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Cell division genes promote asymmetric interaction between Numb and Notch in the *Drosophila* CNS

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SUMMARY

Cell intrinsic and cell extrinsic factors mediate asymmetric cell divisions during neurogenesis in the *Drosophila* embryo. In the NB4-2→GMC-1→RP2/sib lineage, one of the well-studied neuronal lineages in the ventral nerve cord, the Notch (N) signaling interacts with the asymmetrically localized Numb (Nb) to specify sibling neuronal fates to daughter cells of GMC-1. In this current study, we have investigated asymmetric cell fate specifications by N and Nb in the context of cell cycle. We have used loss-of-function mutations in *N* and *nb*, cell division mutants *cyclinA* (*cycA*), *regulator of cyclin A1* (*rca1*) and *string/cdc25 phosphatase* (*stg*), and the microtubule destabilizing agent, nocodazole, to investigate this issue. We report that the loss of *cycA*, *rca1* or *stg* leads to a block in the division of GMC-1, however, this GMC-1 exclusively adopts an RP2 identity. While the loss of *N* leads to the specification of RP2 fates to both progeny of GMC-1 and loss of *nb* results in the specification of sib fates to these daughter cells, the GMC-1 in the double mutant between *nb* and *cycA* assumes a sib fate. These epistasis results indicate that both *N* and *nb* function downstream of cell division genes and that progression through cell cycle is required for the asymmetric localization of Nb. In the absence of entry to metaphase, the Nb protein prevents the N signaling from specifying sib fate to the RP2/sib precursor. These results are also consistent with our finding that the sib cell is specified as RP2 in *N; nb* double mutants. Finally, our results show that nocodazole-arrested GMC-1 in wild-type embryos randomly assumes either an RP2 fate or a sib fate. This suggests that microtubules are involved in mediating the antagonistic interaction between Nb and N during RP2 and sib fate specification.

Key words: *Drosophila*, Neurogenesis, Cell cycle genes, Notch, Numb

INTRODUCTION

The central nervous system (CNS) of the *Drosophila* embryo provides an important paradigm for investigating the mechanisms that generate and pattern the eukaryotic CNS. In the *Drosophila* embryo, the ventral nerve cord consists of a sequence of repeated units called neuromeres. Each neuromere is divided into two hemineuromeres, a total of 16 abdominal, 6 thoracic and 6 gnathal hemineuromeres makes up the ventral nerve cord (Hartenstein and Ortega, 1984; Doe, 1992; Bossing et al., 1996; Schmidt et al., 1997; reviewed in Bhat, 1998a, 1999). The complex array of neurons in a hemineuromere is generated from a relatively uniform two-dimensional epithelial sheet (see Bate, 1976). During neurogenesis, about 30 neuroblast (NB) cells in each hemisegment delaminate from this sheet in ~5 successive waves along the mediolateral (M-L) and anteroposterior (A-P) axes in rows and columns in a stereotyped and spatiotemporal pattern (see Hartenstein and Ortega, 1984; Doe, 1992). Each of these neuroblasts has acquired, by the time it is formed, a unique fate, and the neuroblast that forms in a given position at a given time always acquires the same fate. A neuroblast then functions as a stem cell and divides by asymmetric mitosis, renewing itself with each division and producing a chain of ganglion mother cells (GMCs). A GMC does not self-renew; instead it divides to generate two distinct neurons. These postmitotic neurons then undergo cytodifferentiation. At the end of neurogenesis, each of the hemineuromeres has ~320 neurons and ~30 glia, the other principal cell type in the CNS (Bossing et al., 1996; Schmidt et al., 1997). Thus, a complex array of different cell types is formed from relatively few precursor cells.

While there is ample evidence that neuroblasts divide asymmetrically to self-renew and to generate GMCs (Bate, 1976, Thomas et al., 1984; Hartenstein and Ortega, 1984; reviewed in Cabrera, 1992; Jan and Jan, 1995), recently, genetic and molecular evidence have been obtained that some of the GMCs might also divide by asymmetric mitosis (Hartenstein and Posakony, 1990; Spana and Doe, 1995, 1996; Spana et al., 1995; Knoblich et al., 1995; Hirata et al., 1995; Dye et al., 1998; Skeath and Doe, 1998; Buescher et al., 1998; see also Bhat and Schedl, 1994; Bhat et al., 1995). One of the earliest pieces of evidence comes from a study on the development of the adult sensilla (Hartenstein and Posakony, 1990). Their results also indicate that the neurogenic gene *Notch* (N) plays a role in

generating asymmetric division of secondary precursor cells. Using the temperature-sensitive allele of *N*, they showed that eliminating the N activity in sensillum precursors leads hyperplasia of the sensory neurons at the expense of accessory cells (i.e., shaft, socket cells). More recently, this issue has been also explored in the Midline Precursor 2 (MP2) lineage. The MP2 cell divides once to generate two interneurons, dMP2 and vMP2. In this lineage, the interaction between the transmembrane receptor Notch (N) and the cytoplasmic protein Numb (Nb) regulates the asymmetric division of MP2 to generate dMP2 and vMP2 (Spana and Doe, 1995, 1996; Spana et al., 1995; Knoblich et al., 1995; Hirata et al., 1995; reviewed in Jan and Jan, 1995; Campos-Ortega, 1996). These studies show that loss of function for *nb* results in the duplication of vMP2 at the expense of dMP2 and, therefore, that *nb* is required for the specification of dMP2. Consistent with this result is the finding that Nb is asymmetrically localized during mitosis of MP2 cell to dMP2 (Spana and Doe, 1996). In contrast, the loss of *N* activity results in the MP2 dividing symmetrically to generate two dMP2 cells, indicating that *N* is required for the specification of vMP2. However, loss of both *N* and *nb* (double mutants) results in generating two dMP2 cells. Based on this result, one can conclude that, in order to specify dMP2 identity, neither Nb nor N is required. Nb is required only when there is an intact N; thus, Nb prevents N from specifying vMP2 identity to one of the two daughter cells (see review by Ortega, 1996). These results also indicate that the asymmetric localization of determinants during metaphase plays a central role in specifying asymmetric cell fates to daughters of a precursor cell.

More recently, evidence has been obtained that N and Nb plays a similar role in the asymmetric division of GMC of the RP2/sib lineage, one of the well-studied neuronal lineages in the embryonic ventral nerve cord (Skeath and Doe, 1998; Buescher et al., 1998), a conclusion also reached independently by us (this report) and others (N. Patel, personal communication). These findings are, however, in contrast to the situation in the grasshopper (Kuwada and Goodman, 1985). By ablating cells using laser at different time points during the developmental history, these authors reported that the division of GMCs in the two neuronal lineages, the MP3 lineage and the aCC/pCC lineage, is symmetric. Thus, the sibling cells in these two lineages are equivalent and the identity of one of the two sibling cells is specified first in a stochastic manner. This cell then instructs the other cell to acquire its particular identity, presumably by cell-cell communication.

In this current study, we have investigated the consequence of blocking cell cycle progression to the asymmetric cell fate specification and the link between GMC division and N-Nb pathway in asymmetric cell fate specification in two different neuronal lineages, the GMC-1→RP2/sib lineage and the GMC-1→aCC/pCC lineage. We have used mutations in *N* and *nb*, mutations in cell division genes such as *cyclinA* (*cycA*), *regulator of cyclin A1* (*rca1*) and *string* (*stg*) and the microtubule destabilizing agent nocodazole to investigate these questions. The cell cycle genes that we used in this study are well-studied and their requirements during cell division have been elucidated at the molecular level (see reviews by Edgar and Lehner, 1996; Reed, 1995; Lehner and Lane, 1997). For example, the entry of cells to mitosis in *Drosophila* is regulated by the *stg* gene product (Foe, 1989; Edgar and O'Farrell, 1989, 1990; Jimenez et al., 1990). *stg* encodes the *cdc25* phosphatase and activates the mitosis promoting $p34^{cdc2}$-kinase by dephosphorylating it. The active $p34^{cdc2}$-kinase (by its dephosphorylation at specific tyrosine residue, see Whitfield et al., 1990, and references therein) then acts on a number of downstream targets and regulate entry to metaphase (reviewed in Lehner and Lane, 1997; Johnston, 1998). The *rca1* gene product encodes a novel protein and appears to regulate CycA protein levels (Dong et al., 1997). Mutation in *rca1* gene was initially identified as a dominant suppressor of *roughex* (*rux*) eye phenotype. In *rux*, the cells enter S-phase precociously due to ectopic activation of a CycA/Cdk complex in early G_1 (Dong et al., 1997). In embryos lacking the *rca1* activity, the cells appear to arrest in G_2 of the cell cycle 15-16 similar to *cycA* mutants (Dong et al., 1997). A loss of function for each of these genes would arrest cells in G_2/M transition, due to the loss of $p34^{cdc2}$ activity. Nocodazole, on the contrary, is a microtubule destabilizing drug. It prevents formation of mitotic spindle and arrests cells in a psuedometaphase state (Zieve et al., 1980). However, previous results have shown that the $p34^{cdc2}$ is hypophosphorylated in nocodazole-treated cells, indicating that $p34^{cdc2}$ is active in nocodazole-arrested cells (Morla et al., 1989).

A previous study has reported that the loss of *N* or *nb* leads to the symmetric division of GMC-1 of the RP2/sib lineage. While both the progeny assume RP2 fate in *N* mutants, they assume a sib fate in *nb* mutants (Buescher et al., 1998). We have also independently come to the same conclusion as those of Buescher et al. (1998). In addition, we report that, while the loss of *cycA* (or *rca1*) leads to a block in the division of GMC-1, this GMC-1 assumes an RP2 identity. However, the GMC-1 in the double mutant between *nb* and *cycA* (or *nb* and *rca1*) assumes a sib fate. This result is consistent with the previous observation that Nb is not localized in *stg* mutants (Spana and Doe, 1995). Thus, in the absence of cell division, the Nb protein (which is no longer localized) prevents N from specifying the sib fate. Consequently, the GMC-1 assumes an RP2 fate. Furthermore, our results with nocodazole show that microtubules are likely be involved in mediating the antagonistic interaction of Nb with N.

MATERIALS AND METHODS

Fly strains, genetics

stg^{9A} is a temperature-sensitive allele of *stg/cdc25* phosphatase located on the 3rd chromosome. The *cyclin A* mutant allele used was l(3) 03445, on the 3rd chromosome. *rca1* is a P-element insertion line (*rca1* is on the second chromosome). The *N* allele is a temperature-sensitive allele (N^{ts1}) on the X chromosome. The *nb* allele used was nb^{796} (see Buescher et al., 1998), on the 2nd chromosome. Various double mutants were constructed by standard genetics. The homozygotes were identified using *lacZ*-marked balancer chromosomes or by antibody staining. The *Hs-stg* transgenic line used has been previously described (see Neufeld et al., 1998). To determine the role of *mastermind* (*mam*), we used a weak allele of $-mam^{HD\text{-}1D6}$.

Immunohistochemistry

Embryos from different lines were collected and stained with various antibodies as described previously (Bhat, 1996, 1998b). The antibodies used were against the following proteins: Eve (1:2000 dilution; Frasch, Mt. Sinai), 22C10 (1:4; Goodman, Berkeley) and Zfh-1 (1:400; Lai, Penn State). In double-staining experiments with anti-Eve and mAb22C10, the nuclear Eve staining was developed with DAB reaction using hrp-conjugated secondary antibody and therefore gives a dark-red color and the membrane 22C10 staining was developed with

alkaline phosphate (AP) reaction, which gives a purplish blue color. Zfh-1 staining of a mature aCC or an RP2 neuron is confirmed by double staining embryos with anti-Zfh-1 and mAb22C10. In this case, both are developed with AP reaction since the two antibodies are generated in mouse. However, one can distinguish 22C10 staining as it stains the membrane whereas Zfh-1 is nuclear.

Hs-stg experiment

Hs-stg embryos were collected for 15 minutes, dechorionated, immersed in halocarbon oil and aged for different durations; they were given a heat shock of 20 minutes in a 37°C water bath. These embryos were then allowed to develop at room temperature until they reached late stage 11 (~9 hours old), they were then fixed and stained with anti-Eve. As control, wild-type embryos were similarly treated and stained with anti-Eve.

N^{ts} experiments

Embryos were collected for ~1 hour from the N^{ts1} line at 18°C, thoroughly washed in running water, briefly dried and immersed in halocarbon oil. Embryos of the same stage were hand picked and were aged at 18°C until they reach stage 8 (the age at which the GMC-1 is about to divide). At this point, these embryos were shifted to 29°C, kept at this temperature until they reached ~stage 14 and then quickly washed in heptane and fixed by the formaldehyde-heptane method for immunohistochemistry. Staging was done according to Wieschaus and Nusslein-Volhard (1986).

Hs-N^{intra} experiments

Embryos were collected for ~1 hour from the *Hs-*N^{intra} line at room temperature, thoroughly washed in running water, briefly dried and immersed in halocarbon oil. Embryos of the same stage were hand picked and were aged until they reached stage 8 (the age at which the GMC-1 is about to divide). At this point, these embryos were shifted to 37°C for 20 minutes and allowed to develop for another 2-5 hours at room temperature. The embryos were then quickly washed in heptane and fixed by the formaldehyde-heptane method for immunohistochemistry. Staging was done according to Wieschaus and Nusslein-Volhard (1986).

Nocodazole experiments

Either wild-type, *rca1* or *stg* mutant embryos were collected for 1 hour, aged for an additional 7 hours, dechorionated in 40% bleach for 2 minutes, washed extensively with running water and blotted dry and air dried for 5 minutes. These embryos were then treated with n-butane (Aldritch, Gold label) for 5 minutes to permeabilize them with slow shaking on an orbital shaker, air dried for 10 seconds, and then cultured at 22°C as a monolayer in Sneider's tissue culture medium containing 10 μM nocodazole (Sigma) inside a ziplock bag filled with oxygen for 2-6 hours. At the end of culture, embryos were washed and fixed as above for immunohistochemistry. Examination of nocodazole-treated embryos with acridine orange for evidence of cell death did not reveal any enhancement of general cell death compared to wild-type nor specific cell death in the location where a GMC-1→RP2/sib cells are located (data not shown).

RESULTS

Asymmetric cell fate specification to daughter cells in the GMC-1→RP2/sib lineage

The NB4-2→GMC-1→RP2/sib lineage is one of the well-studied neuronal lineages in the ventral nerve cord of the *Drosophila* embryo (see Thomas et al., 1984; Doe, 1992; Chu-LaGraff et al., 1995; reviewed in Bhat, 1998a, 1999). The NB4-2 is delaminated in the second wave of NB delamination during mid stage 9 (~4.5 hours old) of embryogenesis (Thomas et al., 1984; see Doe, 1992) and is located in the 4th row along the anteroposterior axis and 2nd column along the mediolateral axis within a hemisegment. The NB4-2 generates its first GMC (GMC-1, also known as GMC4-2a) ~1.5 hours after formation. The GMC-1 divides ~1.5 hours later to generate two cells, the RP2 and the sib. The RP2 cell eventually occupies its position in the anterior commissure along with the other RP neurons (RP1, RP2, RP3 and RP4) and projects its anteroipsilateral axon to the intersegmental nerve bundle (ISN) and innervates muscle #2 on the dorsal musculature. The sib cell migrates to a position posterior and more dorsal to RP2 (Chu-LaGraff et al., 1995; Bhat, unpublished). The DiI tracing of the NB4-2 lineage indicates that the sib has no axonal projection at mid stage 17 of embryogenesis (Chu-LaGraff et al., 1995); thus, its ultimate fate has not been determined.

There are several rather well-established ways by which one can distinguish an RP2 neuron from a sib (see Doe, 1992; Bhat and Schedl, 1994; Bhat et al., 1995; Buescher et al., 1998). First is the fact that the cytokinesis of GMC-1 that generates the two daughter cells itself is asymmetric in nearly 97% of the hemisegments (the number of hemisegments examined, n=220). Thus, in ~7.5-hour-old embryos, the cell that is destined to become an RP2 is significantly larger compared to the cell that will eventually become a sib (see Fig. 1b). Second is the level of marker gene expression between an RP2 and a

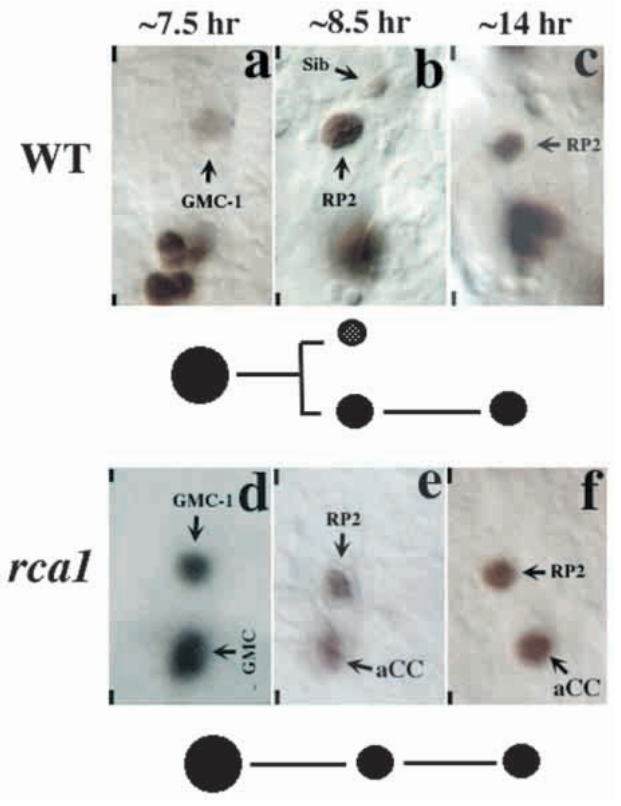

Fig. 1. Loss of *rca1* blocks the GMC-1 division and this GMC-1 assumes an RP2 identity. Ventral views of hemisegments in wild-type (a-c) and *rca1* mutant (d-f) embryos stained with anti-Eve. Anterior end is up, vertical lines mark the midline. Note that the sib cell in ~14 hour embryos can no longer be detected with anti-Eve. The GMC-1 in the mutant fails to divide, however, it enters a differentiation pathway and adopts an RP2 identity.

sib as well as the temporal dynamics of expression of these marker genes. For example, in nearly 99% of the hemisegments, the future RP2 cell has a stronger expression of markers such as Even-skipped (Eve), compared to the cell that is destined to become a sib (Fig. 1b). (We have, however, not encountered a newly formed sib that is the same size as a newly formed RP2 *and* had the same level of expression of marker genes as in an RP2; *n*=>1000). Third, the cell that eventually assumes a sib identity undergoes a size reduction and further downregulation of expression of RP2-specific marker genes. Thus, for instance, examination of ~10-hour-old embryos reveal that all of the hemisegments (*n*=220) have two cells of unequal sizes and different levels of gene expression – the larger RP2 has a higher level of gene expression than the smaller sib. By ~14 hours of development, expression of Eve is completely lost in sib. Therefore, the size reduction and downregulation of marker gene expression are very reliable markers for sib identity specification. Finally, there is a subset of marker genes that only a mature RP2 neuron expresses but not the sib. These include the membrane-specific neuronal marker, 22C10 (see Fujita et al., 1982) or a transcription factor Zfh-1 (Lai et al., 1991). While there are no specific markers for sib cell, a combination of physical appearance and dynamics of gene expression pattern can be effectively utilized to identify these two daughter cell fates of the GMC-1.

Loss of *rca1* blocks GMC-1 division and this GMC-1 becomes an RP2 neuron

To determine the effect of blocking the cell division on the asymmetric division of neuronal precursor cells, we examined the development of the GMC-1→RP2/sib lineage in the cell division mutant, *rca1*. The *rca1* gene was identified in a genetic screen by Zipursky and colleagues (Dong et al., 1997) and was shown to positively regulate CycA protein levels and, thus, required for the G_2/M transition. Loss of *rca1* activity during embryogenesis is shown to prevent cells from entering metaphase at cell cycle 15-16 due to its effect on CycA levels (Dong et al., 1997). Examination of *rca1* null mutant embryos with anti-Eve staining shows that the GMC-1 of the RP2/sib lineage fails to divide (Fig. 1d,e). However, this GMC-1 assumes the identity of the RP2 as judged by its position and gene expression pattern (Fig. 1f). Moreover, in *rca1* mutant embryos, at least one other Eve-positive lineage was similarly affected – the GMC of the aCC/pCC lineage. The aCC/pCC neurons are generated from GMC-1 of NB1-1. The aCC neuron is a pioneering motoneuron for the ISN and projects its axon posteriorly and laterally to the ISN whereas pCC is an interneuron and projects its axon anteriorly along the longitudinal connective (see Fig. 2A,C). Moreover, aCC is slightly larger than pCC and located anterior to pCC in the A-P axis and more ventral in the D-V axis (Fig. 2C). In *rca1* mutants, the GMC fails to divide and takes on the characteristics of an aCC and not the pCC (Fig. 1d-f). These defects in *rca1* mutant embryos are fully penetrant, i.e., every hemisegment in every mutant embryo is affected and, therefore, the *rca1* gene product in these two Eve-positive lineages appears to be indispensable.

Since Eve is expressed in both GMC-1 and RP2, one can argue that the GMC-1 in *rca1* mutants remain as GMC-1 and never differentiate into an RP2 motoneuron. We addressed this possibility by double staining *rca1* mutant embryos with anti-Eve and mAb22C10. This should allow us to determine whether GMC-1 in *rca1* assumes an RP2 identity since only an RP2 neuron expresses 22C10 antigen but not the precursor GMC-1. Moreover, this should also indicate whether the GMC-1 of the aCC/pCC lineage becomes an aCC neuron. As shown in Fig. 2, the 22C10 staining shows that the Eve-positive GMC-1 of the RP2/sib lineage in *rca1* mutants (Fig. 2B,D, dark red color) indeed assumes the identity of an RP2 motoneuron. This is indicated by the fact that this Eve-positive cell projects its axon ipsilaterally to the ISN same as an RP2 motoneuron in wild-type (compare Fig. 2A and C to 2B and D). Similarly, the GMC-1 for the aCC/pCC lineage assumes the identity of the aCC motoneuron is indicated by its posterior and lateral axonal

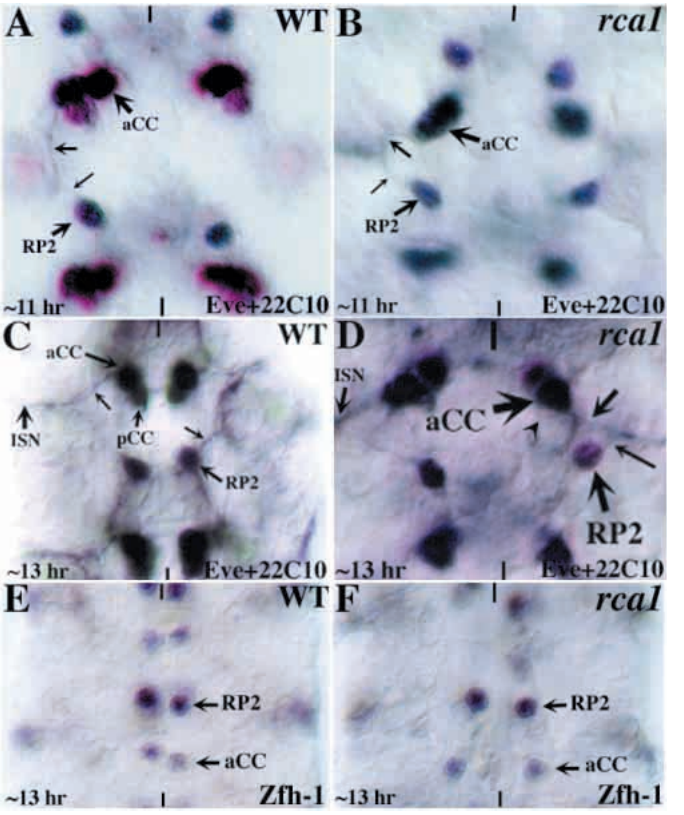

Fig. 2. Loss of *rca1* prevents GMC-1 division and this GMC-1 becomes an RP2 motoneuron. (A-D) Ventral views of segments in wild-type and *rca1* mutant embryos double-stained with anti-Eve (dark-red nuclear staining) and mAb22C10 (purplish membrane staining; the staining of the cell body membrane is somewhat masked by the heavy nuclear Eve staining). Anterior end is up, vertical lines mark the midline. ISN, intersegmental nerve bundle, arrowhead marks the missing pCC neuron (sibling of aCC). (A,C) Wild-type embryos, ~11-hour and ~13-hour-old, respectively. In wild-type, an RP2 begins to project its axon by 10 hours and can be discerned easily (together with the projection from aCC neuron) in a 11 hour embryo. By 13 hours, the axonal projections from an RP2 and aCC neurons become fasciculated. (B,D) *rca1* mutant embryos, ~11 hours and ~13 hours, respectively. The axonal projections from an 'RP2' and an 'aCC' in the *rca1* mutant follow the wild-type pattern in terms of timing and path finding. (E,F) Ventral views of wild-type (E) and *rca1* mutant (F) embryos stained with anti-Zfh-1. Anti-Zfh-1 stains only a mature RP2 neuron but not its precursor GMC or its sibling, RP2-sib; in the GMC-1→aCC/pCC lineage, it stains only the aCC neuron.

projection (Fig. 2B,D). In wild-type, the axonal growth cone from an RP2 begins to project by ~10 hours and can be discerned easily by ~11 hours. The aCC neuron begins to project its axonal growth cone slightly earlier (based on this timing difference, it is believed that aCC is the pioneering neuron for ISN). By 13 hours, the projections from RP2 and aCC neurons fasciculate with the ISN. Examination of timing of axonal projections from an 'RP2' and an 'aCC' neuron in *rca1* mutant embryos indicate that the dynamics of growth cone projections and axonal path finding are same as in wild type. A similar result was also observed in *cycA* mutants (data not shown). Thus, the blocking GMCs at G_2/M transition using *rca1* (or *cycA*) mutants leads to the specification of an RP2 identity to GMC-1 of the RP2/sib lineage and an aCC identity to GMC-1 of the aCC/pCC lineage.

Finally, we also confirmed the above result that the GMC for the RP2/sib cells assumes an RP2 identity and the GMC for the aCC/pCC cells assumes an aCC identity by staining a second RP2/aCC-specific marker, Zfh-1 (see Lai et al., 1991). Zfh-1 is expressed only in a mature RP2 neuron but not its sibling or precursor cell, GMC-1 (Fig. 2E). It is also expressed in the aCC neuron but not its parent GMC-1 or sibling, pCC (Fig. 2E). In *rca1* mutants, consistent with the conclusion that the GMCs in the RP2/sib and aCC/pCC lineages assume an RP2 and aCC identity, respectively (based on the results from anti-Eve and mAb22C10 staining), these cells were positive for the RP2 and aCC neuronal marker, Zfh-1 (see Fig. 2F).

The GMC-1 of the RP2/sib lineage fails to divide in *stg* mutants and this GMC-1 assumes an RP2 identity

The observation that blocking the GMC-1 from entering metaphase induces the GMC-1 to assume an RP2 fate was further examined by using additional cell division mutants. First, embryos mutant for the cell division gene, *stg*, was examined. *stg* encodes the *cdc25* phosphatase and during the entry of cells to mitosis, *stg* is required for the dephosphorylation of $p34^{cdc2}$. The dephosphorylated and active $p34^{cdc2}$ kinase triggers activities of downstream targets and promotes entry to metaphase. Thus, in *stg* mutants, it is known that the cell cycle is blocked at G_2/M transition. To determine the effect of loss of *stg* activity on GMC-1 development, a temperature-sensitive allele of *stg* was used. Embryos at different stages were shifted from the permissive 22°C to the non-permissive 29°C and kept at this non-permissive temperature for various periods of time (see Materials and Methods). At the end of incubation in the non-permissive temperature, these embryos were fixed and stained with anti-Eve and mAb22C10. As shown in Fig. 3, Eve staining of *stg* embryos indicates that, in stg^{ts} embryos that were shifted to restrictive temperatures prior to the division of GMC-1 of the RP2/sib lineage, the GMC-1 assumed an RP2 identity (Fig. 3d-f) as in *rca1* or *cycA* mutants. These results indicate that blocking the GMC-1 in G_2/M transition by eliminating *stg* activity leads to this GMC-1 assuming an RP2 identity. Similarly, we found that the GMC-1 in the aCC/pCC lineage assumes an aCC identity when the activity of *stg* is eliminated prior to GMC-1 division (data not shown). These results present a different consequence of the loss of *stg* activity on cells of the CNS than the one reported for the cells of the wing disc (Neufeld et al., 1998). These authors showed that loss of *stg* activity in cells of the wing disc causes cells to enlarge, indicative of continued cell growth after the arrest, but gradually, these cells were lost from the disc epithelium by cell death. In the CNS, our results show that loss of *stg* activity arrests cells, but these cells *differentiate*. Neufeld et al. (1998) further showed that ectopic expression of *stg* in the cells of the wing disc results in cells spending a much longer period of time in G_1, although the total cell-cycle time was not affected. A similar result was observed in the CNS as well, i.e., we also find that there is a delay in GMC-1 entering G_2-phase in embryos that are ectopically expressing *stg* since GMC-1 becomes Eve-positive at least 30 minutes later than in wild type.

Loss of cyclin A blocks GMC-1 entry into metaphase and this GMC-1 adopts an RP2 identity

Next, we wished to determine the consequence of the loss of *cycA* on GMC-1. Initially, we stained embryos with an antibody against Eve. As shown in Fig. 4B,D, the GMC-1 in *cycA* mutant did not divide; instead, it assumed an RP2 identity similar to loss of *rca1* (or *stg*). Moreover, as in *rca1* mutant embryos, at least one other Eve-positive lineage was similarly affected – the GMC of the aCC/pCC lineage; i.e., in *cycA* mutants, the GMC of the aCC/pCC lineage fails to divide and it takes on the characteristics of an aCC and not the pCC (Fig. 4B,D). These defects in *cycA* (or *rca1*) mutant embryos are

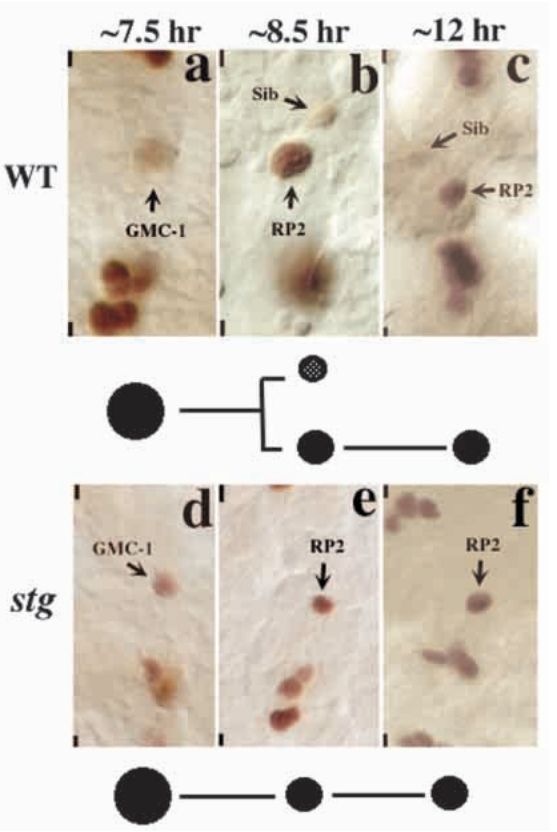

Fig. 3. Loss of string activity blocks the GMC-1 division and this GMC-1 assumes an RP2 identity. Ventral views of hemisegments in wild-type (a-c) and *stg* mutant (d-f) embryos stained with anti-Eve. Anterior end is up, the midline is marked by vertical lines. Note that the sib cell in ~12 hour embryos can be barely seen with anti-Eve.

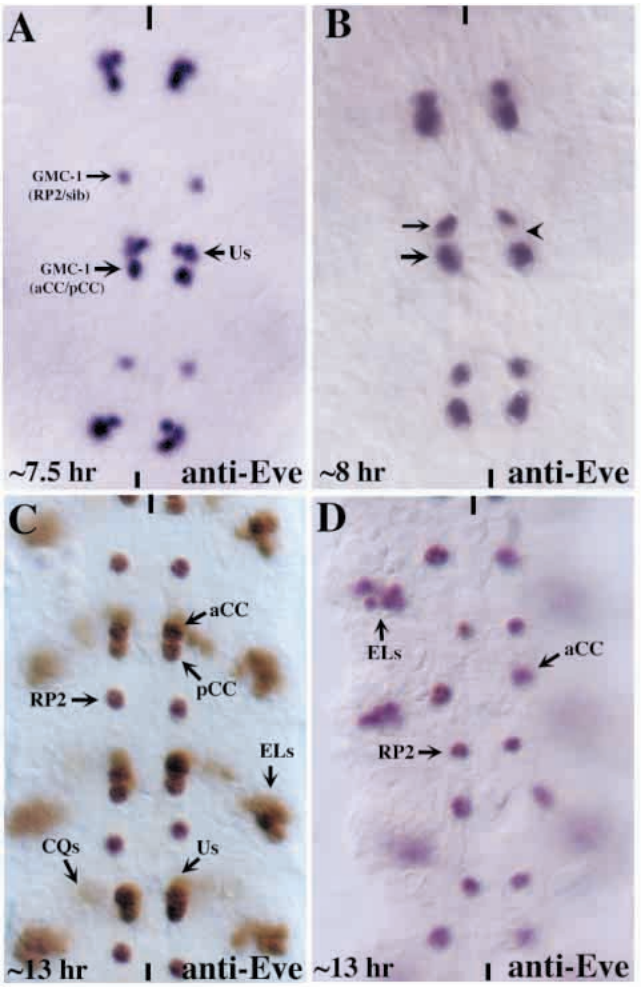

Fig. 4. Loss of cyclin A blocks the GMC-1 division and this GMC-1 assumes an RP2 identity. Ventral views of segments in wild-type (A,C) and *cycA* mutant (B,D) embryos stained with anti-Eve. Anterior end is up, the midline is marked by vertical lines. Note that the sib cell in ~13 hour embryos can no longer be detected with anti-Eve.

fully penetrant and thus every hemisegment in every mutant embryo is affected. The mutant embryos were also examined by double staining with anti-Eve and mAb22C10. As shown in Fig. 5F, the Eve and 22C10 double staining indicate that the GMC-1 assumes the characteristics of an RP2. This RP2 normally projects its axon ipsilaterally to the ISN same as in wild type (compare Fig. 5E and F). Similarly, the GMC-1 for the aCC/pCC lineage assumes the identity of the aCC motoneuron as indicated by its posterior and lateral axonal projection (Fig. 5E,F). These results indicate that blocking GMCs at G_2/M transition using *cycA* leads to the specification of an RP2 identity to GMC-1 of the RP2/sib lineage and an aCC identity to GMC-1 of the aCC/pCC lineage.

Requirement of *cycA* or *rca1* for cell division in the CNS is lineage specific

The anti-Eve staining of *cycA* or *rca1* mutant embryos indicate that loss of these gene products does not affect all the Eve-positive lineages in the ventral nerve cord. Eve is expressed in other neuronal lineages such as the CQs, the Us and the ELs (Fig. 6). The CQs are formed from NB7-1, an S1 neuroblast (Bossing et al., 1996; Schmidt et al., 1997). The GMC for these neurons are formed at the same time as the GMC for the aCC/pCC neurons (generated from another S1 neuroblast, NB1-1) and divide at the same time as GMC for the aCC/pCC lineage. As shown in Fig. 6, the NB7-1 in *cycA* or *rca1* mutants does not divide to generate an Eve-positive GMC for the CQs. However, the effect on CQs is partially penetrant in both the mutants. Thus, ~75% of the hemisegments (n=98 hemisegments) had missing CQs in *cycA* mutants and, in *rca1* mutants, it is ~50% (n=98). The effect on the generation of U neurons is as follows: in *cycA* mutants, the effect is fully penetrant whereas, in *rca1* mutants, 65% of the hemisegments were missing the Us. It must be pointed out that in those hemisegments where these neurons (Us and CQs) are formed, the number of these neurons is fewer than normal. Finally, the effect of loss of *cycA* or *rca1* on another Eve-positive lineage, the EL neurons, was minimal. The EL neurons are formed from NB3-3 (Bossing et al., 1996; Schmidt et al., 1997), an S4 neuroblast (the formation of this neuroblast extends between S3-S5). As shown in Fig. 6, none of the hemisegments had missing EL neurons either in *cycA* mutants or in *rca1* mutants.

The above result indicates that the loss of *rca1* or *cycA* does not affect the division of all neuroblasts. One possibility is that the maternal deposition of these gene products is masking the zygotic loss of these gene products in these lineages. However, this possibility seems to be unlikely since the GMCs for the aCC/pCC or the RP2/sib lineages are generated earlier than the GMCs for the EL neurons. Moreover, the maternal deposition of *cycA*, for example, is completely exhausted before stage 7 (E. van Beers and K. B., unpublished results) and none of the neuroblasts have delaminated from the neuroectoderm at this stage of development (see Doe, 1992). Thus, these results indicate that the effect of loss of *cycA* or *rca1* is lineage specific and every neuronal lineage is not sensitive to the loss of these cell division genes. It is most likely that some other cyclins (i.e., *cyclin B*) complement the loss of *cycA* in these lineages.

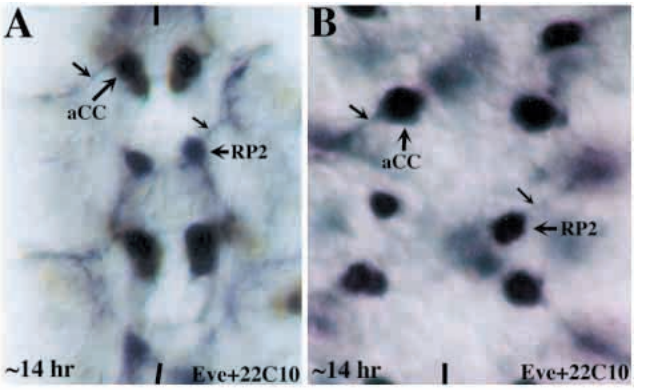

Fig. 5. The GMC-1 in cycA mutants adopts an RP2 motoneuronal identity. Ventral views of segments. Embryos are double stained with anti-Eve (nuclear staining) and mAb22C10 (membrane staining). Anterior end is up, vertical lines mark the midline. ISN, intersegmental nerve bundle. Small arrows mark the axonal projection from an RP2 or an aCC neuron. (A) A ~14-hour-old wild-type embryo. (B) In *cycA* mutant embryo, axonal projection from an 'RP2' and an 'aCC' follows the wild-type pattern in terms of timing and path finding.

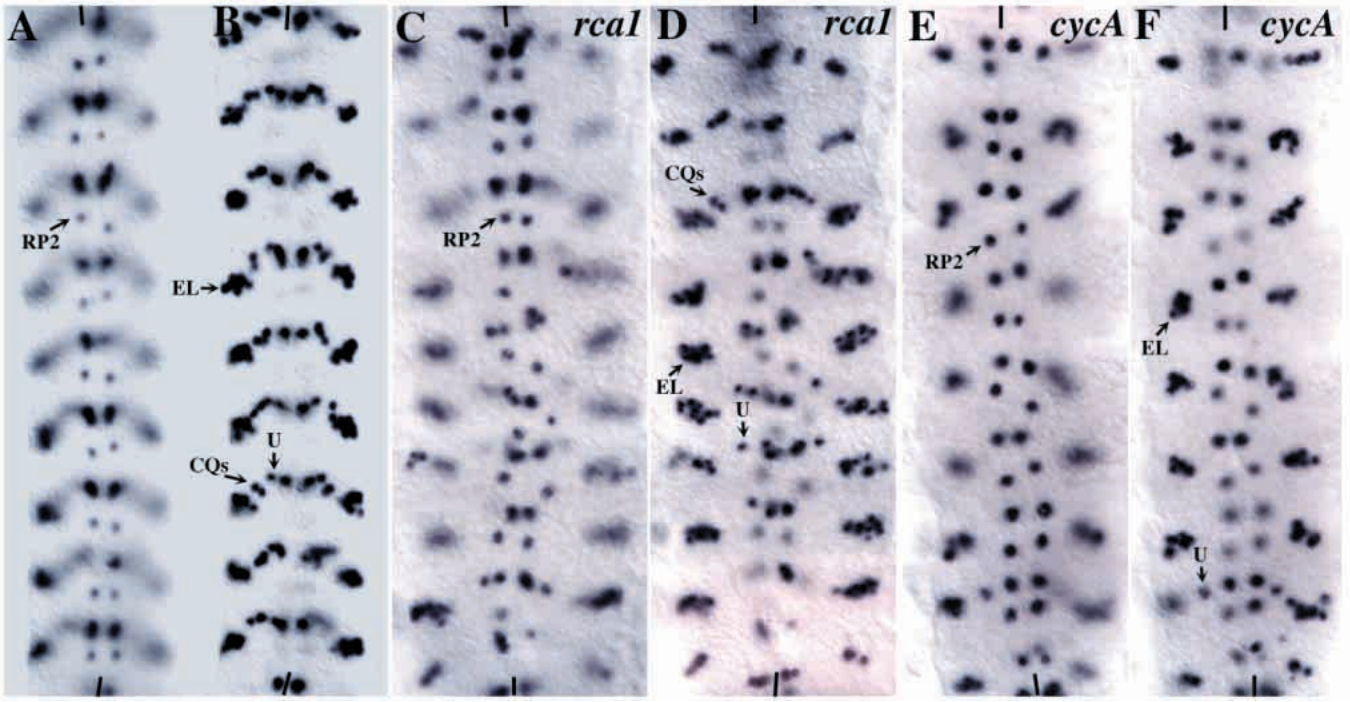

Fig. 6. Loss of cycA or rca1 does not affect the elaboration of several other neuronal lineages. Ventral views of notocord from ~14-hour-old embryos stained with anti-Eve. Anterior end is up. Vertical lines indicate midline. (A,B) A wild-type embryo focussed at two different focal planes. (C,D) An *rca1* mutant embryo focussed at two different focal planes. (E,F) A *cycA* mutant embryo at two different focal planes. A, C and E focus at the RP2 neuronal level and B, D and F focus at the EL neuronal levels.

Notch and Numb interaction specifies sibling neuronal fates

Since previous results show that N and Nb are involved in specifying sibling neuronal fates in the MP2 lineage of the CNS as well as in the PNS and adult sensillum (see Introduction), we sought to determine whether a similar relationship also exists in the RP2/sib lineage. Indeed, two recent reports indicates that the two play a role in the asymmetric specification of the RP2 and sib cell identity (Skeath and Doe, 1998; Buescher et al., 1998). We have also independently come to the same conclusion. As shown in Fig. 7B-C, examination of the embryos from a *ts* allele of *N* (N^{ts1}) indicates that shifting of mutant embryos from the permissive temperature to the restrictive temperature just before the division of GMC-1 of the RP2/sib lineage results in both the progeny of GMC-1 assuming an RP2 identity. In these N^{ts} embryos, as many as 60% of the hemisegments (n=110) had such fate transformations. In ~70% of the hemisegments where the sib is transformed into an RP2, the two had occupied different planes on the D-V (as well as A-P) axis (Fig. 7B). This would suggest that the transformed RP2-sib in these hemisegments still occupies the position of a sib within the CNS. In the remaining 30% of the affected hemisegments, the two cells were occupying the RP2-plane (Fig. 7B,C). The size asymmetry between the two cells was also less faithful than in wild type and frequently two RP2s of the same size were present in these hemisegments (Fig. 7C). Nevertheless, these results indicate that Notch signaling is necessary to generate a sib cell but not needed to specify an RP2 identity. It must be pointed out that the partial penetrance of the phenotype in N^{ts} embryos might be due to the timing of the temperature shift, or represent some degree of redundancy with respect to N signaling. Indeed, this seems to be the case since examination of embryos that are mutant for *mastermind* (*mam*) indicates that the sib cell in these embryos (Fig. 7E) is transformed into an RP2 cell in as many as 65% of the hemisegments (n=30 embryos). This result further indicates that N specifies sib fate via Mam (see also Buescher et al., 1998).

Since Nb functions as a N-signaling antagonist, we next examined the effect of loss of *nb* on the specification of cell identity to the progeny of GMC-1. Initially, we examined a deficiency that removes the *nb* gene. We observed that rarely the RP2 cell was transformed into a sib. More recently, Buescher et al. (1998) showed in an allele of *nb* (nb^{796}) that the RP2 cell transforms into a sib much more frequently (~27% of hemisegments). In this *nb* mutant, while the GMC-1 divides to generate two cells of unequal sizes (data not shown), both cells behave like sib cells; i.e., both these cells begin to lose Eve expression and, as a consequence, the hemisegments in ~13-hour-old embryos were missing the Eve-positive RP2 neuron (Fig. 7F,G; see also Buescher et al., 1998). Interestingly, we observed that often the larger of the two cells in these affected hemisegments lost Eve expression later than the bona fide sib cell (Fig. 7F,G). Finally, the partial penetrance of the phenotype in these embryos appears to be due to maternal deposition of *nb* gene products. This is indicated by the fact that elimination of maternal and zygotic *nb* results in a full penetrance of the RP2-to-sib transformation phenotype (Skeath and Doe, 1998). These results indicate that Nb is preventing N from specifying the sib identity to one of the two daughter cells of GMC-1. These results are also consistent with the finding that expression of a constitutively

active form of N (*Hs-N^{intra}*; only the intracellular domain of *N* is expressed from the heat-inducible *heat shock 70* promoter) during the division of GMC-1 leads to the specification of sib fate to both the progeny of GMC-1 (Fig. 7D). However, we observe that often the smaller cell (the bona fide sib cell) in these affected hemisegments lost Eve expression later than the larger cell, a situation just the opposite of embryos mutant for *nb*. Moreover, for reasons not entirely clear at this moment, the smaller cell in *Hs-N^{intra}* embryos lost Eve expression much later than a sib cell in wild-type. Similarly, we find that the disappearance of Eve expression in the bona fide sib cell and the transformed RP2 cell in *nb* mutants occurs much later than a sib cell in wild-type.

While Nb prevents N from specifying sib fate to one of the daughters of GMC-1 (see also Skeath and Doe, 1998; Buescher et al., 1998), it is not known whether Nb is required for the specification of RP2 identity. Therefore, we also examined the RP2 and sib cell fate specification in *N^{ts}; nb* double mutants. As shown in Fig. 7H, the *N* phenotype was found to be epistatic to the *nb* phenotype. Thus, in these double mutants, the sib cell is found to be transformed into an RP2 cell. However, this transformation was observed in a partially penetrant manner and often a double mutant embryo had some hemisegments showing the *N* phenotype and some other showing the *nb* phenotype (see Fig. 7H). We attribute the mixture of two phenotypes in the double mutant embryo to the partial penetrance of the phenotypes in *N* or *nb* single mutants. Thus, it is likely that in some hemisegments in the double mutant, the N activity is not completely abolished and it is sufficient to specify the sib fate. This result is also consistent with the finding that sib is transformed into an RP2 in *mam* mutants and indicates that neither *N* nor *nb* are required for the specification of RP2 fate. Thus, Nb is required for the specification of RP2 identity only when there is N.

numb phenotype is epistatic to the *cycA* phenotype

Since loss of function for *nb* and *rca1* or *cycA* showed opposing phenotypes in terms of the RP2/sib cell fate specification, we wished to determine the epistatic relationship between these genes in this lineage. Therefore, the fate of GMC-1 in *nb; cycA* double mutants was determined by anti-Eve staining. As shown in Fig. 8B, while the GMC-1 fails to divide to generate two cells in these double mutants, the GMC-1 assumed a sib fate. About ~35% of the hemisegments (between 8 and 10 hemisegments in a double mutant (*n*=15) showed this phenotype. This penetrance of the phenotype is slightly higher than the phenotype observed in *nb* single mutant alone. This suggests that *cycA* mutation has an enhancing effect of the *nb* phenotype. This would argue that normally a small amount of the Nb protein segregates into a sib cell and that, in the absence

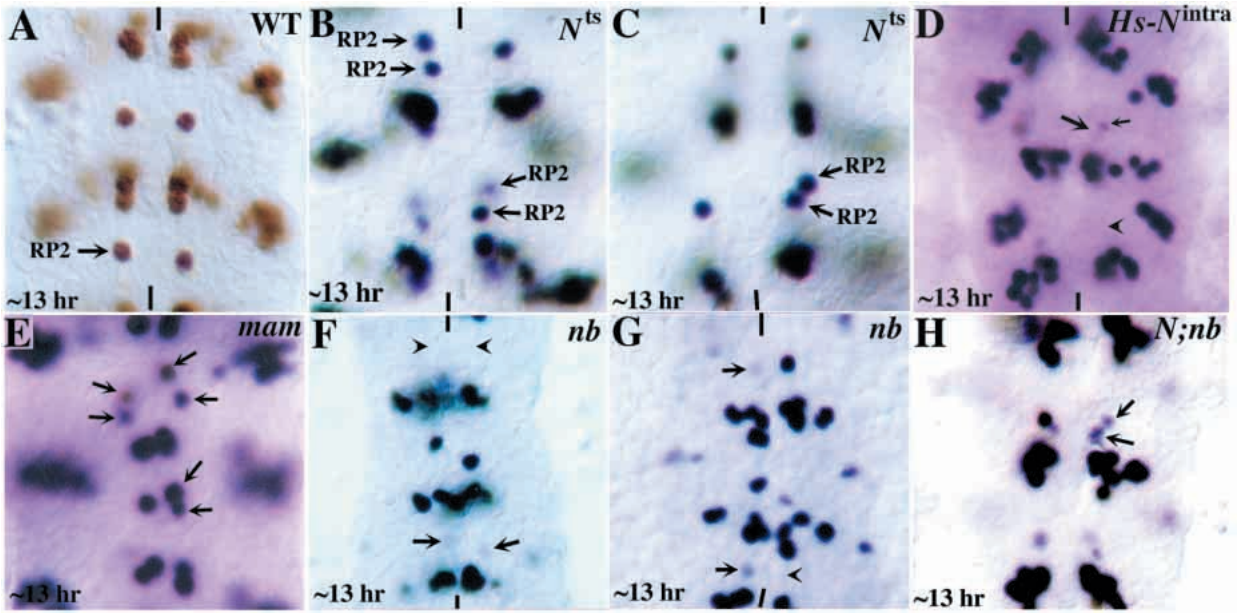

Fig. 7. *N* and *Nb* interact to specify RP2 and sib fates. Ventral views of hemisegments from ~13-hour-old embryos stained with anti-Eve. Anterior end is up. Vertical lines mark midline. (A) Wild-type embryo, note that the sib no longer expresses Eve at this stage. (B) An embryo from the *N^{ts1}* allele; note that both the progeny of GMC-1 express Eve. In the upper-left hemisegment, the two cells are of unequal sizes (the lower cell is presumably the bona fide sib). In the lower hemisegments, the duplicated RP2s occupy different positions on A-P and D-V axis, one is that of an RP2 and the other is that of a sib. (C) An embryo from the *N^{ts1}* allele, note that the duplicated RP2 neurons are of equal sizes. (D) An embryo expressing a constitutively active form of N under the inducible *hsp* promoter (*Hs-N^{intra}*). In the upper-right hemisegment, both the progeny of GMC-1 are losing Eve expression, however, the smaller cell (small arrow) still has Eve expression while the larger cell (arrow) has nearly lost its Eve expression. In the lower-left hemisegment, both cells have lost their Eve expression (arrowhead). (E) An embryo from weak allele of *mam*. The sib cell is transformed into an RP2 cell (arrows) in this *mam* allele. (F) A *nb* mutant embryo. In the uppermost segment, both cells have lost their Eve expression (arrowheads). In the lowermost segment, the sib cells have lost their Eve expression completely while the larger cells (arrows) have nearly lost their Eve expression. (G) Another example of the *nb* mutant. Arrowhead indicates missing Eve-positive RP2 cells (being transformed into sib cells), whereas the arrows indicate the larger, presumably the RP2 cells losing Eve expression due to their transformation into sib cells. (H) A *N^{ts}; nb* double mutant embryo. Hemisegments showing the *N* phenotype (upper right; arrows) as well as the *nb* phenotype (lower right) can be observed in these double mutant embryos (see text).

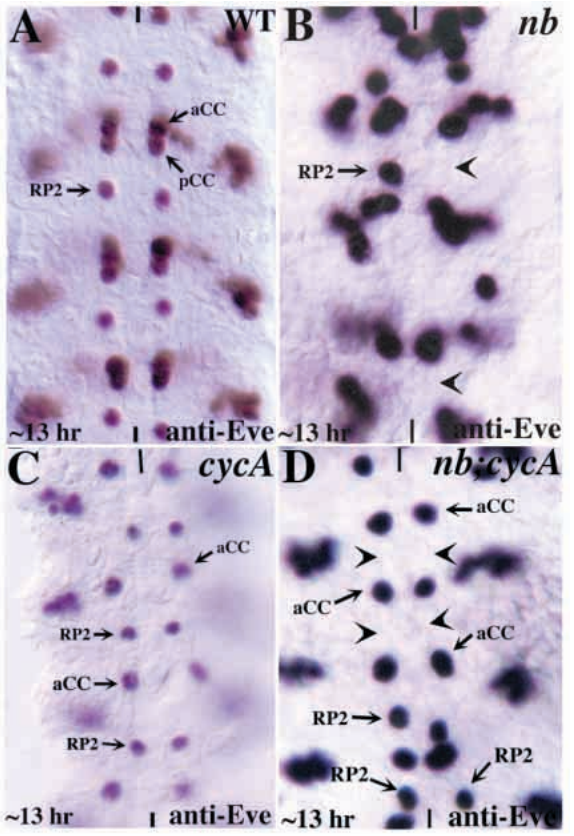

Fig. 8. The *nb* phenotype is epistatic to the *cycA* phenotype. Ventral views of CNS from ~13-hour-old embryos stained with anti-Eve. Anterior end is up. Midline is indicated by vertical lines. (A) A wild-type embryo; (B) a *nb* mutant embryo. In the hemisegment on the right, both progeny of GMC-1 of the RP2/sib lineage have assumed a sib identity and are missing the Eve expression (arrowhead). (C) An embryo from the *cycA* mutant. Note that the GMC-1 fails to divide in this mutant, but it assumes an RP2 identity (the GMC-1 of the aCC/pCC lineage assumes an aCC identity). (D) A *nb; cycA* double mutant. The GMC-1 of the RP2/sib lineage assumes a sib identity as in *nb* single mutant (indicated by arrowheads). The GMC-1 of the aCC/pCC lineage assumes an aCC identity as in *cycA* single mutant.

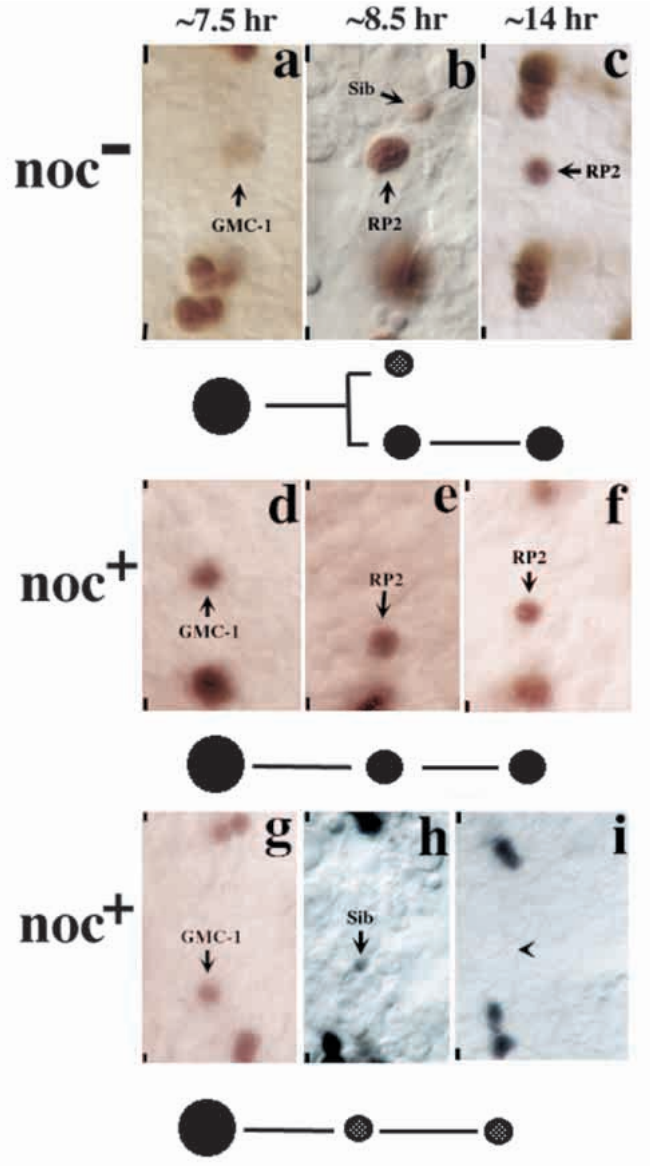

Fig. 9. Treatment of GMC-1 with nocodazole blocks GMC-1 division; however, this GMC-1 assumes either an RP2 identity or a sib identity. Ventral views of hemisegments stained with anti-Eve from wild-type (a-c) and wild-type embryos treated with the microtubule destabilizing drug, nocodazole (d-i). Anterior end is up, the midline is marked by vertical lines. The arrow head in (i) indicates the loss of Eve expression in sib cell. (d-f) The nocodazole-arrested GMC-1 assumes an RP2 fate, whereas in g-i, the nocodazole-arrested GMC-1 adopts a sib fate. The 'sib' phenotype in nocodazole-treated embryos is unlikely to be due to a GMC-1 dying since the dynamic of temporal expression of Eve in these cells is the same as in wild-type.

of cell division, all of Nb is accumulated in one cell, thus, much more effective in blocking the N signaling. Moreover, since the *nb* phenotype is epistatic to the cell division mutant phenotype, Nb must be acting downstream of these genes. This result is consistent with the finding that Nb becomes localized during the metaphase and is not localized in *stg* mutants (Spana and Doe, 1996). Thus, in *rca1* or *cycA* mutants, the absence of a localized Nb prevents the N signaling from specifying sib fate and, as a result, the GMC-1 assumes an RP2 fate.

Nocodazole treatment prevents GMC-1 from dividing and this GMC-1 assumes either an RP2 or a sib identity

It was previously shown that treatment of cells with nocodazole arrests cells in a pseudometaphase state; however, $p34^{cdc2}$ is in a dephosphorylated and, therefore, an active state in these cells (Morla et al., 1989). Nocodazole is a microtubule destabilizing agent that prevents formation of the mitotic spindle (Zieve et al., 1980). The effect of nocodazole is, however, reversible and thus does not cause a permanent injury to treated cells. To determine the fate of a GMC-1 that is blocked from entry to metaphase in a wild-type situation, we cultured permeabilized wild-type embryos with 10 μM nocodazole for various lengths of time (see Materials and Methods). These embryos were fixed at the end of culturing period and stained with Eve antibody. As shown in Fig. 9, nocodazole had an interesting effect on RP2 and sib cell fate specification. We found that the arrested GMC-1 either

assumed an RP2 identity (Fig. 9d-f) or a sib identity (Fig. 9g-i). In ~86% of the hemisegments (*n*=80) where nocodazole had arrested GMC-1 division, the GMC-1 assumed an RP2 identity versus in 14% of the affected hemisegments, the GMC-1 assumed a sib identity. The observation that nocodazole-arrested GMC-1 can assume a sib fate suggests that the destabilization of microtubule by treatment with nocodazole can lead to N signaling despite having Nb in that cell (see Discussion).

DISCUSSION

While neuroblasts self-renew each time they divide, a GMC division is a non-self-renewing type. Therefore, it has been argued that a GMC division is symmetric (i.e. a simple mitosis) and that the two different identities to daughter cells are specified postmitotically (Kuwada and Goodman, 1985). Cell ablation studies in the grasshopper indicates that the specification of one of the two sibling cells identity depends on cell-cell communication *between* sibling cells. Several recent reports, however, argue that the division of GMCs in the aCC/pCC and the RP2/sib lineage in *Drosophila* is asymmetric (c.f., Buescher et al., 1998) and that, at least in the RP2/sib lineage, the asymmetric division involves an asymmetric interaction between N and Nb. We have also independently come to the same conclusion. Thus, loss of N signaling (*N* and *mam*) leads to both sibling cells assuming RP2 fate while loss of *nb* results in these sibling cells assuming sib fate. That N instructs a cell to assume sib fate is also indicated by the fact that expression of the intracellular domain of N results in both the progeny of GMC-1 assuming the sib fate. Thus, this intracellular domain of N can override the negative effect of Nb. We further extend these findings in several ways. First, we have examined the fate specification in *N*; *nb* double mutants. Our results indicate that neither *N* nor *nb* are required for the specification of RP2 fate. *nb* is required for the specification of RP2 fate only when there is an intact *N*, i.e., to prevent N from specifying sib fate. Second, we have addressed the issue of asymmetry in the RP2/sib and the aCC/pCC lineages in the context of progression through cell cycle. We show that blocking GMCs at G_2/M transition using mutations in several cell division genes result in these GMCs assuming the identity of one of their daughter cells. In the RP2/sib lineage, the GMC-1 assumed the RP2 fate whereas in the aCC/pCC lineage, it assumed the identity of an aCC neuron. Interestingly, a double mutant analysis between cell division mutants and *nb*, indicates that the *nb* phenotype is epistatic to the cell division mutant phenotype. Thus *nb* must act after these GMCs exit the G_2 phase of the cell cycle.

While Buescher et al. (1998) have examined the fate of GMC-1 in presumably a cell division mutant called *GA339*, this mutation has not been characterized and consequently nothing is known about this mutation. The corresponding gene has not been cloned and the requirement of this gene product at the molecular level has not been elucidated. Thus, questions such as the nature of the mutation, its gene product, and the point in the cell cycle that this gene is required remain unanswered. Conceivably, it could affect any point in cell cycle, such as G_1, G_2 or M phase. While their results also indicate that the GMC-1 in this mutation assumes an RP2 fate, it must be pointed out that lack of information about *GA339* makes interpretation of their result difficult. The work described in this paper has utilized well-characterized genes to address the question of whether or not progression through cell cycle is required for generating asymmetry.

The interaction between N and Nb in the RP2/sib lineage appears to be similar to the one observed in the MP2 lineage. In the *Drosophila* MP2 lineage, the interaction between N and Nb regulates the asymmetric division of MP2 to generate two distinct interneurons, dMP2 and vMP2 (Spana and Doe, 1995, 1996; Spana et al., 1995; Knoblich et al., 1995; Hirata et al., 1995; reviewed in Jan and Jan, 1995; Campos-Ortega, 1996). The loss of *nb* results in the duplication of vMP2 at the expense of dMP2 and the loss of N results in the duplication of dMP2. This indicates that Nb is required for the specification of dMP2 and N is required for the specification of vMP2. Consistent with these results is the finding that Nb is asymmetrically localized to presumptive dMP2 during mitosis (see Introduction). While the loss of N signaling generates two dMP2 cells, loss of both N and Nb (double mutants) results in also generating two dMP2 cells. Based on this result, one can conclude that, in order to specify dMP2 identity, neither Nb nor Notch is required; Nb is required only when there is an intact N signaling. While it has not been determined whether or not the MP2 cell in *stg* mutants (and other cell division mutants) assumes a dMP2 fate, we have found that, in the RP2/sib lineage, the GMC-1 in *stg* mutants assumes an RP2 fate. The uniformly distributed Nb protein in GMC-1 of the RP2/sib lineage in *stg* mutants must prevent the N signaling pathway from specifying a sib fate to the GMC-1. This is also indicated by the fact that the GMC-1 in double mutants between cell division mutants and *nb* assumes the sib fate.

A question arises as to whether the arrested GMC-1 in cell division mutants (such as *cycA*, *rca1* or *stg*) assumes a mixed identity, i.e., the identity of both RP2 and sib, as opposed to an RP2 identity. Our results show that this cell expresses all the markers for an RP2 cell. Moreover, these cells extend axonal projections that are consistent with an RP2 neuron. Thus, in *cycA* or *rca1* mutants, the ipsilateral axonal projections were observed in every affected hemisegments. Given that a sib cell has no axonal projections (Chu-LaGraff et al., 1995), we think that this cell indeed assumes an RP2 identity. (This scenario also applies to the aCC/pCC lineage – in these cell division mutants, the GMC-1 of the aCC/pCC lineage projects axonal growth cone characteristic of an aCC neuron in each and every affected hemisegment.) However, due to the fact that there are no identified markers for a sib cell, the possibility that the arrested 'GMC-1' in cell division mutants also acquires some characteristics of a sib cannot be ruled out completely.

This study also addresses questions such as what are the roles of cell lineage and cell interactions in the determination of sibling neuronal identity. For example, the results of Kuwada and Goodman (1985) in the grasshopper indicates that the secondary neuronal precursor cells divide asymmetrically to generate two different sibling neurons, instead of generating two equivalent sibling cells, which then differentiate into two distinct neurons. These authors showed that the sibling neurons in two different lineages, aCC/pCC and H-cell/H-cell-sib lineages, are formed equivalent from their parent GMCs. Subsequently, the identity of pCC and H-cell-sib are determined first in a random-stochastic manner. These cells then instruct their sister cells to assume their respective fates (aCC and H-cell). Thus, a cell-cell interaction between postmitotic pairs of

sibling cells appears to play a role in neuronal identity specification. If this scenario in the grasshopper were to hold in the RP2/sib lineage of *Drosophila*, the RP2 and sib cells must be born equivalent and one of the two cells (probably the sib) should assume its identity first. This cell then instructs the identity of the other sibling cell to assume its identity involving a sibling cell-cell interaction. Thus, when a GMC-1 is arrested, this cell should remain as GMC-1. If one considers the possibility that a GMC-1 could not remain as GMC-1 because of certain constraints imposed by the developmental process, one might suppose that the GMC-1 would differentiate into a sib cell in the RP2/sib lineage and pCC cell in the aCC/pCC lineage. Our results, however, indicate that this is not the case. Taken together with the previous results that genes such as *miti*, *pdm1* (Bhat and Schedl, 1994; Bhat et al., 1995), *N*, *nb*, *insc* and *sanpodo* (Spana and Doe, 1995; Dye et al., 1998; Skeath and Doe, 1998; Buescher et al., 1998) are involved in generating asymmetry during GMC division, one can rule out the scenario that has been reported for the grasshopper embryo in the aCC/pCC and H-cell/H-cell-sib lineages. Whether or not this difference between grasshopper and *Drosophila* is due to the evolutionary separation between the two organisms, or the difference is on more trivial grounds, remains to be seen.

While the antagonistic interaction between N and Nb has been shown in several lineages in the CNS as well as in the PNS (see Introduction, see also Hartenstein and Posakony, 1990), it is not known how Nb prevents N from specifying cell fate. It has been recently shown that interaction of N with Delta leads to proteolytic cleavage of the intracellular domain of N (Struhl and Adachi, 1998; see also ref. therein). This intracellular domain of N then translocates to the nucleus and activates downstream target genes (Struhl and Adachi, 1998). Nb can prevent this release of the intracellular domain by blocking access to proteases, or it can physically hold on to the intracellular domain and prevent it from translocating to the nucleus. While our results with nocodazole do not provide evidence to support either of these scenarios, the fact that nocodazole-arrested GMC-1 can adopt a sib fate in wild-type situation suggests that microtubules are likely to have a role in mediating the interaction between Nb and N. We suggest that destabilization of microtubules by nocodazole treatment affects the ability of Nb to antagonistically interact with N.

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