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Mechanism of glia-neuron cell-fate switch in the *Drosophila* thoracic neuroblast 6-4 lineage

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SUMMARY

During development of the *Drosophila* central nervous system, neuroblast 6-4 in the thoracic segment (NB6-4T) divides asymmetrically into a medially located glial precursor cell and a laterally located neuronal precursor cell. In this study, to understand the molecular basis for this glia-neuron cell-fate decision, we examined the effects of some known mutations on the NB6-4T lineage. First, we found that *prospero (pros)* mutations led to a loss of expression of Glial cells missing, which is essential to trigger glial differentiation, in the NB6-4T lineage. In wild-type embryos, Pros protein was localized at the medial cell cortex of dividing NB6-4T and segregated to the nucleus of the glial precursor cell. *miranda* and *inscuteable* mutations altered the behavior of Pros, resulting in failure to correctly switch the glial and neuronal fates. Our results suggested that NB6-4T used the same molecular machinery in the asymmetric cell division as other neuroblasts in cell divisions producing ganglion mother cells. Furthermore, we showed that outside the NB6-4T lineage most glial cells appeared independently of Pros.

Key words: Neuroblast, Glia, Neuron, Asymmetric cell division, *glial cells missing (gcm), prospero, miranda, inscuteable, Drosophila*

INTRODUCTION

During development of multicellular organisms, many different cell types are precisely specified at appropriate time points and positions. Development of the *Drosophila* embryonic central nervous system (CNS) starts with about 30 identified stem cells called neuroblasts (NBs) per hemisegment, and neurons and glia are produced in a stereotyped pattern (Goodman and Doe, 1993). Some of the NBs, so-called neuroglioblasts (NGBs), produce both neurons and glia (Udolph et al., 1993; Bossing et al., 1996; Schmidt et al., 1997), but little is known about how these alternative fates arise from a single stem cell lineage.

A recent study demonstrated the proliferation pattern of an NGB, NB6-4T, and revealed which cell division bifurcates the glial and neuronal lineages (Akiyama-Oda et al., 1999). Preceding the first cell division of the NGB, the mRNA (Akiyama-Oda et al., 1999) and protein (Bernardoni et al., 1999) of the most upstream gene for glial differentiation cascade, *glial cells missing* (*gcm*) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996), which encodes a transcription factor (Akiyama et al., 1996; Schreiber et al., 1997; Miller et al., 1998), appears in the cell. After this cell division, a significant level of Gcm protein is expressed in one daughter cell, which then becomes a glial precursor. The other daughter becomes a neuronal precursor. Thus, the first cell division of NB6-4T is asymmetric, separating the glial fate from the neuronal fate (Fig. 1). In contrast, the corresponding cell in the abdominal segment, NB6-4A, is a glioblast, which divides once to generate two glial cells (Higashijima et al., 1996; Schmidt et al., 1997; Fig. 1).

Asymmetric cell division is one means of generating cell type diversity (Jan and Jan, 1998). In addition to the NGB division, NBs are known to undergo asymmetric cell divisions for production of ganglion mother cells (GMCs), the secondary precursors of neurons. Several components of the molecular machinery involved in the NB divisions have been identified (for reviews, see Knoblich, 1997; Jan and Jan, 1998). During the cell divisions, the cell-fate determinants Numb (Uemura et al., 1989) and Pros proteins (Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992) and *pros* mRNA are localized to the NB cortex and segregated to GMCs (Rhyu et al., 1994; Knoblich et al., 1995; Hirata et al., 1995; Spana and Doe, 1995; Li et al., 1997; Broadus et al., 1998). The cortical localization of Pros protein and mRNA is mediated by Miranda (Mira; Shen et al., 1997; Ikeshima-Kataoka et al., 1997). Mira can directly interact with Pros and Staufen (Stau) proteins; the latter interacts with *pros* mRNA (Li et al., 1997; Shen et al., 1998; Schuldt et al., 1998; Matsuzaki et al., 1998; Broadus et al., 1998). The orientation

of the mitotic spindle and the asymmetric segregation of these proteins and mRNA are directed by *inscuteable* (*insc*) (Kraut et al., 1996). Insc protein itself localizes at the apical cortex of NBs (Kraut and Campos-Ortega, 1996; Kraut et al., 1996), and the correct localization of Insc requires *partner of inscuteable* (Yu et al., 2000) and *bazooka* (Wodarz et al., 1999; Schober et al., 1999). *bazooka* is also involved in polarization of epithelial cells of the blastoderm (Müller and Wieschaus, 1996). On the other hand, the molecular mechanisms responsible for the NGB division that separates glial and neuronal cell fates remain unclear.

In this study, in order to examine the molecular basis for the asymmetric cell division of NB6-4T, we examined known mutations that affect glial differentiation in the lineage. We found that *pros* was required for glial fate determination in the cell. In wild-type embryos, Pros protein was asymmetrically localized at the cell cortex of mitotic NB6-4T and segregated to the nucleus of the glial precursor cell. Similar to the NB divisions that produce GMCs, *mira* and *insc* are required for the correct cortical localization of Pros in NB6-4T. In some other NGB lineages, glial cells appeared independently of Pros, suggesting that there are at least two mechanisms involved in the bifurcation of glial and neuronal fates.

MATERIALS AND METHODS

Drosophila strains

K42, which is a transgenic strain containing *eagle-kinesin-lacZ* (*eg-kinZ*) (Higashijima et al., 1996), was used to identify NB6-4 and its progeny cells. *K42* and Canton-S were used to observe the wild-type phenotypes. The following mutant alleles were used: $pros^{m4}$, $pros^{17}$ (Doe et al., 1991), $pros^{I13}$ (Srinivasan et al., 1998), $mira^{L44}$, $mira^{RR127}$ (Ikeshima-Kataoka et al., 1997), gcm^{e1} (Hosoya et al., 1995), $insc^{p72}$ (Kraut and Campos-Ortega, 1996), $numb^{1}$ (Uemura et al., 1989), $stau^{D3}$ (St Johnston et al., 1991) and $sanpodo^{C55}$ (*spdo*) (Dye et al., 1998). *TM3 [ftz-lacZ]* and *CyO [ftz-lacZ]* balancers were used for identification of homozygotes. We introduced the *eg-kinZ* marker in the $pros^{M4}$, $pros^{17}$, $mira^{L44}$, $mira^{RR127}$ and $spdo^{C55}$ by chromosome recombination. The *K42* $pros^{m4}$ strain was further recombined with $mira^{L44}$ to generate a double mutant, *K42* $pros^{m4}$ $mira^{L44}$. We generated a *Sp/CyO [ftz-lacZ]; K42* strain and used it to introduce the *eg-kinZ* marker in the gcm^{e1}, $insc^{p72}$, $numb^{1}$ and $stau^{D3}$ mutants. An enhancer trap strain, gcm^{p} (Hosoya et al., 1995), was used to detect the transcriptional state of the *gcm* gene. For *insc* exogenous expression, *arm-GAL4* (Sanson et al., 1996), *en-GAL4* (gift from A. H. Brand) and *MZ360* (*eg-GAL4*) (Ito et al., 1995; Dittrich et al., 1997) were used. $insc^{p72}$ *en-GAL4* and *eg-kinZ eg-GAL4* strains were produced by chromosome recombination.

Generation of anti-Insc antisera

A protein, in which the maltose binding protein was fused in-frame to amino acid residues 13-859 of the Insc protein, was prepared as follows: the *Bam*HI-*Xba*I fragment (2664-3100 nucleotides) of *insc* cDNA (Kraut and Campos-Ortega, 1996) was inserted into *Bam*HI and *Hin*dIII sites of the pMAL-c2 vector (New England Biolabs) after end-filling of the *Xba*I and *Hin*dIII termini with Klenow enzyme. Then, into the *Bam*HI site of this plasmid, the *Bam*HI fragment of *insc* cDNA (269-2663 nucleotides) was subcloned, and the resultant plasmid was used to produce the fusion protein in *E. coli*. The protein was purified using amylose resin (New England Biolabs). Rats were injected with the fusion protein emulsified in Freund's adjuvant (Difco) and boosted at 3-week intervals. After the second booster, we obtained anti-Insc antisera.

Staining of embryos

Staining of embryos was performed as described (Akiyama-Oda et al., 2000), using the following antibodies: rat-anti-Gcm (Akiyama-Oda et al., 1998) preincubated with 0-3 hour embryos and used at a dilution of 1:200, rat-anti-Insc at 1:100, rabbit-anti-Repo (Halter et al., 1995) at 1:200, mouse-anti-Engrailed (En) (Patel et al., 1989) at 1:100, mouse anti-Pros (MR1A) (Spana and Doe, 1995) at 1:2, rabbit anti-Mira (Ikeshima-Kataoka et al., 1997) at 1:2500, mouse-anti-β-gal (Promega) at 1:1000 and rabbit-anti-β-gal (Cappel), preincubated with Canton-S embryos and used at 1:5000. The secondary antibodies used were as follows: FITC-conjugated-anti-rat antibody (Jackson), Cy3-conjugated-anti-rat, FITC- and Cy5-conjugated-anti-mouse, and rhodamine- and Cy5-conjugated-anti-rabbit antibodies (Chemicon). TOTO3 (Molecular probes) was used to stain DNA. For observation of stained embryos, we used a Zeiss Axiophot 2 microscope equipped with a Bio-Rad laser confocal system (MRC1024). The ventral side of the embryos was set toward the top so that NB6-4 in either side of one segment could be observed on the same confocal plane. In all figures, ventral views are shown. Anterior is up and the midline is to the left, or indicated by a vertical bar.

Construction of *UAS-insc-GFP* and generation of transformants

Polymerase chain reaction was performed with a 5′-primer containing an *Eco*RV site and a 3′-primer containing an *Xba*I site to amplify the region between nucleotides 232 and 2808 of the *insc* cDNA (Kraut and Campos-Ortega, 1996) using native pfu polymerase (Stratagene). The PCR product was then subcloned into the *Eco*RV and *Xba*I sites of pBluescript II SK− (Stratagene) to make pBS-insc-RVXb. A 0.7-kb fragment of a mutated GFP cDNA excised at the *Nhe*I and *Xba*I sites of pQBI25 (Quantum Biotechnologies) was then inserted into the *Xba*I site of pBS-insc-RVXb to generate pBS-insc-GFP. Then, the *Kpn*I-*Xba*I fragment of the plasmid encoding Insc-GFP (full-length Insc protein fused to GFP) was transferred into pUAST (Brand and Perrimon, 1993), and the resultant plasmid was named pUAST-insc-GFP. In the same way, pUAST-insc-cen-gfp (amino acid residues 252-615 of Insc protein fused to GFP) was produced. S2 cells were cotransfected with pWA-GAL4 (a gift from Y. Hiromi) and either of these plasmids as described (Oda and Tsukita, 1999), and then western blotting was performed using rabbit anti-GFP antibody (Clontech) at a dilution of 1:1000. We obtained signals of the expected sizes (approx. 125 kDa for Insc-GFP and approx. 65 kDa for Insc-cen-GFP).

Transgenic flies were produced by microinjection of the pUAST-insc-GFP and pUAST-insc-cen-GFP plasmids into w^{1118}*; Δ2-3 TM3 Sb/Dr* embryos (Robertson et al., 1988). Established strains, *UAS-insc-GFP4-3*, *UAS-insc-GFP2-1* and *UAS-insc-cen-GFP9-1*, were used for this study. These transformant strains were crossed with $insc^{p72}$ for chromosome recombination.

RESULTS

Loss of glial cells in the NB6-4T and NB7-4 lineages in *pros* mutants

We examined the effects of known mutations on glial fate in NGB lineages of the CNS. The mutations that we first tested were *pros*, *numb* and *spdo*, which are known to be involved in cell-fate determination in some lineages of the CNS and peripheral nervous system (Uemura et al., 1989; Rhyu et al., 1994; Dye et al., 1998; Skeath and Doe, 1998; Doe et al., 1991; Vaessin et al., 1991). The mutant embryos were stained for Repo protein, expression of which is an indicator of glial differentiation (Xiong et al., 1994; Campbell et al., 1994; Halter et al., 1995). In this analysis, we found that a null allele

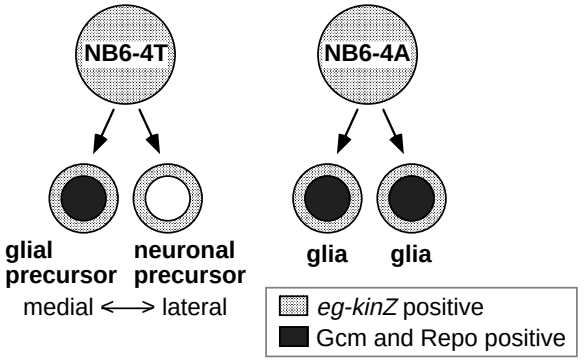

Fig. 1. Schematic representation of the NB6-4T and NB6-4A lineages in the wild-type. The cells in these lineages are all *eg-kinZ* positive. NB6-4T divides asymmetrically to produce a glial precursor cell at the medial side and a neuronal precursor cell at the lateral side. The glial precursor cell exhibits high-level expression of Gcm and Repo, while the neuronal precursor cell expresses neither of the proteins. In contrast to NB6-4T, NB6-4A divides into two glial cells, both of which express Gcm and Repo. Note that expression of Gcm is present before the first cell division in the NBs at weaker levels.

of *pros*, $pros^{17}$, had no Repo-positive cells in the NB6-4T or NB7-4 lineages (Fig. 2B). From most of the other NGB, such as NB1-1A, NB1-3, or 2-5 and NB5-6, and glioblast, GP and NB6-4A, lineages, Repo-positive cells appeared in $pros^{17}$ (Fig. 2B-D). These results indicated that the *pros* mutation led to a loss of glial differentiation in a manner specific to the NB6-4T and NB7-4 lineages. Other *pros* mutant alleles were also examined. With respect to glial differentiation, the phenotypes of $pros^{m4}$ and $pros^{I13}$ were the same as that of $pros^{17}$. In contrast to *pros*, *numb* and *spdo* mutants were relatively normal in their Repo expression (data not shown). Nonetheless, we further examined cell fate of NB6-4T in these mutants using the *eg-kinZ* marker in the same way as shown below for *pros* mutants. After the first cell division of NB6-4T, the medial daughter cell expressed a glial fate determination protein, Gcm, in the mutants (n=25 for *numb* and n=40 for *spdo*; data not shown), as in the wild-type embryos (Fig. 3B). These observations indicated that *numb* and *spdo* were not required for the cell-fate decision at the first cell division of NB6-4T.

We examined expression of Pros protein in NGB and glioblast lineages of wild-type embryos. Longitudinal glia (LG) derived from GP (Jacobs et al., 1989; Schmidt et al., 1997) are known to express Pros (Doe et al., 1991; Ito et al., 1995; Fig. 2H-J). We found that many other Repo-positive cells also expressed Pros transiently or continuously (Fig. 2E-G). These cells included, for example, cells in the NB5-6, NB6-4T, NB6-4A and NB7-4 lineages. This observation indicated that Pros was expressed in glial-fate cells irrespective of whether Repo expression in the cells was affected or not affected by the *pros* mutations.

NB6-4T in *pros* shows rather normal proliferation but produces no glial cells

We examined whether the loss of glial cells in the NGB lineages in *pros* resulted from failure of cell proliferation or of cell-fate determination. We took advantage of *eg-kinZ* to trace progeny cells of the NB6-4T lineage (Higashijima et al., 1996). Most further examinations were performed with this lineage.

In normal development, NB6-4T generates a glial precursor cell at the medial side and a neuronal precursor cell at the lateral side after the first cell division (Akiyama-Oda et al., 1999; Figs 1, 3B). Then, the glial precursor produces three glial cells (Fig. 3C), and the neuronal precursor generates 4-6 neurons (Schmidt et al., 1997). These glial cells move toward the midline, while the neuronal cells are stationary at the lateral region (Fig. 3A). In this study, we observed embryos from the ventral side (see Materials and Methods). Since NB6-4 are delaminated from the most lateral region of the ventral neuroectoderm (Doe, 1992), the medio-lateral body axis and the apico-basal axis of epithelial cells were not orthogonal.

In the $pros^{17}$ mutant, the cell number of NB6-4T progeny was comparable with that in the wild type, but all of the cells remained at the lateral side (Fig. 3D), similar to normal neuronal cells in this lineage (Fig. 3A). Even at earlier stages, neither Gcm nor Repo was detected in any of the NB6-4T progeny cells (Fig. 3E,F). The same results were obtained using the $pros^{m4}$ allele. These observations suggested that NB6-4T in the *pros* mutants showed rather normal proliferation but failed to attain glial fate. Furthermore, we examined gcm^{p}-*lacZ* expression in the $pros^{17}$ and $pros^{M4}$ mutants. The gcm^{p} strain has a P-element insertion at the *gcm* locus (Hosoya et al., 1995), and expression of the *lacZ* gene may mimic transcription of the *gcm* gene. Expression of gcm^{p}-*lacZ* was detected in NB6-4T progeny of the *pros* alleles (Fig. 3K), suggesting the possibility that transcription of *gcm* was initiated in the *pros* mutants. In contrast to NB6-4T, cell fate of a glioblast NB6-4A, the corresponding cell in the abdominal segment, was not affected by the *pros* mutations. Both daughter cells of NB6-4A expressed Gcm and Repo in wild-type (Fig. 3G,H) and mutant (Fig. 3I,J) embryos, and moved toward the midline (Fig. 3A,D).

All progeny cells of NB6-4T show glial features in a *mira* null mutant

Mira protein is known to directly interact with Pros protein during the NB divisions that produce GMCs (Shen et al., 1997; Ikeshima-Kataoka et al., 1997). To examine whether Mira is involved in glial and neuronal cell-fate determination in the NB6-4T lineage, we analyzed two alleles of *mira* mutants, $mira^{L44}$ and $mira^{RR127}$. In the $mira^{L44}$ mutant, which is probably a null mutant (Matsuzaki et al., 1998), all progeny cells of NB6-4T expressed Gcm and Repo, and moved toward the midline (Fig. 4A-C), similar to the normal glial cells of the NB6-4T lineage (Fig. 3A). This observation suggested that the *mira* null mutation led all the NB6-4T progeny cells to a glial fate. On the other hand, $mira^{RR127}$, in which Mira protein with a C-terminal truncation is likely to be produced that causes failure of normal regulation of Pros localization (Ikeshima-Kataoka et al., 1997), showed a phenotype opposite to that of $mira^{L44}$ (Fig. 4D-F). In $mira^{RR127}$, expression of Gcm or Repo was not detected in NB6-4T progeny cells (Fig. 4E,F), all of which remained at the lateral region (Fig. 4D) as in *pros* mutants (Fig. 3D). These results strongly suggested that *mira* and *pros* worked in the same cascade for glial and neuronal cell-fate decision in the NB6-4T lineage. Furthermore, in the context of the glia-neuron cell-fate decision, $pros^{m4}$ $mira^{L44}$ double-mutants showed a phenotype identical to that of *pros*

mutants, but opposite to that of *mira*^{L44} (data not shown). This indicated that *pros* was epistatic to *mira*.

Stau protein is known to interact with Mira protein and *pros* mRNA, and is required for the asymmetric localization of the mRNA during NB divisions that produce GMCs (Li et al., 1997; Broadus et al., 1998; Shen et al., 1998; Schuldt et al., 1998). We examined the NB6-4T lineage in zygotic *stau* mutant embryos bearing the *eg-kinZ* marker. After the first cell division of NB6-4T, expression of *gcm* mRNA and Gcm protein were detected in the medial daughter cell (n=52 and n=42, respectively), and Repo expression pattern was normal in the mutant embryos (data not shown). These results indicated that *stau* was not essential for the cell-fate decision in the NB6-4T lineage.

Asymmetric localization of Mira and Pros during the first cell division of NB6-4T

We further investigated expression patterns of Mira and Pros during the first cell division of NB6-4T. In wild-type embryos observed from the ventral side, Pros protein was localized to the medial cell cortex of the dividing NB6-4T (Fig. 5A). After the cell division, Pros was translocated into the nucleus of the glial precursor cell (Fig. 5B). Mira was also localized to the medial cortex in the mitotic NB6-4T (Fig. 5D). This cortical localization of Mira was not affected by *pros* mutations (Fig. 5E). In contrast, Pros localization was affected by *mira* mutations. In *mira*^{L44}, Pros was detected entirely in the cytoplasm but not at the cortex during the cell division (Fig. 5F), and eventually entered the nuclei of both daughter cells (Fig. 5G). In *mira*^{RR127}, normal cortical localization of Pros was established during mitosis (Fig. 5H), but was not followed by translocation to the nucleus (Fig. 5I,J). Even after cell division, Pros was persistent at the cortices. In addition, Pros was detected in the nuclei of both daughter cells of NB6-4A in wild-type embryos (Fig. 5C). This nuclear localization of Pros was disturbed by the *mira*^{RR127} mutation (data not shown), as observed for NB6-4T daughters (Fig. 5I,J), although neither Gcm nor Repo expression was affected in NB6-4A (Fig. 4D).

In *gcm*^{e1} null mutant embryos, the localization and distribution of Pros and Mira were normal in NB6-4T and NB6-4A (Fig. 6). Since Gcm expression was affected by *mira* and *pros* mutations (Figs 3, 4), *mira* and *pros* seemed to be upstream of *gcm* in the cascade of the cell-fate decision in the NB6-4T lineage.

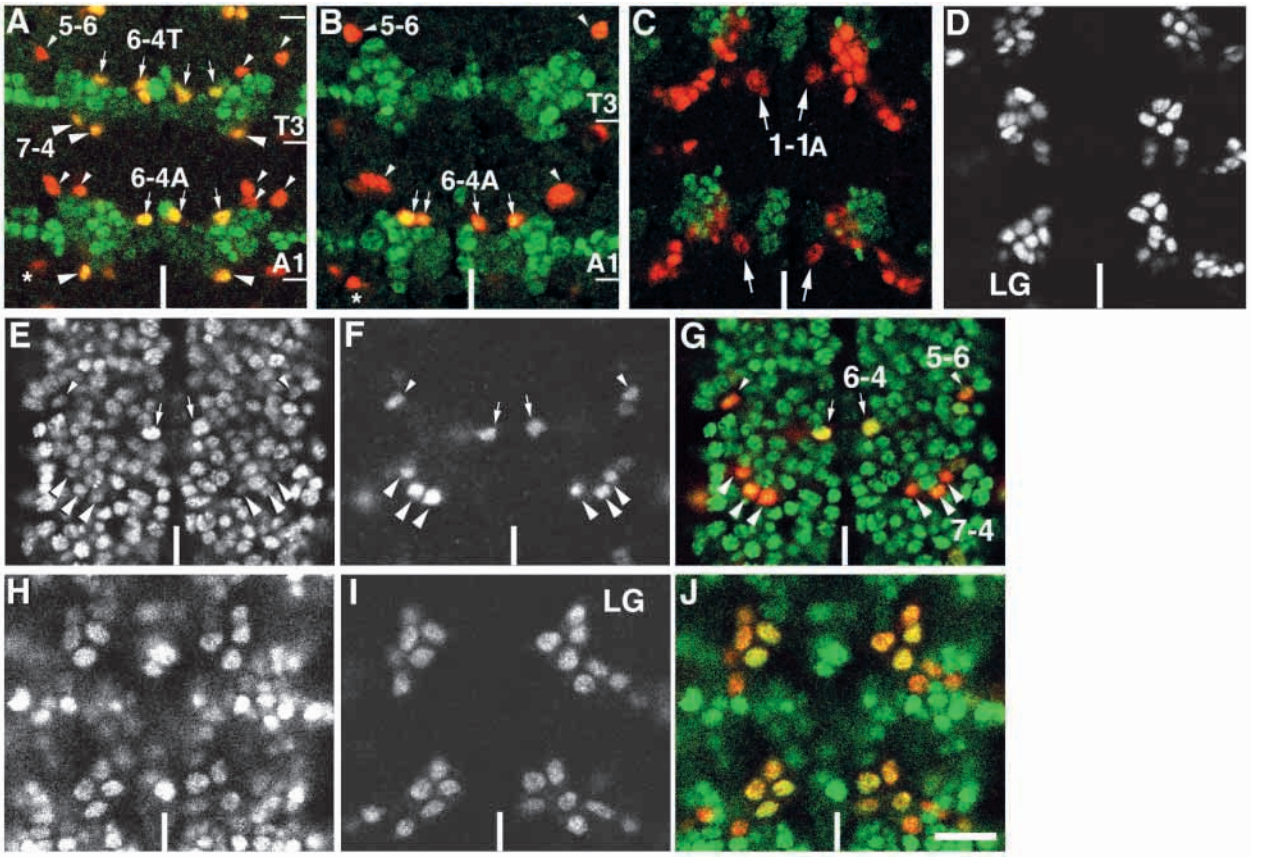

Fig. 2. Glial cells derived from NB6-4T and NB7-4 are lost in *pros* mutants. Wild-type (A,E-J) and *pros*^{17} (B-D) embryos at late stage 11. (A-D) Embryos were labeled for En (green, A-C) and Repo (red, A-C). Only Repo expression is shown in (D). Bars labeled T3 and A1 indicate the boundaries of the segments (A,B). Glial cells derived from NB6-4T, NB6-4A (arrows), NB7-4 (large arrowheads) and NB5-6 (small arrowheads) in the wild-type are shown in (A). An asterisk indicates a glial cell derived from NB2-5 or 1-3 (A). In *pros*, glial cells derived from NB6-4T and NB7-4 were specifically missing (B), while other glial cells derived from NB6-4A (B, arrows), NB5-6 (B, arrowheads), NB2-5 or 1-3 (B, asterisk) and NB1-1A (C, arrows) as well as LG (D) were present. (E-J) Embryos were stained for Pros (E,H; G,J, green) and Repo (F,I; G,J, red). Pros was detected in glial cells derived from NB6-4 (E-G, arrows), NB7-4 (E-G, large arrowheads), NB5-6 (E-G, small arrowheads) as well as LG (H-J). Bar, 20 μm.

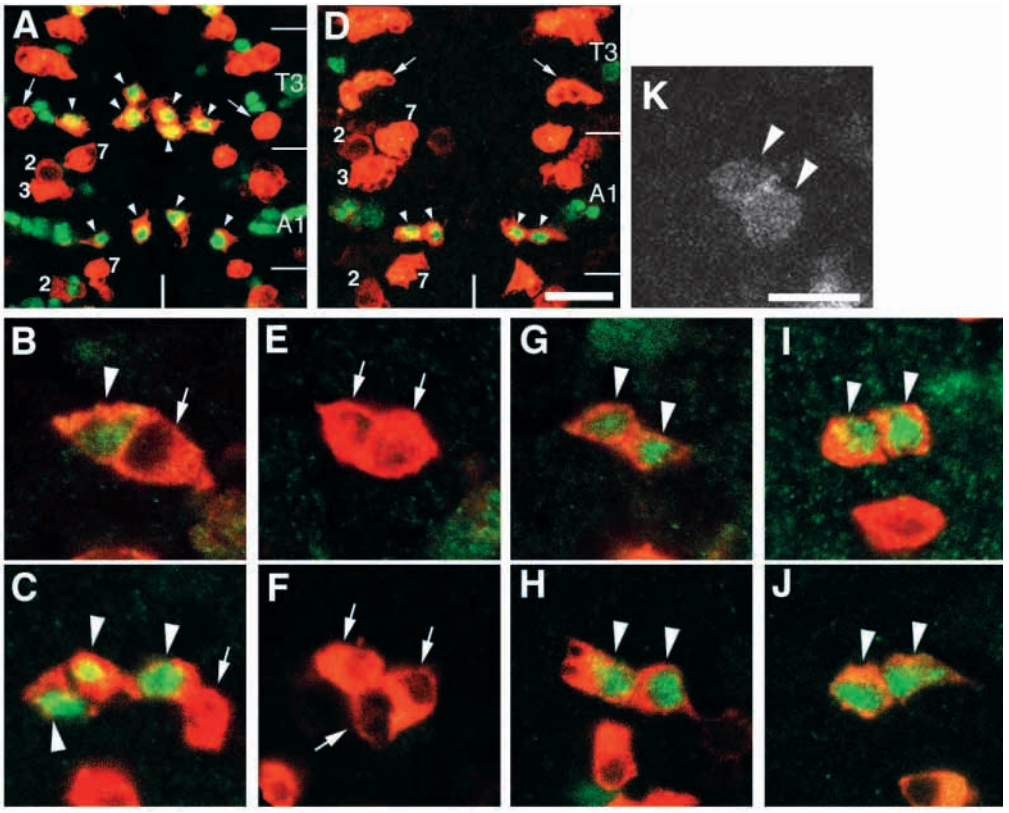

Fig. 3. None of the NB6-4T progeny in *pros* mutants takes glial fate. (A-J) Wild-type (A-C,G,H) and *pros*^{17} (D-F,I,J) embryos bearing *eg-kinZ* were stained for β-gal (red) and Repo (green) (A,C,D,F,H,J), or β-gal (red) and Gcm (green) (B,E,G,I). Stages 12 (A,D) and 11 (B,C,E-J). Gcm/Repo-positive and -negative progeny cells of NB6-4 are indicated by arrowheads and arrows, respectively. 2, 3 and 7 indicate β-gal-positive cells derived from NB2-4, NB3-3 and NB7-3, respectively (A,D). In wild-type embryos, glial progeny cells of NB6-4T and NB6-4A moved toward the midline (A, arrowheads), while neuronal progeny of NB6-4T remained at the lateral region (A, arrows). After the first division of NB6-4T, the medial daughter cell expressed Gcm (B, arrowhead), and all of its three progeny expressed Repo (C, arrowheads). Both daughter cells of NB6-4A expressed these proteins (G, H, arrowheads). In *pros* mutants, all the progeny of NB6-4T remained at the lateral side (D, arrows), and both daughters of NB6-4A moved toward the midline (D, arrowheads). None of the NB6-4T progeny expressed Gcm or Repo (E,F, arrows). NB6-4A daughters expressed the proteins (D,I,J, arrowheads). (K) *gcm*^{p}*/+; pros*^{17} embryos at stage 11 were stained for β-gal (*gcm*^{p}*-lacZ*) and En (not shown). NB6-4T progeny cells (arrowheads) expressed β-gal. Bar in D, 20 μm (A,D); bar in K, 10 μm (B,C,E-K).

Table 1. Expression of Mira, Pros and Gcm proteins in NB6-4T and its daughter cells

Genotype	Protein examined*	Protein localization			Total number of hemisegments examined
		M‡ (%)	L‡ (%)	M and L§ (%)	
eg-kinZ	Mira	17(100)	0 (0)	0 (0)	17
	Pros	78 (99)	1 (1)	0 (0)	79
	Gcm	51 (98)	1 (2)	0 (0)	52
insc; eg-kinZ	Mira	9 (38)	11 (46)	4 (17)	24
	Pros	20 (43)	15 (32)	12 (26)	47
	Gcm	35 (67)	15 (29)	2 (4)	52
insc en-GAL4 / *insc UAS-insc-GFP2-1* ; *eg-kinZ* / +	Pros	50 (96)	2 (4)	0 (0)	52
	Gcm	58 (94)	4 (6)	0 (0)	62
insc en-GAL4 / *insc UAS-insc-GFP4-3* ; *eg-kinZ* / +	Pros	57 (95)	3 (5)	0 (0)	60
	Gcm	53 (96)	2 (4)	0 (0)	55
insc / *insc UAS-insc-GFP2-1* ; *eg-kinZ eg-Gal4* / +	Pros	47 (76)	13 (21)	2 (3)	62
	Gcm	48 (79)	12 (20)	1 (2)	61
insc en-GAL4 / *insc UAS-insc-cen-GFP9-1* ; *eg-kinZ* / +	Pros	48 (87)	7 (13)	0 (0)	55
	Gcm	47 (92)	4 (8)	0 (0)	51

*Localization of Mira was examined in dividing NB6-4T. Pros and Gcm expression were examined in the daughter cells after the first cell division.

‡'M' indicates that the proteins were detected in the medial cortex or the medial daughter cell, and 'L' indicates that they were observed in the lateral cortex or the lateral daughter cell.

§'M and L' indicates that Gcm and Pros were detected in both daughter cells, and Mira was detected at the cortices other than the medial or lateral side.

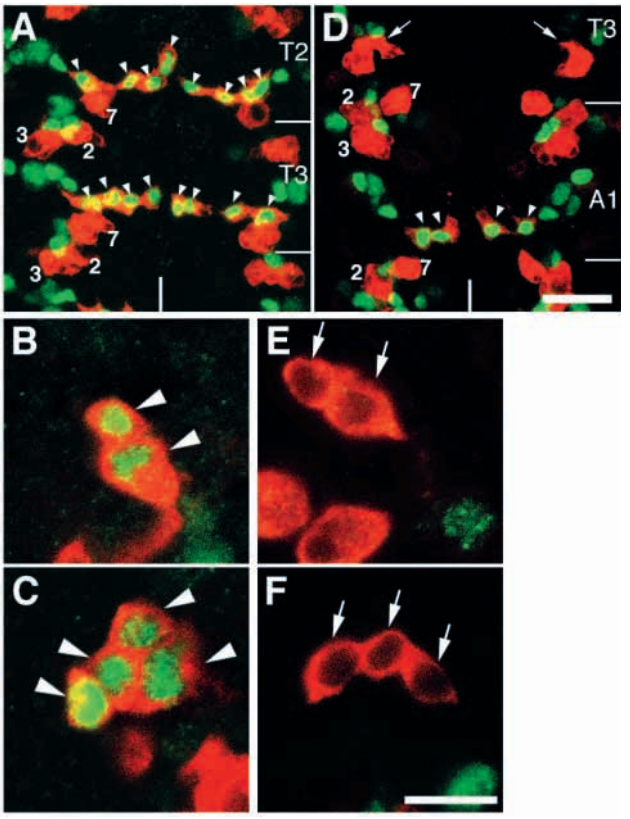

Fig. 4. *mira* mutations affect the cell fate of NB6-4T progeny. *mira*^{L44} (A-C) and *mira*^{RR127} (D-F) embryos bearing *eg-kinZ* were labeled for β-gal (red) and Repo (green) (A,C,D,F), or β-gal (red) and Gcm (green) (B,E). Stages 12 (A,D) and 11 (B,C,E,F). Gcm/Repo-positive and -negative progeny cells of NB6-4 are indicated by arrowheads and arrows, respectively. 2, 3 and 7 indicate β-gal-positive cells derived from NB2-4, NB3-3 and NB7-3, respectively (A, D). (A-C) In *mira*^{L44} embryos, all the NB6-4T progeny expressed Gcm and Repo and moved toward the midline (arrowheads). (D-F) In *mira*^{RR127} embryos, neither Gcm nor Repo expression was detected in the NB6-4T progeny (arrows), which were stationary at the lateral side (D, arrows). Both daughter cells of NB6-4A expressed these proteins (D, arrowheads). Bar in D, 20 μm (A,D); bar in F, 10 μm (B,C,E,F).

Insc determines the direction of Pros and Mira cortical localization during the mitosis of NB6-4T

Next, we investigated whether *insc* is involved in the glial and neuronal fate decision in the NB6-4T lineage. Examination of Insc expression revealed that during the first cell division of NB6-4T in wild-type embryos, Insc protein was localized to the lateral cell cortex (Fig. 7A), which was the opposite side to the Pros crescent (Fig. 7B). In *insc* mutant NB6-4T, the direction of cortical localization of Mira was random (Table 1, Fig. 7C). Similarly, the cortical localization of Pros was frequently displaced (data not shown), followed by randomization of nuclear localization of Pros in either of the daughter cells (Fig. 7E, Table 1). In this situation, the Gcm nuclear appearance was also randomized (Fig. 7F, Table 1) coinciding with Pros in most cases (87%, *n*=53). In less frequent cases, Gcm was detected in one daughter cell while Pros was detected in both (13%, *n*=53). At the later stage, the correct numbers of glial cells derived from NB6-4T and NB6-4A were observed in *insc* mutant embryos. However, the positions of these cells were irregular (Fig. 7G). These observations suggested that *insc* was required for appropriate glia-neuron cell-fate choice of the NB6-4T daughter cells by regulating the direction of Mira and Pros localization.

Rescuing the randomized cell-fate choice of *insc* by restricted expression of Insc-GFP

We further examined whether Insc was required in or outside NB6-4T for the normal glia-neuron cell-fate choice by expressing Insc-GFP fusion proteins in restricted populations of cells in the *insc* mutant background. In this rescue analysis, the *GAL4/UAS* system (Brand and Perrimon, 1993) was used,

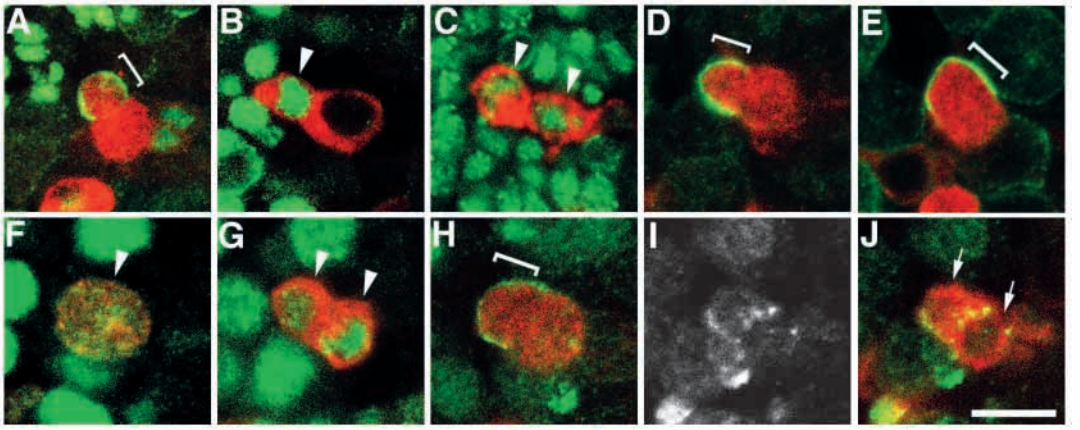

Fig. 5. Localization of Pros at the medial cell cortex during the NB6-4T division. Wild-type (A-D), *pros*^{17} (E), *mira*^{L44} (F, G) and *mira*^{RR127} (H-J) embryos bearing *eg-kinZ* at stage 11 were stained for β-gal (red), Pros (green) and DNA (not shown) (A-C, F-J; I only shows Pros), or β-gal (red), Mira (green) and DNA (not shown) (D, E). (A, B) Pros was localized to the medial cell cortex during the first cell division of NB6-4T (A, bracket) and was segregated to the nucleus of the glial precursor cell (B, arrowhead). (C) Pros was detected in both daughter cells of NB6-4A. (D) Mira was localized to the medial cell cortex of the dividing NB6-4T (bracket). (E) Mira localization was normal in *pros* mutant NB6-4T (bracket). (F, G) In *mira*^{L44}, Pros was detected in the cytoplasm of dividing NB6-4T (F, arrowhead) and was distributed to the nuclei of both daughter cells (G, arrowheads). (H-J) In *mira*^{RR127}, Pros was normally localized during the NB6-4T division (H, bracket), but was detected at the cortex even after the division (I, J). Arrows in J indicate the daughter cells of NB6-4T. Bar, 10 μm.

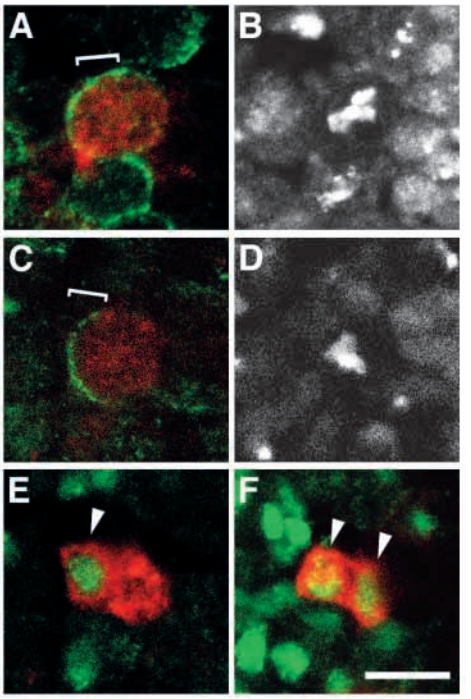

Fig. 6. Pros and Mira expression are normal in *gcm* mutant NB6-4. *gcm*^{e1} embryos bearing *eg-kinZ* at stage 11 were stained for β-gal (red), Mira (green) (A) and DNA (B), or β-gal (red), Pros (green) (C,E,F) and DNA (D). In dividing NB6-4T of *gcm* mutants, Mira and Pros were localized normally to the medial cortex (A,C, brackets). DNA staining is shown in (B,D). After the cell division, Pros was detected in the nucleus of the medial daughter cell of NB6-4T (E, arrowhead) and both daughter cells of NB6-4A (F, arrowheads). Bar, 10 μm.

and two transgenes, UAS-*insc*-GFP and UAS-*insc-cen*-GFP, containing a full-length and a central one-third of the coding region of *insc* cDNA, were constructed. When expressed in the wild-type background using *arm-GAL4*, *en-GAL4* and *eg-GAL4*, these transgenes did not affect the viability of flies or the cell fate of NB6-4. In the following experiments, *en-GAL4* and *eg-GAL4* were used to drive gene expression in posterior compartment cells and in four NBs including NB6-4 (Dittrich et al., 1997), respectively.

Insc-GFP induced by *en-GAL4* in the *insc* mutant background was localized to the lateral cell cortex of dividing NB6-4T (Fig. 8A), similar to the endogenous Insc protein (Fig. 7A,B). After the first cell division, Pros and Gcm were detected correctly in the daughter cells at the medial side in most of hemisegments in these embryos (Fig. 8B,C, Table 1). The positions of glial cells derived from NB6-4 were almost normal (Fig. 8D, compare with Figs 3A, 7G). Similar results were obtained with *eg-GAL4*, although the rates of rescue were not as high as those with *en-GAL4* (Fig. 8E, Table 1). These reduced rescue rates may have been partially due to insufficient amounts of the Insc-GFP protein at the cell division. Compared to *en-GAL4*, *eg-GAL4* was late in inducing gene expression, and exhibited low levels of gene expression. Similarly to Insc-GFP, Insc-cen-GFP exhibited high levels of rescue activity when *en-GAL4* was used (Table 1). This indicated that the central one-third of Insc is sufficient for the normal glia-neuron cell-fate choice of the NB6-4T daughter cells as previously

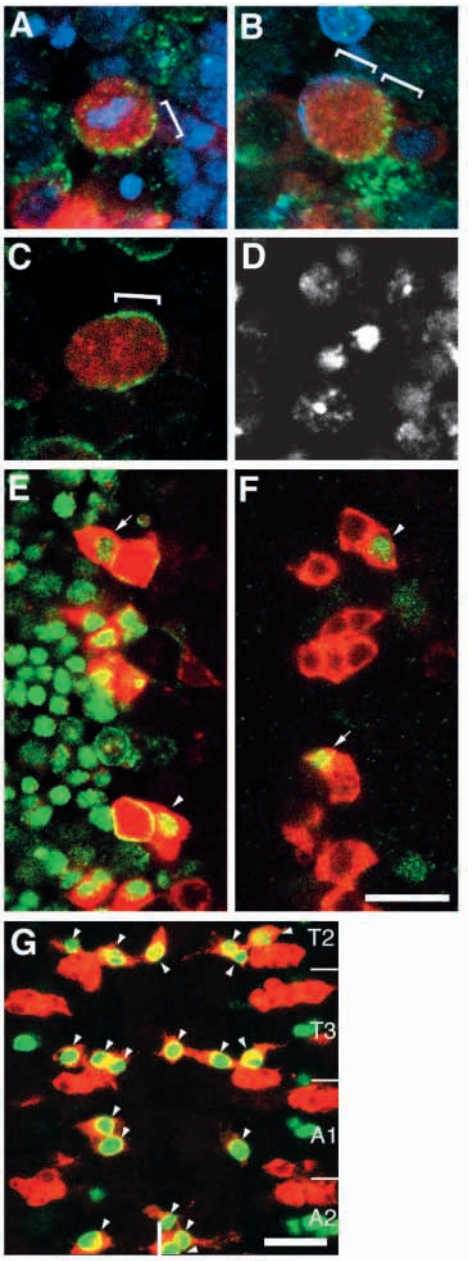

Fig. 7. Insc determines the direction of Mira and Pros localization. Wild-type (A, B) and *insc*^{p72} (C-G) embryos bearing the *eg-kinZ* marker (red), at stages 11 (A-F) and 12 (G). (A) During the cell division of NB6-4T, Insc protein (green) was localized to the lateral cell cortex (bracket). Chromosomes are shown in blue. (B) Insc localization (green) was observed at the opposite side to that of Pros (blue) (brackets). (C,D) Mira (green) localization became random in *insc* mutants; it was observed at the lateral cortex of this dividing NB6-4T (C, bracket). Chromosomes are shown in (D). (E, F) In *insc* mutants, nuclear expression of Pros (E, green) and Gcm (F, green) after the cell division were randomized, as observed in the medial daughter cell in one hemisegment (arrows) and in the lateral daughter cell in the other hemisegment (arrowheads). (G) The number of Repo-positive cells (green) derived from NB6-4 (arrowheads) was normal in *insc* mutant, but the positions of the cells were irregular. Neuronal progeny of NB6-4T in the thoracic segments and one daughter cell of NB6-4A in the A1 segment could be seen, but are out of focus. Bar in F, 10 μm (A-D) and 20 μm (E,F); bar in G, 20 μm.

shown for the NB divisions to produce GMCs (Knoblich et al., 1999; Tio et al., 1999).

DISCUSSION

Mechanisms of the glia-neuron cell-fate switch in the NB6-4T lineage

In normal development, NB6-4T produces a glial precursor cell and a neuronal precursor cell at the medial and lateral sides, respectively, after the first cell division (Fig. 1; Akiyama-Oda et al., 1999). In the present study, we showed that this NGB lineage required activities of *pros*, *mira* and *insc* genes to make correct cell-fate decisions.

In a null allele of *pros*, $pros^{17}$, as well as in other alleles including $pros^{M4}$ and $pros^{I13}$, no cells expressed Gcm or Repo in the NB6-4T lineage (Figs 2B, 3D-F). In contrast, in a null allele of *mira*, $mira^{L44}$, all cells of the lineage expressed the glial proteins (Fig. 4A-C). The double mutant of $pros^{M4}$ and $mira^{L44}$ produced no glial cells in the NB6-4T lineage as the *pros* single mutant did (data not shown). One allele of *mira*, $mira^{RR127}$, also showed the same phenotype (Fig. 4D-F). These results indicated that both *pros* and *mira* are involved in a pathway leading to the glia-neuron cell-fate switch, and that *pros* is epistatic to *mira* in this pathway. The effects of the *insc* mutation on the glia-neuron cell-fate switch in the NB6-4T lineage were slightly different from those of the *pros* or *mira* mutations. In *insc* mutants, both glial and non-glial cells were generated from NB6-4T in many of the hemisegments examined, but glial fate arose randomly from either of the daughter cells (Table 1, Fig. 7F).

These involvements of *pros*, *mira* and *insc* led us to infer an analogy between the first cell division of NB6-4T and the NB cell divisions producing GMCs. We revealed that Pros, Mira and Insc proteins similarly behaved during the first division of NB6-4T (Figs 5, 7A,B), as they do during the usual NB divisions producing GMCs (Knoblich, 1997; Jan and Jan, 1998). In our analyses of wild-type and mutant embryos, the high levels of expression of the earliest glial protein Gcm, and of the later glial protein Repo, were correlated with the nuclear localization of Pros in NB6-4T daughter cells (Figs 3-5). Consistent with this, in $pros^{I13}$ mutant, in which the mutant Pros protein does not enter the nucleus even after cell division (Srinivasan et al., 1998), no glial cells were observed in the NB6-4T lineage (data not shown). These observations suggested an important role of Pros in the onset of glial differentiation in the NB6-4T lineage.

Regulation of the behavior of Pros protein during the first cell division plays a critical role in the glia-neuron cell-fate switch in the NB6-4T lineage. Similar to the well-known asymmetric cell divisions of NBs, Mira was localized at the cell cortex and bound to the Pros protein during the cell division (Fig. 5; Shen et al., 1997; Ikeshima-Kataoka et al., 1997), while Insc determined the direction of the cortical localization of Mira and Pros (Figs 7, 8, Table 1; Kraut et al., 1996). The *insc* rescue experiment using *eg-GAL4* (Fig. 8E, Table 1) showed that Insc was required in NB6-4T but not in other cells, suggesting the cell-autonomous determination of cell polarity. Furthermore, we recently showed that the onset of glial differentiation is correlated with the first cell division in the NB6-4T lineage (Akiyama-Oda et al., 2000). This correlation may be explained by the Pros behavior, since we observed persistent cortical localization of Pros in cell cycle-arrested embryos (data not shown), in which NB6-4T did not start glial differentiation (Akiyama-Oda et al., 2000).

In recent studies *gcm* mRNA was detected before the first cell division of NB6-4T and was asymmetrically distributed during the cell division (Akiyama-Oda et al., 1999; Bernardoni et al., 1999); furthermore, a significant level of Gcm expression was achieved depending on cell cycle progression (Akiyama-Oda et al., 2000). These observations, together with the results in this paper, suggest that the onset of glial differentiation in the NB6-4T lineage is regulated by several steps: (1) initiation of transcription of the *gcm* gene before the first cell division, (2) post-transcriptional regulation during the cell division that includes regulation of distribution and/or stability of *gcm* mRNA, and (3) upregulation of expression level of Gcm protein after the cell division in the medial daughter cell to ensure glial differentiation.

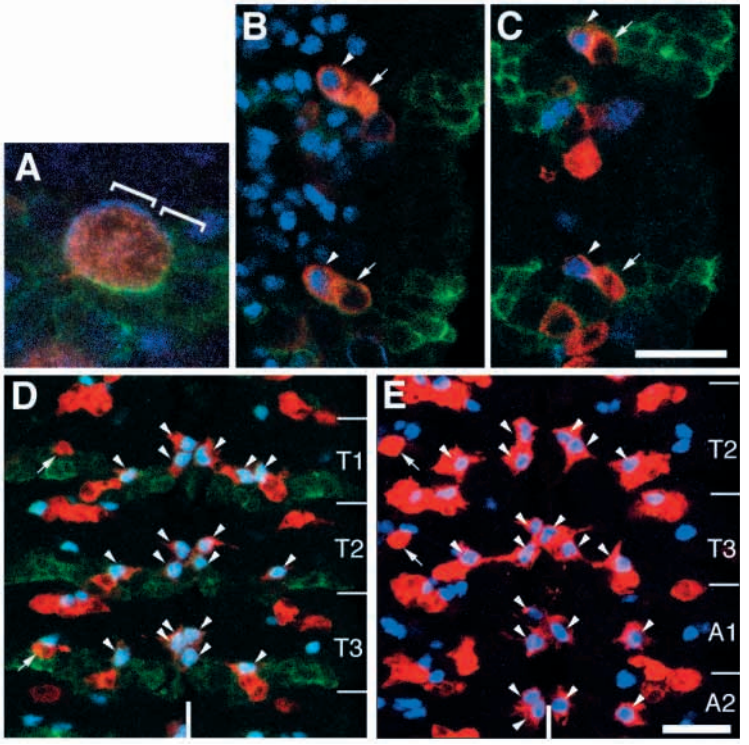

Fig. 8. The randomized phenotypes of *insc* are rescued by Insc-GFP. *insc en-GAL4/insc UAS-insc-GFP; eg-kinZ/+* (A-D) and *insc/insc UAS-insc-gfp; eg-kinZ eg-GAL4/+* (E) embryos at stages 11 (A-C) and 12 (D,E) were stained for β-gal (red) and Pros (blue) (A, B), β-gal (red) and Gcm (blue) (C), or β-gal (red) and Repo (blue) (D,E). Fluorescence of GFP is shown in green (A-D). (A) Insc-GFP (green) was localized to the lateral cortex of dividing NB6-4T. Pros localization (blue) was detected at the opposite side (brackets). (B,C) Pros (B, blue) and Gcm (C, blue) were expressed in the medial daughter cell (arrowheads) of NB6-4T in most of the segments. Arrows indicate the other daughter cell. (D,E) The positions of glial progeny of NB6-4T and NB6-4A (arrowheads) are almost normal. The neuronal progeny of NB6-4T in focus are indicated by arrows. Bar in C, 10 μm (A) and 20 μm (B,C); bar in E, 20 μm (D,E).

Pros protein has a homeodomain (Chu-LaGraff et al., 1991; Matsuzaki et al., 1992), and shows nuclear localization in early interphase cells (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995). It is therefore thought to regulate transcription. Considering this, it is possible that Pros regulates transcription of the *gcm* gene in the medial daughter cell of NB6-4T; however, Pros seems not to be necessary for the first step of transcription initiation of the *gcm* gene, since β-gal derived from the *gcmP-lacZ* gene (Hosoya et al., 1995) was detected, although weakly, in NB6-4T progeny cells even in the *pros* null mutant background (Fig. 3K). It is likely that Pros has an important role in the third step; that is, Pros regulates the level of Gcm protein by upregulating transcription of the *gcm* gene and/or by post-transcriptional regulation in the nucleus of the medial daughter cell.

As yet it is unclear whether *pros* is involved in the second step, although Pros protein was localized asymmetrically (Fig. 5A) and *gcm* mRNA was detected unevenly (Akiyama-Oda et al., 1999; Bernardoni et al., 1999) during the first cell division of NB6-4T. In *stau* mutant embryos, cortical localization of *pros* mRNA does not occur correctly in dividing NBs, but *stau* is not required for the localization of Pros protein during the cell division of NBs and for cell-fate specification of GMCs (Li et al., 1997; Broadus et al., 1998; Shen et al., 1998; Schuldt et al., 1998). We found that *gcm* mRNA and Gcm protein were detected at the medial daughter cell after the NB6-4T division in zygotic *stau* mutant embryos, and cell fates of NB6-4T daughters were correctly specified (data not shown). Considering, however, the effect of the *stau* mutation in the cell division of many other NBs, we cannot exclude the possibility that *stau* has a role in *gcm* mRNA distribution during the NB6-4T division. Even if Stau functions in the distribution of *gcm* mRNA during the cell division, this function may only make a small contribution to the generation of glial and neuronal daughter cells.

Distinct mechanisms that produce glial cells

CNS glial cells, with the exception of midline glia, are produced from seven NGBs, NB1-1A, NB1-3, NB2-2T, NB2-5, NB5-6, NB6-4T and NB7-4, and two glioblasts, NB6-4A and GP, in the *Drosophila* embryo (Bossing et al., 1996; Schmidt et al., 1997). All of these glial fates are governed by *gcm* expression (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). For this reason, Gcm expression must be strictly regulated to produce glial cells in the correct pattern. In this study, we have shown that *pros* is required in two of the NGB lineages, NB6-4T and NB7-4, to start glial differentiation, i.e. to achieve a significant level of Gcm expression (Figs 2, 3), whereas in most other NGB lineages and glioblast lineages, including NB1-1A, NB1-3 or 2-5, NB5-6, NB6-4A and GP, glial cells arise in *pros* mutants (Fig. 2B-D). This indicates that a different molecular mechanism(s), which is independent of Pros, may be used to start expression of Gcm in these glial precursor cells.

NB6-4T and NB6-4A are corresponding cells in the thoracic and abdominal segments, respectively, exhibiting a common gene expression pattern (Broadus et al., 1995), although NB6-4T is an NGB and NB6-4A is a glioblast (Schmidt et al., 1997). As we have shown here (Figs 2, 3) and in the previous study (Akiyama-Oda et al., 2000), the mechanisms of glial fate determination in these cell lineages are different. In NB6-4T, the onset of glial differentiation is dependent on cell cycle progression and Pros function, while that in NB6-4A is not.

In NGB cell lineages, neuronal and glial fate bifurcation is thought to occur at specific points during development. In NB6-4T and probably NB7-4, this cell-fate bifurcation is also based on the molecular machinery involving Pros and Mira, whereas in other NGB lineages, different molecules may be required to generate the two distinct cell types. Further studies are needed in order to understand these mechanisms.

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