

Asymmetric Prospero localization is required to generate mixed neuronal/glia lineages in the *Drosophila* CNS

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SUMMARY

In many organisms, single neural stem cells can generate both neurons and glia. How are these different cell types produced from a common precursor? In *Drosophila*, *glial cells missing* (*gcm*) is necessary and sufficient to induce glial development in the CNS. *gcm* mRNA has been reported to be asymmetrically localized to daughter cells during precursor cell division, allowing the daughter cell to produce glia while precursor cell generates neurons. We show that (1) *gcm* mRNA is uniformly distributed during precursor cell divisions; (2) the Prospero transcription factor is asymmetrically localized into the glial-producing

daughter cell; (3) Prospero is required to upregulate *gcm* expression and induce glial development; and (4) mislocalization of Prospero to the precursor cell leads to ectopic *gcm* expression and the production of extra glia. We propose a novel model for the separation of glia and neuron fates in mixed lineages in which the asymmetric localization of Prospero results in upregulation of *gcm* expression and initiation of glial development in only precursor daughter cells.

Key words: Prospero, *Drosophila*, Glia, *gcm*, CNS

INTRODUCTION

Neurons and glia have dramatically different morphology and function, yet these two cell types frequently arise from common neural precursors. In vertebrates, in vivo clonal analyses and neural stem cell culture experiments have provided strong evidence that single neural stem cells can generate both neurons and glia (Davis and Temple, 1994; Golden and Cepko, 1996; Johe et al., 1996; Qian et al., 1998; Reid et al., 1997; Stemple and Anderson, 1992; Temple, 1989; Walsh and Cepko, 1992). Similarly, a subset of *Drosophila* neural stem cells (here called neuroglioblasts; NGBs) are known to generate mixed neuronal/glia lineages that contribute ~75% of all embryonic CNS glia (Akiyama-Oda et al., 1999; Bossing et al., 1996; Schmid et al., 1999; Schmid et al., 1997).

What are the molecular mechanisms that govern the production of neurons versus glia within a single stem cell lineage? In *Drosophila*, a critical regulator of glial identity is the *glial cells missing* (*gcm*) gene. *gcm* encodes a novel transcription factor that is expressed in all *Drosophila* embryonic glia except for midline/mesectoderm-derived glia (Akiyama et al., 1996; Hosoya et al., 1995; Jones et al., 1995; Schreiber et al., 1997; Vincent et al., 1996). *gcm* loss-of-function mutant embryos show a transformation of CNS glia into neurons (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Conversely, overexpression of *gcm* within the CNS causes nearly every cell to express glial-specific genes, and *gcm* misexpression in identified sensory neurons

transforms them into glia based on both morphological and molecular features (Hosoya et al., 1995; Jones et al., 1995). Thus, within the nervous system *gcm* appears to act as a genetic switch, with cells that are Gcm positive developing as glia, and cells that are Gcm negative developing as neurons. *gcm* is capable of positively regulating its own expression (Miller et al., 1998), which highlights the importance of precisely regulating *gcm* expression patterns and levels.

How is *gcm* expression regulated in neural stem cell lineages that give rise to both neurons and glia? Two recent studies have investigated the cell division profile and patterns of *gcm* expression in an identified precursor, the thoracic NGB 6-4 (NGB 6-4T; Akiyama-Oda et al., 1999; Bernardoni et al., 1999), which gives rise to both neurons and glia. They report that the first division of NGB 6-4T is along the mediolateral axis and generates a larger lateral cell (here called the 'post-divisional NGB') and a smaller medial daughter cell (here called the 'G daughter cell'). The G daughter cell expresses *gcm* and produces three glia; the post-divisional NGB lacks *gcm* expression, switches to an apical-basal division axis, and begins generating neurons. Interestingly, both reports propose that *gcm* mRNA is asymmetrically localized into the G daughter cell during the first division of NGB 6-4T, and that this asymmetric localization of *gcm* triggers glial commitment in G, whereas lack of *gcm* mRNA in the post-divisional NGB allows the production of neural progeny, thereby bifurcating neuron and glial cell fates (Akiyama-Oda et al., 1999; Bernardoni et al., 1999).

This model raises several important questions: how is *gcm*

mRNA localized? What orients the spindle along the mediolateral axis during glia-producing divisions? How is it reoriented along the apical-basal axis for neuron production? Recent work has identified several proteins that may be involved in these processes. The Inscuteable (Insc) protein is apically localized in mitotic neuroblasts, where it is required for the apical-basal orientation of the spindle; indeed, misexpression of Insc in epithelial cells that normally divide perpendicular to the apical-basal axis is sufficient to re-orient their spindles along the apical-basal axis (Kraut et al., 1996). Miranda, Prospero, Staufen and Numb proteins are all localized to the basal cortex of mitotic NBs, and subsequently partitioned into the basal daughter cell (Fuerstenberg et al., 1998a; Matsuzaki, 2000). Miranda is an adapter protein necessary for basal localization of Prospero and Staufen (Ikeshima-Kataoka et al., 1997). Prospero is a transcription factor required for daughter cell-specific gene expression and triggering exit from the cell cycle (Chu-Lagraff et al., 1991; Doe et al., 1991; Li and Vaessin, 2000; Vaessin et al., 1991), while Staufen is an RNA-binding protein necessary for basal localization of *prospero* mRNA (Broadus et al., 1998). Numb is a membrane-associated protein that can affect cell fate by inhibiting *Notch* signaling (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996). Each of these proteins is a candidate for regulating *gcm* expression or mRNA localization in the NGB 6-4T lineage.

We initially set out to investigate whether Insc expression or function regulates spindle orientation in NGB 6-4T, and whether Staufen is required for the asymmetric localization of *gcm* mRNA in NGB 6-4T. We report, in contrast to previous studies, that all divisions of NGB 6-4T are along the apical-basal axis, and that there is no evidence for the asymmetric localization of *gcm* mRNA or protein. We show that there is transient low level *gcm* expression in NGB 6-4T and its progeny during its phase of glial production. We show that the Prospero transcription factor is asymmetrically localized into the daughter cells of at least two NGBs (6-4T and 7-4), where it is required to upregulate *gcm* expression and induce the glial developmental program; failure to localize Prospero leads to ectopic *gcm* expression in these NGBs and the production of extra glia. We propose a novel model for glial production in mixed neuron/glia lineages that requires the asymmetric localization of the Prospero transcription factor.

MATERIALS AND METHODS

Drosophila stocks

All *Drosophila* stocks were grown at 25°C on standard cornmeal-molasses agar medium. Control animals were from a *y w* genetic background. Mutant alleles used in this study were as follows: *pros*¹⁷, *pros*¹⁴ (Doe et al., 1991), *pros*¹¹³ (Srinivasan et al., 1998), *mir*^{ZZ176} (Ikeshima-Katoaka et al., 1997), *stau*^{r9} (St Johnston et al., 1991) and *numb*² (Frise et al., 1996). All *prospero* mutant data presented in figures is from our analysis of *pros*¹⁷; however, similar results were obtained using the *pros*¹⁴ and *pros*¹¹³ alleles. Mutant stocks were maintained over *CyO*, *ftz-lacZ* or *TM3*, *ftz-lacZ* balancer chromosomes.

Immunohistochemistry

Standard methods were used for collection and fixation of *Drosophila* embryos. Immunohistochemistry was performed as described

elsewhere (Spana and Doe, 1995). Primary antibodies were used at the following dilutions: mouse anti-Engrailed, 1:5; rabbit anti-Miranda 1:500 (Fuerstenberg et al., 1998b); mouse anti-Prospero, 1:5 (Spana and Doe, 1995); rabbit anti-Inscuteable, 1:1,000 (Kraut and Campos-Ortega, 1996); mouse anti-Staufen, 1:100 (Broadus et al., 1998); rabbit anti-Numb, 1:250 (Rhyu et al., 1994); rabbit anti-Eagle, 1:1000 (Higashijima et al., 1996); rat anti-Glial cells missing, 1:1000 (Jones et al., 1995); and rat anti-Repo, 1:1000 (Campbell et al., 1994). Actively mitotic cells were identified using rabbit anti-phosphohistone H3 antibodies at 1:2500 (Upstate Biotechnology). Secondary antibodies used for immunofluorescence were conjugated to DTAF, LRSC, or Cy5 (Jackson ImmunoResearch) and were used at 1:400. Embryos were mounted in anti-fade reagent (BioRad) and viewed on a BioRad MRC-1024 confocal microscope. For immunohistochemistry we used the Vectastain HRP detection kit (Vector labs). β -Galactosidase expressed from balancer chromosomes was detected using a rabbit anti- β -galactosidase primary antibody at 1:1000 (Cappel), followed by anti-rabbit secondary antibodies conjugated to alkaline phosphatase, and detected using standard procedures (Boehringer Mannheim). After staining, embryos were mounted in glycerol, viewed on a Zeiss Axioplan microscope, and imaged with a Sony DK-5000 digital camera.

Digoxigenin-labeled RNA probes were generated according to the manufacturer's instructions (Boehringer Mannheim). RNA in situ hybridization to embryos were carried out as described previously (Broadus et al., 1998). Immunofluorescent detection was accomplished using fluorescein-conjugated anti-digoxigenin antibodies (Boehringer Mannheim).

Identification of NGBs and their progeny

NGBs 6-4 and 7-4 are the most lateral Engrailed positive cells in the neuroblast array (Broadus et al., 1995). The most lateral Eagle-positive/Engrailed-positive cell is NGB 6-4T. Eagle is detectable in NGB 6-4T before its first division and subsequently in all NGB 6-4T progeny (Higashijima et al., 1996). Glial progeny of NGBs 6-4 and 7-4 were identified by staining with antibodies to either Gcm or Repo.

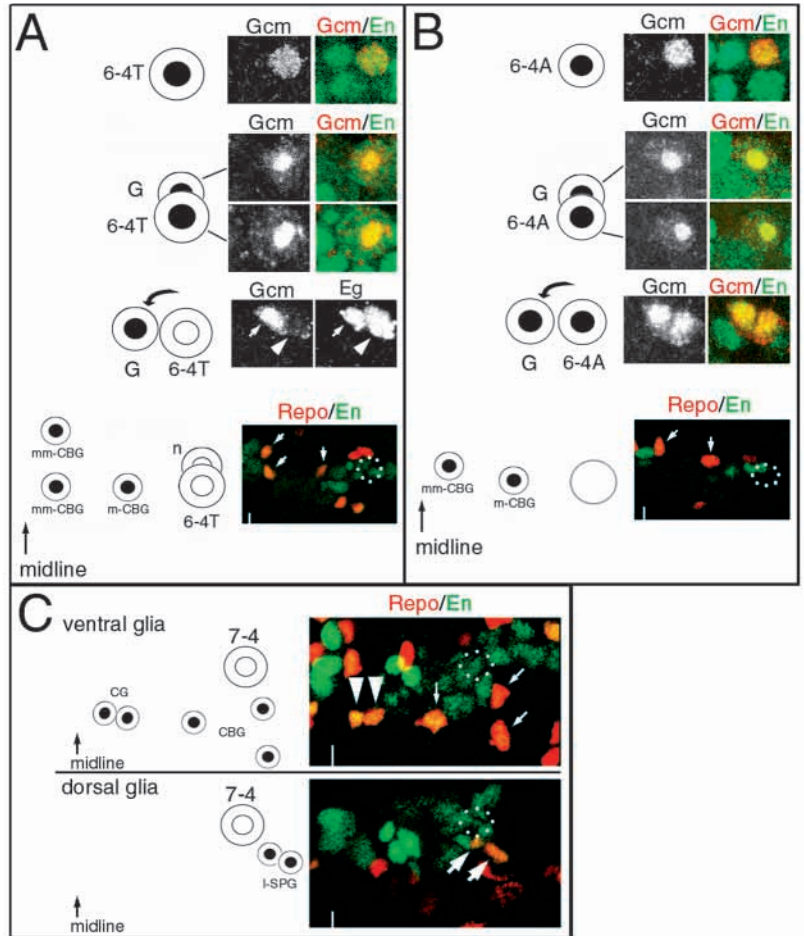
For RNA in situ hybridization with *gcm* mRNA, we used the following criteria to identify NGB 6-4T before, and after, its first division: (1) *gcm* expression in the lateral glial precursor (GP) was used as a positional landmark – the GP is slightly posterior and lateral to NGB 6-4. Prior to the first division of 6-4T the GP is the only cell in the lateral-most region of the CNS to express *gcm*. (2) The first division of NGB 6-4T is tightly correlated temporally with the production of two glial cells by NGB 7-4. Three or more Gcm-positive cells at the position of NGB 7-4 are only observed after NGB 6-4T has undergone its first division. (3) NGB 6-4T consistently divides before the first division of GB 6-4A. (4) In experiments where it was not directly labeled, we identified NGB 6-4T by its position in the neuroblast array relative to NB 6-2, NB 7-2, NGB 7-4 and the lateral GP using either Nomarski optics or background immunofluorescence.

RESULTS

glial cells missing expression and spindle orientation in the NGB 6-4T, 7-4 and GB 6-4A lineages

In thoracic segments the neural precursor 6-4 generates both glia and neurons, and is referred to as NGB 6-4T; in abdominal segments the 6-4 precursor produces only glia, and so we call it GB 6-4A (Akiyama-Oda et al., 1999; Bossing et al., 1996; Schmid et al., 1999). The neural precursor 7-4 generates a lineage composed of both neurons and glia in all segments, and we refer to it as NGB 7-4.

Fig. 1. *Gcm* protein distribution, spindle orientation and cell migration in neural precursor lineages. (A) NGB 6-4T divides along the apical-basal axis and expresses *Gcm* before its first division. In the left column are schematic representations; in the right column are paired confocal images (each pair from the same focal plane) corresponding to the diagrammed cells. Ventral view. Anterior is upwards; midline is towards the left. *Gcm* is expressed in the pre-divisional NGB 6-4T (6-4T). NGB 6-4T divides along the apical-basal axis ($n > 60$ mitotic NGB 6-4T). Both the daughter cell (G) and the post-divisional NGB 6-4T are *Gcm* positive, and *Gcm* is localized to the nucleus (a different focal plane is shown for each of these cells). The G cell (arrow) next undergoes a migration to a position just medial to NGB 6-4T (arrowhead; note that NGB 6-4T and G are in the same focal plane at this stage). By the end of this migration, *Gcm* protein is no longer detectable in NGB 6-4T. The G cell divides twice to generate three glia (one medial cell body glia, m-CBG; and two medial-most cell body glia, mm-CBG), which migrate toward the midline. Subsequent divisions of NGB 6-4T (dotted circle in bottom confocal image) are along the apical-basal axis, and generate neuron-producing daughter cells (n). (B) GB 6-4A divides along the apical-basal axis and expresses *Gcm* before its first division (oriented as in A). *Gcm* is expressed in the predivisional GB 6-4A (6-4A). GB 6-4A divides along the apical-basal axis (a different focal plane is shown for each cell), and both daughter cells are *Gcm*-positive. The G cell next rapidly migrates to a position medial to GB 6-4A (note that these cells are in the same focal plane at this stage); both G and GB 6-4A maintain *Gcm* expression and differentiate as glia. The gray circle in the diagram and the dotted circle in the confocal image approximate the position of the predivisional GB 6-4A. (C) Glial progeny of NGB 7-4. The position of NGB 7-4 (which is just out of the focal plane) is indicated by the dotted circle in the confocal image. Ventral glia: at the ventral surface of the CNS, two glial progeny of NGB 7-4 migrate to the midline and become channel glia (CG, arrowheads); three glia remain close to NGB 7-4 and become cell body glia (CBG, arrows). Dorsal glia: one to two glia migrate laterally and dorsally and become lateral subperineurial glia (l-SPG, large arrows).



NGB 6-4T and GB 6-4A form at early embryonic stage 10 as part of the S3 wave of neuroblasts (Broadus et al., 1995). We find that the first division of NGB 6-4T is oriented along the apical-basal axis, producing a large apical post-divisional precursor (NGB 6-4T) and a smaller basal daughter cell (G) (Fig. 1A; Fig. 2). *Gcm* is expressed before this first division, both daughter cells inherit *Gcm* protein, which enters the nucleus in these cells immediately after NGB 6-4T division (Fig. 1A). Interestingly, shortly after NGB 6-4T completes this division, the G daughter cell migrates from its basal position to a position just medial to the post-divisional NGB 6-4T (Fig. 1A; Fig. 2), which may explain why this division was previously scored as mediolateral (Akiyama-Oda et al., 1999; Bernardoni et al., 1998). By the end of the G cell medial migration, *Gcm* protein is downregulated in NGB 6-4T (Fig. 1A) and maintained only in the G daughter cell. 6-4T continues to divide along the apical-basal axis and subsequently produces neuronal progeny (Fig. 1A). The G daughter cell maintains high levels of *Gcm* protein and produces three glial cells (Fig. 1A). These glia continue to express *Gcm* protein, activate the glial specific gene *reversed polarity* (*repo*) (Fig. 1A) (Campbell et al., 1994; Xiong et al., 1994), migrate medially, and differentiate into cell body glia (CBGs) (Schmid et al., 1999; Schmidt et al., 1997).

These results raise the question of how *Gcm* protein becomes asymmetrically restricted to the G daughter cell after the first division of NGB 6-4T. It has been proposed that *gcm* mRNA is asymmetrically partitioned into the G daughter cell during mitosis of NGB 6-4T (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). To test this model, we scored *gcm* mRNA localization by fluorescent in situ hybridization. We observe low levels of *gcm* mRNA in the predivisional NGB 6-4T, followed by uniform localization in the mitotic NGB 6-4T, and equal distribution to both NGB 6-4T and G sibling cells (Fig. 2). After G cell migration we observe *gcm* mRNA in the G cell and down-regulation of *gcm* mRNA in the post-divisional NGB 6-4T (Fig. 2). We have also scored *gcm*-expressing cells throughout the CNS, and have never observed asymmetric localization of *gcm* mRNA in any mitotic precursor cell ($n=31$). We conclude that *gcm* mRNA is not asymmetrically localized in the mitotic NGB 6-4T, but rather that it becomes transcriptionally upregulated in the G daughter cell soon after it is born.

Similar to NGB 6-4T, GB 6-4A divides along the apical-basal axis and its basal daughter cell rapidly migrates to a position medial to its apical sibling (Fig. 1B). In contrast to NGB 6-4T, GB 6-4A expresses high levels of *Gcm* protein and

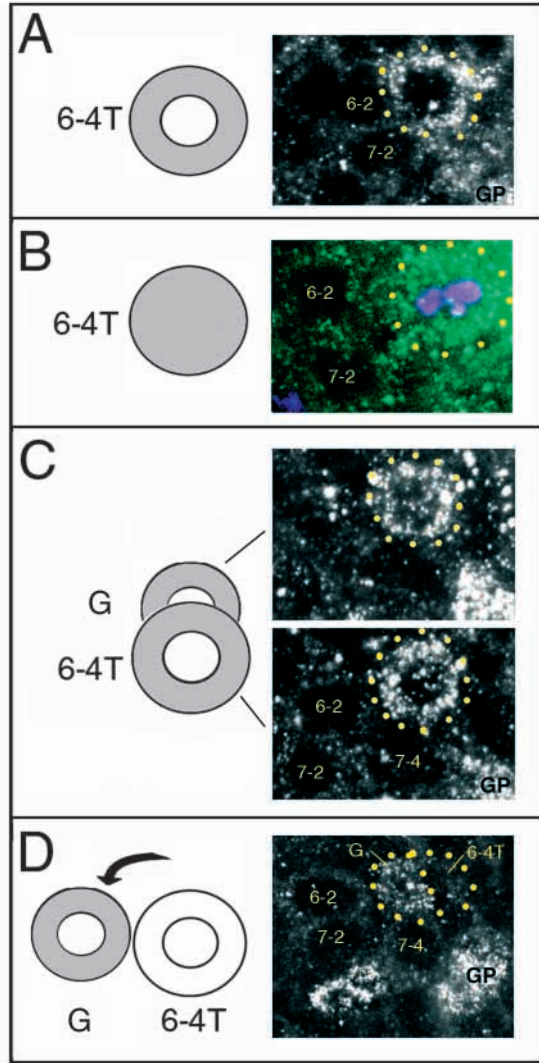
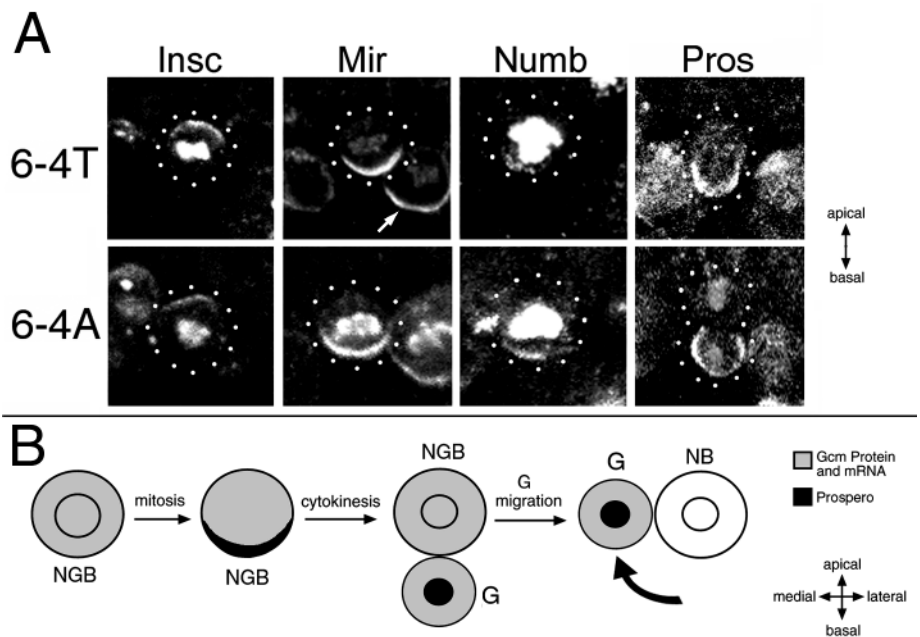


Fig. 2. *gcm* mRNA is not asymmetrically localized during NGB 6-4T cell division. Ventral view *gcm* mRNA localization in the NGB 6-4T lineage; oriented as in Fig. 1. NGB 6-4T (6-4T) and the G daughter cell are circled (yellow dots); the adjacent NB 6-2 (6-2), NB 7-2 (7-2), NGB 7-4 (7-4) and the lateral glial precursor (GP) are labeled when visible and were used as landmarks (see Materials and Methods). (A) *gcm* mRNA is expressed in the predivisional NGB 6-4T (6-4T). (B) Anti-phosphohistone H3 (purple) is a marker for mitotic DNA. *gcm* mRNA (green) is equally distributed throughout the cell in the mitotic NGB 6-4T. (C) *gcm* mRNA is present in both the apical NGB 6-4T and its basally positioned G daughter cell after mitosis. (D) After the medial migration of the G daughter cell, *gcm* mRNA is detected in the G daughter cell but not in the post-divisional NGB 6-4T.

mRNA before its first division, and both daughter cells maintain *gcm* expression (Fig. 1B). These two cells subsequently express *repo*, migrate medially, and differentiate into cell body glia (CBGs; Fig. 1B).

NGB 7-4 forms at late stage 8 as the most lateral En-positive S1 neuroblast (Broadus et al., 1995). The first progeny from NGB 7-4 are Prospero positive and Gcm negative, and differentiate into neurons. At stage 10 (just before the formation of 6-4 neural precursors) NGB 7-4 begins producing several Prospero-positive, Gcm-positive daughter cells that make a total of six to seven glia (Fig. 1C). At stage 12, NGB 7-4 switches back to making Prospero-positive Gcm-negative daughter cells that develop into neurons. All divisions of NGB 7-4 are along the apico-basal axis (Fig. 3A); Gcm-positive progeny are budded off the basal surface of NGB 7-4 but then migrate extensively to their final positions; ultimately, two glia migrate along the ventral surface of the CNS and differentiate as a pair of En-positive midline channel glia, three remain on the ventral surface of the CNS near NGB 7-4 and develop into CBGs posterior to the En-positive stripe; and one or two migrate to a position slightly dorsal and lateral to NGB 7-4 and differentiate as lateral subperineurial glia (Fig. 1D) (Bossing et al., 1996; Schmid et al., 1999). In contrast

Fig. 3. Asymmetric protein localization along the apical-basal axis in NGB 6-4T and GB 6-4A. (A) Lateral view of stage 10 embryos; apical is upwards; basal is downwards; anterior is towards the left. NGB 6-4T (6-4T) and GB 6-4A (6-4A) were identified as described in Fig. 1 and the Materials and Methods. DNA was labeled with anti-phosphohistone H3. Inscuteable (Insc) forms an apical crescent in mitotic NGB 6-4T ($n=7$) and GB 6-4A ($n=8$) and is partitioned to the apical precursor after cytokinesis. Miranda (Mir), Numb and Prospero (Pros) form basal crescents in mitotic NGB 6-4T and GB 6-4A, and are partitioned into the basal G daughter cells in both lineages after cell division ($n>13$ for Mir and Pros; $n>6$ for Numb). The arrow in the 6-4T Mir panel shows an example of a mitotic NGB 7-4 during a glia-producing division; we find these divisions to also occur along the apical-basal axis. (B) Summary of Prospero protein (Pros), *gcm* mRNA and Gcm protein localization in the NGB 6-4T lineage. See text for details.



to the 6-4 neural precursors, we do not detect *gcm* mRNA or protein in NGB 7-4, only in its glial progeny.

In summary, we find that glia-producing divisions of NGB 6-4T and GB 6-4A occur along the apicobasal axis, and that these divisions are followed by medial migrations of glial progeny. *Gcm* mRNA and protein are present in the predivisional NGB 6-4T, and *Gcm* protein enters the NGB and daughter cell nuclei immediately after the first division of this NGB. In addition, we find no evidence for asymmetric localization of *gcm* mRNA in any glial lineage, including NGB 6-4T.

Asymmetric protein localization along the apical-basal axis in NGB 6-4T and GB 6-4A

If *gcm* mRNA and protein are equally distributed into NGB 6-4T and its first-born G daughter cell, how are *gcm* mRNA and protein levels upregulated in G but not NGB 6-4T? To address this issue, we assayed mitotic NGB 6-4T for proteins known to be asymmetrically localized along the apical-basal axis of neuroblasts. Our goal was to identify candidate genes that could differentially regulate *gcm* expression in the NGB 6-4T lineage. *Insc* protein marks the apical side of most or all mitotic neuroblasts and is necessary and sufficient for apical-basal spindle orientation. In NGB 6-4T, *Insc* is localized as an apical crescent at all stages of mitosis (Fig. 3A) and is partitioned into the apically-positioned NGB 6-4T following cytokinesis. The mitotic GB 6-4A also shows apical *Insc* localization (Fig. 3A). Because *Insc* is sufficient to orient the mitotic spindle in all neuroblasts and epithelial cells assayed (Kraut et al., 1996), the apical localization of *Insc* in NGB 6-4T and GB 6-4A provides strong confirmation that both cells divide along their apical-basal axis.

Miranda, Prospero, Staufen, and Numb proteins mark the basal side of many or all mitotic neuroblasts and regulate the fate of daughter cells or their neuronal progeny. In NGB 6-4T, Miranda, Prospero, Staufen and Numb all form basal crescents from metaphase through telophase (Fig. 3A; data not shown for Staufen), and are partitioned into the basally positioned G daughter cell of NGB 6-4T after cytokinesis. The mitotic GB 6-4A also shows basal localization of Miranda, Prospero, Staufen and Numb (Fig. 3A; data not shown for Staufen). These results further confirm the apical-basal division axis of NGB 6-4T and GB 6-4A during glial producing divisions, and show that all of the above proteins are candidates for regulating *gcm* expression in the basal G daughter cell of NGB 6-4T (Fig. 3B).

prospero and *miranda* are required for normal glial development in the NGB 6-4T lineage

To determine if *miranda*, *prospero*, *staufen* or *numb* are involved in the development of glia in the NGB 6-4T lineage, we scored embryos mutant for each gene for the number and position of mature glia derived from NGB 6-4T. The three glia from NGB 6-4T express *repo* and have distinctive positions within the CNS: two near the midline and one between NGB 6-4T and the midline (Fig. 4A). These are the only *Repo*-positive glia adjacent to the midline at the ventral surface of the CNS, and thus are easy to identify unambiguously. We find that mutations in *staufen* and *numb* have no effect on glial development in the NGB 6-4T lineage (Fig. 4B,C; $n > 20$ for each). By contrast, *prospero* mutant embryos show striking loss

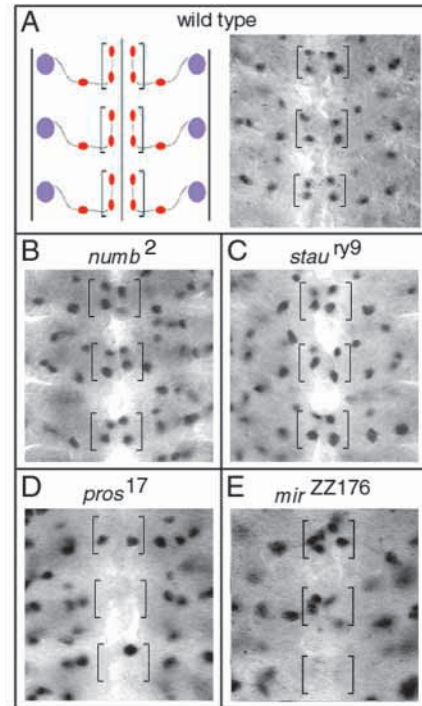
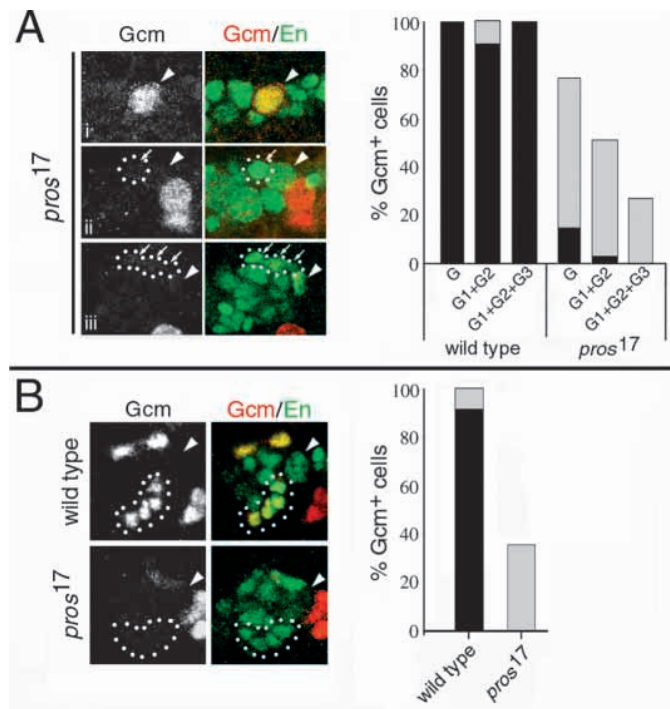


Fig. 4. *prospero* and *miranda*, but not *numb* or *staufen*, are required for glial development in the NGB 6-4T lineage. Ventral view of a stage 13 embryos showing *Repo*-positive glia derived from the NGB 6-4T lineage. Three thoracic segments are shown in all panels; anterior is upwards; $n \geq 20$ for all. (A) Wild-type glial development in the NGB 6-4T lineage. Left: a summary showing the positions of glia (red dots) derived from NGB 6-4T (purple circle) with migration patterns (lines). Right: wild-type embryo stained for *Repo*, brackets indicate NGB6-4T-derived glial progeny. (B,C) *numb* and *staufen* mutants show a wild-type pattern of NGB 6-4T-derived glia. (D) *prospero* mutants show a loss of NGB 6-4T-derived glia. *Repo*-positive cells in the region of 6-4T glia in *prospero* mutants are not derived from NGB 6-4T, based on their lack of expression of the NGB 6-4T marker *eagle* (data not shown, see also Fig. 5). (E) *miranda* mutants have a variable phenotype showing reduced, normal or extra NGB 6-4T-derived glia (see text for details).

of NGB 6-4T-derived glia (Fig. 4D), while *miranda* mutant embryos have a similar but weaker phenotype (Fig. 4E, see below). We conclude that *prospero* and *miranda*, but not *staufen* or *numb*, are required for normal glial development in the NGB 6-4T lineage.

Prospero is necessary to upregulate *glial cells missing* expression in NGB glial progeny

To determine earliest aspect of the *prospero* mutant phenotype in the NGB 6-4T lineage, we assayed whether *gcm* is expressed normally in the G daughter cell. In wild-type embryos, *Gcm* protein is detectable in the predivisional NGB 6-4T and in the sibling NGB 6-4T/G cells immediately after cytokinesis; subsequently, *Gcm* disappears from NGB 6-4T and is upregulated in the G cell and its progeny (e.g. Fig. 1A), which proceed to migrate medially and express *repo* (Fig. 1A). In *prospero* mutant embryos, *gcm* expression is activated normally in the predivisional NGB 6-4T (Fig. 5A) and is detectable in the immediately post-mitotic NGB 6-4T and G cell. Therefore the early induction of *gcm* expression in these



cells is clearly *prospero*-independent. However, Gcm protein levels subsequently decline in the G cell and its progeny and these cells fail to migrate to the midline (Fig. 5A) or express *repo*. These data indicate that Prospero is required in the G daughter cell to maintain or upregulate *gcm* expression levels, induce medial migration, and activate *repo* expression. Surprisingly, these Gcm negative, Repo negative cells do not express the neuron-specific *elav* gene (data not shown), and thus they appear unable to differentiate as glia or neurons.

In *prospero* mutant embryos, *gcm* expression is also greatly reduced in the progeny of NGB 7-4 (Fig. 5B). Low level

Fig. 5. Prospero is required to upregulate *gcm* expression in NGB lineages. (A) Prospero is required for Gcm expression in NGB 6-4T glial progeny. Paired confocal images are from the same focal plane. Ventral view; midline is towards the left; anterior is upwards. Expression of Gcm in the Engrailed-negative lateral glial precursor is seen at the bottom right of most images. (i) *prospero* mutants show normal levels Gcm in the predivisional NGB 6-4T (arrowhead). (ii) By the time the G daughter cell migrates medially, both the G cell (arrow) and post-divisional NGB 6-4T (arrowhead) have down-regulated Gcm. (iii) Later in development all G cell progeny have severely reduced Gcm levels and they fail to migrate to the midline (arrows). Compare with Fig. 1A. The graph shows a quantitation of the percentage of Gcm-positive cells in the NGB 6-4T lineage in wild type ($n=61$) and *prospero* mutants ($n=90$): wild type Gcm levels, black bar; strongly reduced Gcm levels, gray bar; and lack of detectable Gcm, no bar. G, G alone; G1+G2, the first two G-derived cells; G1+G2+G3, all three G-derived cells. (B) Prospero is required for Gcm expression in the NGB 7-4 lineage (oriented as in A). In the wild type, there are 4-5 Gcm-positive progeny (dotted circle) derived from NGB 7-4 (arrowhead). In *prospero* mutants, Gcm expression is severely reduced (dotted circle). The graph shows a quantitation of the percentage of Gcm-positive cells in the NGB 7-4 lineage in wild type ($n=45$) and *prospero* mutant ($n=76$) hemisegments. Bars indicate Gcm levels as described in A.

expression of *gcm* is detectable in many NGB 7-4 progeny shortly after their birth, indicating that in this lineage (as in NGB 6-4T) the induction of *gcm* expression can occur in the absence of *prospero* function. However, *gcm* expression fades rapidly and these cells never express *repo*. Thus, *prospero* is essential for the maintenance of *gcm* expression and normal glial cell fate induction in both the NGB 6-4T and 7-4 lineages.

***miranda* is required to prevent glial cells missing upregulation in NGBs and to limit glial production in NGB lineages**

prospero is clearly necessary for upregulation of *gcm* and glial

Fig. 6. Miranda is required to prevent *gcm* upregulation in NGB 6-4T and NGB 7-4 lineages. (A) Wild type and *miranda* mutant embryos stained for Gcm protein. Anterior is upwards; midline is towards the left. Stage 12 embryos are shown for NGB 6-4T, late stage 11 embryos are shown for NGB 7-4. In wild-type embryos (top row), Gcm is not detected in NGB 6-4T or NGB 7-4 (dotted circles). In *miranda* mutant embryos (bottom row), Gcm protein is present in NGB 6-4T and NGB 7-4 (dotted circles; 60% of hemisegments scored for both; $n=91$ hemisegments). (B) *miranda* mutant embryos can make extra glia from NGB 6-4T. Mutant embryos were stained with antibodies to Repo (to identify glia; red), Eg (to visualize the entire NGB 6-4T lineage, blue) and En (as a second marker for NGB 6-4T, green). Arrowheads, NGB 6-4T; arrows, NGB 6-4T-derived glial progeny; $n=38$ hemisegments. One segment is shown, the midline is indicated by the vertical bar. To the left of the midline, the NGB 6-4T shows high levels of Repo expression and no neuronal progeny were produced by this NGB. By contrast, to the right of the midline the NGB 6-4T is not expressing Repo and several neuronal progeny have been produced (out of plane of focus).

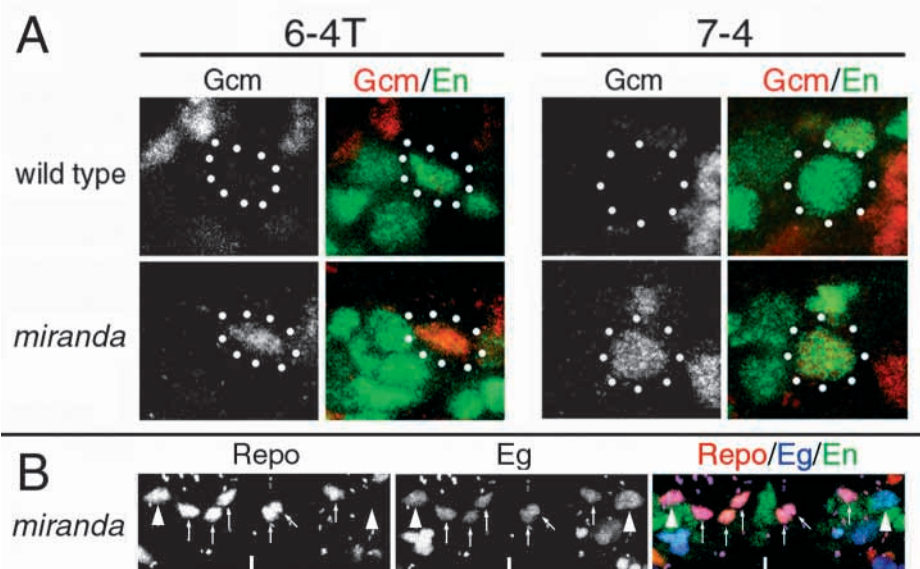
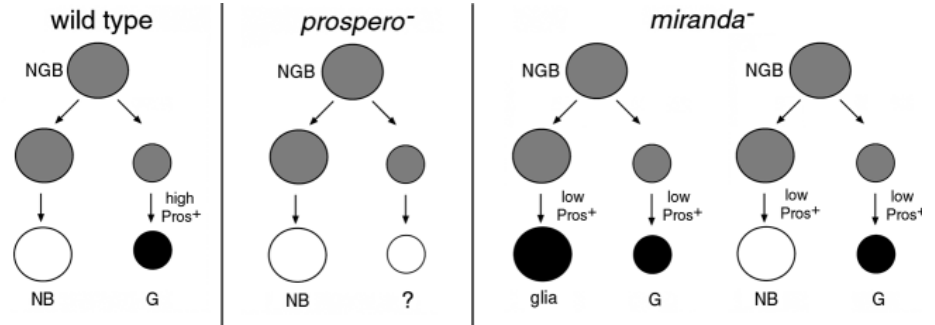


Fig. 7. Requirements for *prospero* in separating glial and neuronal cell fates. In wild type, low level *gcm* mRNA and protein (gray) are evenly segregated to the NGB and the daughter cell. The daughter cell inherits all Prospero, which upregulates *gcm* expression and induces glial fate (black). In *prospero* mutants, low level *gcm* expression (gray) is induced normally in the NGB but fades rapidly and glia are not produced. In *miranda* mutants, Prospero is evenly segregated into the NGB and the daughter cell resulting in either (1) upregulation of *gcm* in the NGB, its transformation into a GB or glial cell, and the truncation of the lineage (black); or (2) insufficient Prospero is present in the NGB, low level *gcm* fades in the NGB, and neuronal progeny are subsequently produced (NB, white).



cell fate induction in the 6-4T and 7-4 lineages, but is it sufficient to induce *gcm* expression in these lineages? In *miranda* mutant embryos, *prospero* mRNA and protein are delocalized during neural precursor cell division, resulting in similar concentrations of Prospero segregating to both NGBs and their daughter cells (Fuerstenberg et al., 1998a; Ikeshima-Kataoka et al., 1997). Interestingly, in *miranda* mutants we find ectopic *gcm* expression in NGB6-4T at stage 13 (Fig. 6A), a time when this NGB is normally making neuronal progeny. *miranda* mutants also show ectopic expression of *gcm* in NGB 7-4 lineage during its window of glial production (Fig. 6A). Thus, mislocalization of Prospero to the NGB by removal of *miranda* function is sufficient to induce ectopic *gcm* expression in these NGBs.

Does the upregulation of *gcm* in NGBs drive the production of extra glial progeny? To address this question, we assayed Repo expression in the NGB6-4T lineage in *miranda* mutants as we can identify the entire NGB 6-4T lineage (see Materials and Methods for details). In *miranda* mutants we typically find only four Repo positive cells in the entire NGB 6-4T lineage, but neuronal progeny are completely absent ($n=45$; 46% of hemisegments scored) (Fig. 6B). We interpret this phenotype to indicate that the G cell produces three glia as usual, but that its sibling NGB differentiates directly into a Repo-positive glia cell, resulting in a termination of the lineage (see Discussion). Thus, it appears that Prospero mislocalized to the NGB can potentially activate *gcm* expression in the NGB and transform it into a glial cell.

We also find two additional phenotypic classes in *miranda* mutants: (1) a variable number of Repo-positive glia are produced (between two and four) and subsequent neuronal progeny are generated normally (12% of hemisegments); or (2) the wild-type pattern of three Repo-positive glia and neuronal progeny are produced (42% of hemisegments scored; e.g. Fig. 6B). These phenotypes indicate that low level Prospero in the NGB is not always sufficient to induce a glial fate, and that reduced Prospero in the G cell may lead to fewer glial progeny.

Mislocalization of Prospero to NGB 7-4 by removal of *miranda* function can also induce Repo expression in this NGB (25% of hemisegments), showing that NGB 7-4 can also be partially transformed towards a glial fate. We do not know if this NGB differentiates as a glial cell (like the Pros-positive NGB 6-4T), generates extra glial progeny, or if it can eventually produce neurons. *miranda*, *prospero* double mutants do not show upregulation of *gcm* in NGBs 6-4T or 7-4 (data not shown), demonstrating that the upregulation of *gcm* in

these NGBs in *miranda* mutant embryos is due to Prospero protein that is delocalized into the NGB. Our results indicate that Miranda, by asymmetrically localizing Prospero to NGB daughter cells, restricts *gcm* upregulation and induction of the glial developmental program to the progeny of NGBs 6-4T and 7-4 during their phases of glial production (Fig. 7).

DISCUSSION

Spindle orientation, cell migration and glial development

The *Drosophila* NGB 6-4T has been reported to divide along the mediolateral axis when producing glia but along the apical-basal axis when producing neurons (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). GB 6-4A was also reported to divide along the mediolateral axis (Akiyama-Oda et al., 1999). From these observations it was suggested that mediolateral cell divisions correlate with glial production while apical-basal division correlate with neuron production. The above studies relied upon the use of general DNA stains and cell position to document the mediolateral division; however, the use of general DNA stains make it hard to distinguish each phase of the cell cycle, and cell position can change rapidly due to cell migration. In this study, we stained for phosphorylated histone to specifically label mitotic cells, and we used antibodies that exclusively mark apical or basal membrane domains of neural precursors. In contrast to previous reports, we find that NGB 6-4T and GB 6-4A always divide along the apical-basal axis, and that in each case the basal daughter cell rapidly migrates medially. We suspect that previous workers missed this apical-basal cell division, and only scored the post-migration mediolateral cell arrangement. From our data we conclude that the glia-producing divisions of NGBs 6-4T and 7-4, and GB 6-4A occur along the apical-basal axis, similar to the division axis of all other *Drosophila* embryonic neural stem cells.

Cell lineage studies show that the grasshopper median neuroblast (MNB) is a multipotent neuronal stem cell that generates both neurons and glia; it too is reported to have a mediolateral division axis when producing glia and an apical-basal division axis when generating neurons (Condrion and Zinn, 1994). In this report, a careful analysis of the MNB at late telophase clearly showed a mediolateral division axis during the time glial progeny are produced. It is not clear if the MNB always, or only occasionally, divides mediolaterally when producing glia; whether there are differences between an

unpaired medial precursor (MNB) and bilateral precursors (NGBs 6-4T and 7-4); or whether there are differences between grasshopper and *Drosophila* in the mechanisms regulating spindle orientation.

The phenomena of apical-basal division followed by medial migration also occurs in the GB 6-4A and lateral GP lineages, with the difference being that all of the cells in these lineages ultimately form glia and migrate medially. Because the medial migration is correlated with *gcm* expression, and does not occur in NGB progeny that lack *gcm* expression, we suggest that high levels of *gcm* expression triggers migration of these glia towards the midline of the CNS. The nature of the cue that orients glial migrations is unknown.

Regulation of *glial cells missing* mRNA and protein localization

Previous reports have suggested that *gcm* mRNA is asymmetrically localized in a medial crescent in the mitotic NGB 6-4T, resulting in the selective partitioning of *gcm* mRNA into the medial glia-producing progeny of NGB 6-4T (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). We believe these conclusions to be in error for three main reasons. First, we show that the mitotic NGB 6-4T contains evenly distributed *gcm* mRNA, and that this mRNA is partitioned equally between NGB 6-4T and its glial-producing G daughter cell after cytokinesis. Second, previous studies did not use a mitosis-specific marker together with probes for *gcm* mRNA localization to prove that the localization was being scored in mitotic NGBs. Third, we show that NGB 6-4T always divides along the apical-basal axis, therefore a medial localization of *gcm* mRNA would not result in it being partitioned unequally into one daughter cell. Indeed, in a recent study that made use of more specific markers for mitotic stage and cell orientation it was found that the first division of NGB 6-4T is not along the mediolateral axis, and that *gcm* mRNA is inherited by both the NGB and the G daughter cell (Ragone et al., 2001). We therefore conclude that the asymmetric localization of *gcm* mRNA is not the mechanism by which neuronal and glial lineages are separated in the NGB 6-4T lineage.

Gcm protein has been reported to be absent from the predivisional NGB 6-4T, perhaps owing to translational repression of *gcm* mRNA prior to its first division (Akiyama-Oda et al., 1999). We show that this is not the case, as Gcm protein is clearly present in the predivisional NGB 6-4T (Fig. 1A; Bernardoni et al., 1999). In addition, it has been reported that Gcm protein is excluded from the nucleus in the postdivisional NGB 6-4T, and that an unidentified mechanism regulates nuclear entry of Gcm specifically in the G daughter cell (Bernardoni et al., 1999). We have shown robust Gcm protein expression in the nucleus of NGB 6-4T (Fig. 1A), arguing strongly against the existence of such a mechanism.

Prospero is necessary to upregulate *glial cells missing* expression in NGB glial progeny

We observe asymmetric localization of Prospero, Miranda, Staufen and Numb proteins into the glial-producing daughter cells during NGB divisions, but only mutations in *prospero* and *miranda* affect the production of glia from NGBs 6-4T and 7-4. These results are consistent with two recent reports of a requirement for *prospero* in the production of glia from selected NGBs (Akiyama-Oda et al., 2000; Ragone et al.,

2001). In *prospero* mutant embryos, NGB 6-4T progeny do not migrate significantly towards the midline or express the glial-specific *repo* gene. These phenotypes are probably due to lack of *gcm* expression, as loss of *prospero* does not affect migration or *repo* expression in GB 6-4A (which has high *gcm* levels even in *prospero* mutant embryos).

We have extended these findings to show that Gcm expression is induced properly in *prospero* mutant embryos, but that both the G daughter cell and the post-divisional NGB downregulate *gcm* expression with a similar timecourse. Thus, the induction of *gcm* expression in NGB 6-4T is *prospero*-independent. Interestingly, *prospero* mutant embryos also fail to upregulate *gcm* to high levels in NGB 7-4 daughter cells. This is consistent with a previous report that NGB 7-4-derived Repo-positive glia are absent in *prospero* mutants (Akiyama-Oda, 2000). Unlike NGB 6-4T, we are unable to detect *gcm* expression in NGB 7-4, only in its new-born (pre-migration) daughter cells. Low level Gcm expression is still present in *prospero* mutant embryos; thus, the induction of *gcm* expression in this lineage also appears to be *prospero*-independent. We propose that *prospero* functions to upregulate low levels of *gcm* in these lineages, but is not sufficient to induce *gcm* expression on its own. Such a mechanism would explain why all neural stem cell progeny in the CNS express high levels of *prospero* but most never induce *gcm* and the glial developmental program. In agreement with this model, we have found that misexpression of low levels of *gcm* throughout the CNS requires Prospero to induce glia in a subset of lineages (M. R. F. and C. Q. D., unpublished).

How might the Prospero transcription factor upregulate low levels of *gcm* expression? Prospero is known to act as a co-factor to stimulate transcriptional activity of several DNA-binding proteins (Hassan et al., 1997). Recent studies show that Gcm can positively autoregulate its own expression in neural tissues (Miller et al., 1998). It is possible that Prospero may act together with Gcm to stimulate expression levels of the *gcm* gene until they become sufficiently high for Gcm to positively autoregulate its own expression. However, embryos homozygous for the *gcm*^{N7-4} allele produce non-functional Gcm protein (Vincent et al., 1996) that is upregulated with normal kinetics in the NGB 6-4T and 7-4 lineages (M. R. F. and C. Q. D., unpublished), indicating that Gcm function is dispensable for its own upregulation. We propose that a lineage-specific co-factor or extrinsic signal converges with Prospero function in these lineages to upregulate *gcm* expression.

How does a NGB know when to make neurons or glia? For example, the first born daughter cell from NGB 6-4T gives rise to glia while all subsequent progeny are neuronal (Akiyama-Oda et al., 1999). By contrast, NGB 7-4 first produces several neuronal progeny, then switches to making glia, and finally switches back to making neurons (M. R. F. and C. Q. D., unpublished). Interestingly, the window of developmental time during which these two neural precursors are making glia are strikingly similar: NGB 7-4 begins making glia at stage 10, shortly after this NGB 6-4T is born (late stage 10) and begins making glia; at stage 11 both precursors terminate glial production and switch to making neurons. The coordinate timing of glial production from these lineages may indicate that a temporally regulated extrinsic cue induces *gcm* expression in these NGBs or their newly born progeny.

Upregulation of *glial cells missing* expression is essential to induce glial cell fate

In *prospero* mutant embryos, NGB 6-4T and its progeny only transiently express low levels of *gcm*. What is the fate of these cells? They never express the glial-specific *repo* gene, and we have found that they also fail to express the neuron-specific *elav* gene, indicating that neither the glial or neuronal developmental program has been initiated. *gcm* is thought to transcriptionally activate genes that promote glial fate or repress neuronal fate (Klamt et al., 1999). We propose that NGB 6-4T in *prospero* mutants produces enough Gcm protein to repress neuron-specific genes, yet insufficient amounts to robustly induce glial-specific genes. This in turn suggests that there may be different *gcm* thresholds for activating glial development (high threshold) and for repressing neuronal development (low threshold).

Asymmetric localization of Prospero is necessary to prevent *glial cells missing* expression in NGBs

In *miranda* mutant embryos, Prospero protein is delocalized at mitosis, allowing NGB/daughter cell siblings to inherit equal concentrations of Prospero. In these embryos, we frequently observe ectopically upregulated *gcm* in NGBs 6-4T and 7-4, and extra glia derived from NGB 6-4T. Akiyama-Oda et al. (Akiyama-Oda et al., 2000) report that in *miranda* mutants all progeny of NGB 6-4T take on a glial fate as determined by Repo staining. By contrast, we find that this transformation takes place in approximately 46% of hemisegments. This difference is not likely to be accounted for by the use of different *miranda* alleles as both appear to completely delocalize Prospero protein in NBs (Fuerstenberg et al., 1998b). Regardless of these differences, these data indicate that *prospero* is a potent activator of *gcm* expression in the NGB 6-4T and 7-4 lineages. The extra glia we observe could come from an extension of the glial portion of the NGB 6-4T lineage, or from a transformation of this NGB into a purely glial progenitor. We favor the latter model, because neurons are never observed in the NGB 6-4T lineage when we observe extra glia. Moreover, high levels of Gcm are correlated with pure glial lineages such as GB 6-4A and the GP, and *gcm* is known to positively autoregulate which may commit precursors with high Gcm to a glial-producing fate.

We also find that in *miranda* mutant embryos, the ectopic expression of *gcm* in NGB 6-4T and 7-4 is in fact due to delocalization of Prospero and not simply the absence of Miranda, as *miranda*, *prospero* double mutants fail to upregulate *gcm* in NGBs. In both the NGB 6-4T and 7-4 lineage, the delocalization of Prospero has relatively little effect on glial production by the daughter cells, presumably because there is sufficient Prospero protein in these daughter cells to upregulate *gcm* expression. Thus, with respect to glial cell fate induction, the asymmetric localization of Prospero may be more important for removing Prospero from the NGB than for enriching Prospero in the daughter cell.

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