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Boss/Sev Signaling from Germline to Soma Restricts Germline-Stem-Cell-Niche Formation in the Anterior Region of *Drosophila* Male Gonads

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SUMMARY

Drosophila **germline stem cells are regulated by the somatic microenvironment, or "niche," which ensures that the stem cells can both self-renew and produce functional gametes throughout adult life. However, despite its prime importance, little is known about how niche formation is regulated during gonadal development. Here, we demonstrate that a receptor tyrosine kinase, Sevenless (Sev), is required to ensure that the niche develops in the anterior region of the male embryonic gonads. Sev is expressed in somatic cells within the posterior region of the gonads. Sev is activated by a ligand, Bride of sevenless (Boss), which is expressed by the germline, to prevent ectopic niche differentiation in the posterior gonadal somatic cells. Thus, we propose that signal transduction from germline to soma restricts expansion of the germline-stem-cell niche in the gonads.**

INTRODUCTION

Stem cells possess the remarkable capacity to generate both daughter cells that retain a stem-cell identity and others that differentiate. Stem cells reside in dedicated cellular microenvironments, termed stem-cell niches, that dictate the stem-cell identity, maintain the stem-cell population, and coordinate proper homeostatic production of differentiated cells (Schoof et al., 2000; Fuchs et al., 2004; Ohlstein et al., 2004; Moore and Lemischka, 2006). Candidate niches have been identified in various tissues, such as intestinal epithelium, epidermis, bone marrow, and the reproductive organs (Fuchs et al., 2004; Ohlstein et al., 2004; Moore and Lemischka, 2006). Recent studies have addressed molecular aspects of how niches define stem-cell identity and behavior through intercellular signaling (Fuchs et al., 2004; Ohlstein et al., 2004; Moore and Lemischka, 2006). However, knowledge of the regulatory mechanism of niche development is limited. In various tissues, spatial location and size of the niches must be precisely regulated (Schoof et al., 2000; Brand et al., 2000; Zhang et al., 2003; Calvi et al., 2003; Ward et al., 2006). The misregulation of niche formation may cause tumors. Ectopic formation or overproduction of a niche results in an enlarged population of stem cells that would produce more daughter cells than the number of differentiated cells needed during tissue development and growth. Thus, the mechanism that restricts niche formation is critical for maintaining tissue homeostasis.

The germline-stem-cell niche in *Drosophila* testes has emerged as a useful model system for studying stem cells, because the cellular components of this niche have been characterized, and important aspects of how the niche influences the properties of residing stem cells are known (Gilboa and Lehmann, 2004; Kiger et al., 2001; Tulina and Matunis, 2001; Yamashita et al., 2003). In the apical tip of the adult testes, the germline stem cells lie in intimate contact with somatic hub cells, known as the niche, which causes the stem cells to retain self-renewing potential (Hardy et al., 1979). Germline stem cells divide to produce one daughter cell that remains associated with hub cells, while the other daughter cell detaches and initiates spermatogenesis (Hardy et al., 1979).

Hub cells are derived from a subset of somatic gonadal cells (SGCs) that is located in the anterior region of male embryonic gonads (Le Bras and Van Doren, 2006). It has been reported that the anteroposterior cellular identities within the gonads are regulated by the homeotic genes *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (DeFalco et al., 2004). However, how the formation of hub progenitors is restricted in the anterior of embryonic gonads remain elusive. Previous observations also suggest that the germline may be required for proper hub formation in male gonads. In the absence of germline cells, expansion of the hub population is observed (Le Bras and Van Doren, 2006). Here, we demonstrate that Sev activity is required to ensure that the niche develops in the anterior region of the male embryonic gonads. Sev is expressed in the posterior SGCs by the function of *Abd-B*, and it is activated by the Boss ligand emanating from the primordial

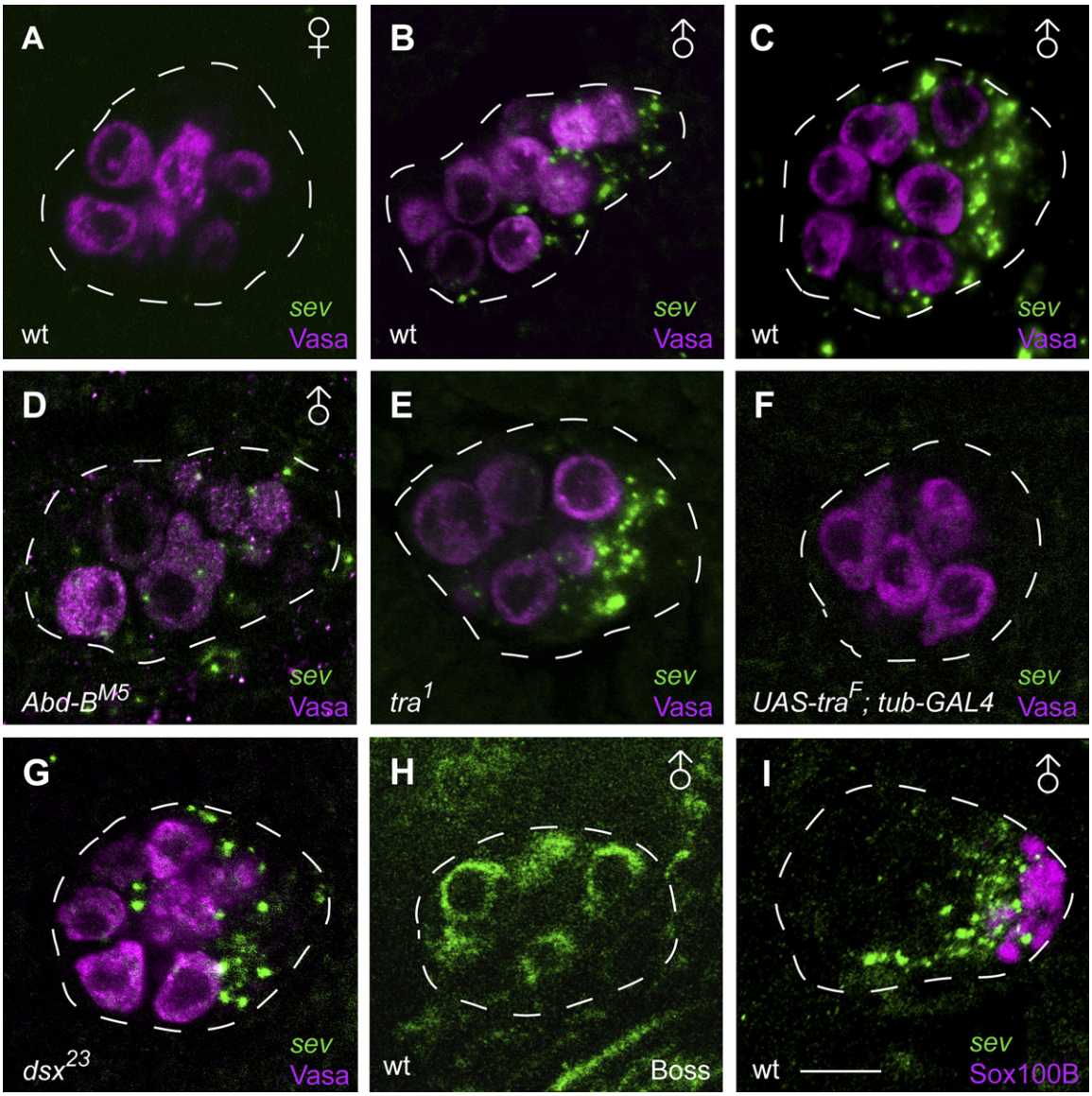

Figure 1. Expression of *sev* and *boss* within the Embryonic Gonads

(A–C) The gonads in (A) a female embryo at stage 16 and in male embryos at (B) stage 13 and (C) stage 16. Embryos were double stained for *sev* mRNA (green) and a marker for the germline, Vasa (magenta). In all panels presented in this paper, anterior is oriented toward the left and the embryonic gonads are outlined by white lines.

(D–G) Distribution of *sev* mRNA (green) and Vasa (magenta) in the gonads of stage-15/16 embryos homozygous for (D) *Abd-B*^{M5}, (E) *tra*^{1}, and (G) *dsx*^{23} and stage-15/16 embryos (F) expressing *tra* (UAS-*tra*^{F}; *tub*-GAL4). Note the *sev* expression in all of the *tra* and *dsx* mutants (n = 54 and 23, respectively), but not in *Abd-B* mutants or in the *tra*-expressing embryos (n = 12 and 22, respectively).

(H) A gonad from a stage-16 male embryo stained for Boss (green). Boss was expressed in all of the pole cells within the gonads (n = 200).

(I) A male gonad at stage 15, stained for *sev* mRNA (green) and Sox100B (magenta). The scale bar is 10 μm.

germ cells (PGCs) to prevent ectopic niche differentiation in the posterior SGCs.

RESULTS AND DISCUSSION

sev Is Expressed in the Posterior SGCs of Male Embryonic Gonads

We expected that the formation of the hub progenitors is regulated by genes expressed within the embryonic gonads in a region-specific and a male-specific manner. While investigating the distribution of transcripts enriched in the embryonic gonads, we discovered that mRNA for the receptor tyrosine kinase Sev (Tomlinson and Ready, 1986; Hafen et al., 1987) was expressed in the SGCs in a male-specific manner (Figures 1A–1C). In male gonads, *sev* was expressed only in a subset of the SGCs in the posterior portion of the gonads. Its expression began at stage 13 and

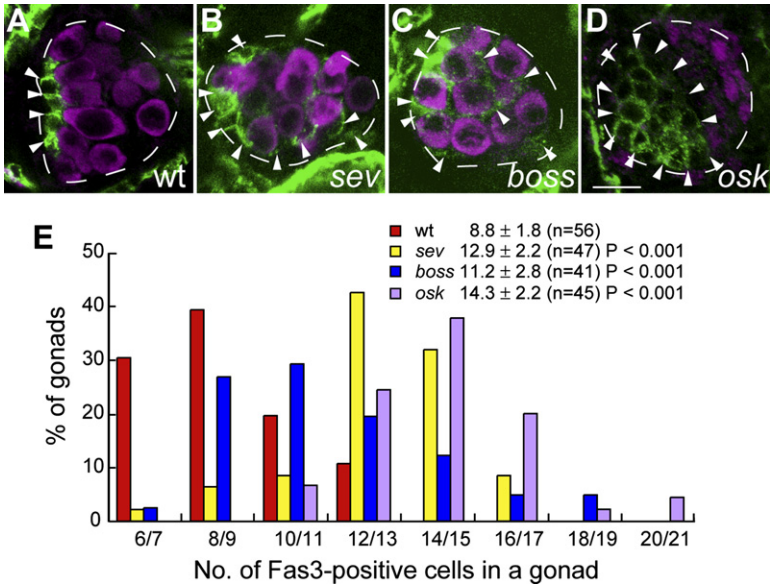

Figure 2. Ectopic Formation of the Hub Progenitors Is Repressed by the Sev Pathway

(A–D) Male gonads from (A) wild-type, (B) *sev*, and (C) *boss* embryos and an (D) agametic embryo derived from an *osk*-homozygous female at stage 16/17. The embryos were stained for Fas3 (green) and either Vasa ([A–C], magenta) or Sox100B ([D], magenta). Arrowheads show Fas3-expressing cells. The scale bar is 10 μm.

(E) Distribution of the number of Fas3-positive cells per gonad in wild-type (red), *sev* (yellow), *boss* (blue), and agametic (violet) embryos at stage 16/17. The average number of Fas3-positive cells per gonad ± SD and the number of gonads observed (n) are also shown. Significance was tested versus wild-type by using Student's t tests. The average numbers of Fas3-positive cells in control embryos are shown in Figure S1.

persisted until at least the end of embryogenesis (Figures 1B and 1C).

We determined that the spatially restricted *sev* expression in the posterior SGCs was dependent on the function of a homeotic gene, *Abdominal-B* (*Abd-B*). *Abd-B* specifies posterior abdominal segments within the embryo and anteroposterior cellular identities within the gonads (Delorenzi and Bienz, 1990; DeFalco et al., 2004). In the absence of *Abd-B* activity, *sev* expression was significantly reduced in the posterior SGCs (Figure 1D). We also found that male-specific *sev* expression was regulated by the sex-determination genes *transformer* (*tra*) and *doublesex* (*dsx*). When genetically female XX embryos were masculinized by depriving them of *tra* activity (DeFalco et al., 2003), *sev* expression was detectable within the posterior SGCs of all embryos (Figure 1E). Conversely, when genetically male XY embryos were feminized by the expression of *tra* (DeFalco et al., 2003), *sev* expression was no longer detectable within the gonads (Figure 1F). Furthermore, when both XX and XY embryos were transformed into intersex embryos by abolishing *dsx* function (DeFalco et al., 2003; Duncan and Kaufman, 1975), *sev* expression was observed in the posterior SGCs of all embryos (Figure 1G). From these results, we conclude that *sev* expression is induced in the posterior SGCs by *Abd-B* function, but that it is repressed by the feminizing pathway downstream of *tra* and *dsx*. Thus, *sev* expression is directly linked with the body-patterning and sex-determination pathways within the gonads.

sev Represses Ectopic Hub Differentiation in the Posterior SGCs

To determine whether *sev* is required for the development of hub cells, we examined the expression of Fasciclin 3 (Fas3) in *sev* mutant embryos. Fas3 is expressed in the hub cells from embryogenesis until adulthood (Brower et al., 1981; Le Bras and Van Doren, 2006); therefore, it is a helpful marker for hub-cell identity. In wild-type embryos, Fas3-positive SGCs were observed only in the anterior region of the male gonads from stage 15 onward (Figure 2A). In the absence of *sev* activity, Fas3-positive SGCs were observed ectopically in the posterior region of the gonads, and their number was significantly increased compared with wild-type embryos (Figures 2B and 2E). This phenotype results from ectopic expression of the Fas3 marker in the posterior SGCs, rather than aberrant proliferation of the anterior SGCs expressing Fas3, because the total number of SGCs detected with a SGC marker, Eyes absent (Eya), was unaffected, and mitosis was not pronounced in the mutant embryos (Table S1; see the Supplemental Data available with this article online).

In addition to Fas3, *escargot* (*esg*) and Armadillo (Arm) have been reported to be expressed predominantly in hub cells within embryonic and adult gonads (Yamashita et al., 2003; Le Bras and Van Doren, 2006). We found that Fas3, *esg*, and Arm signals were detectable in hub clusters within the gonads of wild-type embryos (Figures 3A–3D). Under the staining condition we used, however, weak *esg* and Arm signals were occasionally detected in SGCs other than hub clusters, and weak *esg* signals were also observed in pole cells. Thus, *esg* and Arm signals were not as tightly restricted to hub cells as Fas3 signals (Figures 3A, 3B, and 3D). This made it difficult to confidently identify ectopic hub progenitors within the mutant embryonic gonads by using these markers, although, in the absence of Sev activity, ectopic Fas3-positive cells exhibited a slightly elevated *esg* signal (Figures 3E–3G), and Arm-enriched cells were occasionally observed in the posterior region of the embryonic gonads (Figure 3H).

In contrast, after hatching, *esg* and Arm signals became tightly restricted to hub cells within wild-type gonads (Figures 3I–3L), although a weak *esg* signal remained

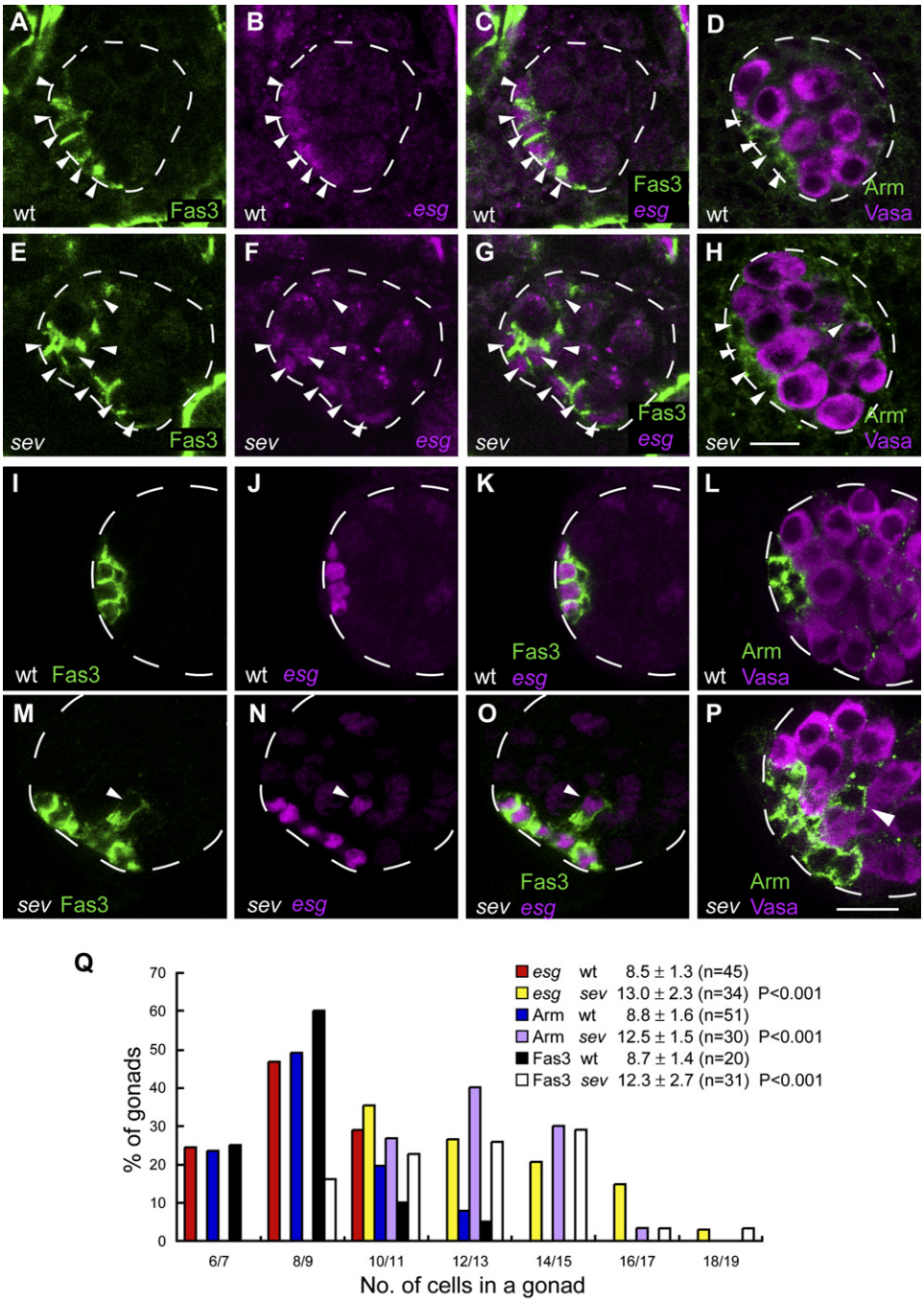

Figure 3. Expression of Fas3, *esg*, and Arm within the Gonads

(A–P) We examined expression of Fas3 ([A, E, I, and M], green), *esg* ([B, F, J, and N], magenta), and Arm ([D, H, L, and P], green) within the male gonads at (A–H) embryonic stage 16/17 and (I–P) immediately after hatching (0–6 hr after hatching). (A–D and I–L) Wild-types and (E–H and M–P) *sev* mutants. (C, G, K, and O) Merged images. An enhancer trap, esg^{K00606} (anti-β-gal antibody), was used to detect *esg* expression. Arrowheads in (A)–(H) point to (A–C and E–G) Fas3-positive and (D and H) Arm-positive SGCs. Fas3/*esg*-double positive and Arm-positive cells were ectopically observed in *sev* male larvae (arrowheads in [M]–[P]). Scale bars are 10 μm.

(Q) The distribution of the number of Fas3-positive cells (black and white), *esg*-positive cells (red and yellow), and Arm-positive cells (blue and violet) per male gonad in wild-type (black, red, and blue) and *sev* (white, yellow, and violet) first-instar larvae is shown. The average number of cells ± SD and the number of gonads examined (n) are also shown. Significance was tested versus wild-type by using Student's t tests. The average number of Fas3-positive cells per gonad in control larvae (sev^{+}) is shown in Figure S1.

detectable in pole cells (Figure 3J). In *sev* first-instar larvae, all of the Fas3-positive cells showed an intense *esg* signal (Figures 3M–3O), and Fas3/*esg*-double positive and Arm-positive cells were ectopically observed (arrowheads in Figures 3M–3P). We also found that the average numbers of Fas3-, *esg*-, and Arm-positive cells were significantly increased compared to wild-type (Figure 3Q). These numbers were almost identical to that of Fas3-positive cells in the gonads of *sev* embryos (Figure 2E). Thus, ectopically formed Fas3-positive cells show intense *esg* and Arm signals after hatching; therefore, we conclude that Fas3 is a reliable marker for identifying ectopic hub progenitors within the gonads.

Taken together, the above-described observations demonstrate that *sev* activity is required to repress hub differentiation in the posterior SGCs of male embryonic gonads.

Boss/Sev Signaling from PGCs to the Posterior SGCs Represses Ectopic Hub Differentiation

In developing eyes, Sev receptor is activated by binding to a ligand, Boss, to initiate a Ras-signaling cascade that eventually phosphorylates the Rolled protein (Hart et al., 1990; Biggs et al., 1994; Raabe, 2000). In the posterior SGCs, the activated form of Rolled (diphosphorylated Rolled, dpRl) was enriched (Figure S2A). The posterior enrichment of dpRl was observed in a male-specific manner and was reduced in *sev* mutants (Figure S2B), consistent with Sev activity in the posterior SGCs. To test whether the ligand activating the Sev receptor was expressed by the SGCs themselves or by the neighboring PGCs, we examined the distribution of Boss within the gonads. Boss expression was detected in almost all of the PGCs, or pole cells, both in male and female embryos from stage 12 onward, but it was undetectable in the SGCs (Figure 1H). In the absence of *boss* activity, Fas3-positive SGCs were ectopically observed in the posterior region of the gonads (Figure 2C), and their number was significantly increased (Figure 2E), consistent with our observations in *sev* mutant embryos. A similar phenotype was also observed in agametic gonads (Figures 2D and 2E) (Le Bras and Van Doren, 2006). Furthermore, in *boss* and agametic gonads, dpRl expression was significantly reduced (Figures S2C and S2D). We conclude that the Boss ligand emanates from the PGCs to activate the Sev receptor in the posterior SGCs, which represses their differentiation into hub-cell progenitors.

In the absence of Sev or Boss, Fas3 was ectopically expressed in the posterior SGCs. However, not all SGCs were able to become the hub progenitors. For example, in the agametic gonads, Fas3 was undetectable in the posterior-most SGCs expressing Sox100B, known as male-specific somatic gonadal precursors (ms-SGPs) (DeFalco et al., 2003) (Figure 2D). We could not detect *sev* expression in ms-SGPs (Figure 1I), and the *sev* mutation did not affect Sox100B expression (Figure S3C). We also found that the *sev* mutation did not alter Eya expression, which was detected at higher levels in the posterior SGCs, including the ms-SGPs (DeFalco et al., 2003) (Figure S3D). From these observations, we propose that Sev activity is not required for posterior patterning, and that hub formation is still restricted to a subset of SGCs by this posterior identity.

Ectopic Hub Differentiation Causes Expansion of the Germline-Stem-Cell Niche in the Adult Testes

Next, we examined whether the ectopic formation of hub progenitors could increase with an increase in size of the germline-stem-cell niche during postembryonic development. In third-instar larvae from *sev*, *boss*, and agametic embryos, hub cells expressing Fas3 were more abundant (Figures S4B–S4E). A similar increase was observed in the mutant testes of adult flies (Figures 4B–4G). The increased number of hub cells formed single or double clusters in the apical regions of *sev* testes (Figures 4B–4D). Single clusters were occasionally displaced from the distal tips of the testes (Figures 4D and 4E). This may result from an ectopic aggregation of the more numerous hub progenitors. We counted the number of germline stem cells associating with the hub cells. An increased number of stem cells were associated with the larger hub clusters in *sev* mutant testes compared to wild-type (Figure 4H). We conclude that the additional hub progenitors in the mutant embryos are maintained during postembryonic development and become functional as the niche. Consequently, the enlarged niche recruits more germline stem cells, resulting in an oversized apex in the testes that contains spermatogonial cells and spermatocytes. This phenotype was exacerbated as spermatogenesis continued (see Supplemental Data; Figures S4G, S4I–S4K, and S5). Thus, Sev is required to repress excess production of germline stem cells, which otherwise produce more daughter cells than are consumed as founder cells for spermatogenesis.

The Role of Boss/Sev Signaling in Male Embryonic Gonads

Our data show that the posterior SGCs, as well as the anterior SGCs, have the capacity to contribute to the germline-stem-cell niche within the male embryonic gonads. However, during development, niche differentiation is normally repressed in the posterior SGCs by Sev. In the absence of Sev activity, posterior SGCs are recruited to form an expanded niche. We also show that Sev is activated in the SGCs by the Boss ligand emanating from pole cells. This implies that varying the number of pole cells will alter the niche size. Our model predicts that a decrease in the number of pole cells should induce ectopic niche formation within the gonads, which consequently increase their chance to recruit pole cells as the stem cells. Thus, we speculate that the interaction between SGCs and PGCs via the Boss/Sev pathway acts as a key component of a negative-feedback loop to maintain an optimal number of germline stem cells in male gonads. A similar feedback mechanism has been reported in the stem-cell system of plant meristem and in *Drosophila* larval ovaries (Schoof et al., 2000; Brand et al., 2000; Gilboa and Lehmann, 2006).

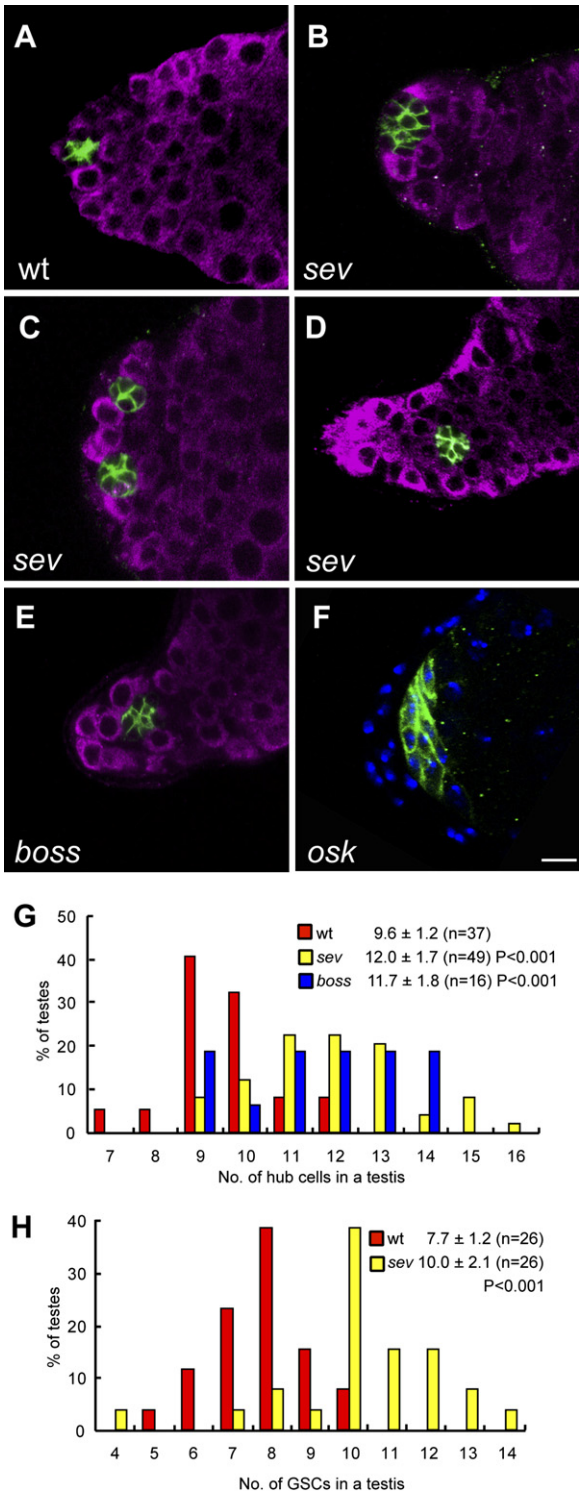

Figure 4. Increase in the Number of Hub Cells and Germline Stem Cells in Adult Testes

(A–F) Testes from (A) wild-type, (B–D) *sev*, (E) *boss*, and (F) agametic male flies (0–3 days after eclosion). Testes were stained for Fas3 (green) and either (A–E) Vasa (magenta) or (F) DNA (Toto-3, blue). (B–D) The increased number of hub cells formed single or double clusters in *sev* testes. (D and E) The single clusters were occasionally displaced from the apex of *sev* and *boss* testes. The scale bar is 10 μm.

(G and H) (G) Distribution of the number of Fas3-positive cells per testis in wild-type (red), *sev* (yellow), and *boss* (blue) adult males. (H) Distribution of the number of germline stem cells per testis in wild-type (red) and *sev* (yellow) adult males. The average number ± SD and the number of testes observed (n) are also shown. Significance was tested versus wild-type by using Student's t tests. The numbers of germline stem cells in testes shown in (A), (B), (C), (D), and (E) are 7, 11, 14, 11, and 9, respectively. The average number of hub cells in agametic testes has been reported to be 13 ± 5, a significant increase from 10.4 ± 2.5 in wild-type testes (Gönczy and DiNardo, 1996). These values are not significantly different from those obtained with the testes of third-instar larvae (Figure S4E).

However, we found that overexpression of a constitutively active Sev results in neither hyperactivation of RI nor repression of hub-cell fate in the anterior SGCs (see Supplemental Data), suggesting that activation of signaling components downstream of Sev is suppressed in the anterior SGCs. It has been widely accepted that the Notch transmembrane receptor and the receptor tyrosine kinase (RTK)/RAS/MAPK pathways antagonize each other in various developmental contexts (Voas and Rebay, 2004; Sundaram, 2005), and that Notch signaling is involved in stem cell maintenance and differentiation in several stem-cell systems (Alexson et al., 2006; Burns et al., 2005; Fre et al., 2005; Luo et al., 2005; Stanger et al., 2005; Woodward et al., 2005; Campos et al., 2006; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ward et al., 2006). In *Drosophila* ovaries, Notch signaling is required for the formation and maintenance of the germline-stem-cell niche (Ward et al., 2006; Song et al., 2007). Overexpression of activated Notch induces expansion of the niche, while a reduction of Notch activity results in loss of the niche. In addition, germline cells express ligands for Notch to induce Notch-receptor activity and thereby to promote their own maintenance and function within the niche (Ward et al., 2006; Song et al., 2007). As the Notch receptor is also expressed predominantly in SGCs of male embryonic gonads (Kidd et al., 1989; Y.K., unpublished data), it is likely that Notch may antagonize Boss/Sev signaling in the anterior region of the gonads. We speculate that the negative- and positive-feedback loops between germline and soma through Sev and Notch signaling act antagonistically to regulate proper niche formation during gonadal development. It will be interesting to test this hypothesis in future experiments. This study provides an important step toward understanding the regulatory mechanisms of niche formation in germline development.

EXPERIMENTAL PROCEDURES

Fly Stocks

The mutants for *sev* and *boss* were viable and fertile. Agametic embryos were obtained from osk^{301}-homozygous mothers raised at 18°C (Lehmann and Nüsslein-Volhard, 1986). We used the sev^{S11} line to express constitutively active Sev protein throughout embryos under the control of a heat-shock promoter (Basler et al., 1991). The 6–9 hr AEL (after egg laying) embryos were heat shocked for 30 min

at 37°C three times with a 2 hr interval at 25°C, and were then allowed to develop to stages 14–17 at 25°C.

The genotypes of the mutant lines used in this study were *sev^{d2}/sev^{d2}* (*sev^{14}*; an amorphic mutation, viable), *boss^{1} cu ca/boss^{1} h ry* (a loss-of-function mutation, viable), *w^{1118} sev^{d2} P{sev5}S11.1/w^{1118} sev^{d2} P{sev5}S11.1*, *mwh^{1} jv^{1} st^{1} red^{1} Sb^{sbd-2} e^{11} ro^{1} ca^{1} Abd-B^{M5}/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}*, *tra^{1}/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}*, and *In(3R)dsx^{23}/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}*. The genotypes of embryos were determined by using the GFP-expressing balancer chromosome.

Antibodies

The antibodies used for immunostaining and in situ hybridization are described in the Supplemental Data.

Immunostaining

Embryos were fixed and devitellinized as described (Kobayashi et al., 1999). For Eya staining, the embryos were devitellinized with a tungsten needle without using methanol. Larval and adult testes were fixed in 4% paraformaldehyde in PBS for 20 min and treated with methanol and detergent as described (Hayashi et al., 2004). The samples were washed three times (20 min each) in PBTx (PBS containing 0.1% Triton X-100) and blocked in PBTxg (PBTx containing 5% goat serum [GIBCO]) for 1.5 hr. Subsequently, the samples were incubated with primary antibodies in PBTxg for 16 hr at 4°C. After washing three times (20 min each) in PBTx, samples were incubated with secondary antibodies in PBTxg for 16 hr at 4°C and rinsed three times (20 min each) in PBTx. Samples were then mounted in Vectashield (Vector Laboratories) and examined under a confocal microscope (Leica Microsystems). For dpRI and Sox100B staining, immunofluorescent signals were enhanced with the TSA Biotin System (PerkinElmer). For Boss and dpRI staining, this protocol was modified as described below.

Boss Staining

The devitellinized embryos were nicked in their anterior portions with a tungsten needle to increase the permeability of the embryos to antibodies. Embryos were incubated with a mouse anti-Boss antibody in PBTxg for 24 hr at 4°C, and then with an Alexa 488-goat anti-mouse antibody in PBTxg for 24 hr at 4°C.

dpRI, Vasa, and Sxl Staining

Embryos were fixed and devitellinized as described (Kobayashi et al., 1999), except that 8% paraformaldehyde in PBS was used. The embryos were nicked as described above and permeabilized with PBTx2 (PBS containing 1% Triton X-100) for 1 hr. After blocking in PBTx2g (PBTx2 containing 5% goat serum) for 1.5 hr, samples were first incubated with a mouse anti-dpRI antibody in PBTx2g for 24 hr at 4°C. After washing three times (20 min each) in PBTx2, the embryos were incubated with an HRP-conjugated goat anti-mouse antibody in PBTx2g for 16 hr at 4°C, washed three times (20 min each) in PBTx2, and rinsed in TNT wash buffer (0.1 M Tris-Cl [pH 7.5], 0.15 M NaCl, 1% Triton X-100). The immunofluorescent signal was enhanced by incubating embryos in biotinyl tyramide (1:50 dilution, TSA Biotin System). Embryos were washed three times (20 min each) in PBTx2, and incubated with streptavidin-FITC (1:500, PerkinElmer) in PBTx2g for 16 hr at 4°C. Next, samples were washed three times (20 min each) in PBTx2, and were simultaneously incubated with rabbit anti-Vasa and mouse anti-Sxl antibodies in PBTxg for 16 hr at 4°C. After the embryos were washed three times (20 min each) in PBTx and incubated with Alexa 568-goat anti-rabbit and Alexa 647-goat anti-mouse antibodies in PBTxg for 16 hr at 4°C, the stained samples were washed three times (20 min each) in PBTx and mounted in Vectashield.

DNA Staining

The immunostained embryos and testes were treated with ribonuclease A (0.04 mg/ml) in PBTxg for 30 min and were incubated with Toto-3 (1:1000, Molecular Probes) in PBTxg containing 0.04 mg/ml ribonuclease A for 20 min. After washing three times (20 min each) in PBTx, the stained samples were mounted in Vectashield.

In Situ Hybridization

DIG-labeled *sev* antisense probes were synthesized from a 7530 bp cDNA fragment. The cDNA was amplified from an embryonic gonad cDNA library (Shigenobu et al., 2006) by using the following pair of primers: CACCATGACCACCACCCACATCAATC and AAGTCGCGA GACTCCCTC. The resulting PCR product was inserted into pENTR/D-TOPO vector (the CACC sequence used for directional cloning is underlined) and transcribed from the T3 RNA polymerase promoter as described (Mukai et al., 2006). Triple staining by in situ hybridization for *sev* RNA and immunostaining with anti-Vasa and either anti-β-gal or anti-GFP antibodies was performed as described (Hayashi et al., 2004). Double staining for *sev* RNA and Sox100B is described below.

Double Staining for sev RNA and Sox100B

The embryos were fixed and devitellinized as described (Kobayashi et al., 1999) and were washed three times (20 min each) in PBTH (PBS containing 0.1% Tween 20, 50 μg/ml heparin, and 250 μg/ml yeast tRNA). Samples were first incubated with rabbit anti-Sox100B antibody in PBTHR (PBTH containing 0.2 U/ml RNase inhibitor [Roche] and 1 mM DTT) for 16 hr at 4°C and washed three times (20 min each) in PBTH. Next, samples were incubated with an HRP-conjugated goat anti-rabbit antibody in PBTHR for 16 hr at 4°C, washed three times (20 min each) in PBTH, and rinsed in TNTH wash buffer (0.1 M Tris-Cl [pH 7.5], 0.15 M NaCl, 0.1% Tween 20, 50 μg/ml heparin, and 250 μg/ml yeast tRNA). The immunofluorescent signals were enhanced by incubating embryos in biotinyl tyramide (1:50 dilution, TSA Biotin System). The embryos were washed three times (20 min each) in PBTH and were incubated with streptavidin-FITC in PBTHR for 16 hr at 4°C. After washing three times (20 min each) in PBTH, the embryos were fixed again in 4% paraformaldehyde in PBS for 40 min and washed five times (5 min each) in PBTw (PBS containing 0.1% Tween 20). Hybridization was performed for 15 hr at 60°C in a solution containing 10 μg/ml *sev* RNA probe, 50% formamide, 5× SSC (1× SSC: 0.15 M sodium chloride/15 mM sodium citrate [pH 7.0]), 0.1% Tween 20, 0.1 mg/ml yeast tRNA, 10 mM DTT, and 10% dextran sulfate. The embryos were washed six times (30 min each) at 60°C in a solution containing 50% formamide, 5× SSC, and 0.1% Tween 20. They were then washed three times (10 min each) in PBTw, blocked in PBTws (PBTw containing 20% sheep serum [Chemicon]) for 1.5 hr, incubated with an alkaline phosphatase-conjugated sheep anti-DIG antibody in PBTws for 1 hr, and washed four times (20 min each) in PBTw. *sev* signals were detected with fluorescent substrates (HNPP/Fast Red) according to the manufacturer's protocol. The stained samples were washed three times (20 min each) in PBTw and mounted in Vectashield.

Sexing and Staging of Embryos

The sex of the embryos was determined either by immunostaining with female-specific anti-Sxl antibodies (Figures 2A–2C, 3D, and 3H; Figures S1, S2A–S2C, S6C, and S6D), immunostaining with male-specific anti-Sox100B antibodies (Figures 1I and 2D; Figures S2D and S3), male-specific *esg* expression (Figures 3A–3C and 3E–3G), or by using an X chromosome carrying *P{Dfd-lacZ-HZ2.7}* (Figures 1A–1C) (DeFalco et al., 2003). The X chromosomes transmitted from male parents were present only in female embryos. Staging of embryos was conducted according to Campos-Ortega and Hartenstein (1997). The sexes of the larvae were determined by using the size of their gonads.

Counting the Numbers of Fas3-Positive Cells and Germline Stem Cells

For counting the number of Fas3-positive cells, embryos, larval gonads, and adult testes were double stained with anti-Fas3 antibody and Toto-3 and were examined with confocal microscopy. The number of nuclei that were immediately surrounded by Fas3 signals was counted.

For counting the germline stem cells, adult testes were triple stained with anti-Vasa, anti-Fas3, and 1B1 antibodies. The germline stem cells were identified as the cells that were positive for Vasa, contained

a single spherical spectrosome marked by the 1B1 antibody, and associated with Fas3-positive hub cells (Yamashita et al., 2003).

Supplemental Data

Supplemental Data include results, experimental procedures, figures, and a table and are available at http://www.developmentalcell.com/cgi/content/full/13/1/151/DC1/.

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