Cell, Vol. 94, 251–260, July 24, 1998, Copyright ©1998 by Cell Press

decapentaplegic Is Essential for the Maintenance and Division of Germline Stem Cells in the *Drosophila* Ovary

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Summary

Stem cells are thought to occupy special local environments, or niches, established by neighboring cells that give them the capability for self-renewal. Each ovariole in the *Drosophila* ovary contains two germline stem cells surrounded by a group of differentiated somatic cells that express *hedgehog* and *wingless*. Here we show that the BMP2/4 homolog *decapentaplegic (dpp)* is specifically required to maintain female germline stem cells and promote their division. Overexpression of *dpp* blocks germline stem cell differentiation. Conversely, mutations in *dpp* or its receptor (*saxophone*) accelerate stem cell loss and retard stem cell division. We constructed mutant germline stem cell clones to show that the *dpp* signal is directly received by germline stem cells. Thus, *dpp* signaling helps define a niche that controls germline stem cell proliferation.

Introduction

In many adult tissues that undergo continuous cell turnover, a population of stem cells is responsible for replacing lost cells. Because of their pivotal role in controlling growth and neoplasia, the mechanisms regulating stem cell function are of great interest (reviewed by Potten and Loeffler, 1990; Doe and Spana, 1995; Lin, 1997; Morrison et al., 1997). Two mechanisms have been proposed to maintain stem cell divisions and to regulate the differentiation of stem cell daughters: intrinsic factors and extracellular signals. Asymmetrically localized intrinsic factors help specify the fates of neuroblast daughters in *Drosophila* embryos (Doe and Spana, 1995). Extracellular signals from surrounding cells mediated by cell surface–associated ligands and diffusible factors are frequently involved (Potten and Loeffler, 1990; Morrison et al., 1997). The identification of several of these factors has made it possible to culture some stem cells in vitro.

The *Drosophila* ovary presents an excellent system for studying two distinct groups of stem cells that remain active during much of adult life (reviewed in Spradling et al., 1997). The adult ovary contains 14–16 ovarioles, each with a germarium at the tip, within which the germline and somatic stem cells are located (Figure 1A). Two or three germline stem cells, located at the anterior tip of the germarium, divide asymmetrically to generate all germline cells in the ovariole (Wieschaus and Szabad, 1979; reviewed in Lin, 1997). Stem cell daughters, known as cystoblasts, undergo four rounds of synchronous division to produce groups of 2, 4, 8, and eventually 16 interconnected cystocytes, the precursors of ovarian follicles (reviewed in de Cuevas et al., 1997). Two somatic stem cells residing in the middle of the germarium give rise to all the somatic follicle cells (Margolis and Spradling, 1995). Three types of mitotically quiescent somatic cells are located in the vicinity of the stem cells: terminal filament and cap cells contact the germline stem cells, while inner sheath cells lie more posteriorly and contact both stem cell types (see Figure 1A).

Germline stem cell division is known to involve intrinsic mechanisms. This division, and subsequent cystocyte divisions, are physically unequal, owing to the segregation of fusomes rich in membrane skeleton proteins such as α-Spectrin, and an Adducin homolog encoded by *hu-li tai shao* (*hts*) (reviewed in de Cuevas et al., 1997; see Figure 1B). The round fusome (or "spectrosome") characteristic of stem cells changes shape as cyst development proceeds, allowing cysts at different stages to be identified. The *bag-of-marbles* (*bam*) gene is highly expressed only in the stem cell daughter (McKearin and Spradling, 1990). The loss of Bam protein in cystoblasts prevents their differentiation, causing germ line tumors to form. *pumilio* (*pum*) and *nanos* (*nos*), encoding translational regulators, also play critical roles in the formation and maintenance of germline stem cells (Lin and Spradling, 1997; Forbes and Lehmann, 1998).

Less is known about the intercellular signals that control stem cell proliferation. Two important signaling molecules, Hedgehog (Hh) and Wingless (Wg) (for review, see Perrimon, 1995; Cadigan and Nusse, 1997), are expressed in terminal filament and cap cells (Forbes et al., 1996a, 1996b). Hh signaling is critical for proliferation and differentiation of follicle cells, but it remains to be determined whether somatic stem cells or their daughters are regulated (Forbes et al., 1996a, 1996b). The role of these signals in the germ line is less clear. Ectopic expression of *hh* did not appear to interfere with the function of germline stem cells (Forbes et al., 1996a).

The transforming growth factor β (TGFβ) family, including TGFβs, activins, and the bone morphogenetic proteins (BMPs), elicits a broad range of cellular responses, including the regulation of cell division, survival, and specification of cell fates (Hogan, 1996; reviewed in Massagué, 1996). TGFβs can repress the proliferation of stem cells as assayed by either in vitro cultures or in vivo ectopic expression (Potten and Loeffler, 1990; Morrison et al., 1997). In *Drosophila*, *dpp* encoding a vertebrate BMP2/4 homolog functions as a local signal as well as a long-distance morphogen to pattern the early embryo and adult appendages by regulating cell proliferation and cell fate determination (Padgett et al., 1987; reviewed in Lawrence and Struhl, 1996). *dpp* is expressed in an anterior subset of follicle cells and is required for establishing egg shape and polarity during late stages of oogenesis (Twombly et al., 1996). Major participants in the Dpp signaling pathway have been identified. *saxophone* (*sax*) and *thick veins* (*tkv*) encode type I serine/threonine kinase transmembrane receptors, whereas *punt* encodes a type II serine/threonine kinase transmembrane receptor (Brummel et al.,

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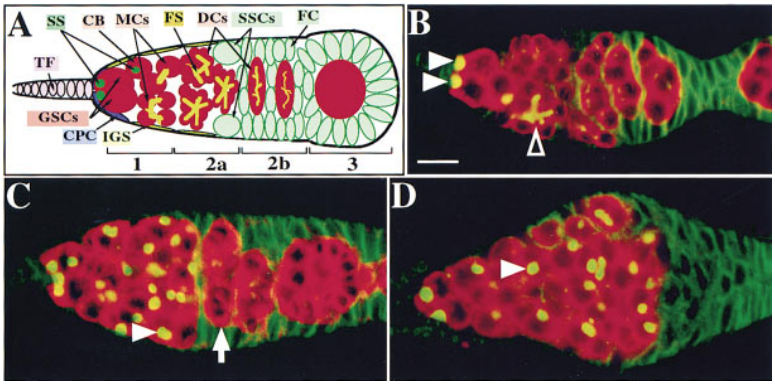

Figure 1. Overexpression of *dpp* in the Germarium Produces Germline Cell Tumors

An explanatory diagram (A) and a micrograph (B) of a wild-type germarium, after immunofluorescent labeling with anti-Vasa (red) and anti-Hts (green) antibodies. Two germline stem cells at the anterior contain spectrosomes (closed arrowheads), while developing cysts lying more posteriorly are connected by branched fusomes (open arrowhead). Tumorous germaria from *hs-GAL4*/*UAS-dpp* females subjected to 3 days (C) and 4 days (D) of heat shock treatments. Many single cells containing spectrosomes lie in the anterior half (see examples indicated by closed arrowhead in [C] and [D]). In (C), but not (D), some 16-cell cysts that had already initiated growth prior to heat shock remain in the posterior half (arrow). All germaria are oriented with the anterior toward the left. The scale bar in (B) represents 10 μm, and all panels are shown at the same scale. Abbreviations: TF, terminal filament; GSCs, germline stem cells; SS, spectrosomes; CPC, cap cells; IGS, inner germarial sheath cells; CB, cystoblast; MCs, mitotic cystocytes; FS, fusomes; DCs, developing 16-cell cycts; SSCs, somatic stem cells; FC, follicle cells.

1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). *mothers against dpp* (*mad*), *Medea* (*Med*), and *Daughters against dpp* (*Dad*) encode a family of conserved TGFβ transducers (Sekelsky et al., 1995; Tsuneizumi et al., 1997; Das et al., 1998; Hudson et al., 1998; Wisotzkey et al., 1998). A paradigm for TGFβ signal transduction has been developed from several experimental systems (Heldin et al., 1997). In *Drosophila*, Dpp binds both type I and II receptors to allow the constitutively active Punt kinase to phosphorylate and activate type I kinases, which phosphorylate Mad. The phosphorylated Mad brings Med into the nucleus as a transcriptional activator to stimulate *dpp* target gene expression.

Here we show *dpp* is essential to maintain female germline stem cells. Overexpression of *dpp* produces ovarian stem cell tumors. Clonal analysis demonstrates that downstream components of the *dpp* signaling pathway are required cell-autonomously in the germline stem cells for their division and maintenance. These results suggest that germline stem cells are regulated by a *dpp* signal that likely derives from surrounding somatic cells.

Results

Ectopic Dpp Expression Induces Germ Cell Tumors

To assess whether Dpp can regulate germline stem cells in the *Drosophila* adult ovary, we ectopically expressed Dpp in the germarium, using hsp70-GAL4 (*hs-GAL4*) and UAS-dpp (Brand and Perrimon, 1993). To distinguish different cell types in the germarium, we used anti-Hts and anti-Vasa antibodies to visualize somatic and germline cells, respectively. The anti-Hts antibody also recognizes spectrosomes and fusomes in the germline cells of the germarium (see de Cuevas et al., 1997). Only germline stem cells and cystoblasts have a big round spectrosome, while cysts have a characteristic branched fusome (Figure 1B).

The germaria from *hs-GAL4* females subjected to heat shock, and from females carrying *hs-GAL4* and *UAS-dpp* in the absence of a heat shock, were indistinguishable from wild type. In contrast, daily heat shock treatment for 3 days after eclosion produced tumorous germaria in the females carrying *hs-GAL4* and *UAS-dpp* (Figure 1C). Large germline cells filling germarial regions 1 and 2a contained spectrosomes but showed no evidence of cyst formation. In regions 2b and 3, 16-cell cysts were observed that probably derived from differentiated cystoblasts, or cysts that had formed before the first heat shock. Consistent with this interpretation, after 4–5 days of heat shocks, single germline cells filled the germarium and 16-cell cysts were rarely detected (Figure 1D). Newly formed cysts are known to require about 4 days to exit the germarium (see Spradling et al., 1997). This phenotype is very similar to that of *bam* and *benign gonial cell neoplasm* (*bgcn*) mutants (Gateff and Mechler, 1989; McKearin and Spradling, 1990). These results suggest that ectopic Dpp inhibits cystoblast differentiation but does not block cyst formation and maturation.

Dpp-Induced Tumor Cells Resemble Germline Stem Cells

Cystoblasts and early mitotic cysts can be distinguished from stem cells because they frequently express cytoplasmic Bam protein (Figure 2A). Staining with anti-cytoplasmic Bam antibodies revealed that the tumor cells had characteristics of germline stem cells (Figure 2B). Rare cells positive for Bam appeared to be growing cysts (Figure 2C). The lack of Bam expression was not caused by growth arrest, because some cells throughout the tumor incorporated the nucleotide analog BrdU (Figures 2D and 2E). These results show that the tumor cells induced by *dpp* overexpression continue to divide and resemble stem cells in their fusome morphology and absence of Bam protein.

To determine if the tumorous stem cells retain the capacity to differentiate, we examined their behavior after flies containing induced tumors were returned to room temperature. Germline cysts started to form 2 days after the downshift and always formed first in the most posterior region of the tumor (Figure 2F). Many 16-cell cysts were seen 4 days after the shift (Figure 2G). Based on their location and number, these cysts must derive from tumor cells, rather than from stem cell divisions that occur after the downshift. However, not all the tumor

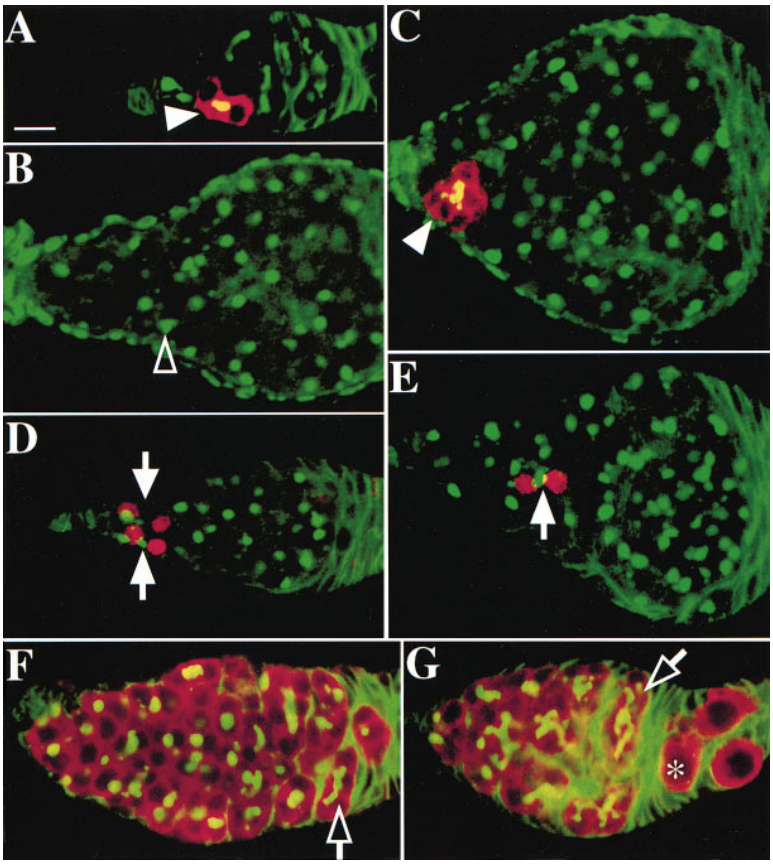

Figure 2. Germline Cells in the *dpp*-Induced Tumors Behave Like Stem Cells

(A) A wild-type germarium stained with anti-BamC (red) and anti-α-Spectrin (green) antibodies shows a single BamC-positive cyst (arrowhead), lying just posterior to the stem cells (indicated by spectrosomes). (B and C) Tumorous germaria induced by *dpp* overexpression stained as in (A) show many stem-like cells, as indicated by spectrosomes and absence of BamC (example, open arrowhead); only rarely are cysts observed (arrowhead in [C]). (D and E) Following incorporation of BrdU for 1 hr, tumorous germaria were stained with anti-BrdU (red) and anti-α-spectrin (green) antibodies. The arrows show mitotically active germline cells that have incorporated BrdU. (F and G) Germaria bearing *hs-GAL4/UAS-dpp* were subjected to 4 days of heat shock treatment, followed by 2 days (F) or 4 days (G) at room temperature prior to staining with anti-Hts (green) and anti-Vasa (red). The open arrows indicate cysts that have formed from tumor cells. The asterisk shows a germ cell derived from the tumor that did not form a 16-cell cyst. All pictures are shown at the same scale, and the bar in (A) represents 10 μm.

cells were able to form complete cysts, since some ovarioles contained cysts with 1, 2, 4, or 8 cells in region 3 (Figure 2G, asterisk).

Overexpressed *dpp* Acts Directly on Germline Stem Cells

Two different models could explain the Dpp effect on germline stem cells: direct signaling to the germline stem cells and relay signaling. The relay signaling model predicts that ectopic Dpp turns on a secondary signal in the somatic cells surrounding germ line stem cells. To test the relay model directly, we took advantage of the hs-GAL4/UAS system and activated type I Dpp receptors. The hs-GAL4/UAS system can express a target gene at high levels in somatic cells of the adult ovary but not in germline cells (Manseau et al., 1997). Both activated *tkv* (*tkv**) (Nellen et al., 1996) and activated *sax* (*sax**) (Das et al., 1998) have been shown to mimic Dpp signaling pathway activation in many developmental processes. When activated *sax** or *tkv**, or both, were overexpressed in the somatic cells of the germarium, using *hs-GAL4*, the same driver for *dpp* overexpression, no germline tumor phenotypes were observed. Egg chamber budding was frequently affected, suggesting that somatic follicle cell function was altered at a later stage (Figures 3A-3C). These results suggest that relay signaling, if it exists, is not sufficient to inhibit germline stem cell differentiation. Since overexpressed Dpp does not appear to act by a relay signal, it likely acts directly on germline cells to inhibit cystoblast differentiation.

dpp and *sax* Are Required for Germline Stem Cell Division and Maintenance

To test the role of *dpp* directly, we examined mutations that reduce its function and that of its receptor, *sax*. *dpp* signaling is essential at many points during *Drosophila* development. Several temperature-sensitive allelic combinations of *dpp* mutants, including dpp^{e90}/dpp^{hr56} and dpp^{hr4}/dpp^{hr56}, can develop to the adult at 18°C (Wharton et al., 1996). These heteroallelic combinations allowed us to examine the mutant phenotypes of *dpp* in the germarium after the shift to 28°C. Of germaria from these genotypes, examined 1 week after the temperature shift, 40%–50% were significantly smaller than heterozygotes, and more severe reductions were seen in older females maintained at the higher temperature. To determine if stem cells were being lost, we stained ovaries from the mutant females with anti-Hts and anti-Vasa antibodies and directly counted the number of stem cells in each ovariole (Table 1). These experiments showed that there was a dramatic reduction in stem cell number in both tested genotypes over a 2-week period. The stronger of the two, dpp^{hr4}/dpp^{hr56}, almost completely eliminated stem cells within 2 weeks. This combination produces significantly fewer adult flies and is known to disrupt embryonic development more severely (Wharton et al., 1996).

If the mutations act specifically on germline stem cells, cystoblasts and cysts should continue to divide and develop. To examine this, we analyzed the morphology of the fusomes in the mutant ovarioles (Figure 4). We

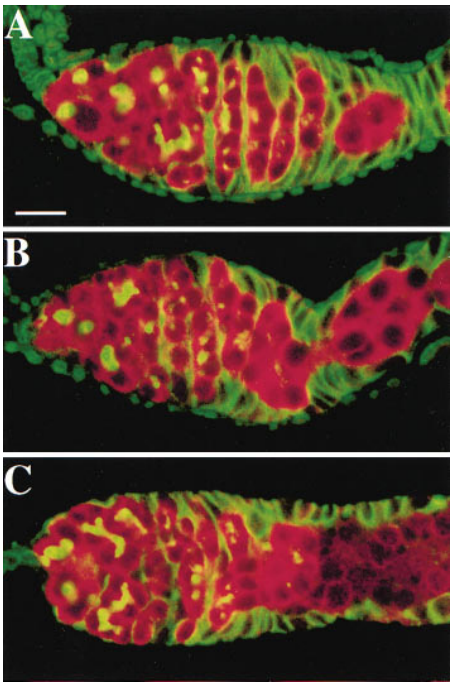

Figure 3. Overexpression of Activated *dpp* Type I Receptors in the Somatic Cells of the Germarium Does Not Mimic the Effect of Ectopic *dpp* Expression

Flies of the indicated genotype were subjected to heat shock treatment for 3 days, and germaria were subsequently labeled with anti-Vasa (red) and anti-Hts (green) antibodies. (A) *hsGAL4/UAS-sax**; (B) *hsGAL4/UAS-tkv**; (C) *UAS-sax*/+;hsGAL4/UAS-tkv**. Two independent lines containing the *UAS-tkv** and *UAS-sax** insertions at different chromosomal sites were tested. None of these combinations produce germline stem cell tumors. The region 3 cysts in (B) and (C) are not budding normally, possibly due to follicle cell defects. The bar represents 10 μm.

also studied ovarioles from the Dpp receptor mutant sax^{P}, which has a weaker effect on stem cell number (data not shown). The effects of stem cell loss are expected to vary among individual germaria since stem cell loss is a random process (Margolis and Spradling, 1995). Germaria from 1-week-old females heterozygous for the *dpp* or *sax* alleles generally contained two stem cells at the anterior (Figures 4A and 4E, open arrows). sax^{P} germaria from 1-week-old females were smaller than wild type and contained either two (Figure 4B) or one (Figure 4C) stem cell, indicating that stem cells were being lost and their division slowed. Many germaria in 2-week-old sax^{P} females had lost both stem cells (Figure 4D, bracket), although cysts and egg chambers at later developmental stages remained. dpp^{e90}/dpp^{hr56} females showed a more rapid loss of stem cells at 28°C (Figures 4F–4H). dpp^{e90}/dpp^{hr56} germaria frequently contained one (Figure 4F) or zero (Figure 4G) stem cells after 1 week. After 2 weeks, most ovarioles lacked stem cells entirely, but some still contained 16-cell cysts or older follicles (Figure 4H).

Because normal cystocyte development continued throughout the germarium, the effects of these mutations appeared to be limited largely to stem cell division and maintenance. Some abnormalities in the later process of egg chamber budding were observed (Figure 4F). Stem cell loss might be caused by either death, developmental arrest, or differentiation. No apoptotic cells were observed in the most anterior region of these germaria, based on DAPI staining, and one or two large germline cells never remained at their anterior tips. These results indicate that a reduction in the level of *dpp* signaling promotes the differentiation of germline stem cells into cysts and thus causes stem cell loss. Consistent with previous studies (Twombly et al., 1996), we observed some partially ventralized eggs with anterior defects in these *dpp* mutants and the sax^{P} mutant (data not shown).

Table 1. *dpp* Is Required for Germline Stem Cell Maintenance

	One Week			Two Weeks		
Genotypes	No GSC	One GSC	Two GSC	No GSC	One GSC	Two GSC
$dpp^{e90}/CyOP23$	0.0% (0)	4.4% (5)	95.6% (108)	0.5% (1)	17.5% (36)	82.0% (168)
$dpp^{hr4}/CyOP23$	0.0% (0)	3.5% (6)	96.5% (165)	1.5% (3)	23.9% (48)	74.6% (150)
dpp^{e90}/dpp^{hr56}	16.0% (17)	29.3% (31)	54.7% (58)	47.3% (140)	39.8% (118)	12.9% (38)
dpp^{hr4}/dpp^{hr56}	18.1% (22)	33.9% (41)	48.0% (58)	98.4% (122)	1.6% (2)	0.0% (0)

The percentage of ovarioles with zero, one, or two germline stem cells is given for each genotype. Actual numbers are given in parentheses.

[a] P23 is a *dpp* transgene on the CyO chromosome (Hursh et al., 1993).

punt, tkv, mad, Med, and *Dad* Are Required Cell-Autonomously for Germline Stem Cell Maintenance

To demonstrate definitively that *dpp* signaling was received by the germ line, we investigated whether components of the signal transduction pathway are autonomously required in these cells. We used Flp-induced mitotic recombination to generate marked clones homozygous for loss-of-function mutations in the germline stem cells of adult ovaries (see Experimental Procedures). Clones were marked using *armadillo-lacZ*, which is strongly expressed in all cells within the germarium (Figure 5A). Stem cell clones can be recognized because only stem cells persist in the germarium more than 5 days after a mitotic recombination event (Margolis and Spradling, 1995; Figure 5B). As recombination events can take place only in mitotically active adult cells, this method will not produce mutant clones in the terminally differentiated terminal filament, cap cells, and/or inner sheath cells. Consequently, this approach excludes potential complications due to mutant clones in these surrounding somatic cells, allowing the autonomous function of genes to be tested in germline stem cells. This method has three major additional advantages. First, the persistent mutant clones can be studied over a long period of time, allowing stem cell maintenance to be quantified. Second, the existence of both a mutant and a wild-type stem cell side by side in the same germarium provides a natural control for the effects of gene removal. Thus, the relative division rates of these two stem cells can be determined simply by counting the number

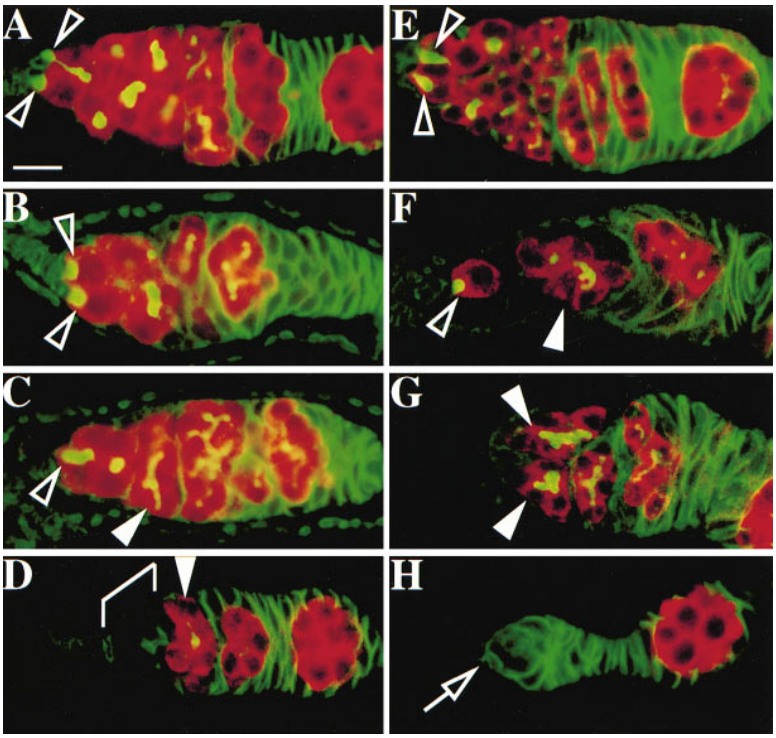

Figure 4. Loss and Differentiation of Germline Stem Cells in *dpp* and *sax* Mutants

Control germaria from a 1-week-old $sax^{P}/+$ (A) and $dpp^{e90}/CyOP23$ (E) females and mutant germaria (B-H) are shown after double labeling with anti-Vasa (red) and anti-Hts (green) antibodies. The stem cells are indicated by open arrowheads. (B) A 1-week old sax^{P}/sax^{P} germarium, two stem cells are present but the number of cysts is reduced. (C) A 1-week old sax^{P}/sax^{P} germarium. Only one stem cell remains and regions 1 and 2a are much reduced, as indicated by the start of region 2b (filled arrowhead). (D) A 2-week-old sax^{P}/sax^{P} germarium. No stem cells or mitotic cysts are present (bracket), and most anterior cyst corresponds to region 2b. (F) A 1-week-old dpp^{e90}/dpp^{hr56} germarium. Only one stem cell (open arrowhead) and no mitotic cysts are found; the most anterior cyst contains 16 cells (filled arrowhead). (G) A 1-week old dpp^{e90}/dpp^{hr56} germarium. No stem cells are present; an 8-cell cyst (lower filled arrowhead) and a 16-cell cyst (upper filled arrowhead) lie at the anterior. (H) A 2-week-old dpp^{e90}/dpp^{hr56} germarium. No germline cells remain in the germarium (arrow). (G) is a projection of several confocal sections, and the rest are single section images. All images are at the same scale, and the bar represents 10 μm.

of mutant and wild-type cysts in germaria with one mutant and one wild-type stem cell. Finally, stem cell–specific effects of the mutations can be assessed by looking at the developmental states of marked cystoblasts, cysts, and egg chambers.

Germline stem cell clones of $punt^{-}$, tkv^{-}, mad^{-}, Med^{-}, and Dad^{-} were generated by subjecting females of the appropriate genotype to heat shock and examining their ovaries beginning 1 week later. Stem cells in the *Drosophila* ovary have a finite life span with a half-life of 4.6 weeks (Margolis and Spradling, 1995; Table 2). In contrast to wild-type clones, stem cells mutant for each of the tested genes (except *Dad*) were lost more rapidly (Table 2). For example, after 1 week, the $punt^{135}$ mutant stem cell was either still present (Figure 5C) or had only recently been lost, as indicated by the presence of relatively young mutant cysts (Figure 5D). However, after 2 weeks, the $punt^{135}$ mutant stem cell had usually been lost, and only a few advanced mutant cysts remained (Figure 5E). mad^{12} mutant stem cells were lost even more rapidly. After 1 week, the mad^{12} mutant stem cell sometimes remained but did not proliferate well, as indicated by the lack of progeny cysts (Figure 5F). More frequently, the stem cell was already lost, and a more developed cyst (or cysts) was observed (Figure 5G). After 2 weeks, mad^{12} mutant stem cells almost never remained (Figure 5H). Surprisingly, we sometimes observed two wild-type stem cells after the mutant stem cell was lost (see Figures 5E and 5G). These results indicate that the *dpp* signal directly acts on germline stem cells to regulate their maintenance. However, no effects were observed on the formation of 16-cell cysts or the subsequent development of germline cells.

Unlike the other tested genes, *Dad* is a negative regulator of *dpp* signaling. The *Dad* gene is induced by the *dpp* pathway and antagonizes the function of *dpp* (Tsuneizumi et al., 1997). The $Dad^{271\text{-}68}$ allele is a severe allele in which the entire C-terminal conserved domain is deleted (Tsuneizumi et al., 1997). Strikingly, stem cells mutant for $Dad^{271\text{-}68}$ were not lost (Figure 5I), even if both stem cells lacked this gene (Figure 5J). No turnover could be detected even 3 weeks after clone induction, suggesting that increasing *dpp* signaling can prolong germline stem cell lifetime.

To compare the magnitude of the effects of different mutations on stem cells, we measured the half-life of the mutant germline stem cells (Table 2; see Experimental Procedures). $punt^{10460}$ is a hypomorphic allele of the *dpp* type II receptor, whereas $punt^{135}$ is a strong allele (Arora et al., 1995; Letsou et al., 1995). In $punt^{10460}$ clones, stem cell half-life was reduced from 4.6 to 0.90 weeks, whereas the stronger $punt^{135}$ allele reduced stem cell half-life to 0.41 weeks. tkv^{8} is a strong allele of the type I receptor (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994). tkv^{8} stem cell clones reduced stem cell half-life to 0.69 weeks. Clones of two alleles of the downstream signal transducer, mad^{9} and mad^{12}, reduced stem cell half-life to 2.5 weeks and 0.25 weeks, respectively. Consistent with this observation, mad^{12} is a much stronger allele than mad^{9} (Sekelsky et al., 1995). Med^{26} is a strong allele of another downstream transducer (Das et al., 1998). Med^{26} stem cells turned over with a half-life of 0.38 weeks.

punt, tkv, mad, and *Med* Are Required Cell-Autonomously to Stimulate Germline Stem Cell Division

To further define the role of the *dpp* pathway in the regulation of germline stem cell division, we compared

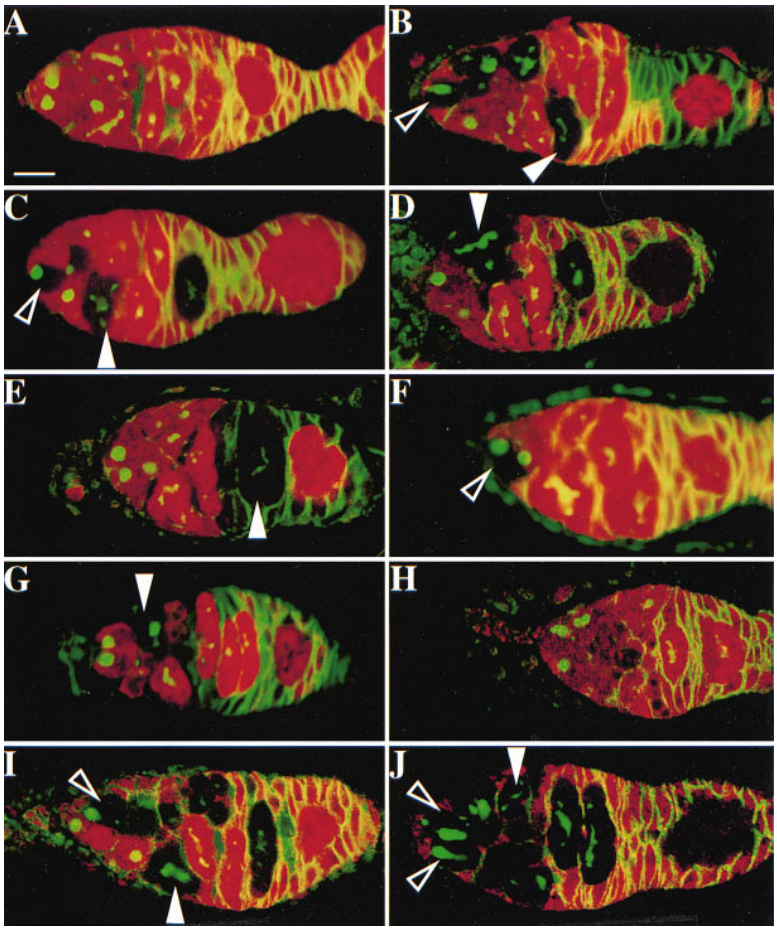

Figure 5. Genes Downstream of *dpp* Are Required in the Germline Stem Cells for Division and Maintenance

Germaria lacking or bearing stem cell clones of the indicated genotypes were generated (Experimental Procedures) and labeled with anti-LacZ (red) and anti-Hts (green) antibodies. Marked stem cells (open arrowheads) and their progeny cysts (some indicated by filled arrowheads) are recognized by the absence of lacZ. (A) Wild type, without heat shock. (B) Wild type, 1 week after heat shock (AHS), showing both a germline and a somatic stem cell clone. (C) A *punt*^{135} germarium 1 week AHS, showing a mutant stem cell and three progeny cysts (one indicated by filled arrowhead). (D) A *punt*^{135} germarium 1 week AHS, a mutant 16-cell cyst (filled arrowhead), and two older cysts remain from the mutant stem cell clone. (E) A *punt*^{135} germarium 2 weeks AHS, only a single mutant region 2b cyst (filled arrowhead) remains from the mutant stem cell clone. (F) A *mad*^{12} germarium 1 week AHS, a mutant stem cell but no progeny cysts are visible (note that the posterior round fusome is within a wild-type cystoblast). (G) A *mad*^{12} germarium 1 week AHS, a mutant two-cell cyst (filled arrowhead) remains from the mutant stem cell clone. (H) A *mad* ^{12} germarium 2 weeks AHS, no mutant cysts remain in the germarium from the mutant stem cell clone (but older mutant egg chamber was present). (I) A *Dad*$^{271–68}$ germarium 1 week AHS, a mutant stem cell, and its progeny cysts are indicated. (J) A *Dad*$^{271–68}$ germarium 1 week AHS, two mutant stem cells, and a normal complement of progeny cysts are present. All the images are at the same scale, and the bar represents 10 μm. Note that in (E) and (G), two wild stem cells are observed.

the number of mutant and wild-type cysts in germaria carrying one mutant and one wild-type germline stem cell. Since each cyst represents one stem cell division, counting the number of wild-type and mutant cysts allowed us to measure relative stem cell division rates. All germaria that still retained a mutant stem cell from all three time points were counted and compared to the number of wild-type cysts (see Experimental Procedures). In controls containing a marked but genetically wild-type stem cell, approximately 50% of cysts were marked, indicating that two germline stem cells are present in 1-week-old adult germaria and divide at similar rates (see Table 2).

As expected based on our previous experiments, *punt*$^{-}$, *tkv*$^{-}$, *mad*$^{-}$, and *Med*$^{-}$ germline stem cells all divided more slowly than wild type (see Table 2 and Figures 5C and 5F). While the relative division rate of marked wild-type stem cells was 0.93, the rates in the tested genotypes ranged from 0.21 to 0.60. The reductions mostly correlated with the known strength of these

Table 2. Downstream Components of the *dpp* Pathway Are Required in Germline Stem Cells for Their Maintenance and Division

Strains	Percent of Germaria[a] with a Marked GSC			GSC[b] Half-Life (Weeks)	Relative[c] Division Rate
	1 Week	2 Weeks	3 Weeks		
Control	37.7 (138)	34.4 (161)	27.5 (160)	4.6	0.93 (1410)
punt^{10460}	43.2 (118)	26.4 (182)	9.5 (116)	0.90	0.36 (1126)
punt^{135}	27.4 (95)	5.1 (138)	0 (114)	0.41	0.37 (329)
tkv^{8}	38.6 (132)	16.4 (176)	6.1 (197)	0.69	0.29 (744)
mad^{9}	43.6 (94)	29.3 (208)	25.8 (155)	2.5	0.60 (1116)
mad^{12}	17.8 (124)	0 (108)	0.7 (136)	0.25	0.21 (214)
Med^{26}	23.8 (172)	7.3 (110)	0 (122)	0.38	0.39 (512)
Dad$^{271-68}$	28.0 (107)	32.6 (86)	32.3 (62)	>>4.6	0.84 (770)

[a] Number of germaria with *lacZ*-negative germline stem cell clone/total germaria × 100. The actual number of germaria counted is given in parentheses.
[b] Calculated as described in Experimental Procedures.
[c] Calculated as described in Experimental Procedures. The number of cysts counted is given in parentheses.

alleles and with their effects on stem cell maintenance. However, both *punt*^{10460} and *punt*^{135} mutant stem cells proliferated about 3-fold slower than the wild type, despite the fact that they differ in strength. Differences between the effects of these mutants on maintenance and division may reflect branchpoints in the pathway and suggest that at least one additional type II receptor also mediates stem cell behavior. Interestingly, *Dad*$^{271\text{-}68}$ mutant germline stem cells, which were more stable than wild type, divided at a similar or slightly slower rate than wild-type ones (Figure 5L). These results demonstrate that components of the *dpp* signaling pathway are required autonomously for the proliferation of germline stem cells.

As shown previously, cysts produced in the presence of overexpressed *dpp* driven by *hs-GAL4* always contained 16 cells. To verify that *dpp* signaling is not involved in regulating the cystoblast and cystocyte divisions, we counted the number of germ line cells in individual cysts mutant for *punt*^{10460}, *mad*^{9}, *mad*^{12}, *Med*^{26}, and *Dad*$^{271\text{-}68}$. In every case, these cysts contained 16 cells, including a single oocyte (data not shown). Therefore, the *dpp* signaling pathway specifically acts on stem cells within the germ line.

Discussion

Stem cells are thought to be regulated by positive and negative diffusible factors, but the function of most of these factors has never been demonstrated in vivo. Our results indicate that Dpp directly signals to maintain *Drosophila* germline stem cells and stimulate their division. These experiments were made possible by a clonal cell marking method that allows the function of stem cells and their progeny to be examined directly over many cell generations. In addition to the *dpp* signal, known components in the *dpp* signaling pathway were shown to be required in these adult stem cells. This action appears to be specific to stem cells, since clones lacking *dpp* pathway components were still able to form 16-cell cysts. Thus, our results clearly demonstrate that a TGFβ-like molecule functions as a stem cell growth factor in vivo.

dpp Signaling Maintains Germline Stem Cells and Controls Their Division

dpp signaling is required for maintaining germline stem cells, on which it may act in at least two distinct ways. First, signaling prevents stem cells from differentiating into cystoblasts. We show that overexpressed *dpp* prevents stem cell differentiation, while reduction of *dpp* function promotes stem cell differentiation. An attractive candidate target of this pathway is the Bam protein, which is normally synthesized at much higher levels in cystoblasts than in stem cells (McKearin and Ohlstein, 1995). The forced expression of Bam in germline stem cells causes them to differentiate in a manner very similar to that caused by reductions in *dpp* signaling (Ohlstein and McKearin, 1997). Thus, *dpp* signaling may negatively regulate Bam protein levels in germline stem cells. Two other genes, *pum* and *nos*, are required to form and maintain germline stem cells (Lin and Spradling, 1997; Forbes and Lehmann, 1998). In the embryo, both proteins work together to repress the translation of target genes such as *hunchback (hb)* (Baker et al., 1992; Murata and Wharton, 1995). In the ovary, *dpp* signaling may down-regulate Bam through effects on the Nos/Pum pathway or by an independent mechanism. However, genes throughout the *dpp* pathway are required, including two nuclear transcription factors, suggesting that the action of the pathway is on target gene transcription.

In addition, *dpp* may function to maintain a specialized association between the stem cells and basal terminal filament cells. Such an association has been postulated to hold the stem cells at the anterior of the germarium, while daughter germline cells all move posteriorly and eventually leave the germarium. Our results indicate that the stem cell loss is due to differentiation. Possibly, the *dpp* signal regulates the expression of adhesion molecules that reside on the cell surface, or of cytoplasmic proteins that indirectly promote stem cell adhesion.

dpp signaling also acts to stimulate stem cell division. *dpp* signaling is already known to stimulate cell proliferation at several points during *Drosophila* development. In the wing imaginal disc, it is essential for cell proliferation and/or survival (Burke and Basler, 1996), whereas it promotes the G2-M transition in the morphogenetic furrow of the developing eye disc (Penton et al., 1997). Consistent with such a requirement, *mad* mutants have greatly reduced imaginal discs, shortened gastric caeca, and small brains (Sekelsky et al., 1995). The requirement for *dpp* signaling that we observe suggests that adult stem cells use strategies similar to those of embryonic and larval somatic cells to regulate proliferation.

dpp Signaling and Stem Cell Aging

During aging, the number and activity of stem cells is thought to be reduced. Our results indicate that the level of *dpp* signaling controls the life span and division rate of germline stem cells. Reduced *dpp* signaling caused premature stem cell loss. Perhaps more surprising was the observation that putative increases in signaling, caused by removal of *Dad* activity from stem cells, caused them to be maintained longer. This finding suggests that *dpp* signaling not only is necessary, but may sometimes be rate limiting for stem cell maintenance. This is the first example where stem cell life span has been extended in an intact organism. These results suggest that it may be possible to extend the life span of stem cells, a process that could be of therapeutic significance.

Does *dpp* Signaling Control Germline Stem Cells in Males?

TGFβ signaling has been shown to limit the growth of germline cysts during *Drosophila* spermatogenesis (Matunis et al., 1997). When *punt* or *schnurri (shn)* function is removed in clones of somatic cells that surround germ cells, cysts continue dividing after four rounds of mitosis (Matunis et al., 1997). However, these authors did not address whether this pathway functions in male germline stem cells. In the embryo and imaginal discs, *punt*

and *shn* can function downstream of *dpp* (Arora et al., 1995; Grieder et al., 1995; Letsou et al., 1995; Ruberte et al., 1995). It is currently uncertain whether *dpp* or another TGFβ family member is utilized to send the signal. Clonal analysis of mutants in *dpp* downstream components in male germline stem cells, like those reported here in the ovary, will be required to determine whether Dpp and/or other TGFβ-like molecules are required for their division and maintenance in testes.

In mouse, the BMP family members BMP2 and −4 are most closely related to *dpp*, with greater than 75% identity, and they can function to rescue *dpp* mutants in embryos (Padgett et al., 1993). Recently, both genes have been knocked out, but the homozygous embryos die too early to assess their possible function in gonads. The roles during spermatogenesis of two other BMP family members, BMP8A and BMP8B, have been tested (Zhao et al., 1996, 1998). BMP8B is required for the resumption of male germline cell proliferation in early puberty and for germline cell survival in the adult, whereas BMP8A plays a role in the maintenance of adult spermatogenesis. Future work is needed to address whether BMP8B acts directly on germline stem cells through an autocrine or paracrine pathway.

Wg and Hh May Cooperate with Dpp to Define a Germline Stem Cell Niche

The "niche" hypothesis postulates that stem cells reside in optimal microenvironments or niches (Schofield, 1978). When a stem cell divides, only one daughter can remain in the niche, while the other becomes committed to differentiate. A stem cell within the niche would have a high probability of self-renewal but a low probability of entry into the differentiation pathway. This model is consistent with the observations that stem cells require the addition of growth factors for proliferation and differentiation in many in vitro culture systems (Potten and Loeffler, 1990; Morrison et al., 1997). The molecular nature of the microenvironment within a niche has yet to be defined in any system; however, the *Drosophila* germarium appears to contain such a niche (Figure 1A). Anteriorly, the stem cells abut terminal filament and cap cells, which both express *hh*, while only the latter express *wg* and *armadillo* (Forbes et al., 1996a, 1996b). Stem cell daughters lie more to the posterior and probably directly contact inner germarial sheath cells, which express *hh* and *patched* (Forbes et al., 1996b). This asymmetry in structure and signals may allow germline stem cells to receive different levels of signals from their daughters. Consistent with the existence of a niche, we sometimes observed two wild-type stem cells in germaria that had recently lost a marked mutant stem cell, suggesting that a vacated niche could be reoccupied.

The existence of the germline stem cell niche is also consistent with stem cell proliferation when *dpp* is overexpressed. Under these conditions, the size of the niche may be substantially enlarged. Conversely, reduction of *dpp* function may weaken the ability of the niche to maintain germline stem cells, leading to accelerated losses. These results suggest that *dpp* is an essential niche signal. However, *dpp* likely interacts with other signals from surrounding somatic cells to make a functional niche for germline stem cells. Nonetheless, the identification of *dpp* as a key niche signal should greatly facilitate efforts to culture *Drosophila* germline stem cells in vitro.

Technical limitations have so far prevented us from identifying the source of the *dpp* signal that is received by germline stem cells. Ideally, analysis of clones of a null *dpp* allele would reveal which cells signal the germ line. However, the somatic cells adjacent to the stem cells cease division early in ovary development, making the induction of specific small clones difficult. The pattern of *dpp* expression in the germarium should also provide some insight into the origins of the signal. However, the only available *dpp-lacZ* fusion line and whole mount in situ experiments failed to detect expression in the germarium, although follicle cell expression in late stage egg chambers was observed, as reported previously (T. X. and A. C. S., unpublished; Twombly et al., 1996). In many systems, low levels of *dpp* expresssion are known to be sufficient for biological effects, so it may be that only low levels are present in the germarium.

In the *Drosophila* leg, antenna, and genital discs, *dpp* and *wg* are induced in the anterior compartment by *hh*, and the mutual repression of *dpp* and *wg* restricts them to their appropriate domains (Brook and Cohen, 1996; Chen and Baker, 1996; Jiang and Struhl, 1996). In vertebrate limb development, *sonic hedgehog* (*shh*) can induce the expression of BMP2 (Johnson and Tabin, 1995). The somatic terminal filament, cap, and inner sheath cells express *hh* and lie adjacent to the germline stem cells (Forbes et al., 1996a, 1996b). *wg* and *dpp* expression may be induced by *hh* and signal to germline stem cells for their proliferation and maintenance. We propose that these and possibly additional signals from the anterior somatic cells define a niche for germline stem cells at the tip of germarium.

Experimental Procedures

Drosophila Stocks and Genetics

The following fly stocks used in this study were described either in FlyBase or as otherwise specified: *tkv*^{8}; *punt*^{10460} and *punt*^{135}; *mad*^{9} and *mad*^{12}; *Med*^{26} (Das et al., 1998); *Dad1*$^{271\text{-}68}$; *sax*^{P}; *dpp*^{hr56}, *dpp*^{hr4}, and *dpp*^{e90}; *UAS-dpp*; *hs-GAL4*; HSFlp; FRT40A *armadillo-lacZ* and HSFLP;FRT82B *armadillo-lacZ* (Lecuit and Cohen, 1997); *UAS-tkv** (activated) and *UAS-sax** (activated) on both chromosomes 2 and 3 (Das et al., 1998). Most stocks were cultured at room temperature. To maximize their effects, *sax*^{P} and *dpp* mutants were cultured at 28°C for 1–2 weeks.

Generating Mutant Germline Stem Cell Clones and Overexpression

Clones of mutant cells were generated by Flp-mediated mitotic recombination, as described previously (Xu and Rubin, 1993). To generate the stocks for stem cell clonal analysis, + FRT40A/CyO, *tkv*^{8}FRT40A/CyO, *mad*^{9}FRT40A/CyO, and *mad*^{12}FRT40A/CyO males mated with virgin females w HSFlp1; *armadillo-lacZ* FRT40A, respectively. FRT82B *Med*^{26}/TM3 Sb, FRT82B *punt*^{135}/TM3 Sb, FRT82B *punt*^{10460}/TM3 Sb, FRT82B *Dad*$^{271\text{-}68}$/TM3 Sb males mated with virgin females w HSFlp1;FRT82B *armadillo-lacZ*, respectively. Two-day-old adult non-CyO or non-Sb females carrying an *armadillo-lacZ* transgene in *trans* to the mutant-bearing chromosome were heat-shocked twice at 37°C for 60 min separated by 8–12 hr. The females were transferred to fresh food every day at room temperature, and

ovaries were removed 1 week, 2 weeks, or 3 weeks after the last heat shock treatment and processed for antibody staining.

To construct the stocks for overexpressing *dpp* and activated receptors, the *hs-GAL4* virgins were crossed with *UAS-dpp, UAS-tkv**/CyO, *UAS-tkv**/TM3 Sb, *UAS-sax**/CyO, *UAS-sax**/TM3 Sb, *UAS-tkv**/CyO; *UAS-sax**/TM6 *UAS-sax**/CyO; *UAS-tkv**/TM6 males, respectively. The females that did not carry balancer chromosomes were heat-shocked at 37°C for 30 min, each time with the interval of 12 hr for 3–5 days.

Calculations

To determine stem cell life spans, stem cells were marked in 1- to 2-day-old females of the appropriate genotype. Subsequently, ovaries were dissected from some of the females 1, 2, and 3 weeks later and stained with anti-Hts and anti-lacZ antibodies. The percentage of germaria containing a marked stem cell was determined by counts of 60–200 germaria at each time point and used to calculate the stem cell half-life.

To measure stem cell division rates, we determined the relative number of wild-type and mutant cysts in germaria that contained one wild-type and one mutant stem cell. A relative division rate of 1.0 would indicate normal division. For a given genotype, these values were similar at each time point, and the average is presented in Table 2. Marked wild-type stem cells gave a value of 0.93 rather than 1.0, probably due to a small fraction of germaria that contained three rather than two germline stem cells.

To measure stem cell loss, germaria with two, one, or no germ line stem cells were counted from the ovaries of the 1- and 2-week-old females. Heterozygous females carrying one copy of the mutant gene in combination with a CyO balancer chromosome containing a *dpp* transgene (Hursh et al., 1993) served as a control. Values are expressed as the percentage of ovarioles with the indicated stem cell compositions.

Immunohistochemistry

The following antisera were used: polyclonal anti-Vasa antibody (1:2000) (Liang et al., 1994); monoclonal anti-Hts antibody 1B1 (1:5) (Zacci and Lipshitz, 1996); polyclonal anti-α-Spectrin antibody (1:100) (Byers et al., 1987); rat anti-Bam antibody (1:100) (McKearin and Ohlstein, 1995); monoclonal anti-BrdU antibody (1:50) (Becton-Dickinson); polyclonal anti-β-galactosidase antibody (1:1000) (Cappel). Labeling with BrdU was carried out for 1 hr at room temperature, as described by de Cuevas and Spradling (1998). All micrographs were taken using a Leica TCS-NT confocal microscope.

Acknowledgments

We would like to give our special thanks to P. Das, S. Cho, and R. W. Padgett for providing FRT-*mad*^{9}, FRT-*mad*^{12}, FRT-*Med*^{26}, *UAS-sax**, and *UAS-tkv** prior to publication. We also thank S. Cohen, R. Dubreuil, F. M. Hoffmann, P. Lasko, H. Lipshitz, D. McKearin, R. W. Padgett, P. Rørth, and T. Tabata for providing fly stocks and antibodies. C. M. Fan, P. Newmark, and the members of the Spradling laboratory generously provided comments on the manuscript. A. C. S. is an Investigator and T. X. is an Associate of the Howard Hughes Medical Institute.

Received April 24, 1998; revised June 5, 1998.

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