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Pdm and Castor close successive temporal identity windows in the NB3-1 lineage

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Neurogenesis in *Drosophila* and mammals requires the precise integration of spatial and temporal cues. In *Drosophila*, embryonic neural progenitors (neuroblasts) sequentially express the transcription factors Hunchback, Kruppel, Pdm1/Pdm2 (Pdm) and Castor as they generate a stereotyped sequence of neuronal and glial progeny. Hunchback and Kruppel specify early temporal identity in two posterior neuroblast lineages (NB7-1 and NB7-3), whereas Pdm and Castor specify late neuronal identity in the NB7-1 lineage. Because Pdm and Castor have only been assayed in one lineage, it is unknown whether their function is restricted to neuronal identity in the NB7-1 lineage, or whether they function more broadly as late temporal identity genes in all neuroblast lineages. Here, we identify neuronal birth-order and molecular markers within the NB3-1 cell lineage, and then use this lineage to assay Pdm and Castor function. We show that Hunchback and Kruppel specify first and second temporal identities, respectively. Surprisingly, Pdm does not specify the third temporal identity, but instead acts as a timing factor to close the second temporal identity window. Similarly, Castor closes the third temporal identity window. We conclude that Hunchback and Kruppel specify the first and second temporal identities, an unknown factor specifies the third temporal identity, and Pdm and Castor are timing factors that close the second and third temporal identity windows in the NB3-1 lineage. Our results provide a new neuroblast lineage for investigating temporal identity and reveal the importance of Pdm and Cas as timing factors that close temporal identity windows.

KEY WORDS: Castor, Pdm (Nubbin), Cell fate, Lineage, Temporal identity, Timer, Hunchback, Kruppel

INTRODUCTION

Normal development of the insect and mammalian central nervous systems (CNS) depends on both the spatial patterning of progenitor domains and the tempo at which individual progenitors generate distinct subtypes of neurons and glia (Berry and Rogers, 1965; Cepko, 1999; Doe and Skeath, 1996; Harris, 1997; Livesey and Cepko, 2001; Rapaport et al., 2001; Reid et al., 1997; Walsh and Reid, 1995). In the mammalian cerebral cortex, individual neural stem cells generate progeny capable of populating all laminar layers (Reid et al., 1997; Walsh and Reid, 1995). Birth-dating studies have revealed that each layer is occupied by neurons of similar birth-order, such that early-born neurons occupy the deepest layers and late-born neurons occupy the most-superficial layers (McConnell, 1995). It has been shown in mouse that the transcription factor Foxg1 is required at a precise moment during neural stem cell proliferation to repress the first-born cell fates and allow progenitors to specify later-born cells in the lineage (Hanashima et al., 2007; Hanashima et al., 2004). These findings highlight the importance of temporal queues during the development and diversification of the nervous system.

The *Drosophila* embryonic CNS has emerged as a powerful model system for dissecting the temporal control of neurogenesis (Cleary and Doe, 2006; Grosskortenhaus et al., 2005; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kanai et al., 2005; Maurange et al., 2008; Novotny et al., 2002; Pearson and Doe, 2004; Pearson and Doe, 2003). Neural stem cells in the embryonic nerve cord, called neuroblasts, delaminate from the epithelium to the interior of the embryo, marking the start of neural differentiation. Individual neuroblasts can be identified based on the time at which they are formed, their position within each hemisegment (NB7-1, for example, is positioned in the seventh row, first column, of the neuroblast array), and their pattern of gene expression (Broadus et al., 1995; Doe, 1992). In addition, each neuroblast generates a unique and invariant cell lineage (Bossing et al., 1996; Karcavich and Doe, 2004; Lundell and Hirsh, 1998; Pearson and Doe, 2003; Schmid et al., 1999; Schmidt et al., 1997) resulting from a series of asymmetric cell divisions in which neuroblasts 'bud-off' ganglion mother cells (GMCs) that typically undergo an additional division to produce two post-mitotic neurons. In this manner, neurons resulting from first-born GMC division are pushed to deep positions in the CNS and express molecular markers for early-born cells, whereas later-born neurons occupy more-ventral positions and express late-born fate markers (Isshiki et al., 2001). The early- and late-born markers are excellent candidates for genes that specify birth-ordered cell fate, also called temporal identity (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003).

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

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temporal identity in both the NB7-1 and NB7-3 lineages (Isshiki et al., 2001). We define the 'second' temporal identity to be that following the Hb-dependent first temporal identity; this can be the second-born or the third-born GMC in a lineage.

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

The best candidate for a multi-lineage fourth temporal identity factor is the zinc-finger protein Castor (Cas), which is detected in neuroblasts just as Pdm levels fade away, and which together with Pdm specifies the fourth temporal identity (U5 neuron) in the NB7-1 lineage (Grosskortenhaus et al., 2006; Isshiki et al., 2001). As with Pdm, it is impossible to know whether Cas has a specific role in specifying U5 identity or a general role as a fourth temporal identity gene without analyzing its function in additional neuroblast lineages. This has been difficult because most neuroblast lineages have not been characterized past the first or second cell division and few molecular markers are known for late-born neurons. For example, NB7-3 generates neurons with well-characterized molecular markers (Isshiki et al., 2001; Lundell et al., 1996; Lundell and Hirsh, 1998; Novotny et al., 2002), but it divides only three times and never expresses Cas. By contrast, NB2-4 divides many times and expresses Pdm and Cas (Isshiki et al., 2001), but there are no molecular markers available to identify late-born neurons in this lineage. Thus, to test the role of Pdm and Cas as multi-lineage late temporal identity factors, and to test any new candidate late-born temporal identity factors, it is necessary to characterize a new neuroblast lineage for both birth-order lineage data and neuronal molecular markers.

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

MATERIALS AND METHODS

Fly stocks

We used the following fly stocks to analyze wild-type and mutant phenotypes at 23°C: *hb^{P1}, hb^{FB}/TM3 hb-lacZ* to remove Hb CNS expression (Hulskamp et al., 1994; Isshiki et al., 2001); *Kr^{1}, Kr^{CD}/CyO hb-lacZ* to remove Kr CNS expression (Isshiki et al., 2001; Romani et al., 1996); *Df(2L)ED773*, which removes both *pdm1* and *pdm2* (Grosskortenhaus et al., 2006), *cas^{24}/TM3 ftz-lacZ* [formerly called *ming^{24}* (Cui and Doe, 1992)], *red e spdo^{ZZ27}/TM3* and *numb^{2} pr cn Bc/Cyo ftz-lacZ* (Skeath and Doe, 1998); *cas-lacZ* (Cui and Doe, 1992); and *svp^{e22}/svp^{z4}* (Miller et al., 2008; Mlodzik et al., 1990). Unless otherwise noted, for misexpression experiments we crossed *insc-gal4* (*1407-gal4,* Bloomington Stock Center) on chromosome II to *UAS-hb* on chromosomes II and III (Wimmer et al., 2000), *UAS-Kr* on chromosomes II and III (Hoch and Jackle, 1998), *UAS-HA:pdm2* on chromosomes II and III (Grosskortenhaus et al., 2006) and *UAS-cas* on chromosomes II and III (W. Odenwald, NIH, Washington DC) at 29°C. Recombinant clones were generated using flies with the following genotype: *y w hs-FLP*/+; *X-15-33*/*X-15-29* (courtesy of Allan C. Spradling, Carnegie Institute, Washington DC).

Molecular markers and immunostaining

Antibody staining was performed according to standard methods. Primary antibodies, dilutions and sources were: rabbit HB9 (Exex – FlyBase) 1:1000 (Odden et al., 2002); guinea pig HB9 1:500, and rat Islet (Isl; Tailup – FlyBase) 1:500 (Broihier and Skeath, 2002); mouse Islet 1:200, mouse Fasciclin 2 (FasII) 1:100, mouse FasIII 1:5 (Developmental Studies Hybridoma Bank, University of Iowa); rabbit Hb 1:200 (this work); guinea pig Kr 1:500 [East Asian Distribution Center for Segmentation Antibodies (EADC), Mishima, Japan]; rat Pdm2 1:10 (Grosskortenhaus et al., 2006); rabbit Cas 1:1000 (Kambadur et al., 1998); rat Zfh2 1:400 (M. Lundell, University of Texas at San Antonio); mouse En 4D9 1:5 (Patel et al., 1989); mouse Late Bloomer 1:4 (C. Goodman, University of California, Berkeley); and mouse β-galactosidase 1:500 (Promega, Madison, WI). Secondary antibodies were purchased conjugated to Alexa 488, Rhodamine RedX or Cy5 (Jackson, West Grove, PA), biotin (Vector, Burlingame, CA) or alkaline phosphatase (Southern Biotechnology, Birmingham, AL) and used at 1:400. Confocal image stacks were collected using a Leica SP2 confocal microscope, processed using ImageJ (NIH) and displayed as two-dimensional projections. Histochemical preparations were acquired using a Zeiss Axioplan microscope.

Identification of NB3-1 and the RP motoneurons

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

RESULTS

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

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

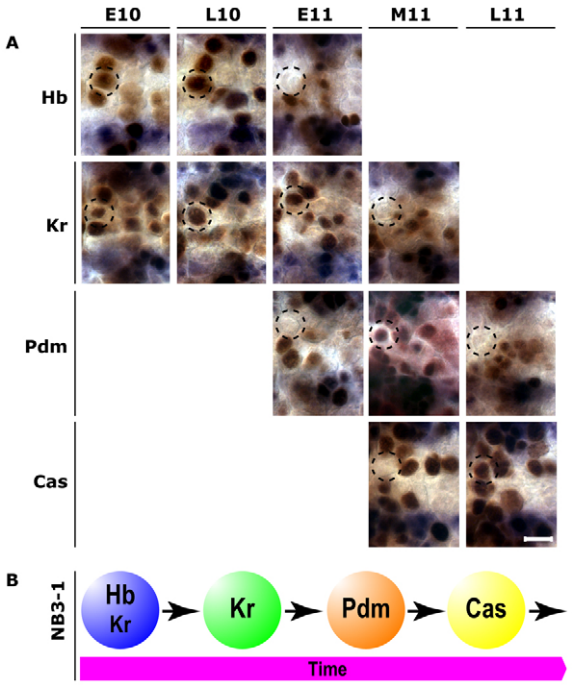

Fig. 1. Temporal identity gene expression in the NB3-1 lineage of the *Drosophila* CNS. (**A**) NB3-1 (encircled with dashed line) sequentially expresses Hb, Kr, Pdm and Cas. A portion of one hemisegment is shown, with Engrailed marking the most-posterior neuroblast (NB) rows 6/7 (blue). Midline, left; anterior, up. Embryonic staging is from Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985): early stage 10 (E10), when NB3-1 forms; late stage 10 (L10); early stage 11 (E11); mid stage 11 (M11); late stage 11 (L11). Scale bar: 10 μm. (**B**) Summary of gene expression in NB3-1.

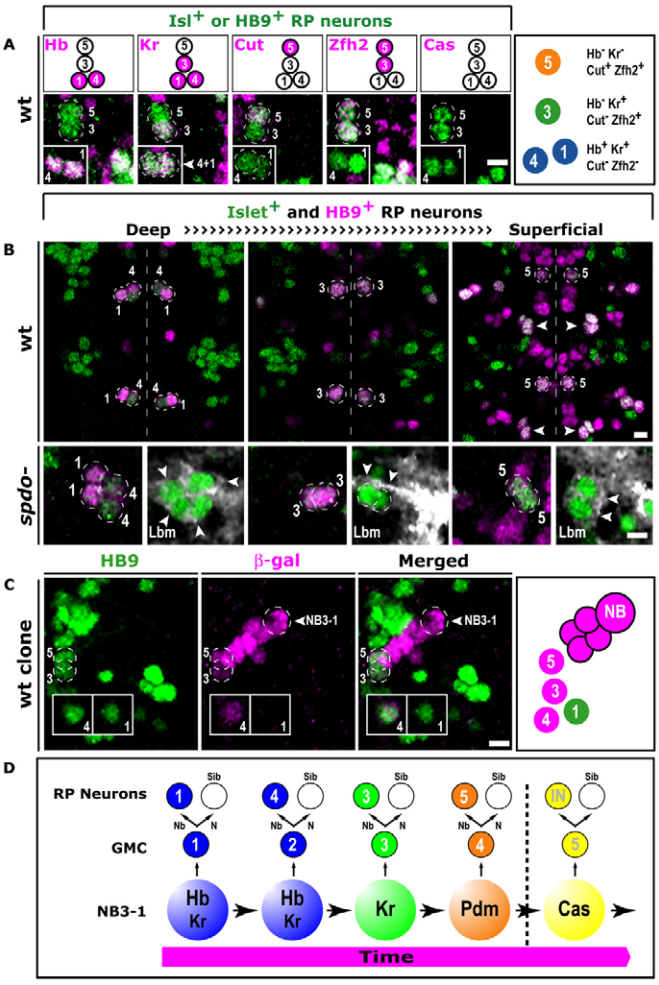

Fig. 2. Molecular markers and cell position can be used to identify each RP motoneuron in the NB3-1 lineage. (**A**) RP1 and RP4 are Hb^+, Kr^+, Cut^-, $Zfh2^-$ and are shown in insets because they are usually obstructed in the projection (n>100). RP3 is Hb^-, Kr^+, Cut^-, $Zfh2^+$ (n>100). RP5 is Hb^-, Kr^-, Cut^+, $Zfh2^+$ (n>100). A single representative hemisegment of a wild-type (wt) stage-16 CNS is shown as a maximum intensity projection. Midline, left; anterior, up. An expression summary is shown above. (**B**) (Top row) RP motoneurons are $Islet^+$ $HB9^+$ (outlined). RP1 and RP4 occupy the deepest layer; RP1 is more dorsal and expresses HB9 at higher levels than does RP4 after stage 15 (n>100). RP3 is directly ventral to RP1 and RP4 (n>100). RP5 is ventral and anterior to RP3 (n>100). White arrowheads, NB7-3-derived EW interneurons. Dashed vertical line, midline. Ventral views of two segments are shown from deep (left) to superficial (right) focal planes. (Bottom row) Each RP neuron has a non-RP neuron sibling, based the duplication of each RP neuron in *sanpodo* mutants. Eight $Islet^+$ $HB9^+$ Late Bloomer$^+$ RP motoneurons are observed in each hemisegment (quantified in Table 1). Midline, between each pair of panels. (**C**) Recombination-induced activation of *lacZ* in NB3-1 labeled RP4, RP3, RP5 and the late-born interneurons but not RP1, showing that RP1 is the first-born neuron in the lineage. HB9, green; β-galactosidase, magenta. RP neurons and NB3-1 are outlined and labeled. One hemisegment of a stage-16 CNS is shown as a maximum intensity projection. Midline, left; anterior, up. A schematic summary of the clone is shown to the right. (**D**) Schematic of NB3-1 gene expression and cell lineage. The vertical dashed line represents the transition between RP neuron and subsequent interneuron specification. Nb, sibling cell fate specified by Numb; N, sibling cell fate requires Sanpodo and active Notch signaling. Scale bars: 3 μm.

Next, we characterized the birth-order of the RP neurons and determined which of the known or candidate temporal identity genes was expressed at the time of their birth. We used both molecular markers and cell body position to identify and distinguish the RP neurons (Fig. 2A,B; see Materials and methods for details). Motoneuron backfills have shown that RP1/4 are the most dorsal, RP3 is intermediate, and RP5 is most ventral in position within the CNS (Landgraf et al., 1997; Schmid et al., 1999). Early-born neurons occupy deeper layers (Isshiki et al., 2001), consistent with a birth-order of RP1/RP4 → RP3 → RP5. Here, we used molecular markers to identify the NB3-1-derived RP neurons, and assayed their Hb, Kr, Pdm and Cas expression profile. We found that RP1/4 are Hb^+ Kr^+, RP3 is Hb^- Kr^+, and RP5 is Hb^- Kr^- (Fig. 2A). This precisely matches the sequence of gene expression within NB3-1 as it progresses through the early portion of its lineage (Fig. 1). We conclude that RP1/4 are born during the early Hb^+ Kr^+ neuroblast expression window, RP3 is born during the Hb^- Kr^+ neuroblast expression window, and RP5 is generated after Hb and Kr expression is lost from the neuroblast.

The RP1 and RP4 neurons express the same molecular markers (Fig. 2A) and have identical axon projections (Landgraf et al., 1997), raising the possibility that they are sibling neurons. To determine whether RP1 and RP4 are sibling neurons, we used *sanpodo* and *numb* mutants to equalize sibling cell fate (Skeath and Doe, 1998). We found that *sanpodo* mutants typically generate a pair of RP1 neurons and a pair of RP4 neurons, whereas *numb* mutants show the opposite phenotype (Fig. 2B, Table 1). These results show that the

Table 1. Summary of phenotypes in the NB3-1 lineage

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

P<0.01 for all experiments.
*All genotypes are described in Materials and methods.
†Average number of each cell type present per hemi-segment based on markers described in Fig. 1.
‡We currently lack the markers to distinguish between RP1 and RP4 in functional analyses.

RP1 and RP4 neurons are not siblings, but rather that they each have a non-RP sibling that assumes the RP fate in *sanpodo* mutants. Furthermore, we generated clones by mitotic recombination that label the entire NB3-1 lineage except RP1 (*n*=4; Fig. 2C). As all four neurons are definitively produced by NB3-1 based on DiI labeling (Bossing et al., 1996; Schmid et al., 1999), this proves that RP1 is derived from the first-born GMC in the lineage. We conclude that NB3-1 sequentially generates the RP1 → RP4 → RP3 → RP5 neurons (and their non-RP siblings), followed by a pool of local interneurons (Fig. 2D).

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

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

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

Kr is known to specify the second temporal identity in the NB7-1 and NB7-3 lineages (i.e. the fate of the GMC born immediately after Hb downregulation) (Cleary and Doe, 2006; Isshiki et al., 2001). We tested whether Kr has a similar function in the NB3-1 lineage. We used *Kr* mutants that were rescued for early segmentation expression (Isshiki et al., 2001), and found a loss of the RP3 neuron, whereas the early-born RP1/RP4 neurons and the late-born RP5 neuron were mostly unaffected (Fig. 4A, Table 1). Thus, Kr is required for the specification and/or survival of the RP3 neuron, i.e. of the RP neuron

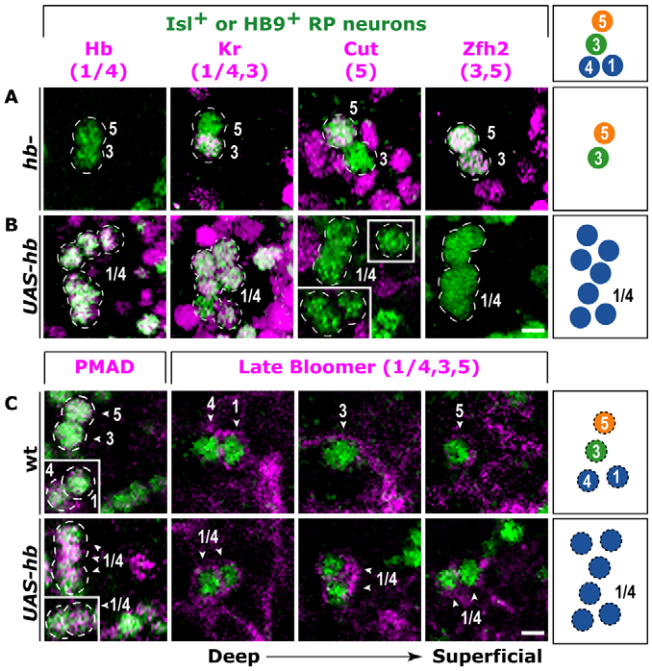

Fig. 3. Hb is necessary and sufficient to specify the first temporal identity. (**A**) *Drosophila hb* mutants lack the RP1 and RP4 neurons, but have normal RP3 and RP5 neurons (Table 1). Wild type has RP1, RP4, RP3 and RP5 neurons (see Fig. 2). (**B**) *hb* misexpression (*insc-gal4 UAS-hb*) generates ectopic RP1/4 neurons based on molecular markers (Table 1). (**C**) (Top row) Wild-type RP motoneurons express the pan-motoneuron markers phosphorylated (p) Mad (PMAD) and Late Bloomer. (Bottom row) *hb* misexpression (*insc-gal4 UAS-hb*) generates ectopic RP1/4 neurons that are double positive for pMad and Late Bloomer (100%, *n*=48). For all panels, a single representative hemisegment of a stage-16 CNS is shown as a maximum intensity projection. Midline, left; anterior, up. A phenotype summary is shown in the right-hand panels, with Late Bloomer expression indicated by dashed circles. Scale bars: 3 μm.

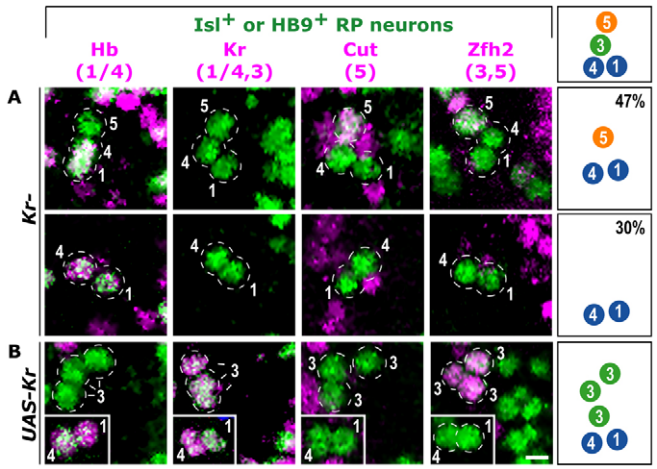

Fig. 4. Kr is necessary and sufficient to specify the second temporal identity. (**A**) In *Kr* mutant *Drosophila* embryos, RP3 is missing in the majority of hemisegments examined (both rows) and RP5 is occasionally missing (second row), whereas RP1/RP4 are normal (Table 1). Wild type has RP1, RP4, RP3 and RP5 neurons (see Fig. 2). (**B**) *Kr* misexpression (*insc-gal4 UAS-Kr*) generates ectopic RP3 neurons, RP5 is usually absent, and RP1/RP4 are normal (Table 1). For all panels, a single representative hemisegment of a stage-16 CNS is shown as a maximum intensity projection. Midline, left; anterior, up. A phenotype summary is shown to the right. Scale bar: 3 μm.

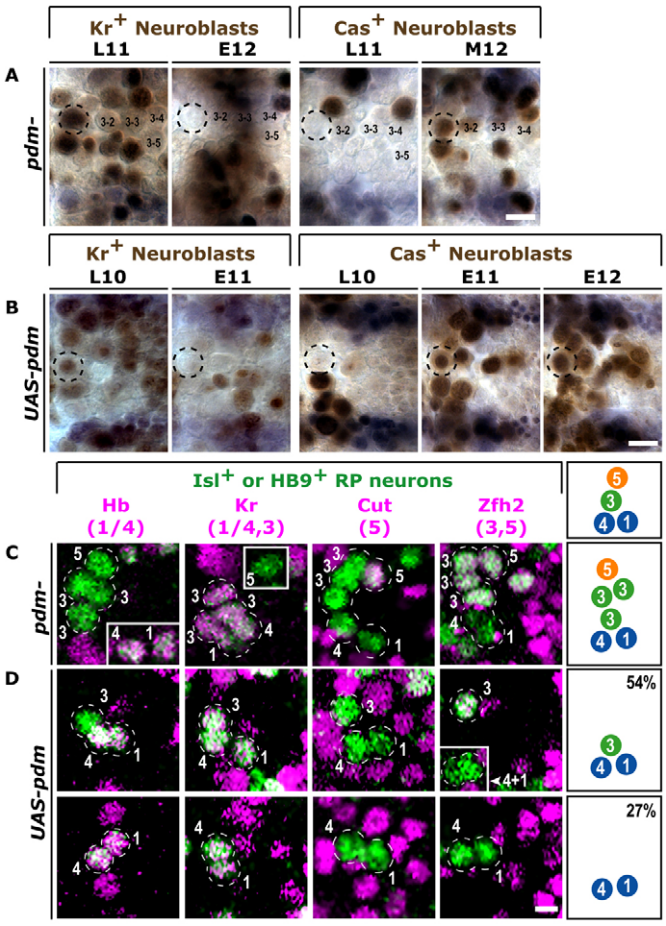

Fig. 5. Pdm closes the second temporal identity window in the NB3-1 lineage. (**A**,**B**) Neuroblast expression of Kr and Cas in *pdm* mutant and *pdm* misexpression embryos. One hemisegment is shown. Kr or Cas, brown; the positional marker Engrailed, blue. NB3-1 is outlined. (A) In *pdm* mutants, Kr expression persists through late stage 11 (it is normally switched off by mid stage 11; see Fig. 1A) and Cas expression is delayed until mid stage 12. (B) In *pdm* misexpression embryos (*insc-gal4 UAS-pdm2*), Kr expression is lost prematurely and Cas is expressed precociously (compare with Fig. 1A). (**C**,**D**) RP neuron specification in *pdm* mutant and *pdm* misexpression embryos. One hemisegment of a stage-16 CNS is shown as a maximum intensity projection. Midline, left; anterior, up. A phenotype summary is shown to the right. (C) In *pdm* mutant embryos there are two to three ectopic RP3 neurons; RP1, RP4 and RP5 neurons are usually normal (Table 1). (D) *pdm* misexpression (*insc-gal4 UAS-pdm2*) results in the frequent loss of the RP5 neuron (both rows) and in the occasional loss of the RP3 neuron (second row); RP1/RP4 are normal (Table 1). Scale bars: 10 μm in A,B; 3 μm in C,D.

born during the Kr neuroblast expression window. To determine whether Kr is sufficient to induce the RP3 identity, we used *insc-gal4 UAS-Kr* to prolong expression of Kr in NB3-1 for the entire length of its cell lineage. We observed a maximum of three RP3 neurons per lineage (Fig. 4B, Table 1). We saw no deleterious effect on the specification of RP1 and RP4, but the Cut$^+$ RP5 neuron was typically missing (Fig. 4B). We conclude that Kr is necessary, within a competence window, to specify the second temporal identity in the NB3-1 lineage (RP3), similar to its role in specifying the second temporal identity in the NB7-1 and NB7-3 lineages (Isshiki et al., 2001).

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

Pdm expression follows Hb and Kr in most neuroblasts, and thus is an excellent candidate for specifying the third temporal identity. Indeed, Pdm is necessary and sufficient to specify the third temporal identity (U4 neuron) within the NB7-1 lineage (Grosskortenhaus et al., 2006). To determine whether Pdm is a multi-lineage temporal identity gene, we assayed its loss-of-function and misexpression phenotype in the NB3-1 lineage. We assayed embryos homozygous for the deficiency *Df(2L)ED773*, which eliminates both *pdm1* and *pdm2* (henceforth referred to as *pdm* mutant embryos). In *pdm* mutant embryos, we observed normal timing of Hb expression in NB3-1 and other neuroblasts (data not shown), a modest extension of Kr expression, and a similar delay in Cas expression (Fig. 5A). Consistent with this change in neuroblast gene expression, *pdm* mutant embryos showed normal specification of the early-born Hb$^+$ RP1 and RP4 neurons, but possessed extra Kr$^+$ RP3 neurons, followed by an apparently normal Cut$^+$ late-born RP5 (Fig. 5C, Table 1). We conclude that Pdm is not required to specify the third temporal identity (the Cut$^+$ RP5 neuron), but is required to limit Kr expression in the neuroblast and thus close the second temporal identity window after the birth of just one Kr$^+$ RP3 neuron.

We next determined whether the continuous expression of Pdm in NB3-1 was sufficient to induce ectopic RP5 neurons (i.e. extend the third temporal identity window). We used *insc-gal4 UAS-pdm2* to generate continuous Pdm expression in neuroblasts, and observed normal timing of Hb expression in NB3-1 and other neuroblasts (data not shown), but premature loss of Kr expression and precocious Cas expression (Fig. 5B). Consistent with this change in neuroblast gene expression, we observed normal specification of the

early-born Hb$^+$ RP1 and RP4 neurons, but a lack of Kr$^+$ RP3 neurons; there was also a loss of the Cut$^+$ late-born RP5 neuron (Fig. 5D, Table 1). We conclude that Pdm is not sufficient to specify the third temporal identity (RP5), but rather it acts as a timer element to define the window of Kr expression and thus the length of the second temporal identity window. The precocious expression of Cas in these Pdm misexpression embryos may result in the precocious formation of Cas$^+$ interneurons at the expense of the RP5 neuron (see below).

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

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

To test whether Cas is required to close the third temporal identity window, we assayed *cas*-null mutant embryos (Cui and Doe, 1992). We found that *cas* mutants have normal Hb and Kr expression in neuroblasts (data not shown), but prolonged Pdm expression (Fig. 6A), consistent with previous work showing that Cas is required to repress *pdm* (Grosskortenhaus et al., 2006; Kambadur et al., 1998). At the neuronal level, we found that *cas* mutants have normal early-born RP1, RP4 and RP3 neurons but possess ectopic RP5 neurons (Fig. 6B, Table 1), consistent with a prolonged third temporal identity window. The ectopic RP5 neurons are not specified by the persistent Pdm protein because *pdm* mutants still formed apparently normal RP5 neurons (Fig. 5) and *pdm cas* double mutants still formed Cut$^+$ RP5 neurons (data not shown). Interestingly, *cas* mutants had a few RP-like (Islet$^+$ HB9$^+$) neurons that lacked expression of the motoneuron marker Late Bloomer and thus might have a mixed interneuron/RP motoneuron identity (see Fig. S2 in the supplementary material). We next examined *insc-gal4 UAS-cas* embryos, which have continuous expression of Cas in NB3-1. We found that RP5 was often missing, but the early-born RP1, RP4 and RP3 were normal (Fig. 6C, Table 1). We conclude that the precocious expression of Cas is sufficient to close the third temporal identity window, in which RP5 is specified. Taken together, our results suggest that Cas is necessary and sufficient to close the third temporal identity window in the NB3-1 lineage.

DISCUSSION

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

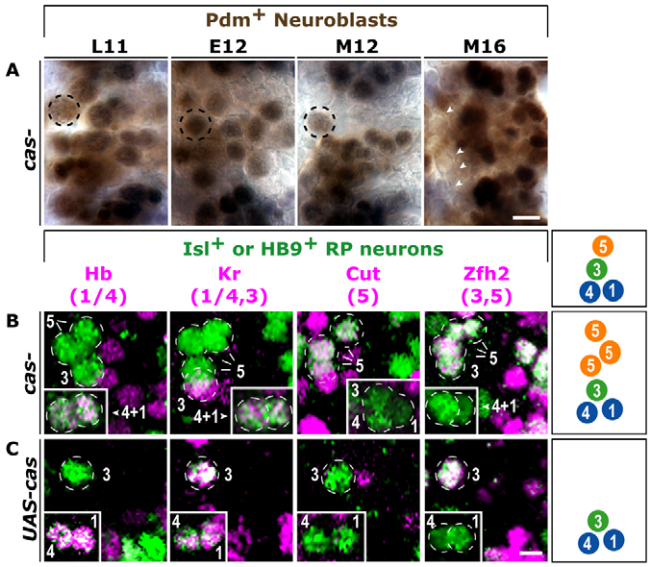

Fig. 6. Cas closes the third temporal identity window in the NB3-1 lineage. (**A**) *Drosophila cas* mutants have persistent Pdm expression in NB3-1 until at least mid stage 12 (M12); in wild type, Pdm is gone from NB3-1 by late stage 11 (see Fig. 1A). At mid stage 16, neuroblasts in the medial column no longer express Pdm (arrowheads); one of these neuroblasts is likely to be NB3-1. (**B**) In *cas* mutants, there are up to four ectopic Cut$^+$ RP5 neurons; RP1, RP4 and RP3 are normal (Table 1). Wild type has RP1, RP4, RP3 and RP5 neurons (see Fig. 2). (**C**) *cas* misexpression (*insc-gal4 UAS-cas*) results in frequent loss of RP5 and occasional loss of RP3; RP1 and RP4 are normal (Table 1). For all panels, a single representative hemisegment of a stage-16 CNS is shown as a maximum intensity projection. Midline, left; anterior, up. A phenotype summary is shown to the right. Scale bars: 10 μm in A; 3 μm in B,C.

Hunchback and Kruppel are multi-lineage temporal identity factors

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

Our data also provide insight into neuroblast competence. When we misexpressed Hb in the NB3-1 lineage, we were able to generate up to nine RP motoneurons; if each has a non-RP sibling, it would be close to the expected number of cells for the entire lineage (Schmid et al., 1999). Thus, Hb seems capable of maintaining at least three very different neuroblast lineages (NB3-1, NB7-1 and NB7-3) in a 'young' state for their entire lineage. By contrast, misexpression of Kr produces only a few RP3 motoneurons before NB3-1 proceeds to make the later-born neurons. The inability of Kr to maintain a second temporal

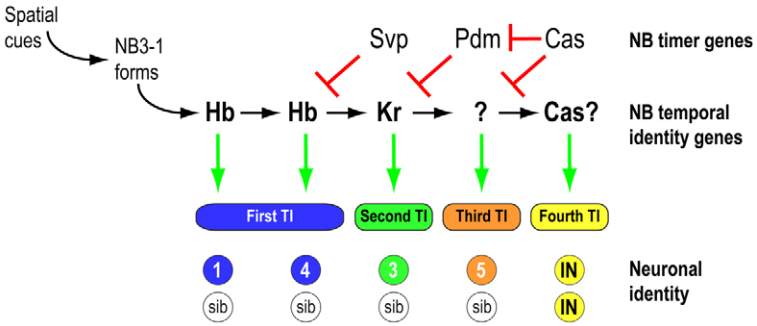

Fig. 7. Hb and Kr specify early temporal identity, whereas Svp, Pdm and Cas act as timer elements within the NB3-1 lineage of the *Drosophila* CNS. Spatial cues specify NB3-1 identity and formation, which allows the neuroblast to respond in a potentially unique way to timer genes and temporal identity genes that are expressed subsequently. Timer elements (top row) include Seven up (Svp), Pdm and Cas. These factors close successive temporal identity windows. Timer genes indirectly control cell fate through the regulation of temporal identity genes. Temporal identity genes (middle row) include Hb and Kr, which specify first and second temporal identities (TIs), respectively, in this and other lineages. Neuronal identity (bottom row) within the lineage: during the first TI, GMC-1 makes RP1/sibling neurons and GMC-2 makes RP4/sibling neurons; during the second TI, GMC-3 makes RP3/sibling neurons; during the third TI, GMC-4 makes RP5/sibling neurons; and during the fourth TI, GMC-5 makes interneurons (IN).

identity state might be due to the initiation of progressive restriction in neuroblast competence in NB3-1, as occurs in NB7-1 (Cleary and Doe, 2006; Pearson and Doe, 2003).

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

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

Pdm does not act as a timer element in all neuroblast lineages. For example, *pdm* mutants do not show extended Kr expression in the NB7-1 or NB7-3 lineages, as judged from the lack of ectopic Kr^+ neurons in these lineages (Grosskortenhaus et al., 2006) (see Fig. S3 in the supplementary material). These results suggest that the spatial identity of a neuroblast can alter its response to timing factors such as Pdm. Although this is counter to the simple model that spatial and temporal factors are independent and act combinatorially to specify birth-order identity within each lineage (Pearson and Doe, 2004), it is consistent with the finding that spatial identity occurs at the time of neuroblast formation (Chu-LaGraff and Doe, 1993; Prokop and Technau, 1994; Skeath et al., 1995), prior to the expression of temporal factors. Taken together, these data suggest that spatial cues allow individual neuroblasts to respond differently to a temporal identity factor expressed at a similar time in all lineages.

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

If Pdm does not specify temporal identity in NB3-1, what is the third temporal identity factor in this lineage? It has recently been reported that the SoxB family member Dichaete is expressed immediately prior to Cas in many embryonic neuroblast lineages (Maurange et al., 2008). However, Dichaete is only transiently expressed in medial column neuroblasts, such as NB3-1, at their time of formation (Zhao and Skeath, 2002) and thus does not have the proper timing for a third temporal identity factor in this lineage. Alternatively, absence of Hb, Kr and Cas might specify the third temporal identity, with Pdm acting solely as a timing factor to establish a gap between Kr and Cas expression. Another possibility is that an as yet unknown factor specifies the third temporal identity in the NB3-1 lineage. Finally, Pdm might specify aspects of RP5 identity that we are not able to detect with our limited number of markers; unfortunately, owing to severe morphological defects in late-stage *pdm* mutant embryos, we have been unable to assay the RP5 axon projection to its target muscle, which would provide a sensitive read-out of its neuronal identity.

Castor closes the third temporal identity window

Cas is expressed right after Pdm in most neuroblasts, and at the time NB3-1 is generating its fourth temporal identity (interneurons). We find that *cas* mutants have an extended window of Pdm neuroblast expression and exhibit production of ectopic RP5 neurons. Thus, Cas is required to close the third (RP5) temporal identity window. In

addition, we find that precocious expression of Cas can prematurely close the third temporal identity window and repress the specification of RP5. We observed comparable phenotypes in the NB7-1 lineage, in which loss of Cas leads to ectopic U4 formation and gain of Cas results in the repression of the U4 identity (Grosskortenhaus et al., 2006). Based on these observations, we predict that Cas functions in multiple neuroblast lineages to close the third temporal identity window. Does Cas specify the fourth temporal identity? We cannot answer this question in the NB3-1 lineage owing to a lack of interneuron markers, but Cas does specify the fourth temporal identity (together with Pdm) in the NB7-1 lineage (Grosskortenhaus et al., 2006). In the future, the role of Cas in the NB3-1 lineage could be examined by making CD8::GFP-marked *cas* mutant clones and assaying neuronal identity by axon projections, or by developing molecular markers for interneurons within the lineage.

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

We propose that there are two classes of genes that regulate neuroblast temporal identity (Fig. 7). One class, of which Hb and Kr are good examples, encodes temporal identity factors that are necessary and sufficient to directly specify a particular temporal identity in multiple neuroblast lineages (Isshiki et al., 2001). A second class encodes timing factors that establish the timing of temporal identity gene expression, but do not directly specify temporal identity. Timing factors, however, may indirectly influence the specification of temporal identities as seen in NB3-1, in which *pdm* is required to restrict the specification of RP3 and properly advance the neuroblast to the Cas-positive state (Fig. 5). Seven up, the one timing factor identified previously, downregulates Hb protein levels and, along with cytokinesis, closes the first temporal identity window to facilitate the Hb → Kr transition (Grosskortenhaus et al., 2005; Kanai et al., 2005). The Kr → Pdm → Cas transitions are independent of cell-cycle progression (Grosskortenhaus et al., 2005). Here, we have shown that Pdm closes the second temporal identity window by repressing Kr expression and activating Cas in NB3-1. Taken together, our observations suggest that Kr and Pdm are involved in a negative-feedback loop in which Kr activates Pdm, which in turns represses Kr and activates Cas to advance neuroblast timing independent of cell-cycle progression. Through its role as a regulator of Kr and Cas timing, Pdm can restrict the production of neuronal cell types and advance the NB3-1 lineage.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/21/3491/DC1

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