Upregulation of Mitimere and Nubbin acts through Cyclin E to confer self-renewing asymmetric division potential to neural precursor cells

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Summary

In the *Drosophila* CNS, neuroblasts undergo self-renewing asymmetric divisions, whereas their progeny, ganglion mother cells (GMCs), divide asymmetrically to generate terminal postmitotic neurons. It is not known whether GMCs have the potential to undergo self-renewing asymmetric divisions. It is also not known how precursor cells undergo self-renewing asymmetric divisions. Here, we report that maintaining high levels of Mitimere or Nubbin, two POU proteins, in a GMC causes it to undergo self-renewing asymmetric divisions. These asymmetric divisions are due to upregulation of Cyclin E in late GMC and its unequal distribution between two daughter cells. GMCs in an embryo overexpressing *Cyclin E*, or in an embryo mutant for *archipelago*, also undergo self-renewing asymmetric divisions. Although the GMC self-renewal is independent of *inscuteable* and *numb*, the fate of the differentiating daughter is *inscuteable* and *numb*-dependent. Our results reveal that regulation of Cyclin E levels, and asymmetric distribution of Cyclin E and other determinants, confer self-renewing asymmetric division potential to precursor cells, and thus define a pathway that regulates such divisions. These results add to our understanding of maintenance and loss of pluripotential stem cell identity.

Supplemental data available online

Key words: Stem cell, Asymmetric division, Mitimere, Nubbin, Cyclin E, *Drosophila*

Introduction

An important aspect of stem cells is that they undergo a self-renewing type of asymmetric division, thus maintaining their number and at the same time generating progeny that are committed to a differentiation pathway. While it is known that differentiated cells can be reprogrammed to re-initiate development (Campbell et al., 1996), or multipotential cells can be induced to differentiate into various lineages, the maintenance of a pluripotent stem cell state has been an intriguing and long standing problem (see Bhat et al., 1988). Questions, such as how many genes are involved in maintaining a stem cell state, or how is a self-renewing asymmetric division regulated, remain unanswered.

In the *Drosophila* CNS, neuroblasts undergo a self-renewing stem cell type of asymmetric division, whereas their progeny, ganglion mother cells (GMCs), undergo a non-self-renewing terminal asymmetric division (Bate, 1976; Thomas et al., 1984; Bhat and Schedl, 1994; Buescher et al., 1998; Wai et al., 1999; Lear et al., 1999). Recently, several genes, such as *inscuteable* (*insc*), *bazooka*, *miranda*, *numb* (*nb*) and *Notch* (*N*), have been shown to be required for the asymmetric division of neural precursor cells (Buescher et al., 1998; Wai et al., 1999; Lear et al., 1999; Lu et al., 1999; Mehta and Bhat, 2001; Yedvobnick et al., 2004). The asymmetric divisions mediated by these proteins appear to be tied to the asymmetric segregation of some of these proteins to one of the two daughter cells during division. For example, during the division of GMC-1 of the RP2/sib lineage, a most intensely studied CNS lineage (reviewed by Bhat, 1999), Insc localizes to the apical end of GMC-1, whereas Nb segregates to the basal end. The cell that inherits Nb is specified as RP2 because of the ability of Nb to block Notch-signaling from specifying sib fate, whereas the cell that inherits Insc is specified as sib by Notch. Thus, in *insc* mutants, both the daughters of GMC-1 adopt an RP2 fate, whereas in *nb* mutants they assume a sib fate (Buescher et al., 1998; Wai et al., 1999). Although there is a good understanding of the terminal asymmetric division, not much is known about how a stem cell undergoes self-renewing asymmetric division. Similarly, it is not known what prevents GMCs from undergoing a stem cell type of asymmetric division. A previous study implicated Prospero (Pros) in inhibiting the ability of GMCs to divide more than once by preventing continued expression of cell-cycle genes (Li and Vaessin, 2000). However, this previous study did not examine any GMCs for multiple rounds of division. Pros is expressed in GMC-1 of the RP2/sib lineage and, in *pros* loss-of-function mutant embryos, this GMC-1 identity is not specified in nearly 95% of the hemisegments (this study). In ~5% of the remaining hemisegments, where GMC-1 identity is normally specified, the GMC-1s divide only once to generate RP2 and sib cells, as in wild type (this study). Moreover, in an hypomorphic allele

of *pros*, GMC-1s are specified properly in ~50% of the hemisegments; however, these GMC-1s do not undergo additional rounds of cell division either. These results suggest that this lineage (and other GMC lineages) is sensitive for the loss of Pros activity in terms of additional cell division.

It has been shown that the two POU proteins, Nubbin (Nub; also known as Pdm1) and Mitimere (Miti; also known as Pdm2), are required for the specification of identity of GMC-1 of the RP2/sib lineage in the *Drosophila* nerve cord (Bhat and Schedl, 1994; Bhat et al., 1995; Yeo et al., 1995). By contrast, a brief ectopic expression of these proteins at high levels prior to GMC-1 division results in a symmetrical division of GMC-1 to generate two GMC-1s, each of which subsequently divide to generate an RP2 and a sib (Yang et al., 1993; Bhat et al., 1995). Consistent with these results is the finding that although the levels of Nub and Miti are very high in a newly formed GMC-1, their levels drop significantly prior to GMC-1 division (Bhat and Schedl, 1994; Bhat et al., 1995). These results suggest that a downregulation of these two POU proteins is necessary for the GMC-1 to exit the cell cycle and to undergo a terminal asymmetric division.

Given these results, we investigated what would happen if *miti* or *nub* genes were overexpressed in GMC-1 at high levels for a prolonged period of time. We show that such an induction in GMC-1 causes it to self-renew several times. However, each of these self-renewing divisions generates a cell that becomes an RP2 or a sib. The self-renewing asymmetric divisions in these embryos are due to a failure in the downregulation of Cyclin E (CycE) in late GMC-1 and its unequal distribution between two daughter cells. An overexpression of CycE in GMC-1 also causes GMC-1 to undergo a similar type of self-renewing asymmetric division. Moreover, loss of function of *archipelago* (*ago*), which downregulates CycE via the degradation of the protein (Moberg et al., 2001), causes a late GMC-1 to accumulate high levels of CycE and results in its unequal distribution between two daughter cells. This causes self-renewing asymmetric division of GMC-1. Finally, overexpression of CycE also causes self-renewing asymmetric division in GMC1-1a of the aCC/pCC lineage, indicating that downregulation of CycE is essential for other GMCs to terminally divide into two distinct cells. These results show that when one of the daughter cell of a GMC acquires high levels of CycE, it behaves as a GMC with the ability to divide again, while the other differentiates into a neuron. These results provide insight into how cells can undergo a stem cell type of asymmetric division and maintain their pluripotency.

Materials and methods

Stocks and genetics

For overexpression of *miti*, *nub* and *CycE*, we used transgenic lines expressing full-length *miti* or *nub* genes under the control of *hsp70* promoter. We also used *UAS-nub* and *UAS-CycE* transgenic lines to overexpress these proteins more specifically in late GMC-1 using *ftz-GAL4*. The *insc* allele used was $insc^{22}$, *nb* allele used was nb^{796}, the *ago* alleles used were the strongest allele ago^{3} and another allele, ago^{1}, and combinations of ago^{3} and a deficiency that eliminates *ago* [Df (3L) GN50]. The *CycE* allele used was $CycE^{AR95}$. For the analysis of GMC-lineage development in *prospero* (*pros*) mutants, the pro^{17} allele was used. The various double mutants were generated by standard genetics.

Overexpression experiments

miti, *nub* or *CycE* transgenic embryos were collected on apple-juice agar plates for 15 minutes and appropriately aged embryos (see text) were heat shocked at 37°C for 20, 25 or 90 minutes. The development of these embryos post-induction was monitored in halocarbon oil and embryos were fixed when they reached appropriate stages. To determine the localization of Insc, 90-minute heat-shocked embryos were either fixed immediately after the induction or allowed to recover for 20 minutes before fixation. Both wild-type (heat shocked and non-heat shocked) and non-heat-shocked transgenic embryos were used as controls. To specifically induce *nub* in GMC-1, we crossed *UAS-nub* and *UAS-CycE* transgenic flies to *ftz-GAL4* flies, and the embryos were collected and stained with appropriate antibodies.

Immunohistochemistry and whole-mount RNA in situ hybridization

Embryos were fixed and stained with various antibodies as described previously (Bhat et al., 1995). The various primary antibodies used, and their dilutions, were as follows: anti-Eve (rabbit, 1:2000; or mouse, 1:5), anti-Zfh1 (mouse, 1:400), anti-Vnd (mouse, 1:1000), Mab22C10 (mouse, 1:4), anti-Spectrin (mouse, 1:50), anti-Insc (rabbit, 1:500), anti-Ftz (rabbit, 1:200) and anti-βGal (rabbit, 1:3000 or mouse, 1:400). For CycE staining, the primary antibody incubation was performed at room temperature. Whole-mount RNA in situ hybridization was performed using standard procedures (Bhat and Schedl, 1994).

Results

Upregulation of Miti or Nub in GMC-1 results in the generation of multiple cells in the RP2/sib lineage

We sought to determine whether the expression of *miti* or *nub* in GMC-1 at high levels for a prolonged period of time would induce the GMC-1 to symmetrically divide multiple times. Transgenic lines expressing *miti* or *nub* genes under the control of the *heat shock protein* (*hsp*) *70* gene promoter were used to overexpress these genes in embryos (this allows high levels of overexpression at desired and precise time points during development). Appropriately aged transgenic embryos (soon after the formation of GMC-1, ~6.45 hours of age at 22°C) were subjected to a 90-minute heat shock at 37°C (henceforth these embryos are called $miti^{P}$ or nub^{P}, P for prolonged induction). The embryos were then allowed to develop for different amounts of time and the development of the GMC-1→RP2/sib lineage was examined at different time points using several cell type-specific markers. A Miti/Nub-negative GMC lineage, GMC1-1a→aCC/pCC, was also examined to determine whether the ectopic expression of these POU genes has any effect on this lineage.

Even-skipped (Eve) staining of late stage 12 (~10 hour) $miti^{P}$ embryos revealed hemisegments with five to seven cells in a closely associated cluster in ~63% of the hemisegments (total number of hemisegments examined, n=510) in the location of RP2 and sib cells, as opposed to an RP2 and a sib in wild type (compare Fig. 1C and 1A). When late stage 14 to early stage 15 (~13 hour) $miti^{P}$ embryos were examined for Eve, duplication of RP2 was observed in ~45% of the hemisegments (n=470), instead of a single RP2 as seen in wild type (compare Fig. 1D and 1B). The multi-cell clusters also generate three RP2s (Fig. 1E,F; 5% of the hemisegments, n=470) or a single RP2 (13% of the hemisegments). These additional cells were indeed RP2s is indicated by the expression of other RP2-specific genes, such as *zfh1* (Fig. 1H), and their axon

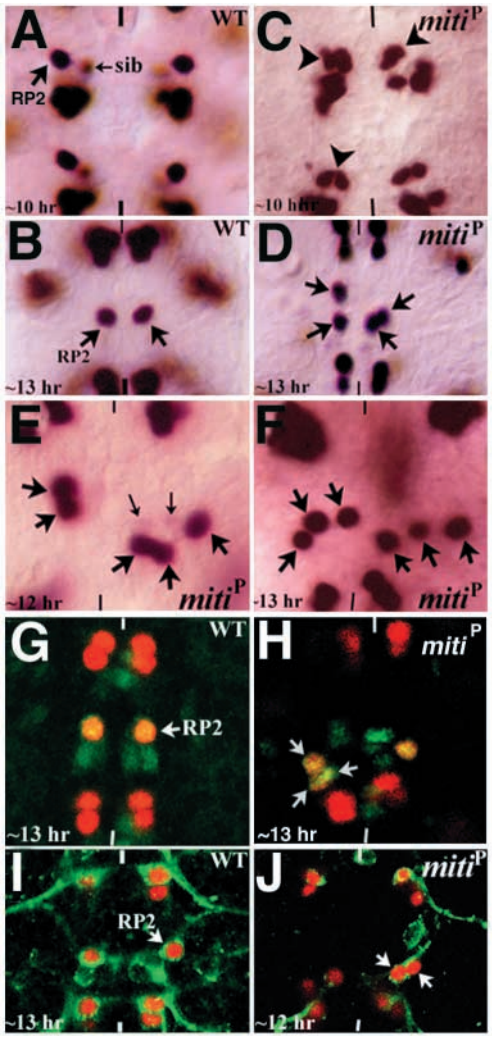


Fig. 1. The GMC-1 division pattern in *miti*^{P} embryos. (A-F) Embryos stained for Eve; (G,H) embryos stained for Eve (red) and Zfh1 (green; yellow indicates co-localization); (I,J) embryos stained for Eve (red) and 22C10 (green). Embryos in A-H were analyzed by light microscopy, those in I and J were analyzed by confocal microscopy. Anterior end is up, vertical lines indicate the midline. Arrow indicates an RP2, small arrow indicates a sib. (A,B) Wild-type embryos, note the smaller sib (with fading Eve expression) and the larger RP2 (A); by 13 hours of age, the sib loses Eve expression and is no longer visible (B). (C-F) *miti*^{P} embryos. Multi-cell clusters are seen in the younger stage embryo (C, arrowheads), whereas in older stage *miti*^{P} embryos (D-F) two to three RP2s were observed per hemisegment. (E) Three RP2s and two sibs can be seen (sibs are barely visible). (G) Wild-type embryo stained for Eve and Zfh1. Note that the RP2 expresses Zfh1 at high levels. (H) *miti*^{P} embryo, all three RP2s co-express Eve and Zfh1. (I,J) Wild-type (I) and *miti*^{P} (J) embryos double stained for Eve and 22C10. The two Eve-positive RP2s also express 22C10 and project normally.

morphology (Fig. 1J). The multi-cell clusters that we observe during stage 12 appear be a collection of GMC-1s (a maximum of two GMC-1s), RP2s and sibs. It seems likely from the above results that there is a preference for the generation of sibs rather than RP2s. Similar results were also observed following overexpression of *nub*. Thus, our analysis indicates that *miti*^{P} and *nub*^{P} embryos can generate as many as three RP2s and two to four sibs (see Fig. 1E). Identical heat-shock experiments of wild-type embryos did not result in any of these phenotypes. Furthermore, no phenotypes were observed in the GMC1-1a→aCC/pCC lineage, a Miti or Nub-negative lineage. Therefore, we conclude that the above phenotypes are not heat-induced artifacts.

Multiple asymmetric self-renewal of GMC-1 with overexpression of *miti* or *nub*

Previous results indicate that between RP2 and sib, a nuclear size asymmetry is generated prior to cytokinesis, and is thus inherent to the lineage. The cytokinesis of GMC-1 is also asymmetric, and the larger cell becomes an RP2, whereas the smaller cell becomes a sib. Moreover, a GMC-1 is generally larger than an RP2, and a sib is smaller than both a GMC-1 and an RP2. We sought to use these characteristics to obtain further evidence that GMC-1 undergoes self-renewing asymmetric division, by staining *miti* and *nub* gain-of-function embryos with the nuclear markers Eve and Zfh1. Zfh1 is not expressed in GMC-1 or sib, but an RP2 expresses Zfh1 soon after its formation and continues to express it (Fig. 2A,B; see also Fig. 1F). Thus, a combination of Eve and Zfh1 is an excellent set of markers to follow the division history of GMC-1. When *miti* (or *nub*) is induced in late stage 10 (~6.75 hours) embryos for 20 minutes and examined for expression of the nuclear markers Eve and Zfh1 during mid stage 11 (~7.5 hour), we observed a GMC-1 and an RP2 (Fig. 2D,E), or a GMC-1 and a sib (Fig. 2F,G). When embryos were induced for 90 minutes and examined during late stage 11 (~8.5 hours), a GMC-1, an RP2 and a sib were observed (Fig. 2H,I). When 90-minute-induced transgenic embryos were analyzed during the stage 12 and stage 13 transition (~10.5 hours), multi-cell clusters were observed in these embryos, with one GMC-1, and several RP2s and sibs (Fig. 2J). Several different combinations consistent with the self-renewal of GMC-1 (such as one GMC-1 and two sibs, two GMC-1s and two sibs, or two GMC-1s and two RP2s in late stage 11; and one to three RP2s and two to four sibs in late stage 12) were also observed in these embryos (data not shown).

Overexpression of *nub* in GMC-1 causes it to adopt a self-renewing type of asymmetric division pattern

We then examined whether overexpression of these POU genes more specifically in GMC-1 could also induce the self-renewing asymmetric division of GMC-1. We employed a UAS×GAL4 strategy (Brand and Perrimon, 1996) and induced a *UAS-nub* transgene with *fushi tarazu* (*ftz*)-*GAL4*, *ftz* being active in GMC-1 (the *ftz* promoter is active in one other Eve-positive neuronal lineage, the Us). This targeted expression would also exclude the possibility that ectopic expression of Miti/Nub in NB4-2 causes GMCs subsequently born from NB4-2 to transform into GMC-1, as could be argued in the case of *hs-miti* or *hs-nub*. First, we examined embryos with Eve staining. In wild type, the asymmetric division of GMC-1 generates the RP2 and the smaller sib with weaker Eve staining by ~7.75 hours of age (Fig. 3A). In ~7.75-hour-old *UAS-nub; ftz-GAL4* embryos, two cells that are of equal or nearly equal size with high levels of Eve are observed (Fig. 3C), a pattern different from wild type. These cells are an RP2 and a GMC (instead of two GMC-1s), as indicated by the fact that a 4-cell

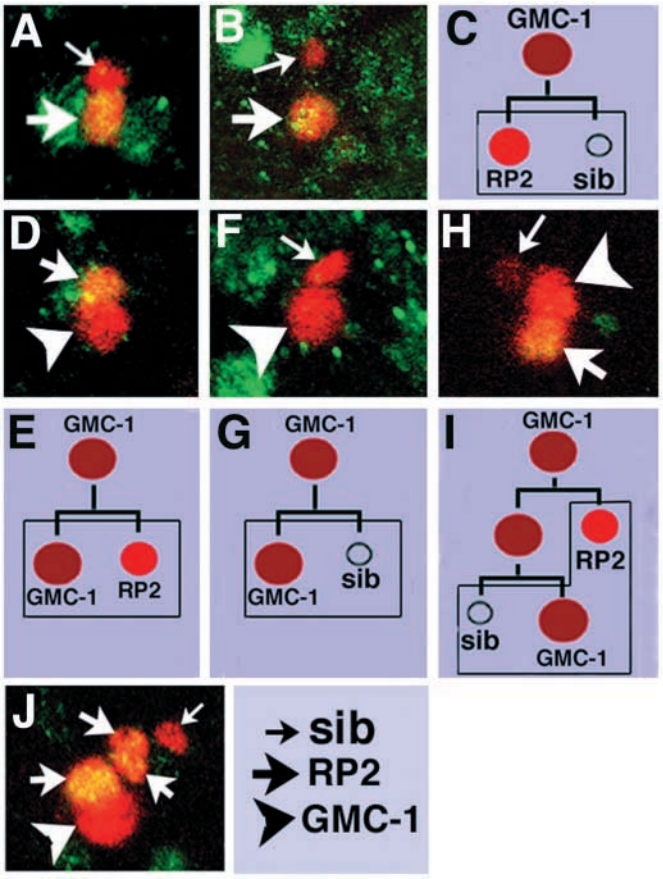


Fig. 2. The GMC-1 adopts a reiterating division pattern in embryos overexpressing *miti* or *nub*. Embryos were stained for Eve (red) and Zfh1 (green). Only merged images are shown, yellow indicates co-expression. The line drawings (C,E,G,I) are interpretations of the data from panels A,B,D,F,H. The boxed area in each line drawing indicates cells observed in the corresponding panels. (A,B) Wild-type embryos. The GMC-1 has terminally divided to generate a sib (Eve positive, Zfh1 negative) and an RP2 (Eve and Zfh1 positive) with no additional cells formed. (D,F) *miti* transgenic embryos in which the gene was induced at 6.75 hours of development for 30 minutes. At the 7.5-hour stage, the GMC-1 appears to have self-renewed (larger of the two cells) and generated an RP2 (D) or sib (F). By the 8.5-hour stage, the GMC-1 is dividing to generate a sib, an RP2 has already been generated (H). (J) An ~10.5-hour stage $miti^{P}$ embryo in which the transgene was induced at 6.75 hours of development for 90 minutes. A multi-cell cluster of several RP2s, one sib and a GMC-1 can be seen.

phenotype, i.e. 2 RP2s and 2 sibs, was not observed at later stages of development (as would be expected from a duplication of GMC-1) (see Yang et al., 1993; Bhat et al., 1995). Instead, in later stages of *UAS-nub; ftz-GAL4* embryos (~8.75 hours), we observed 3-cell phenotypes. An example of a 3-cell phenotype is shown in Fig. 3D, where the GMC-1 has first generated a sib and a self-renewed GMC-1, which then appears to be undergoing a second division to generate another sib and an RP2. As we did not observe the generation of additional cells (or multi-cell clusters) in later stage *UAS-nub; ftz-GAL4* embryos, the GMC-1 appears to undergo only one round of self-renewing asymmetric division (this is most likely due to the lower levels of ectopically produced protein in GMC-1 in these embryos compared with in *hs-miti* or *hs-nub* embryos).

To obtain more direct evidence that GMC-1 adopts a stem cell type of division pattern in an embryo expressing these POU genes, we double stained *UAS-nub*; *ftz-GAL4* embryos for the nuclear marker Eve and the cell cortex marker Spectrin. Spectrin stains the cell cortex throughout the cell cycle (see Prokopenko et al., 1999; Mehta and Bhat, 2001), and thus it can be used to visualize cytokinesis of a dividing cell. As the above experiments revealed that the third cell in embryos overexpressing *nub* appears at ~8.5 hours of development, we sought to visualize the cytokinesis of GMC-1 dividing the second time (a GMC-1 in wild type always completes its division by 7.5-7.75 hours of development). As shown in Fig. 3, in wild type, the asymmetric nuclear division and cytokinesis of GMC-1 (and the unequal sizes of daughter nuclei) to generate the larger RP2 and the smaller sib can be faithfully observed in ~7.5-hour-old embryos (panel E; note the continuity of Eve staining between the two newly forming cells). In ~7.5-hour-old *UAS-nub; ftz-GAL4* embryos, a GMC-1 dividing nearly equally to generate two cells was observed (Fig. 3G). In ~8.5-hour-old *UAS-nub; ftz-GAL4* embryos, hemisegments with a large cell, presumably an RP2 (Fig. 3H), or a smaller sib (Fig. 3I) were observed. In these hemisegments, we also observed a GMC-1 undergoing asymmetric nuclear division and cytokinesis to generate an RP2 or a sib (Fig. 3H; note the continuity of Eve staining between the two newly forming cells), or an equal nuclear division and cytokinesis to generate two RP2s (Fig. 3J; note that elevated levels of Miti or Nub in a late GMC-1 can also result in a symmetrical division of GMC-1 to generate two RP2s; see Fig. 4) (see also Mehta and Bhat, 2001). (We could not employ the method of visualization of GMC-1 division in live embryos using the green fluorescent protein because it takes >3.5 hours for the green florescent protein to become fluorescent after it is produced and, by this time, GMC-1 has completed its division). A summary of the consequences of overexpression of *miti* or *nub* (for 20 minutes and 90 minutes) on GMC-1 division at various developmental time points is presented in Fig. 4. The asymmetric division (which generates the 3-cell phenotypes) and the symmetric division (which generates 2-cell or 4-cell phenotypes) were observed when the transgenes were induced for 20 minutes. However, the multi-cell phenotype – two to three RP2s and three to five sibs – was observed only when the transgenes were induced for 90 minutes.

The levels of Cyclin E in GMC-1 is upregulated in embryos expressing high levels of Miti or Nub

As downregulation of CycE is necessary for the cells to exit from the cell cycle (see Knoblich et al., 1994; Moberg et al., 2001) (reviewed by Edgar and Lehner, 1996; O'Farrell, 2001), we examined whether CycE is upregulated in GMC-1 of embryos expressing Nub or Miti at high levels. First, we examined the expression pattern of CycE in wild type during GMC-1→RP2/sib lineage development. As shown in Fig. 5, a late GMC-1 has very little CycE (Fig. 5A,B), and CycE is nearly undetectable in a newly formed RP2 or sib (Fig. 5C,D). A similar pattern was also observed in non-induced *miti* (or *nub*) transgenic embryos (Fig. 5E-H). However, the induction of *miti* (or *nub*) by a 20-minute heat shock caused a late GMC-

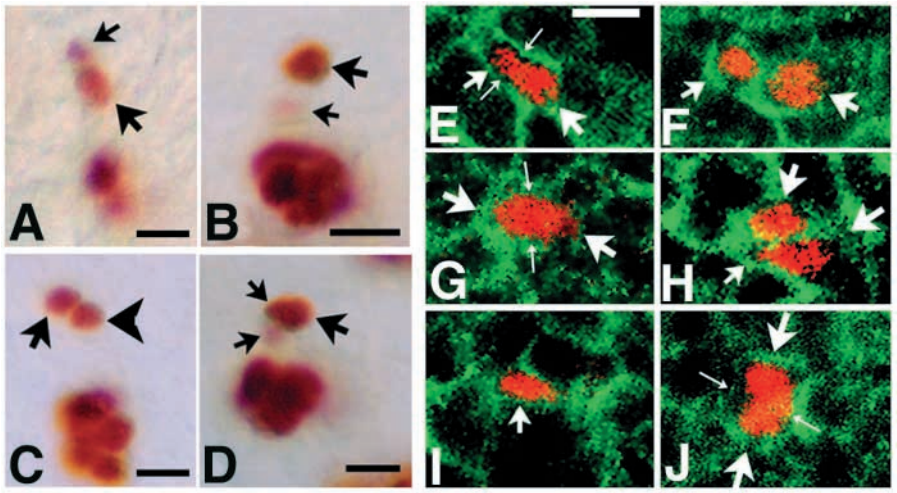


Fig. 3. Self-renewing asymmetric division of GMC-1 in *nub* gain-of-function embryos. (A-D) Embryos stained for Eve; (E-J) embryos double stained for Eve and Spectrin. Arrowhead indicates a GMC-1, arrow indicates an RP2, small arrow indicates a sib. Thin, long arrows (E,G,J) denote indentations indicative of the cell undergoing cytokinesis. (A) ~7.75-hour-old wild-type embryo. The GMC-1 has divided to generate a sib and an RP2. (B) ~9-hour-old wild-type embryo. Note the fading Eve expression in sib. (C) ~7.75-hour-old *UAS-nub; ftz-GAL4* embryo. The GMC-1 has divided to generate two cells. (D) ~9-hour-old *UAS-nub; ftz-GAL4* embryo with a 3-cell phenotype: two sibs and an RP2. (E,F) Wild-type embryos. Both nuclear division and cytokinesis of GMC-1 is asymmetric (E; ~7.5-hours old) and this gives rise to the smaller sib and the larger RP2 (F; ~8-hours old). (G) ~7.5-hour-old *UAS-nub; ftz-GAL4* embryo. The GMC-1 is undergoing a nearly equal nuclear division and cytokinesis to self-renew and to generate an RP2. (H) ~8.5-hour-old *UAS-nub; ftz-GAL4* embryo. The GMC-1 is in the process of dividing to generate a smaller sib; an Eve-positive RP2 has already been generated in this hemisegment (the top cell). (I,J) Two different focal planes of the same area of an ~8.5-hour-old *UAS-nub; ftz-GAL4* embryo, showing a sib cell generated earlier (I) and the GMC-1 dividing to generate two RP2s (J). The long arrow marks the position of the sib. Scale bars: ~7.5μm for A-D; ~5μm for E-J.

1 to accumulate high levels of CycE (Fig. 5I,J; immediately after a 20 minute induction). Moreover, we observed high levels of CycE in one of the progeny of such GMC-1s (Fig. 5K,L; 20 minute induction and 30 minute wait), indicating that this cell is a self-renewed GMC-1 (i.e. it has retained the GMC-1 identity). A one-hour wait following a 20 minute induction usually results in a 3-cell phenotype, and, when those cells were examined, the level of CycE was downregulated and was nearly undetectable (Fig. 5M-P). We also examined embryos where the transgenes were induced for 90 minutes (which causes multiple asymmetric divisions; see Fig. 1). In those embryos one (or two) cells with high levels of CycE were observed (data not shown). These results indicate that the self-renewing cell retains high levels of CycE and that the loss of self-renewing ability coincides with the downregulation of CycE.

Overexpression of Cyclin E results in asymmetric GMC self-renewal

We then investigated whether the basis for the GMC-1 to adopt a self-renewing division pattern in *miti* or *nub* transgenic embryos is entirely due to the upregulation of CycE. Therefore, we overexpressed CycE more specifically in GMC-1 by inducing a *UAS-CycE* transgene with *ftz-GAL4*. As shown in Fig. 6A-C, such an induction of *CycE* in GMC-1 resulted in GMC-1 adopting a self-renewing asymmetric division pattern similar to embryos overexpressing *miti* or *nub*. This phenotype was observed in 11% of the hemisegments (n=507). However, when *CycE* was induced for a prolonged period of time from the *hsp70* promoter, a self-renewing asymmetric division of GMC-1, similar to that observed in $miti^{P}$ or nub^{P} embryos (Fig. 6D-F), was observed in 43% of the hemisegments (n=507). When *CycE* was induced for 30 minutes and double stained with Eve and Zfh1, we observed two sibs and one RP2 (Fig. 6H) or two RP2s and one sib (Fig. 6I). Finally, we found that $miti^{P}$ phenotypes are dependent on *CycE*, thus the $miti^{P}$*; CycE* embryos showed the *CycE* phenotype (in *CycE* mutants, GMC-1 fails to divide, instead it differentiates into an RP2, see supplementary data at http://dev.biologists.org/supplemental/). These results

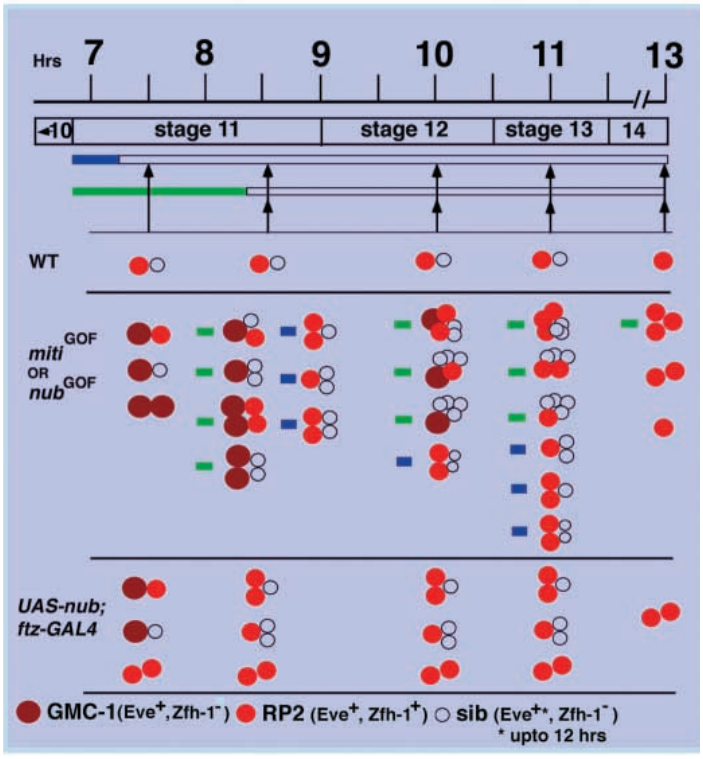


Fig. 4. Summary of the observed GMC-1 division phenotypes due to overexpression of *miti* and or *nub*. WT, wild type; $miti^{GOF}$ or nub^{GOF}, overexpression of *miti* or *nub*, respectively, for 20 minutes (blue bar) or 90 minutes (green bar). The phenotypes that are specific to 20-minute or 90-minute induction times are marked with same colored short bars. Arrows indicate times at which embryos were analyzed. The unmarked phenotypes were observed in both inductions. Note that a 20-minute induction of *miti* or *nub* only in a late GMC-1 (~7.25 hours) induces a symmetrical division of GMC-1 to generate two RP2s (see Mehta and Bhat, 2001). In the *UAS-nub; ftz-GAL4* embryo, the *UAS-nub* transgene was induced with *ftz-GAL4* (the *ftz* promoter is active in GMC-1, see text for details).

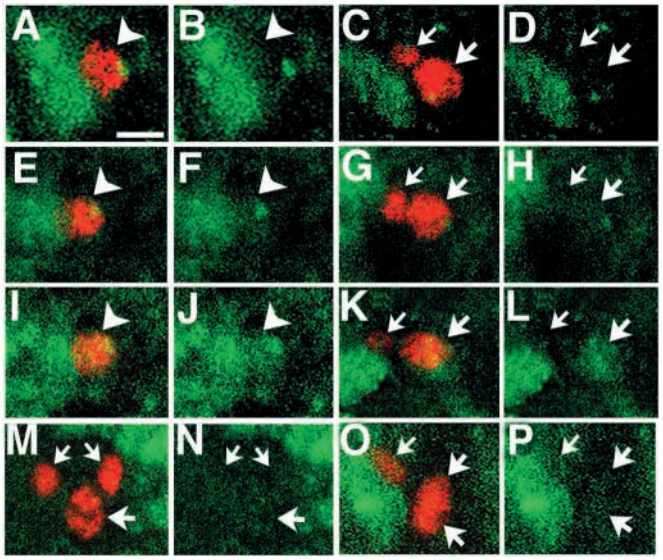


Fig. 5. Cyclin E protein is upregulated in late GMC-1 in embryos overexpressing *miti*. Embryos are double stained for CycE (green) and Eve (red). Only CycE and merged images of the two are shown. Arrowhead indicates a GMC-1, arrow indicates an RP2, small arrow indicates a sib. Only hemisegments are shown. (A,B) A late GMC-1 in a wild-type embryo (~7.25 hours old) with very little CycE. (C,D) A newly formed RP2 and a sib in a wild-type embryo (~7.75 hours old) with undetectable levels of CycE. (E,F) A late GMC-1 in a *hs-miti* embryo (~7.25 hours old) without any induction. (G,H) A newly formed RP2 and a sib in a *hs-miti* embryo (~7.75 hours old) without any induction. (I,J) A late GMC-1 in a *hs-miti* embryo induced for 20 minutes. Note the high levels of CycE. (K,L) A newly formed larger cell (presumably the self-renewed GMC-1, see text) and a sib in a *hs-miti* embryo where the transgene was induced for 20 minutes at the GMC-1 stage. The larger cell has high levels of CycE; CycE is undetectable in the smaller cell. (M,N) Two sibs and an RP2 in a *hs-miti* embryo (~9.5 hours old) in which the transgene was induced for 20 minutes at the GMC-1 stage. (O,P) Three RP2 in a *hs-miti* embryo (~9 hours old) in which the transgene was induced for 20 minutes at the GMC-1 stage. In panels M-P, CycE has been downregulated after the additional round of division. Scale bar: ~7.5 μm.

indicate that upregulation of CycE alone is sufficient for GMC-1 to adopt a self-renewing asymmetric division pattern.

A GMC that does not normally express Miti or Nub, such as GMC1-1a of the aCC/pCC lineage, is insensitive to the ectopic presence of these proteins. Moreover, although gain of function for *miti/nub* elevates the levels of CycE in GMC-1, it does not do so in GMC1-1a. Therefore, we sought to determine whether GMC1-1a is sensitive to the elevated levels of CycE and, if so, what its division pattern would be. Embryos in which *CycE* was induced for 30 minutes were double stained with Eve/Zfh1 and Eve/Ventral Nervous System Defective (Vnd) combinations to examine the GMC1-1a of NB1-1. GMC1-1a asymmetrically divides to generate a pCC [which is Eve-positive but Zfh1 negative (Fig. 6J), or Vnd-positive (Fig. 6M)] and an aCC [which is Eve and Zfh1-positive (Fig. 6J), but Vnd-negative (Fig. 6M). In CycE gain-of-function embryos, we observed hemisegments with two pCCs and one aCC (Fig. 6K,N), or two aCCs and one pCC (data not shown). This indicates that GMC1-1a also undergoes self-renewing asymmetric division when CycE levels are upregulated. The self-renewing asymmetric division of GMC1-1a was observed in 55% of the hemisegments (n=400).

We further explored this in another way. Previous results have shown that Ago downregulates CycE during exit from the cell cycle (Moberg et al., 2001). Thus, in *ago* mutants there is a lack of degradation of CycE protein and, therefore, cells undergo additional rounds of mitosis. We examined the GMC-1→RP2/sib lineage in embryos mutant for *ago*. Several findings were of interest. First, in *ago* mutants the GMC-1 was adopting a self-renewing asymmetric division pattern similar to gain-of-function CycE or Miti/Nub. Thus, in hemisegments with 3-cell phenotypes, two sibs and one RP2 (Fig. 7A), and two RP2s and one sib (Fig. 7B,C) were observed. Second, the late GMC-1 in the mutant had a higher level of CycE than in wild type (Fig. 7D,E). Third, an unequal distribution of CycE between the two daughters of a dividing GMC-1 was observed (Fig. 7F,G; note the continuity of Eve between the two daughter cells). As the transcription of *CycE* (as judged by RNA in situ hybridization) ceases in late GMC-1 (data not shown) and Ago regulates CycE levels at the protein level, the higher levels of CycE in one of the two daughters appears to be due to an unequal distribution of the protein. Fourth, the level of CycE is nearly undetectable among cells in a 3-cell cluster (Fig. 7H,I) indicating that by the time the self-renewed GMC-1 divides again, the level of CycE is downregulated. Finally, we want to point out that the penetrance of the GMC-1 phenotype was low in *ago* mutants. The strongest allele, ago^3, had a penetrance of 5% (n=770), ago^1 had a penetrance of 2% (n=1100) and ago^3/df had a penetrance of 7% (n=770); the upregulation of CycE in late GMC-1 was observed in 7% of the hemisegments in ago^3 embryos. Similarly, the percentage of hemisegments with elevated levels of CycE in GMC-1, or the asymmetric segregation of CycE between GMC-1 daughter cells, was 7% (n=336) in the strongest allele. This correlation indicates that the upregulation of CycE and the observed additional division of GMC-1 in *ago* mutants is not an artifact. The low penetrance can be due to one of several possibilities, such as the hypomorphic nature of the alleles, maternal deposition of *ago* message, or the existence of genetic redundancy for *ago* during embryonic development. Nonetheless, our results show that upregulation of CycE in late GMC-1 results in this GMC adopting a self-renewing asymmetric division pattern.

Asymmetric self-renewal of GMC-1 in $miti^P$ embryos is Insc and Numb independent

When the GMC-1 undergoes a stem cell type of asymmetric division in $miti^P$ embryos, it generates either an RP2 or a sib. Previous results show that Insc and Nb play a central role in the asymmetric division of GMC-1 into RP2 and sib (Buescher et al., 1998; Wai et al., 1999; Lear et al., 1999). Insc protein localizes asymmetrically to the apical end of GMC-1 and Nb to the basal end. The basally localized Nb then segregates into the future RP2 where it prevents Notch-signaling from specifying a sib fate. As a result, this cell becomes an RP2, whereas the other cell becomes a sib. In *insc* mutants the GMC-1 symmetrically divides to generate two RP2s (Fig. 8C,D), whereas in *nb* mutants the GMC-1 symmetrically divides to generate two sibs (Fig. 8H). We wished to determine (1) whether the self-renewal of GMC-1 in $miti^P$ embryos was *insc*

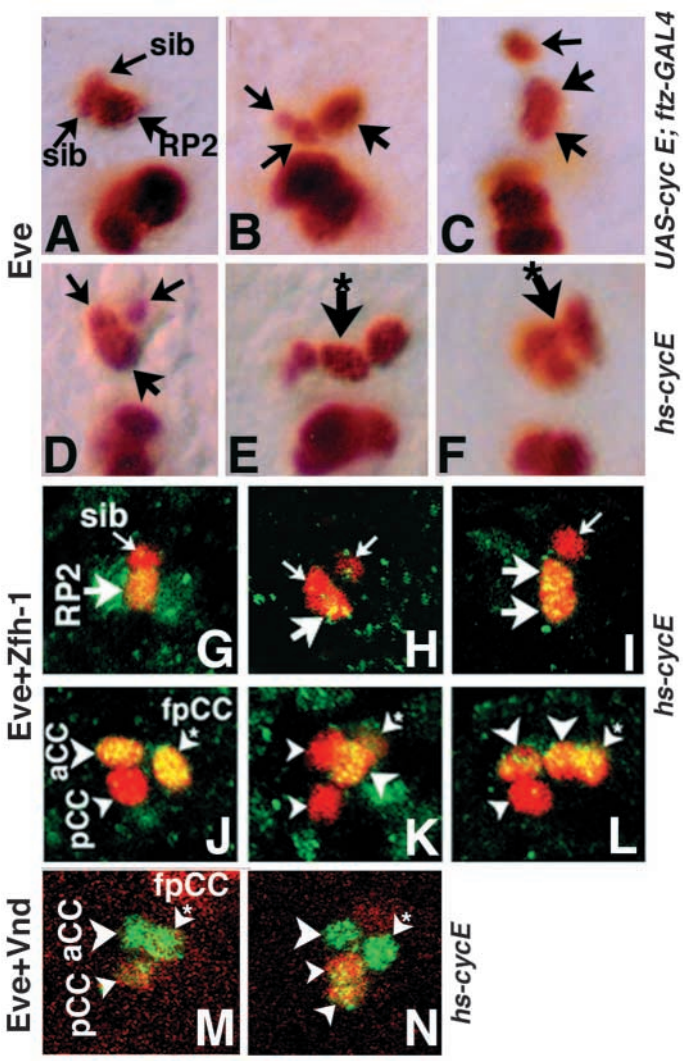


Fig. 6. The upregulation of CycE induces GMCs to undergo a self-renewing asymmetric division. Embryos in panels A-F are stained for Eve (red). Embryos in panels G-L are stained for Eve (red) and Zfh1 (green); co-localization of Eve and Zfh1 is indicated by yellow. Embryos in panels M and N are stained for Eve (green) and Vnd (red); yellow indicates co-localization, only the merged images are shown. Arrow indicates an RP2, small arrow indicates a sib, arrow with a star indicates a multi-cell cluster, arrowhead indicates an aCC, small arrowhead indicates a pCC, arrowhead with a star indicates friend of pCC (fpCC). (A-C) ~9-hour-old *UAS-CycE; ftz-GAL4* embryos showing the 3-cell phenotypes with two sibs and an RP2 (A,B) or two RP2s and a sib (C). (D) A ~9-hour-old *hs-CycE* embryo in which the transgene was induced for 25 minutes at the GMC-1 stage. Note the 3-cell phenotype (2 sibs and an RP2). (E,F) A ~10.5-hour-old *hs-CycE* embryo in which the transgene was induced for 90 minutes at the GMC-1 stage. (G) ~9-hour-old wild-type embryo. (H,I) ~9-hour-old *hs-CycE* embryos in which the embryos were heat shocked for 30 minutes at ~6.75 hours of age. The GMC-1 has self-renewed once to generate either a sib (H) or an RP2 (I), and then terminally divided to generate an RP2 and a sib. (J) Wild-type embryo, showing an aCC (Eve and Zfh1 positive), a pCC (only Eve positive) and a fpCC (Eve and Zfh1 positive). (K,L) ~9-hour-old *hs-CycE* embryos in which the embryos were heat shocked for 30 minutes at ~6 hours of age. The GMC-1 has self-renewed to generate either two pCCs and an aCC (K) or two aCCs and a pCC (L). fpCC is formed later than aCC/pCC, therefore this particular induction timing does not affect its division pattern; a later induction affects fpCC as well (data not shown). (M) Wild-type embryo, showing an aCC (only Eve-positive), a pCC (Eve and Vnd positive) and a fpCC (Eve-positive). (N) ~9-hour-old *hs-CycE* embryos in which the embryos were heat shocked for 30 minutes at ~6 hours of age. The GMC-1 has self-renewed to generate two pCCs and an aCC.

and *nb* dependent, and (2) whether the generation of RP2 versus sib during the stem cell division of GMC-1 is tied to *insc* and *nb*. Therefore, *miti*^{P}; *insc* embryos were generated and examined with several different lineage specific markers. The results indicate that the self-renewal of GMC-1 in *miti*^{P} is *insc*-independent; however, the identity of the progeny that is committed to differentiate from this self-renewing stem-cell division of GMC-1 is *insc*-dependent. Thus, in *miti*^{P}*; insc* embryos the GMC-1 still self-renewed several times, but each time it generated an RP2. In these embryos, only RP2s were observed, no sibs (Fig. 8E,F). Similarly, in *miti*^{P}*; nb* embryos each of the self-renewing asymmetric divisions produced a sib (Fig. 8I).

It has been reported that in embryos mutant for *partner of inscuteable* (*pins*; *raps* – FlyBase), the distribution of Insc is non-asymmetric, which in turn causes a non-asymmetric distribution of Nb, thus both the progeny of GMC-1 adopt an RP2 fate (Yu et al., 2000). To determine the mechanism by which the self-renewing GMC-1 in *miti*^{P} generates an RP2 or a sib, we examined *miti*^{P} embryos using an antibody against Insc. In 37% of the hemisegments (*n*=410), the distribution of Insc in GMC-1 was not asymmetric (Fig. 8K,L). When there is no asymmetric localization of Insc in GMC-1, the asymmetric localization of Nb is also affected, which leads to both daughter cells inheriting Nb and becoming RP2s. Thus, in those GMC-1 in which the localization of Insc is non-asymmetric, the differentiating cell from the self-renewing asymmetric division of GMC-1 will become an RP2 provided it also inherits less CycE (note that in 43% of cases a GMC-1 undergoing a self-renewing division in *miti*^{P} embryos generates an RP2). By contast, in those hemisegments where the Insc localization is asymmetric, localization of Nb must also be asymmetric, and, following division, the cell that does not inherit Nb and at the same time inherits less of CycE will adopt a sib fate. Thus, the combination of asymmetric or non-asymmetric distribution of Insc (and therefore Nb), and the unequal segregation of CycE, appear to determine which cell becomes a GMC-1, an RP2 or a sib.

We also find that the effect of upregulation of Miti on Insc localization is not restricted to GMC-1. This is indicated by the fact that Insc distribution is also non-asymmetric in several other GMCs (see Fig. 8L). However, overexpression of Miti (or Nub) in GMC1-1a, which gives rise to aCC/pCC neurons and is a Miti/Nub-negative GMC, has no effect on Insc localization (Fig. 8N), or on the division pattern of the GMC1-1a. This indicates that GMCs that do not normally express Miti are insensitive to its ectopic expression.

The division pattern of GMC-1→RP2/sib lineage is not affected in embryos mutant for Prospero

A previous study indicated that Prospero (Pros) acts as a repressor of cell-cycle genes and functions to restrict the number of divisions a GMC can undergo to one (Li and

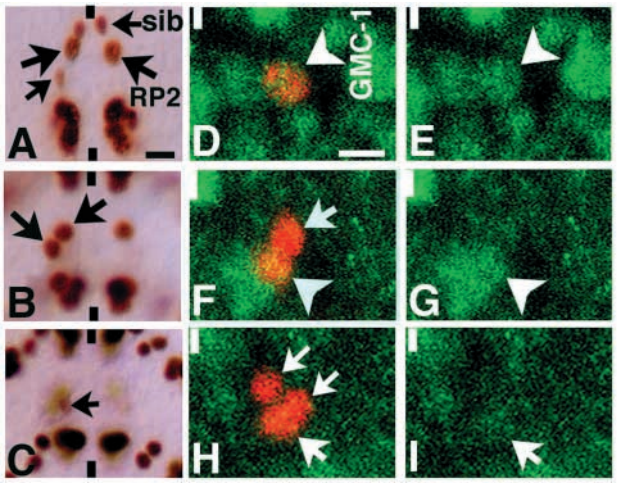


Fig. 7. Self-renewing asymmetric division in *ago* mutant embryos. (A-C) Embryos stained for Eve. (D-I) Merged images of embryos stained for Eve (red) and CycE (green). Arrow indicates an RP2, small arrow indicates a sib, arrowhead indicates a GMC-1. Vertical lines mark the midline. (A) An ~9.5-hour-old *ago* mutant embryo. The hemisegment on the left has the 3-cell phenotype (two sibs and one RP2). (B,C) An ~11-hour-old *ago* mutant embryo with the same segment shown in two different focal planes. The hemisegment on the left has the 3-cell phenotype, two RP2s (B) and one sib with fading Eve expression (C). (D,E) A late GMC-1 in an *ago* mutant embryo (~7.25 hours old) with high levels of CycE protein. (F,G) An ~7.75-hour-old *ago* mutant embryo in which the GMC-1 appears to have nearly completed its division to generate two cells of more or less equal sizes. One of these two cells, presumably the self-renewed GMC-1, has high levels of CycE and the other cell, presumably the RP2, has very low levels of CycE. (H,I) A hemisegment showing a 3-cell phenotype in an ~9-hour-old *ago* mutant embryo. All three cells have undetectable levels of CycE. Scale bars: ~10μm for A-C; ~7.5μm for D-I.

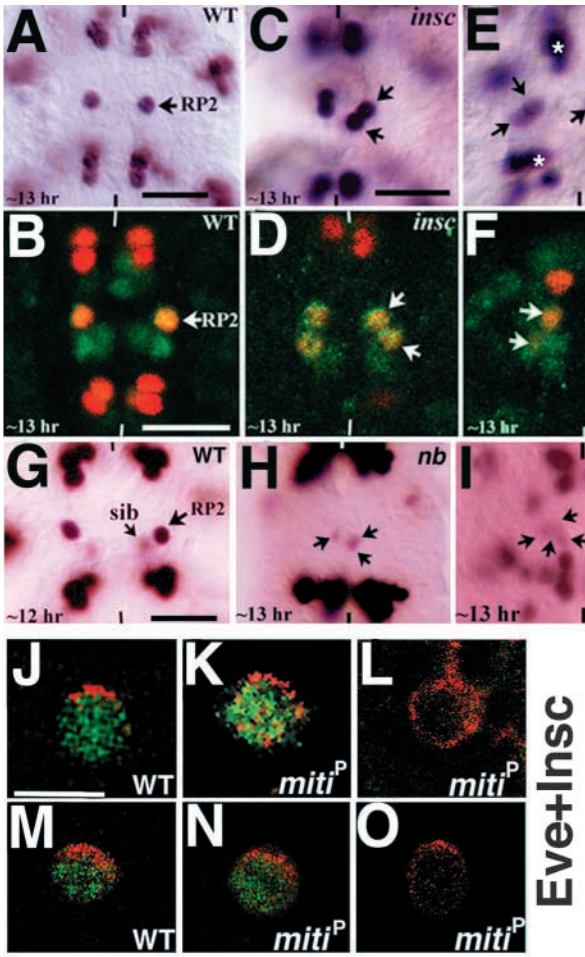
Vaessin, 2000). As Pros is present in GMC-1 of the RP2/sib lineage, we sought to determine (1) whether this GMC-1 undergoes multiple divisions, and (2) whether such divisions are self-renewing asymmetric divisions. If the answer to the above questions is in the affirmative, we can then start examining whether the levels of Pros is affected in GMC-1 of the embryos overexpressing *miti/nub* genes. Therefore, we examined *pros* mutant embryos for the expression of Eve. In these *pros* mutant embryos, we observed an Eve-positive RP2/sib lineage in ~5% of the hemisegments (n=336; we occasionally found *pros* mutant embryos that had a fully penetrant missing Eve-positive RP2/sib, U or CQ lineages). However, the division pattern of GMC-1 in those hemisegments was normal and no additional RP2 or sib cells were observed in *pros* mutant embryos (Fig. 9C,D,F,H). U and CQ lineages are also formed in ~20% and ~15% of the of the hemisegments, respectively (n=336), in *pros* mutant embryos, although there was often a reduction in the number of these neurons. In wild type, the number of Us is four, of which two are Eve and Zfh1 positive (Fig. 9G). In the *pros* mutant embryo where this lineage is formed, we observed usually one Eve- and Zfh1-positive U, and two Eve-positive Us (Fig. 9H). Similarly, the number of CQs observed in the mutant embryo is usually one (Fig. 9J), as opposed to three in wild type (Fig. 9I; only two are visible in this focal plane). These results show that no additional neurons are generated in these lineages in *pros* mutant embryos. If Pros had a role in restricting the number of divisions the GMCs can undergo in these lineages, these GMCs should have divided more than once in the *pros* mutant embryo. As we did not observe such divisions, Pros is unlikely to play a role in the self-renewing asymmetric division pathway described here for GMC-1 and GMC1-1a.

Discussion

Maintenance of a self-renewing fate can be viewed as a state where activities of certain genes maintain that state. Once the activity of such genes is switched off, the cells become committed to a differentiation pathway (see Bhat et al., 1988). Our results indeed support this type of mechanism. That POU genes might be a class of genes that maintain a self-renewing capacity is indicated by the fact that the *Oct4* POU gene (*Pou5f1* – Mouse Genome Informatics), which is expressed in pluripotent stem cells of the mouse early embryo, is turned off when these cells begin to differentiate (Rosner et al., 1990). Similarly, SCIP is expressed in the progenitors of oligodendrocytes, but it is downregulated when these cells are induced to differentiate (Collarini et al., 1992). Our results provide direct evidence that these genes can induce a cell that is committed to a differentiation pathway to acquire a self-renewing capability in a lineage specific manner. Moreover, studies undertaken in the past several years using the *Drosophila* nervous system as a paradigm have revealed how asymmetry can be generated during cell division to produce two distinct postmitotic cells (Rhyu et al., 1994; Spana and Doe, 1996; Knoblich et al., 1995; Hirata et al., 1995; Dye et al., 1998; Buescher et al., 1998; Wai et al., 1999; Schober et al., 1999; Wodarz et al., 1999; Yu et al., 2000; Mehta and Bhat, 2001). However, there is very little information on how an asymmetric self-renewing division pattern is determined. In this paper, we present results that provide insight into this particular process.

The GMC-1 has the potential to undergo a self-renewing asymmetric division

The strongest evidence that a GMC-1 undergoes a self-renewing type of asymmetric division in embryos overexpressing *miti/nub* or *CycE*, and in embryos mutant for *ago*, comes from the presence of hemisegments with two sibs and one RP2. There are two ways the second sib cell can be generated: (1) a self-renewed GMC-1 generates another sib when it divides; and (2) some other cell is transformed into a sib. The following set of evidence indicates the former scenario. First, the second sib cell always appears later in development, i.e. at ~8.5 hours of age (as opposed to in wild type where the GMC-1 terminally divides by ~7.5 hours of age into an RP2 and a sib). Second, the dynamics of Eve expression itself in the sib: expression of *eve* is switched off in a sib during the asymmetric division of GMC-1 and there is no de novo synthesis of Eve thereafter. If a postmitotic cell from an Eve-negative lineage transforms into a sib, it would be negative for Eve and would not be detected. The development of the other Eve-positive neuronal lineages is normal in these embryos, thus it is unlikely that a cell from those Eve-positive lineages is transformed into a sib. Third, the Eve and Spectrin staining of *UAS-nub; ftz-GAL4* embryos provides more direct evidence



for the self-renewal of GMC-1. In ~8. 5-hour-old *UAS-nub; ftz GAL4* embryos, we can observe the larger GMC-1 (this Eve-positive cell is Zfh1 negative, indicating that it is indeed a GMC-1) undergoing asymmetric cytokinesis for the second time. From the heat-shock induction experiments of *nub* or *miti* mutant embryos, it can be argued that higher levels of these proteins in the parental NB4-2 cause later born GMCs to adopt a GMC-1 fate. However, the GMC-1 self-renewing phenotype observed following targeted expression of *nub* using the *ftz-GAL* driver makes this scenario unlikely. Fourth, the results obtained with the *miti*^{P}*; insc* and *miti*^{P}*; nb* double mutant embryos, and

Fig. 8. The cell fate of the differentiating progeny of a GMC-1 self-renewing asymmetric division is Insc and Nb dependent. (B,D,F) Eve is red, Zfh1 is green; (J-O) Eve is green and Insc is red. Anterior end is up, vertical lines mark the midline. In wild type, there is only one Eve- (A) and Eve- and Zfh1 (B)-positive RP2 per hemisegment, whereas in *insc* mutants, the RP2 is duplicated, both being Eve- (B) and Zfh1 (D)-positive. (E,F) In *miti*^{P}*; insc* embryos, the GMC-1 generates an RP2 in each of its self-renewing divisions (the right hemisegments). (G) Wild-type embryo with an RP2 and a sib. (H) *nb* mutant. The GMC-1 has symmetrically divided to generate two sibs. (I) *miti*^{P}*; nb* embryo. In the right hemisegment the sib cells have lost Eve expression (arrowhead), whereas in the left hemisegment several sib cells can be still seen. (J-O) Localization of Insc in GMC-1 is affected in *miti*^{P} embryos. Only the merged images are shown. Insc is asymmetrically distributed in wild-type GMC-1 (J), but its distribution is non-asymmetric in *miti*^{P} embryos (K). Localization of Insc is also affected in several other unidentified GMCs (e.g. a row 4 GMC; L). Localization of Insc is not affected in GMC1-1a (N; a wild type GMC1-1a is shown in M), or several other unidentified Miti/Nub-negative GMCs (O). Scale bars: ~15 μm for A-I; ~10 μm for J-O.

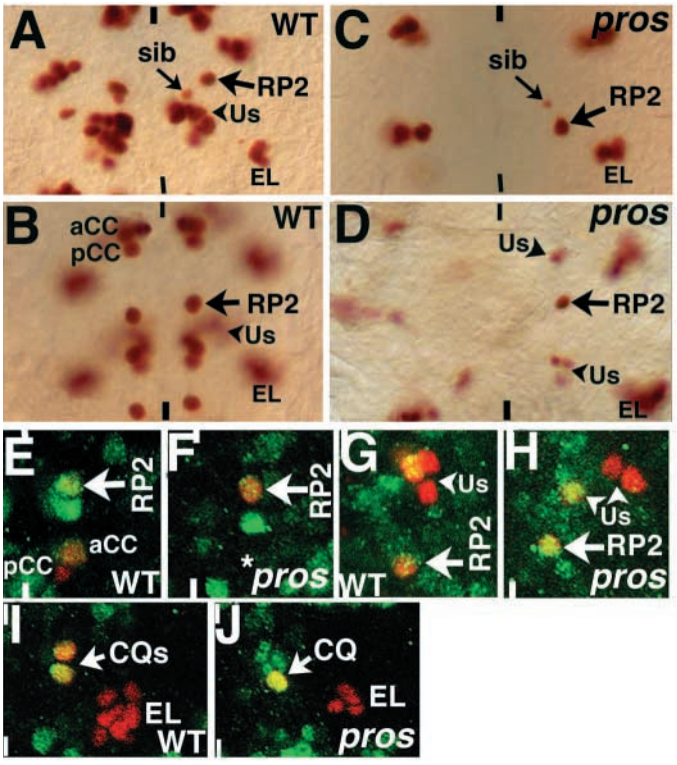


Fig. 9. GMCs for the RP2/sib, U, CQ and EL neuronal lineages do not undergo additional rounds of cell division in *prospero* mutants. Embryos are single stained for Eve (A-D) and double stained for Eve (red) and Zfh1 (green) (E-J; only one half-segment is shown). Midline is marked by vertical lines. Embryos mutant for *pros* have holes in the midline and a mis-routed axon scaffold, thus the neuronal lineages are farther apart. The loss of RP2, U and CQ lineages is partially penetrant in this mutant allele (see text for details). (A) ~10-hour-old wild-type embryo. The number of U neurons is usually four in wild type, CQs are hidden beneath the U cluster, but are visible in the hemisegment on the left. (B) ~13-hour-old wild-type embryo. Us and CQs are out of the focal plane. ELs are also slightly out of the focal plane. (C) ~10-hour-old *pros* mutant embryo. An RP2 and a sib are shown. aCC/pCC, Us and CQs are missing in this segment. (D) ~13-hour-old *pros* mutant embryo. An RP2 is flanked by two Us on top and three Us on the bottom. (E) ~13-hour-old wild type, note that RP2 and aCC are Eve and Zfh1 positive. (F) ~13-hour-old *pros* mutant. Both aCC and pCC are missing (marked by a star). (G) ~13-hour-old wild-type embryo. Out of four Us, two are Eve and Zfh1 positive. The other two are only Eve positive. (H) ~13-hour-old *pros* mutant embryo. There are only three U neurons, one of the two Eve- and Zfh1-positive Us is missing. (I) ~13-hour-old wild-type embryo. In wild type there are three CQs, all are Eve and Zfh1 positive (one of the CQs in this panel is out of the focal plane). The number of ELs varies between eight and ten in the abdominal segments. (J) ~13-hour-old *pros* mutant embryo. There is only one CQ in this hemisegment. The number of ELs is usually the same as in wild type (some of the ELs are out of the focal plane in this panel).

the mis-localization of Insc in GMC-1 of these embryos, are also consistent with this conclusion.

The level, timing and duration of presence of Miti or Nub proteins determine the kinetics of GMC-1 self-renewal

Our results indicate that the level, timing and duration of presence of Miti or Nub proteins determine the dynamics of the GMC-1 division pattern. For example, the asymmetric divisions (which generate the 3-cell phenotypes) and the symmetric divisions (which generate the 4-cell phenotype) were observed when the transgenes were induced for 20-25 minutes. However, the multiple cell-phenotype was observed only when the transgenes were induced for 90 minutes. Once the induction was stopped and the levels returned to normal, the two GMC-1s appeared to exit from the cell cycle to generate postmitotic cells. Similarly, when the transgene was induced with *ftz-GAL4*, only the 3-cell phenotypes, and not the 4-cell or multi-cell phenotypes were observed. Thus, the following picture emerges from these results. Although high levels of Miti and Nub proteins are required for the specification of GMC-1 identity, their level must be downregulated in order for the GMC-1 to divide asymmetrically into postmitotic RP2 and sib. Maintaining a high level of these proteins in GMC-1 commits that cell to adopt a self-renewing stem cell type of division pattern. The results described here also show that Miti and Nub prevent GMC-1 from exiting the cell cycle by upregulation of CycE (see below).

The self-renewal of GMC-1 in embryos expressing high levels of Miti or Nub is due to elevated levels of Cyclin E

Our results clearly show that upregulation of CycE in late GMC-1 is the cause for the adoption of a self-renewing asymmetric division pattern. In other words, presence of high levels of CycE in late GMC-1 and its unequal distribution to one of the two daughter cells prevents this cell from exiting the cell cycle. As this daughter cell still maintains the GMC-1 identity and has sufficient CycE to divide again, a further asymmetric division(s) is ensured. The cell that has lower amounts of CycE becomes committed to a differentiation pathway (RP2 or sib).

What lines of evidence support this conclusion? First, in contrast with wild type, there is a significant amount of CycE present in a late GMC-1 in embryos overexpressing *miti* or *nub*. This CycE preferentially segregates to one of the two daughters of that GMC-1, usually the larger cell. When *miti* or *nub* genes are overexpressed only briefly, the level of CycE is downregulated after just one additional round of division, whereas with prolonged induction, the level is maintained at high levels in one or two cells of the multi-cell cluster for a prolonged duration of time.

Second, upregulation of CycE in a late GMC-1 is also observed in embryos mutant for *ago*, which is known to regulate CycE levels (Moberg et al., 2001). In *ago* mutants, the two daughter cells of such a GMC-1 have unequal CycE levels accompanied by a self-renewing asymmetric division phenotype. The CycE is always downregulated after one additional GMC-1 division, which is consistent with the finding that the self-renewal occurs only once in these embryos. As penetrance in *ago* mutants is partial, and CycE is downregulated in this lineage after just one additional division, there must be additional factors that mediate the downregulation of CycE in this lineage.

Third, embryos expressing high levels of CycE from a *CycE* transgene exhibit the same GMC-1 phenotypes as embryos expressing high levels of Miti or Nub. Thus, these results indicate that upregulation of CycE alone is sufficient for the GMC-1 to adopt a self-renewing type of division pattern. Finally, we find that $miti^{P}$ phenotypes are dependent on *CycE* (data not shown). That is, we did not observe multi-cell clusters in $miti^{P}$*; CycE* double mutant embryos.

In wild type, the downregulation of CycE in GMCs appears to occur through switching off *CycE* transcription and degradation of the protein by factors such as Ago. At what level does Miti or Nub regulate CycE? As these POU genes are thought to be transcriptional activators, they can regulate transcription of *CycE* either directly or indirectly. However, this does not seem to be the case as expressing high levels of *miti* did not have a discernible effect on the levels of *CycE* mRNA in GMC-1, as assessed by whole-mount RNA in situ hybridization (data not shown). In addition, the putative promoter/enhancer region of *CycE* gene does not contain any consensus POU protein-binding sites. Therefore, it seems likely that Miti and Nub regulate factors that are involved in the degradation of CycE in late GMC-1.

The question arises as to how only one cell has a high level of CycE. There are several ways this can happen. There might be an asymmetric degradation of CycE. This scenario seems unlikely as there is only one of two daughter cells with high levels of CycE in *ago* mutants. Given that Ago downregulates CycE via a protein degradation mechanism (Moberg et al., 2001), if there was an asymmetric degradation, in those hemisegments where the levels of CycE was elevated in GMC-1, we would initially expect both the daughter cells to have high CycE levels. However, this was not the case. An asymmetric transcription of the *CycE* gene also seems unlikely as the transcription of *CycE* ceases prior to GMC-1 division, as judged by whole-mount RNA in situ hybridization (data not shown). The most likely possibility is that CycE is unequally distributed between the two daughter cells of GMC-1. The unequal distribution of CycE could be a passive process due to the size difference between daughter cells, especially in the GMC-1→RP2/sib lineage. Moreover, we did not observe a cytoplasmic crescent of CycE during mitosis. By contrast, it could also be an active process. For instance, the size difference between an aCC and a pCC (or between a GMC1-1a and an aCC) is very small, and the fact that GMC1-1a undergoes a self-renewing asymmetric division suggests that the segregation of CycE may not be entirely a passive process.

CycE can induce self-renewing asymmetric divisions in other GMCs

Finally, our results indicate that while a GMC that does not normally express Miti or Nub is insensitive to its ectopic expression (e.g. GMC1-1a of NB1-1; this GMC produces an aCC/pCC pair of neurons), a brief induction of *CycE* in the same GMC causes it to undergo self-renewing asymmetric division. Therefore, CycE can confer a stem cell type of division potential to more than one GMC. Another important conclusion one can draw from this result is that the segregation

of CycE may be an active process. In the case of GMC1→RP2/sib lineage, the cytokinesis of GMC-1 is asymmetric, and the size difference between an RP2 and a sib is significant. Thus, CycE can be asymmetrically segregated because of this size difference. However, the size difference between an aCC and a pCC (or between a GMC1-1a and an aCC) is very small, and the fact that GMC1-1a undergoes a self-renewing asymmetric division suggests that the segregation of CycE may not be entirely a passive process. It is possible that the difference between the levels of CycE needed to retain a cell within the cell cycle and the levels that do not support maintaining the cell within the cell cycle is quite small. Thus, even a minor change in the amount that a cell receives during division might be sufficient to make a difference. Thus, the segregation of CycE can still be a passive process. Nonetheless, these results reveal how a cell can adopt a self-renewing asymmetric division potential through CycE.

A previous study implicated Pros in inhibiting the ability of GMCs to divide more than once by preventing continued expression of cell-cycle genes (Li and Vaessin, 2000). The caveat of this study, however, is that none of the GMC lineage was examined using cell-specific markers to determine whether GMCs continue to divide in embryos mutant for *pros*. The conclusion that Pros inhibits GMC division was mainly based on the presence of additional BrdU-positive cells in late stage (post 15-hours-old) *pros* mutant embryos. Pros is expressed in GMC-1 of the RP2/sib lineage and, in null alleles, this GMC-1 identity is not specified (Doe et al., 1991) We found that in *pros*^{17}, a loss-of-function allele, ~5% of the hemisegments had an RP2/sib lineage specified. In these hemisegments, the GMC-1 divides only once to generate an RP2 and a sib cell as in wild type (Fig. 9). Moreover, we also observed specification of U and CQ lineages in ~20% and ~13% of the hemisegments, respectively, and no additional cell division appeared to occur in these lineages. A previous study found that the aCC/pCC neurons (from GMC1-1a) have an abnormal axon morphology, but it did not find any additional neurons in this lineage (Doe et al., 1991). Similarly, NB6-4 of the thoracic segment produced the normal number of progeny in *pros* mutant embryos (Akiyama-Oda et al., 2000). These results suggest that Pros does not regulate cell division in RP2/sib, U and CQ lineages, and possibly not in many other neuronal lineages, and therefore it is unlikely to function in the *miti/nub* pathway.

Relationship between elevated levels of Miti/Nub and localization of Insc

How is the specification of identity of one of the two progeny, either as an RP2 or as a sib, from a self-renewing asymmetric division of GMC-1 regulated? (Specification of the other progeny as GMC-1 is by high levels of CycE.) Our results indicate that specification of an RP2 versus a sib identity to this differentiating cell is through a combination of low levels of CycE and localization of Insc. This is indicated by the finding that overexpression of Miti and Nub causes localization of Insc to be non-asymmetric. Non-asymmetric Insc also causes non-asymmetric localization of Nb. The cell that has lower levels of CycE and also has Nb becomes an RP2. Whenever the cell with lower levels of CycE fails to inherit Nb (the effect of overexpression of Miti or Nub on the localization of Insc is partially penetrant) that cell will become a sib. That the generation of an RP2 during the asymmetric division of GMC-1 is tied to Nb is also indicated by the analysis of *miti*^{P}*; nb* embryos. Although the self-renewal of GMC-1 in *miti*^{P} embryos is *nb*-independent, the commitment of a progeny to become a sib is *nb*-dependent. Thus, in ~13-hour-old *miti*^{P}*; nb* embryos, we observed multiple cells adopting a sib fate. An often overlooked fact is that in *insc* mutants the GMC-1 division is normal in ~30% of the hemisegments (*n*=280) despite having no *insc*. Similarly, the penetrance of the symmetrical division of GMC-1 in *pins* (where Insc localization is affected as in *miti*^{P} embryos) is also partial, indicating the presence of additional (partially redundant) pathways for Insc that mediate asymmetric fate specification. These very same additional pathways must also influence the choice between a sib and an RP2 when the GMC-1 in *miti*^{P} embryos undergoes a self-renewing type of asymmetric division.

CycE and Ago are part of a mechanism that converts a normal cell into a cancer cell. In *ago* mutants, CycE protein is not degraded and a number of cancer cell lines carry a mutation in *ago* (Moberg et al., 2001). Our results showing that these genes are also involved in a stem cell type of division suggests a commonality between stem cells and cancer cells. Our results also provide a molecular mechanism of how self-renewing asymmetric divisions are possible.

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