

# The germ line regulates somatic cyst cell proliferation and fate during *Drosophila* spermatogenesis

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## SUMMARY

Spermatogenesis relies on the function of germ-line stem cells, as a continuous supply of differentiated spermatids is produced throughout life. In *Drosophila*, there must also be somatic stem cells that produce the cyst cells that accompany germ cells throughout spermatogenesis. By lineage tracing, we demonstrate the existence of such somatic stem cells and confirm that of germ-line stem cells. The somatic stem cells likely correspond to the ultrastructurally described cyst progenitor cells. The stem cells for both the germ-line and cyst lineage are anchored around the hub of non-dividing somatic cells located at the testis tip. We then address whether germ cells regulate the behavior of somatic hub cells, cyst progenitors and their

daughter cyst cells by analyzing cell proliferation and fate in testes in which the germ line has been genetically ablated. Daughter cyst cells, which normally withdraw from the cell cycle, continue to proliferate in the absence of germ cells. In addition, cells from the cyst lineage switch to the hub cell fate. Male-sterile alleles of *chickadee* and *diaphanous*, which are deficient in germ cells, exhibit similar cyst cell phenotypes. We conclude that signaling from germ cells regulates the proliferation and fate of cells in the somatic cyst lineage.

Key words: spermatogenesis, stem cell, germ line–soma interaction, *Drosophila*, cell proliferation

## INTRODUCTION

During the early steps of gametogenesis, germ-line stem cells divide to generate daughter cells, which undergo a limited number of mitotic divisions before entering the meiotic cell cycle. Throughout these steps, germ cells are in contact with specialized somatic cells of the testis or the ovary (reviewed by Bellvé, 1979; Kimble and Ward, 1988; Fuller, 1993; Spradling, 1993). These somatic gonadal cells also differentiate during gametogenesis, as revealed by stage-dependent changes in the expression of molecular markers (Linder et al., 1991; Gönczy et al., 1992). The close association of somatic and germ cells suggests that signaling between the two lineages regulates progression through the early steps of gametogenesis.

Somatic gonadal cells are indeed known to regulate the behavior of early germ cells in several instances. In *C. elegans*, the somatic distal tip cell is necessary for the proliferation of neighboring germ cells (Kimble and White, 1981). The *lag-2* gene encodes the signal produced by the distal tip cell, while *glp-1* encodes the receptor on germ cells (Austin and Kimble, 1989; Yochem et al., 1989; Henderson et al., 1994; Tax et al., 1994). The mechanisms of soma to germ-line signaling during early gametogenesis are not as well characterized in other systems. For instance, specific somatic cells regulate the division rate of germ-line stem cells during *Drosophila* oogenesis, but the signal has not been identified in this case (Lin and Spradling, 1993). In mammals, although Sertoli cells

increase the viability of male germ cells in culture (Tres and Kierszenbaum, 1983), it is not known what molecules mediate this effect, nor whether this reflects an in vivo Sertoli cell function.

Even less is known concerning reciprocal signaling from germ cells to somatic gonadal cells during early gametogenesis. We chose to investigate this question during *Drosophila* spermatogenesis, where early stage germ cells are in contact with two somatic cell populations (Hardy et al., 1979; reviewed by Lindsley and Tokuyasu, 1980). The first is a group of non-dividing cells, which form a hub at the testis tip, around which the presumptive germ-line stem cells are anchored. The second somatic population comprises cyst cells, which surround differentiating germ cells throughout spermatogenesis. Cyst cells are thought to be the daughters of cyst progenitor cells, which are intermingled with the presumptive germ-line stem cells around the hub. Stage-specific enhancer trap lines reveal that early cyst cells progress through discrete differentiative steps as germ cells mature (Gönczy et al., 1992).

Here we verify by lineage tracing the existence and location of stem cells for both the germ-line and the somatic cyst lineage. We then genetically ablate the germ line to test whether signaling from germ cells regulates the behavior of hub cells, cyst progenitors and their daughter cyst cells. It had been established that somatic cells still organize into a gonad following the ablation of germ-line precursors in the embryo and that the resulting 'agametic' testes contain all morphologically recognizable somatic gonadal cells, including those of

the hub and cyst lineage (Geigy, 1931; Aboïm, 1945). However, these studies did not address the behavior of these somatic cells during early spermatogenesis in the absence of a germ line. We show that, in agametic testes, daughter cyst cells ectopically proliferate and cells from the cyst lineage switch to the hub cell fate. We conclude that germ cells normally signal to regulate the proliferation and fate of somatic cyst cells during early spermatogenesis.

## MATERIALS AND METHODS

### Lineage tracing

Marked clones were generated by a technique where recombination through FRT sites joins an  $\alpha$ -tubulin promoter to *lacZ* (Harrison and Perrimon, 1993). We utilized insertion lines located on 2L (D. Harrison, personal communication). Recombination was catalyzed by an hsp-70 FLP recombinase (FLP99; (Golic, 1991; Chou and Perrimon, 1992), whose expression was induced at 38°C for 60 minutes. Testes were fixed and stained for  $\beta$ -galactosidase activity (Gönczy et al., 1992).

### Agametic testes carrying P-*lacZ*

*oskar* (*osk*) is required during oogenesis for embryonic posterior development and pole cell formation; at 18°C, the temperature-sensitive allele *osk*<sup>301</sup> is permissive for posterior development but restrictive for pole cell formation (Lehmann and Nüsslein-Volhard, 1986). *osk*<sup>301</sup> virgin females were collected, aged at 18°C for 4 days and crossed to males from autosomal enhancer trap lines marking specific somatic gonadal cells (Gönczy et al., 1992; Gönczy, 1995). After 4 days, the bottles were shifted to 25°C. The F<sub>1</sub> males had agametic testes carrying one copy of a P-*lacZ* marker, whose expression pattern in early spermatogenesis is given in the appropriate figure legend. Occasionally, an F<sub>1</sub> male had a normally developed testis, indicating that pole cells can sometimes survive in the progeny of *osk*<sup>301</sup> mothers. Indeed, 2-3% of embryos derived from *osk*<sup>301</sup> mothers had at least one pole cell in a gonad, as judged by anti-vasa staining. The exceptional adults were disregarded.

### Counting hub cells

We counted  $\beta$ -galactosidase-positive cells in the hub of wild-type testes carrying the marker S2-11 (see Fig. 7A). For agametic testes, hub cell number was determined using the hub marker M5-4, or S2-11 plus M5-4 (see Fig. 4A). In the latter case, we considered only agametic testes in which the  $\beta$ -galactosidase-positive hub cells could be clearly distinguished from the daughter cyst cell aspect of S2-11 expression. Cell number was rounded to the nearest ten in cases with more than 30 hub cells.

### Immunolocalisation

Testes were dissected in Ringer's, fixed for 40 minutes in 4% formaldehyde-PBX (PBS plus 0.1% Triton X-100), rinsed several times in PBX and blocked overnight at 4°C in PBX-2 (PBX plus 2% Normal Goat Serum). Primary and secondary antibody incubations were for 90 minutes at room temperature (RT), with washes in PBX. Rabbit anti- $\beta$ -galactosidase (Cappel) was used at 1:500; mouse anti-BrdU (Becton Dickinson) at 1:10; anti-Fasciclin III monoclonal 7G10 (Patel et al., 1987) at 1:30. Rhodamine- and fluorescein-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Jackson Laboratories), as well as biotin-conjugated goat anti-rabbit and horse anti-mouse secondary antibodies (Vector) were used at 1:400 after preadsorbing at their final dilution for 2 hours with an equal volume of fixed embryos. Testes were counterstained for 2 minutes in 1  $\mu$ g/ml Hoechst 33258. Immunofluorescent and immunocytochemical detection were carried out as described (Gönczy et al., 1992; Gönczy, 1995).

### BrdU labeling in vitro

Testes were dissected in Ringer's, transferred to a siliconized Eppendorf within 10 minutes of dissection and incubated for 30 minutes at 25°C with 10 mM BrdU (Boehringer Mannheim) in Ringer's. Testes were rinsed twice with Ringer's, fixed for 60 minutes in 4% formaldehyde-PBX, rinsed several times in PBX and blocked overnight at 4°C in PBX-2. Testes were then incubated for 90 minutes at 25°C with anti-BrdU in 66 mM Tris pH 8, 2.66 mM MgCl<sub>2</sub>, 1mM  $\beta$ -mercaptoethanol and 50 units/ml DNase I (Boehringer Mannheim), to promote access of anti-BrdU to DNA. DNase I treatment preserved both morphology and other antigens better than the classical HCl treatment (Schubiger and Palka, 1987). The rest of the procedure was as for other antibodies.

### BrdU pulse-chase in vivo

Males collected on the day of eclosion (day 1) were starved for 24 hours, briefly anaesthetized and transferred to an Eppendorf tube held horizontally. 100  $\mu$ l of 100 mM BrdU in grape juice was pipeted in the tube. Flies recovered and were allowed to feed for 10 minutes; those with grape juice in their gut were selected and aged appropriately before processing. Despite such selection, occasional flies did not incorporate BrdU into any cell within the testis or genital tract and were not analyzed further.

To estimate the biological half-life of BrdU, we used wild-type flies to compare a pulse followed by a 2-day chase with a 30 minute labeling in vitro. After a 2-day chase, germ cells that underwent premeiotic S phase during the BrdU pulse are easily recognized as packets of 16 strongly labeled adjacent cells, now located in the growth phase region. We found an average of 1.4 premeiotic S phase packet labeled per testis in such a pulse-chase ( $n=14$ ). This was about twofold higher than the average number of premeiotic S phase packets labeled during a 30 minute in vitro labeling (0.8;  $n=30$ ). Thus, BrdU must be available for about twice as long (60 minutes) in the pulse-chase experiments.

## RESULTS

### Germ-line and somatic stem cells are located around the hub

We first wanted to establish the location of the progenitor cells for the germ-line and somatic lineages. Spermatogenesis must rely on the activity of germ-line stem cells, as a continuous supply of differentiated spermatids is produced throughout life. In *Drosophila*, the existence of germ-line stem cells has been proven through the analysis of brood patterns following irradiation (Hartl and Green, 1970), but their location in the testis has not been directly established. It has been suggested that five to nine large cells (Fig. 1A,B, arrow) which are anchored around a hub of non-dividing somatic cells at the testis tip (Fig. 1A,B, large arrowhead) are these germ-line stem cells (Hardy et al., 1979).

Since germ cells are accompanied throughout spermatogenesis by two somatic cyst cells, there must also be stem cells for the somatic cyst lineage, to generate a continuous supply of daughter cyst cells. Although the existence of such stem cells has not been proven, ultrastructural work has revealed pairs of elongated cells that contact the hub and flank each presumptive germ-line stem cell (Fig. 1A,B, arrowhead). It has been suggested that these cells act as somatic stem cells and thus are called cyst progenitor cells (Hardy et al., 1979).

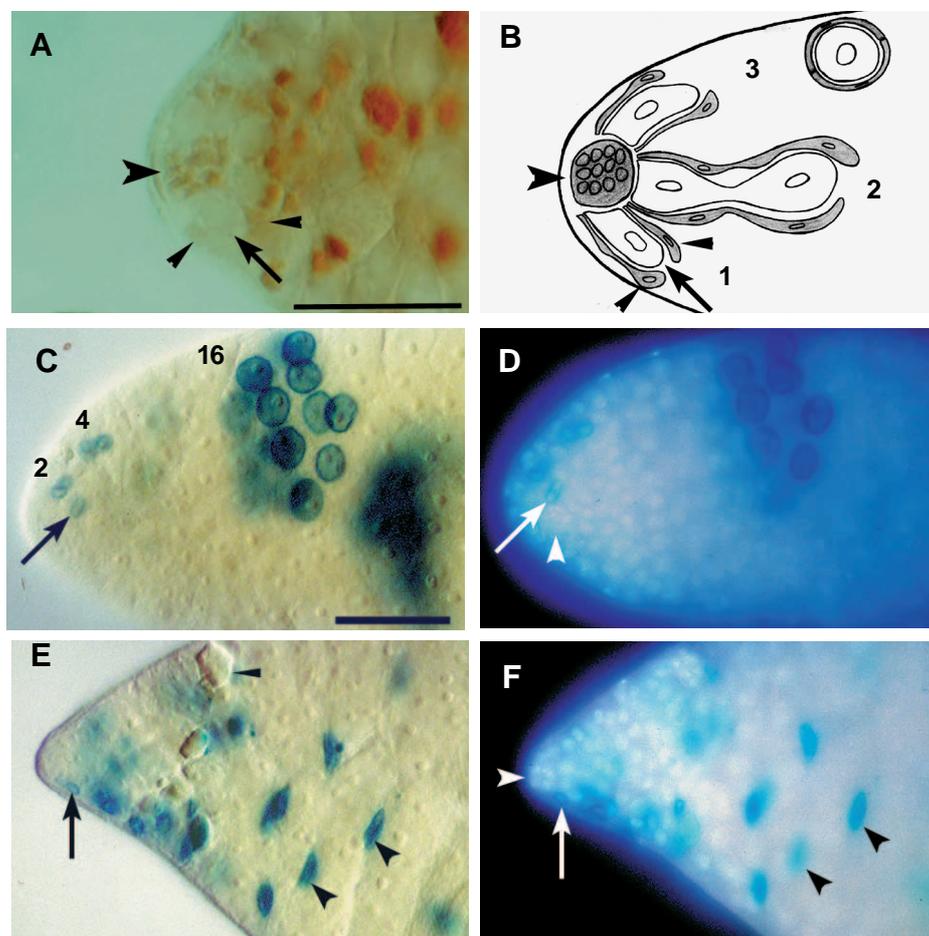
It is thought that one germ-line stem cell and two neighboring cyst progenitors divide in concert (Fig. 1B). One daughter of each of these cells remains in contact with the hub and is

believed to retain parental stem cell fate. The other daughter of each of these cells associates into a cyst, with the two daughter cyst cells surrounding the single daughter germ cell. As the cyst matures and moves away from the hub, the daughter germ cell undergoes four amplificatory mitotic divisions before entry into the meiotic cell cycle. In contrast, the two daughter cyst cells never divide.

We verified the existence and location of germ-line and somatic stem cells by lineage tracing. We used a method in which transient induction of FLP recombinase joins a ubiquitously expressed promoter to *lacZ* coding sequences by recombination through FRT sites (Harrison and Perrimon, 1993; Material and Methods). In this manner, *lacZ* expression permanently marks cells dividing at the time of FLP induction, as well as their subsequent progeny. During spermatogenesis, most cells labeled by this technique are mitotically amplifying germ cells, located in the proliferation region, towards the testis tip (data not shown). Such marked germ cells should mature and move away from the testis tip within 2 days (Lindsley and Tokuyasu, 1980). Therefore, marked cells persisting in the proliferation region significantly after this time must correspond to stem cells and their progeny (see also Margolis and Spradling, 1995). Males were thus aged for 5 days following FLP induction and their testes analyzed by X-gal staining for persistent clones in the proliferation region. In a typical experiment, 84 of 164 testes had a persistent clone of marked germ cells (Fig. 1C), while 22 had a persistent clone of marked cyst cells (Fig. 1E). In both cases, one marked cell (Fig. 1C-F, arrow) was usually identifiable immediately adjacent to the hub, which was visible by DNA counterstaining (Fig. 1D,F, arrowhead). Occasionally, no marked cell was detected at the tip of testes with persistent germ-line or cyst cell clones (data not shown). In these cases, a formerly functional stem cell must have been lost from the testis tip over the course of the 5 days. A similar situation has been reported in *Drosophila* ovaries (Margolis and Spradling, 1995).

The marked cell next to the hub in persistent germ-cell clones must correspond to the stem cell, because other marked cells in the proliferation region

were in packets of 2, 4, 8 or 16 (Fig. 1C), as expected from daughter germ cells undergoing successive rounds of mitotic amplification at increasing distances from the hub. Therefore, germ cells anchored around the hub indeed act as stem cells.



**Fig. 1.** Germ-line and somatic stem cells are located around the hub. Proliferation region of testis. (A) Line 842 stains hub cells (large arrowhead), cyst progenitors (two indicated by thin arrowheads; these stain lightly and are partially out of focus) and early daughter cyst cells (stronger staining toward right). Large unstained cells around the hub are presumptive germ-line stem cells (one indicated by arrow). (B) Schematic illustration of one germ-line stem cell and two neighboring cyst progenitor cells at three successive moments of the division cycle (adapted from Hardy et al., 1979). (1) The germ-line stem cell (arrow) contacts hub cells (large arrowhead), as do flanking cyst progenitors (thin arrowheads). (2) The three cells are thought to divide in concert, although this has not been demonstrated experimentally. (3) The three daughter cells remaining in contact with hub cells retain stem cell fate, while the other three daughters associate into a cyst (upper right), with the two daughter cyst cells surrounding the single daughter germ cell. (C-F) Lineage tracing. (C,E) X-gal staining, Nomarski. (D,F) Combination bright-field / epifluorescence to visualize both X-gal staining and DNA counterstain. (C,D) Persistent germ-line clone. The only individually marked cell (C,D, arrow) must be the germ-line stem cell; this marked cell is adjacent to the hub, a 'rosette' of cells visualized by Hoechst (D, arrowhead). The stem cell is dividing. Three progressively more mature packets of germ cells are also marked: a 2-cell packet, which corresponds to a daughter germ cell after one division; a 4-cell packet, which corresponds to a daughter germ cell after two divisions (2 cells are out of focus); and a 16-cell packet, which corresponds to a daughter germ cell after four divisions (7 cells are out of focus). No 8-cell packet was present in this testis. A more mature 16-cell packet is out of focus toward the right. (E,F) Persistent cyst cell clone. The most apical marked cell (E,F, arrow) is adjacent to the hub (F, arrowhead) and thus corresponds to a cyst progenitor cell, which is the likely stem cell for the cyst lineage. Cyst cell nuclei (two are indicated by large arrowheads) are visible at the edge of more mature packets of germ cells. Some packets of germ cells normally die instead of differentiating (Tates, 1971). One such packet is indicated (E, thin arrowhead). Bar, 50  $\mu$ m; C-F at same magnification.

Our results also unequivocally establish the existence of somatic stem cells that generate the cyst cell lineage. The marked cell next to the hub in persistent cyst cell clones corresponds to the cyst progenitor cell, suggesting strongly that it functions as the stem cell for this lineage. However, we can not formally exclude the possibility that another marked cyst cell in the vicinity of the hub is the cyst lineage stem cell. In contrast to daughter germ cells, daughter cyst cells do not proliferate. Therefore, while daughter germ cells are easily identifiable as packets of 2, 4, 8 or 16 marked cells, daughter cyst cells remain singly marked cells, just like their parental stem cells, from which they can not be unambiguously distinguished.

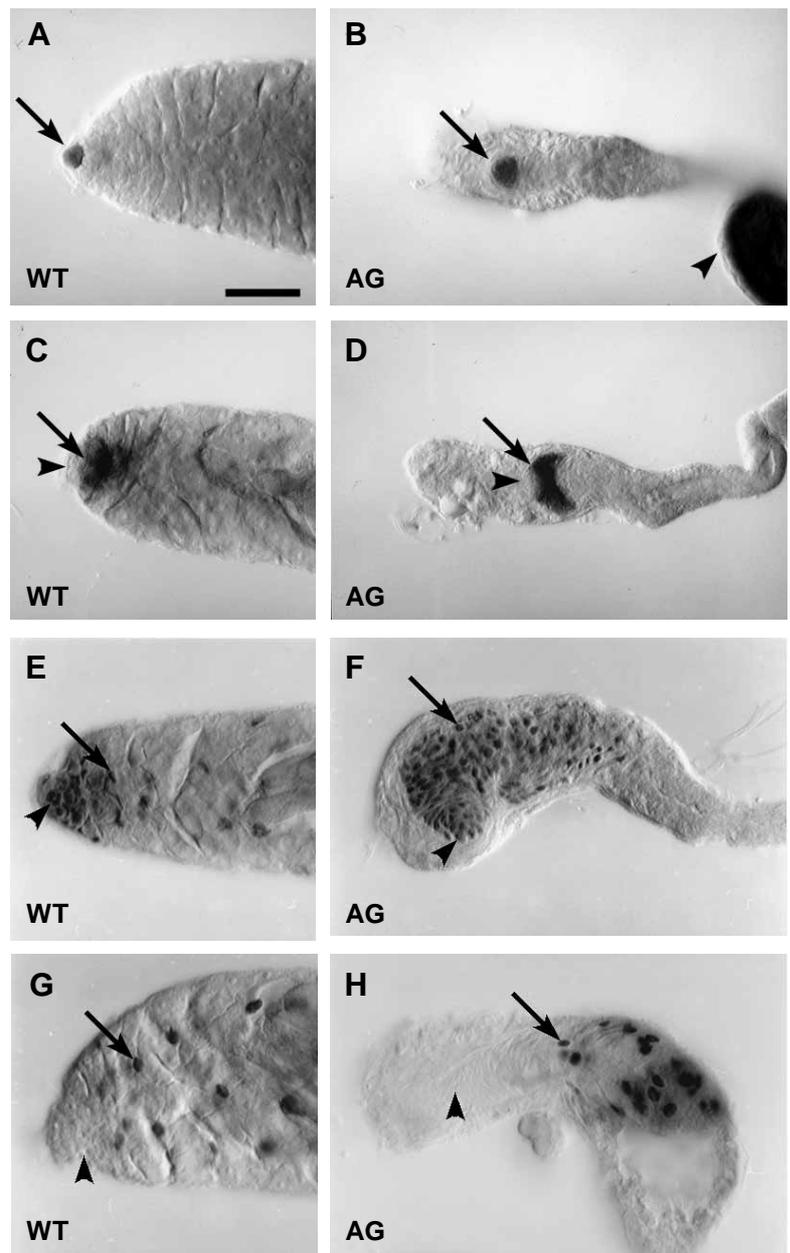
### Somatic cell organization in agametic testes

We next addressed whether signals from germ cells regulate the behavior of somatic hub cells, cyst progenitor and daughter cyst cells during the early steps of spermatogenesis. We analyzed testes of adult males that developed in the complete absence of a germ line (hereafter referred to as agametic testes). We first needed to verify with enhancer-trap markers that the somatic cells are correctly organized in agametic testes, as had been previously reported based on morphological criteria (Aboim, 1945).

Marker lines that express *lacZ* in hub cells (Fig. 2A, arrow) demonstrated that the hub still formed in agametic testes (Fig. 2B, arrow; Gönczy et al., 1992), although it was displaced from the tip by an accumulation of muscle sheath cells (see Fig. 2A,B; data not shown; Aboim, 1945). The hub in agametic testes was also larger, probably as a result of an increase in the volume of individual hub cells, as the number of hub cells in newly eclosed agametic males (13, s.d.=5,  $n=69$  testes) was only slightly elevated compared to wild-type (10.4, s.d.=2.5,  $n=21$  testes; see also Hardy et al., 1979). Overall, germ cells appear to be mostly dispensable for the organization of hub cells in the gonad.

To test for the presence of cyst progenitors, we used a marker expressed both in cyst progenitors and their early daughter cyst cells (Fig. 2C, arrow), since there are no cyst progenitor-specific markers. The marker was expressed in about 40% of agametic testes ( $n=43$ ), in a small area towards the tip (Fig. 2D, arrow), next to the hub (Fig. 2D, arrowhead). This class of agametic testes demonstrates that cyst progenitors can be correctly organized in the gonad and generate early daughter cyst cells in the absence of germ cells.

However, we detected no *lacZ* expression in about 60% of agametic testes analyzed with this marker. Nevertheless, we found evidence of prior cyst progenitor activity in most agametic testes of this second class. Markers that normally label cyst progenitors and daughter cyst cells at all stages of differentiation (Fig. 2E, arrow) revealed that cyst cells usually fill the inside of agametic testes (Fig. 2F, arrow). This mass of cyst cells was also recognizable with Nomarski optics and DNA staining (see Fig. 3),



**Fig. 2.** Somatic cell organization in agametic testes. Apical region of day 1 wildtype (WT) or entire agametic testes (AG) carrying an enhancer-trap marker specific for given somatic cell types. Immunoperoxidase localization of  $\beta$ -galactosidase. (A,B) Line 254, specific for hub cells (A, arrow), locates the hub in the agametic testis (B, arrow). Arrowhead marks stain in seminal vesicle. Cytoplasmic  $\beta$ -galactosidase; lethal insertion at *escargot* (G66B in Kassis et al., 1992). (C,D) Line 1-en-11, specific for cyst progenitors and early daughter cyst cells (C, arrow), labels cells in agametic testes (D, arrow) just below the hub (D, arrowhead). Cytoplasmic  $\beta$ -galactosidase; lethal insertion at *wingless* (Kassis et al., 1992). Other insertions at *wingless* do not express *lacZ* during spermatogenesis (unpublished observations). (E,F) Line D-39, which labels cyst progenitors and daughter cyst cells at all stages of differentiation (general cyst cell marker) (E, arrow) as well as the hub (E, arrowhead), identifies the mass of cells within agametic testes as cyst cells (F, arrow). The arrowhead in F points to the likely position of the hub. Strain lost. (G,H) Line P-573, labeling daughter cyst cells after the proliferation region (maturing cyst cell marker) (G, arrow), labels the daughter cyst cells furthest away from the hub in agametic testes (H, arrow). Cyst progenitors and early daughter cyst cells are not labeled (G,H, arrowhead). Bar, 50  $\mu$ m. All panels at same magnification.

which allowed us to assess that it was often present in the second class of agametic testes. Thus, cyst progenitors must have been initially functional, but lost during larval or pupal stages in these cases. Therefore, there is a partial germ-line requirement for the maintenance of cyst progenitor cell fate.

Except for this partial requirement, our observations confirm that the germ line is dispensable for the organization of hub and cyst progenitors in the gonad. Therefore, we were able to address whether germ cells regulate the behavior of these somatic cells during early spermatogenesis.

### In agametic testes, daughter cyst cells proliferate instead of being quiescent

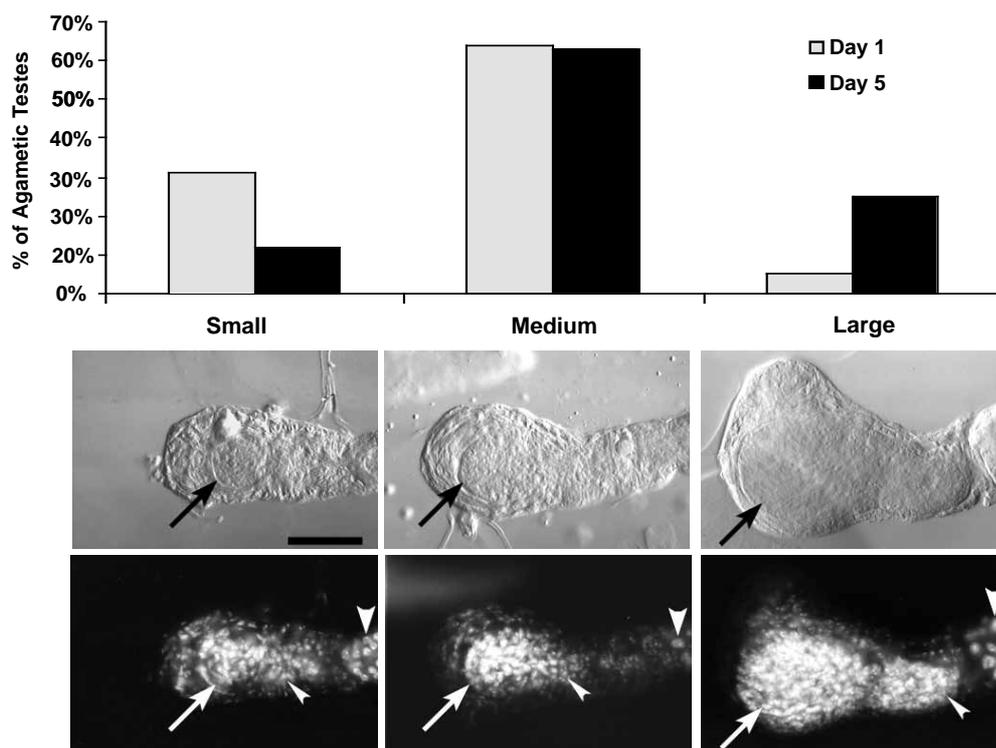
We first investigated whether daughter cyst cells differentiated appropriately in the absence of germ cells. Markers whose expression begins only in maturing cyst cells, past the proliferation region of wild-type testes (Fig. 2G, arrow), were also expressed in agametic testes of newly eclosed males (Fig. 2H, arrow). Thus, daughter cyst cells can initiate their differentiation program in the absence of germ cells.

We next investigated whether the continued absence of germ cells might reveal alterations in the differentiation of cyst cells, by comparing agametic testes of males on the day of eclosion (day 1) to that of males aged for 4 days (day 5). While most agametic testes expressed a maturing cyst cell marker on day 1 (22/22 in one experiment), only about half did so by day 5 (20/36 in the same experiment). Moreover, *lacZ* expression at day 5 was at lower levels and confined to fewer cells than at day 1 (data not shown). These changes were not due to daughter cyst cells being blocked at early stages of differentiation, since there was no increase in the population of cells expressing the cyst progenitor and early daughter cyst cell marker (data not shown). Moreover, daughter cyst cells did not disappear over time. On the contrary, older agametic testes tended to have more cyst cells as judged by Nomarski optics and DNA staining (Fig. 3). The mass of somatic cells also increases with time in agametic ovaries (Margolis and Spradling, 1995). Taken together, our observations indicate that fewer daughter cyst cells progress through differentiation in the continued absence of germ cells.

The increase in cyst cell number over time suggested to us that germ cells may normally restrict the proliferation of the

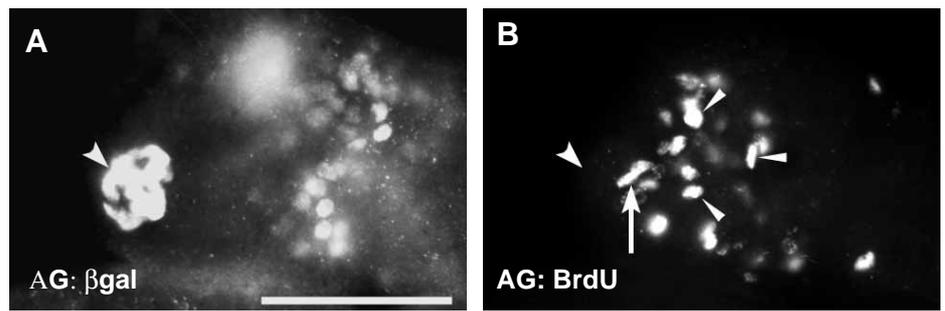
cyst lineage. To investigate this hypothesis, we compared the proliferative behavior of cyst cells in wild-type and agametic testes at day 1, by examining the distribution of cells in S phase during a 30 minute BrdU pulse. As expected, in wild-type testes, most cells in S phase were amplifying germ cells in the proliferation region (see Fig. 6B). Therefore, to identify cyst cells in S phase, we utilized a marker line of the cyst lineage and scored cells doubly labeled for  $\beta$ -galactosidase and BrdU. We observed a mean of 6.1 cyst cells in S phase per wild-type testis (s.d.=4.4,  $n=21$  testes). The marker line also expressed *lacZ* in hub cells, which allowed us to determine that most doubly labeled cells were adjacent to the hub and therefore were probably cyst progenitors (data not shown). Rarely, a doubly labeled cell was located at a short distance from the hub (data not shown). BrdU incorporation in these cases may be due to endoreduplication, as daughter cyst cells do not undergo cell division during wild-type spermatogenesis (Lindsley and Tokuyasu, 1980). Except for these rare cases, daughter cyst cells did not incorporate BrdU, as expected from non-proliferating cells.

In agametic testes, the mean number of BrdU-positive cells within the mass of cyst cells was 12.9 (s.d.=9.4,  $n=47$  testes), more than twice that observed in wild type. The large standard



**Fig. 3.** Cyst cell number tends to increase in older agametic testes. Histogram shows percentage of agametic testes at day 1 ( $n=61$ ) or day 5 ( $n=103$ ) that fall into the small, medium or large classes when scored double-blind for cyst cell mass. Representative examples are below respective bars on histogram. Cyst cell mass can be identified by Nomarski optics (top panels) or Hoechst DNA stain (bottom panels, area between arrow and thin arrowhead). Some agametic testes appeared to have no cyst cells, as judged by Nomarski optics or Hoechst staining, or by the lack of expression of a general cyst cell marker. The fraction of agametic testes without a cyst cell mass varied from 0 to 30% in different genetic backgrounds. Testes entirely devoid of cyst cells were not included in the histogram. The amount of DNA appears comparable in cyst cell nuclei close to the hub (arrow in any panel) and in those distant from it (thin arrowhead in any panel). Wide arrowheads point to cells of the epithelium that connects testis and seminal vesicle. Bar, 50  $\mu$ m. All panels at same magnification.

**Fig. 4.** In agametic testes, daughter cyst cells proliferate instead of being quiescent. Agametic testes (day 1) were incubated in BrdU for 30 minutes. The males carried two hub markers, M5-4 and S2-11. M5-4 is a lethal insertion at *escargot*, also expressing *lacZ* in germ-line stem cells and their progeny. S2-11 also labels daughter cyst cells at the end of the proliferation region. Since the S2-11 P-element is on the X chromosome, an S2-11; *osk*<sup>301</sup>/TM3 stock was generated to obtain sons carrying S2-11. S2-11; *osk*<sup>301</sup> mothers were crossed to M5-4 males to enhance the level of *lacZ* expression in hub cells of the agametic progeny. Indirect immunofluorescent localization of  $\beta$ -galactosidase (A) and BrdU (B). Over 20 cyst cells underwent S phase during the labeling period (B). Note that while some BrdU-labeled cyst cells (B, arrow) contact the hub (A,B, large arrowhead), most do not (B, thin arrowheads). Bar, 50  $\mu$ m. Both panels at same magnification.



deviation probably reflects the heterogeneous development of the cyst lineage among agametic testes. In addition, the majority of cyst cells in S phase in agametic testes (Fig. 4B, thin arrowheads) were distant from the hub (Fig. 4A, arrowhead). Some of these distant cells simultaneously expressed a maturing daughter cyst cell marker (7/22 agametic testes). In contrast, in wild-type, maturing daughter cyst cells were never in S phase (0/21 testes). BrdU incorporation in distant daughter cyst cells of agametic testes is not likely due to endoreduplication, since the amount of DNA seemed comparable in daughter cyst cells close to the hub and in those distant from it (see Fig. 3). Instead, this BrdU incorporation most likely reflects a genuine progression of daughter cyst cells through the cell cycle.

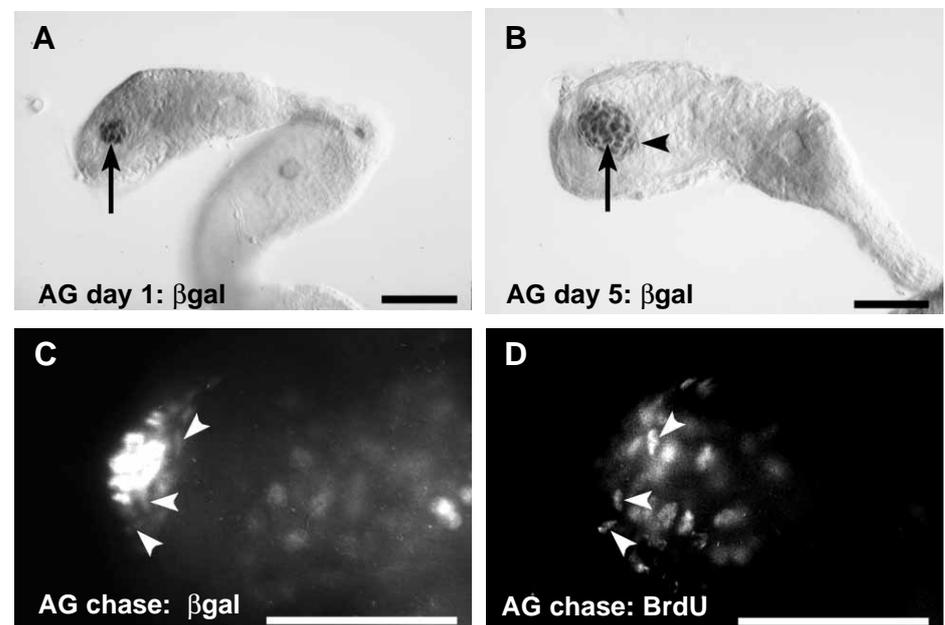
These results demonstrate that normally quiescent daughter cyst cells continue to proliferate in the absence of germ cells. This suggests that germ cells restrict daughter cyst cell proliferation during wild-type spermatogenesis.

#### In agametic testes, formerly proliferating cells are recruited to the hub

We next investigated whether the continued absence of germ cells affected hub cells. We found that the number of cells expressing a hub-specific marker increased significantly over time in agametic testes (compare Fig. 5A and B; day 1 average = 13, s.d.=5,  $n=69$  testes; day 5 average = 20, s.d.=14,  $n=83$  testes;  $P=3 \times 10^{-5}$ ). There was a large standard deviation at day 5 because hub cell number did not increase in a subset of agametic testes (data not shown). In wild-type, the number of hub cells remained constant over time (day 1 average = 10.4, s.d.=2.5,  $n=21$  testes; day 5 average = 10, s.d.=2.4,  $n=26$  testes;

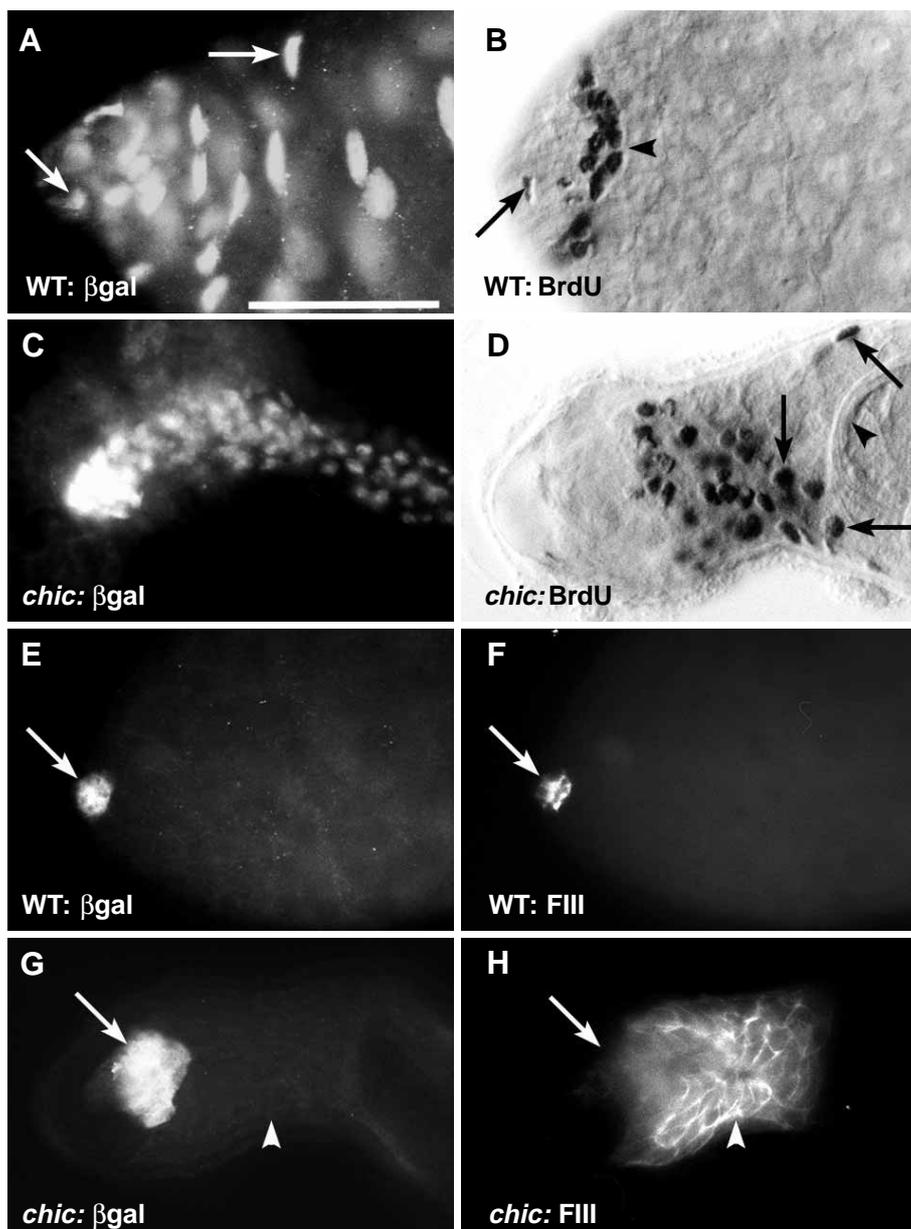
$P=0.26$ ). The increase in the average number of cells expressing *lacZ* in agametic testes most likely reflects a genuine alteration in cell fate, because similar changes were observed with two other hub markers.

In wild-type, hub cells are terminally differentiated and are thought to never divide (Hardy et al., 1979). Accordingly, there was no BrdU incorporation in hub cells of wild-type testes (0/21 testes, data not shown). We considered two possibilities that could explain why more hub cells are present in older agametic testes. First, hub cells could re-enter the cell cycle and divide



**Fig. 5.** In agametic testes, formerly proliferating cells are recruited to the hub. (A,B) Agametic testes with hub marker M5-4, at day 1 (A) or day 5 (B). Immunoperoxidase localization of  $\beta$ -galactosidase. Average hub cell number increases between day 1 (A, arrow) and day 5 (B, arrow). Note that central hub cells at day 5 (B, arrow) express *lacZ* at higher levels than more peripheral ones (B, arrowhead). (C,D) Agametic males (day 2) were fed BrdU and analyzed after a 10 hour chase. Indirect immunofluorescent localization of  $\beta$ -galactosidase (C) and BrdU (D). Three cells that incorporated BrdU during the pulse now express the hub markers (arrowheads). This particular male carried two hub markers, M5-4 and S2-11, in order to enhance  $\beta$ -galactosidase levels in the hub for illustrative purposes. S2-11 also labels daughter cyst cells after the proliferation region (C, cells below the plane of focus toward the right). Similar results were obtained with M5-4 alone and are reported in the text. Bars, 50  $\mu$ m.

**Fig. 6.** *chickadee* mutants have altered cyst cell behavior. Apical regions of day 1 wild-type or *chic*<sup>37</sup> homozygous mutant testes. (A,C) General cyst cell marker M12-41 (P-element is on the X-chromosome). Indirect immunofluorescent localization of  $\beta$ -galactosidase. In wild-type, cyst cells (A, arrows) are spaced apart because they surround packets of unlabeled germ cells. In contrast, cyst cells in *chic* are tightly packed in the absence of early stage germ cells (C). (B,D) Immunoperoxidase localization of BrdU incorporation, following a 30 minute incubation. Only a few cells of the cyst lineage incorporate BrdU in wild-type testes (arrow in B points to a labeled cyst progenitor cell); most cells in S phase are germ cells in the proliferation region (arrowhead in B points to a packet of 16 germ cells in premeiotic S phase; 6 of these cells are out of focus). In *chic*<sup>37</sup> mutant testes, daughter cyst cells distant from the hub undergo S phase (D, arrows). There are some sperm tails in *chic*<sup>37</sup> mutant testes (D, arrowhead), since germ cells are present at earlier developmental stages and progress through part of spermatogenesis. (E–H) Hub marker 254; double labeling with anti- $\beta$ -galactosidase (E, G) and anti-Fasciclin III (F, H). The hub is expanded in *chic*<sup>37</sup> mutant testes (compare E and G, arrow). In wild-type, Fasciclin III protein is restricted to the hub (F, arrow). In contrast, in *chic*<sup>37</sup> mutant testes, fasciclin III is only weakly expressed in hub cells (H, arrow), but ectopically accumulates in cyst cells (H, arrowhead). Bar, 50  $\mu$ m. All panels at same magnification.



to generate more hub cells. Alternatively, neighboring cells from the cyst lineage could switch to the hub cell fate.

If hub cells re-entered the cell cycle, they should incorporate BrdU as they transit through S phase. However, only 5% of day 2 agametic testes had a hub-specific *lacZ*-expressing cell that incorporated BrdU during a 30 minute in vitro labeling ( $n=46$  testes). The rare doubly labeled cell was located at the hub periphery, among cells that express lower levels of the hub marker (see Fig. 5B, arrowhead). Thus, most hub cells do not re-enter the cell cycle in agametic testes. Nevertheless, a peripherally located hub cell occasionally progresses through the cell cycle, which could explain part of the increased hub cell number over time.

Alternatively, the rare doubly labeled cells that are peripherally located may be cells from the cyst lineage executing a last S phase as they begin to express hub-specific markers. Since cyst progenitors and daughter cyst cells proliferate in

agametic testes (see Fig. 4), we pulsed proliferating cells at day 2 and examined whether they could turn on hub-specific markers over time. The duration of the pulse was about 60 minute in these pulse-chase experiments, twice as long as the 30 minute in vitro labeling (see Materials and Methods). Therefore, no more than 10% of agametic testes should have a doubly labeled cell in the pulse-chase experiments if the increase in hub cell number was solely due to the proliferation of peripherally located hub cells. In contrast, this number could be higher if cycling cyst cells switched to the hub fate over time. Cells doubly labeled with BrdU and hub-specific  $\beta$ -galactosidase were observed in 26% of agametic testes after a 10 hour chase ( $n=23$ ) and in 35% of agametic testes after a 28 hour chase ( $n=29$ ). Moreover, there were sometimes more than one doubly labeled cell in such pulse-chase experiments (Fig. 5C,D, arrowheads). Some doubly labeled cells were located more centrally among hub cells, indicating that additional,

BrdU-negative, cells had been incorporated to the hub after the pulse (data not shown).

Taken together, these results indicate that the hub increases in agametic testes mainly or exclusively because formerly cycling cyst cells join the hub. This suggests that germ cells prevent cells of the cyst lineage from adopting the hub fate during wild-type spermatogenesis.

### *chickadee* and *diaphanous* mutants have altered cyst cell behavior

Defects in the cyst lineage similar to those observed in agametic testes were also caused by mutations in *chickadee* (*chic*) and *diaphanous* (*dia*). *chic* encodes the actin-binding protein profilin (Cooley et al., 1992), while *dia* encodes a formin homolog required for cytokinesis (Castrillon and Wasserman, 1994). Severe male-sterile alleles of either of these genes have a decrease in germ-cell number during spermatogenesis (Castrillon et al., 1993; Verheyen and Cooley, 1994). We verified that *chic*<sup>37</sup> mutant males have very few early stage germ cells using marker lines (data not shown). Moreover, rare germ cells in *chic*<sup>37</sup> and *dia*<sup>1</sup> testes did not proliferate properly, since packets of 4, 8 or 16 BrdU-positive germ cells, which are readily observed in wild type (see Fig. 6B, arrowhead), were not detected in mutant testes (see Fig. 6D and data not shown).

The decrease in early germ-cell number in *chic*<sup>37</sup> testes correlated with altered proliferation and fate of cells in the cyst lineage. There was an accumulation of tightly packed cyst cells, as revealed by a general cyst cell marker (Fig. 6C). Like in agametic testes, cyst cells distant from the *chic*<sup>37</sup> testis tip underwent S phases (Fig. 6D, arrows). Moreover, the hub was also expanded in *chic*<sup>37</sup> mutant testes (compare Fig. 6E and G, arrow).

One additional similarity between agametic testes and *chic*<sup>37</sup> mutants was an altered distribution of Fasciclin III in hub and cyst cells. Fasciclin III is a homophilic adhesion molecule (Patel et al., 1987), which is restricted to hub cells of wild-type testes (Figs 6F and 7B, arrow; Brower et al., 1981; Gönczy et al., 1992). In contrast, Fasciclin III was absent or dramatically reduced in hub cells of both *chic*<sup>37</sup> and agametic testes (Figs 6H and 7D, arrow). Furthermore, cyst cells usually ectopically accumulated Fasciclin III protein both in *chic*<sup>37</sup> (in 27/32 mutant testes; Fig. 6H, arrowhead) and in agametic testes (26/32; Fig. 7D, arrowhead).

In *dia*<sup>1</sup> testes, proliferation of cells distant from the testis tip and ectopic Fasciclin III accumulation were also observed (data not shown). However, cyst and hub cell fate could not be directly monitored in this case, since *dia*<sup>1</sup> is an insertional mutant expressing *lacZ* in the testis.

The striking similarity of defects

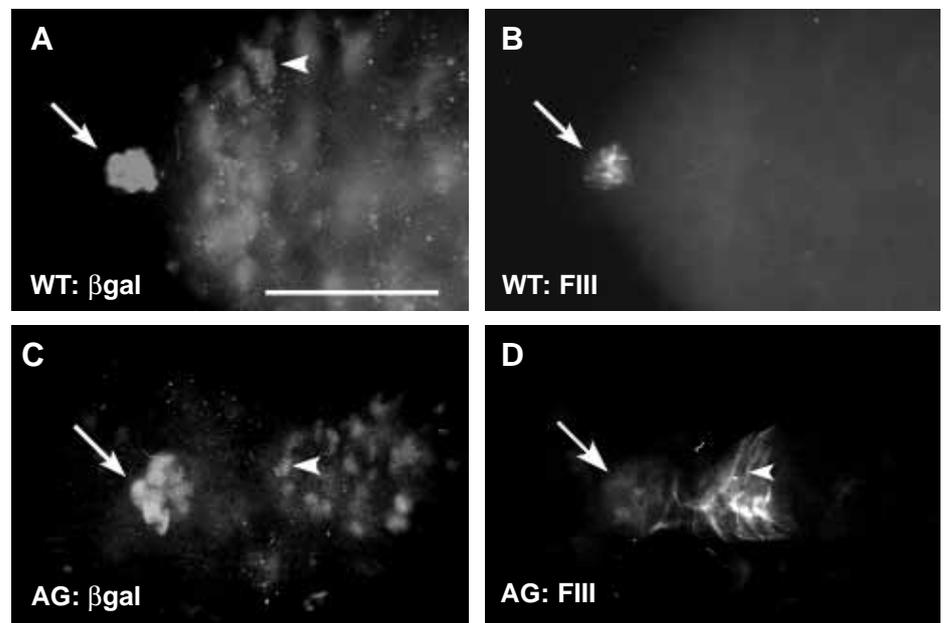
observed in cyst cells of agametic testes and *chic*<sup>37</sup> or *dia*<sup>1</sup> mutant testes further indicates that germ cells are required to regulate the proliferation and fate of somatic cyst cells during spermatogenesis.

## DISCUSSION

### Adjacent germ-line and somatic stem cells are anchored around the hub

During *Drosophila* spermatogenesis, the germ-line and somatic lineages must coordinate their activity, so that each daughter germ cell becomes surrounded by two daughter cyst cells (see Fig. 1B). It has been suggested that this coordination is achieved by the concerted division of one germ-line stem cell and two neighboring cyst progenitor cells (Hardy et al., 1979). We have verified by lineage tracing that the germ-line stem cells are anchored around the hub at the testis tip. We have also established the existence of stem cells for the cyst lineage and shown that they likely correspond to cyst progenitors, which are intermingled with germ-line stem cells around the hub. Therefore, the coordinated production of one daughter germ cell with two daughter cyst cells could indeed involve communication between the two stem cell populations. It is also possible that a coordinating signal emanates from hub cells, which contact the stem cells from both lineages.

Somatic stem cells have also been identified in the *Drosophila* ovary (Margolis and Spradling, 1995). These ovarian follicle stem cells are located in regions 2a and 2b of



**Fig. 7.** Fasciclin III distribution is altered in agametic testes. Apical region of day 1 wild-type testis (A,B) or entire day 1 agametic testis (C,D), both carrying the marker S2-11, which normally labels hub cells (A, arrow) and daughter cyst cells after the proliferation region (A, arrowhead). Double indirect immunofluorescent localization of  $\beta$ -galactosidase and Fasciclin III. In wild-type, Fasciclin III is restricted to the hub (B, arrow). In agametic testes, Fasciclin III expression at the hub is very weak (D, arrow); moreover, cyst cells, some of which express the marker S2-11 (C, arrowhead), ectopically accumulate Fasciclin III (D, arrowhead). Bar, 50  $\mu$ m. All panels at same magnification.

the germanium, far from the germ-line stem cells. Therefore, in contrast to spermatogenesis, there is no direct contact between germ-line and somatic stem cells during oogenesis. Interestingly, there is no need to coordinate the activity of the two stem cell populations in the ovary as stringently as in the testis, because female germ cells undergo mitotic amplification and mature substantially before being surrounded by follicle cells (reviewed in Spradling, 1993).

### Germ cells restrict daughter cyst cell proliferation

It has been previously reported that all morphologically recognizable somatic cell types are present in agametic testes (Aboim, 1945). By using molecular markers, we confirmed that hub and cyst cells are usually present in the absence of germ cells. Moreover, we established that daughter cyst cells are generated and begin their differentiation program in agametic testes. Most agametic testes thus provide an experimental situation in which to probe whether germ cells regulate the function of somatic cells during the early steps of spermatogenesis.

Normally, cyst progenitors give rise to two daughters with distinct proliferative behavior. While one remains a stem cell, which undergoes multiple division cycles, the other daughter becomes a cyst cell, which never divides again (Hardy et al., 1979). We have shown that, in the absence of germ cells, daughter cyst cells continue to proliferate instead of exiting the cell cycle. This suggests that germ cells signal to restrict the proliferation of daughter cyst cells during wild-type spermatogenesis.

Some human testicular tumors result from the overproliferation of Sertoli cells or other somatic gonadal cells (Chomette et al., 1985). Our work raises the possibility that some of these tumors result from the loss of germ-cell signals which normally restrict somatic gonadal cell proliferation. An overproliferation of Sertoli cells is also observed in mice following the targeted deletion of  $\alpha$ -inhibin, a transforming growth factor  $\beta$  family member (Matzuk et al., 1992). However,  $\alpha$ -inhibin is probably not secreted by germ cells, since its mRNA is mainly detected in the Sertoli cells themselves (Vale et al., 1990). Thus,  $\alpha$ -inhibin is thought to be required in Sertoli cells for restricting their own proliferation (Matzuk et al., 1992). Nevertheless, addition of germ cells to cultured Sertoli cells results in augmented  $\alpha$ -inhibin mRNA expression (Pineau et al., 1990). Thus, germ cells may regulate Sertoli cell proliferation by modulating the expression of  $\alpha$ -inhibin within these somatic cells.

Signaling from neighboring cells often controls proliferation. For instance, ablation of the somatic distal tip cell in *C. elegans* results in the cessation of germ-cell proliferation (Kimble and White, 1981). Similarly, removal of a cell at the tip of the developing malpighian tubule in *Drosophila* or *Rhodnius* prevents proliferation of neighboring cells (Skaer, 1989; Skaer, 1992). In both cases, signaling is normally required for continued proliferation. In contrast, ablation of somatic terminal filament cells in the *Drosophila* ovary leads to increased germ-line stem cell proliferation (Lin and Spradling, 1993). In *C. elegans*, lateral hypodermal precursors or spermathecal sheath precursors cells overproliferate when they are isolated from their neighbors (Sulston and White, 1980; Kimble and White, 1981). In these cases, signaling from neighboring cells is normally required to restrict proliferation,

just as signaling from germ cells restricts daughter cyst cell proliferation during *Drosophila* spermatogenesis.

The mechanisms by which extrinsic signals control cell-cycle progression are being unraveled in several systems. For instance, in *S. cerevisiae*, pheromones secreted by cells of the opposite mating type induce cell-cycle arrest of target cells (reviewed by Marsh et al., 1991; Reed, 1991). Pheromones trigger a signal transduction cascade, which results in the inhibitory association of Far1 protein with Cdc28-Cln kinases, thereby preventing the G<sub>1</sub> to S transition (Peter et al., 1993; Peter and Herskowitz, 1994). During *Drosophila* spermatogenesis, signals emanating from germ cells must also lead to the inhibition of cdk kinase activity in daughter cyst cells to restrict cell-cycle progression.

### Germ cells prevent cells of the cyst lineage from adopting the hub cell fate

The factors that permit stem cells to remain selfrenewing are being investigated in many systems (reviewed by Hall and Watt, 1989; Whetton and Dexter, 1993). Such factors can prevent stem cells from dying, from exiting the cell cycle or from differentiating into a cell of the lineage that they normally produce. For instance, erythropoietin is thought to prevent programmed cell death of haematopoietic progenitor cells (Koury and Bondurant, 1990; Williams et al., 1990). A combination of Steel factor and cytokines produced by stromal cells may be required to sustain haematopoietic stem cell proliferation (Lowry et al., 1992). Our analysis suggests for the first time that stem cells could also lose their selfrenewing capacity by switching their fate to that of a related, but distinct, cell type.

Our results indicate that some proliferating cells exit the cell cycle and initiate the expression of hub-specific markers in older agametic testes. The cells switching to the hub fate could be either cyst progenitors or their daughter cyst cells, since both proliferate in agametic testes. Moreover, both cyst progenitor and early daughter cyst cell fates are lost in some agametic testes, possibly because these cells switch to the hub fate. A molecular marker that discriminates cyst progenitors from their daughter cyst cells, and which is not a *lacZ* enhancer trap, is needed to identify unambiguously the type of cyst cell recruited to the hub. In the absence of such a marker, two observations favor cyst progenitors being these cells. First, cyst progenitors are located next to hub cells and, thus, could readily be recruited without migration. Second, there is no apparent reason why daughter cyst cells born in an old agametic testis should have a higher probability to switch fate than those produced in a younger one. Indeed, daughter cyst cells are continuously generated from cyst progenitors. Thus, each daughter cyst cell must lack germ-line signals for the same length of time. In contrast, cyst progenitors are established once during gonadogenesis. Thus, cyst progenitors in old agametic testes have been lacking germ-cell signals for a longer period and may have an increased probability of switching fate. Taken together, these observations lead us to postulate that a signal from germ cells maintains the self-renewing capacity of cyst progenitors. In the absence of this signal, cyst progenitors can switch to a distinct cell fate, possibly because hub and cyst cells are closely related by lineage. Both apparently derive from the same gonadal precursor population during embryogenesis (Boyle and DiNardo, 1995), and most enhancer-trap lines that label cyst

progenitors also label hub cells (unpublished observations). A loss of self-renewing capacity by cell-fate switch may also occur in other systems where cell types lineally related to the stem cells are present.

In contrast to the switch of cyst cells to the hub cell type, the changes in Fasciclin III distribution do not appear to reflect a cell fate transformation, because hub- or cyst cell-specific marker lines are not mis-expressed in agametic testes in the same manner as is Fasciclin III protein (this work and unpublished observations). Alterations in the expression of proteins produced by somatic gonadal cells in the absence of germ cells are also observed in mammals (reviewed by McGuinness and Griswold, 1994). For instance, removal of germ cells from cultured Sertoli cells results in a decreased secretion of androgen-binding protein and transferin by these somatic cells (Welsh et al., 1985; LeMagueresse et al., 1988). Loss of germ cells in vivo or in vitro leads to an increased secretion of testins, a family of structurally related Sertoli cell proteins (Cheng et al., 1989). However, the biological relevance of these changes to mammalian spermatogenesis is unclear. Similarly, the importance of the alterations of Fasciclin III distribution in *Drosophila* is not known, especially since spermatogenesis is not affected in testes null for *fasIII* function (unpublished observations).

### Identifying the signals

Our experiments suggest that cells of the cyst lineage require the germ-line signals continually, rather than just once in development. We infer this because *chic*<sup>37</sup> and *dia*<sup>1</sup> mutants exhibit cyst cell alterations in adult testes, by which time germ cells are lacking, but not in third instar larval gonads, when germ cells are still present (unpublished observations). For instance, the size of the *chic*<sup>37</sup> larval hub is similar to that of heterozygous controls, and Fasciclin III protein is still restricted to the hub of *chic*<sup>37</sup> and *dia*<sup>1</sup> mutant larval gonads (unpublished observations). The cyst cell alterations in *chic*<sup>37</sup> and *dia*<sup>1</sup> mutant animals thus correlate with the disappearance of germ cells, indicative of a continuous requirement for germ-line signals.

The source of these signals could reside with germ-line stem cells or their proliferating daughters. Moreover, germ cells could signal directly to cells of the cyst lineage, or indirectly via other somatic cells. For instance, germ cells could modulate signaling properties of hub cells, which in turn could influence cyst progenitor cells and daughter cyst cells. Mutational analysis should identify components of germ cell to cyst cell signaling. The phenotype of agametic testes predicts that signaling mutants should have continued daughter cyst cell proliferation, increased hub cell number and changes in Fasciclin III distribution. All three phenotypes are exhibited by *chic*<sup>37</sup> and possibly *dia*<sup>1</sup> mutants. Although the alterations are probably only consequences of the absence of germ cells in these two cases, it is formally possible that *dia* and *chic* are bona fide signaling mutants. This is especially plausible for *chic*, since profilin is known to modulate signal transduction in other systems (Goldschmidt-Clermont et al., 1991; Vojtek et al., 1991). Mutants that exhibit the predicted phenotypes while retaining germ cells would identify candidates for loci encoding components of germ cell to cyst cell signaling.

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### REFERENCES

- Aboim, A. N.** (1945). Développement embryonnaire et postembryonnaire des gonades normales et agamétiques de *Drosophila melanogaster*. *Rev. Suisse Zool.* **52**, 53-154.
- Austin, J. and Kimble, J.** (1989). Transcript analysis of *glp-1* and *lin-12*, homologous genes required for cell interactions during development of *C. elegans*. *Cell* **58**, 565-571.
- Bellvé, A. R.** (1979). The molecular biology of mammalian spermatogenesis. In *The Oxford Reviews for Reproductive Biology* (ed. C. A. Finn), Vol. 1, pp. 159-261. Oxford: Clarendon Press.
- Boyle, M. and DiNardo, S.** (1995). Specification, migration and assembly of the somatic cells of the *Drosophila* gonad. *Development* **121**, 1815-1825.
- Brower, D. L., Smith, R. J. and Wilcox, M.** (1981). Differentiation within the gonads of *Drosophila* revealed by immunofluorescence. *J. Embryol. Exp. Morph.* **63**, 233-242.
- Castrillon, D. H., Gönczy, P., Alexander, S., Rawson, R., Eberhart, C. G., Viswanathan, S., DiNardo, S. and Wasserman, S. A.** (1993). Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: Characterization of male-sterile mutants generated by single *P* element mutagenesis. *Genetics* **135**, 489-505.
- Castrillon, D. H. and Wasserman, S. A.** (1994). *diaphanous* is required for cytokinesis in *Drosophila* and shares domains of similarity with the products of the *limb deformity* gene. *Development* **120**, 3367-3377.
- Cheng, C. Y., Grima, J., Stahler, M. S., Lockshin, R. A. and Bardin, C. W.** (1989). Testins are structurally related Sertoli cell proteins whose secretion is tightly coupled to the presence of germ cells. *J. Biol. Chem.* **264**, 21386-21393.
- Chomette, G., Delcourt, A. and Auriol, M.** (1985). Non-germinal tumors of the testis. In *Testicular Cancer*, pp. 35-45. New York: Alan R. Liss.
- Chou, T. B. and Perrimon, N.** (1992). Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. *Genetics* **131**, 643-653.
- Cooley, L., Kelley, R. and Spradling, A. C.** (1992). *chickadee* encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. *Cell* **69**, 173-184.
- Fuller, M. T.** (1993). Spermatogenesis. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias). Vol. 1, pp. 71-147. Cold Spring Harbor, NY: Cold Spring Harbor Press.
- Geigy, R.** (1931). Action de l'ultra-violet sur le pôle germlinal dans l'oeuf de *Drosophila melanogaster*. (Castration et mutabilité). *Rev. Suisse Zool.* **38**, 187-288.
- Goldschmidt-Clermont, P. J., Kin, J. W., Machesky, L. M., Rhee, S. G. and Pollard, T. D.** (1991). Regulation of phospholipase C- $\gamma$ 1 by profilin and tyrosine phosphorylation. *Science* **251**, 1231-1233.
- Golic, K. G.** (1991). Site-specific recombination between homologous chromosomes in *Drosophila*. *Science* **252**, 958-961.
- Gönczy, P.** (1995). Towards a molecular genetic analysis of spermatogenesis in *Drosophila*. Ph.D. Thesis. New York: The Rockefeller University.
- Gönczy, P., Viswanathan, S. and DiNardo, S.** (1992). Probing spermatogenesis in *Drosophila* with *P*-element enhancer detectors. *Development* **114**, 89-98.
- Hall, P. A. and Watt, F. M.** (1989). Stem cells: the generation and maintenance of cellular diversity. *Development* **106**, 619-633.
- Hardy, R. W., Tokuyasu, K. T., Lindsley, D. L. and Garavito, M.** (1979). The germinal proliferation center in the testis of *Drosophila melanogaster*. *J. Ultrastruct. Res.* **69**, 180-190.
- Harrison, D. A. and Perrimon, N.** (1993). Simple and efficient generation of marked clones in *Drosophila*. *Current Biology* **3**, 424-433.
- Hartl, D. L. and Green, M. M.** (1970). Genetic studies of germinal mosaicism in *Drosophila melanogaster* using the mutable *wc* gene. *Genetics* **65**, 449-456.
- Henderson, S. T., Gao, D., Lambie, E. J. and Kimble, J.** (1994). *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. *Development* **120**, 2913-2924.
- Kassis, J. A., Noll, E., VanSickle, E. P., Odenwald, W. F. and Perrimon, N.**

- (1992). Altering the insertional specificity of a *Drosophila* transposable element. *Proc. Natl. Acad. Sci. USA* **89**, 1919-1923.
- Kimble, J. and Ward, S.** (1988). Germ-line development and fertilization. In *The Nematode Caenorhabditis* (ed. W. B. Wood), pp. 191-213. Cold Spring Harbor NY: Cold Spring Harbor Press.
- Kimble, J. E. and White, J. G.** (1981). On the control of germ cell development in *Caenorhabditis elegans*. *Dev. Biol.* **81**, 208-219.
- Koury, M. J. and Bondurant, M. C.** (1990). Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* **248**, 378-81.
- Lehmann, R. and Nüsslein-Volhard, C.** (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in *Drosophila*. *Cell* **47**, 141-152.
- LeMagueresse, B., Pineau, C., Guillou, F. and Jegou, B.** (1988). Influence of germ cells upon transferrin secretion by rat Sertoli cells *in vitro*. *J. Endocrinol.* **118**, R13-R16.
- Lin, H. and Spradling, A. C.** (1993). Germ line stem cell division and egg chamber development in transplanted *Drosophila* germaria. *Dev. Biol.* **159**, 140-152.
- Linder, C. C., Heckert, L. L., Roberts, K. P., Kim, K. H. and Griswold, M. D.** (1991). Expression of receptors during the cycle of the seminiferous epithelium. In *The Male Germ Cell: Spermatogonium to Fertilization*. (ed. B. Robaire). Vol. 637, pp. 313 – 321. New York, NY: The New York Academy of Sciences.
- Lindsley, D. L. and Tokuyasu, K. T.** (1980). *Spermatogenesis*. London: Academic Press.
- Lowry, P. A., Deacon, D., Whitefield, P., McGrath, H. E. and Quesenberry, P. J.** (1992). Stem cell factor induction of *in vitro* murine hematopoietic colony formation by 'subliminal' cytokine combinations: the role of 'anchor factors'. *Blood* **80**, 663-9.
- Margolis, J. and Spradling, A.** (1995). Identification and behavior of epithelial stem cells in the *Drosophila* ovary. *Development* **121**, in press.
- Marsh, L., Neiman, A. M. and Herskowitz, I.** (1991). Signal transduction during pheromone response in yeast. *Annu. Rev. Cell. Biol.* **7**, 699-728.
- Matzuk, M. M., Finegold, M. J., Su, J. G., Hsueh, A. J. and Bradley, A.** (1992). Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature* **360**, 313-319.
- McGuinness, M. P. and Griswold, M. D.** (1994). Interactions between Sertoli cells and germ cells in the testis. *Seminars in Dev. Biol.* **5**, 61-66.
- Patel, N. H., Snow, P. M. and Goodman, C. S.** (1987). Characterization and cloning of Fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* **48**, 975-988.
- Peter, M., Gartner, A., Horecka, J., Ammerer, G. and Herskowitz, I.** (1993). FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. *Cell* **73**, 747-760.
- Peter, M. and Herskowitz, I.** (1994). Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. *Science* **265**, 1228-1231.
- Pineau, C., Sharpe, R. M., Saunders, P. T., Gerard, N. and Jegou, B.** (1990). Regulation of Sertoli cell inhibin production and of inhibin alpha-subunit mRNA levels by specific germ cell types. *Mol. Cell. Endo.* **72**, 13-22.
- Reed, S. I.** (1991). Pheromone signaling pathways in yeast. *Curr. Opin. Genet. Dev.* **1**, 391-396.
- Schubiger, M. and Palka, J.** (1987). Changing spatial patterns of DNA replication in the developing wing of *Drosophila*. *Dev. Biol.* **123**, 145-153.
- Skaer, H.** (1989). Cell division in Malpighian tubule development in *Drosophila melanogaster* is regulated by a single tip cell. *Nature* **342**, 566-569.
- Skaer, H.** (1992). Cell proliferation and rearrangement in the development of the malpighian tubules of the hemipteran, *Rhodnius prolixus*. *Dev. Biol.* **150**, 372-380.
- Spradling, A. C.** (1993). Developmental genetics of oogenesis. In *The Development of Drosophila melanogaster*. pp. 1-70. Cold Spring Harbor NY: Cold Spring Harbor Press.
- Sulston, J. E. and White, J. G.** (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Dev. Biol.* **78**, 577-597.
- Tates, A. D.** (1971). Cytodifferentiation during spermatogenesis in *Drosophila melanogaster*: An electron microscope study. *Ph.D. thesis* Rijksuniversiteit, Leiden.
- Tax, F. E., Yeagers, J. J. and Thomas, J. H.** (1994). Sequence of *C. elegans lag-2* reveals a cell-signalling domain shared with *Delta* and *Serrate* of *Drosophila*. *Nature* **368**, 150-154.
- Tres, L. L. and Kierszenbaum, A. L.** (1983). Viability of rat spermatogenic cells *in vitro* is facilitated by their coculture with Sertoli cells in serum-free hormone-supplemented medium. *Proc. Natn. Acad. Sci. USA* **80**, 3377-3381.
- Vale, W., Hsueh, A., Rivier, C. and Yu, J.** (1990). In *Peptide Growth Factors and their Receptors II* (ed. Sporn, M. B. and Roberts, A. B.), pp. 211-248. Springer, Berlin.
- Verheyen, E. M. and Cooley, L.** (1994). Profilin mutations disrupt multiple actin-dependent processes during *Drosophila* development. *Development* **120**, 717-728.
- Vojtek, A., Haarer, B., Field, J., Gerst, J., Pollard, T. D., Brown, S. and Wigler, M.** (1991). Evidence for a functional link between profilin and CAP in the yeast *S. cerevisiae*. *Cell* **66**, 497-505.
- Welsh, M. K., Ireland, M. E. and Treisman, G. J.** (1985). Stimulation of rat Sertoli cell adenylate cyclase by germ cells *in vitro*. *Biol. Reprod.* **33**, 1050-1056.
- Whetton, A. D. and Dexter, T. M.** (1993). Influence of growth factors and substrates on differentiation of haemopoietic stem cells. *Curr. Opin. Cell Biol.* **5**, 1044-1049.
- Williams, G. T., Smith, C. A., Spooncer, E., Dexter, T. M. and Taylor, D. R.** (1990). Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* **343**, 76-79.
- Yochem, J., Weston, K. and Greenwald, I.** (1989). The *Caenorhabditis elegans lin-12* gene encodes a transmembrane protein with overall similarity to *Drosophila Notch*. *Nature* **335**, 547-550.

