Germ line stem cell differentiation in *Drosophila* requires gap junctions and proceeds via an intermediate state

Lilach Gilboa[1], Alexandria Forbes*, Salli I. Tazuke[2,3,†], Margaret T. Fuller[2,4] and Ruth Lehmann[1,‡]

[1]Developmental Genetics Program, Skirball Institute at NYU School of Medicine and Howard Hughes Medical Institute, 540 First Avenue, New York, NY 10016, USA
[2]Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305-5329, USA
[3]Department of Gynecology and Obstetrics, Stanford University School of Medicine, Stanford, CA 94305-5329, USA
[4]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305-5329, USA

*Present address: Galleon Hedge Fund, New York, NY 10022, USA
†Present address: Center for Research on Reproduction and Women's Health, University of Pennsylvania School of Medicine, 1355 BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6142, USA
‡Author for correspondence (e-mail: lehmann@saturn.med.nyu.edu)

Accepted 10 September 2003

Development 130, 6625-6634
Published by The Company of Biologists 2003
doi:10.1242/dev.00853

Summary

Gap junctions coordinate processes ranging from muscle contraction to ovarian follicle development. Here we show that the gap junction protein Zero population growth (Zpg) is required for germ cell differentiation in the *Drosophila* ovary. In the absence of Zpg the stem cell daughter destined to differentiate dies. The *zpg* phenotype is novel, and we used this phenotype to genetically dissect the process of stem cell maintenance and differentiation. Our findings suggest that germ line stem cells differentiate upon losing contact with their niche, that gap junction mediated cell-cell interactions are required for germ cell differentiation, and that in *Drosophila* germ line stem cell differentiation to a cystoblast is gradual.

Key words: *zpg*, *Drosophila*, Stem cell, Germ line, Gap junction

Introduction

Stem cells can self-renew and generate differentiated progeny. Both of these aspects must be tightly regulated for the correct development and maintenance of many tissues. Control of self-renewal or differentiation could be intrinsic, such as by unequal distribution of determinants, or extrinsic, by signals emanating from surrounding cells, termed 'the niche' (Watt and Hogan, 2000). Communication between stem cells and their niche have been shown to require secretion of extracellular matrix signaling molecules, and interaction via membrane proteins (Spradling et al., 2001; Watt and Hogan, 2000). Here we show that a gap junction protein is required for differentiation of female germ-line stem cells (GSCs) in the fruit fly *Drosophila melanogaster*.

The *Drosophila* ovary is composed of 16-18 units called ovarioles, containing a germarium at the anterior and a chain of developing egg chambers that extend posteriorly. GSCs reside in the anterior of the germarium and closely abut the somatic terminal filament, cap and inner-sheath cells (Fig. 1A), which comprise the GSC niche (Lin, 1998; Spradling, 1993; Xie and Spradling, 2000a). Several extrinsic factors, Decapentaplegic (Dpp), Piwi, Fs(1)Yb and Hedgehog, control GSC or somatic stem cell maintenance and are expressed in different subsets of niche cells (Cox et al., 1998; Cox et al., 2000; Forbes et al., 1996; King and Lin, 1999; King et al., 2001; Xie and Spradling, 1998; Xie and Spradling, 2000b). Not much is known about how external signaling from the niche preserves GSC fate. One protein that is likely to play a role in GSC maintenance is Pumilio, a repressor of translation, which is required within GSCs for GSC maintenance (Forbes and Lehmann, 1998; Lin and Spradling, 1997; Murata and Wharton, 1995; Zamore et al., 1997). However, the targets and partners of Pumilio within GSCs are unknown.

Normally, GSCs divide asymmetrically, such that one daughter cell remains at the anterior, within the niche, and maintains GSC fate; the posterior daughter, one cell diameter removed from the niche (Fig. 1A), differentiates to form a cystoblast (Deng and Lin, 1997). The process of GSC differentiation (here defined as the transition of GSCs to cystoblasts) remains largely unknown. In particular, unlike some mammalian stem cell systems, very few markers are available to follow the steps toward GSC differentiation. In wild-type females, GSCs can be distinguished from cystoblasts by two main criteria: position (GSCs are closer to cap cells), and expression of the cytoplasmic form of the Bag of marbles protein BamC, which appears in cystoblasts and dividing cysts, but not in GSCs. Two genes, *bam* and *benign gonial cell neoplasm (bgcn)* are necessary for the differentiation of the cystoblast (Gonczy et al., 1997; Lavoie et al., 1999; McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Ohlstein et al., 2000; Ohlstein and McKearin, 1997). However, the molecular function of these genes is unknown, and it remains unclear whether the *bam/bgcn* pathway is the only pathway necessary for GSC differentiation.

Recently, we have shown that the gap junction protein Zero population growth (Zpg) is required for an early step of both oogenesis and spermatogenesis (Tazuke et al., 2002). Gap junctions are intercellular, voltage-gated channels that

allow cells to selectively share ions, metabolites and other messenger molecules. They have well-established roles in physiological and developmental processes such as cardiac muscle contraction and ovarian follicle maturation (Phelan and Starich, 2001; Simon and Goodenough, 1998; Simon et al., 1997; White and Paul, 1999). Here we show that GSCs lacking Zpg can divide, but the daughter cell destined to differentiate dies. Our results suggest that *zpg* may be necessary for the differentiation process itself, as well as for survival of differentiated germ cells, and that *zpg* probably acts in parallel to *bam* and *bgcn*. We further show that the differentiation of the GSC to a cystoblast is gradual, and that many of the germ cells in 'stem cell tumors' caused either by strong mutations in *bam* or by overexpression of Dpp may be at an intermediate state between GSCs and cystoblasts.

Materials and methods

Fly strains

For molecular identification of *zpg* as *inx4*, and characterization of zpg alleles zpg^{z5352} and zpg^{z2533} see Tazuke et al. (Tazuke et al., 2002). *Df*(3L)CH20, *Df*(3L)CH12 uncover *zpg* (Hong and Hashimoto, 1995). The *zpg* alleles used are most likely protein nulls, as they contain stop codons at positions 91 and 239, respectively (Tazuke et al., 2002). In newly eclosed flies (1-3 days old), both *zpg* alleles displayed the same phenotypes when homozygous, in trans to each other, or over either deficiency. In old females (three weeks of age), however, differences between flies that carry *zpg* point mutations and those that carry a point mutation over a deficiency were noted (data not shown). To avoid background interference, all our analyses were performed on young females, where the phenotypes of the *zpg* alleles were consistent and indistinguishable from that of the allele over the deficiency.

Three strong *pum* alleles were used in this study: *In*(3R)Msc, pum^{ET7} and pum^{ET1}, described by Forbes and Lehmann (Forbes and Lehmann, 1998). All allelic combinations were tested and produced the described phenotypes. However, *zpg*, pum^{ET1}/*zpg*, *In(3R)Msc* produced mostly empty ovarioles, whereas *zpg*, pum^{ET7}/*zpg*, *In*(3R)Msc produced more ovarioles containing germ line than other allelic combinations. $bgcn^{QS2}$ was described by Ohlstein et al. (Ohlstein et al., 2000) and bam^{86} by McKearin and Ohlstein (McKearin and Ohlstein, 1995). The *hs-bam* transgene on the X chromosome was described by Ohlstein and McKearin (Ohlstein and McKearin, 1997). *hs-dpp* flies were generated by Ron Blackman. The JH1.1 strain carries eight copies of the *hs-dpp* transgene located at 16F-17A, 2B, 77, 96D-97C.

Antibodies

Rabbit anti-Vasa antibody was used at a dilution of 1:5000. Chicken anti-Vasa was a generous gift from Dr K. Howard, and was used at 1:5000. Rabbit anti-Zpg was used at 1:5000 and is described by Tazuke et al. (Tazuke et al., 2002). Rat anti-BamC was a gift of Dr D. McKearin and was used at 1:500. Rabbit anti p-Mad (anti-phosphorylated SMAD1, PS1) (Persson et al., 1998) was a gift of P. ten-Dijke and was used at 1:1000. Rabbit anti-phospho-Histone H3 was from Upstate Biotechnology and was used according to the manufacturer's protocol. 1B1 monoclonal supernatant (Zaccai and Lipshitz, 1996) was from the Developmental Studies Hybridoma Bank and was used at 1:25. In all cases, secondary antibodies, coupled to FITC, Cy3 or Cy5, were purchased from Jackson ImmunoResearch Laboratories and were used at 1:500.

Heat shock

Newly eclosed flies were heat-shocked twice (*hs-bam*) or three times (*hs-dpp*) a day at 37°C, for 1 hour, for four days. Flies were dissected the following day and stained with the appropriate antibodies.

Immunofluorescence

Fixation and immunostaining of ovaries were according to standard protocols. Imaging was performed on a Leica DM RBE confocal microscope, using the Leica TCS NT program.

Stem cell division counts

Newly eclosed flies were dissected and double-stained with chicken anti-Vasa and rabbit anti-phospho-Histone H3 antibodies. Ovarioles were observed under a fluorescent microscope to score for cells that had anti-phospho-Histone staining. Only the cells at the anterior tip of the ovariole were defined as 'GSCs'. The number of cells that double stained for Vasa and phospho-Histone H3 was divided by the total number of ovarioles scored for each genotype.

Clonal analysis

One- to 3-day-old flies of the genotype $hs\text{-}flp^{122}$; *FRT82, armadillo-LacZ* / *FRT82* were heat shocked once for 15 minutes, at 37°C. Under these mild conditions, only 30-50% of the ovarioles, depending on the experiment, were marked, and marked ovarioles mostly had only one marked GSC. Flies were dissected 2-3 days after heat shock and stained with anti-Vasa and anti-β-Gal. Cells that carry no copies, one copy or two copies of the transgene can be distinguished by the intensity of anti-β-Gal staining.

Results

Few *zpg* germ cells reach the cystoblast stage

Most ovarioles from newly eclosed female flies that carry a strong mutation in the gene *zpg* have only a few germ cells, located at the anterior tip of the ovariole; the others contain no germ cells (Tazuke et al., 2002). *zpg* ovarioles that were occupied by germ line harbored 3.8 germ cells on average, as determined by anti-Vasa antibody labeling to mark the germ line (s.d.=1.9, *n*=464). In comparison, wild-type germaria are filled with dividing cysts, the products of cystoblast divisions (Fig. 1C). The stem cell positions (close to cap cells) were occupied by *zpg* cells, but some single germ cells or clusters of germ cells were also located away from the tip (Fig. 1B,D). The single cells in the *zpg* mutant contained a round spectrosome (Fig. 1B,E), and could therefore be a mixed population of GSCs, cystoblasts or an intermediate between the two. To determine the developmental state of *zpg* germ cells, we double-labeled wild-type and *zpg* ovaries with anti-Vasa antibody and an antibody against cytoplasmic Bam protein (BamC), which stains cystoblasts and early cysts, but not GSCs (Ohlstein and McKearin, 1997) (Fig. 1C). Of the 169 *zpg* ovarioles that had germ cells (Fig. 1D), only 2 contained a single cell that was BamC-positive (~1%). By contrast, 13 out of 50 wild-type ovarioles had a single cell stained with anti-BamC (26%; for further analysis of cystoblasts in wild type, see below). We conclude that most *zpg* ovarioles lack cystoblasts.

In some *zpg* ovarioles we observed clusters of 2-3 cells that stained positive with anti-BamC antibodies. We assume that these may be the developing cysts that give rise to the rare egg chambers observed in these mutants (Tazuke et al., 2002). As in wild type, the rare developing *zpg* cysts were interconnected by ring canals. However, the fusome, which normally spreads through the dividing cyst, was either aberrant or absent. The rare egg chambers were almost always composed of less than

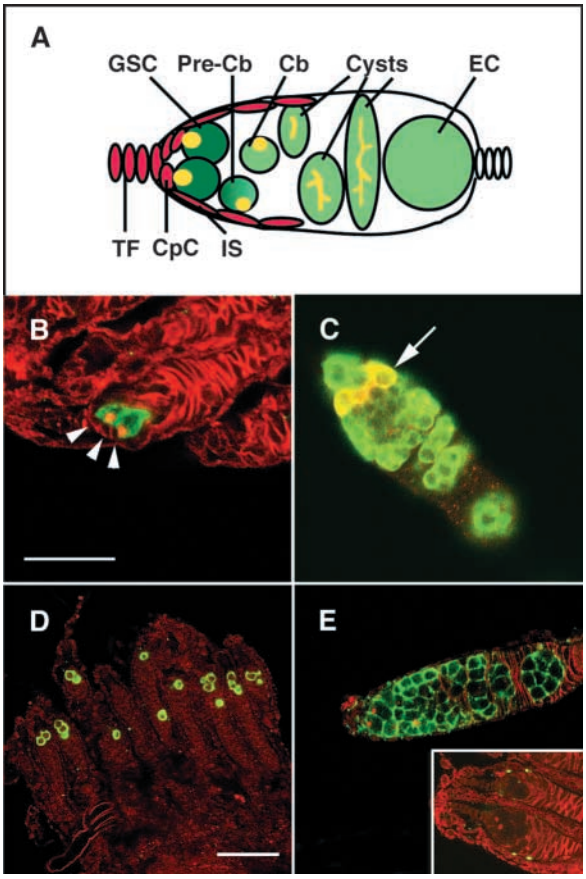

Fig. 1. Germ cells that lack *zpg* fail to differentiate. (A) The *Drosophila* germarium. In all figures, anterior is to the left and posterior to the right. Germ line stem cells (GSCs) are located at the anterior of the ovariole. Progressively more developed germ cells are located posteriorly: a putative intermediate state (or pre-cystoblasts, Pre-Cb), cystoblasts (Cb), dividing cysts and egg chambers (EC). The somatic niche is composed of terminal filament cells (TF), cap cells (CpC) and inner-sheath cells (IS). (B) *zpg* ovaries were stained with anti-Vasa to mark the germ line (green) and with an antibody that recognizes the spectrosome, fusome and the cell cortex (mAb 1B1; red). Only a few germ cells at the anterior tip of the ovarioles could be observed. Some of those germ cells are attached to cap cells (arrowheads). (C,D) Wild-type (C) or *zpg* (D) ovaries stained with anti-Vasa (green) and anti-BamC (red) to mark cystoblasts and dividing cysts. In wild type, a full complement of germ line cells, from GSCs to dividing (yellow; arrow) and budding cysts could be observed. In *zpg* ovaries only a few germ cells at the tip of the ovariole could be observed, mostly lacking BamC staining (panel D depicts many such ovarioles, each containing a few cells). (E) Wild-type and *zpg* ovaries were co-labeled with anti-Zpg (green) and with mAb 1B1 (red). Zpg is expressed on the membrane of germ cells in the germarium. In *zpg* ovaries, no staining with the anti-Zpg antibody was observed, attesting to the specificity of the antibody and to the fact that the alleles used in this study may be protein nulls (E, inset). Scale bars: in B, 20 μm for B,C,E; in D, 50 μm.

16 germ line cells, and DAPI staining indicated degeneration of the chamber (data not shown). As the fusome controls the divisions of the cyst (de Cuevas et al., 1996; Lilly et al., 2000; Lin et al., 1994), the abnormal number of germ cells in the egg chamber is likely to be the result of a severed fusome in the mutants.

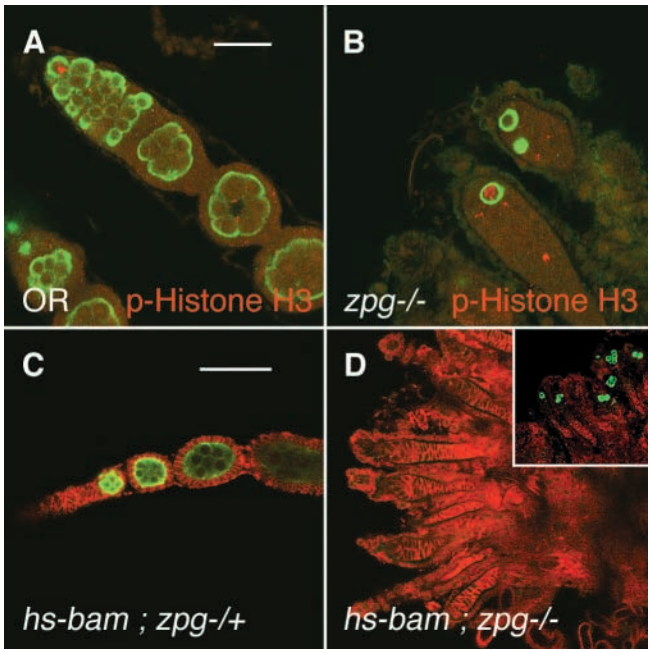

Fig. 2. Differentiation results in death of *zpg* cells. (A,B) Ovaries stained with anti-Vasa to mark the germ line (green) and anti-phospho-Histone H3 to mark mitotic cells (red). Mitotic cells at the GSC position can be observed in both wild-type (A) and *zpg* (B) ovaries. (C,D) Flies carrying a *hs-bam* transgene were heat-shocked, and their ovaries dissected and stained with anti-Vasa to mark the germ line (green), and with the mAb 1B1 antibody to mark the cell cortex, fusomes and spectrosomes (red). *hs-bam, zpg* heterozygous mutant ovaries showed a series of egg chambers attached to empty germaria (C). *hs-bam, zpg* homozygous mutant ovaries lacked germ line (D). Control ovaries of heat-shocked *zpg* mutants showed the *zpg* phenotype (D, inset). Scale bar: in A, 20 μm for A,B; in C, 50 μm for C,D.

Using an antibody raised against the cytoplasmic tail of Zpg (Tazuke et al., 2002), we observed staining in germ cells of every stage of development from GSCs to budding cysts of wild-type germaria. The staining appeared to be at the membrane of the cells and was somewhat stronger in region 2 (Fig. 1E). The presence of Zpg staining in GSCs and dividing cysts correlates with the early defect in germ cell differentiation in *zpg* mutants and suggests that Zpg acts in the germ line.

zpg is required for survival of differentiating early germ cells

The low number of cystoblasts in *zpg* ovarioles could be the result of defects in GSC division or in survival of the GSC daughter cell destined to differentiate. To distinguish between these two possibilities, we stained *zpg* or wild-type germ cells for the mitotic marker phospho-Histone H3 (Fig. 2A,B). The percentage of marked *zpg* cells at the stem cell position per ovariole was not statistically different from that of wild-type GSCs (Table 1). Similarly, the number of wild-type GSCs per ovariole in S phase (detected by BrdU labeling) was comparable to that of *zpg* cells (data not shown). Taken together, these data show that *zpg* and wild-type cells spend a similar proportion of time in M and S phase. Although we cannot rule out the possibility that *zpg* cells have an overall

Table 1. Division rate of *zpg* cells is not different from that of wild-type GSCs

	OR	$Zpg^{z\text{-}2533}$/TM3	$Zpg^{z\text{-}2533}$/CH20	$Zpg^{z\text{-}5352}$/CH20
Number of dividing 'GSCs'	29	36	63	37
Number of total ovarioles counted	831	855	1253	1114
Percentage of dividing 'GSCs' per ovarioles counted	3.5	4.2	5.0 (0.12)	3.3 (0.46)

The numbers in parentheses represent *P* values from a *t*-test comparing each mutant genotype to wild-type (OR) counts.

slower cell cycle, as compared to wild-type GSCs, our data show that *zpg* cells divide. The lack of differentiating cells in *zpg* ovarioles may therefore indicate that *zpg* germ cells die when they commence differentiation.

To test this hypothesis, we forced *zpg* germ cells to differentiate by inducing expression of Bam protein in these cells, using a heat shock construct (Ohlstein and McKearin, 1997). In wild type, overexpression of Bam in GSCs induces the cystoblast differentiation program, resulting in a chain of egg chambers connected to a germarium depleted of germ line (Ohlstein and McKearin, 1997) (Fig. 2C). When Bam was induced in *zpg* flies, no germ cells could be observed with anti-Vasa staining (Fig. 2D). Heat shock itself was not the cause of germ cell death, as ovaries of heat-shocked *zpg* animals, which did not carry the *hs-bam* transgene, still possessed germ cells (Fig. 2D, inset). Thus, *zpg* germ cells die when forced to differentiate. This indicates that Zpg is required for the survival of differentiating germ cells. Taken together, our data indicate that *zpg* cells divide, and that the daughter cells that are destined to differentiate die.

zpg is necessary for differentiation of *pum* mutant GSCs

To further explore the role of Zpg in early germ cell differentiation and survival, we recombined *zpg* alleles with mutant alleles of the gene *pumilio* (*pum*) (Forbes and Lehmann, 1998; Lin and Spradling, 1997). *pum* mutant ovaries exhibit a compound phenotype. Many ovarioles lack germ line completely (Fig. 3B,D), a defect that may be attributed to pre-oogenic defects (Asaoka-Taguchi et al., 1999; Forbes and Lehmann, 1998; Lin and Spradling, 1997; Parisi and Lin, 1999). Ovarioles with germ line have a germ line-depleted germarium connected to a few defective egg chambers (Fig. 3A). This phenotype suggests that Pum has roles in GSC maintenance (Forbes and Lehmann, 1998; Lin and Spradling, 1997). In ovaries from *zpg, pum* double-mutant females, many ovarioles were empty (see Materials and methods). This is consistent with the embryonic and larval requirement for *pum* (Fig. 3D). Ovarioles occupied by germ line exhibited a phenotype more similar to *zpg* than to *pum* mutants: few germ cells at the tip of the ovariole (Fig. 3C,D). Thus Zpg function is required for the differentiation of *pum* mutant germ cells. The apparent difference between the *zpg, pum* and *hs-Bam; zpg* phenotypes may reflect the different roles of Pum and Bam in GSC differentiation. Pum, as a translational repressor may permit GSC maintenance by repressing differentiation, which requires Zpg. By contrast, Bam may have a more instructive role in GSC differentiation, such that its overexpression can overrule GSC-maintenance cues emanating from the niche, independent of *zpg* (Ohlstein and McKearin, 1997) (this study).

In addition to defects in GSC maintenance, *pum* mutants also show defects in cyst development (Forbes and Lehmann, 1998; Parisi and Lin, 1999) (Fig. 3A). This function was also evident in the *zpg, pum* phenotype. Although *zpg, pum* ovarioles occupied by germ line mostly resemble the *zpg* phenotype, we observed that they contained more germ cells, and had more dividing cysts and differentiating egg chambers, than those of the single *zpg* mutant (compare Fig. 3C with Fig. 3D). The double mutant had an average of 0.23 egg chambers per ovariole (n=290), compared with 0.02 (n=500) in flies homozygous for *zpg* and heterozygous for *pum*. The higher number of single cells and egg chambers in *zpg, pum* double mutants may indicate that Zpg function is less essential when cyst development is abrogated, as is the case in *pum* mutants.

bam and *bgcn* 'stem cell' tumors do not develop in the absence of *zpg*

Our analysis suggests that Zpg wild-type function is required for the differentiation of GSCs. At least two other genes, *bam* and *bgcn*, are required for early germ cell differentiation

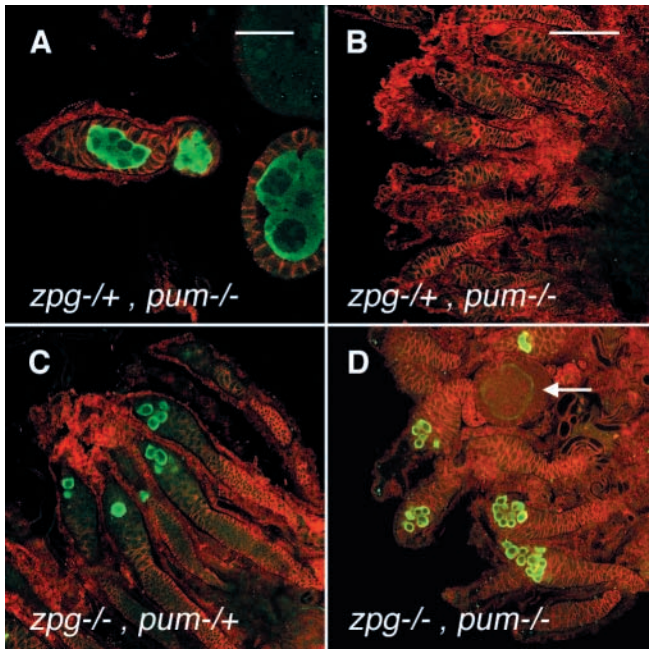

Fig. 3. *pum* mutant germ cells require *zpg* for differentiation. Ovaries from flies of the indicated genotypes were stained with anti-Vasa (green) and the mAb 1B1 antibody (red). *pum* mutant ovarioles were either devoid of germ line (B), or had a few defective, but differentiated, egg chambers and an empty germarium (A). *zpg, pum* double-mutant ovaries resembled *zpg* ovaries, with only a few germ cells at the tip of the ovariole, except that a few more germ cells were present. More cysts and egg chambers were also observed (D, arrow). Scale bar: in A, 20 μm; in B, 50 μm for B-D.

(Gonczy et al., 1997; Lavoie et al., 1999; McKearin and Ohlstein, 1995; Ohlstein et al., 2000). However, the phenotype of *bam* and *bgcn* mutant ovaries is strikingly different from that of *zpg* mutants. Ovaries mutant for *bam* or *bgcn* are filled with many undifferentiated single germ cells harboring a spherical spectrosome, which have been described as GSC tumors (Fig. 4A) (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Ohlstein et al., 2000). By contrast, ovaries from *zpg* flies have only a few germ cells at the tip of the ovariole (Fig. 4B). To determine the functional relationship between these genes, we made flies double mutant for *zpg* with either *bam* or *bgcn*. Ovaries from newly eclosed females were stained to visualize the germ line and the spectrosomes. In the double mutant lacking both *zpg* and *bam*, only a few germ cells were detected at the ovariole tip (Fig. 4C). However, most double-mutant ovarioles had somewhat more germ cells than *zpg* ovarioles (average for *zpg, bam*=6.4, s.d.=3.2, *n*=110; average for *zpg*=3.8, s.d.=1.9, *n*=464). Similar results were obtained with double mutants of *zpg* and *bgcn* (Fig. 4D).

As GSCs can survive in a *zpg* background, the predicted phenotype of a *zpg*, *bam* or *bgcn; zpg* double mutant would have been similar to a *bam* (or *bgcn*) phenotype (i.e. a germarium filled with undifferentiated GSCs). By contrast, the double-mutant phenotype more closely resembles the *zpg* phenotype. To test whether slow division of *zpg* cells accounts for the lack of tumors in young females, we analyzed older (1- to 2-week-old) females, and found a similar phenotype to that of young females (data not shown). Thus, wild-type Zpg function is required for the accumulation of *bam* or *bgcn* mutant germ cell tumors removed from the niche.

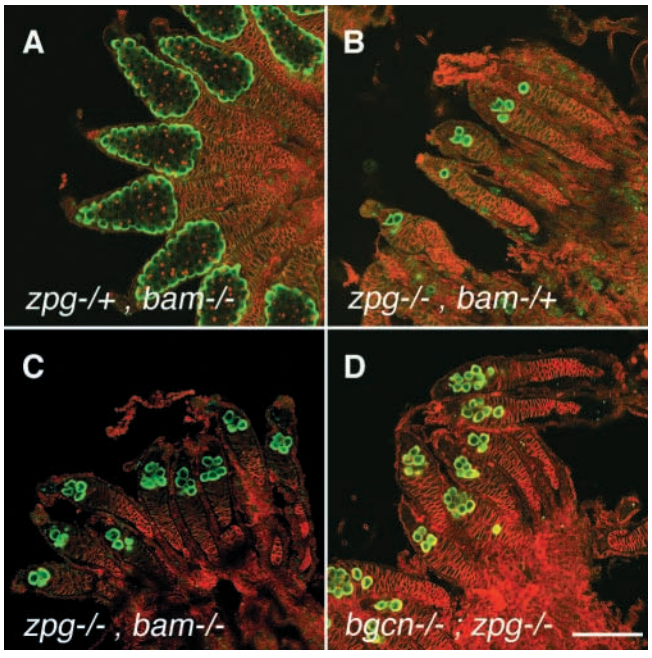

Fig. 4. *zpg* is required for survival of *bam* 'stem-cell tumors'. Ovaries from flies of the indicated genotypes were stained with anti-Vasa (green) and the mAb 1B1 antibody (red). *zpg bam /+ bam* ovaries showed the *bam* phenotype with many 'stem cell-like' germ cells and no egg chambers (A). *zpg bam / zpg+* flies had the *zpg* phenotype with few germ cells at the tip of the ovariole (B). *zpg* and *bam* or *bgcn* double mutant ovaries (C,D) had a similar phenotype to that of *zpg*, with only a few germ cells located at the tip of the ovary. However, the number of germ cells at the tip was higher than in *zpg* single mutants (compare panel B with panels C,D). Scale bar in D: 50 μm for A-D.

Dpp cannot support early germ cell over-proliferation in the absence of *zpg*

To investigate further a possible role for Zpg-mediated intercellular communication in the development of germ cell tumors, we analyzed the genetic interaction between *dpp* and *zpg*. It had been proposed that an increased Dpp signal induced over-proliferation of GSC-like cells (Xie and Spradling, 1998; Xie and Spradling, 2000b). We therefore reasoned that an increased Dpp signal could induce *zpg* cells to over-proliferate. To test this, we crossed flies carrying several insertions of a

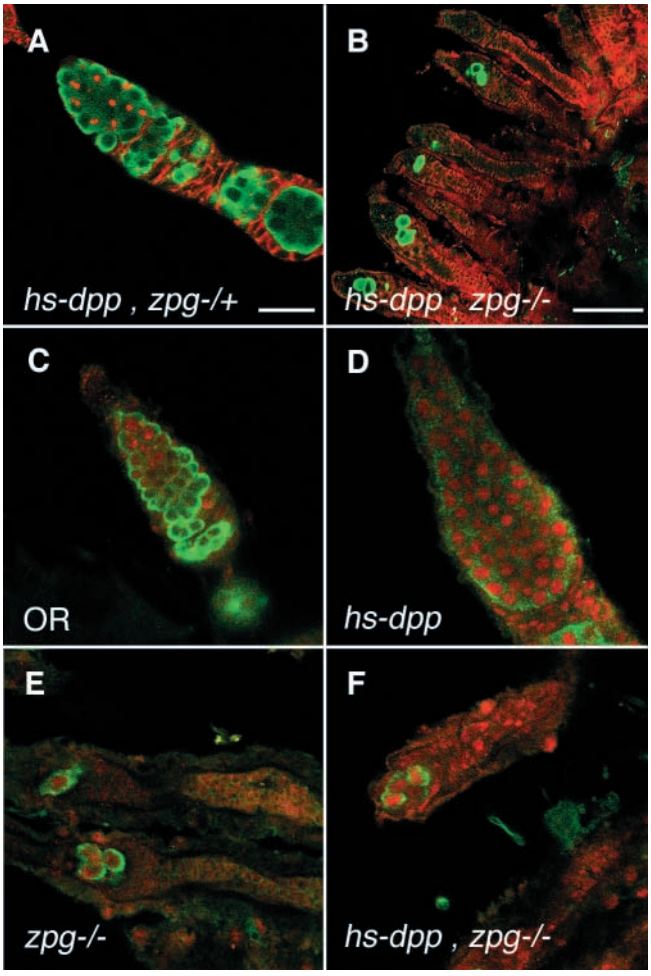

Fig. 5. *zpg* is required for Dpp-induced over-proliferation of early germ cells. Flies of the indicated genotypes were either dissected (C,E), or subjected to heat shock prior to dissection (A,B,D,F), and then stained with anti-Vasa (green) and mAb 1B1 antibody (red; A,B) or anti-pMad (red; C-F). After heat shock, *hs-dpp*; *zpg* heterozygous ovaries were filled with many single germ cells (A). *hs-dpp*, *zpg* homozygous mutant ovaries did not show any proliferation of single germ cells (B). Staining of wild-type (OR) germaria revealed p-Mad staining in early germ cells (C), whereas all germ cells have nuclear p-Mad when Dpp is overexpressed (D). *zpg* cells also have nuclear p-Mad, whether Dpp is overexpressed (F) or not (E). Scale bar: in A, 20 μm for A,C,D; in B, 50 μm.

heat-shock *dpp* transgene (*hs-dpp*) into a *zpg* background. Flies were heat shocked, and then dissected and stained to reveal the germ line and spectrosomes. Control animals heterozygous for *zpg*, carrying a subset of the *hs-dpp* transgenes, had more single germ cells with spherical spectrosomes than did wild type (Fig. 5A). Homozygous *zpg* animals, which carried at least the same number of *hs-dpp* transgenes as the control, did not show an increase in germ cell number (Fig. 5B). To test whether *zpg* cells could respond to a Dpp signal, we stained ovaries of *zpg* animals with antibodies against phosphorylated Mad (p-Mad) (Persson et al., 1998). Mad is a component of the Dpp signal transduction pathway and is phosphorylated upon activation of the pathway (Massague, 1998). In wild type, p-Mad staining could be detected in GSCs, cystoblasts and dividing cysts in region 1 of the germarium. The highest level of staining was observed in early germ cells; staining then gradually declined towards the posterior (Fig. 5C). p-Mad was also detected in the single cells that accumulate following Dpp overexpression (Fig. 5D). Similarly, p-Mad staining was detected in *zpg* germ cells (Fig. 5E,F), suggesting that the mutant cells are able to receive the Dpp signal. There may be several explanations for the failure of *zpg* cells to proliferate or survive in response to a Dpp signal. One is that the Dpp pathway is blocked downstream of Mad in *zpg* cells. Second, *zpg* cells may not be able to survive when unattached to the tip of the ovary. Third, proliferating cells in *hs-dpp* flies, which move away from the niche, are in a more differentiated state than the cells in the niche, and therefore die in a *zpg* background (see Discussion).

An intermediate state between a stem cell and a cystoblast may exist

bam tumor cells and germ cells proliferating after Dpp overexpression (*hs-dpp* tumor cells) are considered to be GSCs because of their round spectrosomes and lack of BamC staining (McKearin and Ohlstein, 1995; Xie and Spradling, 1998; Xie and Spradling, 2000b). Yet, these cells do not accumulate in a *zpg* background. One possible explanation for this observation is that *bam* and *hs-dpp* cells, as they move away from the niche, are at an intermediate state (pre-cystoblast) between a stem cell and a cystoblast, and that cystoblast development and survival requires Zpg (see Discussion). To determine whether an intermediate state between GSCs and cystoblasts exists in wild type, we triple-labeled ovarioles with anti-Vasa, 1B1 monoclonal antibody and anti-BamC, to mark the germ line, spectrosomes and cystoblasts, respectively (Fig. 6B). BamC antibody stained cysts of 4 or 8 cells strongly. Two-cell cysts had notably weaker staining. Only rarely did we observe cystoblasts, i.e. single cells, stained with anti-BamC [data not shown; similar observations were reported by Ohlstein and McKearin (Ohlstein and McKearin, 1997)]. In many ovarioles, we could observe single cells with a spherical spectrosome that were removed from the stem cell position yet did not stain for the cystoblast marker BamC. We counted the number of cells that carried a spherical spectrosome and did not stain with anti-BamC. These cells would comprise the GSC population plus the presumptive intermediate population. Of 100 ovarioles scored, most had between 3 and 5 single cells that did not stain with anti-BamC (Fig. 6A). The average number of these cells was 3.9. This is a greater number than the average number of GSCs that populate an ovariole (between 2 and 3), as determined by cell-lineage analysis and electron microscopy (Carpenter, 1979; Margolis and Spradling, 1995; Wieschaus and Szabad, 1979). These data support the hypothesis that an intermediate state between a stem cell and a cystoblast exists in wild type (see also Ohlstein and McKearin, 1997).

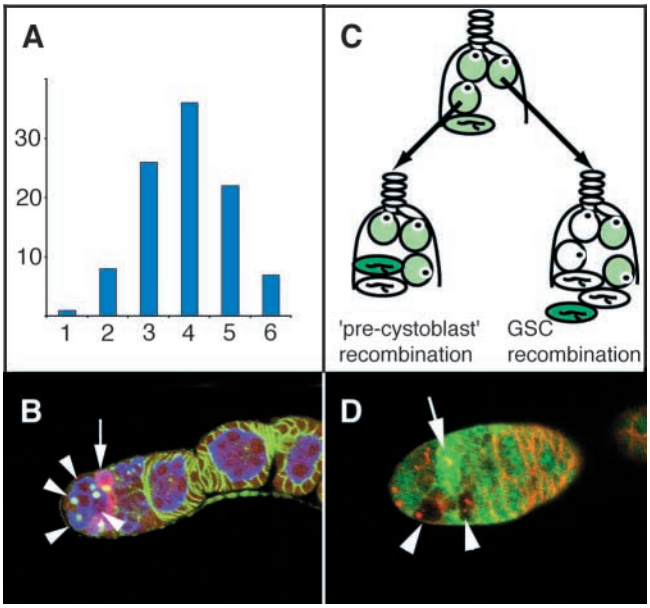

Fig. 6. An average of 3.9 single cells do not stain with BamC antibody. (A) Individual germaria were scored for the number of cells that contain a spherical spectrosome and do not stain with BamC antibodies. The graph illustrates the number of germaria (*y* axis) that contained between 1 and 6 of such cells (*x* axis). (B) Wild-type ovaries were stained with anti-Vasa (blue), with the mAb 1B1 antibody (green) and with anti-BamC (red). Four cells that possess a single spectrosome but do not stain for BamC can be observed at the anterior of the ovariole (arrowheads). A cyst, which does stain with BamC antibody, can be seen just posteriorly (arrow). Scale bar: 20 μm. (C) An illustration of the possible outcomes of mitotic recombination in a pre-cystoblast or a GSC (see arrows). Cells carrying 0 copies of the *lacZ* transgene are depicted in white, whereas those carrying 1 or 2 copies of the transgene are light or dark green, respectively. (D) A germarium representing a GSC mitotic recombination event. Arrowheads mark the GSC and its progeny lacking *lacZ* (only one daughter cell is in plane). An arrow marks the 'twin spot', which strongly expresses *lacZ*.

Wild-type cells at the intermediate state do not amplify

An intermediate cell population between slowly dividing stem cells and differentiating cells, described as transit amplifying cells, is a common feature in stem cell systems, including those giving rise to blood cells, skin and the gut epithelium. These are the products of stem cell division and have limited potential to divide prior to differentiation (Melchers and Rolink, 2000; Watt, 2000; Winton, 2000). In principle, the existence of a transit amplifying cell population in the *Drosophila* germ line could be suggested if the GSC division rate is too low to account for the number of cysts/egg chambers produced (including dying ones) in a set period of time. Acquiring this information, especially in region 1 of the germarium has proved to be difficult (Cox et al., 2000; Drummond-Barbosa

and Spradling, 2001; Lin and Spradling, 1993; Smith et al., 2002). We therefore directly examined the products of possible division of cells at the transition state. If a pre-cystoblast cell amplifies, it should give rise to more than one egg chamber. By contrast, a cystoblast divides incompletely, giving rise to a two-cell cyst and, eventually, to one egg chamber. To distinguish between 'intermediate-state' divisions and cystoblast divisions, we conducted mosaic analysis in wild-type germaria using the FLP-FRT marker system (Xu and Rubin, 1993), in which cells that are produced by mitotic recombination are marked as twin-spots by the copy number of the gene (*lacZ*), encoding the marker β-Galactosidase (0 or 2 copies). Recombination in GSCs would result in a marked GSC, a string of marked cysts arising from its subsequent division, and one cyst that is the twin-spot of the original recombination event (Fig. 6C). A recombination event in the cystoblast would not be observed under our experimental conditions because marked cells within a cyst share the diffusable β-Gal. We reasoned that, if a cell at the transition state divides, we would observe twin-spot cysts even in germaria where GSCs are not marked (Fig. 6C). Such a situation could also arise if a marked GSC left the niche and differentiated. However, GSC loss is unlikely to affect our analysis, as the half-life of wild-type GSCs is 4.6 weeks (Xie and Spradling, 1998), whereas we dissected the females 2-3 days after induction of Flipase. Of 177 ovarioles scored, 104 contained no marked GSCs and no marked cysts (~60%). 73 ovarioles contained marked cysts and a marked GSC (~40%; Fig. 6D). We only observed ovarioles that contained marked cysts and marked GSCs. These results lead us to conclude that cells at the transition state in wild type do not divide at an appreciable rate but proceed to differentiate to a cystoblast. Thus, they do not constitute the equivalent of a transient amplifying population. By contrast, tumor cells in *bam* mutants, or when Dpp is overexpressed, continue to divide, as their differentiation is blocked.

Discussion

zpg ovarioles contain single germ cells at the anterior tip. Most of these cells do not reach the cystoblast stage. As *zpg* cells are not arrested at a particular stage of their cell cycle, and can divide, we conclude that GSCs that lack *zpg* divide to give another stem cell and a daughter cell that dies at early stages of differentiation. Accordingly, overexpression of *bam*, which is necessary and sufficient to promote germ cell differentiation, kills *zpg* cells.

Zpg is a gap junction protein necessary for GSC differentiation

zpg encodes innexin 4 – one of eight innexins in *Drosophila* (FlyBase) (Phelan and Starich, 2001; Stebbings et al., 2002; Tazuke et al., 2002). Innexins are the functional homologues of the vertebrate connexins, or gap junction proteins (Phelan and Starich, 2001). In mammalian oogenesis, gap junctions have been implicated in cell-cell signaling. Early luteinization of granulosa cells is observed either when the oocyte is physically removed from immature wild-type follicles (el-Fouly et al., 1970), or in mice lacking connexin 37 (Simon et al., 1997), suggesting a gap junction-mediated signaling mechanism between the oocyte and granulosa cells.

Here we show that the *Drosophila* gap junction protein Zpg is required for the survival of the germ line stem cell daughter as it moves away from the niche and begins to differentiate. From its expression pattern, and the specificity of its function, it is clear that Zpg acts in a germ line autonomous way. However, we do not yet know whether Zpg facilitates communication between germ cells, or between germ line and soma. The germarium is a compact structure where early germ cells contact each other, the somatic cap cells contact GSCs, and inner-sheath cells contact GSCs and their daughters (King, 1970; Schulz et al., 2002). Further study is needed to clarify which cells communicate with germ cells through Zpg-gap junctions. Likewise, the nature of the requirement for *zpg* in GSC differentiation is still uncertain. Gap junctions may be used to supply the GSC daughter cell with nutrients, or with a survival factor required for its subsequent growth. Alternatively, gap junctions may be used to deposit a factor that is required for the differentiation process itself, rather than for survival, while an accessory mechanism eliminates cells that begin differentiating without that factor. The major argument in favor of a role for Zpg in differentiation at the stem cell stage comes from our phenotypic analysis of *zpg, pum* double mutants in which, unlike in *pum* single mutants, GSCs do not differentiate. Although Pum-mediated repression is removed in the double mutants, GSCs cannot differentiate as they may lack a differentiation signal provided by Zpg. It is harder to imagine how nutritional deficiency per se could block differentiation of the double-mutant cells because single-mutant *zpg* cells do begin to differentiate (and then die).

The nature of GSC differentiation

Recent studies suggest that the niche promotes stem cell fate through Dpp signaling (Xie and Spradling, 1998; Xie and Spradling, 2000b; Zhu and Xie, 2003). This may be achieved through repression of *bam* (Chen and McKearin, 2003). GSCs are also tightly tethered to the niche via adherens junctions (Song et al., 2002). Other, as-yet unidentified mechanisms may be used by the niche to protect GSCs.

Once germ cells leave the niche they activate the differentiation pathway. We propose that differentiation of GCSs to cystoblasts is not direct but proceeds via an intermediate state (Fig. 6). We have shown that most wild-type cells, which by their position within the germarium were judged to be cystoblasts, are not stained with BamC antibody. Our finding concurs with Ohlstein and McKearin, who observed cytoplasmic Bam just before the cystoblasts divide to form a two-cell cyst (Ohlstein and McKearin, 1997), and proposed the existence of an intermediate/pre-cystoblast state between GSCs and cystoblasts. In other stem cell systems, the intermediate cell population has a biological function, namely increasing the progeny of a single stem cell division. Our results indicate that in *Drosophila* females, cells at the intermediate state do not constitute a transit-amplifying cell population. However, the 'pre-cystoblast state' may have a different biological significance. A vacant niche can be filled by a neighboring GSC that divides 'sideways' instead of along the anteroposterior axis (Xie and Spradling, 2000b). An alternative for filling a vacant GSC position might be for a cell at the intermediate state to re-occupy the niche.

The suggested model, adding a transitory state between the stem cell and the cystoblast, raises an interesting question. Is the differentiation of a GSC to a cystoblast a continuous process or a discrete one? It is notable that none of the markers we currently use is specific to the stem cell, the cystoblast or the intermediate. Bam is present (in its fusomal form) already in the stem cell, and BamC gradually accumulates in the cystoblast (Ohlstein and McKearin, 1997) (this study). Pumilio protein and phosphorylated Mad are also detected from GSCs to cystoblasts and early cysts (Forbes and Lehmann, 1998) (this work). In other stem cell systems, including mammalian hematopoiesis, many intermediate cell types are known, and can be isolated by specific marker combinations (reviewed by Melchers and Rolink, 2000). Due to the relative lack of markers, the isolation of these 'cell types' from *Drosophila* ovaries is currently impossible. The overlap of expression patterns of the proteins that are known to have a role in stem cell maintenance and differentiation may indicate that differentiation is gradual, and possibly reversible.

The niche promotes germ line stem cell fate

It has been suggested that in *bam* or *bgcn* mutant ovaries, or when Dpp is overexpressed, the germaria are filled with GSC tumor cells (Gonczy et al., 1997; Lavoie et al., 1999; McKearin and Ohlstein, 1995; Ohlstein et al., 2000; Xie and Spradling, 1998; Xie and Spradling, 2000b). Our findings of an intermediate cell population in wild type raises the possibility that GSC tumor cells share some properties with pre-cystoblasts. Both these populations are single, do not stain for BamC, but exist outside of the niche. Some support to the analogy between pre-cystoblasts and 'GSC tumors' is evident in the fact that the latter do not survive past the niche in a *zpg* background. Thus, the *zpg* double mutants allow us to distinguish two cell populations in 'GSC tumors' – cells that are inside or outside of the niche. We suggest that when Dpp is overexpressed, or in *bam*/*bgcn* mutants, cells outside the niche cannot fully activate the differentiation program and are at an intermediate state between a GSC and a cystoblast. These cells die in a *zpg* background, whereas the tumor cells in contact with the niche survive. Our results thus suggest that beyond Zpg gap junctions and Dpp signaling, there must be additional signaling between GSCs and the niche, which helps maintain GSCs. Additional markers are needed to determine unequivocally whether *bam* tumors are similar to Dpp tumors, and whether they share properties with wild-type pre-cystoblasts.

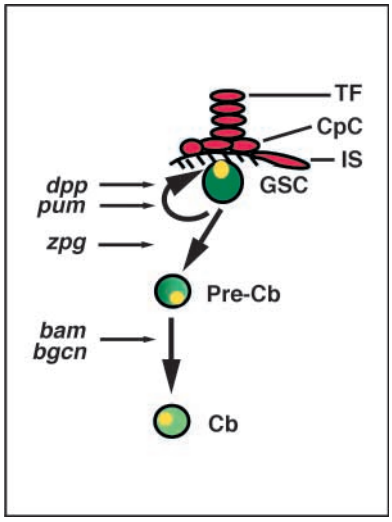

Fig. 7. A model for GSC differentiation. Somatic cells of the niche (TF, terminal filament), cap cells (CpC) and inner sheath cells (IS) are depicted in red. Germ line cells are depicted in shades of green (GSC, germ line stem cell; Pre-Cb, pre-cystoblast; Cb, cystoblast). Stem cell maintenance is controlled by *dpp*, *pum*, and other unidentified factors (dashed lines). Zpg is needed for the differentiation of the stem cell. Bam and Bgcn are needed for the differentiation of the cystoblast and cysts.

Differentiation of the stem cell daughter requires gap junctions. We assume *zpg* is acting in parallel to *pum*, *dpp*, *bam* and *bgcn* because the double mutants showed incomplete epistasis of *zpg* over each of these genes (Fig. 7). Although a role for gap junction proteins has been established in mammalian oogenesis (Ackert et al., 2001; Carabatsos et al., 2000; Juneja et al., 1999; Simon et al., 1997), to our knowledge, this is the first instance where a gap junction protein is shown to control stem cell differentiation. What passes through these gap junctions, and which cells are connected to GSCs through Zpg channels, is still unknown. One intriguing option is that Zpg forms part of the channels that connect GSCs to the surrounding somatic niche cells. If so, that would suggest that the niche in the *Drosophila* germarium is necessary, not only for stem cell maintenance, but also for stem cell differentiation.

Note added in proof

In a recent paper, Kai and Spradling report observations of p-Mad staining in wild-type and Dpp overexpressing germaria similar to our own. In addition, they report that in *bam* mutant-ovaries tumor cells abutting niche cells stain with p-Mad, while cells away from the niche do not (Kai and Spradling, 2003).

We wish to thank members of the Lehmann laboratory, J. Treissman, C. Navarro and, especially, J. Morris for fruitful discussions and critical reading of the manuscript. We thank B. Wakimoto and D. Lindsley for providing *zpg* sterile lines; C. Hashimoto for the deficiency lines uncovering *zpg*; P. ten-Dijke for p-Mad antibody; D. McKearin for BamC antibody and *hs-bam* flies; and K. Howard for chicken anti-Vasa antibody. This work was supported by the Human Frontiers Science Program (L.G.), NIH-Reproductive Scientist Development Program (S.I.T.), Burroughs-Wellcome Fund (S.I.T.), NIH grant U54-HD31398 (S.I.T.), NIH grant P01-DK53074 (sub project #4) (M.T.F), and NIH grant R01 HD27549 (R.L.). R.L. is a Howard Hughes Medical Institute investigator.

References

Ackert, C. L., Gittens, J. E., O'Brien, M. J., Eppig, J. J. and Kidder, G. M. (2001). Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev. Biol.* **233**, 258-270.

Asaoka-Taguchi, M., Yamada, M., Nakamura, A., Hanyu, K. and Kobayashi, S. (1999). Maternal Pumilio acts together with Nanos in germline development in Drosophila embryos. *Nat. Cell Biol.* **1**, 431-437.

Carabatsos, M. J., Sellitto, C., Goodenough, D. A. and Albertini, D. F. (2000). Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev. Biol.* **226**, 167-179.

Carpenter, A. T. (1979). Synaptonemal complex and recombination nodules in wild-type Drosophila melanogaster females. *Genetics* **92**, 511-541.
Chen, D. and McKearin, D. (2003). A discrete transcriptional silencer in the *bam* gene determines asymmetric division of the *Drosophila* germline stem cell. *Development* **130**, 1159-1170.
Cox, D. N., Chao, A., Baker, J., Chang, L., Qiao, D. and Lin, H. (1998). A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev.* **12**, 3715-3727.
Cox, D. N., Chao, A. and Lin, H. (2000). piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503-514.
de Cuevas, M., Lee, J. K. and Spradling, A. C. (1996). alpha spectrin is required for germline cell division and differentiation in the Drosophila ovary. *Development* **122**, 3959-3968.
Deng, W. and Lin, H. (1997). Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in Drosophila. *Dev. Biol.* **189**, 79-94.
Drummond-Barbosa, D. and Spradling, A. C. (2001). Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. *Dev. Biol.* **231**, 265-278.
el-Fouly, M. A., Cook, B., Nekola, M. and Nalbandov, A. V. (1970). Role of the ovum in follicular luteinization. *Endocrinology* **87**, 286-293.
Forbes, A. and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. *Development* **125**, 679-690.
Forbes, A. J., Lin, H., Ingham, P. W. and Spradling, A. C. (1996). *hedgehog* is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in *Drosophila*. *Development* **122**, 1125-1135.
Gonczy, P., Matunis, E. and DiNardo, S. (1997). bag-of-marbles and benign gonial cell neoplasm act in the germline to restrict proliferation during Drosophila spermatogenesis. *Development* **124**, 4361-4371.
Hong, C. C. and Hashimoto, C. (1995). An unusual mosaic protein with a protease domain, encoded by the *nudel* gene, is involved in defining embryonic dorsoventral polarity in Drosophila. *Cell* **82**, 785-794.
Juneja, S. C., Barr, K. J., Enders, G. C. and Kidder, G. M. (1999). Defects in the germ line and gonads of mice lacking connexin43. *Biol. Reprod.* **60**, 1263-1270.
Kai, T. and Spradling, A. (2003). An empty *Drosophila* stem cell niche reactivates the proliferation of ectopic cells. *Proc. Natl. Acad. Sci. USA* **100**, 4633-4638.
King, F. J. and Lin, H. (1999). Somatic signaling mediated by fs(1)Yb is essential for germline stem cell maintenance during *Drosophila* oogenesis. *Development* **126**, 1833-1844.
King, F. J., Szakmary, A., Cox, D. N. and Lin, H. (2001). Yb modulates the divisions of both germline and somatic stem cells through piwi- and hh-mediated mechanisms in the Drosophila ovary. *Mol. Cell* **7**, 497-508.
King, R. C. (1970). *Ovarian Development in* Drosophila melanogaster. New York: Academic Press.
Lavoie, C. A., Ohlstein, B. and McKearin, D. M. (1999). Localization and function of Bam protein require the benign gonial cell neoplasm gene product. *Dev. Biol.* **212**, 405-413.
Lilly, M. A., de Cuevas, M. and Spradling, A. C. (2000). Cyclin A associates with the fusome during germline cyst formation in the Drosophila ovary. *Dev. Biol.* **218**, 53-63.
Lin, H. (1998). The self-renewing mechanism of stem cells in the germline. *Curr. Opin. Cell Biol.* **10**, 687-693.
Lin, H. and Spradling, A. C. (1993). Germline stem cell division and egg chamber development in transplanted Drosophila germaria. *Dev. Biol.* **159**, 140-152.
Lin, H. and Spradling, A. C. (1997). A novel group of *pumilio* mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development* **124**, 2463-2476.
Lin, H., Yue, L. and Spradling, A. C. (1994). The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* **120**, 947-956.
Margolis, J. and Spradling, A. (1995). Identification and behavior of epithelial stem cells in the *Drosophila* ovary. *Development* **121**, 3797-3807.
Massague, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753-791.
McKearin, D. and Ohlstein, B. (1995). A role for the *Drosophila* Bag-of-marbles protein in the differentiation of cytoblasts from germline stem cells. *Development* **121**, 2937-2947.
McKearin, D. M. and Spradling, A. C. (1990). *bag-of-marbles:* a Drosophila gene required to initiate both male and female gametogenesis. *Genes Dev.* **4**, 2242-2251.
Melchers, F. and Rolink, A. (2000). Hematopoietic Stem Cells: Lymphopoiesis and the Problem of Commitment Versus Plasticity. In *Stem Cell Biology* (ed. D. R. Marshak, R. L. Gardner and D. Gottlieb), pp. 307-327. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Murata, Y. and Wharton, R. P. (1995). Binding of Pumilio to maternal *hunchback* mRNA is required for posterior patterning in Drosophila embryos. *Cell* **80**, 747-756.
Ohlstein, B., Lavoie, C. A., Vef, O., Gateff, E. and McKearin, D. M. (2000). The Drosophila cystoblast differentiation factor, benign gonial cell neoplasm, is related to DExH-box proteins and interacts genetically with bag-of-marbles. *Genetics* **155**, 1809-1819.
Ohlstein, B. and McKearin, D. (1997). Ectopic expression of the *Drosophila* Bam protein eliminates oogenic germline stem cells. *Development* **124**, 3651-3662.
Parisi, M. and Lin, H. (1999). The Drosophila pumilio gene encodes two functional protein isoforms that play multiple roles in germline development, gonadogenesis, oogenesis and embryogenesis. *Genetics* **153**, 235-250.
Persson, U., Izumi, H., Souchelnytskyi, S., Itoh, S., Grimsby, S., Engstrom, U., Heldin, C. H., Funa, K. and ten Dijke, P. (1998). The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett.* **434**, 83-87.
Phelan, P. and Starich, T. A. (2001). Innexins get into the gap. *BioEssays* **23**, 388-396.
Schulz, C., Wood, C. G., Jones, D. L., Tazuke, S. I. and Fuller, M. T. (2002). Signaling from germ cells mediated by the rhomboid homolog stet organizes encapsulation by somatic support cells. *Development* **129**, 4523-4534.
Simon, A. M. and Goodenough, D. A. (1998). Diverse functions of vertebrate gap junctions. *Trends Cell Biol.* **8**, 477-483.
Simon, A. M., Goodenough, D. A., Li, E. and Paul, D. L. (1997). Female infertility in mice lacking connexin 37. *Nature* **385**, 525-529.
Smith, J. E., 3rd, Cummings, C. A. and Cronmiller, C. (2002). Daughterless coordinates somatic cell proliferation, differentiation and germline cyst survival during follicle formation in *Drosophila*. *Development* **129**, 3255-3267.
Song, X., Zhu, C. H., Doan, C. and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. *Science* **296**, 1855-1857.
Spradling, A. C. (1993). Developmental genetics of oogenesis. In *The Development of Drosophila melanogaster*, vol. I (ed. M. Bate and A. Martinez Arias), pp. 1-70. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001). Stem cells find their niche. *Nature* **414**, 98-104.
Stebbings, L. A., Todman, M. G., Phillips, R., Greer, C. E., Tam, J., Phelan, P., Jacobs, K., Bacon, J. P. and Davies, J. A. (2002). Gap junctions in Drosophila: developmental expression of the entire innexin gene family. *Mech. Dev.* **113**, 197-205.
Tazuke, S. I., Schulz, C., Gilboa, L., Fogarty, M., Mahowald, A. P., Guichet, A., Ephrussi, A., Wood, C. G., Lehmann, R. and Fuller, M. T. (2002). A germline-specific gap junction protein required for survival of differentiating early germ cells. *Development* **129**, 2529-2539.
Watt, F. M. (2000). Epidermal Stem Cells. In *Stem Cell Biology* (ed. D. R. Marshak, R. L. Gardner and D. Gottlieb), pp. 439-453. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Watt, F. M. and Hogan, B. L. (2000). Out of Eden: stem cells and their niches. *Science* **287**, 1427-1430.
White, T. W. and Paul, D. L. (1999). Genetic diseases and gene knockouts reveal diverse connexin functions. *Annu. Rev. Physiol.* **61**, 283-310.
Wieschaus, E. and Szabad, J. (1979). The development and function of the female germline in Drosophila melanogaster, a cell lineage study. *Dev. Biol.* **68**, 29-46.
Winton, D. J. (2000). Stem cells in the Epithelium of the Small Intestine and Colon. In *Stem Cell Biology* (ed. D. R. Marshak, R. L. Gardner and D. Gottlieb), pp. 515-536. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Xie, T. and Spradling, A. C. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. *Cell* **94**, 251-260.
Xie, T. and Spradling, A. (2000a). The Drosophila Ovary: An In Vivo Stem

Cell System. In *Stem Cell Biology* (ed. D. R. Marshak, R. L. Gardner and D. Gottlieb), pp. 129-148. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Xie, T. and Spradling, A. C. (2000b). A niche maintaining germ line stem cells in the Drosophila ovary. *Science* **290**, 328-330.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Zaccai, M. and Lipshitz, H. D. (1996). Differential distributions of two adducin-like protein isoforms in the Drosophila ovary and early embryo. *Zygote* **4**, 159-166.

Zamore, P. D., Williamson, J. R. and Lehmann, R. (1997). The Pumilio protein binds RNA through a conserved domain that defines a new class of RNA-binding proteins. *RNA* **3**, 1421-1433.

Zhu, C. H. and Xie, T. (2003). Clonal expansion of ovarian germline stem cells during niche formation in Drosophila. *Development* **130**, 2579-2588.