Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing *bam* transcription in the *Drosophila* testis

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Summary

Stem cells are responsible for replacing damaged or dying cells in various adult tissues throughout a lifetime. They possess great potential for future regenerative medicine and gene therapy. However, the mechanisms governing stem cell regulation are poorly understood. Germline stem cells (GSCs) in the *Drosophila* testis have been shown to reside in niches, and thus these represent an excellent system for studying relationships between niches and stem cells. Here we show that Bmp signals from somatic cells are essential for maintaining GSCs in the *Drosophila* testis. Somatic cyst cells and hub cells express two Bmp molecules, Gbb and Dpp. Our genetic analysis indicates that *gbb* functions cooperatively with *dpp* to maintain male GSCs, although *gbb* alone is essential for GSC maintenance.

Furthermore, mutant clonal analysis shows that Bmp signals directly act on GSCs and control their maintenance. In GSCs defective in Bmp signaling, expression of *bam* is upregulated, whereas forced *bam* expression in GSCs causes the GSCs to be lost. This study demonstrates that Bmp signals from the somatic cells maintain GSCs, at least in part, by repressing *bam* expression in the *Drosophila* testis. *dpp* signaling is known to be essential for maintaining GSCs in the *Drosophila* ovary. This study further suggests that both *Drosophila* male and female GSCs use Bmp signals to maintain GSCs.

Key words: Germline, Stem cells, Bmp, Bam, *Drosophila*

Introduction

In adult tissues that are subject to continuous cell turnover, stem cells are responsible for maintaining tissue homeostasis. A stem cell usually undergoes a stereotyped asymmetric cell division: giving rise to one parent stem cell that continues to undergo asymmetric cell divisions and a daughter that commits to differentiation. Stem cell behavior has been shown to be controlled by specialized regulatory microenvironments or 'niches' in several systems (Watt and Hogan, 2000; Spradling et al., 2001; Lin, 2002). However, niche signals in many stem cell systems are still poorly defined. Knowledge of niche signals is also important for expanding stem cells in in vitro cultures for cell replacement therapy. Therefore, the identification of niche signals in different stem cell systems is important not only for a better understanding of how stem cell function is controlled but also for using stem cells in future regenerative medicine.

The *Drosophila* testis has become one of the premier stem cell systems to study molecular mechanisms governing stem cell self-renewal, differentiation and proliferation (Kiger et al., 2000; Tran et al., 2000; Kiger et al., 2001; Tulina and Matunis, 2001). In the testis, there are two types of stem cells, GSCs and somatic stem cells (also known as cyst progenitor cells), which are responsible for producing differentiated germ cells and somatic cyst cells that encapsulate differentiated germ cells, respectively (Fig. 1A). Seven to nine GSCs can be reliably identified by their attachment to hub cells (a group of tightly packed somatic cells) and existence of a spectrosome (Fig. 1A,B). The spectrosome is a spherical fusome that is unique to GSCs and their early progeny, also known as gonialblasts. The fusome is rich in cytoskeletal proteins such as Hu li tai shao (Hts) and α-Spectrin (Lin et al., 1994; de Cuevas and Spradling, 1997). Once a GSC divides, one daughter cell that is in contact with the hub cells retains stem cell identity, whereas the other daughter cell that is not in contact with the hub cells initiates differentiation and becomes a gonialblast. The gonialblast then undergoes four rounds of synchronous cell division to generate a 16-cell germline cluster in which individual germ cells are connected by ring canals and a branched fusome. During the course of germ cell development from a gonialblast to a 16-cell cyst, a pair of somatic cyst cells surrounds the gonialblast, or a developing germ cell cluster, and control proper germ cell proliferation and differentiation (Matunis et al., 1997).

Recent studies indicate that the hub cells and somatic stem cells could function as a niche for GSCs in the testis (Kiger et al., 2000; Tran et al., 2000; Kiger et al., 2001; Tulina and Matunis, 2001). The hub cells are known to express Unpaired (Upd; Os – FlyBase), a secreted ligand that activates the JAK-STAT signaling pathway in GSCs and promotes their self-renewal (Kiger et al., 2001; Tulina and Matunis, 2001). GSCs mutant for *upd* and *Dstat* (Stat92E – FlyBase) are lost rapidly, whereas overexpression of *upd* prevents proper differentiation of gonialblasts, resulting in the accumulation of

undifferentiated germ cells. Moreover, somatic cyst cells are required for the early stage of spermatogonial differentiation (Kiger et al., 2000; Tran et al., 2000). Loss of function of *Egfr* and *raf* in somatic cyst cells disrupts differentiation of gonialblasts, resulting in the accumulation of undifferentiated germ cells in the testis. Somatic cyst cells are also important to ensure that a gonialblast undergoes exactly four rounds of mitosis to generate a 16-cell cluster through a Tgfβ-like signaling pathway (Matunis et al., 1997). Furthermore, *bag of marbles* (*bam*) and *benign gonial cell neoplasm* (*bgcn*) act autonomously in the germline to restrict the proliferation of amplifying germ cells (Gonczy et al., 1997). However, the connection between the Tgfβ signal from somatic cyst cells and *bam/bgcn* in the germline remains unclear.

The *Drosophila* ovary represents another attractive stem cell system in which stem cells and their niche cells can be reliably identified (Xie and Spradling, 2001; Lin, 2002). Germline stem cells have first been demonstrated to be located in the niche, consisting of terminal filament/cap cells and inner sheath cells (Xie and Spradling, 2001; Lin, 2002). *fs(1)Yb* and *piwi* are expressed in the terminal filament/cap cells and are essential for GSC maintenance (King and Lin, 1999; Cox et al., 1998; Cox et al., 2000; King et al., 2001). *decapentaplegic* (*dpp*), a *Drosophila* Bmp member, is expressed in somatic cells such as cap cells, and is essential for GSC maintenance and division in the *Drosophila* ovary (Xie and Spradling, 1998; Xie and Spradling, 2000). Interestingly, another Bmp member, *glass bottom boat* (*gbb*, also known as *60A*), is highly expressed in the male (Wharton et al., 1991; Doctor et al., 1992), but its role in spermatogenesis has not been investigated. Here we show that both *gbb* and *dpp*, are expressed in the somatic cells of the testis and act cooperatively on GSCs to control their maintenance. In addition, *gbb* signaling is essential for repressing *bam* expression in GSCs in *Drosophila*.

Materials and methods

Drosophila stocks and genetics

The following fly stocks used in this study were described either in FlyBase or as otherwise specified: tkv^8 and sax^4 (Brummel et al., 1994); $punt^{10460}$ and $punt^{135}$; Med^{26} (Das et al., 1998); *Dad-lacZ* (Tsuneizumi et al., 1997); dpp^{hr4} and dpp^{hr56}; gbb^4, gbb^{D4} and gbb^{D20}; *bam-GFP* (Chen and McKearin, 2003a); *vasa-GFP* (Nakamura et al., 2001); *c587-gal4*, *hs-gal4* and *nanos-gal4VP16* (Van Doran et al., 1998); *UAS-dpp*, *UASp-bamGFP* (Chen and McKearin, 2003a) and *UAS-gbb* (Khalsa et al., 1998); *hsFLP; FRT$_{82B}$ arm-lacZ*; *FRT$_{40A}$ arm-lacZ*; *FRT$_{G13}$ arm-lacZ*. Most stocks were cultured at room temperature. To maximize their mutant effects, *dpp*, *gbb* and *punt* mutant adult females were cultured at 29°C for 2-7 days.

Generating mutant GSC clones and overexpression

Clones of mutant GSCs were generated by Flp-mediated mitotic recombination, as described previously (Xie and Spradling, 1998). To generate the stocks for stem cell clonal analysis, 2-day-old adult males carrying an *armadillo-lacZ* transgene in trans to the mutant-bearing chromosome were generated using standard genetic crosses and then heat-shocked at 37°C for 3 consecutive days with two one-hour heat-shock treatments daily separated by 8-12 hours. The males were transferred to fresh food every day at room temperature, and the testes were removed 2 days, 1 week and 2 weeks after the last heat-shock treatment, and then processed for antibody staining.

To construct the stocks for overexpressing *dpp* or *gbb*, *nanos-*

gal4VP16 virgins were crossed with *UAS-dpp* and *UAS-gbb* males, respectively. The males that carried *nanos-gal4VP16* and *UAS-dpp* or *UAS-gbb* were cultured at room temperature, or at 29°C, for one week. For examining the expression of *bam-GFP* in the testes overexpressing *dpp* or *gbb*, the *bam-GFP/CyO; nanos-gal4VP16* virgins were used in the crosses.

Measuring GSC loss in *gbb* mutants and marked GSCs, and examining *bam-GFP* expression in *gbb*, *dpp* and *punt* mutant testes

To determine loss of marked mutant GSC clones, GSCs were marked in 1- to 2-day-old males of the appropriate genotype. Subsequently, testes were isolated from some of the males 2 days, 1 and 2 weeks later, and stained with anti-Hts and anti-β-Gal antibodies. The percentage of testes containing one or more marked GSCs was determined by counts of 55-227 testes at each time point.

To measure stem cell loss in *gbb* mutant testes, the testes with different numbers of GSCs were determined based on anti-Hts and anti-Fas3 antibody staining of gbb^4/gbb^{D4} or gbb^4/gbb^{D20} testes of different ages and different treatments. *yw* males carrying no *gbb* mutations served as a control. The 2-day-old control and *gbb* mutant males were cultured at different temperatures after they eclosed at 18°C. Values are expressed as the average GSC number per testis, and/or the percentage of testes carrying no GSCs.

To examine *bam-GFP* expression in *dpp*, *gbb* and *punt* mutant testes, we generated males with the following genotypes at 18°C: *bam-GFP gbb^4/gbb^{D4}*, *bam-GFP gbb^4/gbb^{D20}*, *bam-GFP dpp^{hr56}/dpp^{hr4}* or *$punt^{10460}/punt^{135}$; bam-GFP. bam-GFP* males carrying no mutations for *gbb*, *dpp or punt* served as a control. All the control and mutant males were cultured at 29°C for 4 days before their testes were isolated, stained with antibodies and compared for *bam*-GFP expression at identical conditions.

The TUNEL cell death assay was performed on *punt* mutant testes (Intergen Company).

Immunohistochemistry

The following antisera were used: polyclonal anti-Vasa antibody (1:2000) (Liang et al., 1994); monoclonal anti-Hts antibody (1:3); polyclonal anti-β-Gal antibody (1:1000; Cappel); monoclonal anti-β-Gal antibody (1:100; Promega); polyclonal anti-GFP antibody (1:200; Molecular Probes); polyclonal anti-pMad antibody (1:200) (Tanimoto et al., 2000). The immunostaining protocol used in this study was described previously (Song et al., 2002). All micrographs were taken using a Leica SPII confocal microscope.

Detecting gene expression in purified component cells using RT-PCR

The tips of the testes for the males of the appropriate genotype were dissected off from the whole testes in Grace's media, and were dissociated with collagenase II (Sigma) solution at a concentration of 6 mg/ml. After sorting of GFP-positive cells, using Cytomation MoFlo, from testes with GFP-marked hub cells, somatic cyst cells or germ cells, total RNA was prepared using Trizol (Invitrogen). RNA samples were further amplified using the GeneChip Eukaryotic Small Sample Target Labeling Assay Version II (Affymetrix). After RNA amplification, 100 ng of total RNA was reverse transcribed (RT) using SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen). The following primers were used in this study:

dpp, 5′-AGCCGATGAAGAAGCTCTACG-3′ and 5′-ATGTCGTA-GACAAGCACCTGGTA-3′;

vasa, 5′-ATCGAGGAGGAAATCGAGATGGA-3′ and 5′-GGAA-GCTATGCCACTGCTGAATA-3′;

gbb, 5′-AGATGCAGACCCTGTACATAGAC-3′ and 5′-CTCGT-CGTTCAGGTGGTACAGAA-3′; and

rp49, 5′-GTATCGACAACAGAGTCGGTCGC-3′ and 5′-TTGG-TGAGCGGACCGACAGCTGC-3′.

PCR was performed as follows: 95°C for 4 minutes; 40 cycles of

95°C for 30 seconds, 45°C for 30 seconds and 72°C for 45 seconds; and 72°C for 7 minutes. RT-PCR products were electrophoresed on 2% agarose gel in the presence of ethidium bromide.

Results

Two Bmp genes, *dpp* and *gbb*, function cooperatively to maintain GSCs in the *Drosophila* testis

To investigate the possible role of *dpp* and *gbb* in maintaining male GSCs, we examined GSCs in the testes of temperature-sensitive *dpp* and *gbb* mutant males. Two homozygous allelic combinations used in this study, gbb^4/gbb^{D4} and gbb^4/gbb^{D20}, were allowed to develop to adulthood at 18°C, and subsequently shifted to 22°C or 25°C for 7 days. In this study, an anti-Hts antibody was used to label spectrosomes and fusomes, whereas a DNA dye, DAPI, was used to stain nuclei. The hub can be reliably identified by molecular markers, such as Fasciclin 3 (Fas3), or by DAPI staining (small, DAPI-bright nuclei in the hub cells tightly packed together), whereas GSCs are identified by the presence of a spectrosome and direct contact with the hub cells. The numbers of GSCs in the testes of different mutants were quantified after the testes were immunostained for Hts and Fas3 to visualize spectrosomes in GSCs and hub cells, respectively. A wild-type testis carried an average of 9.1 GSCs ($n=42$, Fig. 1B), and these stem cells would persist for 7 days at 25°C or 29°C. One week after being cultured at 22°C, the testes from gbb^4/gbb^{D4} or gbb^4/gbb^{D20} mutants contained 1.3 ($n=21$) or 1.8 ($n=22$) stem cells, respectively (Fig. 1C). One week after being cultured at 25°C, no GSCs were observed in the mutant testis (Fig. 1D). These results indicate that *gbb* is essential for maintaining GSCs in the testis.

Two temperature-sensitive allelic combinations, dpp^{hr4}/dpp^{hr56} and dpp^{e90}/dpp^{hr56} were used to investigate the role of *dpp* in maintaining GSCs in the testis. Similarly, *dpp* homozygous males were developed to adulthood at 18°C and were then shifted to a restrictive temperature (29°C) for one week. In the *Drosophila* ovary, both mutant combinations lose their GSCs very rapidly at a restrictive temperature (Xie and Spradling, 1998). Surprisingly, one week after being cultured at the restrictive temperature, the testes from *dpp* mutants had no significant GSC loss: dpp^{hr4}/dpp^{hr56} and dpp^{e90}/dpp^{hr56} mutant testes had an average of 7.4 ($n=44$) and 8.8 ($n=49$) GSCs/testis, respectively (Fig. 1E), which is in contrast with the severe GSC loss phenotype in the *dpp* mutant ovary and in the *gbb* mutant testis.

The two allelic *dpp* combinations used in this study represent very weak *dpp* mutants. As there is a stringent requirement for *dpp* during early *Drosophila* development, it is difficult to examine GSC loss in stronger *dpp* mutants because they do not survive to adulthood, even at 18°C. It is still possible that the role of *dpp* in the maintenance of male GSCs can be revealed if *gbb* signaling is comprised, as *dpp* and *gbb* could use the same receptors and downstream components to transduce their signals (Khalsa et al., 1998). To further study the role of *dpp* in the regulation of male GSCs, we constructed two mutant strains homozygous for two *dpp* allelic combinations that were also heterozygous for *gbb*: dpp^{hr4}/dpp^{hr56} gbb^{D4} and dpp^{e90}/dpp^{hr56} gbb^{D4}. The testes from the heterozygous gbb^{D4}, which were cultured at 29°C for one
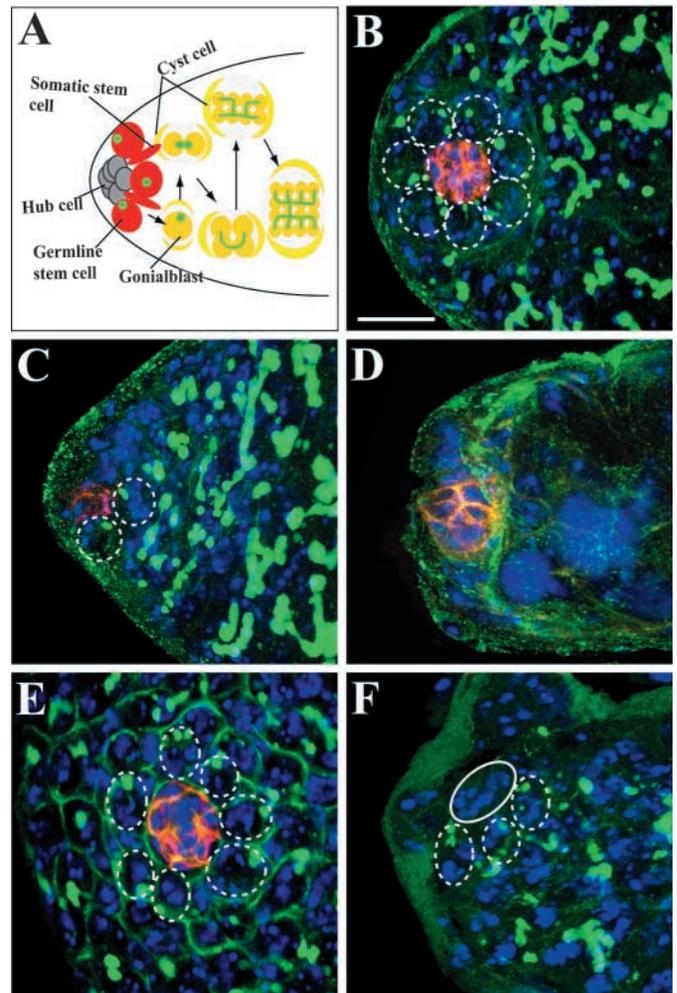
Fig. 1. Dpp and Gbb function cooperatively to maintain GSCs in the *Drosophila* testis. (A) A diagram of the testis tip including GSCs and SSCs. Normally, seven to ten GSCs (three are shown here for demonstration; round red cells) and somatic stem cells (also known as cyst progenitor cells; red elliptical cells) directly contact the hub cells (gray cells). The gonialblast, which is encapsulated by two differentiated somatic cyst cells, moves away from the hub cells and divides to produce a two-cell, four-cell, eight-cell or eventually a 16-cell cluster, which can be identified by the branched fusome (green lines). The testes in B-E are labeled for FasIII (red, hub cells), Hts (green, spectrosomes and fusomes) and DAPI (blue). The testis in F is labeled for Hts (green) and DAPI (blue). The hub cells are labeled red by FasIII in B-E and highlighted by a circle in F, whereas the GSCs are highlighted by broken lines in B-F. (B) The tip of a wild-type testis showing seven GSCs that contact the hub cells. (C,D) The tips of two gbb^4/gbb^{D20} mutant testes showing two remaining GSCs (C) or no GSCs (D) close to the hub cells. (E) A tip of dpp^{hr4}/dpp^{hr56} mutant testis showing seven GSCs near hub cells. (F) The tip of a dpp^{hr4}/dpp^{hr56} gbb^{D20} mutant testis showing three remaining GSCs near the hub cells. All the images are shown at the same magnification. Scale bar: 10 μm.

week, had a normal GSC number (8.6 GSCs/testis, $n=38$). The testes from dpp^{hr4}/dpp^{hr56} gbb^{D4} and dpp^{e90}/dpp^{hr56} gbb^{D4} had an average of 3.0 ($n=13$) and 5.7 ($n=56$) GSCs/testis, respectively (Fig. 1F), in comparison with 7.4 and 8.8

GSCs/testis for *dpp* mutants alone, suggesting that partial removal of *gbb* function can enhance the *dpp*-mutant GSC-loss phenotype in the *Drosophila* testis. These results indicate that *dpp* and *gbb* function cooperatively to regulate male GSCs in *Drosophila*.

To further confirm that Bmp signaling is essential for maintaining male GSCs, we studied mutant phenotypes for one of the Bmp downstream components, *punt*, which encodes a type II serine/threonine kinase receptor for *dpp*, and also possibly for *gbb* (Letsou et al., 1995; Ruberte et al., 1995). A *punt* allelic combination, $punt^{10460}/punt^{135}$, exhibits a temperature-sensitive phenotype: developing to adulthood at 18°C and showing mutant phenotypes at 29°C (Theisen et al., 1996). Interestingly, $punt^{10460}/punt^{135}$ mutant males had normal GSC numbers (8.5 GSCs/testis, $n=20$) after being cultured at 22°C for a week (Fig. 2A). However, one week after shifting to 29°C, almost all the mutant testes completely lost their GSCs (0.1 GSCs/testis, $n=58$; Fig. 2B,C), although wild-type testes still maintained normal GSC number under the same conditions (data not shown). To exclude the possibility that Bmp signaling is important for GSC survival, we applied the TUNEL labeling assay to look for dying GSCs in *punt* mutant testes. During the one-week period at 29°C, no dying GSCs were detected in the *punt* mutant testes, but some rare dying cyst cells or differentiated germ cells were observed ($n=38$, Fig. 2D), suggesting that GSC loss is most likely caused by differentiation triggered by the lack of sufficient Bmp signaling. This result further supports the idea that Bmp signaling is essential for maintaining GSCs in the *Drosophila* testis.

Gbb signaling leads to the transcription of *Dad* and the phosphorylation of Mad protein in GSCs and gonialblasts

The GSC loss caused by defective Bmp signaling could be due to direct and/or indirect signaling to GSCs. To investigate whether Bmp signals are directly received by GSCs, we assessed Bmp signaling activities in GSCs by examining the expression of *Dad*. *Dad* is a *dpp*-responsive gene that negatively regulates *dpp* signaling (Tsuneizumi et al., 1997). Interestingly, *Dad-lacZ*, which reflects *Dad* mRNA expression (Tsuneizumi et al., 1997), was expressed in GSCs and gonialblasts, but not in more differentiated spermatogonial cells (Fig. 3A), indicating that Bmp signals function as short-ranged signals, and their activities are restricted to GSCs and gonialblasts. Moreover, it was also expressed in cyst cells at higher levels but generally not in cyst progenitor cells (Fig. 3A).

To further determine whether mutations in *gbb* affect *Dad* expression in GSCs, we examined the expression of *Dad-lacZ* in the gbb^4/gbb^{D20} mutant background. After *gbb* mutant males were cultured at 22°C for four days, 97% of mutant GSCs ($n=101$) did not express *Dad-lacZ* (Fig. 3B). Interestingly, the *gbb* mutant testes carrying the *Dad-lacZ* mutation (4.8 GSCs; $n=21$) had more GSCs than the *gbb* mutant testes (2.4 GSCs; $n=16$). *Dad-lacZ* is a P element insertion mutation, which augments *dpp* signaling (Tsuneizumi et al., 1997). We also examined the expression of *Dad-lacZ* in the dpp^{hr4}/dpp^{hr56} mutant background. Even after the *dpp* mutant males were cultured at 29°C for 4 days, *Dad-lacZ* expression was only slightly reduced (data not shown), which is consistent with no
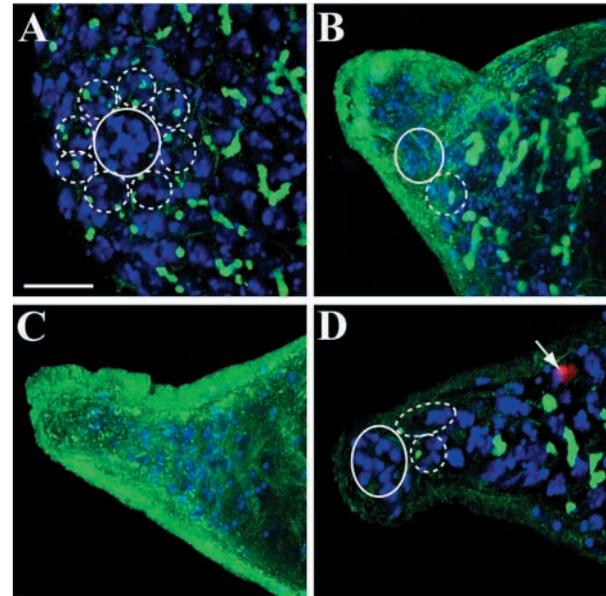

Fig. 2. *punt* is required for maintaining GSCs in the *Drosophila* testis. All the testes are $punt^{10460}/punt^{135}$ mutants and are labeled for Hts (green) and DAPI (blue) except nuclei of dying cells are labeled red in D. The hub cells are highlighted by circles, whereas GSCs are identified by broken lines. (A) The tip of a *punt* mutant testis one week after being cultured at 22°C showing eight GSCs. (B,C) The tips of two *punt* mutant testis one week after being cultured at 29°C showing one remaining GSC (B) or no GSCs (C). (D) The tip of a *punt* mutant testis 4 days after being cultured at 29°C showing that no GSCs undergo apoptosis except a few late somatic cyst cells (arrow). All the images are shown at the same magnification. Scale bar: 10 μm.

obvious GSC loss in the testis of *dpp* mutants. These results suggest that *Dad* is primarily a *gbb*-responsive gene that is also likely to negatively regulate *gbb* signaling in the *Drosophila* testis.

To further test whether *Dad* could inhibit both *gbb* and *dpp* signaling, we overexpressed *Dad* in germ cells using the Gal4-UAS bipartite expression system (Brand and Perrimon, 1993). A germline-specific *nanos-gal4VP16* driver can express a target gene under the control of UAS promoter specifically in germ cells (Van Doran et al., 1998), whereas a *UAS-Dad* transgene can be used to produce *Dad* under a *gal4* driver to inhibit *dpp* signaling (Tsuneizumi et al., 1997). When the *UAS-Dad* transgene was used to overexpress *Dad* in germ cells by *nanos-gal4VP16*, all GSCs were lost in the testes before adulthood (Fig. 3C), indicating that blocking Bmp signaling causes GSC loss or prevents the formation of GSCs. The GSC loss phenotype induced by *Dad* overexpression mimics that of *gbb* mutants, suggesting that *Dad* overexpression probably inhibits not only *dpp* signaling but also *gbb* signaling. Thus, *Dad-lacZ* expression in GSCs may reflect the activities of both *dpp* and *gbb* signaling pathways. Together, these results suggest that Bmp signals appear to function as short-range signals to control GSC maintenance through direct signaling to GSCs.

In *Drosophila*, Dpp brings type I receptors Tkv and Sax, and the type II receptor Punt to form receptor complexes, which in turn phosphorylate Mad (Brummel et al., 1994; Nellen et al.,
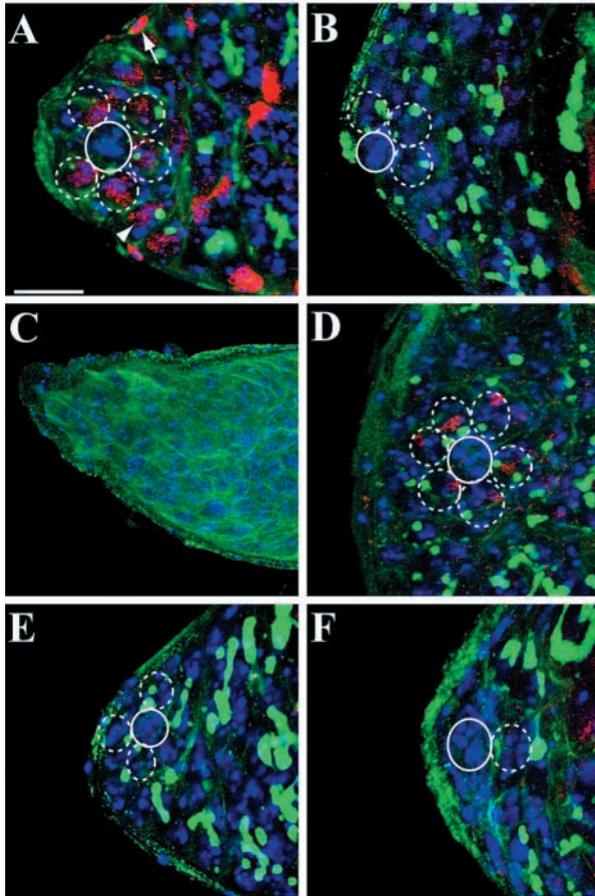
Fig. 3. GSCs and gonialblasts but not other differentiated germ cells are responsive to Bmp signaling in the testis. The hub cells and the GSCs in A, B and D-F are highlighted by circles and broken lines, respectively. (A) The tip of a *Dad-lacZ/+* testis labeled for nuclear *lacZ* (red), Hts (green) and DNA (blue), showing that all five GSCs and all gonialblasts (arrowhead) express *Dad*. Arrows indicate that somatic cyst cells also express *Dad*. (B) The tip of a *gbb⁴/gbbᴰ²⁰*; *Dad-lacZ/+* testis labeled for nuclear *lacZ* (red), Hts (green) and DNA (blue), showing that three remaining GSCs and all the gonialblasts do not have detectable *Dad* expression. (C) The tip of a testis overexpressing Dad specifically in the germ cells labeled for Hts (green) and DAPI (blue), showing no germ cells in the testis. (D) The tip of a wild-type testis labeled for pMad (red), Hts (green) and DAPI (blue), showing pMad accumulation predominantly in GSCs. (E) The tip of a *gbb⁴/gbbᴰ⁴* mutant testis labeled for pMad (red), Hts (green) and DAPI (blue), showing no detectable pMad accumulation in GSCs. (F) The tip of a *punt¹⁰⁴⁶⁰/punt¹³⁵* mutant testis labeled for pMad (red), Hts (green) and DAPI (blue), showing no detectable pMad accumulation in the remaining GSC. All the images are shown at the same magnification. Scale bar: 10 μm.

1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995; Newfeld et al., 1996; Newfeld et al., 1997). The phosphorylated Mad (pMad) is then associated with Medea (Med) and translocated to the nucleus to function as transcriptional activators for *dpp*-responsive genes (Das et al., 1998; Wisotzkey et al., 1998). pMad expression has been directly associated with *dpp* signaling activity in responding cells (Tanimoto et al., 2000). To further determine whether *gbb* signaling is responsible for pMad expression in GSCs, we

examined the pMad expression in wild-type, *gbb* and *punt* mutant GSCs in the testis. pMad preferentially accumulated in GSCs but was absent from gonialblasts and two-cell germ cell clusters (Fig. 3D), which is in contrast to *Dad-lacZ* expression in both GSCs and gonialblasts. This difference could be due to the perdurance of *lacZ* mRNA and/or protein. Alternatively, levels of pMad in gonialblasts are low and undetectable with the existing anti-pMad antibody. In the *gbb* mutant testes that still maintained some GSCs, pMad expression in the GSCs was severely reduced and below the limits of detection (Fig. 3E). In the testes of *punt¹⁰⁴⁶⁰/punt¹³⁵* mutant males cultured at 29°C, pMad levels in GSCs were severely reduced and were sometimes difficult to detect (Fig. 3F). However, pMad expression in late 16-cell germ cell clusters remained high in both the *gbb* and *punt* mutant testes (data not shown). Therefore, *gbb* probably signals through common Bmp receptors, which leads to phosphorylation of Mad and *Dad* transcription.

Bmp signals directly act on GSCs to control their maintenance

To definitely confirm that Bmp signals directly act on GSCs and control their maintenance, we used the FLP-mediated FRT mitotic recombination to generate marked GSC clones mutant for Bmp downstream components (Xie and Spradling et al., 1998; Kiger et al., 2001; Tulina and Matunis, 2001). The *armadillo-lacZ* transgenes that are strongly expressed in all the cells in the tip of the testis were used to mark mutant GSC clones. The marked GSCs were induced in adult testes by heat-shock treatments and identified as *lacZ* negative, spectrosome-containing germ cells that are in direct contact with the hub cells. The percentages of testes carrying one or more marked GSCs were determined at different time points after clone induction. The rate of loss of GSCs mutant for different Bmp downstream components can be used to determine how each Bmp downstream component contributes to the regulation of GSCs.

GSC clones mutant for *punt*, *tkv*, *sax*, *mad* and *Med* were generated as described previously (Xie and Spradling, 1998), and their testes were examined 2 days later. Two days after clone induction, 100% of the testes carried one or more marked wild-type GSCs (Fig. 4A), whereas 2 weeks after clone induction, 63% of the testes still carried one or more marked wild-type GSCs (Fig. 4B, Table 1). Two days after clone induction, over 80% of the testes still carried one or more marked GSCs mutant for *tkv*, *sax*, *punt*, *mad* or *Med* (Fig. 4C,E; Table 1). In contrast to wild-type clones, marked GSC clones mutant for *punt*, *tkv*, *sax*, *mad* and *Med* were lost rapidly 2 weeks after clone induction (Fig. 4D,F; Table 1). For example, none of the testes mutant for *punt¹⁰⁴⁶⁰*, *punt¹³⁵*, *tkv⁸*, *mad¹²* and *Med²⁶* had any GSCs left 2 weeks after clone induction. *punt¹⁰⁴⁶⁰* is a moderate allele, while the rest are strong or null alleles (Brummel et al., 1994; Nellen et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995; Das et al., 1998; Wisotzkey et al., 1998). Interestingly, even though *sax⁴* is a strong or null *sax* allele (Brummel et al., 1994), 2 weeks after the clone induction, 6.3% of the testes carried *sax* mutant GSCs, indicating that *sax* plays a less important role in maintaining GSCs than *tkv*, the other type I receptor. Previous studies suggest that *gbb* preferentially uses *sax* to transduce its signal, whereas *dpp* prefers *tkv* for its signal transduction
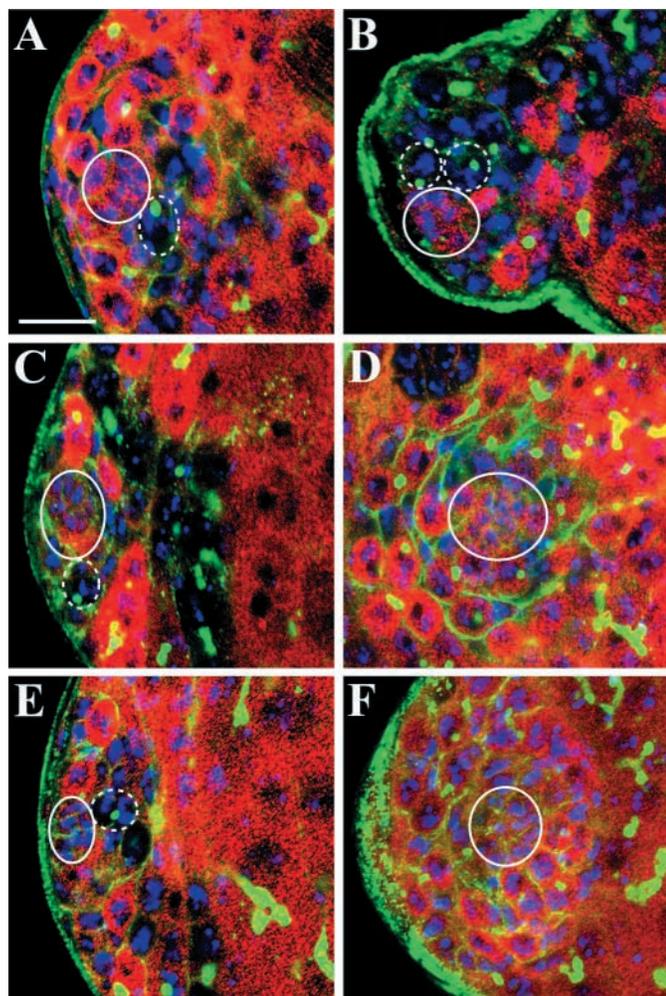
Fig. 4. Bmp downstream components are required in GSCs for their maintenance in the *Drosophila* testis. All the testes are labeled for *lacZ* (red), Hts (green) and DNA (blue). Marked wild-type or mutant GSCs (highlighted by broken lines) are identified as the cells that contain a spectrosome, directly contact hub cells and lack *lacZ* expression, whereas unmarked GSCs are *lacZ* positive (red). (A) The tip of a testis carrying a marked 2-day-old wild-type GSC. (B) The tip of a testis carrying two marked two-week old GSCs. (C) The tip of a testis carrying a marked 2-day-old *tkv* mutant GSC. (D) A testis that has lost a marked *tkv* GSC clone two weeks after clone induction. (E) The tip of the testis carrying a marked 2-day-old *mad* mutant GSC. (F) The tip of a testis that has lost a marked *mad* GSC clone 2 weeks after clone induction. All the images are shown at the same magnification. Scale bar: 10 μm.

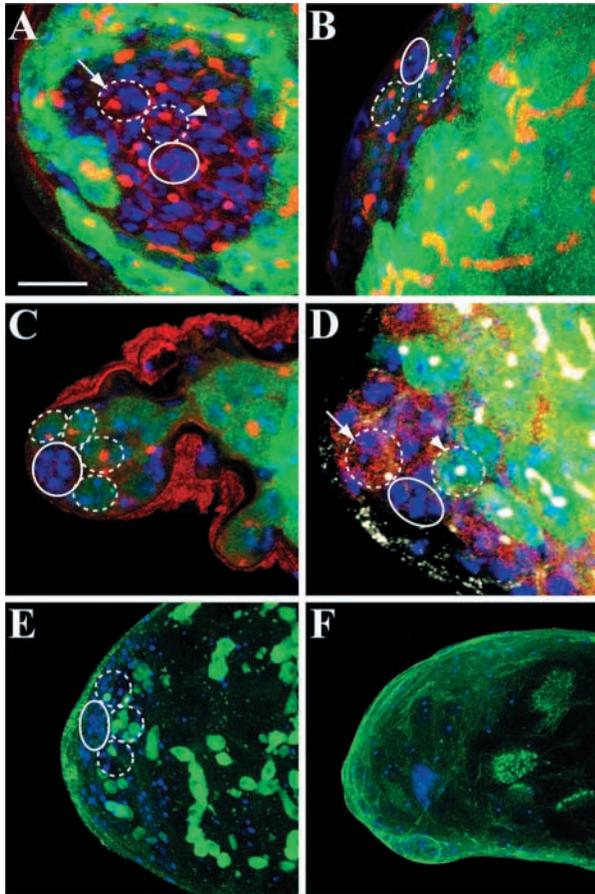**Table 1. Bmp-like downstream components are required in GSCs for their maintenance in the *Drosophila* testis**

	Percentage of testes carrying one or more marked GSC* (total number)		
	Ages		
Genotype	Two days	One week	Two weeks
Wild type	100 (73)	81.8 (77)	63.6 (55)
punt^{10460}	92.3 (78)	14.6 (89)	0.0 (114)
punt^{135}	89.1 (92)	21.2 (99)	0.0 (149)
tkv^{8}	93.3 (90)	1.3 (227)	0.0 (149)
sax^{4}	92.3 (78)	31.9 (72)	6.3 (63)
mad^{12}	82.9 (117)	0.7 (140)	0.0 (120)
Med^{26}	92.2 (76)	1.9 (107)	0.0 (114)

*Percentage of testes carrying marked GSCs = number of testes carrying one or more marked GSCs/total number of testes examined.

(BamC) (Xie and Spradling, 1998). In the ovary, *bam* expression in GSCs is normally transcriptionally repressed, but forced *bam* expression in GSCs leads them to differentiate (McKearin and Spradling, 1990; Ohlstein and McKearin, 1997; Chen and McKearin, 2003a), suggesting the possibility that one function of *dpp* is to prevent *bam* gene expression in GSCs. In wild-type testes, BamC is present in the cytoplasm of two-cell to early 16-cell germline cysts (Kiger et al., 2000). This also raises a similar possibility that Bmp signals directly or indirectly repress *bam* expression in male GSCs.

To determine whether *bam* transcription is repressed in male GSCs, we used a *bam-GFP* transgene (a *bam* promoter fused to the GFP gene) to examine its transcription (Chen and McKearin, 2003a). Interestingly, *bam* was transcribed predominantly in the differentiated germ cells but not in GSCs and gonialblasts in the *Drosophila* testis (Fig. 5A), which is consistent with the BamC expression pattern. Our result that *Dad* is expressed only in GSCs and gonialblasts supports the idea that Bmp signaling suppresses *bam* expression in GSCs and gonialblasts. If *bam* repression in GSCs is mediated by Bmp signaling, we would predict that *bam* expression in GSCs defective for Bmp signaling would be upregulated. To test this hypothesis, we generated *dpp*, *gbb* or *punt* homozygous mutant males that also carried the *bam-GFP* transgene to monitor *bam* expression. As predicted, *bam-GFP* was not obviously upregulated in *dpp*^{hr56}/*dpp*^{hr4} mutant GSCs just like in wild-type ones (data not shown), consistent with the fact that the *dpp* mutations have little effect on the maintenance of male GSCs. Interestingly, *bam-GFP* expression was elevated in the *gbb*^{4}/*gbb*^{D4} or *gbb*^{4}/*gbb*^{D20} mutant GSCs (Fig. 5B), indicating that *gbb* signaling is essential for repressing *bam* transcription in GSCs. Furthermore, at 22°C, *bam* expression was undetectable in the *punt* mutant GSCs, but it was elevated in *punt* mutant GSCs at 29°C (Fig. 5C). To further confirm this observation, we generated marked *Med* mutant GSCs that carried the *bam-GFP* transgene. Consistently, 66% of 3-day-old marked *lacZ*-negative *Med* mutant GSCs expressed *bam-GFP*, while neighboring unmarked *lacZ*-positive wild-type GSCs failed to express it (Fig. 5D). These results demonstrate that Bmp signaling is required to suppress *bam* transcription in GSCs in the *Drosophila* testis.

To further investigate whether forced *bam* expression causes GSC loss in males, we used two different means to ectopically

(Haerry et al., 1998). Our results argue that *gbb* preferentially uses *tkv* instead of *sax* to transduce its signal in male GSCs. Therefore, we conclude that Bmp signals directly act on GSCs and control their maintenance in the *Drosophila* testis.

Bmp signaling represses *bam* expression in GSCs and forced *bam* expression drives GSC differentiation

In the *Drosophila* ovary, overexpression of *dpp* causes accumulation of GSC-like cells, which contain a spectrosome and do not express the cytoplasmic form of Bam protein

Fig. 5. Gbb signaling is essential for repressing *bam* transcription in GSCs in the *Drosophila* testis. The testes in A-C are labeled for GFP (green), Hts (red) and DNA (blue); the testis in D is labeled for *lacZ* (red), GFP (green), Hts (white) and DNA (blue); the testes in E and F are labeled for Hts (green) and DAPI (blue). The hub cells are highlighted by circles, whereas some GSCs are highlighted by broken lines. (A) The tip of a *bam-GFP* wild-type testis showing no *bam* expression in GSCs (arrowhead) and gonialblasts (arrow). (B) The tip of a *gbb⁴/gbbD²⁰* mutant testis (after being cultured at 29°C for one week) showing elevated *bam-GFP* expression in GSCs. (C) The tip of a *punt¹⁰⁴⁶⁰/punt¹³⁵* mutant testis (after being cultured at 29°C for a week) showing elevated *bam-GFP* expression in GSCs. (D) The tip of a testis carrying a marked *Med* mutant GSC (arrowhead, lacZ negative) and unmarked wild-type GSCs (arrow, lacZ positive), showing elevated *bam-GFP* expression in the mutant *Med* GSC. (E) The tip of a *hs-bam* testis showing three remaining GSCs 1 week after heat-shock treatments. (F) The tip of a *nos-gal4VP16;UAS-bam* testis showing no GSCs. All the images are shown at the same magnification. Scale bar: 10 μm.

express *bam* in male GSCs: *hs-bam* (the *bam* gene under the control of a *hsp70* promoter) and *nanos-gal4VP16* driven *UASp-bamGFP* expression (Ohlstein and McKearin, 1997; Chen and McKearin, 2003a). Two days after heat-shock treatments (4 hours per day for 3 days), GSCs in all the testes without *hs-bam* remained normal with an average of 9.3 GSCs per testis ($n=26$), and a week later they retained 7.6 GSCs per testis ($n=30$). By contrast, 2 days after heat-shock treatments, GSCs in most of the testes carrying *hs-bam* was reduced to an average of 3.9 GSCs per testis ($n=31$) (Fig. 5E), whereas 1

week later, about 70% of the testes completely lost their GSCs with an average of 2.0 GSCs ($n=33$) (Fig. 5F). Similarly, *nanos-gal4*-driven germ cell-specific *bam* expression caused GSC loss in testes before adulthood, whereas forced *bam* expression in somatic cyst cells had no effect on GSC maintenance (data not shown), supporting the idea that *bam* overexpression triggers GSC loss in a germ cell-specific manner. The *UASp-bam* we used here has been used previously to induce GSC differentiation in the *Drosophila* ovary (Chen and McKearin, 2003a). These results indicate that *bam* misexpression can cause GSC loss in males like in females, and further suggest that GSC loss caused by defective Bmp signaling could be, at least in part, caused by elevated *bam* expression in GSCs.

dpp or *gbb* overexpression cannot completely block the differentiation of GSCs and their progeny in the male

In the *Drosophila* ovary, *dpp* overexpression completely blocks germ cell differentiation, resulting in the formation of GSC-like tumors (Xie and Spradling, 1998). To determine whether *dpp* or *gbb* overexpression can also prevent germ cell differentiation in the testis, we overexpressed *dpp* or *gbb* using the *nanos-gal4VP16* driver. In the testes overexpressing *dpp*, the hub appeared to be bigger with more cells, and there were slightly more single germ cells with a spectrosome around the hub cells (Fig. 6A), whereas the testes overexpressing *gbb* appeared to be normal (Fig. 6B). In the *dpp*-overexpressing testes, the gonialblasts could still differentiate and divide but failed to stop after four rounds of cell division for normal gonialblasts, resulting in the formation of the spermatogonial clusters with more than 16 germ cells (data not shown). These results suggest that overexpression of either *dpp* or *gbb* does not block gonialblast differentiation. These observations suggest that the contribution of *dpp* signaling to the regulation of the GSC lineage differentiation is different in males and in females. It seems that *dpp* overexpression directly or indirectly influences hub cell formation during early development as the *nanos-gal4VP16* driver is expressed in germ cells during early gonadal development. Extra single germ cells in the *dpp*-overexpressing testes are probably a consequence of more hub cells, as the bigger hub could potentially produces more Upd molecules, which are known to influence germ cell differentiation.

In the *Drosophila* male, loss of bam function results in unrestricted proliferation of spermatogonial cells (Gonczy et al., 1997). In females, *dpp* overexpression completely prevents BamC accumulation in the germ cells (Xie and Spradling, 1998). Possibly, the unrestricted proliferation of spermatogonial cells in the *dpp* overexpressing testis could result from the suppression of *bam* expression. To test whether hyperactive Bmp signaling can inhibit *bam* expression in the testis, we used the *nanos-gal4VP16* driver to overexpress *dpp*, an activated *tkv* (*tkv**) and *gbb* in the germ cells of testes. Interestingly, all the germ cells including differentiated germ cell clusters in the testes overexpressing *dpp* failed to express *bam-GFP* (Fig. 6C). Similarly, overexpression of *tkv** using *nos-gal4VP16* and *UAS-tkv** resulted in complete suppression of *bam* expression in all the germ cells (data not shown). However, in the testis overexpressing *gbb*, *bam-GFP* expression was repressed in some two-cell and four-cell germ
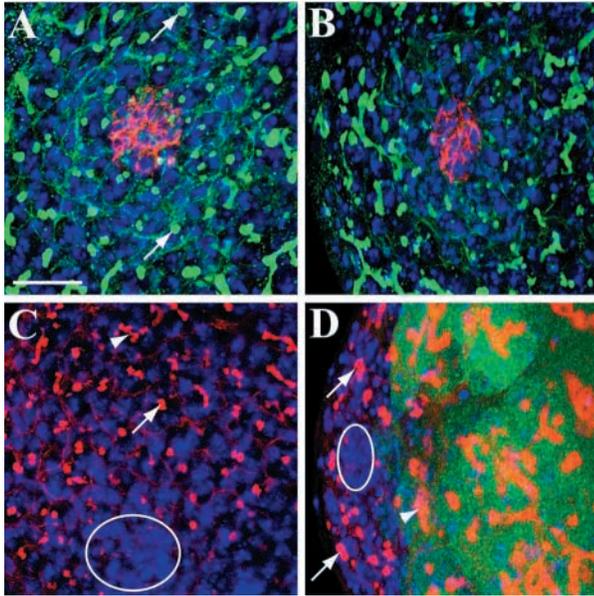
Fig. 6. Overexpression of *dpp* but not *gbb* completely represses *bam* transcription in the testis. The testes in A and B are labeled for Fas III (red), Hts (green) and DNA (blue), whereas the testes in C and D are labeled for GFP (green), Hts (red) and DAPI (blue). The hub cells are identified by FasIII staining (red) in A and B, and are highlighted by circles in C and D. (A) The tip of a *dpp*-overexpressing testis showing more hub cells and slightly more single germ cells with a spectrosome (arrows) two or three cells away from the hub cells. (B) The tip of a *gbb*-overexpressing testis showing a normal hub and normal germ cell development. (C) The tip of a *dpp*-overexpressing testis showing that late differentiated germ cells (a two-cell cluster indicated by an arrow; a 16-cell cluster indicated by an arrowhead) fail to express *bam-GFP*. (D) The tip of a *gbb*-overexpressing testis showing that *bam-GFP* expression in the two-cell clusters (arrows) is delayed but is expressed in late differentiated germ cells (arrowhead). All the images are shown at the same magnification. Scale bar: 10 µm.

cell clusters where it is normally expressed in wild-type testes (Fig. 6D). These results indicate that elevated *dpp* signaling but not *gbb* signaling is sufficient to inhibit *bam* transcription in the germ cells of the testis.

Both *dpp* and *gbb* are expressed in the somatic cells that are in close association with GSCs in the *Drosophila* testis

To determine the sources for Gbb and Dpp in the testis, we used RT-PCR to study the presence of *gbb* and *dpp* mRNAs in the purified hub cells, somatic cyst cells and germ cells using fluorescent-activated cell sorting (FACS). The hub cells were marked by the *upd-gal4* driven *UAS-GFP* expression (Fig. 7A). The somatic cyst cells and somatic stem cells were marked by the *c587-gal4*-driven *UAS-GFP* (Fig. 7B). *vasa* is a germline-specific gene (Lasko and Ashburner, 1988; Hay et al., 1988). The germ cells were marked by a *vasa-GFP* transgene (Nakamura et al., 2001) (Fig. 7C). The tips of the testes were isolated and dissociated, and the GFP-positive cells were purified from the dissociated testicular cells by FACS. As a control, *vasa* mRNAs were present in the whole testis and isolated germ cells but were absent in the somatic cyst cells

and hub cells (Fig. 7D). Interestingly, *gbb* and *dpp* mRNAs were present in the hub cells and the somatic cysts/somatic stem cells but were absent in the germ cells (Fig. 7D). In addition, *dpp* mRNAs appeared to be less abundant than *gbb* mRNAs in the testis. These results indicate that both Dpp and Gbb are probably somatic cell-derived Bmp signals that directly regulate GSC maintenance in the testis.
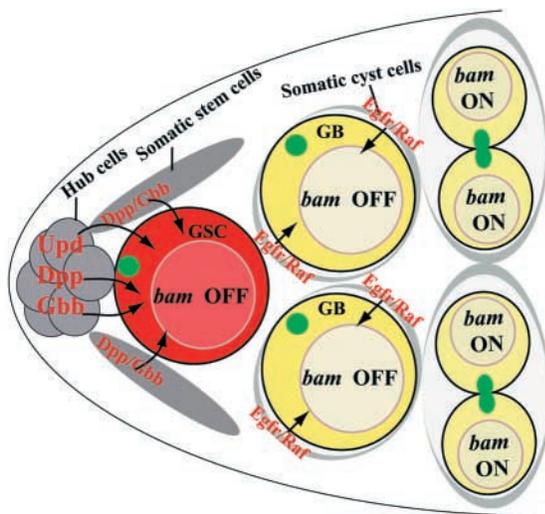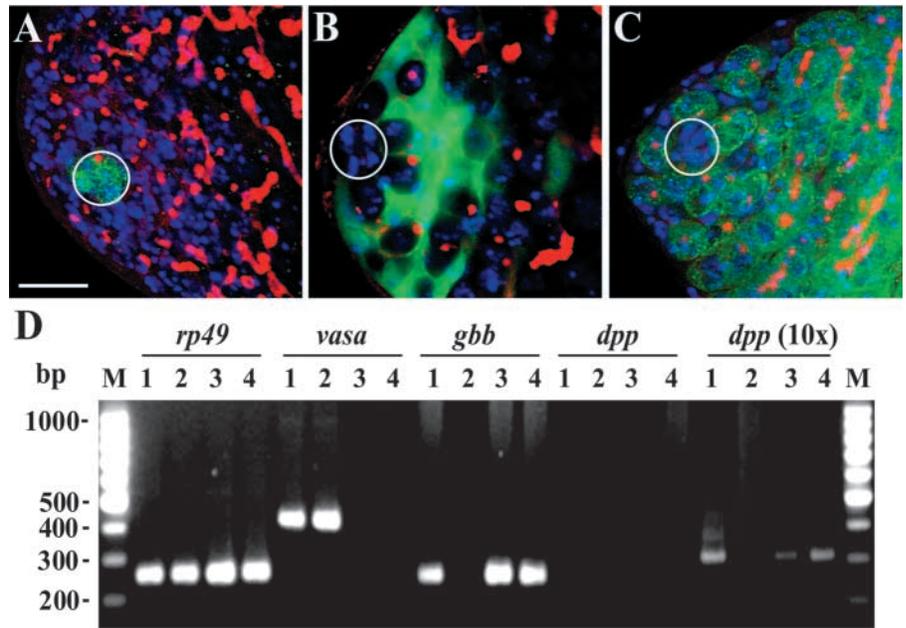
Discussion

It has been proposed that the hub cells and the somatic stem cells function as a niche for maintaining GSCs in the *Drosophila* testis. The Upd signal from the hub cells activates the Jak-Stat signaling pathway in GSCs and controls their maintenance in the testis (Kiger et al., 2001; Tulina and Matunis, 2001). The unknown signal regulated by EGFR and Raf in the somatic cysts also appears to be important for the proper differentiation of gonialblasts in the testis (Kiger et al., 2000; Tran et al., 2000). Here, we propose that the Bmp signals from somatic cells, Dpp and Gbb, are essential for maintaining GSCs in the *Drosophila* testis (Fig. 8). In this study, we also show that Gbb is essential for keeping *bam* repressed in GSCs and gonialblasts, and that *bam* misexpression causes GSC loss in the testis. Moreover, this study reveals similarities between *Drosophila* males and females with regards to GSC regulation: both male and female GSCs require Bmp signaling for their maintenance and for repressing *bam* transcription in GSCs.

Bmp signaling mediated by Dpp and Gbb is essential for maintaining GSCs in the *Drosophila* testis

dpp signaling has been shown to be essential for controlling GSC maintenance and division in the ovary (Xie and Spradling, 1998). The Bmp signaling pathway mediated by *punt* and *shn* in the somatic cyst cells is known to be important for controlling the proliferation of spermatogonial germ cells (Matunis et al., 1997). However, it is not known whether *dpp* and *gbb* are involved in the regulation of GSCs in the testis. In this study, we have provided molecular and genetic evidence that suggests both *gbb* and *dpp* are expressed in the somatic cells of the testis and work cooperatively to maintain GSCs and to repress *bam* transcription in GSCs.

In the *gbb* mutant testis, GSCs are lost very rapidly but gonialblasts still develop into 16-cell cysts, suggesting that *gbb* functions specifically to control GSC maintenance during germ cell development in the testis. Surprisingly, mutations in *dpp* have very little effect on GSC maintenance, which is in contrast to the role of *dpp* in the ovary (Xie and Spradling, 1998). However, a mutation in one copy of the *gbb* gene greatly enhances the stem cell loss phenotype of *dpp* mutants even though heterozygous *gbb* males have normal GSC number, indicating that *dpp* and *gbb* work cooperatively to control GSC maintenance. In the *Drosophila* testis, *dpp* plays a less important role than does *gbb* with regards to GSC regulation, which could be due to much lower *dpp* expression. In the *Drosophila* ovary, both *dpp* and *gbb* are equally important for maintaining GSCs and repressing *bam* transcription in GSCs (Xie and Spradling, 1998; Song et al., 2004). Although *dpp* overexpression in the ovary completely blocks cystoblast differentiation and causes the accumulation of GSC-like germ cells (Xie and Spradling, 1998), overexpression of either *dpp*

Fig. 7. Two Bmp like molecules, Dpp and Gbb, are expressed in the somatic cells in the *Drosophila* testis. The testes in A-C are labeled for Hts (red), GFP (green) and DAPI (blue), and their hub cells are highlighted by circles. (A) The tip of a *upd-gal4;UAS-GFP* testis showing GFP-labeled hub cells. (B) The tip of a *c587-gal4;UAS-GFP* testis showing GFP-labeled somatic stem cells and somatic cyst cells. (C) The tip of a *vasa-GFP* testis showing GFP-labeled germ cells including GSCs. (D) A DNA gel with RT-PCR products showing that *gbb* and *dpp* mRNAs are primarily present in the somatic cells of the testis. In this gel, mRNAs from the whole testes, purified germ cells, somatic cyst cells/somatic stem cells and hub cells are marked as templates 1, 2, 3 and 4, respectively. *vasa* serves as a positive control, while *rp49* is an internal control. For *dpp* (10×), approximately 10-fold more RNA template was used because of its low abundance. A-C are shown at the same magnification. Scale bar: 10 µm.

production of pMad. This study suggests that *gbb* signals through previously defined *dpp* receptors to regulate the phosphorylation of Mad. We show that pMad in *gbb* mutant GSCs is severely reduced just like in *punt* mutant GSCs. *Dad* has been established as a *dpp*-responsive gene in other developmental processes (Tsuneizumi et al., 1997). In this study, we show that *Dad-lacZ* expression in GSCs and gonialblasts is beyond detection in the *gbb* mutant testis. Interestingly, partial removal of *Dad* function can also partially suppress the stem cell loss phenotype of *gbb* mutants, suggesting that *Dad* negatively regulates *gbb* signaling. However, *Dad-lacZ* expression is only slightly reduced in *dpp* mutant GSCs and gonialblasts. These results indicate that *Dad* is primarily a *gbb* responsive gene in the *Drosophila* testis. They also argue that *gbb* indeed signals through common *dpp* receptors, promotes Mad phosphorylation and activates *Dad* transcription in GSCs in the same way as *dpp* does.

Dpp can function as a long-range gradient, which elicits different responses at different concentrations (reviewed by Podos and Fugerson, 1999). In the tip of the *Drosophila* testis, Gbb and Dpp appear to function as short-range signals and their signaling activities are restricted to GSCs and gonialblasts based on expression of *Dad-lacZ* and pMad. *gbb* and *dpp* mRNAs appear to be expressed in both the hub cells and the somatic cyst cells. In the ovary, Bmp signals also appear to function as short-range signals (Kai and Spradling, 2003; Song et al., 2004). Gbb and Dpp must be produced and/or activated around the hub cells and the somatic stem cells in order for them to signal locally to GSCs and gonialblasts. It would be very interesting to see whether Gbb and Dpp are localized and/or activated around the hub cells.

Repression of *bam* transcription in GSCs by Bmp signaling may help maintain GSCs in the testis

Stem cells must remain undifferentiated and continue self-renewal at every cell division. The relationship between the undifferentiated state and self-renewal remains to be defined.

Fig. 8. A current working model for how Bmp signals maintain GSCs in the *Drosophila* testis. In this model, Upd from hub cells, Gbb/Dpp from hub cells/somatic stem cells are important for GSC maintenance. An unknown signal initiated by EGFR/Raf signaling from somatic cyst cells is important for the proper differentiation of gonialblasts. Dpp/Gbb signaling also helps repress *bam* expression in GSCs and in gonialblasts (GBs). Two-cell germ cell clusters distant from hub cells/somatic stem cells receive less Bmp signaling and begin to express *bam* and promote further differentiation.

or *gbb* has little effect on differentiation of gonialblasts in the testis. These observations suggest that Bmp signaling is essential for maintaining GSCs in both sexes but *gbb* and *dpp* contribute differently.

Even though *gbb* has been shown to work synergistically with *dpp* potentially through the use of common Bmp receptors in patterning wing imaginal discs (Khalsa et al., 1998), it is not known whether *gbb* signaling directly contributes to the

Even though several signals have been identified for stem cells in different systems, there is little known about their direct target genes in stem cells, which could help us to understand how these signals are translated into the self-renewal or undifferentiated state of GSCs. In order for a stem cell to maintain its identity, it at least requires the repression of the genes that are important for differentiation of stem cell daughters. In this study, we show that Bmp signals from the niche cells are involved in repressing *bam* transcription in GSCs in the testis.

In the present study, we demonstrate that Bmp signaling mediated by Dpp and Gbb is essential for maintaining GSCs in the testis. *bam* is known to be both necessary and sufficient for the differentiation of a cystoblast in the *Drosophila* ovary (McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). In the *bam* mutant testis, GSCs are well maintained, and gonialblasts still differentiate but overproliferate into clusters with more than 16 germ cells (Gonczy et al., 1997), suggesting that *bam* is not necessary for the initial differentiation of gonialblasts. In this study, we show that forced expression of *bam* in GSCs causes them to be lost, which may be due to differentiation and/or cell death. Normally, *bam* transcription is absent in GSCs, suggesting that an active mechanism exists to repress *bam* expression in GSCs. The mechanism appears to be mediated by Bmp signals that originate from the surrounding somatic cells – the niche cells. In the testis, the GSCs mutant for *gbb*, *punt* and *Med* have elevated *bam* transcription. *dpp* overexpression leads to *bam* transcriptional repression in all the germ cells of the testis. These results demonstrate that Bmp signaling is essential for keeping *bam* repressed in GSCs. In the *Drosophila* ovary, Bmp signaling appears to directly repress *bam* expression in GSCs (Chen and McKearin, 2003b; Song et al., 2004). Whether the repression of *bam* transcription in GSCs mediated by Bmp signaling in the testis is direct remains to be determined. This study indicates that niche signals maintain the undifferentiated or self-renewal state of stem cells, at least in part, by repressing the expression of the genes that are important for the differentiation of their progeny.

How are Bmp and Jak-Stat signaling pathways integrated in male GSCs?

Upd is another known signal for GSCs in the *Drosophila* testis, and activates the Jak-Stat signal transduction cascade in GSCs to maintain their stem cell identity (Kiger et al., 2001; Tulina and Matunis, 2001). *upd* overexpression disrupts normal differentiation of gonialblasts, leading to the accumulation of stem cell-like germ cells in the testis. As both Jak-Stat and Bmp signaling pathways are required in GSCs for their maintenance, they must be integrated and interpreted collectively in GSCs. There are several possible ways both signaling transduction pathways could interact with each other. First, Jak-Stat and Bmp signaling pathways regulate each other in GSCs. In the mammalian system, Bmps can regulate Stat function by controlling the activity of the FRAP serine-threonine kinase in neural stem cells (Rajan et al., 2003). This may also happen in the GSCs of the testis. It is possible that Jak-Stat signaling regulates Bmp signaling through a yet unidentified mechanism. Second, both signaling pathways activate their own transcription factors, which together activate the expression of the genes that are important for maintaining

GSCs in the undifferentiated or self-renewal state. Third, both signaling pathways could activate expression of different genes that are important for maintaining GSCs while repressing different genes that cause GSC differentiation. These different scenarios await to be investigated in the future.

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