Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, *bag of marbles*, in germline stem cells in the *Drosophila* ovary

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Summary

The *Drosophila* ovary is an attractive system to study how niches control stem cell self-renewal and differentiation. The niche for germline stem cells (GSCs) provides a Dpp/Bmp signal, which is essential for GSC maintenance. *bam* is both necessary and sufficient for the differentiation of immediate GSC daughters, cystoblasts. Here we show that Bmp signals directly repress *bam* transcription in GSCs in the *Drosophila* ovary. Similar to *dpp*, *gbb* encodes another Bmp niche signal that is essential for maintaining GSCs. The expression of phosphorylated Mad (pMad), a Bmp signaling indicator, is restricted to GSCs and some cystoblasts, which have repressed *bam* expression. Both Dpp and Gbb signals contribute to pMad production. *bam* transcription is upregulated in GSCs mutant for *dpp* and *gbb*. In marked GSCs mutant for *Med* and *punt*, two essential Bmp signal transducers, *bam* transcription is also elevated. Finally, we show that Med and Mad directly bind to the *bam* silencer in vitro. This study demonstrates that Bmp signals maintain the undifferentiated or self-renewal state of GSCs, and directly repress *bam* expression in GSCs by functioning as short-range signals. Thus, niche signals directly repress differentiation-promoting genes in stem cells in order to maintain stem cell self-renewal.

Key words: germline, stem cells, male, Bmps

Introduction

Stem cells in adult tissues have the ability to self-renew and generate differentiated cells that maintain tissue homeostasis. Specific regulatory microenvironments, also known as niches, are thought to regulate many stem cell types by producing signals important for stem cell proliferation and differentiation (Watt and Hogan, 2000; Spradling et al., 2001). Stem cells usually divide asymmetrically to generate parent stem cells and differentiated cells, although they can also undergo symmetric cell division to replenish lost stem cells or expand the stem cell pool (Xie and Spradling, 2000; Zhu and Xie, 2003). Even though many niche signals have been identified for different stem cell types in many systems, it is still largely unknown how niche signals control stem cell self-renewal and differentiation (Spradling et al., 2001). Therefore, it is essential to reveal the link between niche signals and intrinsic factors that are essential for stem cell self-renewal and differentiation in order to gain a better understanding of how stem cell behavior is controlled.

The *Drosophila* ovarian germline stem cells (GSCs) have become an attractive system to study stem cells and their relationship with niches (Xie and Spradling, 2001; Lin, 2002).

Two or three GSCs are located at the tip of the ovariole, also known as the germarium, and are surrounded by terminal filament cells, cap cells and inner sheath cells that form a niche for GSCs. GSCs and their progeny in the germarium can be reliably identified and distinguished by a germ cell-specific structure, called the fusome, which is rich in membrane skeletal proteins, such as Hu li tai shao (Hts) and α-Spectrin (Lin et al., 1994; de Cuevas et al., 1997). In GSCs and their immediate differentiating daughters, cystoblasts, the fusome is spherical in shape, and is also known as the spectrosome. A cystoblast will undergo synchronous mitotic divisions with incomplete cytokinesis to generate two-, four-, eight- and sixteen-cell cysts, in which the fusome is branched to interconnect individual cystocytes (Lin et al., 1994). GSCs are invariably anchored to cap cells through adherens junctions (Song et al., 2002). Loss of adherens junctions between cap cells and GSCs causes GSCs to migrate away from cap cells and undergo differentiation (Song et al., 2002). Upon GSC division, the original GSC remains anchored to cap cells and retains stem cell identity, whereas the cystoblast moves away from cap cells and undergoes differentiation. As a GSC is lost, a neighboring GSC can generate two daughter cells that both contact cap cells and remain as GSCs, thus replenishing a

vacant niche space (Xie and Spradling, 2000). Functioning as a GSC niche, terminal filament/cap cells express *piwi*, *dpp*, *fs(1)Yb* (also known as *Yb*) and *hedgehog* (*hh*), which are essential for maintaining GSC asymmetric cell division (Xie and Spradling, 1998; King and Lin, 1999; Cox et al., 2000; Xie and Spradling, 2000; King et al., 2001). Intrinsic factors in GSCs, including *pumilio*, *nanos*, *dpp* receptors and downstream components, are also important for GSC maintenance (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Xie and Spradling, 1998). Two intrinsic factors, *bag of marbles* (*bam*) and *benign gonial cell neoplasm* (*bgcn*), are required in cystoblasts for their proper differentiation (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995; Lavoie et al., 1999). However, in GSCs the interplay between genes involved in self-renewal versus differentiation remains unclear.

The functions of *dpp* signaling and *bam* in the maintenance of GSCs and the differentiation of cystoblasts seem to be directly opposing. Loss of *bam* function completely eliminates cystoblast differentiation, similar to that caused by *dpp* overexpression (McKearin and Spradling, 1990; Xie and Spradling, 1998). By contrast, forced overexpression of *bam* in GSCs causes their elimination, similar to that observed when *dpp* signaling is disrupted in GSCs (Ohlstein and McKearin, 1997; Xie and Spradling, 1998). These observations can be explained by a simple model wherein *dpp*, functioning as a short-range signal, directly promotes GSC self-renewal and suppresses *bam* expression in GSCs, while allowing cystoblasts to express *bam* and differentiate.

Several studies have supported this model (Xie and Spradling, 1998; Chen and McKearin, 2003a; Kai and Spradling, 2003). *bam* mRNA is absent in GSCs, but quickly accumulates in cystoblasts and mitotic cysts (McKearin and Spradling, 1990). Overexpression of *dpp* completely suppresses the expression of BamC protein in germ cells, thus preventing cystoblasts from differentiating (Xie and Spradling, 1998). A recent study by Kai and Spradling showed that *dpp* signaling activity is restricted to GSCs and cystoblasts (Kai and Spradling, 2003).

The asymmetric distribution of *bam* between GSCs and cystoblasts could be due to transcriptional regulation and/or mRNA stability. The recent elegant *bam* promoter analysis has revealed that its transcription is actively repressed through a silencer (Chen and McKearin, 2003a). However, whether and how *dpp* signaling directly represses *bam* transcription remains unknown. In this study, we provide genetic and molecular evidence to support the model proposing that Bmp signaling represses *bam* transcription through binding of its downstream transcriptional effectors, Mad and Medea (Med), to the defined *bam* silencer.

Materials and methods

Drosophila stocks and genetics
The following fly stocks used in this study were described either in FlyBase or as otherwise specified in the Results section:

  punt^{10460};
  punt^{135}; Med^{26}; Dad-lacZ; dpp^{hr4};
  dpp^{hr56}; gbb^4, gbb^{D4};
  gbb^{D20}; bam-GFP (GFP gene driven by the *bam* promoter);
  vasa-GFP; c587-gal4;
  hs-gal4; UAS-dpp; and
  UAS-gbb; hsFLP; FRT$_{82B}$ armadillo-lacZ.

Most stocks were cultured at room temperature. To maximize their mutant phenotypes, *dpp*, *gbb* and *punt* mutant adult females were cultured at 29°C for 2-7 days. To achieve the uniform GSC-like phenotype, *c587-gal4;UAS-dpp* females were also cultured at 29°C for 7 days.

Generating mutant GSC clones and overexpression
Clones of mutant GSCs were generated by Flp-mediated mitotic recombination, as described previously (Xu and Rubin, 1993; Xie and Spradling, 1998). To generate the stocks for making mutant GSC clones and examining *bam-GFP* expression, 2-day old *hsFLP; bam-GFP/+; FRT$_{82B}$ punt^{135}/FRT$_{82B}$ armadillo-lacZ* and *hsFLP; bam-GFP/+; FRT$_{82B}$ Med^{26}/FRT$_{82B}$ armadillo-lacZ* females were heat-shocked at 37°C for 3 consecutive days with two one-hour heat-shock treatments separated by 8-12 hours. The ovaries were removed 3 days after the last heat-shock treatment and then processed for antibody staining.

To construct the stocks for overexpressing *dpp* or *gbb*, the females that carried *hs-gal4*, and either *UAS-dpp* or *UAS-gbb*, were heat-shocked at 37°C for different lengths of time for particular experiments, as indicated in the Results. The females that carried *c587-gal4* and *UAS-dpp* or *UAS-gbb* were cultured at room temperature or at 29°C for 7 days. For examining the expression of *bam-GFP* in the ovary overexpressing *dpp* or *gbb*, the females that carried *c587-gal4* or *hs-gal4*, and *UAS-dpp or UAS-gbb*, also carried a *bam-GFP* transgene.

Measuring GSC loss in *gbb* mutants and examining *bam-GFP* expression in *gbb*, *dpp* or *punt* mutant germaria
To measure stem cell loss in *gbb* mutant and control ovaries, the germaria with different numbers of GSCs, ranging from three to none, were counted from the ovaries of 2-day- and one-week-old *bam-GFP gbb^4/gbbD4, bam-GFP gbb^4/gbb^{D20}* or *bam-GFP* (control) females. The 2-day-old control and *gbb* mutant females were cultured at room temperature after they eclosed at 18°C, whereas the one-week-old control and *gbb* mutant females were cultured at 29°C. Values are expressed as the average GSC number per germarium and the percentage of germaria with no GSCs.

To examine *bam-GFP* expression in *dpp*, *gbb* or *punt* mutant germaria, we generated females 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}*, *bam-GFP; punt^{10460}/punt^{135}* or *bam-GFP* (control) females. All the control and mutant females were cultured at 29°C for 4 days before their ovaries were isolated and immunostained, to compare *bam*-GFP expression under identical conditions.

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-galactosidase antibody (1:100; Cappel); polyclonal anti-GFP antibody (1:200; Molecular Probes); and 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.

Examining gene expression using the Affymetrix microarray
Total RNA from the ovaries of different genotypes or treatments was isolated using Trizol (Invitrogen), and biotin-labeled cRNA probes were produced using an RNA transcript labeling kit (Enzo BioArray). The *Drosophila* GeneChips were purchased from Affymetrix, and were hybridized, stained and detected according to the manufacturer's instructions.

Detecting gene expression in purified component cells using RT-PCR

After sorting GFP-positive cells by using Cytomation MoFlo, total RNA was prepared using Trizol (Invitrogen) from these isolated cells. The RNA samples were further amplified using the GeneChip Eukaryotic Small Sample Target Labeling Assay Version II (Affymetrix). After the RNA amplification, 100 ng of total RNA was reverse-transcribed (RT) using the SuperScriptIII First-Strand Synthesis System for RT-PCR, according to manufacturer's protocol (Invitrogen). The following primers were used in this study:

dpp, 5′-AGCCGATGAAGAAGCTCTACG-3′ and 5′-ATGTCG-TAGACAAGCACCTGGTA-3′;

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

gbb, 5′-AGATGCAGACCCTGTACATAGAC-3′ and 5′-CTCGTC-GTTCAGGTGGTACAGAA-3′; and

rp49, 5′-GTATCGACAACAGAGTCGGTCGC-3′ and 5′-TTGGT-GAGCGGACCGACAGCTGC-3′.

PCR was performed as follows: 94°C for 2 minutes; 35 cycles of 94°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 a 2% agarose gel in the presence of ethidium bromide.

Electrophoretic mobility shift assays for the binding of Mad and Med to the *bam* silencer

The GST-Mad construct was described previously (Kim et al., 1997). Med was PCR-amplified from its cDNA, with the introduction of *Xho*I sites at both ends, then subcloned into a pGEX-4T2 vector (Amersham Pharmacia Biotech). Its sequence was confirmed by sequencing. GST-Mad, GST-Med and GST proteins were purified by affinity chromatography using Glutathione Sepharose™ 4B according to the manufacturer's protocol (Amersham Pharmacia Biotech), and confirmed by western blots.

A Cy5 5′-modified oligonucleotide containing the bipartite *bam* silencer element (+17 to +54) was used as a probe. Binding reactions were performed according to the published protocol (Kim et al., 1997). Specificity of binding was determined by the addition of 100-fold molar excess of unlabeled competitor DNA corresponding to the *bam* silencer element, with site A and/or B. DNA-protein complexes were resolved on a 5% (w/v) non-denaturing polyacrylamide gel using 0.5×TBE running buffer at 150 V for 3 hours at 4°C. Gels were imaged on a Typhoon 8700 (Amersham Biosciences).

Results

dpp signaling activity is correlated with *bam* transcriptional repression in GSCs and cystoblasts

Previous studies have shown that a *dpp* signal produced by somatic cells is essential for maintaining GSCs but not for cystoblast development (Xie and Spradling, 1998; Xie and Spradling, 2000). *bam* transcription is active in young, differentiating germ cells but is repressed specifically in GSCs in the ovary (Chen and McKearin, 2003a). This raises the interesting possibility that *dpp* signaling and *bam* expression directly oppose each other. To investigate this possibility, we examined the correlation between *dpp* signaling activity and *bam* expression in the germarium. *dpp* signaling activity is usually monitored by *Dad* and phosphorylated Mad (pMad) expression (Tsuneizumi et al., 1997; Tanimoto et al., 2000). *Dad* is a *dpp* target gene, and a *Dad-lacZ* line recapitulates its expression (Tsuneizumi et al., 1997). A *bam-GFP* transgene (with the GFP gene driven by the *bam* promoter) has been generated to study *bam* transcription (Chen and McKearin, 2003a). Throughout this study, an anti-Hts antibody was used to label spectrosomes and fusomes, and a DNA dye, DAPI, was used to label nuclei. Cap cells can be reliably identified by bright DAPI staining, and by their unique position and nuclear morphology. GSCs are identified by the presence of a spectrosome (a spherical fusome) on their anterior side and by their direct contact with cap cells; cystoblasts also contain a spectrosome but fail to be associated with cap cells (Fig. 1A).

Similar to the observations recently made by Kai and Spradling (Kai and Spradling, 2003), *Dad-lacZ* was expressed in GSCs and some cystoblasts at high levels, but in the other cystoblasts and mitotic cysts at much lower levels (Fig. 1B). As reported by Chen and McKearin (Chen and McKearin, 2003a), *bam* transcription was repressed in GSCs and some cystoblasts, but was active in the other cystoblasts and dividing cystocytes (Fig. 1C). In germaria carrying *bam-GFP* and *Dad-lacZ*, GSCs and the cystoblasts that had strong *Dad* expression did not show *bam-GFP* expression (Fig. 1D), whereas the cystoblasts and mitotic cysts that had weak or no *Dad*
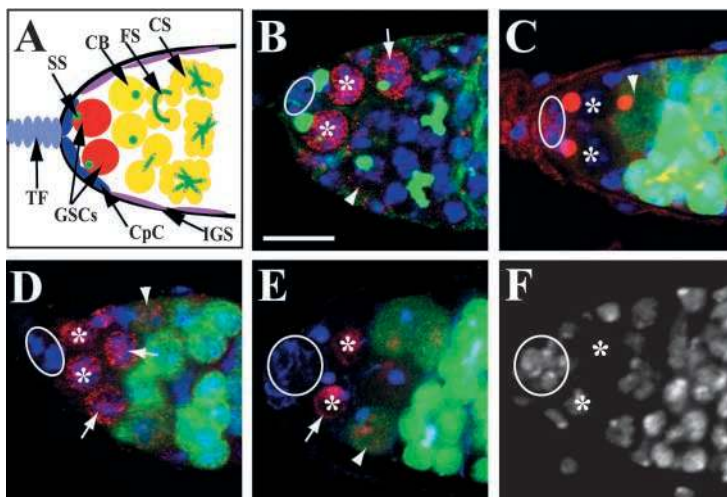

Fig. 1. *dpp* signaling activity is restricted to GSCs and some cystoblasts where *bam* transcription is actively repressed. (A) Diagram showing GSCs, their differentiated progeny and surrounding somatic cells. In panels B-F, cap cells are highlighted by circles, whereas GSCs are indicated by asterisks. (B) Tip of the *Dad-lacZ* germarium labeled for nuclear β-Gal (red), Hts (green, fusomes) and DAPI (blue), showing high *Dad* expression in GSCs and in a cystoblast (arrow), but not in another cystoblast (arrowhead). (C) Tip of the *bam-GFP* germarium labeled for GFP (green), Hts (red, fusomes) and DAPI (blue), showing *bam* expression in a cystoblast (arrowhead) and cysts, but not in GSCs. (D) Tip of the *bam-GFP;Dad-lacZ* germarium labeled for β-Gal (red), GFP (green) and DAPI (blue), showing that GSCs and two cystoblasts (arrows) express high *Dad* but no *bam*, and that a cystoblast (arrowhead) has low *Dad* and begins to express *bam*. (E,F) Tip of the *bam-GFP* germarium labeled for pMad (red), GFP (green), Hts (blue, fusomes) and DAPI (white, F), showing high pMad accumulation but no *bam* expression in GSCs, and low pMad but *bam* expression in a cystoblast (arrowhead, E). TF, terminal filament; GSCs, germline stem cells; SS, spectrosome; Cpc, cap cells; CB, cystoblast; FS, fusome; IGS, inner sheath cells; CS, cysts. All micrographs are shown at the same scale. Scale bar: 10 μm.
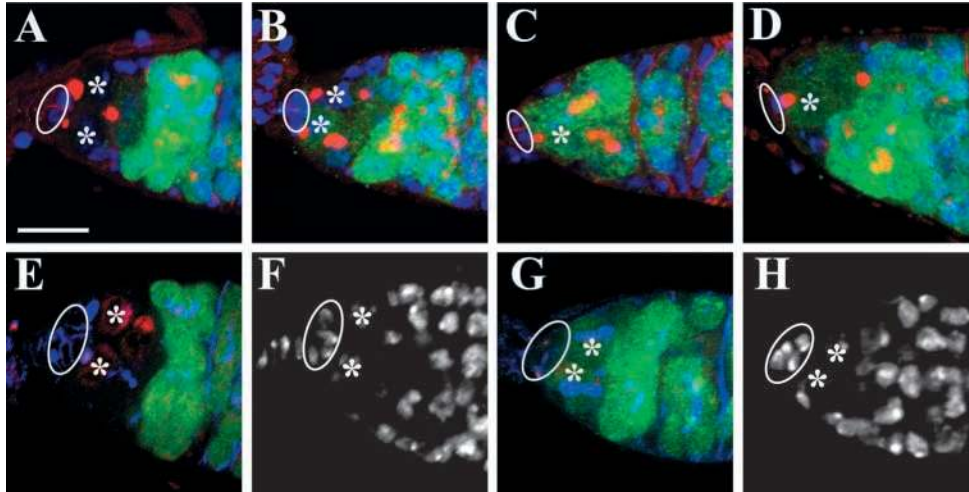
Fig. 2. *dpp* is essential for repressing *bam* transcription in GSCs. Germaria in panels A-D are labeled for Hts (red, fusomes), GFP (green) and DAPI (blue), whereas the germaria in E-H are labeled for pMad (red), GFP (green), Hts (blue, fusomes) and DAPI (white; F,H). All the GSCs are indicated by asterisks, and cap cells in all the panels are marked by circles. (A) A germarial tip from a *bam-GFP* female cultured at 29°C for one week, showing no *bam* expression in GSCs. (B) A germarial tip from a *bamGFP dpp^{hr56}/dpp^{hr4}* female cultured at 29°C for 2 days, showing that one of the two GSCs begins to express *bam*. (C,D) Germarial tips from *bamGFP dpp^{hr56}/dpp^{hr4}* females cultured at 29°C for 4 (C) or 7 (D) days, showing that the only remaining GSC starts to express *bam*. (E,F) Germarial tip from a *bam-GFP* female cultured at 29°C for 4 days, showing high pMad accumulation and no *bam* expression in GSCs. (G,H) A germarial tip from a *bamGFP dpp^{hr56}/dpp^{hr4}* female cultured at 29°C for 4 days, showing that two mutant GSCs have low pMad levels and begin to express *bam*. All micrographs are shown at the same scale. Scale bar: 10 µm.

expression showed obvious *bam-GFP* expression (Fig. 1D). Similarly, GSCs and the cystoblasts that showed strong pMad expression did not express *bam-GFP*, whereas the cystoblasts and mitotic cysts that showed weak or no pMad expression expressed *bam-GFP* (Fig. 1E,F). These results further support the idea that the *dpp* signaling pathway is activated in GSCs at high levels, whereas *bam* transcription is actively repressed.

dpp signaling is essential for repressing *bam* transcription in GSCs

The *dpp^{hr56}/dpp^{hr4}* temperature-sensitive mutant was chosen to investigate the expression of *bam-GFP* in *dpp* mutant GSCs because it shows gradual loss of GSCs within two weeks at a restrictive temperature (29°C) (Xie and Spradling, 1998). After *bam-GFP* and *bam-GFP dpp^{hr56}/dpp^{hr4}* females were cultured at 29°C for 2, 4 or 7 days, the ovaries were immunostained with anti-GFP and anti-Hts antibodies to visualize *bam-GFP* and fusomes, respectively. In the germaria from the *bam-GFP* females, the *bam-GFP* expression pattern was completely normal, and was absent in GSCs even one week after being cultured at 29°C (Fig. 2A). However, even two days after being cultured at 29°C, 28% of the *bam-GFP dpp^{hr56}/dpp^{hr4}* germaria that contained GSCs started to express *bam-GFP* in one or more GSCs (*n*=283; Fig. 2B). After 4 days and 7 days, 66% (*n*=35) and 89% (*n*=19) of the mutant germaria that still had at least one GSC expressed *bam-GFP* in one or more GSCs, respectively (Fig. 2C,D). To further confirm the role of *dpp* signaling in repressing *bam* transcription, we also compared the levels of *bam* mRNA in wild-type and *dpp* mutant ovaries using a microarray approach. *bam* mRNA was dramatically upregulated in *dpp^{hr4}/dpp^{hr56}* mutant ovaries in comparison with wild type (Table 1; samples were normalized with an internal control, the *Actin 42A* gene). These results demonstrate that the *dpp* signal is required to repress *bam* transcription in GSCs.

Next, we investigated whether elevated *bam* transcription in *dpp* mutant GSCs can be correlated with reduction of pMad expression. After 4 days at 29°C, control germaria maintained the normal number of GSCs and showed the normal pMad expression pattern (Fig. 2E,F). By contrast, many *dpp* mutant germaria completely lost their GSCs, and in the remaining GSC-containing germaria in which *bam-GFP* was also upregulated in GSCs, pMad was severely reduced but not completely eradicated in the GSCs (Fig. 2G,H). In the germaria in which *bam-GFP* was not obviously upregulated, levels of pMad were relatively higher but less than normal (data not shown). These results indicate that *dpp* signaling contributes, at least in part, to pMad production in GSCs, and could be responsible for repressing *bam* transcription.

dpp overexpression is sufficient for repressing *bam* transcription in the cystoblast

Our previous study showed that overexpression of *dpp* throughout the germarium completely inhibits cystoblast differentiation and causes the accumulation of GSC-like cells that fail to express BamC (Xie and Spradling, 1998). Our experiments described above suggest that the *dpp* signal is likely to be restricted to the tip of the germarium, adjacent to cap cells. To test whether GSCs are competent to respond to *dpp* signaling outside their niches, *dpp* was specifically overexpressed in somatic cells other than cap cells, using the *c587-gal4* line to drive a *UAS-dpp* transgene. The *c587-gal4*

Table 1. *dpp* signaling is necessary and sufficient for repressing *bam* expression in GSCs in the *Drosophila* ovary

Genes	Wild type	hs-gal4/UAS-dpp	C587-gal4/UAS-dpp	dpp^{hr4}/dpp^{hr56}
Actin 42A	1028	2476* (1028)[†]	4686 (1028)	1554 (1028)
dpp	4.7	354.2 (147.6)	271.9 (59.6)	−3.8 (−2.5)
Dad	24.0	103.0 (42.9)	296.0 (64.9)	4.9 (3.2)
bam	110.0	−9.3 (−3.8)	−3.2 (−0.7)	326.2 (217.5)

[a]The numbers shown in this table are the arbitrary ones that were quantified by the Affymetrix scanner.

[†]The numbers in parentheses are normalized based on the number of the house-keeping gene *Actin 42A* in wild-type ovaries.
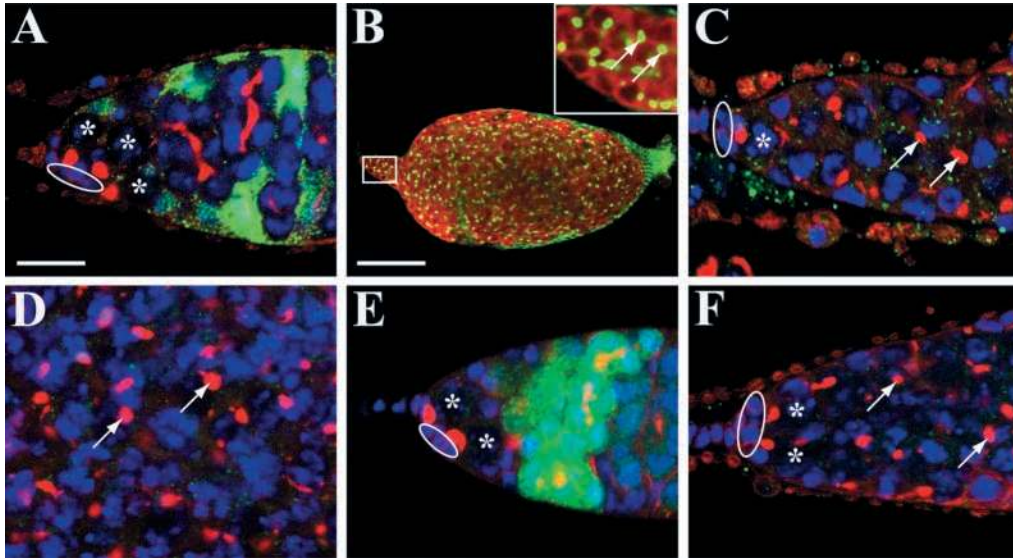
Fig. 3. *dpp* overexpression is sufficient for repressing *bam* transcription in single germ cells. Germaria in panels A and C-F are labeled for Hts (red, fusomes), GFP (green) and DAPI (blue), whereas the germarium in B is labeled for Vasa (red, germ cells) and Hts (green, fusomes). Circles highlight cap cells and asterisks indicate GSCs. (A) A germarial tip showing *c587-gal4*-driven *UAS-GFP* expression in inner sheath cells but not in cap cells. (B) A *c587-gal4;UAS-dpp* germarium resulting from *dpp* overexpression is filled with single germ cells with a spectrosome. The inset shows the tip of the germarium (highlighted by a rectangle in B) at a higher magnification (4×), containing only germ cells with a spectrosome (arrows). (C) Tip of the *c587-gal4;bam-GFP;UAS-dpp* germarium showing that the accumulated spectrosome-containing germ cells (two indicated by arrows) a few cells away from the tip of the germarium fail to express *bam-GFP*. (D) Middle portion of the *c587-gal4;bam-GFP;UAS-dpp* germarium showing that spectrosome-containing germ cells (two indicated by arrows) fail to express *bam-GFP*. (E) Tip of the *hs-gal4;UAS-dpp* germarium showing no *bam* expression in GSCs, but expression in differentiated germ cells without any heat-shock treatments. (F) Tip of the *hs-gal4;UAS-dpp* germarium showing no *bam* expression in GSCs and in spectrosome-containing germ cells (two indicated by arrows) distant from the tip after three days of heat-shock treatments. Scale bars: in A, 10 μm for A,C-F; in B, 60 μm.

line can drive expression of a UAS-GFP transgene in inner sheath cells and early follicle cells (Fig. 3A). When *UAS-dpp* expression was driven in inner sheath cells and follicle cells, germaria were filled with single germ cells with a spectrosome, suggesting that germ cells distant from their niche are still capable of responding to *dpp* (Fig. 3B). To further test whether *dpp* overexpression is sufficient to inhibit *bam* expression, we examined *bam-GFP* expression in *dpp*-induced GSC-like tumors. In *dpp*-overexpressing ovaries, *bam-GFP* was not expressed in the single germ cells either close to (Fig. 3C) or away from (Fig. 3D) the germarial tip. These results indicate that *dpp* signaling is sufficient to inhibit *bam* transcription.

The *c587-gal4* driver is expressed in somatic cells during early gonadal development, and overexpression of *dpp* also inhibits germ cell differentiation at early developmental stages (Zhu and Xie, 2003). To exclude the possibility that early *dpp* overexpression produces abnormal GSCs whose progeny cannot differentiate normally and thus fail to express *bam*, we examined *bam-GFP* expression at the adult stage when *dpp* was overexpressed using *UAS-dpp* driven by the *hs-gal4* driver (the promoter of a heat-shock protein 70 gene fused with the *gal4* gene). Without any heat-shock treatments, all the germaria had the normal GSC number and the normal *bam-GFP* expression pattern (Fig. 3E). After three consecutive days of 2-hour heat-shock treatments, the anterior half of the germaria were filled with single spectrosome-containing germ cells, and showed no obvious *bam-GFP* expression (Fig. 3F). These results further support the idea that *dpp* signaling is sufficient for directly or indirectly repressing *bam* transcription. Owing to the fact that GFP protein is stable, we could not determine how fast *dpp* overexpression can diminish *bam* mRNA using the *bam-GFP* transgene. Thus, we measured the quantity of *bam* mRNA 2 hours after a pulse of heat-shock-induced *dpp*

overexpression using the microarray approach. Interestingly, 2 hours after a pulse of *dpp* overexpression, *bam* mRNA was below detection (Table 1), indicating that *dpp* signaling rapidly represses *bam* transcription and/or causes rapid degradation of *bam* mRNA. This result further suggests that *dpp* signaling might directly repress *bam* transcription.

gbb is expressed in the somatic cells of the germarium and is essential for maintaining GSCs and repressing *bam* transcription in GSCs in the *Drosophila* ovary

In addition to Dpp, another Bmp-like molecule, Glass bottom boat (Gbb), exists in *Drosophila* and resembles human BMPs 5, 6, 7 and 8 (Wharton et al., 1991; Doctor et al., 1992). It has been shown that synergistic signaling by *dpp* and *gbb* controls wing growth and patterning in *Drosophila* (Haerry et al., 1998; Khalsa et al., 1998). To investigate the possibility that *gbb* could also be involved in the regulation of GSCs, we first used RT-PCR to determine whether *gbb* mRNA was present in different cell types of the germarium. Inner sheath cells and early follicle cells were isolated from *c587-gal4;UAS-GFP* females using fluorescent-activated cell sorting (FACS). Agametic ovaries were isolated from newly eclosed females that developed from *ovo*[D1rS1] homozygous embryos lacking germ cells (Oliver et al., 1990). The agametic ovary is composed of terminal filament cells, cap cells and early follicle cells but lacks inner sheath cells (Margolis and Spradling, 1995). Single germ cells, resembling GSCs, were isolated from *c587-gal4; vasa-GFP/UAS-dpp* females using FACS. *vasa* is a germ cell-specific gene (Hay et al., 1988; Lasko and Ashburner, 1988), and *vasa-GFP* is specifically expressed in the germ cells (Nakmura et al., 2001). *dpp* is expressed in the somatic cells of the germarium but not in germ cells (Xie and
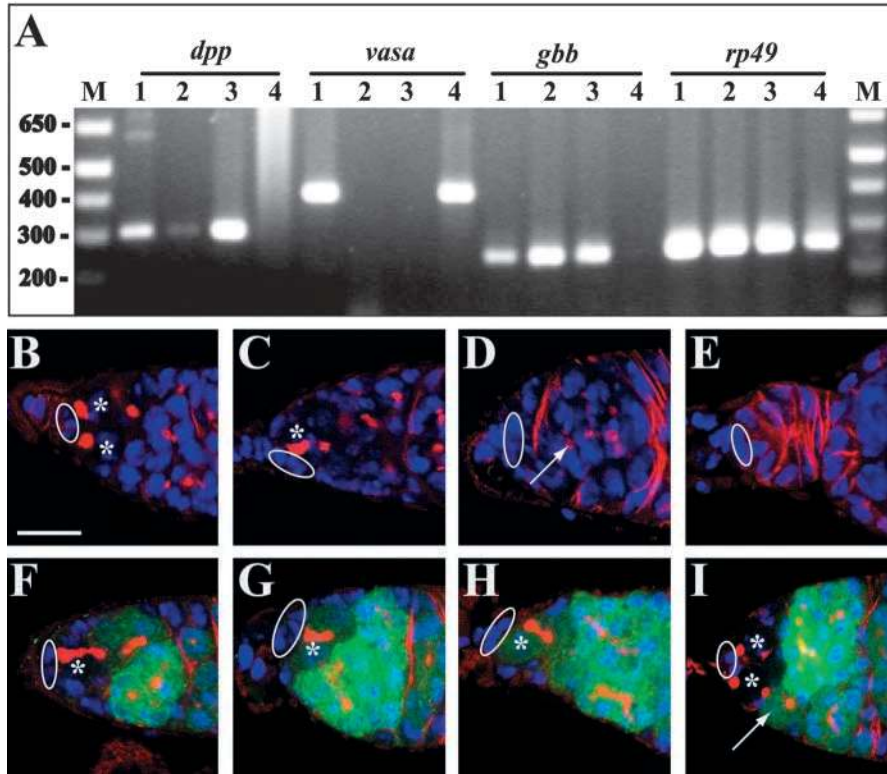
Fig. 4. *gbb* is expressed in the somatic cells of the germarium and is essential for maintaining GSCs and for repressing *bam* transcription in GSCs. (A) A DNA gel with RT-PCR products showing that *gbb* is expressed in the somatic cells of the germarium but not in GSCs. In this gel, mRNA for whole ovaries, agametic ovaries, inner sheath cells and GSC-like germ cells are marked by templates 1, 2, 3 and 4, respectively. *vasa* and *dpp* genes are positive controls, whereas *rp49* is an internal control. Germaria in B-E are labeled for Hts (red, fusomes) and DAPI (blue), whereas germaria in F-I are labeled for Hts (red, fusomes), GFP (green) and DAPI (blue). Circles highlight cap cells, whereas asterisks indicate GSCs. (B) Germarial tip from a wild-type *bam-GFP* female cultured at 29°C for 1 week showing two GSCs. (C-E) Germarial tips from the *bam-GFP gbb⁴/gbbᴰ⁴* females cultured at 29°C for one week showing one GSC (C), no GSC but 16-cell cysts (one indicated by arrow; D) and no GSCs and no cysts (E). (F) Germarial tip from a *bam-GFP gbb⁴/gbbᴰ⁴* female cultured at room temperature for 2 days showing that the remaining GSC does not express *bam*. (G,H) Germarial tips from *bam-GFP gbb⁴/gbbᴰ⁴* (G) and *bam-GFP gbb⁴/gbbᴰ²⁰* (H) females cultured at 29°C for one week, showing that the remaining single GSC expresses *bam*. (I) A germarial tip from a *c587-gal4/UAS-gbb; bam-GFP* female showing a normal number of GSCs, and normal *bam-GFP* expression in cystoblasts (arrow) and other differentiated germ cells. Scale bar in B: 10 μm for B-I.

Spradling, 2000). *vasa* mRNA was present in germ cells but not in inner sheath cells and agametic ovaries (Fig. 4A), whereas *dpp* mRNA was present in inner sheath cells and agametic ovaries but not in germ cells (Fig. 4A), indicating that the different cell types in germaria were properly isolated. *gbb* mRNA was detected in inner sheath cells and agametic ovaries but not in the GSC-like germ cells (Fig. 4A), indicating that *gbb* is expressed in the somatic cells. These results indicate that *gbb* could be another somatic signal for controlling GSCs.

We next determined whether mutations in *gbb* cause GSC loss in the ovary. Two allelic combinations of *gbb*, *bam-GFP gbb⁴/gbbᴰ⁴* and *bam-GFP gbb⁴/gbbᴰ²⁰*, and a wild-type strain carrying *bam-GFP* were allowed to develop to adulthood at 18°C and were then shifted to room temperature or 29°C. The germaria from the wild-type females 2 days after being cultured at room temperature, or 7 days after being cultured at 29°C, had a normal number of GSCs, two or three GSCs (Fig. 4B). However, 2 days after being shifted to room temperature, the germaria from the *gbb⁴/gbbᴰ⁴* and *gbb⁴/gbbᴰ²⁰* females had an average of 1.0 and 1.5 GSCs, respectively (Table 2). One week after being shifted to 29°C, 88% of the *gbb⁴/gbbᴰ⁴* mutant germaria and 60% of the *gbb⁴/gbbᴰ²⁰* mutant germaria completely lost their GSCs in comparison with 36% and 7% 2 days after being cultured at room temperature, although the rest usually had one GSC left (Fig. 4C-E). These results demonstrate that *gbb* is essential for maintaining GSCs in the *Drosophila* ovary.

As *dpp* and *gbb* can function synergistically in other developmental processes, we examined whether *bam-GFP* expression was upregulated in *gbb* mutant germaria. As

described earlier, *bam-GFP* was not expressed in GSCs in the wild-type females after being cultured either at room temperature or at 29°C (Fig. 2A). Two days after being cultured at room temperature, the GSCs rarely expressed *bam-GFP* in the mutant *gbb⁴/gbbᴰ⁴* germaria (one out of the total 49 germaria) and in the *gbb⁴/gbbᴰ²⁰* mutant germaria (two out of the total 55 germaria) (Fig. 4F). By contrast, one week after being cultured at 29°C, most of the GSCs expressed *bam-GFP* in the *gbb⁴/gbbᴰ⁴* (five out the six germaria carrying one or more GSCs) and *gbb⁴/gbbᴰ²⁰* (13 out of the 16 germaria carrying one or more GSCs) mutant germaria (Fig. 4G,H). These results demonstrate that *gbb* is also essential for repressing *bam* transcription in GSCs.

Having established that *dpp* is sufficient to repress *bam* expression in GSCs, we then asked whether *gbb*

Table 2. *gbb* is essential for maintaining GSCs in the *Drosophila* ovary

	GSCs	
Genotypes	2 days (room temperature)	7 days (29°C)
bam-GFP	2.5±0.5* (0%)† (66)‡	2.5±0.5 (0%) (56)
bam-GFP gbb⁴/gbbᴰ⁴	1.0±0.8 (36.5%) (76)	0.2±0.6 (88.2%) (34)
bam-GFP gbb⁴/gbbᴰ²⁰	1.5±0.7 (7.0%) (86)	0.6±0.8 (60.0%) (45)

*Means and standard deviations were calculated using the Microsoft Excel program.

†The percentage of the germaria that carry no GSCs was calculated by dividing the number of the germaria that carry no GSCs by the number of the total germaria examined.

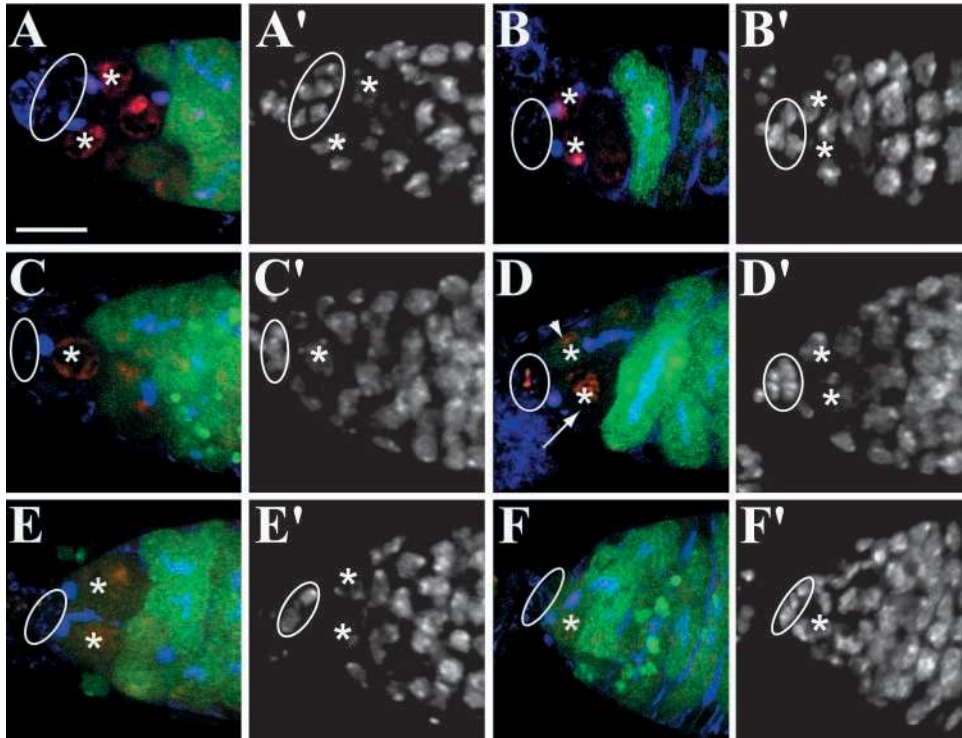‡The number of the total germaria examined for a given genotype at a particular treatment.

Fig. 5. Reduction of pMad is correlated with upregulated *bam* transcription in *gbb* mutant GSCs. All the germaria are labeled for pMad (red), GFP (green), Hts (blue, fusomes) and DAPI (white). The panels A′-F′ represent the corresponding DAPI images for panels A-F. Circles highlight cap cells, whereas asterisks indicate GSCs. (A) A germarial tip from a *bam-GFP* female cultured at 29°C for 4 days showing normal pMad expression and no *bam-GFP* expression in GSCs. (B-F) Germarial tips from either *bam-GFP gbb⁴/gbbᴰ⁴* (C,F) or *bam-GFP gbb⁴/gbbᴰ²⁰* (B,D,E) females cultured at 29°C for 4 days, showing reduced pMad expression. *gbb* mutant GSCs with easily detected pMad do not express *bam-GFP* (B,C; one indicated by an arrow in D), whereas the other GSCs with severely reduced pMad show *bam-GFP* expression (one indicated by an arrowhead in D). All the micrographs are shown at the same scale. Scale bar: 10 µm.

overexpression was sufficient to repress *bam* transcription in germ cells. Similarly, *bam-GFP* expression was studied in germaria overexpressing *gbb* using the C587 driver and the *UAS-gbb* transgene, which has been used to effectively overexpress *gbb* in the wing disc (Khalsa et al., 1998). The germaria overexpressing *gbb* had the normal number of GSCs and cysts (Fig. 4I), indicating that GSC maintenance and division, and germ cell differentiation, appeared to be normal. Similarly, the *bam-GFP* expression pattern was also normal in the *gbb*-overexpressing germaria (Fig. 4I). These results suggest that *gbb* overexpression, unlike that of *dpp*, is not sufficient to inhibit *bam* transcription.

Loss of *gbb* signaling results in a reduction of pMad in GSCs that is related to *bam* upregulation in GSCs

It appears that *gbb* uses the same downstream components as *dpp* does in regulating wing development (Haerry et al., 1998; Khalsa et al., 1998). *dpp* signaling results in the production of pMad (Newfeld et al., 1997; Tanimoto et al., 2000). To investigate whether *gbb* is also involved in the production of pMad in GSCs, we examined pMad accumulation in *gbb* mutant GSCs, and the relationship between pMad accumulation and *bam* transcription. As expected, pMad and *bam-GFP* expression patterns in GSCs and cystoblasts remained normal four days after the control females were cultured at 29°C (Fig. 5A,A′). Four days after being cultured at 29°C, the expression of pMad in the GSCs in both *gbb⁴/gbbᴰ⁴* and *gbb⁴/gbbᴰ²⁰* females was generally reduced (Fig. 5B-F′). Some of the mutant *gbb* germaria that had moderately reduced pMad expression in GSCs showed no *bam-GFP* expression in GSCs (Fig. 5B-D′), whereas the germaria that had severely reduced levels of pMad in GSCs showed significant *bam-GFP* upregulation in GSCs (Fig. 5E-F′). There appeared to be a good correlation between levels of

pMad and *bam-GFP* expression in *gbb* mutant GSCs. These results indicate that *gbb* signaling also results in the phosphorylation of Mad and that levels of pMad in GSCs seem to correlate with levels of *bam* repression.

punt and *Med* are required cell-autonomously to repress *bam* transcription in GSCs

punt encodes a type II serine/threonine kinase receptor for *dpp* and also possibly for *gbb* (Letsou et al., 1995; Ruberte et al., 1995). A temperature-sensitive *punt* allelic combination, *punt¹⁰⁴⁶⁰/punt¹³⁵*, can develop to adulthood at 18°C and exhibits mutant phenotypes at 29°C (Theisen et al., 1996). Newly eclosed *punt¹⁰⁴⁶⁰/punt¹³⁵* females at 18°C had a normal number of GSCs and a normal *bam-GFP* expression pattern in their germaria (Fig. 6A). Some *punt¹⁰⁴⁶⁰/punt¹³⁵* mutant GSCs started to express *bam-GFP* two days after being shifted to 29°C (Fig. 6B). One week after being cultured at 29°C, the GSCs in 75% of the mutant germaria (a total of 97 germaria were examined) that still carried one or more GSCs had already expressed *bam-GFP* (Fig. 6C), and 53% of the mutant germaria (a total of 123 germaria were examined) had only one or no GSC (Fig. 6C,D). After four days at the restrictive temperature, pMad in most *punt* mutant GSCs was severely reduced and *bam-GFP* was upregulated (Fig. 6E-H). These results further show that defective Bmp signaling results in the derepression of *bam* transcription in GSCs and that levels of pMad are correlated with the repression status of *bam* transcription in GSCs.

So far, *bam* expression has been examined only in *dpp*, *gbb* and *punt* mutant germaria in which Bmp signaling is defective in both somatic cells and germ cells. To determine whether direct Bmp signaling in GSCs is necessary for repressing *bam* transcription, we used the FLP-mediated FRT recombination technique to generate *punt* and *Med* mutant GSCs marked by loss of expression of the *armadillo* (*arm*)-*lacZ* transgene, and
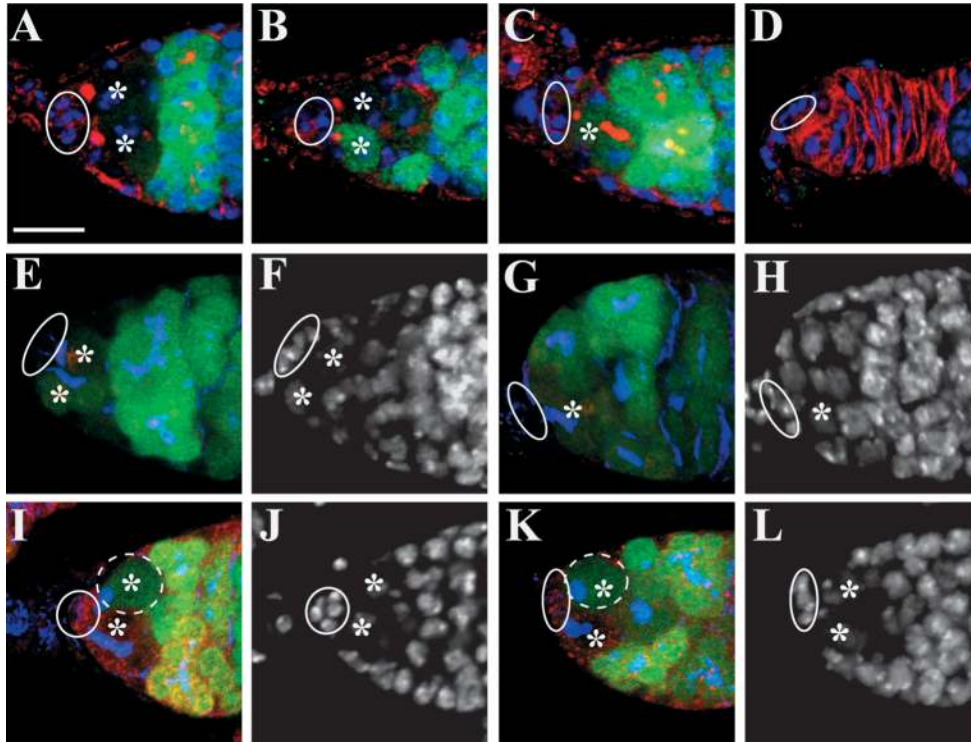
Fig. 6. *punt* and *Med* are required cell-autonomously in GSCs to repress *bam* transcription. Germaria from *punt10460/punt135* mutant females cultured at 18°C for 2 days (A), or at 29°C for 2 (B) or 7 (C,D) days, are labeled for Hts (red, fusomes and somatic follicle cells), *bam-GFP* (green) and DAPI (blue). Germaria in E-H from the *punt10460/punt135* mutant females cultured at 29°C for 4 days are labeled for pMad (red), *bam-GFP* (green), Hts (blue, fusomes) and DAPI (white). Germaria in I-L are labeled for *arm-lacZ* (red), GFP (green), Hts (blue) and DAPI (white). F,H,J and L represent corresponding DAPI stainings for E,G,I and K, respectively. Circles highlight cap cells, whereas asterisks indicate GSCs. (A-D) Germarial tips showing two GFP-negative GSCs (A), one GFP-positive and one GFP-negative GSC (B), one GFP-positive GSC (C) and no GSC (D). (E-H) Germarial tips showing two GFP-positive GSCs with severely reduced pMad (E,F) and one GFP-positive GSC with severely reduced pMad (G,H). (I-L) Germarial tips showing a *bam-GFP*-positive marked *punt135* GSC (outlined by a dashed line; I) and a *bam-GFP*-positive marked *Med26* GSC (dashed line; K). The marked GSCs are identified by loss of *arm-lacZ* expression. All micrographs are shown at the same scale. Scale bar: 10 μm.

then examined *bam-GFP* expression in the marked mutant GSCs (Xu and Rubin, 1993; Xie and Spradling, 1998). *Med* encodes a common Smad 4 for Tgfβ-like signaling pathways, and *Med26* is a strong *Med* mutant (Das et al., 1998; Wisotzkey et al., 1998). 54% of the marked three-day old *punt135* GSCs expressed *bam-GFP* (a total of 37 marked GSC clones were examined), and 65% of the marked three-day old *Med26* mutant GSCs showed obvious *bam-GFP* upregulation (a total of 48 marked GSC clones were examined) (Fig. 6I-L). These results demonstrate that direct Bmp signaling is necessary for repressing *bam* transcription.

Mad and Med directly bind to the silencer in the *bam* promoter *in vitro*

We have so far shown that Bmp signaling mediated by Dpp and Gbb is essential for repressing *bam* transcription in GSCs. This *bam* transcriptional repression could be directly or indirectly controlled by Bmp signaling. As shown recently by Chen and McKearin, a silencer located at the 5′ UTR of the *bam* gene is both necessary and sufficient for repressing *bam* transcription in GSCs (Chen and McKearin, 2003a). In *Drosophila*, the *brinker* (*brk*) gene is actively repressed by *dpp* signaling through a transcriptional silencer (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999; Marty et al., 2000; Muller et al., 2003). Interestingly, *bam* and *brk* silencers show remarkably similar sequences: 13 out of 19 base pairs are identical in A and B sites (Fig. 7A). The *brk* silencer has been shown to be directly occupied by a complex containing Mad, Med and Schnurri (Shn), and its repression requires *shn* and functional *dpp* signaling (Muller et al., 2003). *shn* is known to be required in GSCs for their maintenance,

and loss of *shn* function results in GSC loss (Xie and Spradling, 2000). All the evidence suggests that the *bam* silencer could be directly occupied by a complex containing Mad, Med and possibly Shn.

We performed electrophoretic mobility shift assays to test whether Med and Mad can bind directly to the *bam* silencer in vitro using a Cy5-labeled *bam* silencer element (Fig. 7B), and purified bacterially expressed GST-Mad and GST-Med (Fig. 7C). GST-Mad (a fusion between GST and the N-terminal DNA-binding domain and linker region of Mad) was shown to bind to the *dpp* responsive elements in vitro (Kim et al., 1997), whereas GST-Med is a fusion of GST with the full-length Med. Interestingly, both Mad and Med could bind to the silencer but with different affinities. It appeared that Med bound to the silencer with a higher affinity than Mad (Fig. 7D). The binding specificity of Mad and Med to the silencer was demonstrated by a competition experiment with an unlabelled DNA fragment containing A and B sites (Fig. 7B,D). The unlabeled DNA fragment containing either an A or a B site could almost completely compete for binding of Mad to the labeled silencer. However, the unlabeled DNA fragment with the A site, but to much less extent, with the B site could compete for binding of Med to the labeled silencer. These data suggest that Mad occupies both the A and B sites, whereas Med preferentially binds to the A site. pMad accumulates in the GSC nucleus at high levels (Kai and Spradling, 2003) (this study). As described earlier, *Med* is also required in GSCs for repressing *bam* transcription. These in vitro binding results suggest that a protein complex containing Mad and Med, stimulated by Bmp signaling, directly binds to the *bam* silencer to repress its transcription in GSCs.
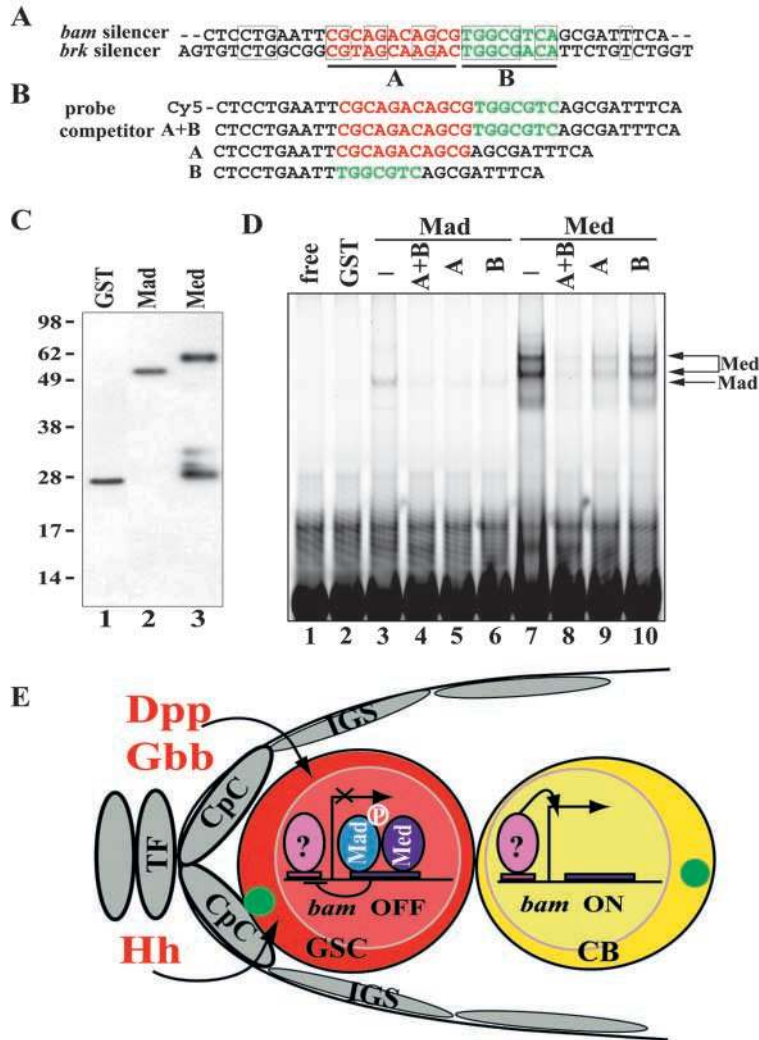
Fig. 7. Mad and Med bind directly to the *bam* silencer in vitro. (A) Sequence alignment of the bipartite *bam* and *brk* silencers, in which conserved base pairs are boxed. Sites A (red) and B (green) of the *bam* silencer are as previously described (Chen and McKearin, 2003a). (B) A Cy5 5′-modified oligonucleotide containing the *bam* silencer and unlabeled competitors in electrophoretic mobility shift assays (competitor A+B, the unlabeled silencer with sites A and B; competitor A, same flanking sequences with site A only; competitor B, same flanking sequences with site B only). (C) Immunoblot analysis of purified recombinant GST-tagged proteins with a mouse anti-GST antibody. Lane 1, 50 ng of GST; lane 2, 200 ng of GST-Mad; lane 3, 200 ng GST-Med. The ~30 kDa bands in lane 3 are probably C-terminal degradation products of GST-Med protein. (D) Gel shift assay showing that Mad or Med bind to the *bam* silencer in vitro. Approximately 10 nmol of protein was used in each binding reaction. The double shift bands for Med may result from partially degraded proteins. The labeled probe without protein (lane 1) or with GST protein (lane 2) serve as negative controls. The labeled probe binds to Mad (lane 3) and Med (lane 7). The unlabeled competitors A+B (lane 4), A (lane 5) or B (lane 6) could effectively compete away Mad binding. The unlabeled competitors A+B (lane 8) or A (lane 9) could effectively compete for Med binding but B (lane 10) could only partially compete. (E) Current model for how Bmp niche signals control GSC identity by directly repressing *bam* transcription. Bmp signals from cap cells produce the highest levels of pMad, which associates with Med and directly occupies the *bam* silencer to repress its transcription in GSCs. As a cystoblast moves away from cap cells, levels of pMad are reduced to below the critical threshold level, *bam* transcription is then derepressed and activated by an unknown activator (indicated by '?').

Discussion

Stem cells are located in a niche, which provides extracellular cues that control stem cell self-renewal, division and differentiation. Dpp is a signaling molecule that originates from the niche and is necessary for maintaining GSCs in the *Drosophila* ovary (Xie and Spradling, 1998; Xie and Spradling, 2000). This study has identified another Bmp-like molecule, Gbb, as an essential niche signal for maintaining GSCs. It is likely that Dpp and Gbb function cooperatively as short-range signals in the GSC niche, as their signaling activities, monitored by pMad and Dad expression, are restricted to GSCs and some cystoblasts. Previous studies have demonstrated that *bam* is known to be necessary and sufficient for cystoblast differentiation (McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). This study shows that upregulation of *bam* in GSCs is associated with stem cell loss in *dpp* and *gbb* mutants. Here we propose a model to possibly explain how Bmp signaling directly represses *bam* transcription in *Drosophila* ovarian GSCs (Fig. 7E). The Bmp signals from the GSC niche activate their signaling cascade in GSCs, which leads to Mad phosphorylation, and then the translocation of the Mad and Med complex into the nucleus, which probably directly binds to the *bam* promoter and represses *bam*

transcription in GSCs. This study demonstrates that Bmp signals maintain GSCs, at least in part, by repressing *bam* transcription in GSCs in the *Drosophila* ovary.

Gbb is another niche signal that is essential for maintaining GSCs

In this study, a new function of *gbb* in the regulation of GSCs in the *Drosophila* ovary is revealed. Loss of *gbb* function leads to GSC differentiation and stem cell loss, similar to *dpp* mutants. *gbb* is expressed in somatic cells but not in germ cells, suggesting that *gbb* is another niche signal that controls GSC maintenance. Like *dpp*, *gbb* contributes to the production of pMad in GSCs and also functions to repress *bam* expression in GSCs. As in the wing imaginal disc (Haerry et al., 1998; Khalsa et al., 1998; Ray and Wharton, 2001), *gbb* also probably functions to augment the *dpp* signal in the regulation of GSCs through common receptors in the *Drosophila* ovary. In both *dpp* and *gbb* mutants, pMad accumulation in GSCs is severely reduced but not completely diminished. As the *dpp* or *gbb* mutants used in this study do not carry complete loss-of-function mutations, it remains possible that complete elimination of either *dpp* or *gbb* function is sufficient for eradicating pMad accumulation in GSCs. Alternatively, both

dpp and *gbb* signaling are required independently for full pMad accumulation in GSCs, and thus disrupting either one of them only partially diminishes pMad accumulation in GSCs. The lethality of null *dpp* and *gbb* mutants, and the difficulty in completely removing their function in the adult ovary, prevent us from further testing these possibilities directly.

Interestingly, *dpp* overexpression results in complete suppression of cystoblast differentiation and complete repression of *bam* transcription in the germ cells, whereas *gbb* overexpression does not have obvious effects on cystoblast differentiation or *bam* transcription. Even though the *UAS-gbb* transgene and the *c587* driver for *gbb* overexpression have been demonstrated previously to function properly (Khalsa et al., 1998; Kai and Spradling, 2003; Zhu and Xie, 2003), it is possible that active Gbb proteins are not produced in inner sheath cells and somatic follicle cells because of a lack of proper factors that are required for Gbb translation and processing in those cells, which could explain why the assumed *gbb* overexpression does not have any effect on cystoblast differentiation. However, as active Dpp proteins can be successfully achieved using the same expression method, and Dpp and Gbb are closely related Bmps, it is unlikely that active Gbb proteins are not produced in inner sheath cells and follicle cells. Alternatively, *dpp* and *gbb* signals could have distinct signaling properties, and *dpp* may play a greater role in regulating GSCs and cystoblasts. Recent studies have indicated that Dpp and Gbb have context-dependent relationships in wing development (Ray and Wharton, 2001). In the wing disc, duplications of *dpp* are able to rescue many but not all of the phenotypes associated with *gbb* mutants, suggesting that *dpp* and *gbb* have not only partly redundant functions but also distinct signaling properties. In the wing and ovary, *gbb* and *dpp* function through two Bmp type I receptors, *sax* and *tkv* (Khalsa et al., 1998; Xie and Spradling, 1998) (this study). The puzzling difference between *gbb* and *dpp* could be explained by context-dependent modifications of Bmp proteins, which render them different signaling properties in different cell types. It will be of great interest to better understand what causes Bmps to have distinct signaling properties in the future.

Dpp and Gbb function as short-range signals in the GSC niche

All the defined niches share a commonality, structural asymmetry, which ensures stem cells and their differentiated daughters receive different levels of niche signals (Watt and Hogan, 2000; Spradling et al., 2001). In order for a niche signal to function differently in a stem cell and its immediately differentiating daughter cell that is just one cell away, it has to be short-ranged and localized. This study, and a recent study by Kai and Spradling (Kai and Spradling, 2003), show that Bmp signaling mediated by Dpp and Gbb results in preferential expression of pMad and Dad in GSCs. In this study, we show that Bmp signaling appears to elicit different levels of responses in GSCs and cystoblasts, suggesting that the cap cells are likely to be a source for active short-ranged Bmp signals. These observations support the idea that Bmp signals are only active around cap cells. Consistently, when GSCs lose contact with the cap cells following the removal of adherens junctions they move away from the niche and then are lost (Song et al., 2002). As *gbb* and *dpp* mRNAs are broadly expressed in the other somatic cells of the germarium besides cap cells, localized active Bmp proteins around cap cells could be generated by localized translation and/or activation of Bmp proteins. As they can function as long-range signals (Podos and Ferguson, 1999), it remains unclear how Dpp and Gbb act as short-range signals in the GSC niche.

Bmp signals probably directly repress *bam* transcription in GSCs

Previous studies, and this study, have shown that Bmp signaling and *bam* expression are directly opposing in *Drosophila* ovarian GSCs (McKearin and Spradling, 1990; Ohstein and McKearin, 1997; Xie and Spradling, 1998). *bam* is actively repressed in GSCs through a defined transcriptional silencer (Chen and McKearin, 2003a). These observations lead us to propose a model in which Bmp signals from the niche maintain adjacent germ cells as GSCs by actively suppressing *bam* transcription and thus preventing differentiation into cystoblasts (Fig. 7E).

In this study, we show that the levels of pMad are correlated with the amount of *bam* transcriptional repression in GSCs and cystoblasts. In the wild-type germarium, pMad is highly expressed in GSCs and some cystoblasts where *bam* is repressed. In other cystoblasts and differentiated germline cysts, pMad is reduced to very low levels, and thus *bam* transcriptional repression is relieved. In the GSCs mutant for *dpp*, *gbb* or *punt*, pMad levels are severely reduced, and *bam* begins to be expressed. The repression of *bam* transcription as a result of *dpp* overexpression seems to be a rapid process as *bam* mRNA was reduced to below detectable levels two hours after *dpp* was overexpressed. This suggests that repression of *bam* transcription by Bmp signaling could be direct. Furthermore, Med and Mad can bind to the defined *bam* silencer in vitro, which also supports the idea that Bmp signaling acts directly to repress *bam* transcription. Similar results have been obtained in a recent study (Chen and McKearin, 2003b). Similarly, Dpp signaling has been shown to repress *brk* expression in the wing imaginal disc and in the embryo (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). The repression of *brk* expression by Dpp signaling is mediated by the direct binding of Mad and Med to a silencer element in the *brk* promoter (Muller et al., 2003). As the *brk* silencer is very similar to the *bam* silencer, our results suggest that *bam* repression in GSCs is also mediated directly by Dpp and Gbb in a similar manner.

It remains unclear how the binding of Med and Mad to the *bam* silencer results in *bam* transcriptional repression in GSCs. For the *brk* silencer, Dpp signaling and Shn are both required to repress *brk* expression in the *Drosophila* wing disc and embryo (Marty et al., 2000; Torres-Vazquez et al., 2001; Muller et al., 2003). Mad and Med belong to the Smad protein family, which are known to function as transcriptional activators by recruiting co-activators with histone acetyltransferase activity (reviewed by Massague and Wotton, 2000). In the wing disc, Shn is proposed to function as a switch factor that converts the activating property of Mad and Med proteins into a transcriptional repressor property (Muller et al., 2003). Possibly, the Mad-Med complex could also recruit Shn to the *bam* repressor element. Consistent with the possible role of Shn in repressing *bam* expression in GSCs is the observation that GSCs that lose *shn* function differentiate, and thus are lost (Xie and Spradling, 2000). Also, it remains possible that Mad

and Med could recruit a repressor other than Shn when binding to the *bam* repressor element. In the future, it will be very important to determine whether Shn itself is a co-repressor for Mad/Med proteins or whether it directly recruits a co-repressor to repress *bam* transcription in GSCs.

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