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The *Drosophila* Ovarian and Testis Stem Cell Niches: Similar Somatic Stem Cells and Signals

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Summary

The stem cell niches at the apex of *Drosophila* ovaries and testes have been viewed as distinct in two major respects. While both contain germline stem cells, the testis niche also contains "cyst progenitor" stem cells, which divide to produce somatic cells that encase developing germ cells. Moreover, while both niches utilize BMP signaling, the testis niche requires a key JAK/STAT signal. We now show, by lineage marking, that the ovarian niche also contains a second type of stem cell. These "escort stem cells" morphologically resemble testis cyst progenitor cells and their daughters encase developing cysts before undergoing apoptosis at the time of follicle formation. In addition, we show that JAK/STAT signaling also plays a critical role in ovarian niche function, and acts within escort cells. These observations reveal striking similarities in the stem cell niches of male and female gonads, and suggest that they are largely governed by common mechanisms.

Introduction

Stem cells are controlled within local tissue microenvironments known as niches that are generated by nearby stromal cells (reviewed in Ohlstein et al., 2004; Yamashita et al., 2005). One of the best-characterized niches supports germline stem cells (GSCs) within the *Drosophila* ovary (Xie and Spradling, 2000; see Figure 1A). A GSC niche is located at the tip of each ovariole within the germarium, a generative region that is divided into regions: 1, 2a, 2b, and 3 (Figure 1A). The niche itself contains five to seven nondividing somatic cap cells that anchor two or three GSCs via adherens junctions (Song et al., 2002) and stimulate reception of an essential BMP signal (Xie and Spradling, 1998). Following each GSC division, the posterior daughter cell leaves the niche, differentiates into a "cystoblast," undergoes four synchronous, incomplete divisions to form a 16-cell germline cyst (de Cuevas and Spradling, 1998), and steadily moves in a posterior direction through the germarium. The niche signal directly regulates stem cell fate by repressing transcription of the cystoblast determinant gene *bag-of-marbles* (*bam*) in their proximal, but not their distal, daughters (Chen and McKearin, 2003a, 2003b; Kai and Spradling, 2003; Song et al., 2004). Less is known about the anatomy and regulation of a second niche that controls the somatic stem cells (SSCs) located midway along the germarium near the start of region 2b. SSCs divide in response to somatic Hedgehog signals to produce follicle cells that encapsulate passing cysts (Margolis and Spradling, 1995; Forbes et al., 1996; Zhang and Kalderon, 2000).

Inner germarium sheath (IGS) cells, which line the surface of regions 1 and 2a (Figure 1A), support germ cell differentiation and somatic cell production. Thin cytoplasmic processes from IGS cells envelop cystoblasts and cysts for several days prior to follicle formation (Mahowald and Strassheim, 1970; King, 1970; Mahowald and Kambysellis, 1980). In addition, IGS cells located halfway down the germarium anchor SSCs via adherens junctions and are postulated to play a critical role in defining the SSC niche (Song and Xie, 2002). Both anterior IGS cells and those near the SSCs have been reported to be differentiated and immobile but capable of maintaining parity with cyst number by undergoing sporadic division or death (King, 1970; Margolis and Spradling, 1995; Xie and Spradling, 2000). Following GSC loss, IGS cells gradually disappear by apoptosis as preexisting cysts leave the anterior germarium and acquire follicle cells (Xie and Spradling, 2000; Kai and Spradling, 2003). This destroys the SSC niches, but the released SSCs can often associate with cap cells in the vacated GSC niche and continue to divide (Kai and Spradling, 2003).

The stem cell niche located at the apical tip of the *Drosophila* testis shows both similarities and differences to this model of the ovarian niche (compare Figures 1A and 1B; reviewed by Lin, 2002; Gilboa and Lehmann, 2004). As in the ovary, critical signals are sent by a small cluster of somatic cells, the hub, that directly contact GSCs, but not their gonialblast (cystoblast) daughters. In both males and females, newly formed cysts can revert to the stem cell state under certain circumstances (Kai and Spradling, 2004; Brawley and Matunis, 2004). However, testis GSCs require JAK/STAT signals from the hub (Tulina and Matunis, 2001; Kiger et al., 2001), while this signaling pathway in the ovary has been reported to act later, during follicle formation (Ghiglione et al., 2002; Baksa et al., 2002; McGregor et al., 2002). Recently, BMP signals that repress *bam* have also been shown to contribute to testis stem cell maintenance, but do not control cyst initiation (Shivdasani and Ingham, 2003; Kawase et al., 2004; Schulz et al., 2004). A further accepted difference between the two gonadal tips concerns the origin of the thin somatic cells surrounding the developing cysts. Contrary to the reported quiescence of female IGS cells, the somatic cells surrounding male cysts descend from stem cells known as "cyst progenitor cells" that are interspersed between the GSCs and that maintain their own attachments to the hub (Hardy et al., 1979; Gonczy and DiNardo, 1996; Figure 1B). Thus, the male GSC niche con-

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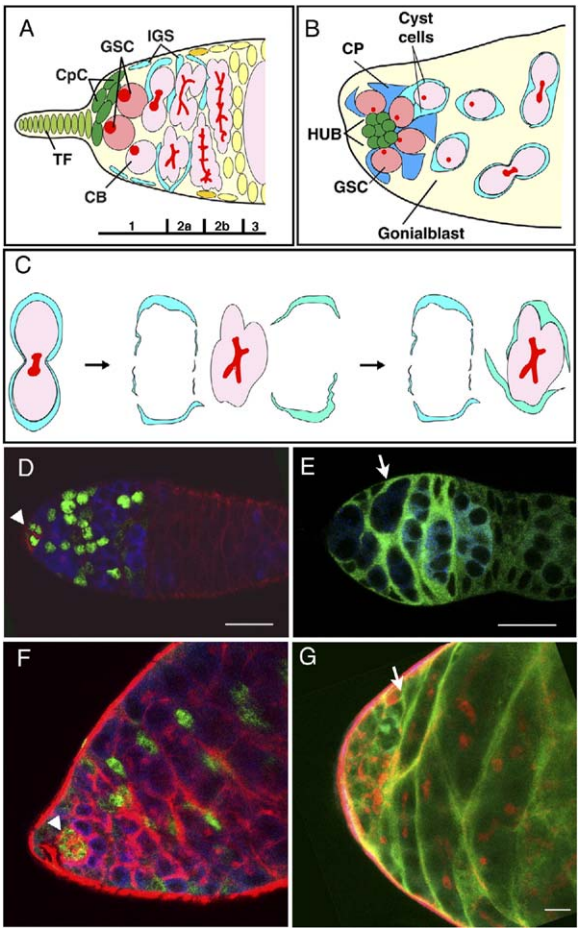

Figure 1. Encapsulation of Forming Cysts in Ovariole and Testis

(A) A schematic drawing of a *Drosophila* germarium showing germline cells (pink) including the GSC, cystoblast (CB), and developing cysts. Somatic cells include terminal filament (TF; green), cap cells (CpC; green), IGS cells (blue), stem cells (gold), spectrosome/fusome (red), and follicle cells (yellow).

(B) A schematic drawing of a *Drosophila* male testis showing the hub (green), germline (pink), cyst progenitor cells (CP; blue), spectrosome/fusome (red), and developing cysts.

(C) Model of stationary IGS cells showing the need for displacement or breakage of the IGS cells as the germline cysts move along the germarium.

(D) *Drosophila* germarium from line PZ1444 which directs β-galactosidase expression in cap (arrowhead) and IGS cell nuclei (β-gal, green; Vasa, blue; actin, red). Cap cells are distinguished from IGS cells by location and abundant actin staining.

(E) *fax*-GFP expression in IGS cell cytoplasm. The extensions that wrap developing cysts are labeled (arrow; GFP, green; Vasa, blue).

(F) *Drosophila* testis from line PZ1444 which directs β-galactosidase expression in hub (arrowhead) and somatic cyst cell nuclei; compare with (D) (β-gal, green; Vasa, blue; actin, red).

(G) *fax*-GFP staining in testis somatic cyst cell cytoplasm; compare with (E). The extensions that wrap developing cysts are labeled (arrow; GFP, green; actin, red). The scale bars for (F) and (G) are the same. The scale bars represent 10 μm.

tains two types of stem cells, while the female niche is thought to contain only one.

The idea that ovarian cystoblasts interact with stationary IGS cells rather than newly generated, mobile somatic cyst cells as in the testis presents several difficulties. Permanent IGS cells would have to stretch and periodically break their cytoplasmic processes to allow cysts to move and contact more posterior IGS cells (Figure 1C). How would permanent IGS cells keep pace with changes in the size and number of cysts within the anterior germarium that occur as a function of age (Xie and Spradling, 2000)? Finally, how can this model be reconciled with the fact that IGS cells are labeled quite frequently in lineage tracing experiments under conditions where cyst numbers are not changing (Margolis and Spradling, 1995)?

To address these questions, we reinvestigated IGS cell behavior using lineage labeling. We find that a distinct subset of 12–18 IGS cells, which we term "escort cells," are maintained by 4–6 terminally located "escort stem cells" (ESCs) that extensively contact GSCs within the niche. Escort stem cells strongly resemble the cyst progenitor cells of the testis in morphology, location, and behavior. Following a single division, escort stem cell daughters move with cysts through the germarium until they are lost by apoptosis and replaced by follicle cells. Moreover, we find that, as in the testis, JAK/STAT signaling is essential for the maintenance and division of both the GSCs and ESCs. Our findings reveal striking similarities in the cellular organization at the apex of both male and female gonads, and show that the anatomy and regulation of male and female germline stem cell niches are more similar than previously believed.

Results

IGS Cells Resemble Testis Cyst Cells

We used gene markers expressed in IGS cells to study their morphology and to look for evidence of stretching and breakage. We found that a *failed axon connections*-GFP (*fax*-GFP) strain (see Experimental Procedures) strongly labels the IGS cell cytoplasm (Figure 1E), and observed that IGS processes do not angle toward the posterior, as expected if cysts moved past stationary IGS cells (Figure 1C). Instead, the morphology of these cells appeared very similar to that of the testis somatic cyst cells (Figure 1G). We also noticed parallels in gene expression between testis cyst cells and ovarian IGS cells that held for this and several other tested markers. For example, the lacZ enhancer trap PZ1444 (Kai and Spradling, 2003) labels the cap and IGS cell nuclei in the ovary (Figure 1D) as well as the hub and somatic cyst cell nuclei in the testis (Figure 1F). These observations motivated us to reexamine whether IGS cells, like cyst cells in the testis, are a stem cell-based population that migrates along with cysts.

IGS Cells Cycle and Move Posteriorly

In order to address whether IGS cells are maintained by stem cells, we followed their cell lineage using integrated FLP/FRT constructs that generate random lacZ-positive cells following a brief heat shock (Harrison and Perrimon, 1993; Margolis and Spradling, 1995). IGS cells are known to divide sporadically (Margolis and

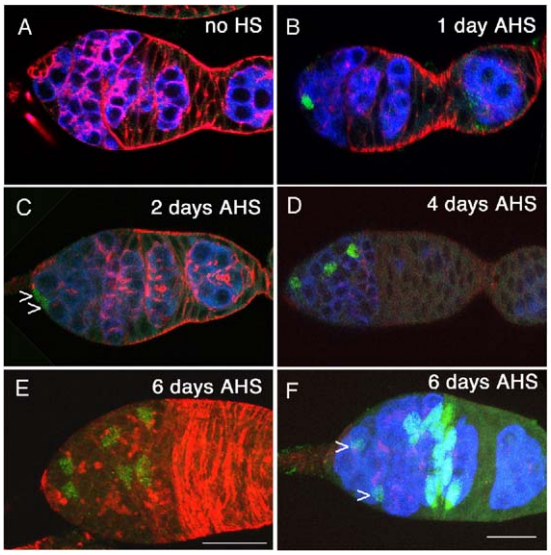

Figure 2. Lineage Analysis of Randomly Marked Dividing Cells

Ovaries were stained for β-galactosidase (β-gal) expression a variable number of days after heat shock (AHS) to induce FRT-mediated recombination.

(A) No heat-shock treatment, no β-gal-positive cells are observed (in this and all subsequent pictures, β-gal is in green, Vasa in blue, and actin in red).

(B) One day AHS.

(C) Two days AHS. Nuclei juxtaposed probably belong to the same clone (arrowheads).

(D) Four days AHS.

(E) Six days AHS. A putative double clone (no Vasa staining performed).

(F) Six days AHS. Two labeled IGS cells in region 1-2a (arrowheads) accompanied by an SSC clone. The scale bars represent 10 μm.

Spradling, 1995; Song and Xie, 2002) and we sought to distinguish several possible sources for such cells. First, quiescent IGS cells might occasionally be stimulated to divide and replenish a recently lost neighbor. Second, undifferentiated SSC daughter cells might respond to a signal from dying IGS cells and migrate anteriorly to replace them. Finally, there might exist a small population of IGS stem cells whose progeny maintain the IGS cell population in response to cell movement out of region 1.

FLP recombinase was activated briefly by a short heat shock, and ovaries were stained at various times after heat shock (AHS) to reveal the number and location of β-gal-positive IGS cells. Ten different experiments were carried out and a total of 535 germaria were analyzed (see Experimental Procedures). The same results were obtained using 5 day old or 10 day old females. Somatic cells in region 1 and region 2a were recorded as IGS cells, but more posterior cells that contact a "lens-shaped 16-cell cyst" were regarded as part of the SSC lineage. Germaria containing labeled IGS cells in regions 1 and 2a were extremely common (>80%) at all times after heat shock (Figures 2B and 2C), but absent in non-heat-shocked controls (Figure 2A). The labeled cells we scored as IGS cells are located anterior to the SSCs and can be distinguished from prefollicle cells even when an SSC clone is also present (Figure 2F). At 1 day AHS, one to two anterior labeled somatic cells were observed, and these were almost always located near the cap cells (Table 1; Figure 2B). By 2 days AHS the average number of labeled IGS cells increased to about three cells, and this value changed very little thereafter (Figure 3A), even after 2–3 weeks (not shown). The labeled cells usually consisted of a cell near the cap cells, along with either one or two more posterior cells (Figure 2D). Less frequently, up to about six cells were labeled, possibly due to the induction of two clones in the same germarium (Figure 2E). These studies show that at least some IGS cells divide regularly during normal oogenesis.

The total number of IGS cells does not increase significantly during adult life (Xie and Spradling, 2000), and somatic cell apoptosis is rare within region 1-2a (Margolis and Spradling, 1995; Drummond-Barbosa and Spradling, 2001). Because labeled cells were first observed near the anterior of the germarium, and only later seen in region 2a, the daughters of an anterior division must move toward the posterior. Therefore, the dividing cells likely exist to balance posterior IGS cell movement.

Cycling IGS Cells Do Not Exit Region 2b

By observing germaria lacking SSC clones (and old enough that transient follicle cell clones have departed), we found that labeled IGS cell daughters do not move past the region 2a/2b boundary (Figures 2D and 2E). Thus, labeled IGS cells are never observed very far past the position of the SSCs and do not give rise to other types of somatic cells found in region 2b and beyond. Consequently, the labeled cells must turn over within this region. Consistent with this, a small number of somatic cells have been observed previously to enter apoptosis near the region 2a/2b junction (Drummond-Barbosa and Spradling, 2001).

To examine directly where IGS cells turn over, we stained wild-type germaria for apoptotic cells using a TUNEL labeling assay (ApopTag). Few if any IGS cells underwent apoptosis in region 1 or early region 2a. However, single somatic cells positive for the apoptosis marker were regularly observed (16% of germaria) near the boundary of regions 2a and 2b (Figures 3B and 3C). These cells were always located near the surface of the germarium. By the time the apoptotic cells were positive in this assay they stained poorly with cell-type markers, and thin cytoplasmic extensions could not be seen. However, the location and frequency of apoptotic somatic cells support the idea that IGS cells turn over early in region 2b, as cysts acquire a layer of prefollicle cells.

Dividing IGS Cells Are Not Derived from SSCs

We considered the hypothesis that dividing IGS cells derive from the SSCs located at the region 2a/2b junction. If SSC daughter cells can migrate anteriorly from their birth sites near the region 2a/2b junction to replenish IGS cells, then germaria containing an SSC clone should more frequently include labeled IGS cells than germaria without such a clone, and should accumulate labeled IGS cells with time. However, we found no difference in the number of labeled IGS cells in germaria

Table 1. Lineage Analysis of Somatic Clones in the Anterior Germarium

Days AHS	1	2	4	6
Germaria analyzed	44	14	12	36
Percent with labeled IGS	79	86	83	89
Number of IGS cells labeled/germaria	2.17 ± 1.2	2.6 ± 1.3	2.5 ± 1.3	3.00 ± 1.5
Percent of labeled germaria with IGS cells at the tip	97	80	80	97

Flies were heat shocked for 60 min and germaria were dissected and analyzed at the indicated times thereafter. A labeled IGS was considered to be at the tip if it contacted cap cells or GSCs. The number of labeled IGS cells/germaria corresponds to the number of such cells in region 1-2a. Labeled cells in region 2a-2b that might have been IGS or derived from SSC were excluded from counts.

lacking or containing SSC clones, even 6 days or more after heat shock (Figure 3E). Moreover, there was no increase in IGS cell labeling with time, even several weeks AHS (Figure 3D) or in germaria that contained two labeled SSCs (Figure 3F).

Dividing IGS Cells Are Maintained by Stem Cells Located near the Cap Cells

Because the dividing IGS cells do not derive from SSCs, their progenitors evidently reside within the IGS cell population. The inferred posterior movement of labeled cells strongly suggests that these progenitors are the anterior cells located near the GSCs and cap cells in each clone. This would explain why we usually saw at least one labeled cell near the cap cells 1 day after heat shock, and in a similar position among the two to three cells within the clones at all later times AHS (Table 1).

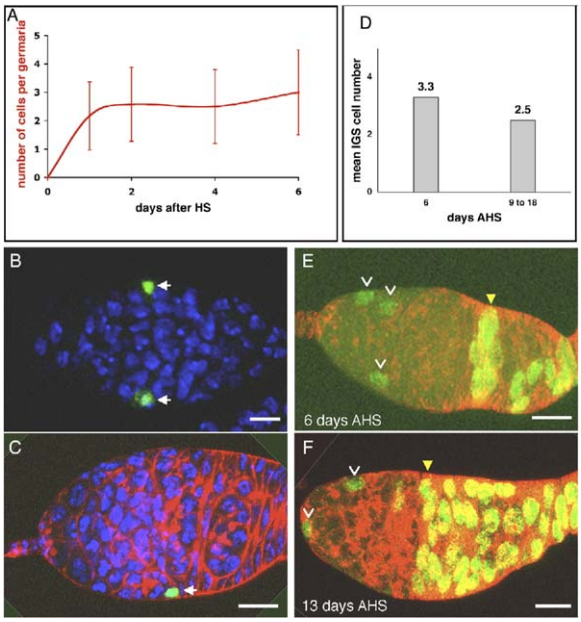

Figure 3. IGS Cell Lineage and Posterior Movement

(A) The mean number of labeled IGS cells in region 1-2a plateaus by 2 days AHS, indicating the absence of transit divisions.

(B and C) TUNEL staining of wild-type germaria. Somatic cell(s) undergoing apoptosis (arrows) near the region 2a/2b junction. Somatic cell apoptosis was not observed in region 1-2a (ApopTag, green; DAPI, blue; actin, red).

(D) The mean number of labeled region 1-2a IGS cells from germaria also containing one or two SSC clones at 6 days and 9–18 days AHS. Despite the presence of labeled SSC(s) and their progeny, labeled IGS cells do not increase with time.

(E) Only three IGS cells (open arrowheads) are present 6 days AHS, despite the labeled SSC clone (yellow arrowhead).

(F) Only two IGS cells (open arrowhead) are present 13 days AHS, even though both SSCs are labeled (yellow arrowhead; β-gal, green; actin, red). The scale bars represent 10 μm.

The IGS progenitors might function as true stem cells by dividing to produce one daughter cell that remains as a stem cell and one that moves posteriorly to become an IGS cell. Alternatively, the progenitors might sometimes divide to produce two progenitor cells and other times divide to produce two IGS cells. These two possibilities can be distinguished by examining whether the clonal labeling patterns depend on the fraction of IGS progenitors that are labeled (see Margolis and Spradling, 1995). We found that the pattern of IGS cell labeling did not depend on the labeling frequency (see Figure S1), indicating that the IGS progenitors are true stem cells.

We can also conclude that the daughters of the IGS cell progenitors differentiate without further division. During the approximately 2 days required for a labeled daughter cell to move to region 2a-2b, its labeled progenitor sometimes does not divide again, generating a stem cell clone of two cells (Figures 2F, 3F, and S1). When a second division does occur prior to the loss of the first daughter by apoptosis, the clone size reaches three (Figure 2D). Consistent with this, only IGS cells positioned at the tip of the germarium labeled with cell cycle markers (BrdU, cyclinB); however, they do so at a low frequency (1/196 germaria for BrdU, 1/22 germaria for cyclin B). This may be due to cell impenetrability to these reagents or more likely to difficulties in detection given the thin cytoplasm of these cells.

These results demonstrate the existence of a previously unrecognized somatic stem cell lineage within germarium regions 1 to early 2b. These cells move from the vicinity of the cap cells to region 2b in about 2 days, very similar to the transit time of germline cysts through the same region. Consequently, we propose that the squamous IGS cells move posteriorly along with associated germline cysts and are actively maintained by anterior stem cells. For this reason, we name these cells “escort cells” and refer to their progenitors as “escort stem cells” (ESCs).

Escort Stem Cells Are Morphologically Distinctive and Resemble Testis Cyst Progenitor Cells

Analyzing the location of the anterior-most cells in labeled escort cell clones positioned the ESCs adjacent

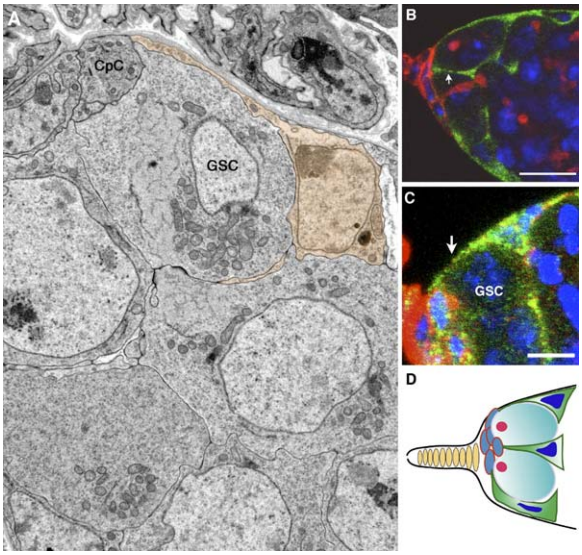

Figure 4. Escort Stem Cells Are Morphologically Distinctive

(A) A transmission electron micrograph showing the tip of a germarium. An ESC (orange pseudocolor), showing its cytoplasmic extension that wraps a GSC and contacts its cap cell. See also Supplemental Data.

(B) A *fax*-GFP-labeled germarium visualized by confocal microscopy reveals a GFP-labeled ESC (arrow) between two GSCs.

(C) A MAP-kinase-labeled germarium reveals an ESC extension surrounding a GSC and contacting the cap cell (compare with [A]; MAP-K, green; spectrin, red; DAPI, blue).

(D) Model showing the anatomical relationships between cap cells (blue), GSCs (light green), and ESCs (dark green). Filled red circles represent spectrosomes. The scale bars represent 5 μm.

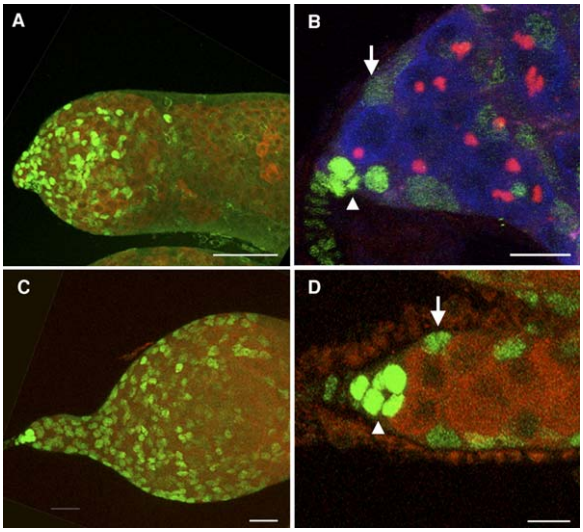

Figure 5. Escort Cells but Not ESCs Respond to Altered Cyst Development

(A) A germarium from an *Sxl*^{f4} female homozygous for PZ1444. Cyst formation is incomplete and the numbers of germ cells (Vasa, red) and escort cells (β-gal, green) are greatly increased.

(B) Cap cells (arrowhead), GSCs, and ESCs (arrow) at the tip of an *Sxl*^{f4} ovariole are normal (Vasa, blue; β-gal, green; Hts, red).

(C) A germarium from a *bam*$^{\Delta 86}$ female heterozygous for PZ1444. Cyst formation is blocked and the numbers of germ cells (Vasa, red) and escort cells (β-gal, green) are greatly increased.

(D) Cap cells (arrowhead), GSCs, and ESCs (arrow) at the tip of a *bam*$^{\Delta 86}$ germarium are normal (Vasa, red; β-gal, green). The scale bars represent 20 μm (A), 10 μm (C), and 5 μm (B and D).

to the GSCs and cap cells. Examination of the somatic cells in this location by fluorescence and electron microscopy revealed that ESCs are structurally distinctive and arranged in an organized manner. Within each germarium, between four and six (5.2 ± 0.7) elongated, thin ESCs surround and extensively contact their adjacent GSCs (Figures 4A–4C, arrows). Characteristic features of all such cells are extensions as long as 12 μm, often quite thin, which contact cap cells adjacent to their junction with the GSCs (Figures 4A and S2). The distinctive shape of the ESCs, their contact with GSCs and cap cells, their single division lineage, and their production of cells that envelope developing cysts are all strikingly similar to the somatic cyst progenitor cells of the testis (compare Figures 4D and 1B). Further data supporting the model in Figure 4D are presented in Figure S2.

The finding that ovariole tips contain 4–6 ESCs allowed us to estimate the total number of escort cells supported by the ESCs from our clonal data. The total cell population at steady state supported by 6 ESCs would be about 18 cells (number of stem cells times number of cells per clone) or perhaps slightly higher, because IGS cells in region 2b were not scored if they could be confused with prefollicle cells. Because 5–6 cysts are found on average in region 1-2a, 12 escort cells would provide for about 2 escort cells per cyst. However, we were unable to release individual cysts with two associated escort cells from dissected germaria, as can be done in the testis. Consequently, the escort cells may link the cysts in region 1-2a into larger units, or even into a single cellular network that moves progressively to the posterior.

Eighteen escort cells is lower than the 30 ± 3 IGS cells defined using the PZ1444 enhancer trap. Thus, escort cells likely represent a majority but not all of the PZ1444-positive cells in wild-type germaria. Further studies will be required to verify the existence of this second class of IGS cells, to identify specific markers, and to determine whether these cells permanently differentiate during ovarian development, like cap cells.

ESCs Are Not Affected by Disrupting Early Germ Cell Development

To determine whether the organization and activity of ESCs was affected by changes in early germ cell differentiation, we analyzed several mutations that disrupt cyst development. The adult viable *Sxl* ^{f4} allele blocks germ cell development at the cyst stage, leading to a substantial accumulation of arrested cysts and escort cells (Figure 5A). Despite this, no change in the architecture of the tip region of the germarium was observed (Figure 5B). Evidently, ESCs continue to divide and their daughter escort cells remain closely associated with the incomplete cysts that accumulate in *Sxl* ^{f4} germaria (Figure 5A).

To examine the effect of disrupting germ cell devel-

opment at an even earlier stage, we studied *bam*$^{\Delta 86}$ females (Figure 5C). A normal complement of ESCs (Figure 5D) was found to surround the GSCs which remain at the tip of *bam*$^{\Delta 86}$ mutant germaria (Kai and Spradling, 2003). These germaria contain an average of 402 ± 139 GSCs accompanied by 178 ± 83 escort cells. The large increases in the number of escort cells in both the *Sxl*^{f4} and *bam*$^{\Delta 86}$ germaria indicate that escort cell turnover is not simply programmed by their age or position in the germarium, but is linked to the continued development of the accompanying germ cells.

The Ovarian Niche Requires JAK/STAT Signaling

Male and female germ cell niches are revealed by these studies to be strikingly similar in cellular architecture. Consequently, we examined whether the JAK/STAT pathway, the major signaling pathway controlling male GSCs and cyst progenitor cells, might also play a previously unappreciated role in the female GSC niche. Previous studies established that JAK/STAT signals were necessary for polar cell specification and follicle formation (Silver and Montell, 2001; Ghiglione et al., 2002; Baksa et al., 2002; McGregor et al., 2002). However, a role for JAK/STAT signaling within region 1 of the germarium has not been reported. In *Drosophila*, JAK/STAT signaling initiates when the ligand *unpaired* (*upd*; or its close relatives) binds to the *domeless* receptor and activates *hopscotch* (the Janus kinase). The JAK kinase in turn phosphorylates the *Stat92E* transcription factor, which translocates to the nucleus (reviewed in Rawlings et al., 2004). The *Stat92E*^{06936} lacZ enhancer trap is strongly expressed in the cap and IGS cells, but only weakly in prefollicle cells (Figure 6A; Silver and Montell, 2001; Baksa et al., 2002).

To study whether this pathway has a role in the ovarian niche, we shifted temperature-sensitive *Stat92E*^{06936}/*Stat92E*^{F} (*Stat92E*^{ts}) females to 30°C, conditions that drastically reduce STAT function (Baksa et al., 2002). GSCs, defined by their cap cell contact and rounded spectrosome, fell in number from about 2 per ovariole at day 0, to an average of 1.3 per ovariole at day 2, and to just 0.6 per ovariole at day 6 (Figures 6B–6D). By this time, only 9% of ovarioles still retain 2 anterior germ cells, while nearly half (48%) lack GSCs entirely (Figure 6C). Even the remaining anterior germ cells may no longer function as GSCs, as their spectrosomes no longer associate with cap cells (Figure 6B, asterisk) and new cyst production appears to be blocked. Conversely, when we increased JAK/STAT signaling by driving the ligand *upd* in germarial somatic cells using the c587-GAL4 driver, the number of cysts increased, and the germarium became disorganized (Figure 6E). Rare ovarioles (3.5%) became completely filled with stem-like cells (Figure 6F). These tumors resemble those reported in the testis following *upd* overexpression (Tulina and Matunis, 2001). Thus, in addition to previously reported effects (Baksa et al., 2002), JAK/STAT signaling is required to maintain GSCs in the niche and high levels can stimulate them to proliferate.

To determine whether the JAK/STAT pathway also influences the structure and behavior of ESCs, we carried out similar experiments using ESC markers. We found that anti-MAP-kinase antibody labels escort and follicle

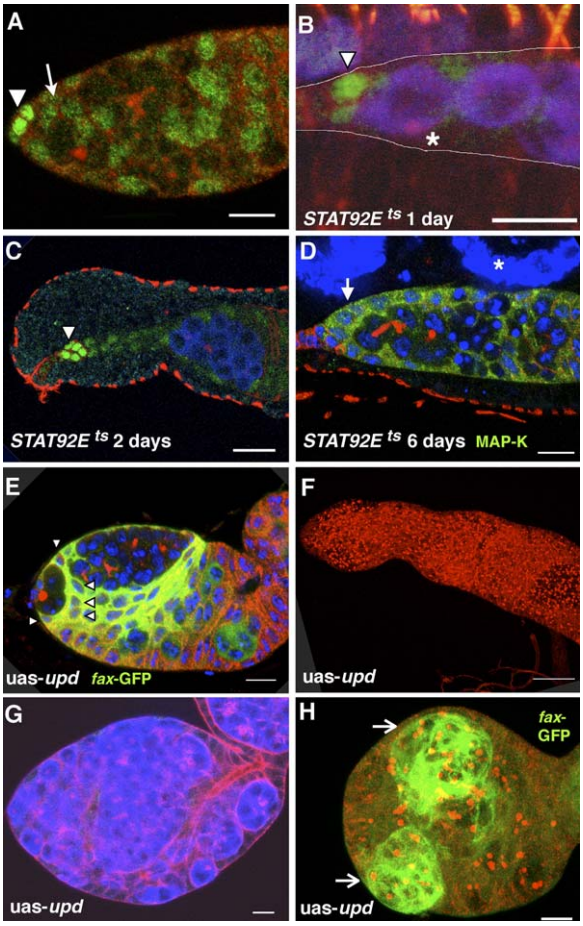

Figure 6. JAK/STAT Mutations Affect Stem Cell Maintenance and Germarium Structure

(A) A germarium from the *STAT92E*^{06936} lacZ enhancer trap showing expression in cap cells (arrowhead) and IGS cells (arrow) (β-gal, green; actin, red).

(B) A germarium from a *STAT92E*^{06936}/*STAT92E*^{F} female shifted to 30°C for 1 day; one GSC remains associated with the cap cell cluster (arrowhead). Note the unusual position of the spectrosome (*) (β-gal, green; actin, red; Vasa, blue).

(C) A germarium from a *STAT92E*^{06936}/*STAT92E*^{F} female shifted to 30°C for 2 days. The cap cells are clustered (arrowhead) and GSCs have been lost (β-gal, green; actin, red; Vasa, blue).

(D) MAP-kinase staining of *STAT92E*^{06936}/*STAT92E*^{F} female after 6 days at 30°C showing altered ESC morphology (arrow; MAP-K, green; 1B1, red; DAPI, blue). "*", polyploid sheath nucleus.

(E–H) *upd* overexpression driven by c587-GAL4.

(E) An increased number of ESCs (arrowheads) contacting GSCs and projecting extensions toward the tip within a single section are revealed by *fax*-GFP. Five additional ESCs are found in other sections.

(F) The swollen germarium contains hundreds of single germ cells and partial cysts (fusomes [Hts], red).

(G) The germarium is wider than normal and contains an increased number of abnormally organized germline cysts (Vasa, blue; actin, red).

(H) A stack view of a germarium overexpressing *upd* reveals two organizing centers (arrows) containing escort cells marked with *fax*-GFP and fusome-containing germ cells (arrows; GFP, green; 1B1 marks fusomes, red). The scale bars represent 10 μm except for (F), where it represents 50 μm.

cell membranes (Figure 4C). Within a few days after shifting *STAT92E^{ts}* females to 30°C, ESCs and their progeny lost their extended shape, and formed an epithelial monolayer surrounding the remaining aggregated germ cells (Figure 6D). After 6 days, an average of only 2.3 somatic cells remained at the tip, and these lacked the normal morphology of ESCs. Conversely, up to 11 ESCs (mean = 8.2, n = 6) were observed contacting the GSCs and extending projections toward the cap cells in ovarioles subjected to *upd* overexpression (Figure 6E). Therefore, JAK/STAT signaling controls the morphology and proliferation of ESCs as well as GSCs.

Anterior Germarium Structure Depends on JAK/STAT Signaling

These experiments further suggested that JAK/STAT activity controls the shape of the germarium independently from its effects on GSC and ESC numbers. Following a shift to the restrictive temperature, the space between the basement membrane and the muscle sheath surrounding the ovariole swells dramatically (Figure 6C). Within 2 days, the ovariole shrinks in diameter within the sheath, while the cap cells tightly cluster but do not change in number. The vacated region fills with amorphous material (Figures 6C and 6D), and some sheath-associated cells undergo extra endocycles to form giant polyploid nuclei (Figure 6D, asterisk).

Different effects are seen when *upd* expression is driven within IGS cells using the c587 GAL4 line. The diameter of the anterior germarium increases and more than two cysts (the usual limit) span the width of the germarium (Figure 6G). Frequently, the ovarioles take on a "double" appearance, with two tips (Figure 6H) and two parades of downstream cysts within a single sheath. It is known that the c587 driver has two centers of maximal expression on opposite sides of the 2a-2b region in normal germaria (D. Drummond-Barbosa and A.C.S., unpublished data). Thus, changes in JAK/STAT signaling alter ovariole morphology and influence the number and location of cell proliferation centers.

Stat92E Is Required in the Escort Cell Lineage

In order to address the specific cell requirements of JAK/STAT signaling, we examined germaria that had been clonally made homozygous for strong *Stat92E* alleles. Loss of STAT activity in germ cells, indicated by the absence of GFP, did not affect normal development (n = 24; Figure 7A). Similarly, germaria bearing SSC clones did not present any of the phenotypes associated with the *Stat92E^{ts}* mutants and were morphologically wild-type in regions 1 and 2a (n = 17; Figure 7B). However, cysts containing mutant follicle cells (i.e., lacking GFP) frequently did not become properly encapsulated, as expected (McGregor et al., 2002; Baksa et al., 2002). Thus, neither the GSC nor the SSC lineages appear (at least alone) to explain the requirement for *Stat92E* function in the anterior germarium.

Eight days after clone induction, some germaria did contain defects very similar to those observed previously in the *Stat92E^{ts}* mutant at restrictive temperature. Most of these (33 out of 39) exhibited multiple escort cells that lacked GFP, indicating the presence of *Stat92E*-deficient clones in two or more ESCs. (The six exceptional germaria likely contained ESCs lacking GFP that were hidden from view by the angle of mounting.) Near the site of the mutant ESCs and escort cells, germaria were distorted, lacked normal patterns of developing cysts, and were surrounded by an expanded muscle sheath delimiting amorphous material (Figures 7C and 7D). Germaria with *Stat92E* mutant ESCs maintained a reduced number of GSCs that frequently contained mislocalized spectrosomes (Figures 7C and 7D). These results show that *Stat92E* is required in the ESCs and escort cells to maintain the normal organization of the anterior germarium and its surrounding sheath, and to prevent premature GSC loss.

Discussion

The Female GSC Niche Contains a Second Type of Stem Cell

Our studies reveal that a previously unknown type of stem cell, escort stem cells, closely contacts the GSCs within the niche at the tip of each *Drosophila* ovariole. ESC daughters encase newly produced cystoblasts and remain tightly associated as they grow into 16-cell cysts and enter meiosis. The existence of thin somatic cells that interact with early female germ cells was known previously. However, these inner germarium sheath cells were believed to divide only rarely, and to remain attached in place along the wall of the germarium. The discovery that escort cells are a dynamic cell population supported by distinctive stem cells provides essential information for the further study of early germ cell differentiation, including the processes of GSC regulation, cyst formation, oocyte determination, meiosis, and germline sex determination. All these events take place between the stem cell stage and early region 2b, where we find that germline cysts lose their escort cells by apoptosis and acquire a follicle cell covering.

Escort Cells Resemble Testis Somatic Cyst Cells

Our findings indicate that the cellular architecture within which early germ cell development occurs is more similar between males and females than previously appreciated. ESCs resemble testis cyst progenitor cells, which are precursors of the two thin somatic cyst cells that surround each developing male 16-cell cyst. There are already indications that escort and somatic cyst cells are involved in common processes and express similar genes (Figure 1). Early male and female germ cells may send common signals to these overlying somatic cells, because both require the activity of *stem cell tumor* (*stet*), a rhomboid-like membrane protease that activates Egfr ligands in the signaling cell (Tran et al., 2000; Kiger et al., 2000; Wasserman et al., 2000; Schulz et al., 2002; Figure 4C). Reciprocal signals between germline and escort or somatic cyst cells may terminate cyst growth (Matunis et al., 1997), prevent reversion toward the stem cell state (Kai and Spradling, 2004; Brawley and Matunis, 2004), and mediate germline sex determination (reviewed in Oliver, 2002). Thin somatic cell processes also surround mouse ovarian germ cells as they form interconnected cysts and enter meiosis (Pepling and Spradling, 1998), suggesting that

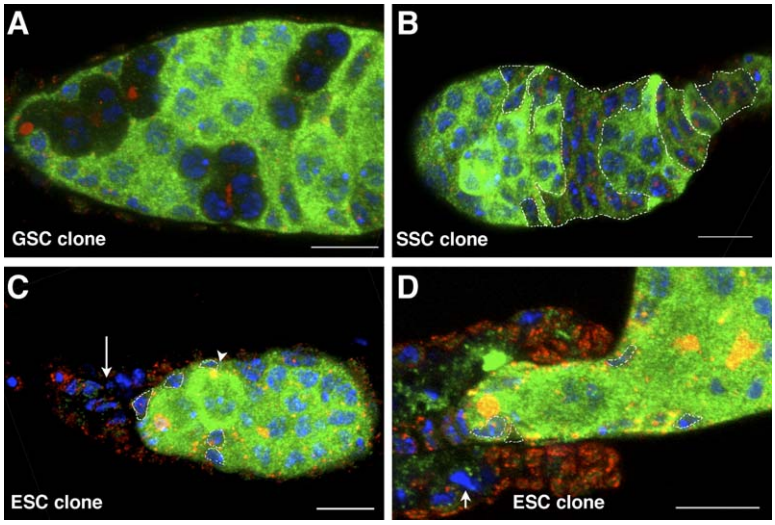

Figure 7. STAT Is Required Cell Autonomously in ESCs but Not in GSCs

Germaria bearing GSC, SSC, and ESC clones of *Stat92E*^{j6C8} were stained with antibodies for GFP (green), HTS (red), and DAPI, 8 days after clones were induced. Similar results were obtained with *Stat92E*^{0634}.

(A) A *Stat92E* GSC clone shows no phenotype indicating that the GSC loss defects seen in the *Stat92E*^{ts} allele are non-cell autonomous.

(B) An SSC clone shows defects in region 3 and posterior to it but displays a normal germarial tip. White dots mark the somatic mutant cells.

(C) Double ESC clone (white dots) shows phenotypes reminiscent of the *Stat*^{ts} allele. Regions 1 and 2 are greatly disorganized, the muscle sheath is separated from the germarium (arrow), and spectrosomes can be seen mislocalized (arrowhead).

(D) Double ESC clone, in this case beside the loosened sheath, extrachromosomal material can be seen between the germarium and the sheath (arrow), and the shrunken tip has only one GSC.

some functions mediated by escort-like cells may occur during vertebrate as well as invertebrate gametogenesis.

Escort Cells Require JAK/STAT Signals to Maintain the Structure and Function of the Anterior Germarium

We found that JAK/STAT signaling is required during early female gametogenesis, as it is in the male. Moreover, clonal analysis showed that the escort lineage is a major mediator of this requirement. When escort cells (but not germ cells or follicle cells) lack STAT activity, the shape of the germarium tip, and the organization of its epithelial and muscle sheath, is greatly altered. The sheath swells and fills with material whose nature remains unknown. Moreover, large polyploid nuclei arise in this region, either by division of normal sheath cells or possibly by migration from another location within the ovariole. Escort cells line the outer surface of the anterior germarium and may exert these influences through changes in their cytoskeletal organization, by altering the structure of the basement membrane, or through relay signals to the affected cell types.

GSCs were also subject to a STAT-dependent influence from escort cells. When their associated escort cells lacked STAT activity, one or both GSCs lost their attachment to cap cells, failed to maintain their spectrosomes in an anterior orientation, and ceased division. GSCs and ESCs contact each other over a large portion of their surfaces, but it is not known what direct signals and adhesive interactions occur. We found that ESCs divide in a manner that is at least generally coordinated with the rate of cyst production. Even when later stages of cyst development are disrupted, as in *Sxl*^{f4} and *bam*$^{\Delta 86}$ germaria, the number and morphology of ESCs is little changed. The stem cell-stem cell interactions likely act in both directions, because ESCs are absent in agametic germaria, and existing ESCs are lost within a few days after forced GSC differentiation (Margolis and Spradling, 1995; Xie and Spradling, 2000; Kai and Spradling, 2003).

Male and Female GSC Niches Are More Similar Than Previously Recognized

Strong similarities have long been recognized between niches that support GSCs in ovarioles and at the tip of the testis. In both sexes, GSCs form tight, cadherin-based junctions with cap or hub cells, and undergo mostly asymmetric divisions to yield cyst-forming cells that embark on a program of rapid, incomplete divisions. The structure and behavior of fusomes and ring canals during GSC divisions are highly similar (de Cuevas and Spradling, 1998; Yamashita et al., 2003). However, it has previously been thought that differences in anatomy, such as the presence of cyst progenitor cells only in the testis, and in distinct regulatory signals, BMP (Xie and Spradling, 1998) versus JAK/STAT (Tulina and Matunis, 2001; Kiger et al., 2001), indicated that these niches displayed fundamental differences. Recently, however, BMP signaling was shown to be required in male as well as female GSCs (Shivdasani and Ingham, 2003; Kawase et al., 2004; Schulz et al., 2004), and to act by repressing *bam* expression. Now, by showing that ovariole tips contain ESCs analogous to cyst progenitor cells, and require JAK/STAT signaling, our studies strongly suggest that male and female GSC niches develop and operate using fundamentally similar cells and regulatory mechanisms. Remaining differences include the fact that in male GSCs, STAT activity is required cell autonomously (Tulina and Matunis, 2001; Kiger et al., 2001). Moreover, *bam* expression is required to initiate cyst development in females, but not males.

Germline Sex Determination and Sexually Dimorphic Gonad Development

Sex-specific gonadal morphology ultimately arises due to autonomous aspects of germline sex determination

that first manifest during embryogenesis (Janzer and Steinmann-Zwicky, 2001), and to differences in the structure of the embryonic male and female somatic gonad (DeFalco et al., 2003, 2004). Recently, a major difference between the male and female gonads during early larval development was shown to be the male-specific expression of JAK/STAT ligand(s) in anterior somatic cells and the consequent activation of JAK/STAT signaling within male but not female germ cells (Wawersik et al., 2005). Induced activation of JAK/STAT signaling in female germ cells at this time was sufficient to elicit male-specific germ cell responses such as cell division (Wawersik et al., 2005).

Differing levels of JAK/STAT and BMP signaling during the development of male and female gonads may program and pattern the corresponding GSC niches, prior to the emergence of significant autonomous germ cell sexual differences. We found that the level of JAK/STAT signaling affects the overall shape of the distal end of the gonad, possibly through interactions with muscle and sheath cells. Elevated levels of *upd* expression produce a more testis-like ovariole, with a rounded tip containing cysts that move away in a less-ordered manner. Reduction of STAT activity generates a hyper thin ovariole within an abnormal organization of its sheath. The number of ESCs near the cap cells increases when JAK/STAT signaling is elevated, and decreases when it is reduced. All these observations suggest that the location and strength of JAK/STAT signaling within the somatic cells of the developing gonad help program its morphology and niche structure. Our findings open the way for a detailed analysis of how male- and female-specific niche development is programmed.

Experimental Procedures

Drosophila Strains and Culture

Fly stocks were maintained at 22°C–25°C on standard medium. X-15-29, X-15-33, and hs-FLP (flipase) strains used for clonal analysis are described in Harrison and Perrimon (1993). *failed axon connections*-GFP (*fax*-GFP) was a generous gift from Dr. Michael Buszczak. PZ1444 is a lacZ enhancer trap line expressed in cap and IGS cells (Margolis and Spradling, 1995; Xie and Spradling, 2000; Kai and Spradling, 2003); PZ1444 heterozygotes were used to determine IGS cell number. c587 is a GAL4 driver line expressed in cap and IGS cells that has been described (Kai and Spradling, 2003). Mutant mitotic clones were generated according to Xu and Rubin (1993). Females of genotypes hs-FLP; FRT82B, Ubi-GFP/*Stat92E*^{j6C8} FRT82B or hs-FLP; FRT82B, Ubi-GFP/*Stat92E*^{0634} FRT82B were produced by standard crosses. Four-day-old females were heat shocked for 1 hr at 38°C three times, about half a day apart. Flies were transferred to fresh food and kept at 25°C for 8 days, to ensure that clones analyzed were stem cell clones. The same results were obtained for both *Stat92E* alleles. Other genes and balancer chromosomes used are described in FlyBase (http://flybase.bio.indiana.edu).

Generation and Analysis of IGS Cell Clones

Mitotic clones were generated according to Margolis and Spradling (1995). Further details can be found in Supplemental Data.

Immunostaining and Fluorescence Microscopy

Immunofluorescence was carried out as previously described (Drummond-Barbosa and Spradling, 2001). For activated MAP-kinase, ovaries were dissected in buffer B with the addition of phosphatase inhibitors (16.7 mM KH_2PO_4/K_2HPO_4, 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$ [pH 6.8], 50 mM NaF, 1 mM $NaVO_4$, 10 mM β-glycerophosphate) and fixed in the same medium plus 5% formaldehyde (Ted Pella, Redding, CA). Primary antibodies were diluted as follows: rabbit anti-β-galactosidase (1:3000; Cappel, Irvine, CA), mouse anti-β-galactosidase (1:200; Promega, Madison, WI), rabbit anti-GFP (1:5000; Torrey Pines Biolabs, Houston, TX), mouse anti-*Drosophila* 1B1 (specific for Hts protein; 1:100), rabbit and rat anti-*Drosophila* Vasa (1:2000 and 1:200, respectively; gift of Dr. Paul Lasko), rabbit anti-*Drosophila* α-spectrin (1:400), and mouse anti-MAP-kinase (1:200; Sigma, St. Louis, MO). Actin was labeled using rhodamine phalloidin (1:200; Molecular Probes, Eugene, OR).

Electron Microscopy

Electron microscopy of ovaries was carried out as described previously (Cox and Spradling, 2003).

ApopTag Staining

ApopTag staining was carried out as described in Drummond-Barbosa and Spradling (2001).

Supplemental Data

Supplemental figures and other data are available at http://www.developmentalcell.com/cgi/content/full/9/4/501/DC1/.

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