A Misexpression Screen Reveals Effects of *bag-of-marbles* and TGFβ Class Signaling on the Drosophila Male Germ-Line Stem Cell Lineage

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ABSTRACT

Male gametes are produced throughout reproductive life by a classic stem cell mechanism. However, little is known about the molecular mechanisms for lineage production that maintain male germ-line stem cell (GSC) populations, regulate mitotic amplification divisions, and ensure germ cell differentiation. Here we utilize the Drosophila system to identify genes that cause defects in the male GSC lineage when forcibly expressed. We conducted a gain-of-function screen using a collection of 2050 EP lines and found 55 EP lines that caused defects at early stages of spermatogenesis upon forced expression either in germ cells or in surrounding somatic support cells. Most strikingly, our analysis of forced expression indicated that repression of *bag-of-marbles* (*bam*) expression in male GSC is important for male GSC survival, while activity of the TGFβ signal transduction pathway may play a permissive role in maintenance of GSCs in Drosophila testes. In addition, forced activation of the TGFβ signal transduction pathway in germ cells inhibits the transition from the spermatogonial mitotic amplification program to spermatocyte differentiation.

THE mechanisms that control maintenance of stem cells and amplification and differentiation of cells derived from stem cell lineages play a critical role in tissue homeostasis for short-lived but highly differentiated adult cell types such as blood, skin, and intestinal epithelium. Stem cells must reliably both self-renew and produce differentiating daughter cells, and these alternate fates must be kept in balance to produce populations of differentiating cells over the lifetime of an individual. In addition, the number of transit-amplifying divisions that daughter cells committed to differentiation undergo before ceasing mitosis and entering terminal differentiation plays a profound role in the ability of relatively small numbers of stem cells to produce the huge number of differentiated progeny typical in many stem cell lineages. Stem cell behavior can be regulated by both extrinsic signals from the surrounding microenvironment and intrinsic mechanisms mediated by molecules that act in the stem cells or their descendants

(WATT and HOGAN 2000; SPRADLING *et al.* 2001). Discovery of the mechanisms that regulate stem cell self-renewal, amplification divisions, and differentiation is crucial to the effort to maintain stem cell function in disease and aging and to harness the potential of stem cells for regenerative medicine.

The male germ line in Drosophila has emerged as a powerful genetic system in which to study mechanisms that regulate tissue replenishment from stem cells. As in mammals, differentiating male gametes are produced from a relatively small stem cell population. In Drosophila testes, the outcome of a stem cell division is usually asymmetric: one daughter self-renews stem cell identity while the other daughter becomes a gonialblast and initiates differentiation (Figure 1A). As in many other stem cell systems, the stem cell daughter committed to differentiation (the gonialblast) goes through a series of transit-amplifying mitotic divisions before differentiation. Cytokinesis is incomplete during the amplifying mitotic divisions, so that the resulting spermatogonia remain interconnected by cytoplasmic bridges and divide and differentiate in synchrony. In wild-type *Drosophila melanogaster*, the number of amplifying mitotic divisions is exactly four, so that each gonialblast produces a cluster of 16 interconnected spermatogonia (Figure 1A). The spermatogonia then cease mitosis and initiate the spermatocyte differentiation program. The spermatocytes grow 25 times in volume, turn on a new gene expression program, and then undergo meiosis to produce 64 interconnected spermatids, which terminally differentiate (FULLER 1993).

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Recent work has demonstrated that Drosophila male germ-line stem cells (GSCs) are maintained by signals produced by a somatic support cell niche, the hub, located at the apical tip of the testis. In young adult males, an average of nine GSCs lie in a rosette surrounding and contacting the cluster of somatic hub cells (Figure 1B; Yamashita *et al.* 2003). The hub cells express a ligand, *unpaired* (*upd*), that activates the JAK-STAT signal transduction pathway in the adjacent GSCs and induces the cells to maintain male GSC identity (Kiger *et al.* 2001; Tulina and Matunis 2001). When a GSC divides, the mitotic spindle orients perpendicular to the hub (Yamashita *et al.* 2003) so that one daughter maintains contact with the hub and self-renews stem cell identity. The GSCs are flanked by somatic stem cells, the cyst progenitor cells, which also divide asymmetrically, resulting in self-renewal of cyst progenitor cells and production of differentiating somatic cyst cells (Figure 1A). Two somatic cyst cells surround the gonialblast and its progeny during all the remaining stages of germ cell differentiation (Hardy *et al.* 1979) and codifferentiate with the germ-line cells that they enclose (Gönczy *et al.* 1992; Gönczy and DiNardo 1996). Signaling between germ cells and surrounding somatic cyst cells is required for the normal program of spermatogonial-amplifying mitotic divisions (Kiger *et al.* 2000; Tran *et al.* 2000; Schulz *et al.* 2002), as well as for the switch from the spermatogonial mitotic division program to spermatocyte differentiation (Gönczy *et al.* 1997; Matunis *et al.* 1997).

Although outlines of the events that regulate stem cell behavior, the transit-amplifying divisions, and the switch from mitosis to terminal differentiation in the Drosophila male germ line are beginning to emerge, the underlying molecular mechanisms remain largely unknown. Some of the important players are germ-line specific and have been identified in screens for viable but sterile mutations (Gönczy *et al.* 1997; Schulz *et al.* 2002; Tazuke *et al.* 2002). However, many of the pathways implicated play crucial roles at multiple earlier stages of development, so null mutants are lethal (Matunis *et al.* 1997; Kiger *et al.* 2001). In addition, animals carrying loss-of-function mutations in genes required for stem cell maintenance may undergo initial rounds of successful gametogenesis and so may not be sterile. To circumvent these limitations, we have carried out a gain-of-function screen as an alternative approach for identifying genes with potential roles in the regulation of male GSC maintenance, GSC division, and the early stages of germ cell differentiation, including the transit-amplifying divisions and the switch to spermatocyte differentiation. Genes that normally specify stem cell self-renewal or the mitotic program in transit-amplifying cells may cause accumulation of early germ cells at the expense of differentiation when forcibly expressed in gonialblasts or spermatogonia. Indeed, previous studies showed that forced expression of the *upd* ligand in early

germ cells caused accumulation of cells with stem cell characteristics (Kiger *et al.* 2001; Tulina and Matunis 2001). Conversely, genes that normally act to induce early germ cells to differentiate may force differentiation or induce a mixed identity and apoptosis if forcibly expressed in GSCs, leading to stem cell loss.

Here we identify a number of genes that cause defects in the GSC lineage when forcibly expressed either specifically in early male germ cells or in cyst progenitor cells and cyst cells surrounding male germ cells by utilizing a collection of previously generated EP lines carrying random *P*-element insertions (Rørth 1996; Rørth *et al.* 1998) to drive expression of nearby genes. Strikingly, forced expression of *bam* in germ cells caused GSC loss, while forced expression of the TGFβ class signaling molecule *decapentaplegic* (*dpp*) in germ cells increased numbers of germ-line stem cells and blocked the transition from the spermatogonial transit-amplifying divisions to the spermatocyte differentiation program.

In the Drosophila female germ line, *bam* is required for stem cell daughters to initiate differentiation (McKearin and Spradling 1990), and forced expression of *bam* in female GSCs led to differentiation (Ohlstein and McKearin 1997). TGFβ class signaling from the somatic niche appears to maintain the female GSC population by suppressing *bam* (Chen and McKearin 2003a,b). In male germ cells, wild-type function of *bam* and TGFβ class signaling were previously shown to be required at a later step for the transition from the spermatogonial-amplifying mitotic division program to the spermatocyte fate (Gönczy *et al.* 1997; Matunis *et al.* 1997). Our data suggest that *bam* and TGFβ class signaling also play a role in maintenance of male GSCs. We find that forced premature expression of *bam* in early male germ cells (stem cells, gonialblasts, and spermatogonia) leads to accumulation of male germ cells at the single-cell stage and then death of the early male germ cells. Thus, expression of *bam* must be tightly regulated for survival of male GSCs. Loss-of-function analysis suggests that TGFβ class signaling is required for long-term maintenance of the stem cell population, possibly by suppressing *bam*. However, the phenotype of *bam* loss of function clearly shows that *bam* plays a different role in the male *vs.* female germ lines. In females, TGFβ signaling, via repression of *bam*, appears to instruct female GSCs to self-renew rather than to differentiate. In male GSCs, repression of *bam* expression, possibly under control of TGFβ signaling, appears to play a permissive role by allowing GSC survival.

MATERIALS AND METHODS

Fly strains: Flies were raised on standard cornmeal-molasses-agar medium. The EP collection was obtained from the Berkeley *Drosophila* Genome Project (unpublished data; Rørth 1996; Rørth *et al.* 1998), flies carrying a *tubulin*-green fluorescent protein (*tub*-GFP) construct were obtained from A.

Spradling, an *UAS-bam*-GFP and a *Pro-bam*-GFP were obtained from D. McKearin, *dpp* temperature-sensitive alleles *hr2, hr56,* and *e90* were obtained from K. Wharton, and *UAS-heph* lines were generated in our laboratory as follows: a *heph* cDNA was isolated from a testes library and cloned into the *pUAST* vector (BRAND and PERRIMON 1993). The *UAS-heph* transgene was introduced into flies by *P*-element-mediated germ-line transformation (SPRADLING 1986). A total of 10 lines were made and tested for effects when forcibly expressed in testes. All other Drosophila mutants, marker, and balancer chromosomes are as described in FLYBASE CONSORTIUM (2003) and were obtained from the Bloomington stock center.

Gain-of-function screen and *gal4; UAS* expression studies: To test the effects of forced expression, flies carrying one of two *gal4* transgene drivers, *nanos-gal4-VP16* (VAN DOREN *et al.* 1998) and *patched-gal4* (HINZ *et al.* 1994), were crossed to flies from the collection of 2050 strains carrying EP inserts described in RØRTH *et al.* (1998). For EP inserts on the second and third chromosome, males bearing the EP insert were crossed to females carrying the *gal4* transgene driver. For EP lines on the first chromosome, females bearing the EP insert were crossed to males carrying the *gal4* transgene driver. Crosses were set and the resulting progeny were initially raised at 18°. Crosses involving the *nanos-gal4-VP16* transgene driver were shifted to 29° after 7 days. Crosses involving the *patched-gal4* transgene driver were shifted to 29° after 10 days. Testes from 5–10 males from each cross were dissected 7–10 days after the temperature shift. Flies carrying *UAS*-cDNA constructs were raised at 18° and shifted to 29° as adults. However, phenotypes upon forced expression of target genes from cDNA constructs were also obtained when flies were kept at 18°.

Testes squashes, immunofluorescence, and histochemistry: Testes were dissected in testis buffer (10 mM Tris-HCl, pH 6.8, 180 mM KCl) and examined for a phenotype with a Zeiss Axiophot microscope in phase and fluorescent microscopy. Images were taken with a CCD camera using IP-LabSpectrum software. Immunofluorescence on whole testes was performed as in ASHBURNER (1989). Testes used for anti-mitogen-activated protein (anti-MAP) kinase immunohistochemistry were dissected in testes buffer with phosphatase inhibitors (50 mM NaF, 10 mM NaVO₄, 10 mM β-glycerophosphate) prior to the staining procedure. Immunofluorescense experiments on squashed testes were performed as described in HIME *et al.* (1996). The hybridoma/monoclonal antibodies mouse anti-α-spectrin 3A9 (1:5) developed by D. Branton and R. Dubreuil, mouse anti-fasciclin III 7G10 (1:10) developed by C. Goodman, and mouse anti-Armadillo N27A1 (1:10) developed by E. Wieschaus were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences. The monoclonal rat anti-E-cadherin antibody was obtained from T. Uemura. The monoclonal mouse anti-MAP kinase antibody (Sigma-Aldrich, clone MAPKyt) was used at 1:200, and polyclonal rabbit antiphosphorylated histone-H3 antibody (Upstate Biotechnology, Lake Placid, NY) was used at 1:100. Secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1:200. 4′,6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used at 1 μg/ml, acridine orange (Sigma, St. Louis) at 5 μg/ml.

In situ **hybridization:** Whole-mount *in situ* hybridizations were performed as described in TAUTZ and PFEIFLE (1989), with the modifications for RNA probes described in KLINGLER and GERGEN (1993). Ribonucleotide probes were generated from linearized plasmid using the Roche Molecular Biochemicals (Indianapolis) RNA-labeling kit. Drosophila expressed sequence tag clones containing cDNAs of TGFβ homologs were obtained from Research Genetics, Invitrogen (San Diego),

plasmid-containing *glass bottom boat* cDNA was obtained from K. Wharton, and plasmid-containing *bam* cDNA from D. McKearin.

Clonal analysis: Males carrying the *FRT-G13-sax* chromosome and control animals carrying the *FRT-G13* chromosome were crossed to females carrying the *flippase* recombinase gene under control of a heat-shock promotor and a *FRT-G13* chromosome marked with a nuclear-targeted GFP (*FRT-G13*-GFP). Progeny were heat-shocked as adults for 2 hr in a 37° water bath. Testes from adult males were dissected 3, 7, and 10 days after heat shock.

RESULTS

A gain-of-function screen of 2050 EP lines (MATERIALS AND METHODS) identified 55 EP lines that affected proliferation, differentiation, or survival of early male germ cells when forcibly expressed either in early germ cells or in surrounding somatic support cells (Table 1). Expression of nearby genes from the EP inserts was achieved in separate crosses to two different transgene drivers: *nanos-gal4-VP16* (*nos-gal4*) and *patched-gal4* (*ptc-gal4*). To avoid possible lethality due to forced expression of target genes during development (especially an issue with the *ptc-gal4* transgene driver), the level of activation was controlled temporally by temperature shifts: crosses were set and flies were initially grown at 18°, where activity of the *gal4* transgene driver is relatively low, and then shifted to 29° to induce high levels of expression.

The expression pattern of the *gal4* transgene drivers in testes was first characterized by crossing flies carrying the *gal4* transgene driver to flies carrying a *UAS*-GFP reporter transgene and monitoring GFP expression. The *nos-gal4* driver activated expression of GFP in germ cells at the testis apical region, including GSCs visible as single GFP-positive cells directly adjacent to the hub (Figure 1B, large arrow), gonialblasts visible as the next tier of single GFP-positive cells (Figure 1B, small arrow), and spermatogonia, visible as clusters of 2, 4, or 8 cells (Figure 1B, large arrowheads) displaced from the apical tip. GFP was also detected in early spermatocytes, visible as 16-cell clusters of larger cells (Figure 1B, small arrowhead), although the GFP signal faded during the early stages of spermatocyte differentiation. The *ptc-gal4* transgene driver activated expression of GFP in the cytoplasm of somatic cyst progenitors and cyst cells (Figure 1C, arrows) during all stages of spermatogenesis. The *ptc-gal4* transgene driver may also produce some expression of target genes in early germ cells, as low levels of GFP were occasionally detected in germ cells. To demonstrate that the *gal4* drivers strongly activated expression of target genes from the EP lines, we probed embryos carrying the *ptc-gal4* activator and an EP insert in *bam* (*EP(3)0667bam*) for expression of *bam* mRNA in embryos by *in situ* hybridization. Although *bam* mRNA was not detected in somatic cells of control embryos lacking the EP insert (Figure 1D), *bam* mRNA was strongly expressed in the segmental striped pattern characteristic of the *ptc* gene in *EP(3)0667bam; ptc-gal4* embryos (Figure 1E, arrows).

TABLE 1

EP lines causing defects at early stages of spermatogenesis when forcibly expressed

nos-gal4 (germ cell driver)	*ptc-gal4* (somatic cyst cell driver)
EP lines causing a reduced number of early germ cells	
EP(X)1457: CG14447	*EP(X)0325: CG1583*
EP(2)0605: wunen2[b]	*EP(X)1547: BcDNA: LD22118*
EP(3)0416: CG11520	*EP(2)0303: CG13211*
EP(3)0610: Limpet	*EP(2)0639: CG630*
EP(3)0667: bag-of-marbles[a,b]	*EP(2)2031: Glutathione S transferase S1*
EP(3)0775: corto[b]	*EP(2)2182: l(2)06655*
EP(3)0917: EST: RE29262	*EP(2)2198: mastermind[b]*
EP(3)1230: CG3308	*EP(2)2363: spinster*
EP(3)3204: hephestus[a,b]	*EP(2)2431: CG8704[b]*
EP(3)3252: CG6783	*EP(2)2506: ABC transporter expressed in trachea*
EP(3)3281: CG10164	*EP(2)2522: CG15161*
EP(3)3629: lama ancestor	*EP(2)2622: bunched[b]*
EP(3)3662: CG6328	*EP(3)0569 (nearby locus not identified)*
EP(3)3690: CG8121	*EP(3)0809: Neurexin*
	EP(3)0410: Gliolectin
	EP(3)0500: Malic enzyme
	EP(3)0648: SNF4/AMP activated protein kinase gamma subunit
	EP(3)0659: Cysteine string protein
	EP(3)0709: CG5376
	EP(3)1230: CG3308
	EP(3)3015 (nearby locus not identified)
	EP(3)3091: Protein kinase 61C
	EP(3)3187: Ubiquitin-specific protease 64E[b]
	EP(3)3252: CG6783
	EP(3)3260: CG18005
	EP(3)3269: CG10823
	EP(3)3289: Ets at 97D
	EP(3)3365: CG8833
	EP(3)3367: CG12313
	EP(3)3455: BcDNA:GH12663
	EP(3)3582 (nearby locus not identified)
	EP(3)3683: BcDNA:32148
	EP(3)3740: CG1475
EP lines causing cell death at the end of mitotic amplification divisions	
	EP(3)3645[a] (nearby locus not identified)
	EP(3)3652: gilgamesh[a]
	EP(3)3658: cyclin H[a]
EP lines causing an increased number of early germ cells	
EP(3)3519: tribbles[a,b]	*EP(3)0746: squid[a,b]*
EP(3)35191: tribbles[a,b]	*EP(X)1388[c] (nearby locus not identified)*
EP(2)2232: decapentaplegic[a,b,c]	
EP(3)0581: dally[c]	
EP(2)2505: numb[c]	

[a] Displayed defects in at least 50% of testes examined.

[b] A role in male or female gametes has previously been shown for this line.

[c] Appeared to cause an increase in the number of stem cells and/or gonialblasts. Nearest open reading frame based on sequence data (FLYBASE CONSORTIUM 2003).

Testes from adult EP line; *gal4* transgene driver males were dissected and assessed by phase-contrast microscopy to score effects on early male germ cells. This method allows rapid scoring of alterations in quantity and/or quality of multiple germ cell stages on the basis of observed differences in cell size and shape and the stereotypical number of germ cells per cyst. Testes from newly eclosed wild-type males have a developmental gradient of differentiating germ cells along the apical to basal axis of the testis. GSCs and mitotically amplifying spermatogonia appear as small cells near the apical tip of the testis (Figure 2, A and B, arrowheads). Spermato-
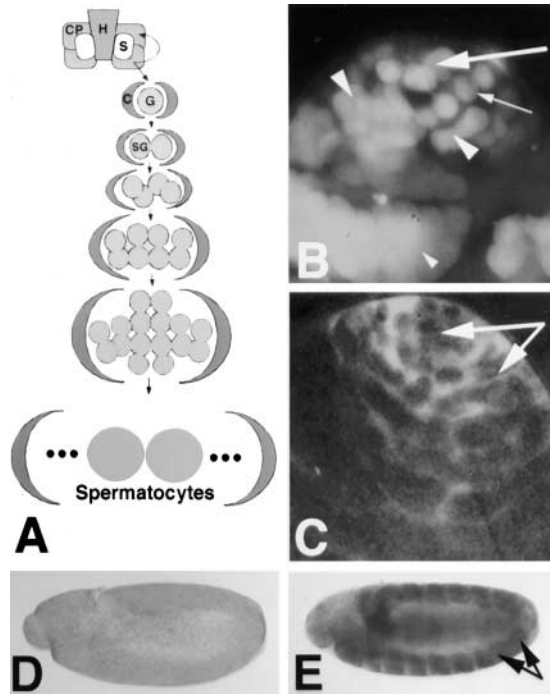
FIGURE 1.—Expression from EP lines. (A–C) Modified from KIGER and FULLER (2001). (A) Schematic of the cell types at the apical tip of wild-type testes. Somatic hub cells, H; GSC, S; cyst progenitor cell, CP; gonialblast, G; spermatogonia, SG; and cyst cell, C. (B and C) Apical, top; magnification ×400. Expression of *UAS*-GFP at the testes tip is shown in (B) germ cells under control of the *nos-gal4* transgene driver, single GSC (large arrow), gonialblast (small arrow), spermatogonia (large arrowheads), spermatocytes (small arrowhead), and in (C) somatic cyst cells (arrows) under control of the *ptc-gal4* transgene driver. (D and E) Anterior, left; magnification ×200. *In situ* hybridization with *bam* mRNA to (D) wild-type embryos and (E) embryos from *EP(3)0667bam; ptc-gal4* flies is shown. Note expression of *bam* in stripes (arrows).

cytes appear as increasingly larger cells, arranged in clusters of 16 cells per cyst, and are displaced away from the apical tip (Figure 2A, black arrow; Figure 2B, white outlines indicating increasingly more mature spermatocyte cysts). Germ cells at later stages of terminal differentiation normally fill the middle and basal part of the testis, with elongating spermatids extending up the testis lumen (Figure 2A).

In the initial screen, 182 of the 2050 EP lines examined were associated with defects at early stages of spermatogenesis in at least two testes of 5–10 EP line; *gal4* transgene driver progeny when crossed to one or the other of the *gal4* transgene drivers. When these 182 lines were retested under the same conditions, 55 EP lines (Table 1) caused reproducible defects in spermatogenesis, although usually with variable penetrance.

Genes causing reduced numbers of early male germ cells upon forced expression: The most common defect (45 EP lines) caused by forced expression of target genes was a reduction in the number of early germ cells (stem cells, gonialblasts, and spermatogonia) at the apical tip

(Table 1). For example, *EP(3)3629; nos-gal4* males, which carry an EP insert near the *lama ancestor* gene, had very few early germ cells (Figure 2D, arrowhead) compared to *EP(3)3629; TM6* control siblings (Figure 2C, arrowhead). Spermatocytes and later germ cell stages were commonly present (Figure 2D, arrow), possibly due in part to the temperature-shift regimen used to temporally control forced expression. Mature spermatocytes and later-stage germ cells may have derived from early germ cells that initiated differentiation while the animals were still at 18°, where the *gal4* transgene driver is less active. In many such lines, reduction in the number of early germ cells was accompanied by signs of cell death, indicating that forced expression of the target genes in germ line or soma may adversely affect early germ cell viability.

Among the 45 EP lines resulting in reduced numbers of early male germ cells in the testis, 14 caused a reduction in the number of early germ cells when forcibly expressed in male germ cells using the *nos-gal4* transgene driver, consistent with a germ cell intrinsic effect. Target genes in this class may normally affect early germ cell proliferation or survival. However, it is possible that forced expression of some of these genes may be detrimental to cell division and/or survival no matter what the cell type and that the loss of early germ cells is a trivial consequence of forced expression specifically in early germ cells. Strikingly, 31 EP lines caused reduced numbers of early germ cells when forcibly expressed using the *ptc-gal4* transgene driver. As the *ptc-gal4* transgene driver activates expression primarily in somatic cells (Figure 1C), it may be that defects in somatic support cells have a non-cell-autonomous adverse affect on early male germ cell survival or proliferation. Alternatively, effects on early germ cell survival or proliferation could be due to low levels of early germ-line expression under control of the *ptc-gal4* transgene driver. This second possibility is unlikely for most of the EP lines, however, as all lines were tested with both *gal4* transgene drivers and most that had defects with the *ptc-gal4* transgene driver did not show defects with the *nos-gal4* transgene driver (Table 1). Only two EP lines, *EP(3)1230* (inserted in *CG3308*) and *EP(3)3252* (inserted in *CG6783*), both underlined in Table 1, caused reduced numbers of early germ cells when forcibly expressed in either germ-line or somatic cells.

Among the 14 EP lines causing a reduction in the number of early germ cells when forcibly expressed in male germ cells, two lines [containing an insert upstream of *hephestus* (*heph*) and an insert upstream of *bam*] caused complete loss of early germ cells. This suggests that the products of these genes may have strong adverse effects on stem cell survival or promote stem cell daughter differentiation. Effects of forced expression of *bam* are presented in detail below (Figures 3 and 4). Forced expression of *heph* in early germ cells from line *EP(3)3204* or from a *UAS-heph* cDNA construct under

the temperature-shift regimen of the screen caused loss of early germ cells. Affected testes contained mostly differentiated spermatids and lacked early germ cells (Figure 2E, elongated spermatid bundles commonly extended all the way up to the testis tip). Surprisingly, the effect of forced expression of *heph* in germ cells appeared to be different, depending on the expression level. When *UAS-heph; nos-gal4* animals were shifted to 21° rather than to 29°, we occasionally observed testes with clusters of >16 small cells (Figure 2F, circled area), suggesting that *heph* may also play a role in the transition from the spermatogonia to spermatocyte differentiation program.

Genes affecting the spermatogonia-to-spermatocyte transition upon forced expression: Among the 55 EP lines causing a phenotype in the GSC lineage when forcibly expressed, 10 lines appeared to affect the transition from the spermatogonia to spermatocyte differentiation program. EP inserts upstream of two known genes, *gilgamesh* (*gish*) and *cyclin H*, and one unknown locus, (*EP(3)3645*), caused 16-cell spermatogonial cysts to undergo cell death rather than to differentiate into spermatocytes (Figure 2G). Strikingly, forced expression from these EP lines caused germ cell death only when activated by the somatic cell driver, but not upon activation by the germ cell driver (Table 1). This is consistent with previous work indicating a requirement for certain genes expressed in somatic cyst cells for survival and differentiation of early spermatocytes (FABRIZIO *et al.* 2003).

In contrast, forced expression from seven EP lines caused spermatogonia to continue mitotic proliferation at the expense of differentiation. Two EP lines, *EP(3) 0746* (inserted upstream of *squid*) and *EP(X)1388* (an insert for which a downstream gene has yet to be identified), caused spermatogonia to proliferate beyond the 16-cell stage when expressed in somatic cyst cells, indicating a potential role in signaling between germ cells and surrounding somatic cyst cells. This is consistent with previous data showing that signals from somatic support cells regulate the transition from spermatogonia to the spermatocyte stage (MATUNIS *et al.* 1997; KIGER *et al.* 2000). Five EP lines, *EP(3)3519* and *EP(3)35191* (both inserted upstream of *tribbles*), *EP(3)0581* (inserted upstream of *dally*), *EP(2)2505*, and
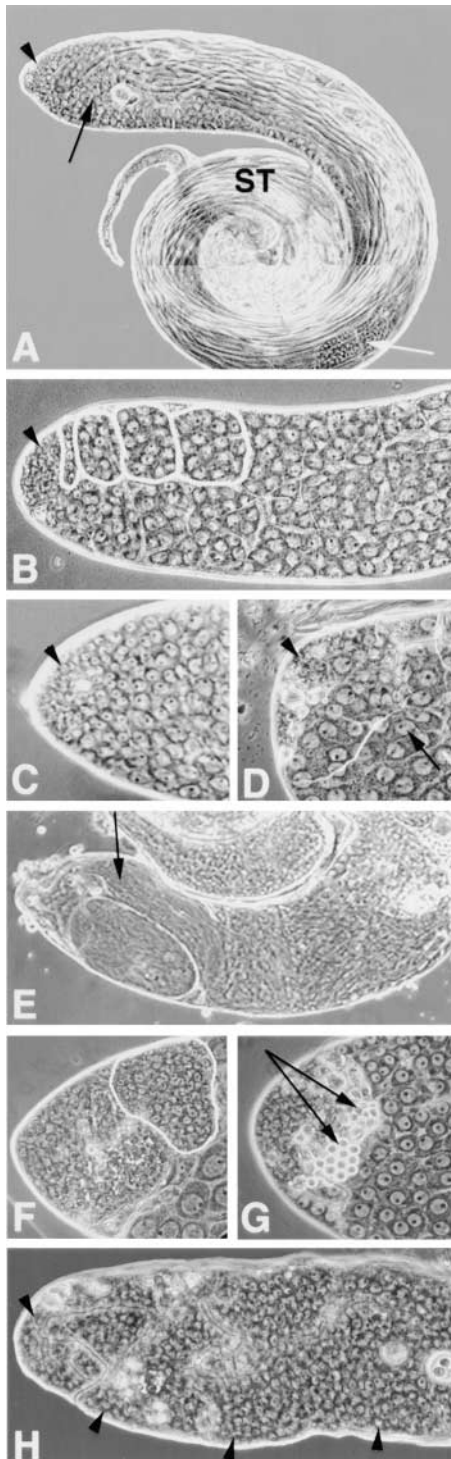

FIGURE 2.—Gain-of-function screen phenotypes. (A–H) Phase-contrast microscopy images, apical to the left; magnifications: (A) ×50; (B, G, and H) ×200; (C–F) ×400. (A) Whole wild-type testes, small early germ cells (arrowhead) at the apical tip, larger-sized spermatocytes (black arrow) displaced away from the tip, differentiating round spermatids (white arrow) along the coil of the testis, and bundles of elongated spermatids (ST are shown). (B) Wild-type apical testes region: developing germ cell clusters are indicated by circles, small early germ cells by arrowhead. (C) Apical testes tip of a control animal containing many small early germ cells (arrowhead). (D) Few early germ cells (arrowhead) at the apical testes tip from an *EP(3)3629lama ancestor*; *nos-gal4* animal; spermatocytes, arrow. (E) Example for loss of all early germ cells: whole testes from an *UAS-heph; nos-gal4* animal. Note sperm bundles (arrow) near the apical tip. (F) Cluster of >16 small germ cells (circle) in testes from an *UAS-heph; nos-gal4* animal raised at 21°. (G) Cell death at the end of the mitotic proliferation area (arrows) in testes from an *EP(3)3652gish; ptc-gal4* animal. (H) Apical testes region from an *EP(X)1388*; *ptc-gal4* animal. Note many small germ cells (arrowheads).

EP(2)2232 (inserted upstream of *dpp*, discussed in detail below; Figures 5 and 6), caused spermatogonia to undergo additional rounds of transit mitotic amplification division when forcibly expressed in early germ cells.

Among the seven EP lines that caused spermatogonia to continue mitotic proliferation at the expense of differentiation, four lines (inserts upstream of *dally, numb, dpp,* and *EP(X)1388*) appeared to also affect the number of GSCs and/or gonialblasts when forcibly expressed. On the basis of phase-contrast microscopy, many of the accumulating small cells at the apical tip from *EP(X) 1388; ptc-gal4* animals appeared to occur singly, characteristic of stem cells and gonialblasts, rather than in clusters as do the more differentiated spermatogonia (Figure 2H).

To explore possible mechanisms underlying effects on early male germ cell survival, proliferation, and differentiation caused by forced expression of candidate genes identified in the screen, we carried out more indepth analysis of two genes, *bam*, which causes loss of early germ cells, and *dpp*, which causes massive overproliferation of spermatogonial cysts when forcibly expressed in the germ line.

Forced expression of *bam* in early male germ cells causes stem cell loss: Testes from males carrying *EP(3) 0667*, an insert in the *bam* locus, and the *nos-gal4* transgene driver frequently showed loss of early male germ cells (Figure 3, A and C; Table 1). Control siblings from the same cross and temperature regimen had abundant early germ cells at the testis apical tip on the basis of phase-contrast microscopy (Figure 3B) and expression of a *UAS*-GFP transgene marker in early germ cells under control of *nos-gal4* (not shown). In contrast, in *EP(3)-0667bam; nos-gal4; UAS*-GFP males, 40% of the testes scored 8 days after the shift to 29° and 70% of the testes scored 10 days after the shift to 29° showed elongated spermatid bundles extending almost all the way up into the testis apical tip and few or no detectable spermatocytes (Figure 3, A and C) or GFP-positive early germ cells (data not shown).

To better understand why forced expression of *bam* in early male germ cells might cause GSC loss, we characterized the normal expression pattern of *bam* in wildtype testis. *In situ* hybridization to wild-type testes revealed high levels of *bam* mRNA in spermatogonia in the region of mitotic amplification divisions (Figure 3D, arrow). Strikingly, *bam* transcripts were not detected at the apical tip, in the position of the GSCs, and did not appear to accumulate substantially until a few cell diameters away from the tip. Some signal also appeared in spermatocytes and throughout the testes, although this was not different from background levels detected in control testes labeled with sense mRNA. Immunofluorescence staining with anti-BamC antibody revealed accumulation of BamC protein in the cytoplasm of spermatogonia starting a few cell diameters away from the hub. Accumulation of BamC was first detected in four-
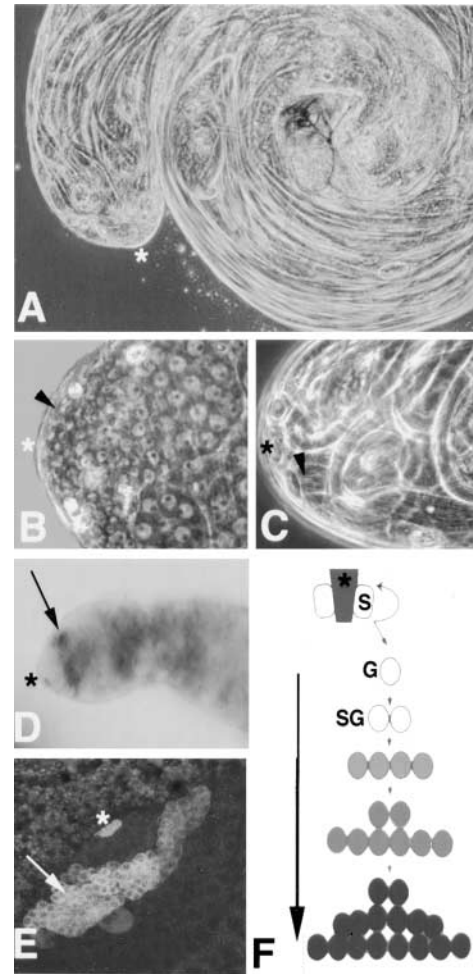

FIGURE 3.—Forced expression of *bam* causes early germ cell loss. (A–E) Apical testes tips (asterisks); magnifications: (A and D) ×200, (B, C, and E) ×500. (A) Whole testes from an *EP(3)0667bam; nos-gal4* animal filled with sperm bundles. (B and C) Apical testes tip from (B) a wild type with small germ cells (arrowhead) at the tip and (C) an *EP(3)0667bam; nos-gal4* animal 10 days after the shift to 29°. Note sperm bundles at apical tip in C (arrowhead). (D and E) *bam* expression in spermatogonia (arrows) of wild-type testes by (D) *in situ* hybridization with *bam* mRNA and (E) immunofluorescence with anti-BamC antibody. The testis in E is also labeled with anti-fasciclin III antibody to point out the apical hub cells (below asterisk). (F) Accumulation of *bam* expression in spermatogonia. GSC, S; gonialblast, G; spermatogonia, SG; hub, asterisk.

cell cysts, with levels apparently increasing in later-stage spermatogonia (Figure 3, E and F). BamC disappeared abruptly in the region of the testis where spermatogonia transition to spermatocyte differentiation. Most strikingly, BamC was not detected in the cytoplasm of male GSCs or in gonialblasts in wild-type testes (Figure 3E). The strict, cell-type-specific high-level expression of *bam* mRNA and BamC in spermatogonia but not in stem cells or gonialblasts raises the possibility that high levels of expression of *bam* may be incompatible with male GSC identity or survival.

TABLE 2

Loss of early germ cells upon forced expression of *bam* in the germ line

Time	Normal appearing (%)	Increased no. of cells at single-cell stage (%)		Few or no early germ cells (%)
		−degeneration	+degeneration	
Day 1	100			
Day 2	40	60		
Day 3	20		60	20
Day 4	20		60	20
Day 5	0		70	30
Day 6	20		45	35
Day 7	10		40	50
Day 8	15		45	40
Day 9	10		35	55
Day 10	10		20	70

100% = 30 testes.

To investigate the cause of loss of male germ cells upon forced germ-line expression of *bam,* we carried out a time course experiment, dissecting testes from *EP(3)0667bam; nos-gal4; UAS*-GFP males each day after the shift to 29° for a period of 10 days (Table 2). Our observations suggest that upon forced expression from the *bam* EP insert, early male germ cells first arrest in differentiation, accumulate as single cells, and then die.

At day 1 after the temperature shift, test animals were indistinguishable from their *TM6; nos-gal4; UAS*-GFP sibling controls by both phase-contrast microscopy and expression of GFP in the germ line. However, by 2 days after the temperature shift, 60% of testes from *EP(3)066-7bam; nos-gal4; UAS*-GFP animals contained increased numbers of single GFP-positive cells and decreased numbers of GFP-positive spermatogonial cell clusters at the apical tip compared to sibling control testes. Staining with the molecular marker α-spectrin confirmed that testes from *EP(3)0667bam; nos-gal4; UAS*-GFP animals contained increased numbers of cells with a ball-shaped spectrosome at the apical tip 2 days after the shift to 29°. In wild type, α-spectrin localizes to a spherical subcellular structure called the spectrosome in male GSCs and gonialblasts. In contrast, in spermatogonia and spermatocytes, α-spectrin localizes to the fusome, a branched, linear structure that extends through the intercellular bridges that connect the mitotically related germ cells within a cyst. Testes from control siblings contained on average 14 (ranging from 10 to 18) spectrosome dots next to and close to the hub at the apical tip (Figure 4A), as in wild type. In contrast, 2 days after the temperature shift, 60% of testes from *EP(3)0667bam; nos-gal4; UAS*-GFP animals had increased numbers of cells with a single spectrosome dot in the apical region (Figure 4B; on average 22 spectrosome dots, ranging from 10 to 32; 30 testes examined). Strikingly, many of the testes with increased numbers of spectrosomes contained either no or only a few (1–5, compared to >10 in wild type) small fusomes, suggesting that under the influence of forced expression of *bam,* early germ cells may arrest in a single-cell state rather than differentiate as spermatogonia.

Analysis of dividing cells by staining with antiphosphorylated histone-H3 antibody also suggested that forced expression of *bam* did not channel early germ cells into spermatogonial divisions. In wild type, male GSCs and gonialblasts divide as single cells, while spermatogonia divide synchronously in groups of two, four, or eight. Control siblings from the cross 2 days after the shift to 29° commonly displayed single antiphosphorylated histone-H3-positive cells directly adjacent to the somatic hub (stem cell position) or slightly displaced from the hub (gonialblast position), as well as groups of cells staining for phosphorylated histone-H3 (dividing spermatogonia) more distant from the hub (Figure 4C). In testes from *EP(3)0667bam; nos-gal4; UAS*-GFP animals 2 days after the temperature shift, single antiphosphorylated histone-H3-positive cells were detected next to the hub and displaced away from the hub (Figure 4D), but clusters of antiphosphorylated histone-H3-positive cells were rarely detected.

The effects of forced expression of *bam* became more severe over time (Table 2). By 5 days after the temperature shift, 70% of the testes from *EP(3)0667bam; nos-gal4; UAS*-GFP animals showed an increased number of single GFP-positive cells at the tip (Figure 4F) and decreased numbers of GFP-positive spermatogonial cell clusters. In addition, by 3–5 days after the temperature shift, early germ cells in many testes had abnormal morphology and appeared refractile in phase contrast, characteristic of dying cells. Staining with acridine orange revealed many dying cells and cell clusters in testes from *EP(3)0667bam; nos-gal4; UAS*-GFP animals (Figure 4H). In contrast, in testes from sibling controls, only a few dying germ cells were commonly detected near the apical tip by acridine-orange staining (Figure 4G). By 10 days after the temperature shift, the majority of the testes from *EP(3)0667bam; nos-gal4; UAS*-GFP animals

lacked all earlier-stage germ cells on the basis of phase-contrast microscopy (Figure 3, A and C), GFP, and lack of staining with anti-α-spectrin (Figure 4J).

In addition to loss of early germ cells, forced expression of *bam* in male germ cells also caused massive increase in the size of the somatic hub. Apical hub cells in testes from sibling control males expressed the cell-surface marker fasciclin III (Figure 4A) and formed a tight cluster ∼15 μm in diameter, as in wild type. In contrast, in testes from *EP(3)0667bam; nos-gal4* animals, cells displaying the cell-surface marker fasciclin III were detected in a broad area at the apical tip 10 days after the temperature shift (Figure 4J). In many cases, the hub appeared enlarged up to 20 times compared to the hub in testes from sibling controls subjected to the same temperature-shift regimen (Figure 4I). The dramatic enlargement of the hub was mainly observed several days after the temperature shift to induce high levels of *bam* expression, after most early germ cells had been lost from the testes. At 2 days after the shift to 29°, the hub appeared to be only slightly enlarged in testes from *EP(3)0667bam; nos-gal4* males (Figure 4D) compared to sibling controls (Figure 4C).

Forced expression of *bam* in early germ cells from a UAS-*bam*-GFP cDNA construct encoding *bam* protein fused to GFP caused an even more dramatic loss of early male germ cells than did forced expression of *bam* from the EP insert. Testes from *UAS-bam*-GFP; *nos-gal4* animals were tiny and had no detectable germ cells on the basis of phase-contrast microscopy or GFP staining (data not shown). Staining with antifasciclin III and anti-E-cadherin revealed that the somatic apical hub was greatly enlarged in testes from *UAS-bam*-GFP; *nos-gal4* males (data not shown), as in *EP(3)0667bam; nos-gal4* males shifted to 29° for several days.

Forced expression of *dpp* in early male germ cells blocks the transition from spermatogonial amplification divisions to spermatocyte fate: Forced expression of the TGFβ homolog *dpp* either from the *EP(2)2232* insert or from a *UAS-dpp* cDNA transgene under control of the early germ-cell-specific *nos-gal4* transgene driver produced testes filled with large numbers of early germ cells, as seen in phase contrast microscopy (Figure 5B), and in DAPI-stained preparations (Figure 5D). Testes
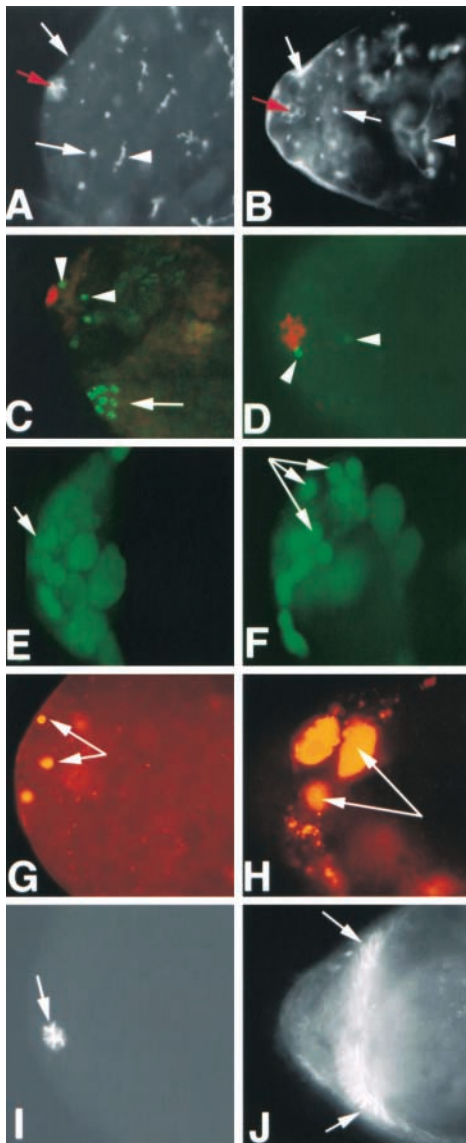

FIGURE 4.—Single early germ cells initially accumulate in testes from *EP(3)0667bam; nos-gal4* animals. (A–J) Apical testes tips; magnification: ×500; apical, left. (A and B) Immunofluorescence with anti-fasciclin III (red arrows) and anti-α-spectrin antibodies 2 days after the shift to 29°. (A) Testis from a *TM6; nos-gal4* animal showing spectrosome dots (arrows) at the apical tip and small fusomes (arrowhead) displaced away from the tip. (B) Testis from an *EP(3)0667bam; nos-gal4* animal with many spectrosome dots (arrows) in the apical region and lacking small fusomes. Note large fusomes (arrowhead) connecting spermatocytes. (C and D) Immunofluorescence with anti-fasciclin III (red) and antiphosphorylated histone-H3 (green) antibodies 2 days after the shift to 29°. (C) Testis from a control animal with single antiphosphorylated histone-H3-positive cells (arrowheads) close to the hub and a cluster of eight antiphosphorylated histone-H3-positive cells (arrow) displaced away from the hub. (D) Testes from an *EP(3)0667-bam; nos-gal4* animal with single antiphosphorylated histone-H3-positive cells (arrowheads) next to the hub and displaced away from the hub. (E and F) Expression of *UAS*-GFP in early germ cells 2 days after the shift to 29°; single cells (arrows) in testes from (E) a *TM6; nos-gal4* animals and (F) an *EP(3) 0667bam; nos-gal4* animal. Note increased number of single cells in F. (G and H) Acridine orange staining 5 days after the shift to 29° in testes from (G) a *TM6; nos-gal4* animals with (arrows) few dying cells at the tip and (H) an *EP(3)0667-bam; nos-gal4* animal with single and clusters (arrows) of dying cells. (I) Immunofluorescence with anti-fasciclin III to testes from a *TM6; nos-gal4* animal 10 days after the shift to 29°; hub, arrow. (J) Immunofluorescence with anti-fasciclin III and anti-α-spectrin to testes from an *EP(3)0667bam; nos-gal4* animal 10 days after the shift to 29°. Note large hub (arrows; compare to A and I) and lack of anti-α-spectrin-positive cells (compare to A).
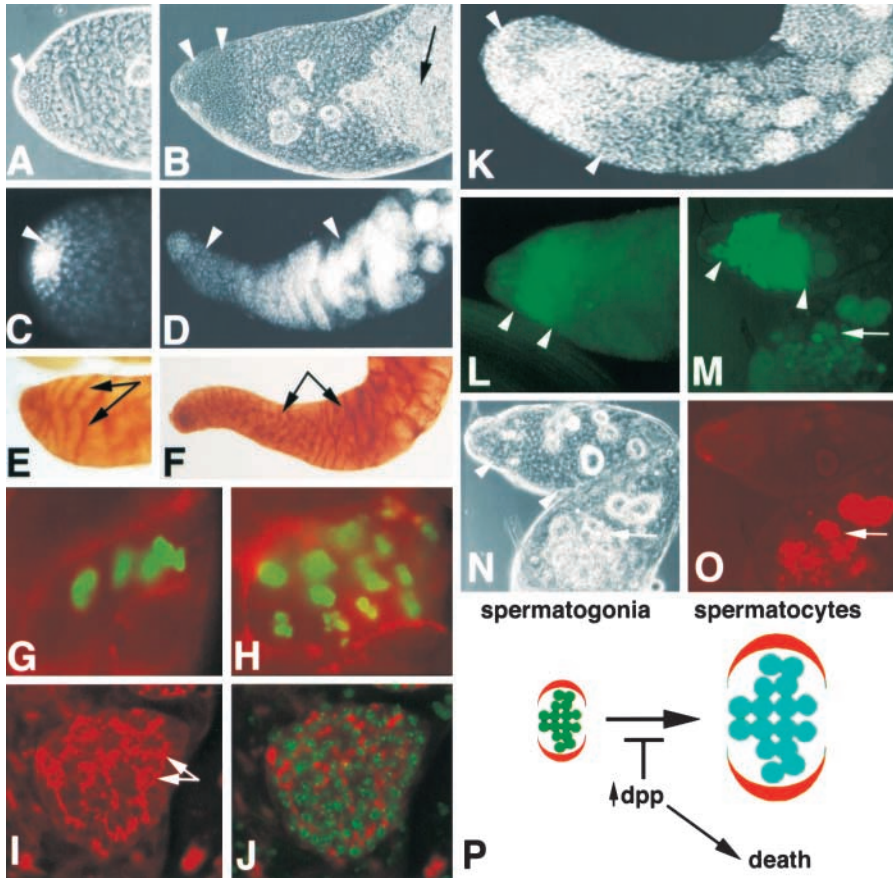
FIGURE 5.—Forced expression of *dpp* causes spermatogonial overproliferation and cell death. (A–O) Apical, left; magnifications: (A–F) ×200; (G and H) ×2000; (I and J) ×1000; (K and L) ×300; and (M–O) ×230. (A and B) Phase-contrast microscopy images. Note small early germ cells (arrowheads in A) at the apical tip in testes from a *CyO; nos-gal4* animal and (B) filling the apical region of testes from an *UAS-dpp; nos-gal4* animal. Note dying cells (arrow in B). (C and D) DAPI-stained preparations. Small bright staining nuclei (arrowheads in C) at the apical tip in testes from a *CyO; nos-gal4* animal and (D) filling the entire testis from a *UAS-dpp; nos-gal4* animal. (E and F) Immunohistochemistry with anti-MAP kinase (brown) outlining the somatic cyst cells surrounding early germ cells (E) at the apical tip of testes from a *CyO; nos-gal4* animal and (F) throughout testes from an *UAS-dpp; nos-gal4* animal. (G and H) Immunofluorescence with anti-Armadillo antibody (red) to outline one germ cell cluster and antiphosphorylated histone-H3 antibody (green). (G) Cyst from a testis from a *CyO; nos-gal4* animal with eight antiphosphorylated histone-H3-positive cells undergoing mitosis in synchrony (only five antiphosphorylated histone-H3-positive cells are in the plane of focus). (H) Cyst from a testis from an *UAS-dpp; nos-gal4* animal with 16 antiphosphorylated histone-H3-positive cells (only 11 antiphosphorylated histone-H3-positive cells are in plane of focus). (I) Immunofluorescence staining with anti-α-spectrin antibody (red) to a cluster of germ cells in testes from an *UAS-dpp; nos-gal4* animal; note branched fusomes (arrows). (J) Same cluster of germ cells shown in I double labeled for anti-α-spectrin antibody (red) and DAPI (green); note >16 DAPI-positive small nuclei interconnected by fusome. (K) Testes from an animal expressing *UAS-tkv** in early germ cells are filled with bright-staining DAPI-positive cells (arrowheads). (L and M) Expression of a *Pro-bam*-GFP reporter gene construct (arrowheads) in testes from (L) a *TM3; nos-gal4* animal and (M) a *UAS-tkv*; nos-gal4* animal. Note much broader GFP-positive region in M; dying cells, arrow. (N) Phase-contrast image of the testis in M. Note small germ cells in region of *Pro-bam*-GFP expression (arrowheads) and dying cells (arrow). (O) Same testis shown in M but taken in another channel to visualize autofluorescing, dying cells (arrow). (P) Model demonstrating the effect of forced *dpp* expression. High levels of *dpp* block the transition from spermatogonia to spermatocyte stage and eventually push the cells into death.

from sibling controls raised under the same temperature regimen had the normal gradient of a few small cells with brightly DAPI-staining nuclei at the apical tip transitioning to clusters of spermatocytes, which are much larger cells with more diffuse nuclear DAPI staining (Figure 5, A and C). In contrast, testes from *UAS-dpp; nos-gal4* animals contained massive numbers of small early germ cells with brightly DAPI-staining nuclei (Figure 5, B and D). The affected testes had fewer spermatocytes than normal and abundant signs of cell death, visible as refractile regions by phase-contrast microscopy, distal to the accumulating early germ cells (Figure 5B, arrow). The bulk of the early germ cells accumulating in testes from *EP(2)2232dpp; nos-gal4* or *UAS-dpp; nos-gal4* males were clustered in cysts (Figure 5D) and surrounded by somatic cyst cells. In sibling control testes, as in wild type, staining with anti-activated MAP kinase (Figure 5E) or anti-Armadillo (Figure 5G)

revealed somatic cyst cells surrounding clusters of developing germ cells. Staining of testes from *UAS-dpp; nos-gal4* animals with anti-activated MAP kinase (Figure 5F) or anti-Armadillo (Figure 5H) revealed large numbers of clusters of germ cells surrounded by somatic cyst cells.

The dramatic increase in number of early germ cells in testes from *UAS-dpp; nos-gal4* animals appeared to be largely due to failure of spermatogonia to exit the amplifying mitotic divisions and initiate differentiation as spermatocytes. Immunofluorescence staining with an antibody against anti-phosphorylated histone-H3 to detect dividing cells revealed that testes from *UAS-dpp; nos-gal4* animals frequently contained clusters of more than eight cells undergoing synchronous mitosis and packaged in a single cyst surrounded by anti-Armadillo-positive cyst cells (Figure 5H). In contrast, testes from sibling controls had clusters of two, four, and eight cells but

never more spermatogonia undergoing synchronous division (Figure 5G), as in wild type. Staining with DAPI and anti-α-spectrin revealed that cysts containing >16 spermatogonia interconnected by highly branched fusomes (Figure 5, I and J) were common in testes from *UAS-dpp*; *nos-gal4* animals. Thus, forced expression of *dpp* in early germ cells caused spermatogonia to continue the amplifying mitotic division program beyond the fourth mitosis instead of becoming spermatocytes. The same effect on accumulation of early germ cell clusters was detected upon forced expression of an activated form of the TGFβ type I receptor *thick veins* (*tkv*) (*UAS-tkv**) in early germ cells, as assessed by phase-contrast microscopy (not shown), DAPI staining (Figure 5K), and labeling with early germ cell markers as described above (data not shown).

As the continuing proliferation of spermatogonial cysts observed in testes from *UAS-dpp*; *nos-gal4* or *UAS-tkv**; *nos-gal4* males resembled the phenotype of loss of function of *bam* (GÖNCZY *et al.* 1997) and *bam* has been shown to be negatively regulated by TGFβ pathway signaling in female germ cells (CHEN and MCKEARIN 2003a,b), we assayed expression of a reporter transgene carrying genomic regulatory sequences of *bam* driving expression of GFP (*Pro-bam-GFP*). In control animals, the *Pro-bam-GFP* transcriptional reporter was expressed in spermatogonia in the region of mitotic amplification, but not in male GSCs, gonialblasts, or differentiating spermatocyte cysts (Figure 5L), reflecting the accumulation of *bam* transcript in wild-type testes (Figure 3D). Similarly, the *Pro-bam-GFP* transcriptional reporter was not expressed in early germ cells in the stem cell or gonialblast region in testes from *UAS-tkv**; *nos-gal4* (Figure 5M) or *UAS-dpp*; *nos-gal4* (data not shown) males. Expression of the *Pro-bam-GFP* reporter was detected in spermatogonial cysts in testes from freshly hatched *UAS-tkv**; *nos-gal4* (Figure 5M) or *UAS-dpp*; *nos-gal4* (data not shown) animals, although the signal was frequently variable and often weak. Even within the same testis, often some spermatogonial cysts expressed the *Pro-bam-GFP* reporter while other spermatogonial cysts did not. The observed expression from the *Pro-bam-GFP* reporter suggests that TGFβ signaling may suppress *bam* expression in early germ cells; however, a cell- or stage-specific mechanism may exist in spermatogonia to counteract repressive effects of TGFβ signaling on transcription of *bam*.

Expression of the *Pro-bam-GFP* reporter commonly faded in the more distal spermatogonial cysts that accumulated in *UAS-tkv**; *nos-gal4* (Figure 5M) or *UAS-dpp*; *nos-gal4* (data not shown) testes. It is possible that the aberrant spermatogonial cysts that accumulate in the affected testes have passed beyond the stage at which the proposed mechanism counteracts repressive effects of TGFβ signaling on transcription of *bam*. Alternatively, the aberrant spermatocyte cysts may not express the *Pro-bam-GFP* reporter because they have already initiated

a cell death program. Examination by phase-contrast microscopy (Figure 5N), by autofluorescence in the non-GFP channels (Figure 5O), or by acridine-orange staining (data not shown) revealed signs of massive cell death in the more distal regions of the testes from freshly hatched *UAS-tkv**; *nos-gal4* or *UAS-dpp*; *nos-gal4* males. In testes from older males, often only dead or dying material was detected, suggesting that high levels of TGFβ signaling ultimately cause cell death (Figure 5P), perhaps due to failure of spermatogonia to proceed to the spermatocyte differentiation program. Loss of function of *bam* also leads to continued proliferation of spermatogonia, failure to proceed to spermatocyte differentiation, and eventually germ cell death (GÖNCZY *et al.* 1997).

The TGFβ signal transduction pathway affects GSC number and the size of the stem cell niche: In addition to blocking the transition from spermatogonial mitotic amplification divisions to spermatocyte differentiation, forced expression of *dpp* or *tkv** in early male germ cells also resulted in a mild increase in GSC number. Immunofluorescence staining with anti-α-spectrin revealed increased numbers of cells with a spectrosome dot next to the apical hub, a characteristic of stem cell identity, in testes from *UAS-dpp*; *nos-gal4* animals (Figure 6B) compared to sibling controls (Figure 6A). Examination of testes from *UAS-dpp*; *nos-gal4; UAS-tub-GFP* flies confirmed the increased number of germ cells in the stem cell position next to the hub. The *UAS-tub-GFP* construct allows visualization of germ cells around the apical hub (Figure 6, C and D). As the somatic hub was often displaced away from the tip in adult testes from *UAS-dpp*; *nos-gal4* animals (see below), we examined testes from third instar larvae grown at 18°. Larval testes from sibling controls had an average of nine *tub*-GFP-positive male germ cells around the hub at the testis apical tip (500 testes examined), compared to an average of 12–13 *tub*-GFP-positive male germ cells around the apical hub in *UAS-dpp*; *nos-gal4* animals of the same age (100 testes examined, Figure 6E). The difference in the number of stem cells in testes from *UAS-dpp; nos-gal4* and control animals was statistically significant ($P < 0.0001$)

The increase in GSC number may be due to an increase in the size of the stem cell niche. The diameter of the somatic apical hub was increased in larval and adult testes from *UAS-dpp*; *nos-gal4* and *UAS-tkv**; *nos-gal4* animals, on the basis of the expression of hub markers such as anti-Fasciclin III (Figure 6, A and B) or *upd* mRNA (data not shown). Expansion of the somatic hub was less extreme in adult testes from *UAS-dpp*; *nos-gal4* and *UAS-tkv**; *nos-gal4* animals than in adult testes from *EP(3)0667bam*; *nos-gal4* animals (see above). In addition, in 38% of the adult testes from *UAS-dpp*; *nos-gal4* animals ($N = 100$), the somatic hub was displaced away from the apical tip. In some cases (6/100), two somatic hubs were detected at different positions
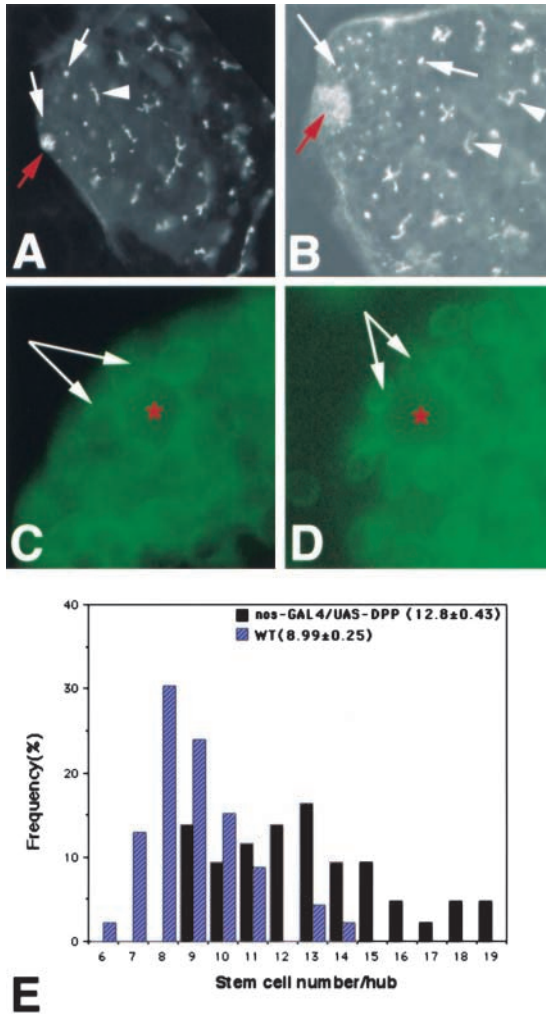
Figure 6.—Forced expression of *dpp* causes an increase in the number of cells in the stem cell position. (A–D) Apical testes tips; magnification: ×500. (A and B) Immunofluorescence staining with anti-fasciclin III and anti-α-spectrin antibodies, showing spectrosome dots (arrows) next to the apical hub (red arrows) and small fusomes (arrowheads) displaced away from the hub in testes from (A) a *CyO; nos-gal4* animal and (B) an *UAS-dpp; nos-gal4* animal. (C and D) A single row of *tub*-GFP-positive cells around the hub (asterisks). Note (C) seven *tub*-GFP-positive cells in testes from a *CyO; nos-gal4* animal and (D) 10 *tub*-GFP-positive cells in testes from an *UAS-dpp; nos-gal4* animal. (E) Number and frequency of *tub*-GFP-positive cells in the stem cell position of larval testes from control animals (blue bars) and *UAS-dpp; nos-gal4* (black bars) animals. *P*-value of Student's test: *P* < 0.0001.

Figure 7.—The TGFβ signal transduction pathway may play a role for stem cell maintenance. (A) Wild-type testis tip *in situ* hybridized with a *Maverick* mRNA probe. Note strong staining (arrow) in early germ cells in the apical region. (B and C) *sax*-mutant germ cell cluster (circle) lacking (B) GFP expression and (C) showing large spermatocytes in phase-contrast microscopy. Magnification: (A) ×200; (B and C ×500).

(compare Figure 6A and 6B). The increase in the number of differentiating gonialblasts and spermatogonia could be due to either increased stem cell number or a delay in differentiation during the mitotic amplification stage.

To further assess the possible role of TGFβ signaling in early male germ cell differentiation, we analyzed the expression pattern of TGFβ family members in testes and the effects of mutants in components of the signal transduction pathway on spermatogenesis. *In situ* hybridization of wild-type testes with antisense-mRNA against the TGFβ homologs *dpp, glass bottom boat, screw*, or *Myoglianin* did not reveal expression in early germ cells at the tip of wild-type testes. However, mRNA for the TGFβ homolog *Maverick* was detected in early male germ cells of wild-type testes (Figure 7A), raising the possibility that a TGFβ homolog, other than *dpp*, might play a role in early germ cell behavior.

Testes from males carrying *dpp* temperature-sensitive alleles showed displacement of the somatic hub and reduction in the number of early germ cells (data not shown). However, these effects were observed in *dpp/+* heterozygotes as well as in *dpp/dpp* animals, and at both permissive and restrictive temperatures, making it difficult to interpret their meaning.

As an alternative approach to explore possible requirements for TGFβ class signaling for normal early male germ cell behavior, we analyzed germ cell clones homozygous mutant for a loss-of-function allele of the TGFβ type I receptor component, *saxophone* (*sax*). Germline clones were generated using the FLP-FRT mitotic recombination system (Xu and Rubin 1993) and identified by lack of expression of a nuclear-targeted GFP

within the same testes from adult *UAS-dpp; nos-gal4* animals. In larval testes from *UAS-dpp; nos-gal4* animals, the somatic hub had an average diameter of 18 μm, compared to 15 μm in larval testes from sibling controls.

In addition to an increased number of GSCs, testes from *UAS-dpp; nos-gal4* animals had mildly increased numbers of germ cells with a spectrosome dot displaced away from the hub characteristic of gonialblasts, as well as increased numbers of early germ cells interconnected by small fusomes, characteristic of spermatogonial cysts
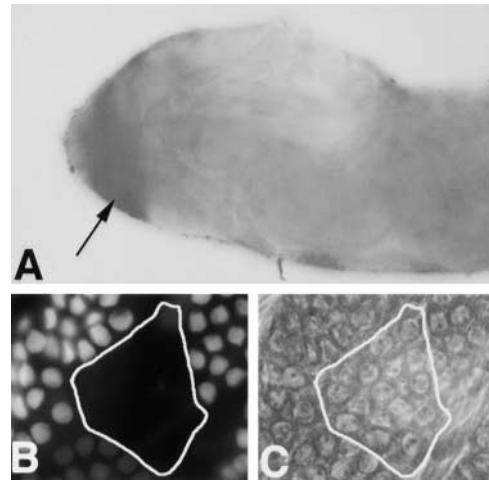
TABLE 3

Mosaic analysis of wild-type and *sax* mutant germ cell clones

	Genotype	
	FRT-wt	*FRT-sax*
3 days after heat shock		
No. of testes	50	50
Testes with clones	50	7
Clones/testes	>5	1 (6 testes)
	2 (1 testis)	
7 days after heat shock		
No. of testes	100	94
Testes with clones	90	13
Clones/testes	>10	1 (8 testes)
	2 (4 testes)	
	3 (1 testis)	
10 days after heat shock		
No. of testes	40	40
Testes with clones	35	2
Clones/testes	>10	1

carried on the chromosome bearing the wild-type *sax* allele (MATERIALS AND METHODS). In control animals, clones of marked, GFP-negative germ cells wild type for *sax* were induced at high frequency (90–100% of testes examined had at least one germ-line clone) under the heat-shock conditions utilized. Marked GSCs wild type for *sax* persisted and continued to produce abundant cysts of differentiating germ cells for 3 days, 1 week, and 10 days after heat shock (Table 3). In contrast, clusters of marked differentiating germ cells homozygous mutant for *sax* were detected in only 15% of the testes examined. In each case, the *sax* mutant germ cells developed normally into spermatocytes (Figure 7, B and C), suggesting that *sax* is not required cell autonomously for spermatogonia to make the transition to spermatocyte fate. However, wild-type function of *sax* may be required in germ cells for GSCs to divide at a normal rate. Whereas control testes containing marked, GFP-negative GSC clones wild type for *sax* routinely had several differentiating marked, GFP-negative germ cell cysts of differentiating germ cells, testes with persistent *sax*-mutant germ cell clones normally had only one or two cysts of differentiating *sax*-mutant germ cells (Table 3). GSCs homozygous mutant for *sax* may either be lost over time or divide less frequently than wild-type stem cells.

DISCUSSION

A gain-of-function screen led to identification of a number of candidate genes that affect early male germ cell differentiation, division, or survival when forcibly expressed either in early germ cells or in surrounding somatic cyst cells. Genes that reduced the number of early germ cells when forcibly expressed comprised the most common class. For some, forced expression may simply be toxic. However, some genes, such as *bam*, may play important regulatory roles in germ cell differentiation that compromise GSC or gonialblast survival when they are forcibly expressed in earlier stages. Strikingly, many of the EP lines that caused early germ cell loss did so when expressed in somatic cells but not when expressed in the germ line, reinforcing the emerging picture of dependency of early germ cells on surrounding somatic support cells (MATUNIS *et al.* 1997; KIGER *et al.* 2000; SCHULZ *et al.* 2002; TAZUKE *et al.* 2002).

A particularly interesting set of genes caused stage-specific loss of cysts of spermatogonia at or around the time of the transition from spermatogonial to spermatocyte identity. Of the three EP lines in this class identified in the screen, all resulted in the death of spermatogonial cysts when expressed in somatic cells but not when expressed specifically in early germ cells. *EP(3)3652* is located near *gish*. In eye development, *gish* acts in combination with the transcription factor *sine oculis* (*so*) to regulate glial cell migration (HUMMEL *et al.* 2002). Function of *so* in somatic cyst cells has recently been shown to be required for survival of germ cell cysts at the spermatogonia-to-spermatocyte transition (FABRIZIO *et al.* 2003). As the three EP lines affect a similar specific step in spermatogenesis, they may drive expression of genes that either inhibit *so* expression or function or interfere with the pathway downstream of this transcription factor in somatic cyst cells.

With only two exceptions among the 55 EP lines that caused a phenotype under the conditions of the screen, phenotypes were observed only when forcibly expressed in one of the two tissues tested. The exceptions, EP inserts near the predicted genes *CG3308* and *CG6783*, both caused germ cell loss when forcibly expressed under control of either the *nos-gal4* (germ-cell-specific) or the *ptc-gal4* (expressed mainly in somatic cyst cells) transgene driver. If the proteins encoded by *CG3308* and *CG6783* are secreted into or affect molecules secreted into the extracellular space, it may not matter in which cell type the gene is forcibly expressed for there to be an effect on germ cell behavior. However, no motifs or predicted structural features suggesting that either *CG3308* or *CG6783* encode secreted proteins have been identified. Alternatively, as the *ptc-gal4* transgene driver may also activate low-level expression of target genes in germ cells, the effects of forced expression of *CG3308* and *CG6783* could be cell autonomous in the germ line, with germ cells being particularly sensitive to the products of these genes.

One technical caveat was that the majority of the lines tested showed a high degree of variability in both penetrance and expressivity. One source of variability may be the temperature-shift regimen employed for temporal control. As vials contained progeny at differ-

ent stages of development at the time of the shift, one may expect differences in phenotype among males from the same vial. For example, as discussed in detail below, testes in which *bam* was forcibly expressed displayed different phenotypes, depending on the time after the shift to high temperaure. Also, different levels of expression may cause different phenotypes, as seen for forced expression of *heph*. The *heph* gene, which encodes a predicted RNA-binding protein of unknown function, is normally expressed in early germ cells of wild-type testes (C. Schulz, unpublished data). Forced expression of high levels of *heph* in early germ cells appeared to cause early germ cell loss, while forced expression of lower levels of *heph* in early germ cells blocked the transition from the spermatogonial mitotic division program to spermatocyte differentiation, resulting in cysts containing clusters of >16 spermatogonia. For future screens, use of a system that drives forced expression at consistently higher levels may alleviate some of the variability we observed and may also lead to identification of a higher relative number of genes that have effects when forcibly expressed.

Mechanisms that repress *bam* in male GSCs may permit stem cell maintenance: Expression of the *bam* gene is normally tightly controlled. *bam* mRNA is expressed in mitotically amplifying spermatogonia and accumulation of the protein in the cytoplasm (BamC) was detected only in clusters of spermatogonia and not in male GSCs or gonialblasts. BamC protein normally disappears abruptly as germ cells make the transition to spermatocyte differentiation. Wild-type function of *bam* is required cell autonomously in the male germ line for spermatogonia to cease mitotic amplification divisions and initiate the spermatocyte differentiation program. In males lacking *bam* function, spermatogonia fail to cease mitosis, producing cysts of 32, 64, and more spermatogonia before eventually undergoing cell death (Gönczy *et al.* 1997). For some alleles, even testes from *bam*/+ heterozygous males often contained many clusters of >16 small germ cells (Gönczy *et al.* 1997; Fly-Base Consortium 2003; C. Schulz, unpublished data). The *bam* expression pattern and loss-of-function mutant phenotype suggest that in males *bam* acts primarily to limit the number of mitotic spermatogonial amplification divisions and that a certain threshold level of *bam* function may be required to trigger the switch to spermatocyte fate (Gönczy *et al.* 1997).

Our data on the effects of forced *bam* expression suggest that regulation of the *bam* expression pattern is important for both maintenance of GSCs and differentiation of gonialblasts and spermatogonia. When expression of *bam* in early germ cells was forced under control of the *nos-gal4* transgene driver, early germ cells initially accumulated as single cells at the apical testes tip. Longer or higher forced expression of *bam* in early germ cells resulted in early germ cell death, first apparent in spermatogonial clusters but also occurring in single cells

by 6 days after the temperature shift. By 10 days after the shift, early germ cells were often completely lost, indicating that high levels of *bam* expression are lethal to early germ cells and that spermatogonia are more sensitive to this lethal effect, possibly because they also express *bam* protein intrinsically. Although early male germ cells are sensitive to forced expression of *bam*, it appears that high levels of forced *bam* expression are necessary to have an effect. Contrary to the female germ line, where pulses of *bam* expression under the control of a heat-shock promotor forced differentiation of GSCs, no strong effects on male germ cells were noted in initial experiments where pulses of heat shock were applied to male flies carrying the same *hs-bam* transgene (Ohlstein and McKearin 1997).

We propose that sustained ectopic expression of high levels of *bam* in early germ cells blocks gonialblasts from initiating or carrying out the spermatogonial differentiation program, perhaps by eliciting prematurely the mechanism through which *bam* normally causes cessation of the spermatogonial mitotic amplification divisions at the 16-cell stage. Alternatively or in addition, forced expression of *bam* may prematurely activate aspects of the spermatocyte differentiation program that are incompatible with survival of stem cells, gonialblasts, and spermatogonial cysts.

The effects of forced expression of *bam* suggest that the mechanisms that keep *bam* expression turned off in early male germ cells may play a role in maintenance of the stem cell population by shielding stem cells from the effects of inappropriate expression of a gene involved in the differentiation pathway for the lineage. Recent studies in the female germ line have identified a silencer element located just downstream of the *bam* start of transcription that is required to block *bam* transcript expression in female GSCs (Chen and McKearin 2003a). If this same silencer element blocks expression of *bam* in stem cells and gonialblasts in the male germ line, then the factors that bind to it are likely to play a role in maintenance of the stem cell population. Transcriptional and post-transcriptional mechanisms that negatively regulate expression of differentiation genes may be a general feature of the mechanisms that maintain stem cell populations in many adult stem cell systems.

TGFβ signaling and the transition from spermatogonial to spermatocyte fate: The TGFβ signal transduction pathway clearly plays a role in regulating the transition from the spermatogonial-amplifying mitotic division program to spermatocyte differentiation. However, exactly how TGFβ signaling acts to govern this transition remains a puzzle. Mosaic analysis by Matunis *et al.* (1997) demonstrated that cysts of wild-type spermatogonia undergo extra rounds of mitotic divisions and fail to become spermatocytes when associated with a somatic cyst cell mutant for either *punt*, the TGFβ type 2 receptor, or *schnurri*, a transcription factor downstream of

TGFβ signaling during embryogenesis (ARORA *et al.* 1995). These data suggested that receipt of a TGFβ class signal by somatic cyst cells induces the somatic cells to send a signal of unknown nature to the germ cells that they enclose, either inducing or permitting the spermatogonia to initiate differentiation as spermatocytes.

Here we find that forced expression of the TGFβ class signaling molecule *dpp* specifically in germ cells has effects similar to loss of function of the signal transduction pathway in somatic cyst cells: failure of spermatogonia to stop mitotic divisions and become spermatocytes. Similar findings were recently described by SHIVDASANI and INGHAM (2003). This result was surprising, as one would expect that forced expression of a ligand might cause a phenotype opposite from loss of function of a receptor. One explanation might be that precise levels of the *dpp* ligand may be critical, for example, for proper temporal or spatial control of activation of the pathway in somatic cyst cells. Another possibility is that *dpp* may not be the normal ligand, but that high levels of *dpp* secreted from germ cells may bind to TGFβ receptors on cyst cells and block their ability to respond to the normal ligand. Consistent with this hypothesis, the TGFβ type II receptor *punt* and both TGFβ type I receptors *sax* and *tkv* have been demonstrated to bind *dpp* in transfected Cos cells (LETSOU *et al.* 1995). The TGFβ homolog *Maverick*, rather than *dpp*, may be the ligand normally expressed in spermatogonia for activation of the TGFβ signal transduction pathway in surrounding cyst cells, as we detected *Maverick* mRNA but not *dpp* mRNA in early germ cells in wild-type testes by *in situ* hybridization.

Alternatively, TGFβ signaling may be required in germ cells. Indeed, forced expression of the activated *tkv* receptor in early germ cells also caused spermatogonia to continue mitotic proliferation rather than differentiate as spermatocytes. The apparently cell autonomous effect of forced expression of the activated *tkv* receptor in germ cells suggests a direct role for the TGFβ signaling pathway in germ cells. However, our results that marked clones of germ cells mutant for the TGFβ receptor *sax* differentiate as spermatocytes, along with similar findings by MATUNIS *et al.* (1997) and SHIVDASANI and INGHAM (2003) that marked clones of germ cells mutant for *punt, schnurri,* or *Mothers against dpp* differentiate as spermatocytes with the normal number of 16 spermatocytes per cyst, indicate that the TGFβ signaling pathway may not normally be required in germ cells for proper execution of the spermatogonia-to-spermatocyte transition. These observations raise the possibility that forced expression of *dpp* or the activated *tkv* receptor in early germ cells blocks the transition from the spermatogonial mitotic division program to spermatocyte differentiation by artificial and abnormal interference with the germ cell autonomous mechanisms that regulate this critical cell fate transition. The only Drosophila

genes previously known to be required cell autonomously in the germ line for spermatogonia to exit the spermatogonial division program and become spermatocytes are *bam* and its partner, *bgcn* (GÖNCZY *et al.* 1997). As discussed above, the phenotype of males haplo-insufficient for *bam* suggests that the level of *bam* expressed in male germ cells is important for the correct transition from spermatogonia to spermatocytes. One model proposed for the female germ line is that *dpp* secreted from somatic cap cells at the tip of the germarium blocks expression of *bam* in GSCs, allowing stem cell maintenance (CHEN and MCKEARIN 2003a,b). Strikingly, the *Pro-bam*-GFP reporter was expressed at reduced levels in spermatogonia from males in which *UAS-tkv** or *UAS-dpp* were forcibly expressed in early male germ cells under control of the *nos-gal4* germ-line-specific transgene driver, suggesting that activated Tkv or Dpp may suppress *bam* expression in males as well. It is tempting to speculate that, in the male, forced expression of *dpp* in spermatogonia may alter levels of *bam* expression so that *bam* protein does not reach a critical threshold required for the transition to spermatocyte differentiation. However, consistent with the production of many cysts of differentiating interconnected spermatogonia in *UAS-tkv**; *nos-gal4* and *UAS-dpp; nos-gal4* males, some expression of the *Pro-bam*-GFP reporter was detected. The expression of the *Pro-bam*-GFP reporter even in the presence of the activated *tkv* receptor suggests that there may be mechanisms at work in spermatogonia that can override silencing of *bam* expression by the TGFβ signaling pathway. Because the *Pro-bam*-GFP transcriptional reporter was expressed in spermatogonia even in cells expressing the activated *tkv* receptor, these mechanisms are likely either to interfere with the TGFβ signal transduction pathway downstream of receptor activation or to act independently of and/or override the TGFβ signaling effect. Forced expression of activated *tkv* in spermatogonia may also somehow affect expression or stability of Bam protein, as SHIVDASANI and INGHAM (2003) did not detect accumulation of BamC protein in spermatogonial cysts in testes from *UAS-tkv**; *nos-gal4* animals.

TGFβ signaling and male GSCs: Forced expression of the TGFβ class signaling molecule *dpp* or the activated *tkv* receptor in early male germ cells led to a mild increase in the number of male GSCs and gonialblasts around the apical hub and to reduced expression levels of the *Pro-bam*-GFP transcriptional reporter in spermatogonia. If TGFβ signaling normally acts on the silencer element in the *bam* gene to repress expression of *bam* in male GSCs, as has been shown for female GSCs (CHEN and MCKEARIN 2003a,b), then forced activation of TGFβ signaling in male early germ cells might delay the transition from stem cell to spermatogonial differentiation by delaying the accumulation of *bam* protein. However, the effect of activation of TGFβ class signaling on male GSCs was much more subtle than the effects

noted in female germ cells (XIE and SPRADLING 1998). The difference between the sexes in this regard may reflect the fundamental difference in the role of *bam* in male *vs.* female early germ cells discussed above. Loss of function of TGFβ class signaling in male GSCs had a subtle, but opposite, effect. Germ-line clones homozygous mutant for the TGFβ class receptor *sax* appeared at lower frequency and tended to produce fewer differentiating cysts compared to control clones. Of course, data from clonal analysis must always be interpreted with caution because of the possibility of effects from secondary recessive mutations on the chromosome arm. However, given our observations on the effects of forced expression of *bam*, it is tempting to speculate that loss of function of *sax* from germ cells allows *bam* to be expressed too early in male GSCs and gonialblasts, slowing or arresting differentiation of spermatogonial cysts and eventually leading to early germ cell loss. We note that some *sax* mutant germ-line clones did persist over time, again suggesting that male GSCs appear less sensitive than female early germ cells to either loss of function of TGFβ signaling or overactivation of the receptor.

Although parallels between the male and female GSC systems are beginning to emerge, *bam* and the TGFβ signaling pathway appear to play fundamentally different roles in male *vs.* female early germ cells. In both cases, male GSCs appear to be less sensitive than female GSCs to perturbations . We propose that this difference relates, at least in part, to the difference in the primary role of *bam* in the two sexes. In the female germ line, expression of *bam* appears to be the key event that produces a cystoblast and drives it to embark on cystocyte differentiation (McKEARIN and OHLSTEIN 1995; OHLSTEIN and McKEARIN 1997). Thus the mechanisms that suppress *bam* expression in GSCs and allow it in cystoblasts are likely to be key instructive determinants in the decision between stem cell self-renewal and the onset of differentiation. In contrast, in the male germ line, wild-type function of *bam* is primarily required at a later step in the differentiation pathway for cessation of the amplifying mitotic spermatogonial divisions and the transition to spermatocyte differentiation (GÖNCZY *et al.* 1997). In this case, the mechanisms that block *bam* protein expression in the GSCs may play a permissive rather than instructive role in allowing stem cell maintenance.

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