

## ***bag-of-marbles* and *benign gonial cell neoplasm* act in the germline to restrict proliferation during *Drosophila* spermatogenesis**

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### **SUMMARY**

Stem cells divide asymmetrically, regenerating a parental stem cell and giving rise to a daughter cell with a distinct fate. In many stem cell lineages, this daughter cell undergoes several amplificatory mitoses, thus generating more cells that embark on the differentiation program specific for the given lineage. Spermatogenesis in *Drosophila* is a model system to identify molecules regulating stem cell lineages. Mutations at two previously identified loci, *bag-of-marbles* (*bam*) and *benign gonial cell neoplasm* (*bgn*), prevent progression through spermatogenesis and oogenesis, resulting in the overproliferation of undifferentiated germ cells. Here we investigate how *bam* and *bgn* regulate the male germline stem cell lineage. By generating FLP-mediated clones, we demonstrate that both *bam* and *bgn* act autonomously in the germline to restrict proliferation during spermatogenesis. By using enhancer

trap lines, we find that the overproliferating germ cells express markers specific to amplifying germ cells, while at the same time retaining the expression of some markers of stem cell and primary spermatogonial cell fate. However, we find that germ cells accumulating in *bam* or *bgn* mutant testes most resemble amplifying germ cells, because they undergo incomplete cytokinesis and progress through the cell cycle in synchrony within a cyst, which are two characteristics of amplifying germ cells, but not of stem cells. Taken together, our results suggest that *bam* and *bgn* regulate progression through the male germline stem cell lineage by cell-intrinsically restricting the proliferation of amplifying germ cells.

Key words: *Drosophila*, spermatogenesis, stem cell, proliferation control, *bag-of-marbles*, *bam*, *benign gonial cell neoplasm*, *bgn*

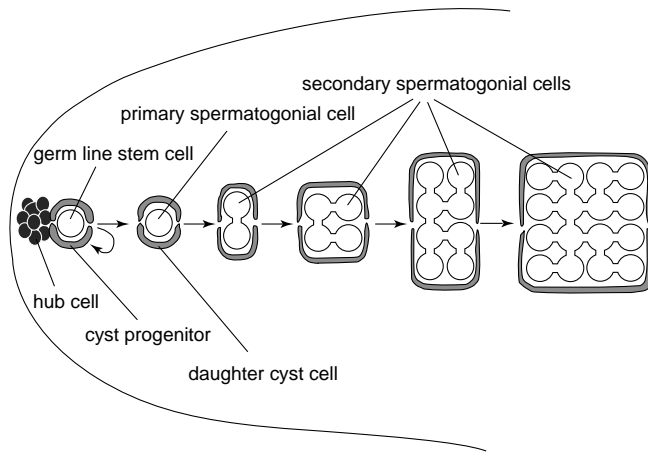
### **INTRODUCTION**

A stereotyped sequence of events takes place in stem cell lineages to ensure that an adequate number of differentiated cells is produced (reviewed by (Hall and Watt, 1989; Evans and Potten, 1991). Initially, a stem cell divides asymmetrically, regenerating a parental stem cell and giving rise to a daughter cell with a distinct fate. This daughter cell then undergoes several amplificatory mitoses, thus generating more cells that embark on the differentiation program specific for the given lineage. In theory, the proliferation and fate of stem cells, daughter cells and their amplifying progeny could be regulated cell-intrinsically, or, alternatively, by signaling from neighbouring cells. The actual mechanisms as well as the molecules that control progression through stem cell lineages are still poorly understood in most systems.

Spermatogenesis in *Drosophila* presents an opportunity to analyze the regulation of stem cell lineages in vivo (reviewed by Lindsley and Tokuyasu, 1980; Fuller, 1993). Spermatogenesis begins as a germline stem cell divides asymmetrically, regenerating a parental stem cell and giving rise to a daughter cell, called a primary spermatogonial cell (Fig. 1). The primary spermatogonial cell is the mitotic founder of a cyst of secondary spermatogonial cells. There are four mitotic divisions, producing sixteen spermatogonial cells. Cytokinesis

is incomplete during the four amplificatory mitoses and the arrested cleavage furrows leave the sixteen spermatogonial cells interconnected by intercellular bridges called ring canals. The fusome, a structure composed of both membrane and cytoskeletal components, runs continuously through the ring canals between amplifying germ cells at the 2-, 4-, 8- and 16-cell stages (Lin et al., 1994; McKearin and Ohlstein, 1995; Hime et al., 1996). The sixteen germ cells then enter the meiotic cell cycle and proceed through the differentiation program to become mature spermatozoa.

Progression through the germline stem cell lineage occurs in a well-defined environment of somatically derived cells. In particular, germ cells are accompanied throughout spermatogenesis by cells from the somatic cyst lineage. Cyst progenitors act as stem cells for this lineage and generate the two daughter cyst cells that surround each newly formed primary spermatogonial cell (Fig. 1; Hardy et al., 1979; Gönczy and DiNardo, 1996). Unlike primary spermatogonial cells, daughter cyst cells withdraw from the cell cycle and, thus, do not form a mitotically amplifying population. As a consequence, two daughter cyst cells continue to surround the amplifying progeny of the primary spermatogonial cell, as well as differentiating germ cells. Both germline stem cells and cyst progenitors are anchored at the testis tip around a cluster of somatic cells referred to as the hub (Hardy et al., 1979). The



**Fig. 1.** Schematic representation of a wild-type testis tip. Two kinds of stem cells are anchored around 8–16 hub cells at the tip of the testis. First, there are 9–12 germline stem cells (one is represented here for clarity). Second, each germline stem cell is surrounded by two cyst progenitors, which act as stem cells for the cyst lineage. Spermatogenesis begins as a germline stem cell divides asymmetrically, regenerating a parental stem cell and giving rise to a primary spermatogonial daughter cell. The primary spermatogonial cell is the mitotic founder of a cyst of secondary spermatogonial cells. There are four mitotic divisions to produce sixteen of these cells, which have left the vicinity of the hub. Cytokinesis is incomplete during the four amplificatory mitoses, and the arrested cleavage furrows leave the sixteen spermatogonial cells interconnected by ring canals. The sixteen cells then enter the meiotic cell cycle, and are now called spermatocytes, before differentiating into mature spermatozoa. Two cyst progenitors are thought to generate the two daughter cyst cells that surround each newly formed primary spermatogonial cell. Daughter cyst cells do not divide; thus, two daughter cyst cells also surround amplifying spermatogonial cells, as well as germ cells in subsequent stages of spermatogenesis.

association of somatic and germline cells throughout spermatogenesis suggests that cell-extrinsic signaling, together with cell-intrinsic factors, will regulate progression through these stem cell lineages.

A genetic approach can identify such cell-extrinsic and cell-intrinsic components. In particular, mutations that result in the overproliferation of undifferentiated germ cells should identify loci involved in one of three key decisions along the germline stem cell lineage. First, such a mutant phenotype may identify loci that assign the primary spermatogonial cell fate. In the mutant, primary spermatogonial cells would be unable to adopt a fate different from their parental stem cell, and thus indefinitely proliferate as stem cells. Second, such a mutant phenotype may identify loci that restrict the number of amplificatory mitoses, or, third, that are required for entry into the meiotic cell cycle. In these cases, mutant spermatogonial cells would be unable to limit the number of mitoses to four, or be unable to enter the meiotic cell cycle, and thus indefinitely proliferate as spermatogonial cells.

Mutations in two previously identified loci, *bag-of-marbles* (*bam*) and *benign gonial cell neoplasm* (*bgn*), result in the absence of mature germ cells from testes and ovaries, and in the overproliferation of small cells with morphological characteristics of undifferentiated germ cells (Gateff, 1982;

McKearin and Spradling, 1990). Therefore, *bam* and *bgn* are candidate regulators of the progression through the male and female germline stem cell lineages. It is not unexpected that some regulators are shared between spermatogenesis and oogenesis, since aspects of early gametogenesis are similar in both sexes. Thus, female germline stem cells also divide asymmetrically, regenerating a parental stem cell and giving rise to a cystoblast with a distinct fate (reviewed in Spradling, 1993). Like the primary spermatogonial cell, the cystoblast undergoes four amplificatory mitoses characterized by incomplete cytokinesis, generating sixteen interconnected germ cells that embark on the remainder of the oogenic differentiation program.

In *bam* mutant ovaries, the overproliferating germ cells appear to behave as stem cells by two criteria. First, germ cells overproliferating in *bam* mutant ovaries are either not connected to a neighbouring cell, or are connected to a single neighbouring cell, with a fusome passing between the two cells (McKearin and Ohlstein, 1995). This pattern of connections is identical to that of wild-type germline stem cells, but different from that of wild-type amplifying germ cells, which are connected to several neighbouring germ cells as a result of incomplete cytokinesis. Similarly, branched fusomes are rarely observed in *bgn* mutant ovaries (Lin et al., 1994). Second, germ cells overproliferating in *bam* mutant ovaries undergo S phase asynchronously (McKearin and Ohlstein, 1995). Again, this behavior is that observed among germline stem cells, but not among clonally amplifying germ cells, which progress synchronously through the cell cycle. These observations led to the conclusion that *bam* mutant germ cells behave as stem cells and to the postulate that *bam* function is normally required in females to assign the fate of the cystoblast and, by extension to males, that of the primary spermatogonial cell (McKearin and Ohlstein, 1995). However, in males, the *bam* or *bgn* mutant phenotype has not been examined in detail and, despite the apparent phenotypic similarity during spermatogenesis and oogenesis, *bam* or *bgn* may still play a distinct role in the male germline stem cell lineage. Moreover, although the small cells overproliferating in mutant ovaries and testes are likely to be germ cells, their identity has not been tested directly and possible effects on the somatic lineages have not been examined.

*bam* encodes a protein with no strong homologies to other proteins (McKearin and Spradling, 1990). Bam protein is present in the cytoplasm and the fusome of the cystoblast, the primary spermatogonial cell and their amplifying progeny (McKearin and Ohlstein, 1995). Thus, while the distribution of Bam protein is consistent with its postulated role in assigning the primary spermatogonial and cystoblast cell fates, the presence of Bam protein in amplifying germ cells raises the possibility that it may also play a role during that stage of gametogenesis. Although these localization studies suggest that *bam* is required intrinsically in the germline to restrict germ cell proliferation, this has not been directly tested. *bgn* has not yet been characterized molecularly, precluding a prediction as to where *bgn* function might be required. However, reciprocal pole-cell transplant experiments have shown that, in females, *bgn* function is required in the germline to prevent germ cell proliferation (Mahowald and Wei, 1994). Whether this is also the case in males has not been examined.

Here we test whether *bam* and *bgn* function are required

intrinsically in the germline during spermatogenesis. We also characterize the identity of the cells accumulating in *bam* or *bgcn* mutant testes, to define the step at which *bam* and *bgcn* regulate progression through the male germline stem cell lineage. Our results indicate that *bam* and *bgcn* act cell-intrinsically to restrict the amplification of spermatogonial cells or to promote their entry into the meiotic cell cycle.

## MATERIALS AND METHODS

### Fly strains

*bam*<sup>86</sup> is an excision allele of the original *bam* P-element-induced mutation; *bam*<sup>86</sup> removes most of the *bam* coding sequence and is therefore probably a null allele (McKearin and Ohlstein, 1995). *bgcn*<sup>Q52</sup> is an ethyl-methane-sulfonate-induced allele of *bgcn* (Schüpbach and Wieschaus, 1991); the testis phenotype of *bgcn*<sup>Q52</sup> homozygous flies was similar to that of *bgcn*<sup>Q52</sup> hemizygous flies, suggesting that *bgcn*<sup>Q52</sup> is a strong loss-of-function, and possibly null, allele. *w*<sup>1118</sup>, *bgcn*<sup>Q52</sup>/CyO and *bam*<sup>86</sup>/TM3Sb flies were used as controls. Enhancer-trap lines marking specific stages of somatic and germ cell differentiation during spermatogenesis, as well as the X-gal-staining procedure have been described elsewhere (Gönczy et al., 1992; Gönczy, 1995).

### Counting hub cells

The hubs were located in testes stained for Fasciclin III (see below) and hub cell number was estimated by counting nuclei revealed by Hoechst counterstaining.

### Generation of *bam* or *bgcn* mutant germline clones

Marked germline clones were generated by FLP-mediated recombination at FRT sites (Golic, 1991; Chou and Perrimon, 1992; Xu and Rubin, 1993). We used *P{hs neo; ry<sup>+</sup>; FRT}* inserted at 43D and *P{hs neo; ry<sup>+</sup>; FRT}* at 82B. Clones were induced in a background of germ cells heterozygous for a P-element driving *lacZ* expression in germ cells almost throughout spermatogenesis. In this manner, clones of mutant germ cells are recognizable as *lacZ*-negative cells. Males were collected on the day of eclosion, heat shocked for 60 minutes at 38°C, returned to food vials and aged for 5–7 days at 25°C prior to X-gal staining. The *lacZ*-expressing strains utilized were *ms(2)27CD* (P-element at 27CD) for clones on 2R (*bgcn* clones) and *eff<sup>7</sup>* (P-element at 88D) for clones on 3R (*bam* clones) (Castrillon et al., 1993).

### Whole-mount indirect immunofluorescence and immunocytochemistry

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). Testes were then incubated either for 90 minutes at room temperature or overnight at 4°C with primary antibodies at the appropriate dilution: 1:1500 for mouse anti-Bam polyclonal Bam.m2 (a gift from the McKearin laboratory; McKearin and Ohlstein, 1995); 1:50 for mouse anti-adducin-like protein monoclonal mAb 1B1 (a gift from the Lipshitz laboratory; Zaccari and Lipshitz, 1996); 1:50 for mouse anti-Fasciclin III monoclonal 7G10 (a gift from the Goodman lab; Patel et al., 1987). Testes were then rinsed three times for 10 minutes in PBX, incubated for 90 minutes at room temperature with secondary antibodies at the appropriate dilution: 1:100 Cy3-conjugated goat anti-mouse antibodies (Jackson Laboratories) or 1:400 Biotin-conjugated horse anti-mouse antibodies (Vector), which had been preadsorbed at their final dilution for 2 hours against an equal volume of fixed embryos. For indirect immunofluorescence, testes were counterstained for 2 minutes in 1 µg/ml Hoechst 33258 in PBX, washed three times for 10 minutes in PBX and once in PBS. For immunocytochemistry, testes were incubated for 50 minutes with

avidin-biotinylated horseradish peroxidase (Vectastain ABC kit, Vector), washed three times for 10 minutes, once for 30 minutes in PBX and processed for immunocytochemistry (Kellerman et al., 1990). Testes were counterstained with Hoechst as described above. Finally, testes were mounted in a drop of Vectashield (Vector) or 80% glycerol, and viewed by confocal (BioRad) or standard epifluorescence microscopy.

### Indirect immunofluorescence of individual cysts

This procedure was adapted from Hime et al. (1996). Testes were dissected in TB1 (15 mM potassium phosphate (equimolar di- and mono-basic), pH 6.7, 80 mM KCl, 16 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% PEG 6000) and transferred to a 7 µl drop of TB1 on a poly-L-lysine-coated glass slide. Testes were gently teased open towards the tip with a fine tungsten needle and cysts of germ cells were allowed to flow out for a few seconds, before being flattened under a siliconized 18 mm<sup>2</sup> coverslip. The preparation was frozen in liquid nitrogen and the coverslip was flipped off with a razor blade; usually, most of the cysts remained affixed to the slide. The slide was placed for 10 minutes in ethanol precooled to –20 °C. The cells were then covered with freshly prepared 3.7% formaldehyde-PBS for 10 minutes, and rinsed in slide carriers successively with 0.1 M glycine (2× 5 minutes) and PBS (2× 5 minutes). The cysts were then incubated with 1:500 rabbit anti-α spectrin polyclonal serum, (rabbit #905; Pesacreta et al., 1989) and 1:100 mouse anti-phosphotyrosine monoclonal 4G10 antibodies (Upstate Biotechnology Ltd., Lake Placid, NY) for 60 minutes at room temperature. Washes were 3× 5 minutes in PBS in a slide carrier, followed by a 60 minutes incubation with 1:200 Cy3-conjugated goat anti-rabbit and 1:200 FITC-conjugated goat anti-mouse secondary antibodies (both from Jackson Laboratories). The slides were then washed 3× 5 minutes in PBS, counterstained for 2 minutes with 1 µg/ml Hoechst 33258 in PBS and rinsed again in PBS. A drop of 80% glycerol was placed onto the tissue, which was then covered with a coverslip.

### BrdU labeling and immunocytochemistry

Testes were dissected in Ringer's (Ashburner, 1989) and incubated within 10 minutes of dissection for 30 minutes at 25°C with 10 µM BrdU (Boehringer Mannheim). Testes were rinsed with Ringer's, fixed for 60 minutes in 4% formaldehyde-PBX, rinsed several times in PBX, and processed for anti-BrdU and anti-Fasciclin III immunocytochemistry as described (Gönczy and DiNardo, 1996).

## RESULTS

### The fate and proliferation of somatic cyst and hub cells are not affected in *bam* or *bgcn* mutant testes

Although the small cells accumulating in *bam* or *bgcn* mutant testes have morphological characteristics of undifferentiated germ cells, we wanted to ascertain, by using enhancer-trap marker lines, whether the fate and proliferation of the somatic cyst and hub cells were affected in *bam* or *bgcn* mutant testes.

In wild-type testes, cyst progenitors behave as stem cells for the cyst lineage. It is thought that a pair of cyst progenitors gives rise to the two daughter cyst cells which surround each newly formed primary spermatogonial cell (Hardy et al., 1979; Gönczy and DiNardo, 1996). The two daughter cyst cells never divide, but continue to surround each packet of maturing germ cells throughout spermatogenesis (Fig. 2A, arrows). In *bam* or *bgcn* mutant testes, most markers of cyst progenitor and daughter cyst cell fates were appropriately expressed (Fig. 2B and data not shown). Importantly, only two daughter cyst cells surrounded each packet of small cells accumulating in *bam* or

*bgn* mutant testes (Fig. 2B, arrows and data not shown). Thus, initial progression through the cyst lineage appears unaffected and daughter cyst cells do not overproliferate in the absence of *bam* or *bgn* function.

In wild-type testes, germline stem cells (Fig. 2C, arrowheads) and cyst progenitors are anchored at the testis tip around a hub of 8–16 somatically derived cells (Hardy et al., 1979; Fig. 2C, arrow). In *bam* or *bgn* mutants, the hub was correctly positioned at the testis tip (Fig. 2D, arrow and data not shown). Hub cell number in *bam* homozygous mutant testes (12.9 cells; s.d.=2.8;  $n=33$  hubs counted) was indistinguishable from that in heterozygous siblings (12.7 cells; s.d.=1.9;  $n=33$  hubs counted). Similarly, hub cell number in *bgn* homozygous mutant testes (10.4 cells; s.d.=2.5;  $n=43$  hubs counted) was equivalent to that in heterozygous siblings (10.4 cells; s.d.=2.2;  $n=35$  hubs counted). Moreover, hub cells in *bam* or *bgn* mutant testes expressed Fasciclin III (see Fig. 7B, arrowhead), a cell adhesion molecule whose expression is restricted to the hub of wild-type testes (Brower et al., 1981; Gönczy et al., 1992). We did note that the Fasciclin III signal was more diffuse in 62% (20/32) of the hubs of *bgn* homozygous mutant testes (see Fig. 7B, arrowhead), perhaps suggesting a looser arrangement of cells. Except for this minor change in *bgn* mutants, these results indicate that the fate and proliferation of somatic cyst and hub cells are not affected by the absence of *bam* or *bgn* function.

#### ***bam* and *bgn* are required cell-intrinsically to restrict germ cell proliferation**

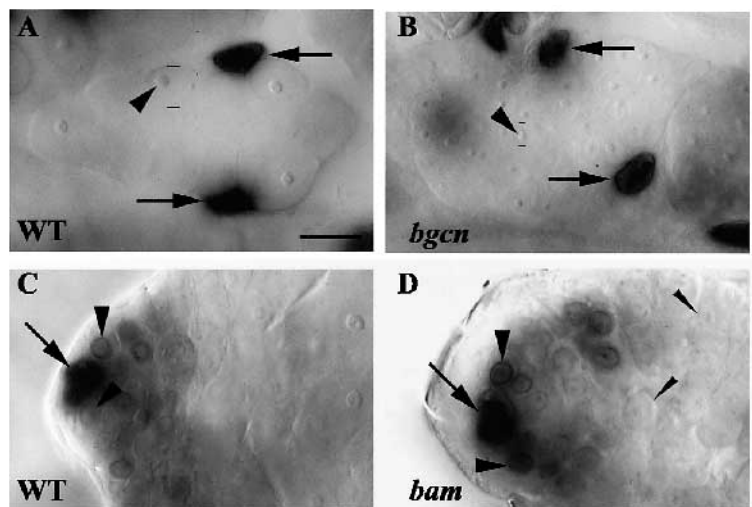
Given that somatic cyst and hub cells are largely unaffected in *bam* or *bgn* mutant testes, and that a variety of marker lines demonstrated that the small accumulating cells were indeed germ cells (see below and data not shown), we wanted to test whether *bam* and *bgn* are required cell-intrinsically to restrict germ cell proliferation. To this end, we generated marked mutant germline clones by FLP-mediated recombination at FRT sites (Golic, 1991; Xu and Rubin, 1993). Clones were generated in such a way that homozygous mutant germ cells were recognized by loss of *lacZ* expression (see Materials & Methods). Since FLP-induced recombination and chromosome segregation occur in dividing cells, during spermatogenesis, most clones are induced in individual mitotically amplifying spermatogonial cells, thus generating mosaic cysts that contain both *lacZ*-negative and *lacZ*-positive cells (data not shown). These mosaic cysts mature and move away from the testis tip within 2 days (Lindsley and Tokuyasu, 1980). Some of the testes that are analyzed 3–5 days later have cysts towards the tip that contain solely *lacZ*-negative cells. These persistent clones must be the progeny of a germline stem cell that was made homozygous at the time of FLP induction (see Gönczy and DiNardo, 1996). About 50% of testes had such germline stem cell clones.

Young males were heat-shocked to induce FLP expression, and aged for 5–7 days prior to X-gal staining. When control clones were induced, cysts with only *lacZ*-negative cells contained, at most, 16 large germ cells, each having the typical morphological features of maturing germ cells in the extended G<sub>2</sub> phase that precedes the meiotic divisions (Fig. 3B, arrows). When

homozygous *bgn* mutant clones were induced, cysts with only *lacZ*-negative cells usually contained many more than 16 small germ cells, each having the morphological features of amplifying germ cells (Fig. 3C, arrows). These cysts were indistinguishable from those found in mutant testes completely lacking *bgn* function. Moreover, *lacZ*-negative cysts containing 16 large germ cells were never observed ( $n=15$  testes with *lacZ*-negative cysts). Thus, *bgn* is required cell-intrinsically to restrict germ cell proliferation during spermatogenesis. Similarly, when homozygous *bam* mutant clones were induced, cysts with *lacZ*-negative cells usually contained many more than 16 germ cells, which were small and had the morphological features of amplifying germ cells (Fig. 3D, arrow). These cysts were indistinguishable from those found in mutant testes completely lacking *bam* function. In addition, *lacZ*-negative cysts containing 16 large germ cells were never observed ( $n=10$  testes with *lacZ*-negative cysts). Thus, *bam* is also required cell-intrinsically to restrict germ cell proliferation during spermatogenesis.

#### **Germ cells accumulating in *bam* or *bgn* mutant testes express markers of both stem cell and gonial cell fates**

Since *bam* or *bgn* mutant testes had essentially indistinguishable characteristics by the remaining criteria examined, we



**Fig. 2.** Somatic cyst and hub cells are not affected in *bam* or *bgn* mutant testes. X-gal staining. (A,B) A dissected cyst from a wild-type (A) or *bgn* mutant (B) testis carrying a cyst cell marker line (473), which normally labels daughter cyst cells associated with amplifying spermatogonial cells and more mature germ cells. There are two cyst cell nuclei (arrows) per cyst of germ cells in both cases. Note that the wild-type cyst contains 16 large germ cells (10 in this focal plane), each having a large nucleus (region between thin lines) and prominent nucleolus (arrowhead); while in the *bgn* mutant cyst, there are many more germ cells (at least 40 in this focal plane), each with a small nucleus (between thin lines) and nucleolus (arrowhead). An identical result was obtained with *bam* mutant testes. (C,D) Testis tip from a wild-type (C) or *bam* mutant (D) testis carrying a marker line (M5-4) which normally labels hub cells (C, arrow), as well as germline stem cells (C, arrowheads) and their immediate daughters. This marker line demonstrates that hub cells (D, arrow) are present and correctly located at the tip of *bam* mutant testes. In addition, this marker line demonstrates that *lacZ* expression is not maintained in *bam* mutant germ cells located away from the testis tip (D, thin arrowheads). A similar result was obtained with *bgn* mutant testes. Bar, 50  $\mu$ m; all panels are at the same magnification.

discuss subsequent results obtained with both mutants jointly in the text. The data is usually illustrated by a single figure panel for either *bam* or *bgcn*, while occasional minor differences between the two mutants are reported in the figure legends.

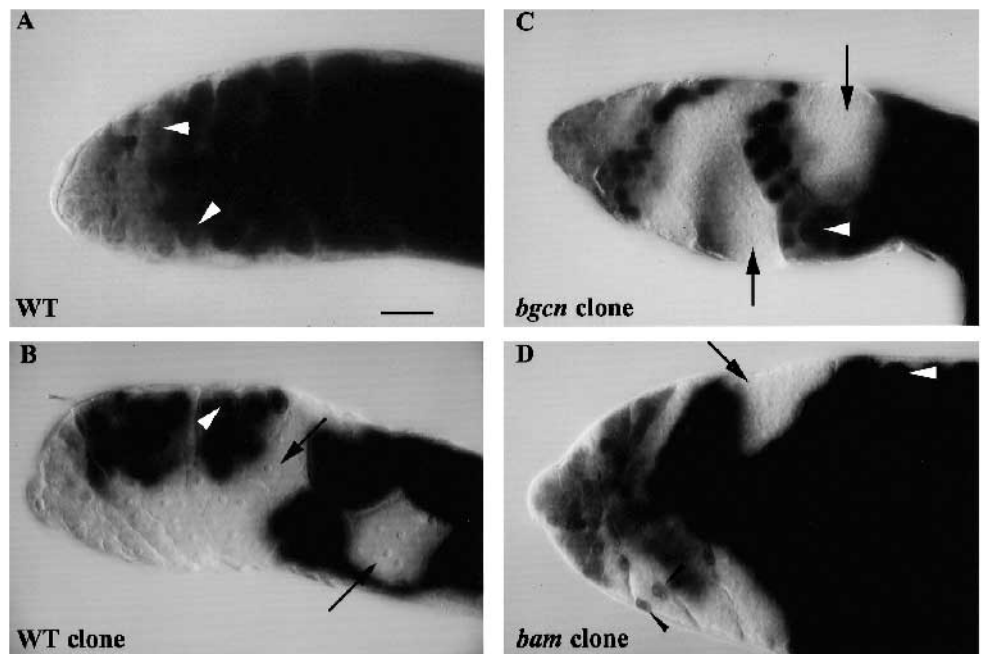
It had been postulated that mutations in *bam* or *bgcn* may interfere with germline sex determination (Mahowald and Wei, 1994; Wei et al., 1994), since mutations in the female germline sex determination genes *Sxl*, *otu* or *snf* also result in the accumulation of undifferentiated germ cells in the ovary (Gollin and King, 1981; King and Riley, 1982; Oliver et al., 1993). Therefore, we examined whether germ cells in *bam* or *bgcn* mutant testes were sexually transformed by using germline sex-specific enhancer-trap markers. Three independent marker lines, which are expressed in early male germ cells (Fig. 2C, arrowheads and Fig. 4A, arrow) but not in early female germ cells (Gönczy et al., 1992; Gönczy, 1995), were expressed in *bam* or *bgcn* mutant testes (Fig. 2D, arrowheads and Fig. 4B, arrow). This indicates that germ cells in *bam* or *bgcn* mutant testes are not sexually transformed.

An overproliferation of male germ cells in mutant testes could reflect a requirement for *bam* or *bgcn* function during spermatogenesis in assigning the primary spermatogonial cell fate, in restricting the number of amplificatory mitoses to four or in promoting entry into the meiotic cell cycle. We utilized enhancer-traps that help distinguish the fate of germline stem cells from that of primary and secondary spermatogonial cells as markers to determine what step of the progression through the male germ lineage was affected in mutant testes.

Some markers which normally express *lacZ* strongly in germline stem cells (Fig. 4C, arrow), and to a weaker extent in primary spermatogonial cells, were still robustly expressed in germ cells accumulating some distance away from the tip of *bam* (3 independent marker lines) or *bgcn* mutant testes (1 marker line; Fig. 4D, solid arrowhead), at a position in the testis where *lacZ* expression has been long turned off in wild type (compare Fig. 4C with Fig. 4D). Germ cells located even further away from the testis tip often appeared to be dying, as judged by their cellular morphology and fragmented DNA (data not shown). In summary, *bam* or *bgcn* mutant

germ cells accumulating some distance away from the testis tip retain some characteristics of stem cell fate.

However, other markers that normally express *lacZ* strongly in germline stem cells (Fig. 2C, arrowhead), and to a weaker extent in primary spermatogonial cells, were not expressed in germ cells accumulating some distance away from the tip of *bam* (3 marker lines tested) or *bgcn* mutant testes (2 marker lines tested; Fig. 2D, thin arrowheads). Thus, accumulating mutant germ cells have also lost some characteristics of germline stem cells and have probably progressed past the very early stages of spermatogenesis. Accordingly, such germ cells in mutant testes expressed a marker that is specific to secondary spermatogonial cells in late stages of amplification and to germ cells early in premeiotic G<sub>2</sub> (Fig. 4E, arrow and 4F, arrowhead).



**Fig. 3.** *bam* and *bgcn* are required cell autonomously to restrict germ cell proliferation. Germline clones are revealed as *lacZ*-negative cells in the background of *lacZ*-positive germ cells. X-gal staining. (A) Negative control; no heat-shock (relevant genotype FRT{43D} *ms*-6491(*lacZ*)/FRT{43D} *bgcn*; *hs*-FLP99/+). In the absence of heat-shock, no germline clone was induced in this testis, and all germ cells expressed *lacZ* (white arrowheads). (B) Positive control; heat-shock (relevant genotype FRT{43D} *ms*-6491(*lacZ*)/FRT{43D} +; *hs*-FLP99/+). A clone of wild-type germ cells is visible as cysts of *lacZ*-negative germ cells (arrows) in an otherwise *lacZ*-positive background (white arrowhead). The more mature cysts of the clone, for example the one indicated by the right-most arrow, contain 16 large, *lacZ*-negative germ cells (6 visible in this focal plane), which have a size and morphology typical of maturing germ cells in the extended G<sub>2</sub> phase that precedes the meiotic divisions. (C) *bgcn* clone; heat-shock (relevant genotype: FRT{43D} *ms*-6491(*lacZ*)/FRT{43D} *bgcn*; *hs*-FLP99/+). A clone of *bgcn* mutant germ cells is visible as cysts containing *lacZ*-negative germ cells (arrows) in an otherwise *lacZ*-positive background (white arrowhead). Cysts of mutant germ cells contain many more than 16 germ cells (arrows), which have a size and morphology typical of amplifying germ cells. Less mature cysts (closer to the tip) consist of the usual 2, 4 and 8 germ cells. (D) *bam* clone; heat-shock (relevant genotype: FLP99; FRT{82B} *ms*-1011(*lacZ*)/FRT{82B} *bam*). A clone of *bam* mutant germ cells is visible as cysts of *lacZ*-negative germ cells (arrow) in an otherwise *lacZ*-positive background (white arrowhead). Cysts of mutant germ cells contain many more than 16 germ cells (arrow), with the morphology typical of amplifying germ cells. Although *lacZ*-expressing germ cells, which are heterozygous for *bam*<sup>86</sup>, were usually phenotypically normal, we observed cases where they overproliferated. This haploinsufficient effect was observed in the presence or absence of FLP-expression, and in several other strains carrying the *bam*<sup>86</sup> allele, and did not interfere with the interpretation of the germline clones. The *ms*-1011(*lacZ*) marker line is expressed in cyst cells (thin arrowheads) as well as germ cells. Bar, 50  $\mu$ m; all panels are at the same magnification.



Taken together, these observations suggest that germ cells accumulating in *bam* or *bgn* mutant testes have a mixed identity, simultaneously retaining some aspects of stem cell fate while expressing markers of amplifying spermatogonial and primary spermatogonial cell fates.

### Cytoplasmic Bam protein is present in *bgn* mutant germ cells

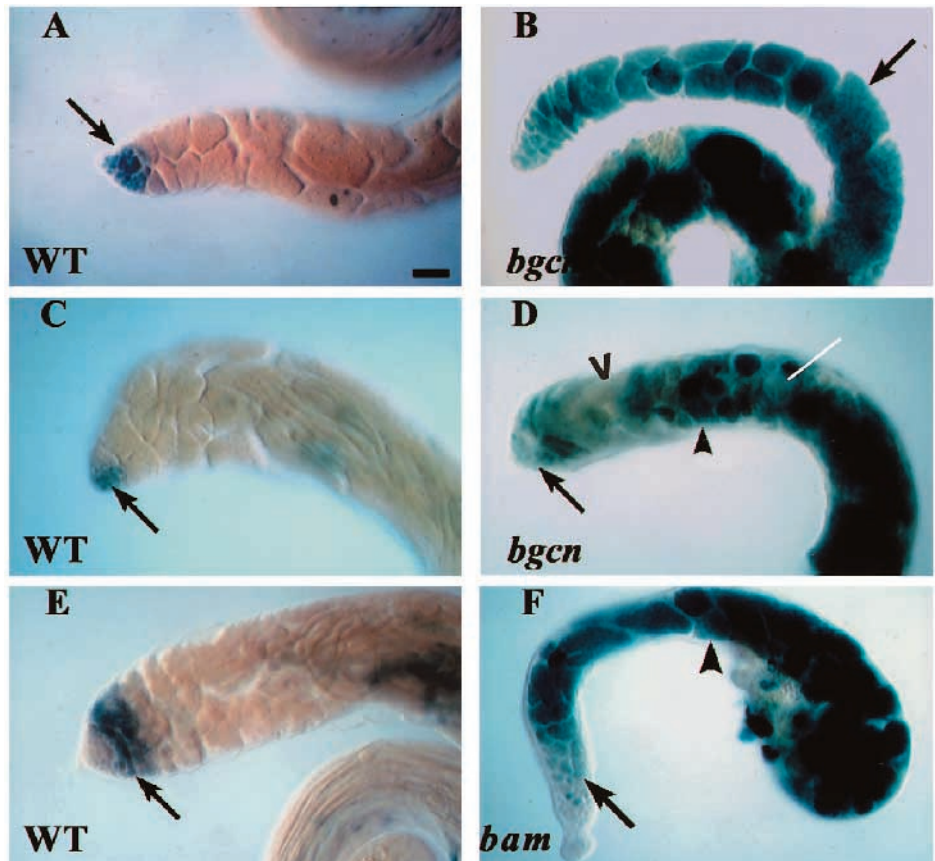
We sought additional ways to investigate whether accumulating germ cells in mutant testes have adopted aspects of the spermatogonial cell fate. To this end, we tested whether cytoplasmic Bam protein was expressed in *bgn* mutant germ cells. In wild-type testes, cytoplasmic Bam protein is present in the cytoplasm of the primary spermatogonial cell and its amplifying spermatogonial progeny (Fig. 5A, arrow; McKearin and Ohlstein, 1995), but not in stem cells (Fig. 5B, arrowhead). In testes bearing homozygous *bgn* mutant clones, cytoplasmic Bam protein was present in the accumulating germ cells (Fig. 5C, large arrow). Cytoplasmic Bam protein was similarly present in germ cells from *bgn* homozygous mutant flies (data not shown). These observations further indicate that accumulating *bgn* mutant germ cells behave as amplifying germ cells. These observations also suggest that *bgn* function is not required for the proper expression of Bam protein.

### Many *bam* or *bgn* mutant germ cells within a cyst are interconnected

Another behavior that distinguishes a germline stem cell from a primary spermatogonial cell and its amplifying spermatogonial progeny is that cytokinesis is complete only during stem cell divisions. In contrast, incomplete cytokinesis takes place in primary and secondary spermatogonial cells at each of the four amplifying mitotic divisions, generating packets of 16 germ cells interconnected by ring canals (Hardy et al., 1979). The fusome forms a continuous and branched structure in such a packet, extending cytoplasmically between ring canals (Lin et al., 1994; McKearin and Ohlstein, 1995; Hime et al., 1996). We addressed whether accumulating *bam* or *bgn* mutant germ cells undergo incomplete cytokinesis by determining if branched fusomes extend between several neighbouring mutant cells within a cyst. In wild type, the fusome was labeled with the F-actin binding drug phalloidin, as well as with antibodies to an adducin-like

protein and to alpha-spectrin (Fig. 6A, arrows and arrowhead, and data not shown; Hime et al., 1996). In *bam* or *bgn* mutant testes, staining with phalloidin (data not shown), anti-adducin like antibodies and anti-alpha spectrin antibodies revealed that groups of several neighbouring mutant germ cells within a cyst were linked via fusome-like cytoplasmic extensions (Fig. 6B, arrows and arrowhead and Fig. 6C, arrowheads). However, some mutant germ cells were only connected by a short structure labeled with these antibodies (see Fig. 6B, small arrowheads), perhaps because this subset of germ cells are connected to a single neighbour.

We estimated the fraction of germ cells within one cyst that were connected to neighbouring germ cells by examining well-isolated cysts in squashed preparations stained with anti-alpha



**Fig. 4.** Germ cells accumulating in *bam* or *bgn* mutant testes are not sexually transformed, and express markers of both stem cell and amplifying germ cell fates. X-gal staining. (A,B) A marker line (606) labeling early germ cells in the male (A, arrow) but not in the female (Gönczy et al., 1992), expresses *lacZ* in *bgn* mutant testes (B, arrow). An identical result was obtained with *bam* mutant testes. (C,D) A marker line (M34a) normally labeling germline stem cells (C, arrow) and their immediate daughters, is expressed in *bgn* mutant germ cells, whether they are located close to the testis tip (D, arrow) or away from it (D, solid arrowhead). Expression appeared in some cases to be lower in earlier stage cysts (open arrowhead) compared to later stage cysts (solid arrowhead). Cysts located even further away from the tip (white line) often contained dying germ cells which look refractile by Nomarski optics and have fragmented DNA as visualized by staining with the DNA dye Hoechst (not shown). Similar results were obtained with *bam* mutant testes, although the maintenance of *lacZ* expression away from the testis tip was less pronounced than in *bgn* mutant testes. (E,F) A marker line (ms-987) normally labeling a stripe of germ cells as they exit the amplification phase and enter the extended G<sub>2</sub> phase preceding the meiotic divisions (E, arrow) is maintained in *bam* mutant germ cells (F, arrowhead) located away from the testis tip. A similar result was obtained with *bgn* mutant testes. Bar= 50 µm; all panels are at the same magnification.

spectrin antibodies. In a wild-type cyst with 16 germ cells, 8 germ cells are connected to a single neighbour, while 8 are connected to more than one (see Fig. 1). We found that 95% of *bam* mutant germ cells ( $n=82$  germ cells examined within one cyst) had a fusome that extended into at least one neighboring cell, and that 25 to 50% of them had a fusome that in fact extended into more than one neighbouring cell. Similar results were observed in *bgcn* mutant testes ( $n=$  about 50 germ cells examined within one cyst). Although serial electron micrograph reconstructions will be needed to exactly quantify the extent of interconnection in mutant testes, our present observations unequivocally indicate that most overproliferating *bam* or *bgcn* mutant germ cells are interconnected, and have thus undergone only incomplete cytokinesis.

We verified that ring canals, the intercellular bridges through which the fusome normally extends, were also present between interconnected germ cells in *bam* or *bgcn* mutant testes. In the wild type, phosphotyrosine-containing epitopes become apparent in ring canals towards the end of the spermatogonial amplification stage (Hime et al., 1996). In *bam* or *bgcn* mutant testes, phosphotyrosine-containing epitopes similarly revealed the presence of ring canals (Fig. 6D, arrows). Double-labeling with anti-spectrin and anti-phosphotyrosine antibodies confirmed that the fusome passes through ring canals in *bam* or *bgcn* mutant testes (Fig. 6C,D), as it does in the wild type (Hime et al., 1996). Also, cells containing multiple ring canals were seen, verifying that some cells are connected to more than one neighbour (see Fig. 6D).

Taken together, the distribution of both fusome and ring canal components indicates that *bam* or *bgcn* mutant germ cells are interconnected as a result of incomplete cytokinesis during the divisions leading to overproliferation. This lends further support to the hypothesis that *bam* or *bgcn* mutant germ cells behave as amplifying primary or secondary spermatogonial cells, and not as stem cells.

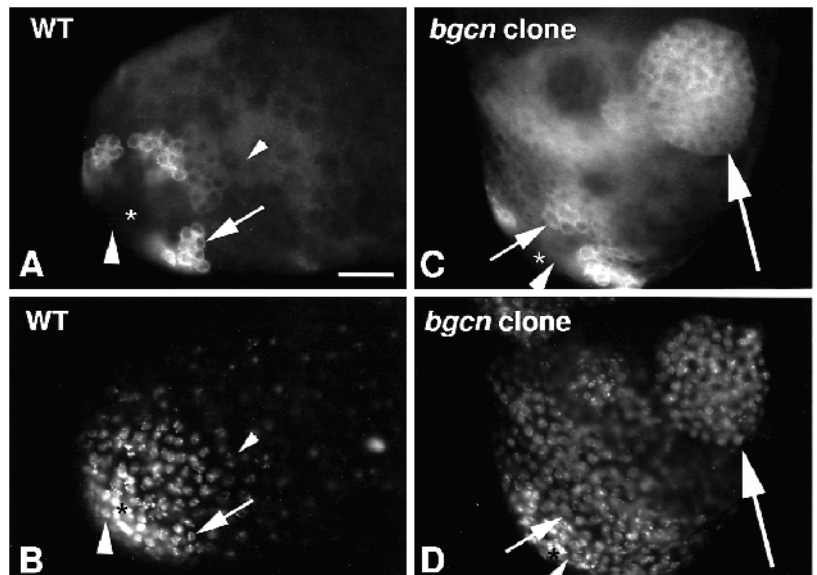
#### ***bam* and *bgcn* germ cells within a cyst undergo S phase in synchrony**

In contrast to what we observed in testes, germ cells in *bam* mutant ovaries are largely not physically connected to a neighbouring cell, and none is connected to more than one (McKearin and Ohlstein, 1995). Accordingly, neighbouring germ cells in *bam* mutant ovaries do not undergo S phase in synchrony (McKearin and Ohlstein, 1995). Instead, individual mutant germ cells incorporate BrdU at any given time, as would individual germline stem cells in a wild-type ovary. Since we have shown that accumulating germ cells within *bam* or *bgcn* mutant testes are extensively connected to their neighbours, we wanted to determine whether mutant germ cells within a cyst would also progress through the cell cycle in synchrony.

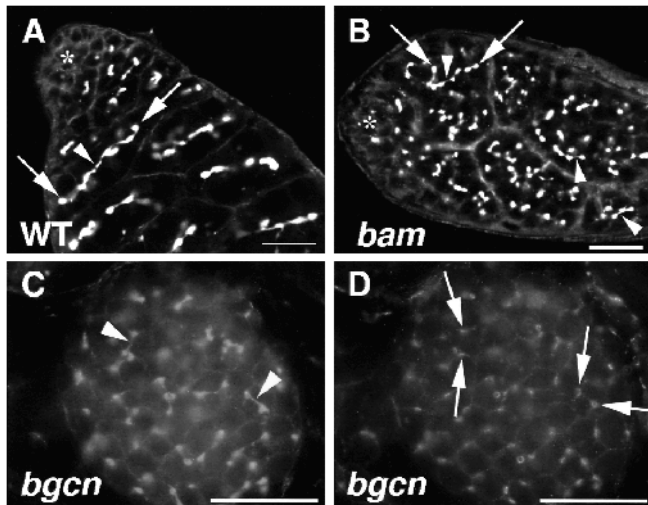
Wild-type and mutant testes were incubated with BrdU for 30 minutes and processed for anti-BrdU and anti-Fasciclin III immunocytochemistry to reveal cells that had undergone S phase and cells of

the hub, respectively. In the wild type, BrdU-positive nuclei (Fig. 7A, arrow) were restricted to the testis tip, in the vicinity of the hub (Fig. 7A, arrowhead). All germ cells within a wild-type cyst underwent S phase in near synchrony (Fig. 7A, arrow). The cyst furthest from the hub to incorporate BrdU contained 16 germ cells, presumably in premeiotic S (Fig. 7A, arrow). Cysts with 16 labeled germ cells were always located close to the tip, indicating that premeiotic S closely follows the four rounds of amplifying mitotic divisions. No incorporation was observed away from the tip, as wild-type germ cells entered the extended G<sub>2</sub> period leading to the meiotic divisions.

In contrast, in *bam* or *bgcn* mutant testes, BrdU-positive nuclei were observed not only in the vicinity of the hub (Fig. 7B, arrowhead), but also away from it (Fig. 7B, arrows), past the region to which proliferation is normally restricted (see Fig. 7A). Most or all mutant germ cells within a cyst underwent S phase in near-synchrony (Fig. 7B, arrows). As expected, there were more than 16 germ cells within most BrdU-positive cysts (Fig. 7B arrows). Mutant germ cells within a cyst similarly underwent M phases in near-synchrony (data not shown). Thus, accumulating *bam* and *bgcn* mutant germ cells within a cyst indeed progress through the cell cycle in synchrony, perhaps



**Fig. 5.** Cytoplasmic Bam protein is present in *bgcn* mutant germ cells. A wild-type testis (A,B) and a testis containing clones of *bgcn* mutant germ cells (C,D), processed for indirect immunofluorescence with Anti-Bam cytoplasmic antibodies (A,C) and counterstained with the DNA dye Hoechst (B,D). The arrows and arrowheads in A and B point to the same location, as do those in C and D. (A) Bam protein is normally expressed in the cytoplasm of primary and secondary spermatogonial cells (arrow), but not in that of germline stem cells (large arrowhead), which are located adjacent to the hub, the approximate position of which is indicated by the asterisk in A and C. (B) The nuclei of stem cells (large arrowhead), primary and secondary spermatogonial cells (arrow) fluoresce brightly with Hoechst, whereas the nuclei of later maturing germ cells (small arrowhead; spermatocytes) fluoresce weakly. (C) Cytoplasmic Bam protein is produced in clones of *bgcn* mutant germ cells (large arrow). The arrowhead marks the position of a germline stem cell adjacent to the hub (asterisk), the small arrow that of the normal domain of primary and secondary spermatogonial cell Bam expression. (D) The clone of *bgcn* mutant germ cells producing cytoplasmic Bam protein is comprised of early germ cells that fluoresce brightly with Hoechst (large arrow). Bar, 50 µm; all panels are at the same magnification.



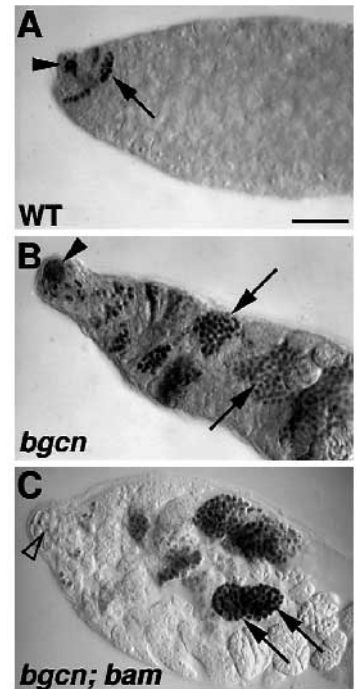
**Fig. 6.** Groups of *bam* or *bgcn* mutant germ cells are interconnected within a cyst. (A,B) Wild-type (A) and *bam* mutant (B) testes processed for indirect immunofluorescence with anti-adducin-like mAb 1B1 antibodies and analyzed by confocal microscopy. (A) The antigen recognized by mAb 1B1 is present in the fusome that runs through the ring canals and extends cytoplasmically in germ cells within a cyst. Like other fusome antigens, the one recognized by mAb 1B1 is present both along the thinner portion of the fusome which runs between neighbouring cells (arrowhead), as well as on the knob-like structure (arrows) found at the distal part of the fusome in each cell. For clarity, the arrows and arrowhead point to the relatively large fusome in a cyst of maturing germ cells. Note, however, that the branched fusome is present in younger cysts located closer to the testis tip. The asterisk in A and B indicates the probable position of the hub. (B) mAb 1B1 shows that the fusome runs through several neighbouring germ cells within a *bam* mutant cyst, with both the thinner portion (arrowheads) and the knob-like structure (arrows) being present. A similar result was obtained with *bgcn* mutant testes. Some cells that do not appear to be extensively connected by a fusome are in fact connected to cells above or below this focal plane; however, we also noted in both mutants that cysts also contain germ cells with a small fusome extending into only a single neighbouring cell. (see text for estimated connectedness) (C,D) Isolated *bgcn* mutant cysts processed for double indirect immunofluorescence with anti-alpha spectrin (C) and anti-phosphotyrosine (D) antibodies and viewed by epifluorescence. (C) alpha-spectrin is present in the fusome that extends cytoplasmically in germ cells within a cyst (arrowheads). Note that the fusome connects several neighbouring germ cells within a *bgcn* mutant cyst. Some cells that do not appear to be extensively connected by a fusome are in fact connected to cells above or below this focal plane (see text for estimated connectedness). (D) phosphotyrosine epitopes are present on ring canals in *bgcn* mutant cysts (arrows). Note that the fusome (panel C, arrowheads) connects several neighbouring germ cells within a *bgcn* mutant cyst as it passes through ring canals (D, corresponding arrows). Bars, 50  $\mu$ m.

because cell cycle regulators can freely diffuse between interconnected mutant cells.

#### ***bgcn; bam* double mutant germ cells also are interconnected and undergo S phase in synchrony**

Germ cells singly mutant for *bam* or *bgcn* behave as amplifying primary or secondary spermatogonial cells, since they undergo incomplete cytokinesis and progress through the cell

**Fig. 7.** *bam* or *bgcn* singly mutant germ cells and *bgcn;bam* doubly mutant germ cells within a cyst undergo S phases in synchrony. (A,B) 30 minute BrdU incorporation, followed by anti-BrdU and anti-Fasciclin III immunocytochemical detection. (C) 30 minute BrdU incorporation, followed by anti-BrdU immunocytochemical detection. (A) Apical region of wild-type testis. BrdU incorporation occurs at the tip, in the region where stem cells and amplifying germ cells are located. Some nuclei in the vicinity of the hub (arrowhead) undergo S phase; slightly away from the hub, cysts of germ cells at the 4-, 8- and 16- (arrow) cell stage incorporate BrdU; note that germ cells within a cyst undergo S phase in synchrony. No S phases occur further away from the hub, during the remainder of spermatogenesis. (B) Apical region of *bgcn* mutant testis. Some nuclei in the vicinity of the hub (arrowhead) undergo S phase; cysts that contain up to over one hundred synchronously replicating nuclei (arrows) are found away from the tip, in an area where no S phases are observed in the wild type. Since it is difficult to identify all the germ cells that belong to a given mutant cyst, we can not formally exclude that a few cells within a mutant cyst do not undergo S phase in synchrony. (C) Apical region of *bgcn;bam* double mutant testis. Again, cysts that contain up to over one hundred synchronously replicating nuclei (arrows indicate two overlapping cysts) are found away from the tip. The location of the hub has been determined in this case by counterstaining with the DNA dye Hoechst (not shown) and is indicated by the empty arrowhead. Bar, 50  $\mu$ m; all panels are at the same magnification.



cycle in synchrony. However, this behavior could be due to partial redundancy between *bam* and *bgcn* function, and germ cells doubly mutant for *bam* and *bgcn* may behave as stem cells.

To address this possibility, we first determined whether *bgcn;bam* double-mutant germ cells had branched fusomes that extend between several neighbouring cells within a cyst. Staining with anti-adducin-like and anti-spectrin antibodies showed that groups of several *bgcn;bam* double-mutant germ cells within a cyst were linked via fusome-like cytoplasmic extensions, in a manner indistinguishable from either single mutant (data not shown). Again, we found that 95% of *bgcn;bam* double mutant germ cells ( $n=32$  germ cells examined within one cyst) had a fusome that extended into at least one neighboring cell and that 25-50% of them had a fusome that in fact extended into more than one neighbouring cell. We then determined by BrdU labeling experiments that *bgcn;bam* double-mutant germ cells within a cyst underwent S phase in near synchrony (Fig. 7C, arrows), in a manner indistinguishable from either single mutant (see Fig. 7B).



Taken together, these observations indicate that *bgcn*; *bam* double-mutant germ cells undergo incomplete cytokinesis and progress through the cell cycle in synchrony. Therefore, *bgcn*; *bam* double-mutant germ cells, just like germ cells in either single mutant, behave as amplifying primary or secondary spermatogonial cells, and not as stem cells, indicating that *bam* and *bgcn* are not partially redundant with each other.

## DISCUSSION

Spermatogenesis in *Drosophila* is a model system to dissect the mechanisms regulating progression through stem cell lineages. In the testis, a germline stem cell divides asymmetrically to generate a daughter primary spermatogonial cell that undergoes four amplificatory mitoses before entry into the meiotic cell cycle and differentiation. *bam* and *bgcn* regulate progression through the germline stem cell lineage as mutations in either locus result in the overproliferation of undifferentiated germ cells.

### ***bam* and *bgcn* may restrict the proliferation of amplifying germ cells during spermatogenesis**

To determine the step affected by the loss of *bam* or *bgcn* function, we asked whether the cells overproliferating in mutant testes resembled stem cells or instead primary or secondary spermatogonial cells. By utilizing enhancer-trap lines as cell fate markers, we found that overproliferating germ cells have a mixed identity, expressing some markers of stem cell fate as well as markers of amplifying germ cell fates. Which step, then, does *bam* and *bgcn* regulate during progression through the germline stem cell lineage? Although the mixed identity of overproliferating germ cells precludes a definitive answer, we discuss two possible scenarios.

First, *bam* and *bgcn* may play a role in assigning the primary spermatogonial cell fate. If this were the case, two stem cells should be generated by each stem cell division in mutant testes, resulting in the overproliferation of cells harboring stem cell characteristics. Why, then, do overproliferating germ cells in *bam* or *bgcn* mutant testes not maintain the expression of all stem cell markers, and why do they express markers of later amplifying germ cells? This could be because, although playing a role in assigning the primary spermatogonial cell fate, *bam* or *bgcn* may not be strictly necessary for the primary spermatogonial cell fate, thus explaining the partial adoption of amplifying germ cell fates in mutant testes. Alternatively, the maintenance of stem cell fate may depend on signals present at the testis tip, for instance emanating from the somatic hub cells. This would cause mutant germ cells to lose some aspects of stem cell fate as they move away from the hub, and, possibly by default, adopt that of amplifying germ cells. The existence of an extrinsic signal to maintain stem cell fate is suggested by analogy to other systems in which stem cell function is thought to require an appropriate stromal cell environment. For instance, various cytokines as well as the cell signaling ligand Steel appear to be required for hematopoietic stem cell activity (Lowry et al., 1992).

Alternatively, *bam* and *bgcn* may be required to restrict the proliferation of amplifying germ cells or to promote their entry into the meiotic cell cycle. If this were the case there should

be an accumulation of cells harboring the characteristics of amplifying germ cells in mutant testes. Why, then, would overproliferating germ cells in *bam* or *bgcn* mutant testes retain the expression of some stem cell markers? One possibility is that overproliferating mutant germ cells initially behave like amplifying germ cells, but then adopt again some aspects of the stem cell fate. In accordance with this hypothesis, we noted that some markers of stem cell fate which remain expressed in mutant testes are expressed at higher levels some distance from the hub, as if expression might have been shut off at first (see Fig. 4D, open arrowhead), but then re-initiated (see Fig. 4D, solid arrowhead). A similar reversion to an earlier stage in a stem cell lineage has been observed in mutations in *gld-1*, a *C. elegans* germline-specific tumor suppressor gene (Francis et al., 1995). In *gld-1* mutants, female germ cells initially enter the meiotic cell cycle, but then adopt again a proliferative behavior characteristic of germ cells at earlier stages of oogenesis (Francis et al., 1995).

We favor this second scenario, in which *bam* and *bgcn* are required to restrict the proliferation of amplifying germ cells or to promote their entry into the meiotic cell cycle, because germ cells accumulating in *bam* or *bgcn* mutant testes have several additional characteristics of amplifying germ cells. We found that cytoplasmic Bam protein is expressed in *bgcn* mutant germ cells, that *bgcn* and *bam* mutant germ cells undergo incomplete cytokinesis and that they proliferate in synchrony within a cyst. All three aspects are characteristic of amplifying germ cells, but not of germline stem cells. Therefore, we conclude that *bam* and *bgcn* most likely do not regulate the decision to adopt a primary spermatogonial cell fate, but, rather, act to restrict the proliferation of amplifying germ cells or promote their entry into the meiotic cell cycle.

### ***bam* and *bgcn* may regulate a different step in the male and female germline stem cell lineages**

The nature of germ cells overproliferating in *bam* or *bgcn* mutant testes is clearly distinct from that of germ cells overproliferating in *bam* or *bgcn* mutant ovaries. In *bgcn* mutant ovaries, branched fusomes are rarely observed (Lin et al., 1994) and serial reconstruction of *bam* mutant ovaries revealed that cytokinesis is complete in overproliferating mutant germ cells, as it is in wild-type germline stem cells (McKearin and Ohlstein, 1995). In addition, neighbouring *bam* mutant germ cells do not undergo S phase in synchrony, but as independent cells, as do wild-type germline stem cells (McKearin and Ohlstein, 1995). These observations indicate that, in ovaries, *bam*, and possibly *bgcn*, mutant germ cells proliferate as stem cells. This suggests that *bam*, and possibly *bgcn*, are necessary for the cystoblast cell fate during oogenesis. Accordingly, *bam* gene transcription is activated and cytoplasmic Bam protein first appears in cystoblasts, and not stem cells (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995). Moreover, forcing Bam expression in female germline stem cells leads to their loss, perhaps by their conversion into cystoblasts (Ohlstein and McKearin, 1997). This suggests that *bam* is not only necessary, but also sufficient for the cystoblast cell fate during oogenesis. By contrast, forcing Bam expression in male germline stem cells does not appear to affect them (Ohlstein and McKearin, 1997), further indicating that *bam* acts differentially during spermatogenesis and oogenesis.

It is surprising that *bam*, and possibly *bgcn*, would play a

different role in males and females, since the early steps in the two germline stem cell lineages are so similar. One possible explanation is that *bam* and *bgn* act at two steps in both sexes, initially specifying the primary spermatogonial or cystoblast cell fate, and subsequently restricting germ cell amplification, but that mutational analysis reveals their activity at a different step in each sex. For instance, in males, *bam* and *bgn* may participate in assigning the primary spermatogonial cell fate but the essential determinant could be a signal from neighbouring somatic cells. However, in amplifying male germ cells, *bam* and *bgn* would play an essential role in restricting proliferation. In females, *bam* and *bgn* may similarly participate in restricting proliferation of amplifying germ cells, but this role would be obscured by the earlier and essential requirement in assigning the cystoblast fate.

The function of *bam* and *bgn* may have been adapted to play an essential role in the cystoblast due to a unique requirement of the female germline stem cell lineage. In females, only one of the sixteen amplifying germ cells adopts the oocyte fate, while its fifteen sisters become nurse cells. Oocyte determination relies on polarized microtubule-dependent intercellular transport from the nurse cells into the oocyte through ring canals (Suter and Steward, 1991; Yue and Spradling, 1992; Theurkauf et al., 1993). The basis for this polarity appears to be established as early as in the cystoblast (Lin and Spradling, 1995). Since the fusome is associated with microtubules, it has been suggested that the fusome helps establish and maintain polarity during cystoblast and amplifying germ cell divisions (Lin et al., 1994; Lin and Spradling, 1995). Since a form of Bam protein is associated with the fusome (McKearin and Ohlstein, 1995), it is possible that Bam itself plays a role in establishing polarity in the cystoblast. In *bam* mutant ovaries, polarity could not be established, and the cystoblast cell fate would not be assigned, resulting in the overproliferation of cells with stem cell characteristics. By contrast, in males, all sixteen amplifying germ cells are equivalent, and there is no evidence of polarity analogous to that observed in females. This might explain why *bam* and *bgn* are not strictly necessary for assigning primary spermatogonial cell fate.

### Cell-intrinsic and cell-extrinsic restriction of germ cell proliferation

In many cell types, cooperation between cell-intrinsic and cell-extrinsic factors regulates cell-cycle progression. For instance, in budding yeast, cell-cycle arrest can be triggered by exposure to pheromones secreted by a cell of the opposite mating-type (reviewed by (Marsh et al., 1991; Reed, 1991). The cell-extrinsic pheromone signal is then transduced by cell-intrinsic components that eventually lead to an inhibition of Cdc28-Cln kinases and G1/S arrest (Peter et al., 1993; Peter and Herskowitz, 1994).

During spermatogenesis, the close association of germline cells with specific somatic cells raises the possibility that both cell-extrinsic and cell-intrinsic factors regulate progression through the germline stem cell lineage. We have shown that *bam* is required cell-intrinsically to restrict germ cell proliferation. This was not unexpected, because Bam protein is detected exclusively in germ cells of both ovaries and testes (McKearin and Ohlstein, 1995). In contrast, it is not known where *bgn* is expressed since this gene has not been cloned. However, in the female, reciprocal pole cell transplant experi-

ments had indicated that *bgn* is required in the germline to restrict germ cell proliferation in ovaries (Mahowald and Wei, 1994). Our mosaic analysis of *bgn* demonstrates a germline requirement for this gene during spermatogenesis as well. Thus, *bam* and *bgn* act intrinsically in the germline to restrict germ cell proliferation, and can be thought of as germline-specific tumor-suppressor genes.

In addition to the cell-intrinsic requirement for *bam* and *bgn* function, we have recently found that a signal from the somatic cyst cells is required to restrict germ cell proliferation (Matunis et al., 1997). If this cell-extrinsic signal is absent, germ cells overproliferate and behave as primary or secondary gonial cells as well, just like *bam* or *bgn* mutant germ cells. This raises the possibility that the cell-extrinsic signal affects the function of the germline intrinsic factors *bam* and *bgn*. Alternatively, the cell-extrinsic and cell-intrinsic pathways might be acting in parallel to modify components of the cell-cycle machinery, and thus regulate progression through the germline stem cell lineage.

### Spermatogenesis as a stem cell system

It has proven difficult to characterize the mechanisms that govern progression through complex stem cell lineages, such as mammalian hematopoiesis. This comes in part from the tedium of identifying and analyzing stem cells in these systems in their natural cellular environment. In contrast, during *Drosophila* spermatogenesis and oogenesis, the germline stem cells are clearly identified, as are neighbouring supporting somatic cells (Hardy et al., 1979; King and Riley, 1982; Margolis and Spradling, 1995; Gönczy and DiNardo, 1996). Experimental manipulation and genetic analysis of *Drosophila* gametogenesis have begun to uncover roles for intrinsic regulators as well as cell signaling in these relatively simpler stem cell lineages (McKearin and Spradling, 1990; Wei et al., 1994; Lin and Spradling, 1995; Margolis and Spradling, 1995; Forbes et al., 1996a,b; Gönczy and DiNardo, 1996; Matunis et al., 1997; this work). Some of these insights will likely extend to more complex systems, where it will remain difficult to systematically identify the regulators that govern progression through each step of the stem cell lineage.

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