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Drosophila Neuroblasts Sequentially Express Transcription Factors which Specify the Temporal Identity of Their Neuronal Progeny

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Summary

Neural precursors often generate distinct cell types in a specific order, but the intrinsic or extrinsic cues regulating the timing of cell fate specification are poorly understood. Here we show that *Drosophila* neural precursors (neuroblasts) sequentially express the transcription factors Hunchback → Krüppel → Pdm → Castor, with differentiated progeny maintaining the transcription factor profile present at their birth. Hunchback is necessary and sufficient for first-born cell fates, whereas Krüppel is necessary and sufficient for second-born cell fates; this is observed in multiple lineages and is independent of the cell type involved. We propose that Hunchback and Krüppel control early-born temporal identity in neuroblast cell lineages.

Introduction

Development of the central nervous system (CNS) requires precise spatial and temporal patterning of cell fates. Spatial patterning is well studied, but we know relatively little about the mechanisms controlling the sequential generation of different cell types. In the vertebrate retina, multipotent precursors generate neuronal and glial cell types in a stereotyped order, due to a progressive restriction in precursor cell fate that is modulated by both intrinsic and extrinsic factors (Cepko, 1999; Harris, 1997; Ohnuma et al., 1999). Similarly, cell intrinsic and extrinsic mechanisms regulate the timing of oligodendrocyte differentiation (Durand and Raff, 2000). In the mammalian cortex, neurons that share a laminar position also share common molecular, physiological, and morphological features. Lineage analyses show that single neural precursors divide asymmetrically to generate neurons that populate all cortical layers; birthdating studies show that deep layer neurons are produced first, while superficial layer neurons are generated last; and transplantation experiments show that the laminar fate of a neuron is specified at the time of its birth (McConnell, 1995). Thus, the laminar identity of a neuron is tightly linked to its birth order, but the molecular mechanism of birth order-dependent specification of neuronal identity is unknown.

The *Drosophila* CNS is an attractive model system to study birth order-dependent specification of cell fate. The stem cell-like precursors of the CNS, called neuroblasts, can be individually identified, and each has a unique, invariant cell lineage to produce a diverse population of neurons and glia (Bossing et al., 1996; Schmid et al., 1999). Neuroblasts divide asymmetrically to "bud off" a series of smaller daughter cells (ganglion mother cells; GMCs), which each typically make two postmitotic neurons. Cell lineage, ablation, transplantation, in vitro culture and genetic studies in *Drosophila* and other insects indicate that GMC birth order is the primary determinant of its identity (Doe and Goodman, 1985; Furst and Mahowald, 1985; Prokop and Technau, 1994; Schmid et al., 1999; Weigmann and Lehner, 1995).

Genes that regulate GMC birth order identity in multiple neuroblast lineages have not yet been identified, but good candidates include the genes *hunchback* (*hb*), *pdm1/pdm2* (subsequently called *pdm*), and *castor* (*cas*; also called *ming*). *hb* is expressed in early-born deep layer neurons, *pdm* is expressed in middle layer neurons, and *cas* is expressed in late-born superficial layer neurons (Cui and Doe, 1992; Kambadur et al., 1998; Mellerick et al., 1992). In addition, we show in this paper that the *Krüppel* (*Kr*) gene is also expressed in early-born deep layer neurons, and thus is another candidate for regulating temporal identity within neuroblast lineages. *hb*, *Kr*, and *pdm* encode nuclear transcription factors that first function during segmentation and subsequently in the developing CNS (Gaul et al., 1987; Romani et al., 1996; Yang et al., 1993; this paper). *cas* encodes a nuclear zinc finger transcription factor detected during segmentation (this paper) and required for CNS development (Cui and Doe, 1992; Mellerick et al., 1992).

In this paper, we precisely define the timing of *hb* → *Kr* → *pdm* → *cas* expression in several identified neuroblast lineages. We show that *hb* → *Kr* → *pdm* → *cas* are sequentially expressed in neuroblasts, with GMC/neuronal progeny maintaining the transcription factor profile present at their birth. We show that Hb and Kr are necessary and sufficient for early-born cell fates; this is observed in multiple lineages and is independent of the cell type involved. We conclude that Hb and Kr specify early-born temporal identity in *Drosophila* neural stem cell lineages.

Results

Early-Born Neurons Express Hb and Kr, Assume Deep Layer Positions, and Have Long Axon Projections

To begin investigating birth order dependent cell fate specification in the *Drosophila* CNS, we characterize the morphological and molecular features that distinguish early- versus late-born neurons. We examine the axon projections and cell positions of first-born neurons from datasets of DiI-labeled neuroblast clones (Schmid et al., 1999), and find that first-born neurons typically occupy the deepest (most internal) position in the clone and have the longest axon projections of any cell in the clone

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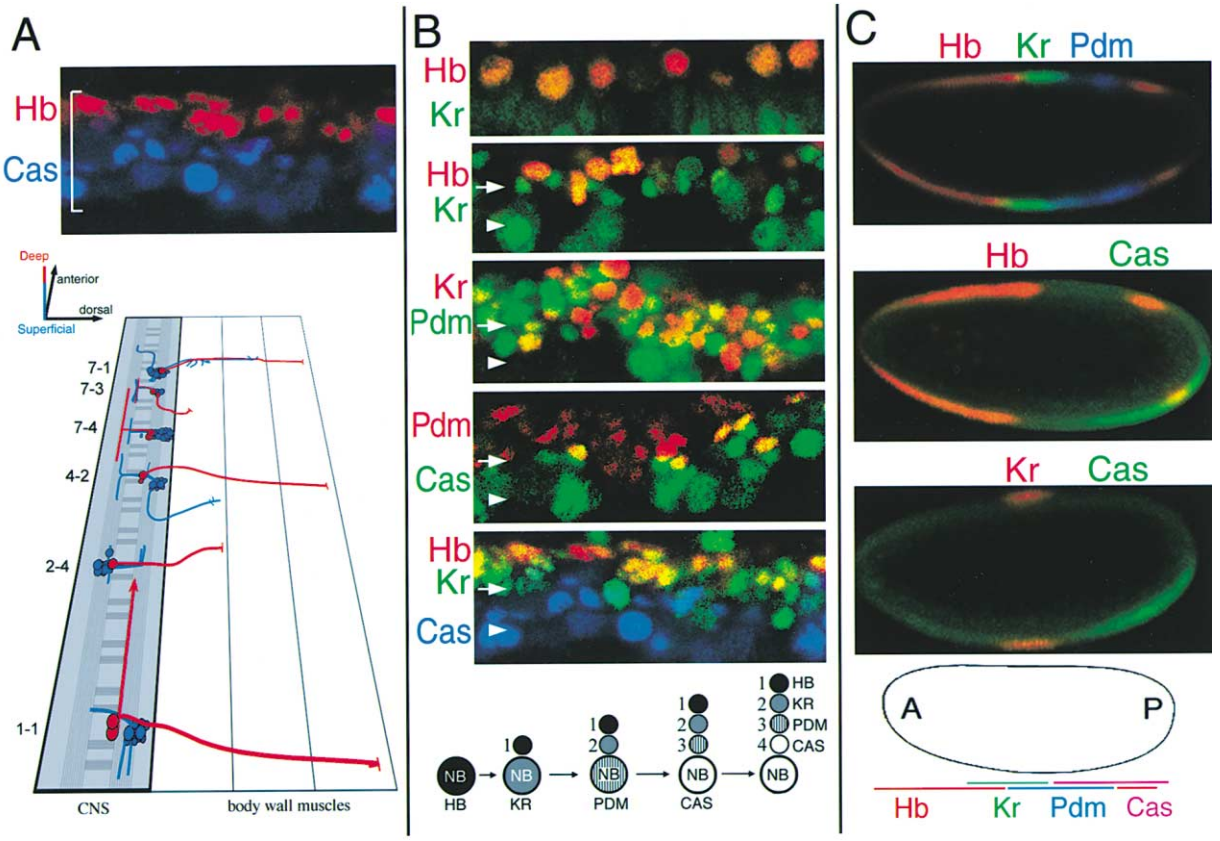

Figure 1. Relationship between Hunchback, Krüppel, Pdm, and Castor during Neurogenesis and Segmentation

(A) Hb^+ neurons occupy a deep layer and have long projections. Top: optical cross-section of a stage 12 CNS; deep layer at top; CNS thickness, bracket. Hb^+ neurons (red) occupy the deepest layer, and Cas^+ neuroblasts and GMCs (blue) are in a superficial layer. Bottom: schematic of six DiI neuroblast clones (Schmid et al., 1999). Early-born Hb^+ neurons (red) are in the deepest layer and have the longest axons of the clone; later-born neurons (blue) are superficial and have shorter axons.

(B) Hb, Kr, Pdm, and Cas are sequentially expressed in neuroblasts. Top: Optical cross-sections of embryos stained for the indicated proteins. Neuroblast layer, arrowhead; GMCs/neuron layer, arrow. Embryo stages from top to bottom: 9, 10, 11, 12, and late 12. Bottom: summary showing transient Hb, Kr, Pdm, and Cas expression in neuroblasts, followed by stable Hb, Kr, Pdm, and Cas expression in neuroblast progeny (gene expression overlaps not shown for clarity; see text for details).

(C) Hb, Kr, Pdm, and Cas are expressed in progressively more posterior domains in cellular blastoderm embryos. Anterior, left; ventral, down. Bottom: summary of expression domains.

(Figure 1A). Conversely, later-born neurons lie in more superficial positions (nearest the ventral epithelium) and have relatively short projections (Figure 1A).

What genes might regulate these birth order-specific neuronal properties? It is known that deep layer neurons are Hb^+, middle layer neurons are Pdm^+, while superficial layer neurons are Cas^+ (Kambadur et al., 1998). Here we confirm these findings and identify Kr as a new deep layer transcription factor. Kr is weakly detected in Hb^+ neurons, and strongly Kr^+ neurons define a deep layer that lies between the Hb^+ and Pdm^+ layers (Figure 1B). We also observe some neurons showing coexpression of Kr/Pdm and Pdm/Cas (see below). We tested whether Hb, Kr, Pdm, and Cas were expressed in a temporal order within neuroblasts at the time each layer of neurons is being generated. Indeed, we observe sequential, transient expression of Hb → Kr → Pdm → Cas in neuroblasts (Figures 1B and 2), which is "stabilized" in progeny born during each window of gene expression (described in more detail in the next section). In addition, Pdm is transiently expressed in a subset of newborn Hb^+ neuroblasts and their first-born GMCs (Yang et al., 1993), probably due to persistence of Pdm from the neuroectoderm, but it is usually not maintained in their Hb^+ neuronal progeny (data not shown; Kambadur et al., 1998).

Interestingly, the temporal expression pattern of Hb, Kr, Pdm, and Cas within neuroblasts parallels the spatial pattern of these genes during segmentation. Hb, Kr, Pdm, and Cas are detected in progressively more posterior domains at cellular blastoderm, respectively (Figure 1C). Thus, the spatial order of these genes during segmentation is the same as their temporal order in neuroblasts, raising the possibility of a conserved gene cassette used in both segmentation and neurogenesis (see Discussion).

Hunchback, Krüppel, Pdm, and Castor Are Transiently Expressed in Neuroblasts but Maintained in Their Neural Progeny

In this section we assay identified neuroblast lineages to test the hypothesis that transient Hb → Kr → Pdm →

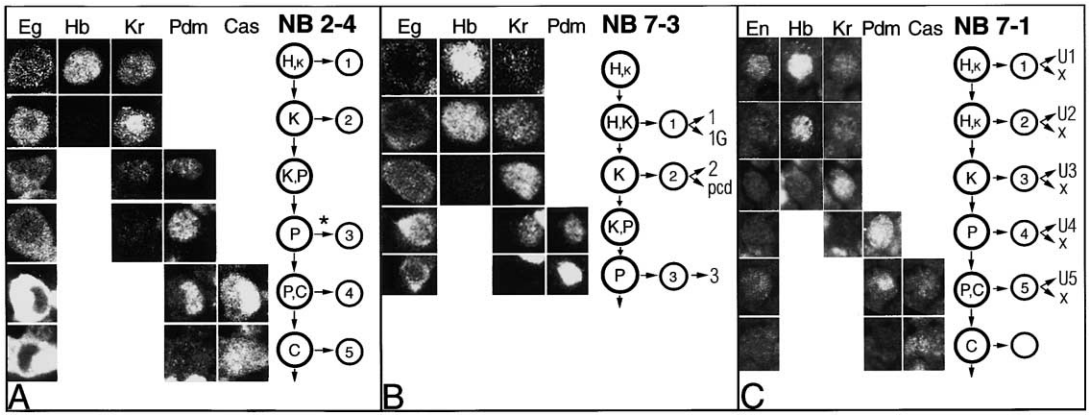

Figure 2. Hunchback, Krüppel, Pdm, and Castor Are Sequentially Expressed in Identified Neuroblasts

Ventral views of progressively older neuroblasts (NB) triple labeled for the proteins shown. Summaries show timing of gene expression relative to GMC birth order. Hb, H; Kr, K; Pdm, P; Cas, C (letter size reflects expression level).

(A) NB 2-4 and its GMCs are identified by Eagle-kinesin-lacZ transgene expression (Eg). Embryo stages from top to bottom: 10, early 11, mid 11, late 11, early 12, and late 12. *, Pdm$^+$ GMC rarely detected.

(B) NB 7-3 and its progeny are identified by Eagle-kinesin-lacZ expression. Embryo stages from top to bottom: early 11, late 11, early 12, mid 12, and late 12. NB 7-3 is transiently Pdm$^+$ at formation, but Pdm does not persist in early-born progeny (data not shown). Neurons: EW1, 1; GW, 1G; EW2, 2; EW3, 3 (in this and subsequent figures). Programmed cell death, pcd.

(C) NB7-1 can be identified as the most posterior/medial Engrailed$^+$ neuroblast (En; first four rows) or the most posterior Gooseberry$^+$ neuroblast (last two rows). Embryo stages: 8, early 9, mid 9, 10, early 11, and 11, from top to bottom. U1–U5, Eve$^+$ neurons; x, Eve$^-$ sibling neurons.

Cas expression in neuroblasts is stably maintained in neuronal progeny born during each window of gene expression. We characterize three model neuroblast lineages, an early forming neuroblast (7-1) and two late-forming neuroblasts (7-3 and 2-4), where we can track specific neuronal progeny from birth to differentiation. Although early- and late-forming neuroblasts begin their cell lineages hours apart (Broadus et al., 1995), all show the same sequential, transient Hb → Kr → Pdm → Cas pattern of expression (Figure 2).

Neuroblast 2-4 sequentially expresses Hb/Kr, Kr, Kr/Pdm, Pdm, Pdm/Cas, and Cas (Figure 2A). GMCs and neurons with most of these expression patterns can be detected in deep to superficial layers of the CNS, respectively, except we rarely observe Kr$^+$/Pdm$^+$ or Pdm$^+$ GMCs (data not shown). In addition, GMC-1 is transiently Pdm$^+$ (data not shown).To track gene expression patterns at the level of identified neuronal/glial progeny, we examined gene expression in the neuroblast 7-3 and 7-1 lineages (see below).

Neuroblast 7-3 produces only three GMCs: GMC-1 generates the EW1 interneuron and the GW motoneuron, GMC-2 produces the EW2 interneuron and an EW2 sibling that rapidly undergoes programmed cell death, and GMC-3 differentiates directly into the EW3 interneuron (Lundell and Hirsh, 1998; R. Karcavitch and C.Q.D., submitted). Subsequently, we refer to these neurons by the abbreviated names of 1/1G, 2, 3 (respectively) to reflect their birth order. Neuroblast 7-3 sequentially expresses Hb, Hb/Kr, Kr, Kr/Pdm, Pdm; it is never Cas$^+$ (Figure 2B). In addition, the newborn neuroblast 7-3 and GMC-1 are transiently Pdm$^+$ (data not shown). GMC-1 is Hb$^+$ Kr$^+$ and generates the Hb$^+$ Kr$^+$ 1/1G neurons, GMC-2 is Kr$^+$ and generates the Kr$^+$ interneuron 2, and GMC-3 is Pdm$^+$ and generates the transiently Pdm$^+$ interneuron 3 (Figure 3A).

Neuroblast 7-1 generates over 20 GMCs, but only the first five GMCs express the nuclear marker Even-skipped (Eve) (Bossing et al., 1996; Broadus et al., 1995). Each of the five Eve$^+$ GMCs produces one Eve$^+$ motoneuron (named U1, U2, U3, U4, U5, based on birth order) and one Eve$^-$ sibling neuron which we cannot track (Schmid et al., 1999; Skeath and Doe, 1998). Neuroblast 7-1 sequentially expresses Hb/Kr, Kr, Kr/Pdm, Pdm, and Pdm/Cas (Figure 2C). The Hb$^+$ Kr$^+$ neuroblast produces two Hb$^+$ Kr$^+$ GMCs which develop into Hb$^+$ Kr$^+$ U1 and U2 motoneurons; the Kr$^+$ neuroblast generates a Kr$^+$ GMC-3 which produces the Kr$^+$ U3 motoneuron; and GMC-4 and GMC-5 are born during the Pdm$^+$ and Pdm$^+$ Cas$^+$ phases of gene expression, respectively, and produce the Pdm$^+$ U4 motoneuron and the Pdm$^+$ Cas$^+$ U5 motoneuron (Figure 4A).

We draw three conclusions from our detailed gene expression analysis. First, nearly all of the 30 known neuroblasts go through an invariant temporal pattern of Hb → Kr → Pdm → Cas gene expression, including early-forming and late-forming neuroblasts (see Discussion). Second, Hb → Kr → Pdm → Cas gene expression is transient in neuroblasts, but is maintained in differentiated neuronal progeny. Third, Hb → Kr → Pdm → Cas gene expression is correlated with birth order and not a particular cell type. For example, Hb$^+$ progeny are all early-born, but can differentiate as interneurons, motoneurons, or glia depending on their parental neuroblast (see below).

Hunchback Is Necessary and Sufficient for Specifying First-Born Cell Fates

In the wild-type neuroblast 7-3 lineage, the first-born GMC and its 1/1G neuronal progeny are Hb$^+$ Kr$^+$; later-born interneurons 2 and 3 do not express Hb (Figure 3A). In addition, the molecular markers Zfh-1, Zfh-2, and

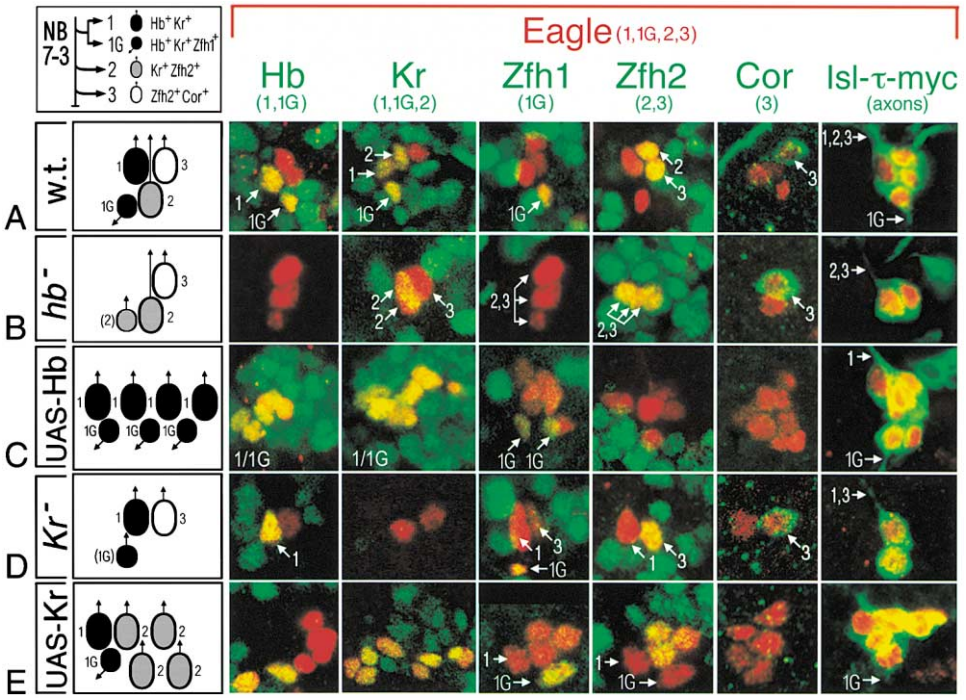

Figure 3. Expression and Function of Hunchback and Krüppel in the Neuroblast 7-3 Lineage

Embryos double labeled for the 7-3 lineage marker Eagle (Eg; red) and the indicated cell fate/axon projection markers (green). Each neuron can be uniquely identified by marker expression, cell size, and axon projection (see top left schematic; additional details provided upon request). Left column: phenotype summaries (axon projections, arrows; variable neuron survival, parentheses). Anterior up, midline at left, stage 15.

(A) Wild-type. Neurons maintain the Hb and Kr profile that they inherited at the time of their birth, and can be uniquely distinguished by the markers shown.

(B) *hb* CNS mutant (*hb*$^{-}$). The first-born 1/1G neurons are missing or partially transformed to a later-born interneuron 2 fate; later-born neurons are normal. Some cells contain nonfunctional cytoplasmic Hb protein (not shown).

(C) Ectopic Hb throughout the 7-3 lineage (*UAS-hb*). There are extra cells in the lineage and all differentiate as first-born 1/1G neurons.

(D) *Kr* CNS mutant (*Kr*$^{-}$). One or both of the 1/1G neurons are occasionally missing, interneuron 2 is always missing, and interneuron 3 is normal.

(E) Ectopic Kr throughout the 7-3 lineage (*UAS-Kr*). There are extra cells in the lineage and all have the interneuron 2 fate, except the normal first-born 1/1G neurons.

the neurotransmitter corazonin label different subsets of these neurons (Figure 3). In embryos specifically lacking Hb in the CNS (*hb* CNS mutants; see Experimental Procedures), we observe either a duplication of interneuron 2 at the expense of the first-born 1/1G neurons (11%, n = 27), consistent with a duplication of GMC-2 fate, or a specific loss of the first-born 1/1G neurons (89%, n = 27) (Figure 3B). We currently cannot distinguish whether loss of first-born 1/1G neurons is due to cell death or due to "skipping" of the GMC-1 fate (i.e., the lineage begins with GMC-2; see Discussion). In contrast, when neuroblast 7-3 is forced to continuously express Hb ("*UAS-hb*"; see Experimental Procedures), there are additional neurons in the lineage (as many as 11 cells; average of 6.3 cells, n = 43), and all exhibit first-born 1/1G fates based on molecular markers, neurotransmitter expression, and characteristic axon projections (Figure 3C). We conclude that Hb is necessary for normal GMC-1 development, but not later-born cell fates; that continuous Hb can transform all progeny toward a GMC-1 fate; and that continuous Hb expression leads to an extension of the neuroblast cell lineage (see Discussion).

In the wild-type neuroblast 7-1 lineage, the first two GMCs and their two Eve^{+} U1, U2 motoneuron progeny are Hb^{+}; later progeny do not express Hb (Figure 4A). In *hb* CNS mutants, we rarely detect Eve^{+} GMC-1/GMC-2 (data not shown) or their Eve^{+} U1, U2 motoneuron progeny (both detected 0%, one detected 11%; n = 53); however, later-born GMCs and neurons develop normally based on molecular marker expression (Figure 4B). We currently cannot distinguish whether loss of the Eve^{+} U1, U2 motoneurons is due to cell death or due to skipping of the first two GMCs fates (see Discussion). In contrast, when neuroblast 7-1 is forced to continuously express Hb, there are extra Eve^{+} cells (as many as 19 cells; average of 14.4 cells, n = 21), and all differentiate as early-born U1/U2 motoneurons based on molecular marker expression (Figure 4C). Thus, Hb is necessary for normal GMC-1 and GMC-2 development without affecting later-born cell fates, and continuous Hb can transform many or all progeny toward a GMC-1/GMC-2 fate.

Hunchback Regulates First-Born Motoneuron Fate in Multiple Neuroblast Lineages

Is Hb required for the specification of all first-born neurons? To broaden our analysis, we assayed the first-

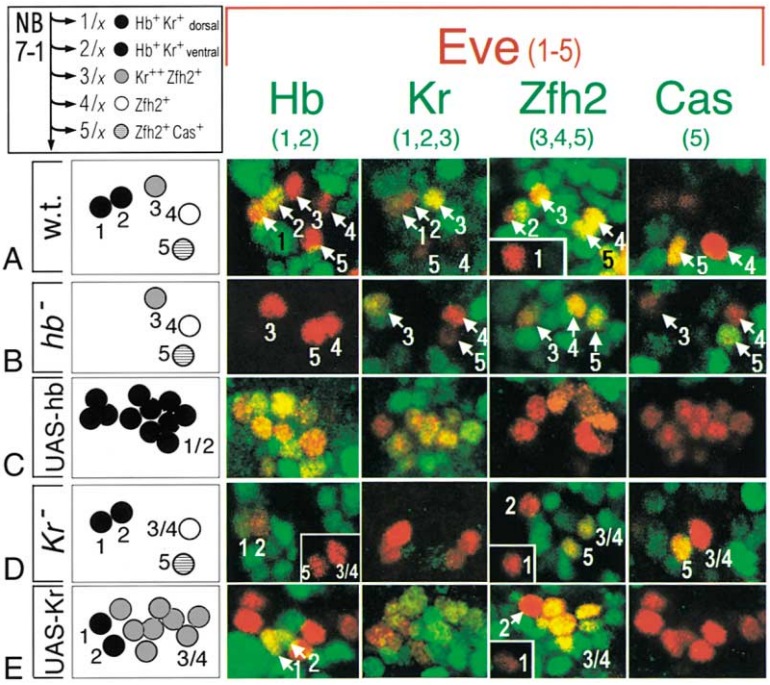

Figure 4. Expression and Function of Hunchback and Krüppel in the Neuroblast 7-1 Lineage

Embryos double labeled for the 7-1 progeny marker Eve (red) and the indicated cell fate markers (green). In wild-type embryos, the U1, U2, U3, U4, and U5 motoneurons (labeled 1, 2, 3, 4, and 5) can be uniquely identified by marker expression and cell position (see top left schematic; additional details provided upon request). All panels show projections of multiple focal planes; insets are used when projections would superimpose two cells. Left column: phenotype summaries. Anterior up, midline at left, stage 15.

(A) Wild-type. Neurons maintain the Hb/Kr/Cas profile that they inherited at the time of their birth, and can be uniquely distinguished by the markers shown.

(B) *hb* CNS mutant (*hb*$^{-}$). The U1/U2 neurons are missing, but later cells in the lineage are normal. Some cells contain nonfunctional cytoplasmic Hb protein (not shown).

(C) Ectopic Hb throughout the 7-1 lineage (*UAS-hb*). There are extra Eve^{+} cells in the lineage, and all differentiate as U1/U2 neurons. Hb is ubiquitous.

(D) *Kr* CNS mutant (*Kr*$^{-}$). The U3 or U4 neuron is frequently missing (we cannot distinguish them with available markers), but earlier- and later-born cells in the lineage are normal.

(E) Ectopic Kr throughout the 7-1 lineage (*UAS-Kr*). There are extra Eve^{+} cells in the lineage, and all differentiate as U3 or U4 neurons, except that the Hb^{+} U1/U2 neurons are normal.

born progeny from the well-characterized 1-1 and 4-2 lineages. Both lineages produce an Eve^{+} GMC-1; in the 1-1 lineage, it generates the Eve^{+} aCC motoneuron/pCC interneuron siblings, whereas in the 4-2 lineage, it produces the Eve^{+} RP2 motoneuron and its Eve^{-} sibling (Figures 5A and 5D) (Broadus et al., 1995). In *hb* CNS mutants, the first-born Eve^{+} neurons typically survive (>90%, n > 60 for each) but abnormally express Zfh-2, a marker for later-born neurons (Figures 5B and 5E), and the aCC and RP2 motoneurons fail to project to their proper dorsal muscle target (see below); both phenotypes are consistent with a transformation of GMC-1 to a later-born GMC fate. In contrast, when all neuroblasts are forced to continuously express Hb, there are duplications of the first-born aCC/pCC (17%, n = 58; Figure 5F), duplications of the first-born RP2 (6%, n = 54; Figure 5C), or triplications of RP2 (4%; n = 54; data not shown). These results show that *hb* regulates first-born motoneuron and interneuron cell fates in the neuroblast 1-1 and 4-2 lineages.

We would predict that if *hb* controls first-born identity in all neuroblast lineages, then we should observe severe motoneuron axon projection defects because many motoneurons derive from Hb^{+} first-born GMCs. Wild-type embryos have about 35 motoneurons with a stereotyped projection pattern to ventral and dorsal bodywall muscles (Figure 5G), including the Hb^{+} aCC, RP2, U1, and U2 motoneurons that innervate dorsal muscles (Schmid et al., 1999). *hb* CNS mutants show a reduction in the number of motoneuron projections, particularly to the dorsal muscles (Figure 5H). Embryos where *hb* is misexpressed in all neuroblasts show the opposite phenotype: a striking increase in the number of motoneurons, particularly to the dorsal muscles (Figure 5I). These results suggest that *hb* regulates first-born cell fate and/or axon projection patterns in most or all of the many neuroblast lineages that produce first-born motoneurons.

Hunchback Regulates First-Born Glial Cell Fates

Results described above suggest that *hb* is required for specifying first-born GMC identity in lineages where first-born GMCs produce motoneurons or interneurons. To determine whether *hb* specifies first-born cell fate in lineages that produce glia, we assayed the thoracic 6-4 (6-4T) neuroblast lineage, in which the first-born GMC produces glia, and the 7-4 lineage, in which only later-born GMCs generate glia. The 6-4T lineage produces a Hb^{+} GMC-1, which produces the two Hb^{+} MM-CB glia that migrate to the midline (Table 1; M. Freeman and C.Q.D., unpublished results), whereas neuroblast 7-4 first generates Hb^{+} interneurons, and then produces many Hb^{-} glia, including two dorsoventral channel glia located at the midline (Table 1; M. Freeman and C.Q.D., unpublished results). *hb* CNS mutants lack the first-born MM-CB glia at the midline, but have no change in the number of later-born channel glia at the midline (Table 1). In contrast, forced misexpression of *hb* in all neuroblasts results in extra MM-CB glia at the midline and a decrease in the number of midline channel glia (Table 1). These results, together with the neuronal phenotypes described above, strongly suggest that Hb regulates first-

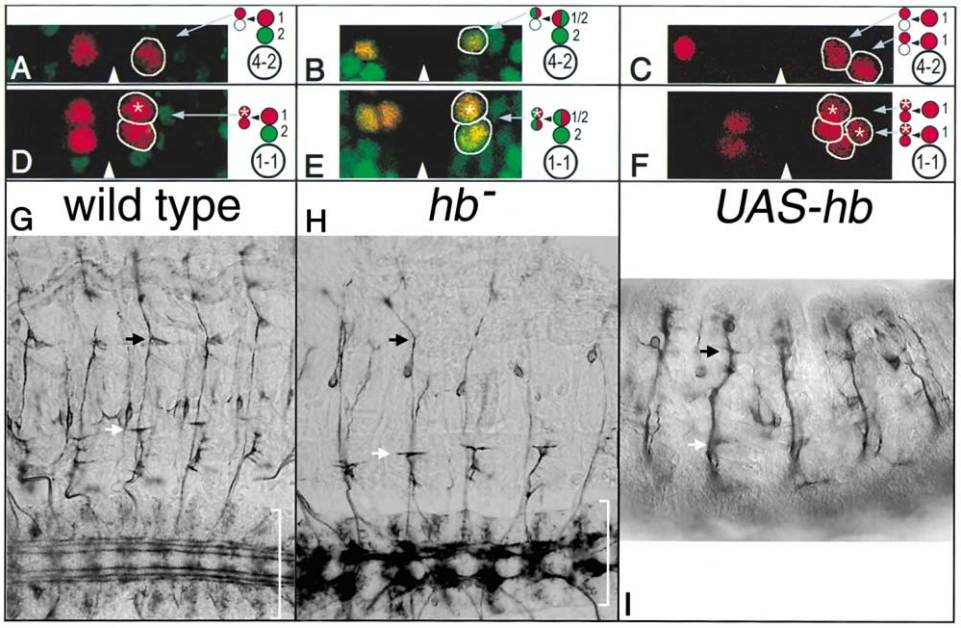

Figure 5. Hunchback Regulates First-Born Motoneuron Projection Patterns and Cell Fate

(A–F) Hb regulates first-born neuron fate in the 4-2 and 1-1 lineages. Confocal double label for Eve (red; marks the neuronal progeny of GMC-1) and Zfh-2 (green; marks later-born progeny); anterior, up; ventral midline, arrowhead; *, aCC motoneuron (identified by Zfh-1 expression; data not shown). (A and D) Wild-type. The Hb^+ GMC-1 generates an Eve^+ $Zfh\text{-}2^-$ RP2 motoneuron and an Eve^- sibling in the 4-2 lineage (A) or the Eve^+ $Zfh\text{-}2^-$ aCC motoneuron/pCC interneuron siblings in the 1-1 lineage (D). (B and E) *hb*$^-$ CNS mutant. In both lineages, Eve^+ first-born neurons survive but abnormally express Zfh-2. (C and F) Ectopic Hb in all neuroblasts. Zfh-2 expression is suppressed and the Eve^+ first-born neurons are occasionally duplicated. (G–I) Hb regulates the number and extent of motoneuron axon projections. Late stage 16 embryos; anterior, left; CNS, bracket; dorsal muscle 3/11 synapse, black arrow; lateral muscle synapse 12/13, white arrow. (G) Wild-type flat mount embryo showing $FasII^+$ motoneuron projections (vertical bundles) and three CNS fascicles (horizontal bundles). (H) *hb* CNS mutant flat mount embryo showing reduced motoneuron axon projections. (I) *hb* misexpression whole-mount embryo showing supernumerary motoneuron projections.

born temporal identity, rather than cell type identity, within multiple neuroblast lineages.

Krüppel Is Necessary and Sufficient for Second-Born Cell Fates

In the wild-type neuroblast 7-3 lineage, the first-born GMC-1 and its 1/1G neuronal progeny are Hb^+ Kr^+, while the second-born GMC-2 and interneuron 2 are Hb^- Kr^+ (Figure 3A). In embryos lacking *Kr* CNS expression (*Kr* CNS mutants; see Experimental Procedures), the first-born 1/1G sibling neurons are variably affected: both can be missing (17%, n = 64), 1G can be missing (73%, n = 64), or both can be normal (10%, n = 64); however, the second-born interneuron 2 is always missing (100%, n = 64) and the third-born interneuron 3 is almost always normal (90%, n = 64). Absence of interneuron 2 could be due to cell death or due to skipping of the GMC-2 fate (see Discussion). In contrast, when neuroblast 7-3 is forced to continuously express Kr ("*UAS-Kr*," see Experimental Procedures), there are extra cells in the lineage (4–8; average of 5.5, n = 27), and all but two cells differentiate as GMC-2 derived interneuron 2; the two unaffected cells are the GMC-1 derived 1/1G neurons (Figure 3E). We conclude that Kr contributes to GMC-1 development (where it is expressed with Hb) and is essential for GMC-2 development (where it is expressed without Hb), that continuous Kr can transform all progeny except GMC-1 toward a GMC-2 fate, and that continuous Kr leads to an extension of the neuroblast cell lineage (Figure 7B).

In the wild-type neuroblast 7-1 lineage, the first two GMCs and their U1/U2 motoneuron progeny are Hb^+ Kr^+, GMC-3 and its U3 motoneuron progeny are Kr^+, and subsequent GMCs do not express Kr (Figure 4A). In *Kr* CNS mutants, one of the U3/U4 motoneurons is frequently missing (73%, n = 63), although all earlier- and later-born neurons develop normally (Figure 4D). We suspect that the missing neuron is the normally Kr^+ U3, based on cell position, but we lack markers to distinguish U3/U4 in *Kr* mutant embryos. As in the 7-3 lineage, the *Kr* phenotype may arise through cell death or a skipping of the GMC-3 fate (see Discussion). In

Table 1. Hb Regulates First Born GMC Development in Neuronal/Glial Neuroblast Lineages

	Wild-Type	*hb*$^-$	*uas-hb; pros-gal4*
First born glia[a] (6-4T lineage)	36 (n = 18)[b]	5 (n = 30)	70 (n = 28)
Later born glia[c] (7-4 lineage)	36 (n = 18)	36 (n = 30)	2 (n = 28)

[a] First-born Hb^+ MM-CB glia that migrate to the CNS midline (M. Freeman and C.Q.D., unpublished results).
[b] Number of hemisegments scored.
[c] Later-born Hb^- channel glia at the CNS midline (M. Freeman and C.Q.D., unpublished results).

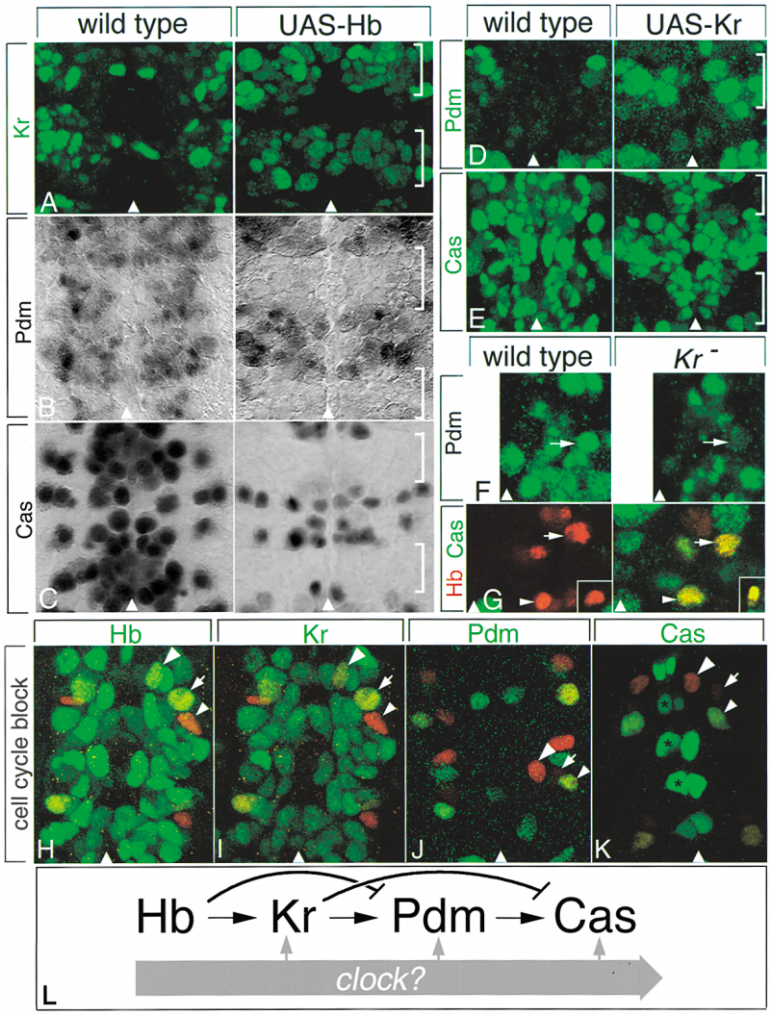

Figure 6. Regulatory Interactions between Hunchback, Krüppel, Pdm, and Castor during Neurogenesis

(A–G) Genotypes at top; proteins assayed at left; *engrailed-GAL4* misexpression domain, bracket; anterior, up; ventral midline, triangle; all embryos are stage 11, except (C) and (E) are at stage 12. (A–C) Ectopic Hb activates Kr and represses Pdm and Cas. (D and E) Ectopic Kr activates Pdm and represses Cas. (F) Kr is necessary to activate Pdm in some neuroblasts, including neuroblast 2-4 (arrow; identified by *eg-kinesin-lacZ* expression, not shown). (G) Kr is necessary to repress Cas (green) in neuroblasts 5-4 (arrowhead), 2-4 (arrow), and its first GMC (inset), but not in neuroblasts 7-1 or 7-3 (not shown). Hb expression (red) is normal. (H–K) Cell cycle-arrested neuroblasts are Hb^+ (H), Kr^+ (I), Pdm^- (J), and Cas^- (K). Early stage 12 *string*^{7M53} embryos; two segments shown; anterior, up; ventral midline, triangle; stained for Hb, Kr, Pdm, or Cas (green) and Eg (red) to identify neuroblasts 7-3 (large arrowhead), 2-4 (arrow), 3-3 (small arrowhead), and 6-4. Cas^+ neuroblasts are 2-1, 5-1, 6-1 (*, from top to bottom), and 3-3; Pdm^+ neuroblasts include 3-3. (L) Summary of regulatory inputs driving sequential Hb, Kr, Pdm, and Cas expression in neuroblasts. Arrows, positive regulation; bars, negative regulation. Gray arrows indicate regulation of each gene expression transition. See text for details.

contrast, continuous expression of Kr in neuroblast 7-1 results in extra Eve^+ neurons (8–14; average of 10.0, n = 23) with all differentiating as U3 or U4 motoneurons, except the normal pair of early-born U1/U2 motoneurons (Figure 4E). We suspect all neurons have the normally Kr^+ U3 fate, but we lack a marker to distinguish U3/U4 fates. We conclude that Kr is necessary for U3 motoneuron development, and that continuous Kr can transform most or all 7-1 progeny, except the first-born U1/U2 neurons, toward a U3 neuron fate (Figure 7B).

Regulation of Hunchback, Krüppel, Pdm, and Castor CNS Expression

Accurate temporal regulation of Hb, Kr, and Cas is critical for proper CNS development, so it is important to determine the mechanisms that regulate sequential gene expression in neuroblasts. Regulatory interactions between Hb, Kr, Pdm, and Cas can be detected using misexpression assays: overexpression of Hb activates Kr and represses Pdm and Cas (Figures 6A–6C); overexpression of Kr activates Pdm, represses Cas, but has no effect on Hb expression (Figures 6D and 6E; data not shown); and Pdm positively regulates Cas expression (Brody and Odenwald, 2000), leading to the model that each gene can activate the next gene in the pathway and repress the "next plus one" gene (Figure 6L). These interactions are not necessary for driving sequential gene expression, however, as we observe that *hb*, *Kr*, or *cas* mutations have only subtle alterations in the remaining gene expression profiles (Figures 6F and 6G and data not shown), and loss of *hb* or *Kr* does not appear to affect the fate of cells born later in the lineage based on existing markers (Figures 3 and 4). Thus, an independent pathway must also drive the sequential expression of Hb → Kr → Pdm → Cas in neuroblasts. This mechanism involves cell cycle progression, directly or indirectly, because newly formed neuroblasts remain Hb^+ Kr^+ if they are cell cycle-arrested before their first division and rarely if ever make the transition to Kr^+ Hb^-, Pdm^+, or Cas^+ (Figures 6H–6K; Cui and Doe, 1995; Weigmann and Lehner, 1995). We conclude that a cell cycle-dependent "clock" is required to drive the transitions in Hb → Kr → Pdm → Cas gene expression (gray arrows in Figure 6L).

Discussion

Establishing Sequential Hunchback → Krüppel → Pdm → Castor Expression in Neuroblasts

Our data do not support a model in which global temporal cues trigger gene expression transitions simultaneously in all neuroblasts (Brody and Odenwald, 2000).

Most of the 30 neuroblasts in each segment, including the earliest neuroblasts to form (e.g., 7-1 and 7-4) and some of the latest neuroblasts to form (e.g., 2-4 and 7-3), go through the same Hb → Kr → Pdm → Cas gene expression cascade. Exceptions are neuroblasts 2-1, 3-3, 5-1, and 6-1 (which start with Kr, Pdm, or Cas; Cui and Doe, 1992 and data not shown). Thus, early-forming neuroblasts can generate Cas^+ progeny at the same time that late-forming neuroblasts produce Hb^+ progeny.

We favor a model in which the timing of Hb → Kr → Pdm → Cas expression is regulated primarily by a cell cycle-dependent clock but also by regulation within the Hb → Kr → Pdm → Cas pathway. Evidence for the latter mechanism is that misexpression studies show that Hb, Kr, Pdm, and Cas typically activate the next gene in the pathway and repress the "next plus one" gene in the pathway (Figures 6A–6G); and that loss of function mutations can result in premature expression of later genes in the pathway and the skipping of GMC fates. Evidence for the cell cycle-dependent clock mechanism is that *hb* and *Kr* mutants have relatively subtle changes in *hb*, *Kr*, *pdm,* or *cas* expression or in later-born GMC fates; that cell cycle arrested neuroblasts remain Hb^+ Kr^+ and fail to make a transition to Hb^- Kr^+, Pdm^+, or Cas^+; and that when neuroblast 1-1 is cell cycle arrested for several hours prior to its first cell division and then triggered to divide, it will produce an Eve^+ GMC-1 instead of a later-born Eve^- GMC (Weigmann and Lehner, 1995), highlighting the importance of the cell cycle progression rather than developmental time in regulating GMC identity.

The molecular nature of the cell cycle-dependent clock is unknown. A cell cycle/cytokinesis block could affect neuroblast gene expression in many ways, either directly or indirectly, by phosphorylating an unknown regulatory factor in the neuroblast; by preventing a regulatory factor from being segregated out of the neuroblast (e.g., Prospero); or by blocking a GMC-dependent "feedback signal" to the neuroblast, similar to that proposed for mammalian cortical or retinal neurogenesis (Cepko, 1999; Qian et al., 2000). The feedback signal model is inconsistent with our observation that GMCs can be skipped in *hb* or *Kr* mutants without affecting later-born fates, but it is consistent with misspecified or dying GMCs signaling effectively if the signal is generic (e.g., simple cell-cell contact).

All early developing neuroblasts, such as 7-1, produce two Hb^+ GMCs before downregulating Hb expression, whereas many late developing neuroblasts, such as 7-3, produce just one Hb^+ GMC. How do neuroblasts regulate the number of Hb^+ GMCs produced? All neuroblasts could express Hb for the same length of time but have different cell cycle rates, or all neuroblasts may have the same cell cycle rate but vary the length of Hb expression.

Differential Regulation of Hunchback, Krüppel, and Castor Expression in Neuroblasts and GMCs

Hb, Kr, and Cas are transiently expressed in neuroblasts but maintained in GMC and neural progeny (Pdm can be transient or stable in neuroblast progeny, depending on the lineage). By inheriting and maintaining the gene expression profile of their parental neuroblasts, GMCs can "memorize" their birth order. This seems a powerful and efficient way for stem cells to make a variety of fate-restricted progeny in invariant sequence. A similar mechanism may be used during vertebrate cortical and retinal development, where precursors transiently express genes that are maintained in a subset of differentiated progeny (Frantz et al., 1994b; Perron et al., 1998). It is currently unknown what distinguishes transient neuroblast expression from persistent GMC/neuronal expression.

Function of Hb and Kr in Birth Order Specification

Loss of Hb or Kr from early-born GMCs results in loss or transformation of neurons normally derived from these GMCs, but later-born neurons develop normally based on nuclear markers, neurotransmitter expression, and axon projections. Neuronal loss in *hb* and *Kr* mutants is likely due to multiple mechanisms: (1) Cell death. We can detect necrotic Eve^+ or Eg^+ neurons in the 7-1 or 7-3 lineages of *Kr* CNS mutants, supporting a cell death model. Although we have not detected necrotic Eve^+ or Eg^+ neurons *hb* mutants, we do see more early 7-3 lineages in which three $Prospero^+$ GMCs are born (44%; $n = 27$) than mature 7-3 lineages with progeny from all three GMCs (11%; $n = 27$), suggesting that cell death also occurs in *hb* mutants. (2) GMC skipping (i.e., the neuroblast skips a GMC fate without duplicating a later-born fate). In *Kr* mutants, neuroblast 7-3 typically produces two $Prospero^+$ GMCs at the time GMC-1 and GMC-2 are normally born, consistent with a skip of GMC-2 fate. Similarly, *hb* mutants often produce only two $Prospero^+$ GMCs in the 7-3 lineage, consistent with a skip of the GMC-1 fate. We cannot assay for GMC skipping in the 7-1 lineage. (3) GMC transformation. *hb* mutants clearly show transformation of GMC-1 to GMC-2 fate in the 7-3 lineage, based on the observed duplication of interneuron 2 at the expense of the 1/1G sibling neurons. This phenotype may arise if the endogenous Kr in GMC-1 is sufficient to induce GMC-2 fate in the absence of Hb. In *Kr* mutants, a similar transformation of GMC-2 into the GMC-3 fate is never observed, perhaps because the Kr^- GMC-2 does not prematurely express Pdm. Thus, we have evidence that *hb* mutants show all three phenotypes, GMC death, skipping, and transformation, while *Kr* mutants show only GMC death and skipping phenotypes.

Misexpression of Hb or Kr can transform all GMCs toward a first-born or second-born fate, respectively. We are convinced that this is a transformation of GMC identity, rather than an increase in the proliferation of early-born GMCs, because the extra early-born neurons are produced at the expense of the later-born neurons in the lineage, and the extra cells are not reduced in size (extra embryonic divisions lead to reduced cell size; Lane et al., 1996). Interestingly, Kr misexpression fails to transform early-born Hb^+ GMCs into the later-born Kr^+ fate (Figures 3E and 4E). Kr is induced early enough to affect GMC-1 in the 7-3 lineage because we observe GMC transformations in other lineages (e.g., 7-1) prior to the time GMC-1 is born in the 7-3 lineage, yet it has no effect. We favor the model that first-born Hb^+ fates are dominant over second-born Kr^+ fates. In the future, cell type-specific Hb misexpression studies could be

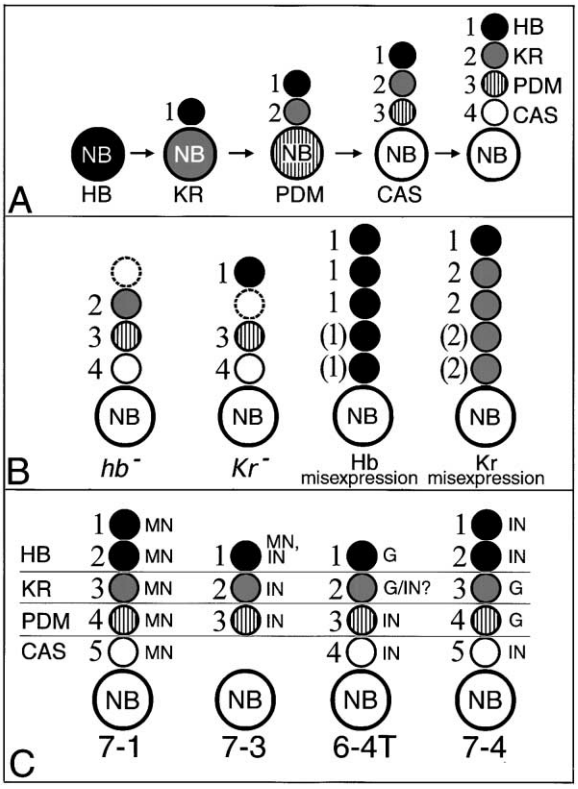

Figure 7. Hunchback and Krüppel Control Temporal Identity within Neuroblast Lineages

(A) Neuroblasts transiently express Hb → Kr → Pdm → Cas, but their progeny maintain the transcription factor profile present at their birth (gene expression overlaps not shown for clarity; see text for details).

(B) Hb and Kr loss of function and misexpression phenotypes. GMCs are labeled according to birth order, with "1" representing all Hb^+ GMCs (there are one or two Hb^+ GMCs depending on the lineage) and "2, 3, 4" representing the following GMCs in the lineage. Dashed circles represent abnormal GMC development (GMC death, GMC skipped, or GMC transformed to a later-born fate; see text for details).

(C) Hb, Kr, Pdm, and Cas regulate temporal identity rather than cell type identity. NB, neuroblast; MN, motoneuron; IN, interneuron; G, glia.

used to determine precisely when birth order-specific cell fates become fixed: in neuroblasts, GMCs, or neurons? It would also be interesting to determine if a pulse of Hb expression midway through a neuroblast lineage is sufficient to induce first-born cell fates, and if so, does the temporal program resume or reset to the beginning of the lineage after the Hb pulse ends?

hb, *Kr*, *pdm*, and *cas* are not the only genes controlling temporal identity in neuroblast lineages. Some markers for first-born fate occur normally in *hb* mutants, such as Eve expression in the 1-1 and 4-2 lineage. Moreover, Hb misexpression may not fully transform every cell in the 7-3 lineage to a first-born fate: a full transformation would produce equal numbers of interneuron 1/motoneuron 1G siblings, but we typically observe only 2–3 motoneurons and 5–6 interneurons, suggesting that only the first 2–3 GMCs are fully transformed to a first-born fate. Finally, in most lineages, there are GMCs produced after Cas expression ends; additional genes such as *grainyhead* (Brody and Odenwald, 2000) may specify the temporal identity of these GMCs.

Hb and Kr Regulate Temporal Identity, Not Cell Type Identity

Hb is expressed in virtually all first-born GMCs, and these can differentiate into motoneurons, interneurons, or glia, depending on the neuroblast lineage (Figure 7C). Similarly, high level Kr is detected in virtually all second-born GMCs (i.e., the GMCs following the Hb^+ GMCs), and they can differentiate into motoneurons, interneurons, or glia (Figure 7C and data not shown). Not only are Hb and Kr expressed in multiple cell types, but they are necessary and sufficient for the proper cell fate specification of motoneurons, interneurons, or glia, depending on the neuroblast lineage (Figures 7B and 7C).

An extremely interesting question is how GMC "temporal identity" (regulated by Hb and Kr) is coordinated with individual "neuroblast identity" to achieve the proper sequence of cell types that characterizes each neuroblast lineage. The Hb protein, its putative mammalian ortholog Ikaros, and the mammalian Kr-related EKLF protein all associate with chromatin remodeling proteins (Muller and Leutz, 2001), and both Hb and Kr are thought to regulate chromatin-mediated heritable expression of homeotic genes (Farkas et al., 2000). Thus, Hb and Kr may modulate chromatin structure such that different subsets of genes are accessible for transcription in first-born versus second-born GMCs, with the palette of genes expressed by a first-born or second-born GMC, dependent on the neuroblast-specific transcription factors they inherit. In this manner, neuroblast identity might be integrated with GMC temporal identity to create the unique cell types characterizing each neuroblast lineage. This is conceptually similar to homeotic genes and tissue-specific genes working together to uniquely specify distinct cell types in each tissue at different anterior-posterior levels of the body axis (Graba et al., 1997).

Neuroblast Proliferation Versus Quiescence

We find that when neuroblast 7-3 is forced to continuously express Hb or Kr, it generates an extended lineage of up to ten neurons instead of four neurons and one programmed cell death. The increase does not appear to be due to an extra round of cell division by the normally postmitotic neurons because the extra cells are produced at the expense of later-born cell types and because we do not see smaller cells (as expected, since extra cell divisions in the embryo lead to smaller cell size; Lane et al., 1996). We propose that Hb or Kr misexpression results in production of extra GMCs, and suggest that each neuroblast has an intrinsic mechanism for triggering quiescence that is related to successful transition from Hb and Kr expression to later genes in the hierarchy. Our data also show that Hb and Kr can regulate features of neuroblast cell biology (cell cycle control) in addition to regulating GMC temporal identity.

Evolution of Hunchback → Krüppel → Pdm → Castor Expression in the CNS

We show that the temporal gene expression in neuroblasts (early to late: Hb → Kr → Pdm → Cas) mimics

the major domains of gene expression at cellular blastoderm (anterior to posterior: Hb → Kr → Pdm → Cas). Additional studies will be needed to discern common and distinct regulatory features between Hb, Kr, Pdm, and Cas expression during segmentation and neurogenesis. The expression of all genes at both stages of development raises the question of which function is ancestral. Hb is detected in the CNS of various arthropod, leech, and *C. elegans* embryos (Fay et al., 1999; Iwasa et al., 2000; Sommer et al., 1992). In mammals, Hb-related genes of the Ikaros family are best known for regulating immune development, but some also show CNS expression (Cortes et al., 1999; Honma et al., 1999). A mammalian Pdm homolog, SCIP/Oct-6, is expressed in specific cortical layers of the brain (Frantz et al., 1994a), and a mammalian Cas ortholog exists but has not been characterized. It will be interesting to determine whether genes regulating temporal identity in *Drosophila* neuroblasts have similar functions in the mammalian CNS or immune system.

Experimental Procedures

Fly Stocks

hb^{KM}; hb^{14F}/TM3 *ftzlacZ* (or hb^{P1}, hb^{FB}/TM3 *ftzlacZ* for Figure 3 *islet-tau-myc* column) were used to remove *hb* CNS expression. hb^{14F} and hb^{FB} are genetically null *hb* mutations; hb^{14F} makes stable nonfunctional cytoplasmic Hb protein that persists in the CNS (Hulskamp et al., 1994). The hb^{KM} and hb^{P1} transgenes are expressed in the anterior Hb gap domain but not in neuroblasts or their progeny (J. Margolis and J. Posakony, personal communication; Margolis et al., 1995; Wimmer et al., 2000). To misexpress *hb,* we crossed *UAS-hb* (Wimmer et al., 2000) at 29°C to *engrailed-GAL4* (for scoring 7-1 and 7-3 lineages), *prospero-GAL4* (for scoring 6-4T and 7-4 glial lineages), *scabrous-GAL4* (for scoring 1-1 and 4-2 lineages), or *engrailed-GAL4 patched-GAL4* (for scoring motoneuron projection phenotypes). Kr^{CD}, Kr^{1}/CyO *hb-lacZ* was used to selectively remove *Kr* CNS expression; Kr^{1} is a null mutant and Kr^{CD} is expressed in the central Kr gap domain and weakly in the neuroectoderm, but not in neuroblasts or their progeny (Romani et al., 1996). To misexpress Kr, we crossed either *engrailed-GAL4* or *Scabrous-GAL4* to *UAS-Kr* (Hoch and Jackle, 1998) at 29°C. An *Eagle-kinesin-lacZ* line was used for identifying neuroblasts 2-4 and 7-3 and their progeny, including axon projections. An *islet-tau-myc* line was used for labeling postmitotic neurons from neuroblast 7-3. $string^{7M53}$/TM3 *hb-lacZ* was used for cell cycle arrest experiments.

Molecular Markers and Immunostaining

Antibody staining was performed according to Broadus et al. (1995). Primary antibodies: mouse anti-β-galactosidase (1:400; Promega); rat anti-β-galactosidase (1:4000), mouse anti-myc monoclonal 9E10 (1:100), rat anti-Hb, guinea pig anti-Hb, guinea pig anti-Kr (1:500), rabbit anti-Pdm1 (1:500), mouse anti-Pdm1 monoclonal (1:7), mouse anti-Even-skipped monoclonal 2B8 (1:10), rabbit anti-Corazonin (1:2500), mouse and rabbit anti-Castor (1:1000, 1:2000), rabbit anti-Eagle (1:700), mouse anti-FasII monoclonal 1D4 (1:10), mouse anti-Zfh-1(1:300), rabbit anti-Zfh-1 (1:7500), mouse anti-Zfh-2 (1:500), and rat anti-Zfh-2 (1:200); references and specific sources provided on request. Species-specific secondary antibodies conjugated to Alexa green, LRSC, RedX, Cy5, or HRP (Jackson) were used at 1:300. Fluorescent preparations were imaged on a BioRad 1024 confocal microscope, HRP preparations were imaged on a Zeiss Axioplan; all data except Figures 6D and 6E are from abdominal segments 2–6.

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