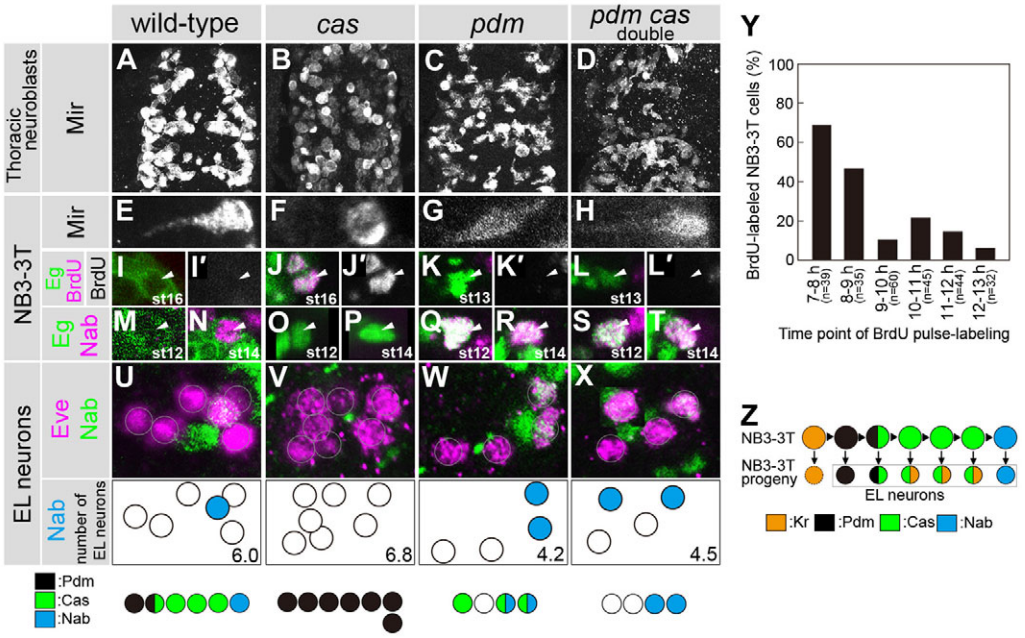

Fig. 4. Temporal transcription factors regulate the timing of entry to quiescence. (A-X) *Drosophila* embryos were labeled for the indicated markers. Genotypes are shown at the top. NB3-3T was identified by *eg*>GFP or Eg (green). (**A-H**) Thoracic NBs (A-D) and NB3-3T (E-H) at stage 16. In wild-type (A,E), *pdm* (C,G) and *pdm cas* (D,H) embryos, NBs become elongated, whereas *cas* mutant NBs remain round (B,F). (**I-L′**) NB3-3T (white arrowhead) in embryos pulse labeled with BrdU (I-L, magenta, I′-L′,white) at the indicated stages. In *pdm* (K,K′) and *pdm cas* (L,L′) embryos, BrdU incorporation is only occasionally observed in NB3-3T at stage 13. In *cas* embryos (J,J′), BrdU incorporation is still observed at stage 16. (**M-T**) Nab expression (magenta) in NB3-3T. (**U-X**) Thoracic EL neurons at stage 16 or 17. In *cas* embryos (V), the number of EL neurons increases and Nab expression is not detected, whereas in *pdm* (W) and *pdm cas* (X) embryos, the number of EL neurons decreases and Nab-positive EL neurons are observed precociously. Bottom: phenotype summaries. (**Y**) The number of BrdU-positive NB3-3T in *pdm* embryos. n, number of NB3-3T scored. (**Z**) Schematic model of formation of thoracic EL neurons.

is temporally expressed in many NB lineages, and that *cas* mutants lose Nab expression from all but a medial NB (Clements et al., 2003), Nab function in NBs has not been investigated.

To test the function of Nab in NBs with respect to NB quiescence, we examined the patterns of Nab expression within the NB3-3 lineage (Fig. 5). NB3-3T started expressing Nab just after Cas became absent (at the time NB3-3T became quiescent), and then Nab expression was maintained in the quiescent NB during embryogenesis (Fig. 2F; Fig. 5A). Although the *nab* transcript was expressed predominantly in NBs, Nab protein was also detected in GMCs and neurons (presumably inherited from the parental NB). In addition to Nab-positive NB3-3T, we detected Nab protein in the sixth-born EL neuron in the lineage (Fig. 2F; Fig. 5C; see Fig. S3 in the supplementary material). This suggests that Nab expression starts just before NB3-3T produces its last embryonic GMC that gives rise to the sixth-born EL neuron. Nab was expressed precociously in *pdm* mutant and in *pdm cas* double mutant NB3-3T, and was not detectable in *cas* mutants (Fig. 4M-T). Thus, the onset of Nab expression correlates with NB3-3T entry into quiescence in wild type and several mutant genotypes.

To determine whether Nab is actually involved in triggering NB quiescence, we assayed the NB3-3T lineage using BrdU pulse-labeling (Fig. 6). In *nab* mutant embryos, NB3-3T continued to incorporate BrdU well beyond its normal time of quiescence (Fig. 6A); consequently, NB3-3T generated additional EL neurons (as many as eight cells, an average of 7.1 cells, *n*=23) (Fig. 7B). This effect was not limited to NB3-3T; *nab* mutant first instar larvae showed delayed quiescence and prolonged proliferation of many thoracic NBs, based on their round morphology and ability to incorporate BrdU, which was not seen in wild type (Fig. 6B). We conclude that *nab* is a Pdm-regulated gene that is required for triggering NB entry into quiescence (Fig. 6E).

Squeeze, a putative Nab partner, is required for timely triggering of NB quiescence

Nab function requires the presence of a co-factor. In *Drosophila*, two transcription factors, Squeeze (Sqz) and Rotund, can associate with Nab (Terriente Felix et al., 2007). Because *sqz* was transcribed in a temporal pattern within the CNS, we focused our attention on Sqz. As with Nab, Sqz protein level was controlled by temporal factors (see Fig. S7 in the supplementary material). In NB3-3T, we detected Sqz protein slightly prior to Nab (Fig. 2F; Fig. 5A), with Sqz detected in the fifth-born EL neuron (Fig. 2F; Fig. 5A,C,E), one cell division prior to Nab (Nab is detected in the sixth-born EL neuron; see above).

Consistent with the idea that Nab acts as a co-factor of Sqz, Sqz and Nab were co-expressed at high levels when NB3-3T entered quiescence (Fig. 2F; Fig. 5A). Sqz expression declined over time in proliferating larval stages. By contrast, Nab expression continued.

To investigate Sqz function, we examined two genotypes: a null sqz^{ie} mutant and a strong hypomorphic *sqz* mutant (in which Sqz protein can barely be detected) (Figs 6 and 7). We found that the loss or reduction of Sqz function led to a delay in NB3-3T entering quiescence, and a corresponding increase in the number of EL neurons (as many as eight cells, an average of 7.1 *n*=31) (Fig. 6A,E; Fig. 7D,E). Despite the delay, NB3-3T eventually entered quiescence, as we found no proliferating NBs in the thorax of newly hatched *sqz* mutant larvae (Fig. 6B). As expected, the extra seventh-born EL neurons in *sqz* mutants were occasionally DmLin29

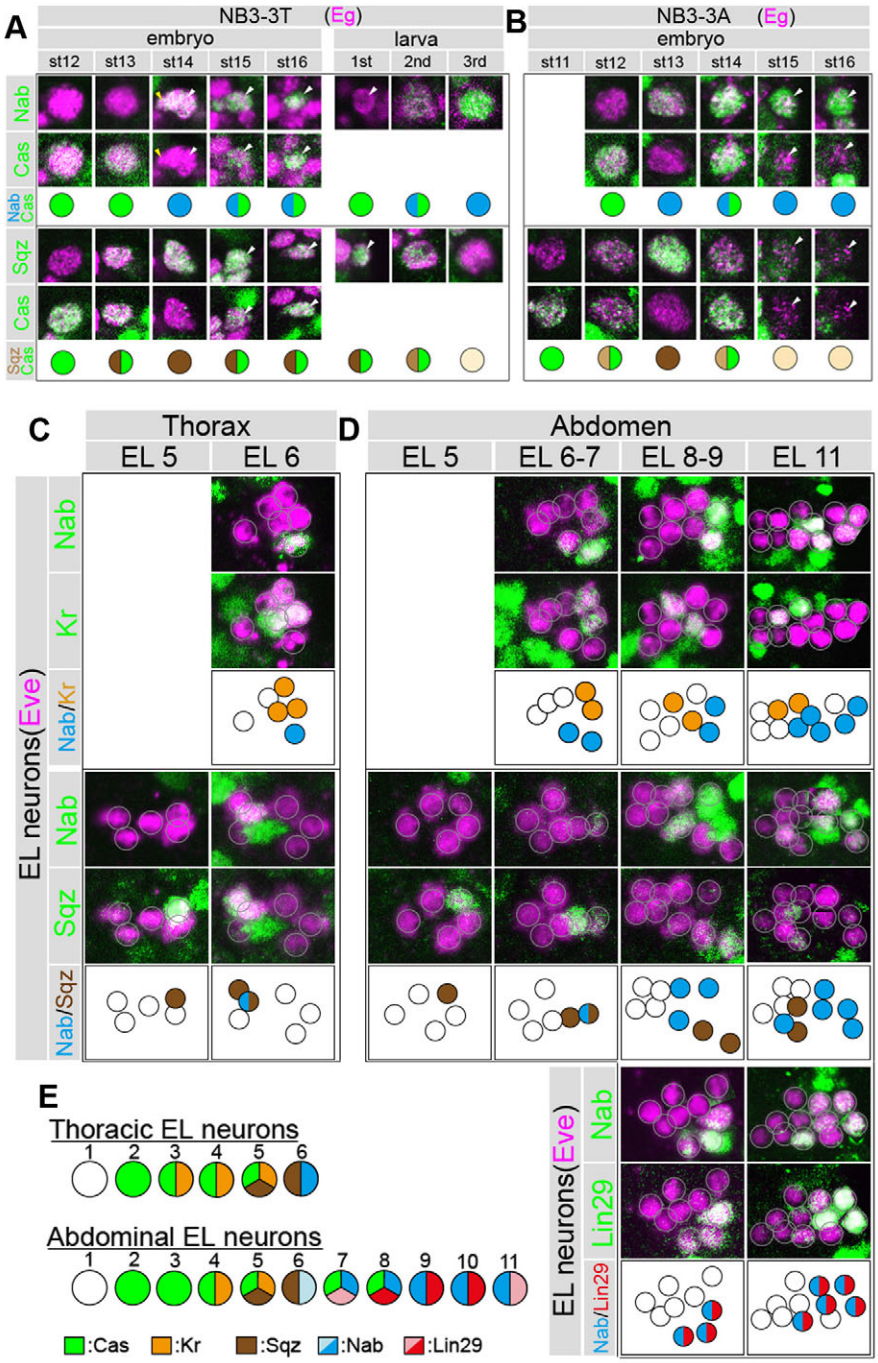

Fig. 5. Expression of Nab and Sqz in the *Drosophila* NB3-3 lineage. (**A**,**B**) Nab (green, upper row in the upper box) and Sqz (green, upper row in the lower box) expression in thoracic (A) and abdominal (B) NB3-3 (white arrowhead), compared with Cas expression (green, middle row in each box) at the indicated stage. NB3-3 lineage is visualized by Eg (magenta in embryonic stage) or *eg*>LacZ (magenta in larval stage). Yellow arrowhead, Nab-positive NB3-3 progeny. (**C**,**D**) Nab, Sqz, Kr and DmLin29 expression (green) in thoracic (C) and abdominal (D) EL neurons (identified by Eve, magenta) at the point when the indicated number of EL neurons exist. Schematic diagrams of the expression patterns are shown at the bottom row in each box. Nab expression in abdominal sixth-born EL neuron was often reduced at late stages. (**E**) Schematic diagrams of gene expression in EL neurons.

positive (Fig. 7D,E). Sqz is unlikely to regulate NB quiescence by controlling the expression of Nab, because *sqz* mutants usually showed normal timing of Nab expression in the sixth-born EL neuron (79%, *n*=29, data not shown).

To test whether Sqz is indeed an obligatory co-factor for Nab in inducing quiescence, we examined whether Nab and Sqz can rescue the failure of NB quiescence in *cas* mutants. Although expression of either Nab or Sqz alone did not rescue *cas* mutant phenotype, co-misexpression of Nab and Sqz was able to induce quiescence in the absence of Cas (Fig. 6F; see Fig. S8 in the supplementary material). These results are consistent with the idea that Nab and Sqz – two novel downstream mediators of Pdm – act together to regulate gene expression to initiate NB quiescence.

Nab and other downstream mediators of Pdm cooperate to regulate NB quiescence

Despite the ability of Nab/Sqz to bypass the requirement of Cas to induce NB quiescence, several lines of evidence suggest that additional factors downstream of Pdm are involved in determining the timing of entry into quiescence. First, precocious expression of Nab alone and precocious co-expression of Nab and either Sqz or Rotund were all insufficient to accelerate the entry into quiescence (Fig. 6C and data not shown). Second, when co-misexpression of Nab and Sqz rescued the NB quiescence phenotype of the *cas* mutant, this manipulation also rescued the prolongation of Pdm expression that occurs in *cas* mutant (Fig. 6F; see Fig. S8 in the supplementary material). This was

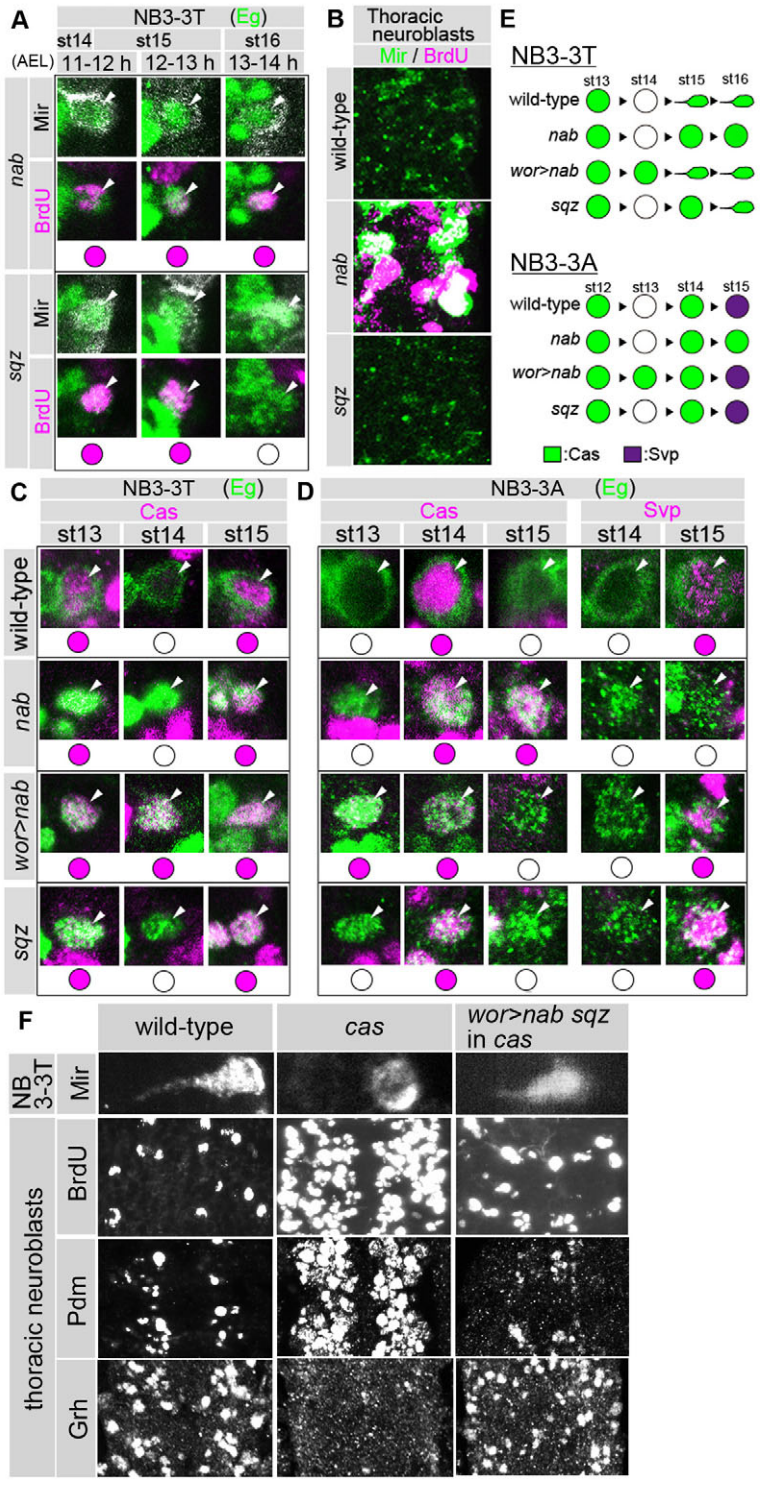

Fig. 6. Nab and Sqz regulate temporal state and NB quiescence. (**A**) BrdU incorporation (magenta) in NB3-3T (white, visualized by Mir) of *nab* and *sqz*^{02102} *Drosophila* embryos pulse-labeled at the indicated times. NB3-3 lineage is visualized by Eg (green). (**B**) BrdU incorporation (magenta) in the thoracic hemisegment of wild-type, *nab* and *sqz*^{02102} newly hatched larva (0-6 hours ALH). (**C**,**D**) Cas expression (magenta) in NB3-3T (C) and Cas and Svp expression (magenta) in NB3-3A (D) from embryos. Svp expression is not detected in NB3-3T (data not shown). Genotypes are shown on the left. The NB3-3 lineage is visualized by *eg*>GFP or Eg (green). Arrowhead indicates NB3-3. (**E**) Phenotype summaries. In *sqz*^{ie} *nab* double mutants, NB quiescence was inhibited as in *nab* mutants (data not shown). (**F**) NB3-3T (visualized by Mir), BrdU incorporation, Pdm expression and Grh expression in thoracic NBs of wild-type, *cas* and *wor>nab sqz* in *cas* embryos at stage 16. The co-expression of Nab and Sqz rescues the defect in Grh expression, indicating that NBs maintain stem cell characteristics and undergo temporal change.

unexpected because the loss of function of either *nab* or *sqz* does not affect Pdm expression (see Fig. S9 in the supplementary material). Thus, co-misexpression of Nab and Sqz might have resulted in proper expression of other genes downstream of Pdm, through an artificial downregulation of Pdm. Taken together, these results suggest that the pathway that controls NB quiescence branches at Pdm, and that Nab/Sqz and another yet unidentified factor cooperate to regulate the timing of quiescence (see Fig. S10 in the supplementary material).

Nab function in specifying temporal identity

Because Nab is expressed temporally in thoracic as well as abdominal NBs, Nab may have a dual function to trigger NB quiescence and also to regulate temporal identity. We tested this hypothesis by assaying temporal identities within thoracic and abdominal NB3-3 lineages. Wild-type NB3-3 sequentially expressed Cas, then Sqz, then Nab/Cas (Fig. 2F; Fig. 5A,B). Loss of *nab* did not affect the timing of the first Cas expression phase, or the initiation of the second Cas expression (Fig. 6C-E). However, the late phase of the lineage was severely

affected in the *nab* mutant, as assayed by expression of the neuronal temporal identity markers Kr (which is expressed in the middle-born EL neurons) and Dmlin29 (which is expressed in late-born EL neurons) (Fig. 2F; Fig. 7A,E). Loss of *nab* led to Kr de-repression in all EL neurons that are born subsequent to normal Kr-positive neurons, with concomitant loss of the late-born identity marker DmLin29 (Fig. 7B,E). Moreover, in the *nab* mutant, Svp expression was never observed, and Sqz expression and the second Cas expression phase were prolonged (Fig. 6D,E; see Fig. S9 in the supplementary material). We conclude that Nab is required for proper specification of late temporal identity within the NB3-3 lineage.

We next investigated whether precocious expression of Nab can switch temporal fates. When Nab was misexpressed in all embryonic NBs from late stage 11 and beyond, the middle phase of the lineage was affected; the second Cas expression phase was initiated precociously (Fig. 6C-E), Kr was absent from EL neurons (only 5% left, n=20) (Fig. 7C,E) and the sixth-born thoracic EL neuron was abnormally DmLin29 positive (Fig. 7C,E). We conclude that precocious expression of Nab can shift the temporal identity towards that of later stages. By contrast, precocious expression of Nab was insufficient to trigger NB quiescence at earlier stages (Fig. 6C; Fig. 7C and data not shown). The *sqz* mutant also showed Kr de-repression, whereas no gross abnormalities in transcription factor switching and DmLin29 expression were seen (Fig. 6C,D; Fig. 7D,E; see Fig. S9 in the supplementary material). We conclude that Nab both regulates temporal identity and induces NB quiescence in the NB3-3T lineage; these phenotypes were not perfectly correlated, suggesting that Nab likely acts via distinct pathways to regulate each process.

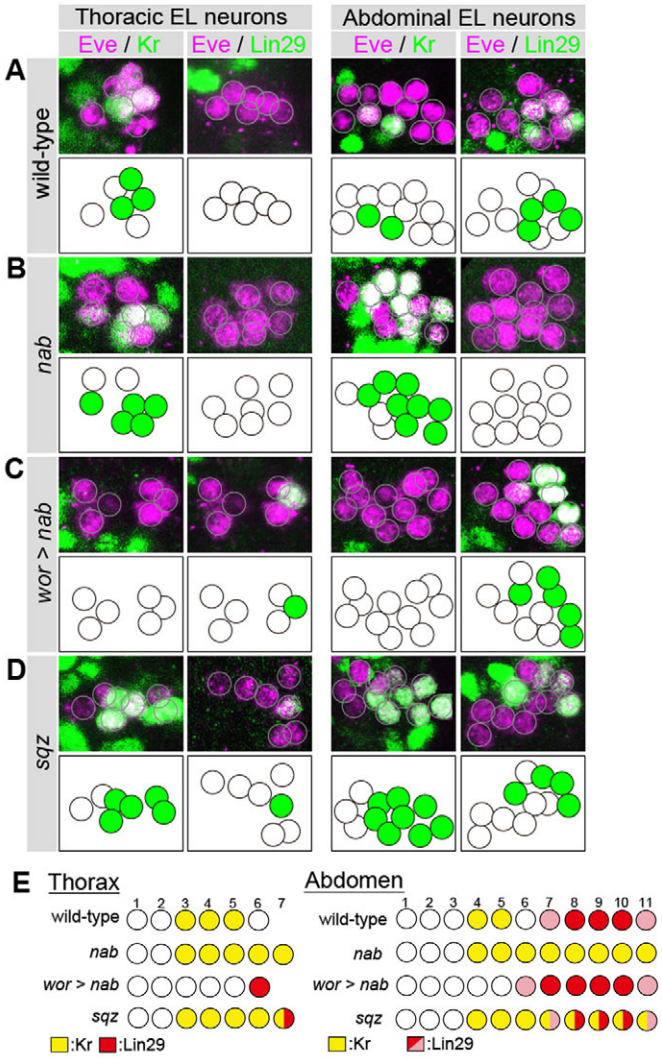

Fig. 7. Nab and Sqz regulate temporal cell fates of NB3-3 progeny. (**A-D**) Thoracic and abdominal EL neurons (Eve, magenta) of wild-type (A), *nab* (B), *wor>nab* (C) and sqz^{02102} (D) stage 17 *Drosophila* embryos. Kr and DmLin29 expression is shown in green. In *nab* (B) and sqz^{02102} (D) embryos, the number of thoracic EL neurons increased. In *nab* embryos (B), DmLin29 expression is not detected. In *wor>nab* embryos (C), although the number of EL neurons is mostly unchanged, Kr-positive EL neurons are missing and DmLin29-positive EL neurons are observed in the thorax. (**E**) Phenotype summaries.

DISCUSSION

Establishing the NB3-3T and NB3-3A lineages from embryos to larvae

We have revealed for the first time the temporal changes in a *Drosophila* NB lineage from embryonic NB formation, through entry into quiescence, to resumption of proliferation in larval stages. Using a model NB system with which we can reproducibly trace the complete lineage formation at the resolution of individual cell divisions, we have shown that despite considerable differences in extracellular environment the temporal changes – as defined by the switching of transcription factor/co-factor expression – proceeded continuously in each NB throughout the embryonic and larval stages. Moreover, we have found mutual regulation between quiescence and the series of the temporal transcription factors/co-factor; the temporal transcription factors/co-factor endogenously control the timing of triggering NB quiescence, which in turn suspends the switching of late temporal transcription factor expression.

Spatial regulation of NB entry into quiescence

In the *Antp* mutant and following ectopic expression of Abd-A there was a lack of NB quiescence, and consequently we observed what appeared to be a precocious generation of larval neurons during embryogenesis. This strongly supports the notion that temporal changes in NBs actually continue in sequence before and after quiescence, i.e. through embryogenesis and larval stages, and in the absence of quiescence the changes occur precociously. In addition, this indicates that spatial and temporal factors control NB quiescence through independent routes (see Fig. S10 in the supplementary material).

Antp mutants did not exhibit NB3-3T quiescence in all thoracic T1-T3 segments. In *Antp* mutants, epidermis in T2 and T3 segments transform into that in the T1 segment, and some thoracic NB lineages retain thoracic-specific features (Berger et al., 2005; Martinez-Arias, 1986; Wakimoto and Kaufman, 1981). These facts indicate that the inhibition of NB3-3T quiescence by *Antp* mutation is not just a consequence of global transformation of thoracic-to-abdominal segments but rather results from specific effects on individual NBs. NB-specific misexpression of Abd-A also repressed Antp and inhibited NB3-3T quiescence (Fig. 3K and data not shown). This also provides evidence that the effect is specific to NBs. Furthermore, because the effect could be observed even when Abd-A was induced after several divisions of the NB, thoracic NBs probably maintain plasticity of their identities during lineage formation.

Temporal regulation of NB entry into quiescence

We showed that the temporal transcription factors/co-factor Pdm, Cas, Sqz and Nab play a role in triggering NB quiescence intrinsically in NBs (Figs 4 and 6; see Fig. S10 in the supplementary material). All of

these factors also controlled temporal specification within late lineages of embryonic NBs in both thoracic and abdominal segments. We confirmed this by further examining the relationships of the temporal factors (Figs 4, 6 and 7; see Fig. S7 and Fig. S9 in the supplementary material). For example, the loss of Pdm function in NB3-3T resulted in precocious transcription factor switching and precocious quiescence. Conversely, in *cas* mutant embryos, in which Pdm expression was de-repressed, quiescence was inhibited and expression of late-stage-specific temporal factors disappeared. Similar to Pdm upregulation, loss of *nab* function resulted in loss of both transcription factor switching and quiescence.

Although Nab and Sqz can form a complex, *nab* and *sqz* mutants displayed very different phenotypes. Both mutants showed de-repression of Kr expression; however, *sqz* mutants showed no other abnormality in transcription factor switching, whereas *nab* mutants showed the above-mentioned defects in transcription factor switching and timing of quiescence. These mutant phenotypes revealed that regulation of late temporal events is distributed into multiple pathways. Pdm probably coordinately regulates all factors involved in the timing of NB quiescence, because the loss of Pdm alone is sufficient to cause precocious entry into quiescence.

Function of Nab and Sqz in lineage formation

We showed that Nab and Sqz work for NB quiescence in NBs. The Nab/Sqz-mediated repression of Kr may be controlled in NBs due to changes in NB temporal identity, or in both NBs and their neurons. Nab might inhibit transcription by recruiting the nucleosome remodeling and deacetylase chromatin remodeling complex as mammalian Nab does (Srinivasan et al., 2006). Mammalian Nab acts with EGR-1, EGR-2 to determine the fate of cells in hematopoiesis (Laslo et al., 2006; Svaren et al., 1996), but whether it can act with the mammalian homolog of LIN-29/Sqz has not been reported. Loss of *lin-29*, a *C. elegans* homolog of *sqz*, causes a heterochronic phenotype in which adulthood is not reached and molting is repeated (Ambros and Horvitz, 1984; Rougvie and Ambros, 1995). *C. elegans* has a *nab* homolog gene, *mab-10*, that acts with *lin-29* in a heterochronic genetic cascade (D. Harris and H. R. Horvitz, personal communication).

Quiescent NBs memorize temporal identity during quiescence

It is unclear what molecular mechanisms enable NBs to suspend the switching of transcription factor expression and maintain temporal identity during quiescence. We know that the mechanisms for switching expression of early temporal transcription factors can be either cell division dependent or independent (Grosskortenhaus et al., 2005; Mettler et al., 2006). Irrespective of the mechanism used, a NB can 'memorize' its temporal state before quiescence and resume the intrinsic temporal changes once cell cycle progression is reactivated. Embryonic stem cells may maintain multipotency during a slow proliferation state by staying in S phase (Andang et al., 2008). When quiescent NBs re-entered the cell cycle, their initial progeny incorporated BrdU fed since hatching (Fig. 2C), indicating that quiescent NBs stay either prior to S phase or early in S phase. It will be important to identify the point in the cell cycle at which NB enters quiescence.

Evolution of temporal lineage development

Another well-established mechanism that governs temporal aspects of lineage formation is the heterochronic gene cascade in *C. elegans*. This cascade contains one each of the *hunchback* homolog and *lin-29* genes and generates five distinct temporal cell identities within a single cell lineage (Moss, 2007). *Drosophila* NB lineage formation uses two types of Zn-finger proteins, namely the Hb/Cas class [Cas shares DNA-binding characteristics with Hb (Kambadur et al., 1998)] and the Kr/LIN-29 class. These pairs are expressed three times in NB lineages in the following order: (1) Hb and Kr→(2) Cas, Kr and Sqz→(3) Cas and DmLin-29→end of lineage. This sequence seems to produce at least ten distinct temporal identities within an NB lineage. The repetitive use of these temporal transcription factors in three distinct phases appears to have made the NB lineage longer and more diverse. Lack of Hb also generates NB lineage variety; the NB3-3 and NB2-1 lineages lack Hb and initiate their lineage with Kr. Although the model NB employed in this study lacks Hb, the sequence and entry into quiescence we described here are common to many typical NB lineages that start with Hb. Interesting questions from the perspective of evolution are how do the three phases combine to form a single lineage and how has NB quiescence evolved in the middle of the NB lineages?

Neural stem cells in the mouse cerebral cortex go through ~11 divisions and some enter quiescence in late embryo (Merkle et al., 2004; Takahashi et al., 1995; Ventura and Goldman, 2007). We thus consider the possibility that mammalian neural stem cell and *Drosophila* NB share a similar intrinsic mechanism that induces quiescence.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/23/3859/DC1

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