Accumulation of a differentiation regulator specifies transit amplifying division number in an adult stem cell lineage

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Abstract

A key feature of many adult stem cell lineages is that stem cell daughters destined for differentiation undergo several transit amplifying (TA) divisions before initiating terminal differentiation, allowing few and infrequently dividing stem cells to produce many differentiated progeny. Although the number of progenitor divisions profoundly affects tissue (re)generation, and failure to control these divisions may contribute to cancer, the mechanisms that limit TA proliferation are not well understood. Here, we use a model stem cell lineage, the Drosophila male germ line, to investigate the mechanism that counts the number of TA divisions. The Drosophila Bag of Marbles (Bam) protein is required for male germ cells to cease spermatogonal TA divisions and initiate spermatocyte differentiation [McKearin DM, et al. (1990) Genes Dev 4:2242–2251]. Contrary to models involving dilution of a differentiation repressor, our results suggest that the switch from proliferation to terminal differentiation is triggered by accumulation of Bam protein to a critical threshold in TA cells and that the number of TA divisions is set by the timing of Bam accumulation with respect to the rate of cell cycle progression.

The anatomy of developing germ cell cysts makes the Drosophila germ line especially well suited for investigating how the number of TA divisions is controlled. Because TA sister cells descended from a common gonialblast are contained within a common somatic cell envelope and divide in synchrony, the number of rounds of TA division executed prior to differentiation can be assessed by counting the number of differentiated spermatocytes per cyst (Fig. 1A). Also, because TA cells are easily distinguishable from spermatocytes, genetic manipulations that perturb the counting mechanism can be discriminated from those that cause failure of the switch from TA divisions to differentiation.

Two genes required in germ cells for spermatogonia to stop TA divisions and differentiate into spermatocytes are bag of marbles (bam) and benign gonial cell neoplasms (bgcn) (9, 10). Male germ cells mutant for bam or bgcn undergo several extra rounds of mitotic TA division (Fig. 1D and E), fail to differentiate, and eventually die. Here we show that, in addition to being required for the switch from mitotic proliferation to meiotic differentiation, Bam also plays a role in counting the number of TA divisions. Our data suggest that timing of the switch from proliferation to differentiation is specified by accumulation of Bam protein to a critical level and the number of TA divisions is set by a combination of Bam accumulation and cell cycle length.

Results

The number of TA divisions before differentiation in the Drosophila melanogaster male germ line is tightly controlled. Counts of the number of spermatocytes per intact cyst confirmed that in wild-type, 99% of spermatocyte cysts counted had 16 cells (n = 112) (Fig. 1F and G), indicating exactly four rounds of TA division. Comparisons between species suggested that the number of TA divisions is under genetic control; in Drosophila pseudoobscura, 96% of spermatocyte cysts scored (n = 49) had 32 cells, indicating five rounds of TA division.

In absence of bam, TA cells continued to proliferate and failed to differentiate, undergoing several extra rounds of mitotic division as reported in refs. 9 and 10. While wild-type testes incubated for 2 min with the nucleotide analog 5-ethyl-2'-deoxyuridine (EdU) before fixation showed only 1-, 2-, 4-, 8-, and 16-cell clusters, testes from bam mutant males showed EdU incorporation into cells that were 32-, 64-, 128-, and 256-cell clusters.

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The authors declare no conflict of interest.

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incorporate EdU (arrow). (F normal cyst of differentiating spermatocytes. (H) Phase contrast image of compared to spermatocytes in panel G. 31% had exactly 32 cells/cyst and 12% had >32 cells/cyst (it was difficult to count spermatocyte number exactly in large cysts). Thirty-two-cell cysts expressed the spermatocyte-specific marker, Sa-GFP, indicating the germ cells had differentiated as spermatocytes (Fig. 1 J and K). The number of TA divisions was not affected in bgen/+ males (100% 16-cell cysts, n = 49), suggesting that bam may be the limiting component.

Turnover of Bam protein may help control the rate of Bam accumulation. The Bam protein has a predicted C-terminal PEST sequence (10), a motif thought to target proteins for rapid turnover (11). Flies with one copy of a bamΔPEST transgene and wild-type at the endogenous bam locus had 9% (n = 102) to 18% (n = 101) (depending on the transgenic line) of cysts prematurely differentiate with eight cells (Fig. 1L). Flies with one copy of the stronger bamΔPEST transgene and heterozygous for bam had 39% eight-cell spermatocyte cysts (n = 101) (Fig. 1M). Flies with two copies of the stronger bamΔPEST transgene and wild-type for the endogenous bam locus had 68% eight-cell spermatocyte cysts (n = 100) (Fig. 1N). The eight-cell cysts had large cells with the typical spermatocyte morphology (Fig. 1O), suggesting that the switch to differentiation occurred successfully, although prematurely. Postmeiotic cysts were observed with 32 instead of the normal 64 elongating spermatids (Fig. 1P), confirming that germ cells from bamΔPEST animals progressed through the meiotic divisions and into spermatid differentiation. In contrast, flies that were wild-type for endogenous bam and carried two copies of a wild-type bam transgene (a 2.9-kb genomic fragment that rescues bam mutant male and female sterility) (10) had no eight-cell spermatocyte cysts (99% 16-cell cysts, n = 100), suggesting that the early differentiation observed upon deletion of the PEST sequence might be due to premature accumulation of the stabilized Bam protein rather than to increased gene dosage. Two extra copies of wild-type bam may not accumulate Bam protein early enough to cause a premature switch to differentiation, because bam is likely transcriptionally repressed in early germ cells via the TGFβ signaling pathway in males (12, 13), as has been documented in the female germ line (14, 15). Evasion of early transcriptional repression of bam by expression under control of a heat shock promoter caused premature differentiation (18% eight-cell cysts, n = 91) after heat shock (2 h at 37 °C on days 7 and 8, dissected day 11 after starting cultures). Flies of the same genotype without heat shock (n = 104) and wild-type flies heat-shocked using the same protocol (n = 100) produced no eight-cell cysts.

The pattern of Bam protein expression in testes was consistent with Bam accumulation setting the timing of the switch from spermatogonial proliferation to spermatocyte differentiation. By immunofluorescence staining on wild-type testes, Bam protein was detected in the cytoplasm of spermatogonia in 4-, 8-, and 16-cell cysts in S phase at the tip of the testis, bam mutant testes also showed large cysts of 32 or more cells undergoing S phase far from the testis tip (Fig. 1 D and E compared to B and C; see also H compared to G). Two such overproliferating S-phase cysts scored had at least 150 cells, indicating more than seven rounds of TA division. The number of TA divisions appears to be set by the timing of Bam protein accumulation. Male germ cells lacking one copy of bam (bamΔPEST) usually made the transition from mitotic proliferation to spermatocyte differentiation, but often did so late; 43% of spermatocyte cysts had completed one or more extra TA divisions. Of 101 bam/+ spermatocyte cysts scored (Fig. 1J), 31% had exactly 32 cells/cyst and 12% had >32 cells/cyst (it was difficult to count spermatocyte number exactly in large cysts). Thirty-two-cell cysts expressed the spermatocyte-specific marker, Sa-GFP, indicating the germ cells had differentiated as spermatocytes (Fig. 1J and K). The number of TA divisions was not affected in bgen/+ males (100% 16-cell cysts, n = 49), suggesting that bam may be the limiting component.

Timing of the switch from TA cell divisions to terminal differentiation depends on Bam dosage. (A) Scheme of normal differentiation of Drosophila melanogaster male germ cells. (Blue) Bam protein, (red) somatic cyst cells, and (CySC) cyst stem cells, (GSC) germ-line stem cell, (GB) gonialblast, (H) hub. (B-E) Cysts in S phase in wild-type or bamΔPEST/bamΔPEST mutant testes marked by a short pulse of EdU nucleotide analog staining (green) and colabeled with DAPI (red) in panels C and E. (B and C) Wild-type: Only germ cells at the anterior tip incorporate EdU (arrow). (D and E) bamΔPEST/bamΔPEST: Germ cells undergo extra rounds of synchronous S phase in large cysts away from the tip (arrows). (F, L, M-N) Number of spermatocytes per cyst in indicated D. melanogaster genotypes. (Black bar) Normal 16 cells/cyst, indicating four rounds of division before differentiation. (F and G) Wild-type. (G) Phase contrast image of normal cyst of differentiating spermatocytes. (H) Phase contrast image of overproliferating spermatogonial cysts in bam mutant (note small size of cells compared to spermatocytes in panel G). (I-K) bamΔPEST/+ heterozygotes. "Other" in panel I refers to one 26-cell cyst and one 30-cell cyst. (L and K) Cyst with 32 spermatocytes visualized by (J) phase contrast and (K) fluorescence imaging of Sa-GFP, a differentiation marker. (L–P) A bamΔPEST transgene caused premature spermatocyte differentiation. (L) bamΔPEST/+ . (M) bamΔPEST/bamΔPEST; (N) bamΔPEST/bamΔPEST; (O) and (P) Phase contrast images of bamΔPEST/bamΔPEST;/+ . (Q) Eight-cell spermatocyte cyst. (P) Postmeiotic cyst with 32 spermatids instead of the normal 64 undergoing early elongation. (Scale bars, 50 μm.)
early 16-cell cysts, but not in stem cells, gonia, blast, or two-cell cysts (Fig. 2A–F). Bam protein levels dropped abruptly in 16-cell cysts soon after premeiotic DNA replication. In wild-type testes costained with anti-PCNA to mark S phase, Bam protein was detected in 6/6 16-cell cysts undergoing premeiotic DNA replication, indicated by the punctate pattern of PCNA in the nucleus.
individual cysts were separated were stained with anti-Bam and

Fig. 2. (A–C, arrow). Bam protein levels dropped abruptly to background after premeiotic S phase, concomitant with or immediately preceding loss of PCNA staining.

Bam protein accumulated to levels detectable by antibody staining early in four-cell cysts. Bam protein was not detected in any (0/6) two-cell mitotic anti-phospho histone H3 (PH3) positive cysts (Fig. 2D, arrowheads), but was detected in all (4/4) four-cell mitotic cysts scored from whole mount preparations (Fig. 2E, arrowheads). Consistent with this, in squashed preparations where cysts were separated from each other and somatic cells were labeled with ptc-Gal4/UAS-GFP to outline cysts, 0/5 two-cell cysts and 9/9 four-cell cysts had Bam protein (Fig. 2G).

The four-cell cysts outlined with the somatic cell marker showed a variety of Bam levels from low to high, consistent with early onset and then increasing levels of Bam protein during the four-cell stage.

In immunofluorescence images of squashed preparations with ptc-Gal4/UAS-GFP, Bam immunofluorescence appeared to be low in four-cell cysts, higher in eight-cell cysts, and lower again in 16-cell cysts (Fig. 2F). To quantify anti-Bam immunofluorescence in different stage cysts, squashed testis preparations where individual cysts were separated were stained with anti-Bam and anti-PH3 to mark mitosis. The mean anti-Bam fluorescence signal per pixel (see Materials and Methods) was quantified in the cytoplasm of individual 4-, 8-, and early 16-cell interphase or mitotic (anti-PH3 positive) cysts (Fig. 2H).

In wild-type, Bam levels were low in interphase four-cell cysts (note, only Bam positive cysts at this stage could be scored), increased in mitotic four-cell cysts, reached a peak during interphase eight-cell cysts, decreased slightly in early 16-cell cysts, and then abruptly decreased in later 16-cell cysts (Fig. 2K).

Measurements of anti-Bam immunofluorescence in individual males carrying the bamΔPEST transgene indicated that Bam protein accumulated to a peak earlier in this genotype, consistent with the earlier switch from spermatogonia to spermatocytes. In bamΔPEST/bamΔPEST; +/+ testes, one out of the five 2-cell PH3 positive cysts scored had positive Bam staining, although levels were low (Fig. 2I) and levels of Bam protein peaked in four-cell interphase cysts in bamΔPEST/bamΔPEST; +/+ testes (Fig. 2L). Although it was not possible to compare absolute levels of Bam protein between wild-type and bamΔPEST/bamΔPEST; +/+ testes in the two separate experiments, the timing of Bam accumulation was clearly shifted earlier in flies carrying the bamΔPEST transgene.
In bam/+ males with half the bam gene dosage, the Bam protein accumulated to detectable levels later in the four-cell stage than in wild-type. Using ptc-Gal4/UAS-GFP to outline cysts so they could be identified regardless of Bam status, 0/7 two-cell and only 8/13 four-cell cysts scored had Bam protein in bam/+ males (Fig. 2J) as compared with all four-cell cysts in wild-type. In the same preps, Bam fluorescence was observed in PH3+ 16-cell and >16-small-cell cysts. Measurements of anti-Bam immunofluorescence in cysts from bam/+; ptc-Gal4/UAS-GFP males showed peak Bam levels at the eight-cell and again at the 16-cell stage (Fig. 2M), consistent with two populations of cells, one population that would differentiate as 16-cell cysts and another population that would differentiate as 32 or >32-cell cysts as observed by phase contrast microscopy (Fig. 1I).

Manipulation of cell cycle regulators in spermatogonia indicated that the number of TA divisions in the male GSC lineage may be specified by interplay between the timing of Bam accumulation and cell cycle length. A transgene expressing Gal4 (16) under control of the bam promoter drove expression of a UAS-GFP reporter starting in late spermatogonial cysts (Fig. S1). When a positive regulator of the G2/M transition, string (stg), a homolog of S. pombe cdc25 (17), was forcibly expressed in late TA cells using bamGal4, cells in 97% of cysts completed one or two (but never more) extra rounds of TA division before differentiating (n = 100 cysts). Sixty-five percent of the spermatocyte cysts had exactly 32 cells/cyst. In addition, 21% had between 16 and 32 cells/cyst, suggesting that a subset of cells underwent an extra division (Table S1), and 11% had >32 cells/cyst (Fig. 3A and B).

Conversely, when the cell cycle inhibitor fruhsart (frs) (18) was forcibly expressed using bamGal4, 36% of cysts differentiated with eight spermatocytes rather than 16 (n = 106) (Fig. 3C). Similarly, forced expression of the cell cycle inhibitor tribbles (trb), which causes degradation of Stg (19), resulted in 63% eight-cell cysts (n = 104 cysts) (Fig. 3D and E). The effect of forced expression of trb was suppressed by reducing the bam dosage by half; the frequency of eight-cell spermatocyte cysts decreased from 63 to 10%, and the frequency of 16-cell spermatocyte cysts increased from 37% to 87% in bamGal4/bam+/+; UASfrs testes (Fig. 3F).

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(Figs. 2L and 4A) may allow the switch to occur early, resulting in eight-cell spermatocyte cysts. In wild-type, Bam levels appear to peak in the eight-cell stage (Fig. 2K), before the last mitotic division and considerably before Bam is down-regulated. We suggest that accumulation of Bam to a threshold in G2 of the eight-cell stage may cause germ cells to initiate the spermatocyte program after completing the eight-cell M-phase. As a result, 16-cell cysts execute premeiotic DNA replication, then enter meiotic prophase. The subsequent abrupt down-regulation of Bam protein may be a downstream effect of initiating the spermatocyte program.

Threshold Bam levels may be required for differentiation, because Bam may need to fully titrate an inhibitor to trigger the switch to differentiation. Recent studies indicate that Bam and its partner Bgcn act by translational repression and suggest an antagonistic role with the translational initiation factor eIF4a (23, 24). As cysts from eIF4a+/−; bam+/+ testes overproliferate less than cysts from bam+/+; sibling control testes [8/94 (9%) as compared to 28/87 (32%); Table S3], it will be interesting to explore whether eIF4a, some other component involved in translational control, or an mRNA encoding an inhibitor of differentiation might set the threshold requirement for Bam. Interestingly, although bam is not required for male germ cells to initiate TA divisions, forced early expression of bam is sufficient to drive GSCs to differentiation (25) or at high sustained levels, to death (26). These observations suggest that Bam may have a variety of targets and that timing and levels of expression of this potent regulator must be tightly controlled for normal differentiation in the male GSC lineage.

Accumulation of a critical differentiation factor to a threshold may also limit TA divisions and trigger the switch to differentiation in certain mammalian stem cell lineages. In oligodendrocyte precursor cells, which also have a restricted number of divisions, accumulation of a cdk inhibitor triggers differentiation. Although the molecular identity of the factor accumulating is different, the regulatory logic is similar (27).

**Materials and Methods**

**Fluorescence Quantification.** Testes from newly eclosed males for all genotypes were fixed in squashed preparations and stained with anti-Bam. A sample subset was also stained with anti-Ph3. Another sample subset expressed the somatic cell marker ptcGAL4/UAS-GFP. Cysts (as in Fig. 2 F–J) were imaged in a single plane. The mean fluorescence intensity/pixel of several nonoverlapping 12 × 12 pixel areas in the cyst cytoplasm was measured and averaged for each cyst. The mean background fluorescence/pixel of tissue in which Bam was not expressed was subtracted from each cyst measurement. Finally, for each cyst type (four-cell, four-cell dividing, eight-cell, eight-cell dividing, 16-cell), the measurements of mean cyst pixel fluorescence were averaged, and the standard error of the mean (SEM) calculated.

**See SI Text** for more details on fly strains and husbandry, heat shock, generation of transgenic flies with the Bam,PEST, live cyst dissection, immunostaining, fluorescence quantification, measuring cell cycle timing by double S-phase label of dissected testes, and measuring cell size.

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Supporting Information

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SI Text

Fly Strains and Husbandry. Drosophila melanogaster were raised on standard molasses medium at 25 °C unless stated otherwise. Drosophila pseudoobscura were raised at 22 °C. Wild-type flies were Oregon R for routine breeding experiments, and yw for immunostaining experiments. bam<sup>bgcn1</sup>, UASStg, bgcn<sup>1</sup>, enc<sup>II</sup>, UAS-GFP, and hs-bam flies were acquired from the Bloomington Stock Center. bam<sup>1</sup> (1), UASStb (2), UASfys (3), bamGal4 (4), bgcn<sup>2-0702</sup>(5), ptcGal4 (6), enc<sup>II</sup> (7), sa-GFP (8), and eIF4a<sup>iso106</sup> (9) flies were described previously. bgcn<sup>1</sup>+/flies. bgcn<sup>1</sup>/bgcn<sup>2-0702</sup> males had a bgcn null phenotype.

Heat Shock. hs-bam/TM3 or yw larvae and pupae were heat-shocked by immersing vials in a 37 °C water bath for 2 h on days 7 and 8 after starting fly cultures. Flies were dissected on day 3 after heat shock (day 11 after starting cultures).

Generation of Transgenic Flies with the Bam<sup>PEST</sup>. Site-directed mutagenesis was performed using the Bio-Rad Laboratories Muta-Gen In Vitro Mutagenesis kit according to the manufacturer’s protocol (10) in the plasmid pCasper-bam, a wild-type <i>bam</i> construct that includes the <i>bam</i> promoter. The BamPEST sequence was mutated using the oligonucleotide, 5′-gtg age tgg cag cag cag gag cag gaa gga-3′, producing a frameshift that changed the identity of 7 aa and deleted 22 more subsequent amino acids (most of the PEST sequence and the rest of the C terminus). Before use in the mutagenesis reaction, the oligonucleotide was phosphorylated using 1× kinase buffer containing 10 U T4 polynucleotide kinase per microgram of oligonucleotide and 1 mM ATP for 1 h at 37 °C. Single-stranded DNA was made according to ref. 11. Mutations at the targeted site were confirmed by sequencing. The mutated bam sequence, named BamPEST, was isolated from puc as a SacI/blunted-KpnI fragment and subcloned into the vector pCasper-4 using the SacI/blunted-BamHI site. The resulting construct was introduced into flies as a transgene by P-element mediated transformation via embryo injection (12).

Live Cyst Dissection. Testes were dissected into TBI (15 mM potassium phosphate, pH 6.7, 80 mM KCl, 16 mM NaCl, 5 mM MgCl<sub>2</sub>, and 1% PEG 6000), and a single testis was moved to a clean slide with ~20 μL 1× PBS, pH 7.4. The end of the testis containing sperm was removed with forceps, the remaining portion of the testis was peeled open, and spermatocyte cysts were pushed away from the testis sheath. A coverslip was then lowered over the cysts, and PBS was removed with a Kimwipe until the cysts were one-cell-layer thick. The number of cells in each intact cyst was counted using phase contrast microscopy on a Zeiss Axioskop microscope. Images were taken with Kodak Ektachrome 64T film or a Spot RT3 digital camera using Spot software (both from Diagnostic Instruments).

Immunostaining. For testis squashes, five to six pairs of testes from 0- to 2-day-old (d.o.) adult males were dissected from the desired genotype, placed on siliconized slides under a coverslip, and frozen in liquid nitrogen. The coverslip was popped off and the entire sample immersed in 95% ethanol at −20 °C for 10 min followed by 4% formaldehyde in 1× PBS for 7 min at room temperature (RT). For whole mount samples, at least 10 testis pairs from 0 to 2 d.o. adult males were dissected into 1× PBS. The testis pairs were spun down, the 1× PBS removed, and 95% ethanol at −20 °C was added for 10 min followed by 4%

Fluorescence Quantification and Statistics. All images were analyzed using Image J. To quantify fluorescence/cyst, the mean fluorescence intensity/pixel of a 12 × 12 pixel area in the cyst cytoplasm (where Bam staining is found) was measured as many times as possible within each cyst without redundancy and the
means were averaged for each cyst. A screen capture was taken of any cyst quantified as a visual record to avoid measuring the same cyst twice. In addition, the fluorescence/pixel of cells located immediately before and after Bam positive cysts was measured by the same method. The mean background tissue fluorescence/pixel (measured from tissue that did not stain for Bam) was measured from a 250 by 250 pixel square from each image and subtracted from the mean fluorescence/pixel for each cyst. The final measurement of mean cyst fluorescence/pixel minus background fluorescence/pixel was averaged for each cyst type [before Bam expression, 2-cell dividing (for bamΔPEST), 4-cell, 4-cell dividing, 8-cell, 8-cell dividing, 16-cell early, 16-cell dividing (for UASstg and bam/+), >16-cell (for UASstg and bam/+), and after Bam expression]. The SEM was calculated for each sample group.

**Measuring Cell Cycle Timing by Double S-Phase Label of Dissected Testes.** Testes from 0 to 1 d.o. males of each genotype were dissected into separate small culture dishes each containing 50 μL Schneider’s Media (Invitrogen). To each dish, 0.5 μL fresh 10 mM BrdU was added to the Schneider’s Media (final concentration 100 μM). The solutions were mixed, and the dishes were incubated in a dark humidified chamber at 25 °C for 15 min. The testes in each dish were washed twice initially, then six more times over the next 45 min in fresh Schneider’s Media. Then the testes in each dish were further incubated in 50 μL Schneider’s Media in a humidified chamber at 25 °C. Seven hours after BrdU was first added, the testes in each dish were removed from the Schneider’s Media drop and placed into another 5-μL drop of Schneider’s media with 0.5 μL 10 mM EdU added (final concentration 100 μM). The solutions were mixed, and the dishes were incubated in a humidified chamber at 25 °C for 5 min. Testes were immediately transferred into a tube containing 4% formaldehyde for an hour, permeabilized, stained with Alexa488-azide (from Invitrogen), and rinsed in PBST as described above. Testes were then stained with anti-BrdU (B8434; Sigma) following manufacturer instructions. All steps were completed in the dark to protect BrdU and the Alexa488-azide. EdU positive cysts were scored for the presence/absence of BrdU.

**Measuring Cell Size.** Squashed testes from wild-type (yw) and UASstg/bamGal4 flies were immunostained as described above with anti-Bam and anti-PH3. Images were taken on the confocal with the same hardware settings given above. Individual cells (where they could be confidently defined) in a cyst were measured at the smallest diameter that passed through the cell center. The average size of the smallest diameter of the cells was calculated for each cyst. Several cysts of each of the four categories were measured: Wild-type four-cell PH3, wild-type eight-cell PH3, UASstg/bamGal4 four-cell PH3, and UASstg/bamGal4 eight-cell PH3. SEM was calculated for each sample group.

Fig. S1. Expression of UAS-GFP driven with bamGal4 begins in late TA cells. (⁎) hub. (Scale bar, 50 μm.)
Fig. S2. String forcibly expressed with the bamGal4 driver caused overproliferation of TA cells before becoming spermatocytes. In a bam\textsuperscript{86}/+ background, the overproliferation was enhanced (note the shift from the >16 column to the >32 column). In addition, both genotypes showed a greater percentage of cysts overproliferating than the bam heterozygote. ‘‘>16’’ refers to cysts that had >16 but <32 spermatocytes/cyst.
Table S1. Counts of number spermatocytes/cyst from UASstg/bamGal4 males

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Some cysts had only part of the cyst go through an extra division. These cysts have > 16 (and < 32) cells/cyst. This is the only *D. melanogaster* genotype studied in this paper in which many cysts had > 16 and < 32 cells/cyst. Cysts that had ~64 cells/cyst were difficult to accurately count in live preps.
Table S2. Counts of number of spermatocytes/cyst

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<td>Cysts, %</td>
<td>0 100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>D. virilis—male (n = 109)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cells/cyst</td>
<td>32</td>
</tr>
<tr>
<td>Cysts, %</td>
<td>0 100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>D. virilis—female (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cells/cyst</td>
<td>32</td>
</tr>
<tr>
<td>Cysts, %</td>
<td>0 100</td>
</tr>
</tbody>
</table>

Similarities (frs) and differences (enc, D. pseudoobscura, and D. virilis) between males and females in number of rounds of transit amplifying division.
Table S3. Counts of number of spermatocytes/cyst in *elF4a*/*; bam*/* males as compared to *bam*/* sibling controls

<table>
<thead>
<tr>
<th></th>
<th><em>elF4a</em>/<em>; bam</em>/* male (n = 94) <em>T</em> = 25 °C</th>
<th><em>bam</em>/* male (n = 87) (sibling controls) <em>T</em> = 25 °C</th>
<th><em>elF4a</em>/<em>; bam</em>/* male (n = 43) <em>T</em> = 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cells/cyst</td>
<td>8 16 &gt;16 32 &gt;32</td>
<td>8 16 &gt;16 32 &gt;32</td>
<td>8 16 &gt;16 32 &gt;32</td>
</tr>
<tr>
<td>Cysts, %</td>
<td>0 91 1 4 3</td>
<td>0 68 1 15 16</td>
<td>0 100 0 0 0</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

One mutant copy of *elF4a* suppressed the *bam*/* overproliferation phenotype.