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Jak–STAT regulation of male germline stem cell establishment during *Drosophila* embryogenesis

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ABSTRACT

Germline stem cells (GSCs) in *Drosophila* are descendants of primordial germ cells (PGCs) specified during embryogenesis. The precise timing of GSC establishment in the testis has not been determined, nor is it known whether mechanisms that control GSC maintenance in the adult are involved in GSC establishment. Here, we determine that PGCs in the developing male gonad first become GSCs at the embryo to larval transition. This coincides with formation of the embryonic hub; the critical signaling center that regulates adult GSC behavior within the stem cell microenvironment (niche). We find that the Jak–STAT signaling pathway is activated in a subset of PGCs that associate with the newly-formed embryonic hub. These PGCs express GSC markers and function like GSCs, while PGCs that do not associate with the hub begin to differentiate. In the absence of Jak–STAT activation, PGCs adjacent to the hub fail to exhibit the characteristics of GSCs, while ectopic activation of the Jak–STAT pathway prevents differentiation. These findings show that stem cell formation is closely linked to development of the stem cell niche, and suggest that Jak–STAT signaling is required for initial establishment of the GSC population in developing testes.

Introduction

Stem cells harbor the potential to produce healthy cells to replace lost, damaged or diseased cells because they have the ability to both self-renew and give rise to daughter cells that differentiate. It is often necessary to establish a large number of stem cells for therapeutic purposes, but identifying, isolating, and maintaining these cells have proven difficult. Furthermore, stem cells that have been successfully isolated and maintained in culture may fail to integrate properly when transferred into damaged tissues. Understanding how stem cells function in their cellular microenvironment, or niche, is critical to generating methods for manipulating them. One of the underlying aspects of stem cell biology that remains largely unexplored is how and when adult stem cells are established during normal development.

Stem cell establishment is defined as the process by which a non-self-renewing precursor cell becomes an asymmetrically dividing cell that both self-renews and also produces differentiating daughters. This process has previously been studied in a limited number of systems including blood, neural and epithelial development (reviewed in Chia et al., 2008; Fuchs, 2008; Slack, 2008). In these systems, the formation of stem cells involves dramatic changes in cell signaling, extensive cellular migration, and is closely coordinated with organogenesis as niche formation frequently occurs with stem cell establishment. Factors required for stem cell establishment are often, but not always, essential in maintaining self-renewing adult stem cells depending on the stem cell type. For example, while *SCL/tal*-1, *LMO2*, and *Runx1* are all required for mouse blood lineage specification, inactivation of these genes in adult HSCs does not abolish maintenance or self-renewal (Orkin and Zon, 2008). In contrast, bulge stem cells in the hair follicle require Sox9 for their specification as well as maintenance (Nowak et al., 2008; Slack, 2008).

Spermatogenesis is one of the most accessible systems used to study stem cell formation as germline stem cells (GSCs) can often be assayed functionally. Primordial germ cells (PGCs) are the precursors of GSCs, and their development is remarkably similar between vertebrates and invertebrates (Seydoux and Braun, 2006). In many organisms, PGCs are specified early in embryogenesis, and divide and migrate extensively before assimilating with somatic cells in the gonad (Santos and Lehmann, 2004). In *Drosophila*, PGCs form at the posterior of the syncytial embryo, migrate through the epithelium after gastrulation, split into two groups and finally coalesce with the somatic gonad in parasegment 10 (reviewed in Dansereau and Lasko, 2008). Female GSCs are formed at the larval to pupal transition (Gilboa et al., 2003; Zhu and Xie, 2003), preferentially from PGCs at the anterior of the gonad (Asaoka and Lin, 2004). Dpp signaling

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maintains GSCs in the adult ovary, and is similarly required in the larval gonad during the PGC to GSC transition (Gilboa et al., 2003; Zhu and Xie, 2003). In contrast, male GSCs are thought to be specified much earlier in development.

While previous studies have indicated that male GSCs may be formed in *Drosophila* at the end of embryogenesis (Aboim, 1945; Kerkis, 1931), the exact timing and cellular behavior of PGCs transitioning to GSCs have not been examined. In the adult *Drosophila* testis, GSCs are maintained by Jak–STAT signaling initiated from a group of somatic cells at the testis apex called the hub (Kiger et al., 2001; Tulina and Matunis, 2001). 5–9 GSCs are anchored to the hub by cell adhesion molecules at the hub–GSC interface, and the orientation of their division is regulated by cortically localized Adenomatous Polyposis Coli tumor suppressor (APC) proteins (Yamashita et al., 2003). It is believed that physical displacement of the stem cell daughter from the hub causes it to initiate differentiation.

As the gonialblast moves away from the hub, it is enveloped by two cyst cells produced by cyst progenitor cells (CPCs, also referred to as somatic stem cells) also docked at the hub, and undergoes four rounds of cell division to produce a 16-cell spermatogonial cyst. Spermatogonial divisions are marked by incomplete cytokinesis, and result in the step-wise development of 2-, 4-, 8- and 16-cell syncytia with stabilized ring canals that serve as intracellular bridges. Specialized organelles known as fusomes extend through the cytoplasm of interconnected spermatogonia (Fuller, 1993; Hardy et al., 1979). Furthermore, late 2-cell to early 16-cell spermatogonia express the differentiation factor Bag-of-marbles (Bam), which is required for spermatogonia to mature (Chen and McKearin, 2003; Song et al., 2004). However, whether any of these mechanisms of adult GSC maintenance and regulation are observed in nascent GSCs remains to be determined.

Recently, hub formation in male embryonic gonads has been characterized during embryogenesis (Le Bras and Van Doren, 2006). Initially, the gonad is formed from the coalescence of PGCs and somatic gonadal precursors (SGPs) at stage 14 of embryogenesis. While a group of *Abdominal-B*-specified male-specific SGPs (msSGPs) is maintained in male gonads at the posterior (DeFalco et al., 2003), a subset of *escargot* expressing SGPs coalesces at the anterior of the gonad to form the embryonic hub, which expresses adult hub markers and is associated closely with a rosette of germ cells at the anterior of the gonad by the end of embryogenesis. These anterior germ cells may be specified to self-renew by the hub at this time, although no direct method has been used to determine this.

Here, we directly examine the timing and regulation of GSC establishment in developing *Drosophila* testes. We show that a subset of PGCs associated with the hub begin to function as GSCs by the end of embryogenesis. Furthermore, we find that the Jak–STAT pathway is required to prevent differentiation of these hub-associated PGCs, and is likely required for the initial establishment of the GSC population. Taken together, these data show that niche formation is closely associated with stem cell establishment, and that mechanisms regulating stem cell maintenance in the adult are likely to control the process of stem cell establishment.

Materials and methods

Fly stocks

w^{1118} and *ru st faf-lacZ e ca flies* were used as wild-type. Either *nanos-Gal4::VP16* on II (E. Selva) or *nanos-Gal4::VP16* on III (M. Van Doren) were used to drive expression of *UAS-GMA* on III (D. Kiehart) (Bloor and Kiehart, 2002), *UAS-Ecadherin::GFP* (H. Oda), *UAS-upd* (D. Harrison) and *UAS-GFP::nls*. Other stocks include *escargot-GFP* (L. Cooley Flytrap) (Kelso et al., 2004; Morin et al., 2001) and the enhancer trap lines M5-4 (Steve DiNardo) and *Mgm-1* (Janzer and Steinmann-Zwicky, 2001). *mwh red e* $stat^{[Frankenstein]}$ or $stat^{F}$ (C. Dearolf) and $stat92E^{06346}$ were balanced over Tm3,Sb,Kr-GFP for genotype selection in embryos. Flies carrying Kr-GFP on the X chromosome (Casso et al., 2000) were used to determine the sexual identity of embryos or larvae as described (Wawersik et al., 2005). Fly stocks were obtained from the Bloomington Stock Center (http://flystocks.bio.indiana.edu/) unless specified otherwise.

Embryo collection

For the STAT92E immunostaining experiments, embryos ranging from 0–19, 19–21, 21–23, 23–25, and 25–27 h after egg laying (AEL) were collected. For PGC division orientation and fusome staining experiments embryos were collected at 12–16 (16/17E), 16–20 (St 17E/M), 20–24 (17L/L1E), 26–30 (L1E/M) and 48–52 (L2) hours AEL. For STAT92E loss of function experiments, *mwh red e* $stat^{[Frankenstein]}$/Tm3, Sb,Kr-GFP virgins were crossed to $stat92E^{06346}$/Tm3,Sb,Kr-GFP males at 18 °C; resulting embryos were collected at 8–12 h AEL then incubated at 29 °C in a humidified chamber for 24 h.

Immunostaining and in situ hybridization

Immunostaining of adult testes, embryos and larvae was performed as described (Matunis et al., 1997; Wawersik et al., 2005). Fluorescence *in situ* hybridization for *upd* was also performed as described (Wawersik et al., 2005). Primary antibodies used were: rabbit anti-Vasa at 1:5000, a gift from P. Lasko (Lasko and Ashburner, 1990); chick anti-Vasa at 1:5000 (from K. Howard, personal communication); rabbit anti-STAT92E at 1:1000 (from S. Hou); rabbit anti-phospho-STAT92E at 1:1000 (Cell Signaling Technologies); mouse anti γ-tubulin GTU-88 at 1:200 (Sigma); rabbit anti-GFP at 1:10,000 (Torrey Pines); rabbit anti-β-Gal at 1:10,000 (Cappel); mouse anti-β-Gal at 1:1000 (Promega); mouse anti-1B1 at 1:25 (Developmental Studies Hybridoma Bank [DSHB]); mouse anti-Fasciclin III at 1:50 (DSHB); mouse anti-Sex-lethal M18 (DSHB); rabbit anti-Sox100B at 1:1000 (S. Russel); rat anti-DE-Cadherin DCAD2 (DSHB); rabbit anti-phospho-histone H3 at 1:200 (Upstate Cell Signaling Solutions); rabbit anti-Anillin at 1:1000 (C. Field); rat anti-Bam at 1:1000 (D. McKearin); and rabbit anti APC2 at 1:10,000 (M. Bienz). Alexa secondary IgG (H + L) antibodies used were (Molecular Probes): goat anti-rabbit 405 at 1:200, goat anti-rabbit 488 at 1:400 or 1:500, goat anti-mouse 488 at 1:400 or 1:500, goat anti-mouse 555 at 1:200 or 1:500, goat anti-rabbit 568 at 1:200 or 1:500, goat anti-mouse 568 at 1:200 or 1:500, and goat anti-chick 568 at 1:200 or 1:500. Nuclei were stained with DAPI at 1 μg/mL (Roche). Sexual identity of embryos and larvae was determined by immunostaining for either Sxl expression, presence of hub markers (DN-Cad or Fasciclin III) (Le Bras and Van Doren, 2006), the msSGP and pigment cell marker, Sox100B (DeFalco et al., 2008, 2003), or by examining inheritance of an X-linked Kr-GFP transgenes (Wawersik et al., 2005) detected with anti-GFP antibodies.

Analysis of confocal images

Samples were mounted in Vectashield (Vector Laboratories) and viewed with Zeiss LSM 5 Pascal or BioRad Radiance2000 confocal systems. Z-series were analyzed using the Pascal software or NIH ImageJ. Graphs were prepared and statistical analysis (ANOVA, unpaired Student's *t*-test assuming unequal variances, Fisher exact text, and χ^2-test) performed in Microsoft Excel and Graphpad Prism. Ratios in Supplementary Fig. 2 graph were determined in anteriorly located germ cells by dividing the average pixel intensity in either a crescent (stage 16/17E) or elliptical (stage 17L/L1) region at the hub-proximal cell cortex by the average pixel intensity in a similar-shaped region at the hub-distal cell cortex. GFP signal intensity was first normalized to the intensity of Vasa staining in the region.

Analysis of division planes

Supplementary Fig. 3E shows a schematic of division plane analysis. In stage 16–17E embryos where the hub was not yet detectable by Fasciclin III expression, division orientations were scored with respect to the A–P axis as determined by the presence of male-specific SGPs (msSGPs) at the posterior of the gonad (DeFalco et al., 2003). Gonads were partitioned into anterior, middle, and posterior regions, and cell division plane scored as "perpendicular" if within 20° of being aligned along the gonad A–P axis, "parallel" if within 20° of being aligned orthogonal to the A–P axis, or "intermediate" in any other orientation. In stage 17L-L1 embryos where the hub has formed, cell divisions were identified as "perpendicular" if the division plane was within 20° of being perpendicular to the hub, "intermediate" if between ~20–70° of being perpendicular, or "parallel" if between ~70–90° of being perpendicular.

Results

Jak–STAT pathway activation becomes restricted to anterior germ cells at the embryo to larval transition

Jak–STAT signaling is a key regulator of GSC behavior in adult testes. STAT92E activation is necessary for GSC maintenance in adult testes (Kiger et al., 2001; Tulina and Matunis, 2001), and stabilized STAT92E protein accumulates in GSCs and daughter gonialblasts located adjacent to the hub (Boyle et al., 2007; Leatherman and Dinardo, 2008). To identify nascent male GSCs during development, we examined the gonads of varying stages for germ cells with increased STAT92E immunoreactivity, which is an indicator of Jak–STAT pathway activation (Chen et al., 2002; Johansen et al., 2003; Read et al., 2004). In newly-formed gonads (stage 13; ~10 h AEL), all germ cells throughout male (but not female) gonads accumulate STAT92E, which promotes male-specific germ cell development (Wawersik et al., 2005). While all male PGCs retained STAT92E by stage 15 of embryogenesis (~12 h AEL, Figs. 1A,E; (Wawersik et al., 2005), STAT92E accumulation became restricted to anteriorly localized germ cells by the end of embryogenesis (stage 17L, ~22 h AEL, Fig. 1B). This process began at mid-stage 17 of embryogenesis (~20 h AEL), after which time the number of germ cells with STAT92E dropped to 6–7 cells localized to the anterior portion of the gonad, while the total number of germ cells in each gonad continued to increase (Fig. 1E). Furthermore, restriction of germ cells containing STAT92E correlated with restricted expression of the Jak–STAT activating ligand, *upd*, from SGPs in the anterior half of the gonad (Fig. 1C), to a tight cluster of anterior SGPs resembling the newly-formed hub (Fig. 1D; Le Bras and Van Doren, 2006). These results are consistent with previous analyses showing restricted expression of an *upd* enhancer trap to the developing hub (Le Bras and Van Doren,

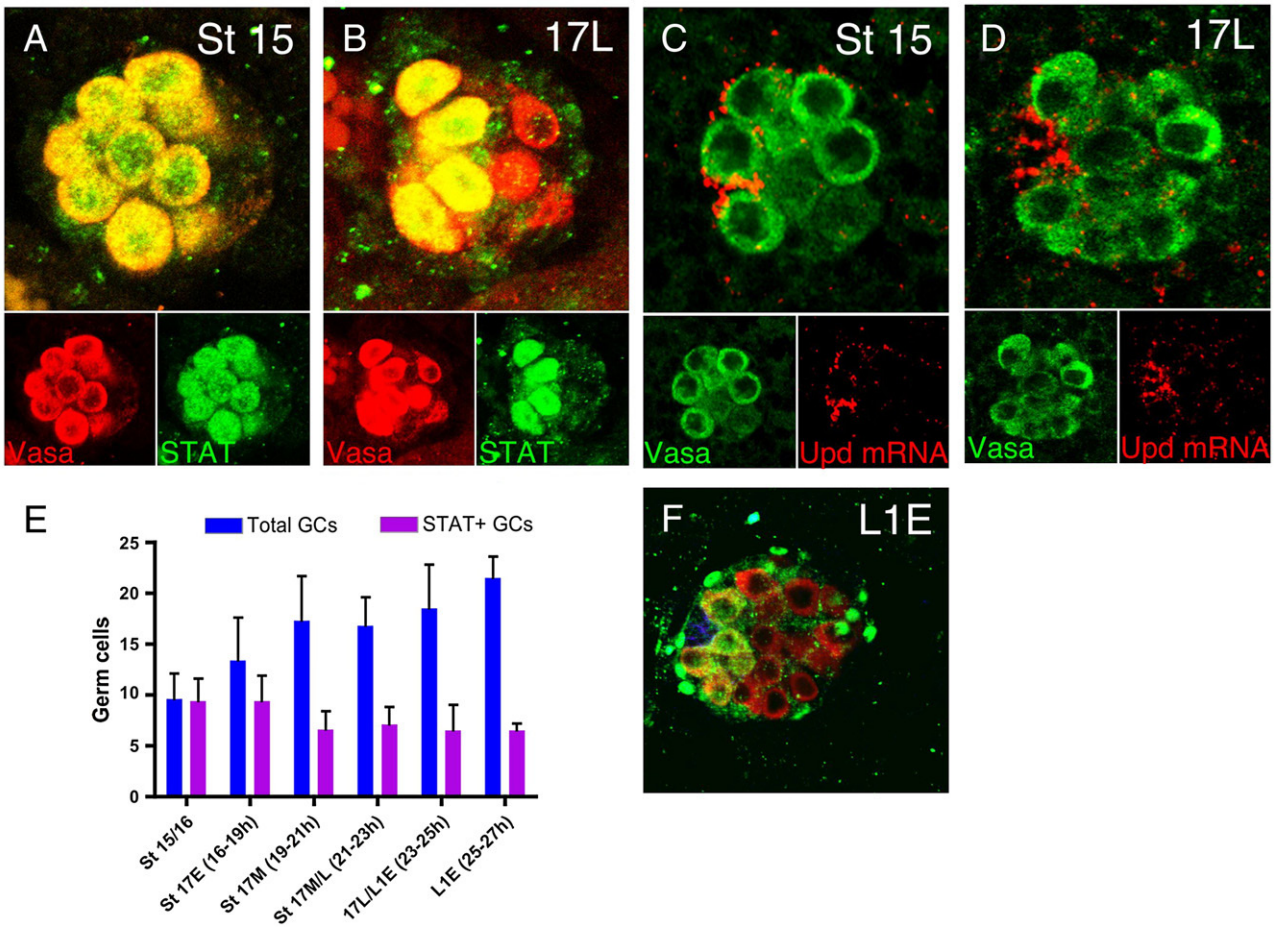
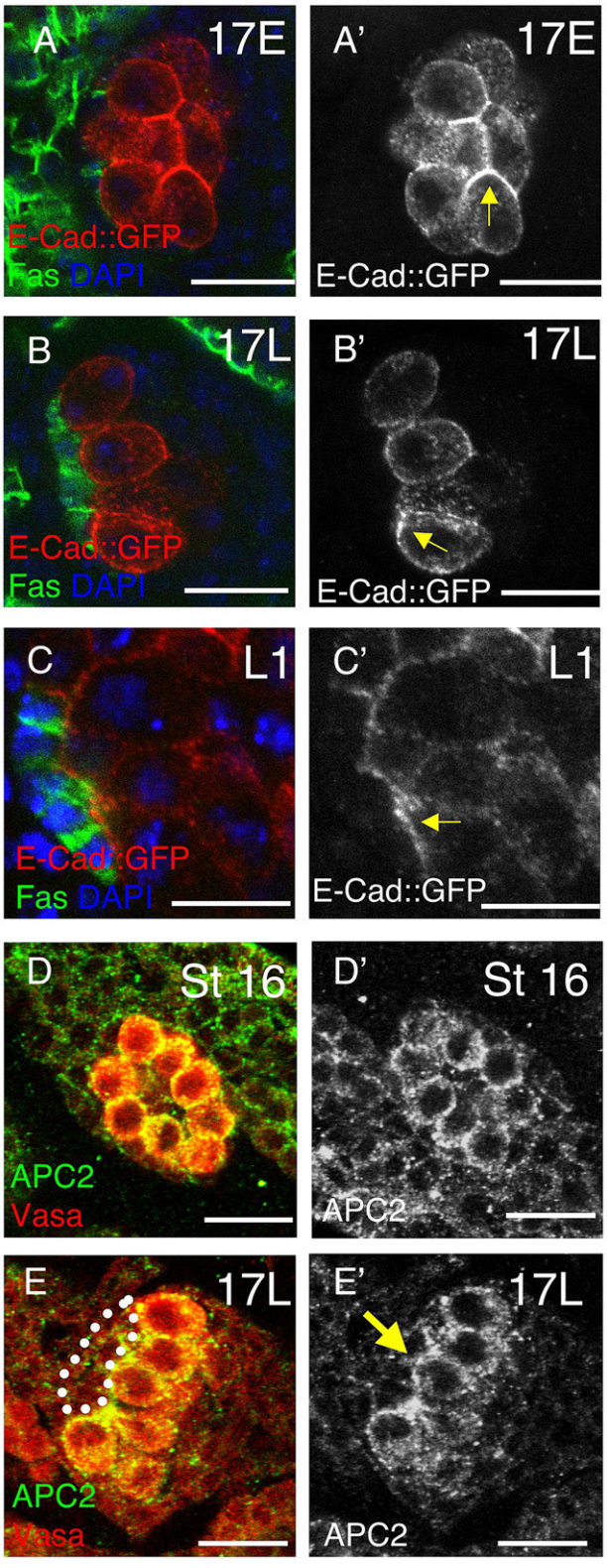


Fig. 1. Jak–STAT signaling becomes restricted to anterior germ cells in late stage 17 embryos. (A, B) Wild-type embryos immunostained for STAT92E (green) and Vasa (red) after gonad coalescence. Anterior of the gonads is to the left. Insets show individual channels. (A) Gonad from male stage 15 embryo showing STAT92E accumulation in the nucleus and cytoplasm of all PGCs. (B) Late stage 17 male gonad with STAT92E accumulation restricted to the anterior-most germ cells. (C, D) Fluorescent *in situ* hybridization performed on wild-type embryos with an anti-sense probe to *upd* mRNA (red), along with immunostaining for Vasa (green). (C) Stage 15 male gonad with *upd* expression slightly enriched at the anterior. (D) Late stage 17 male wth *upd* detected robustly in a tight cluster of SGPs at the gonad anterior. (E) Graph showing the total number of germ cells (blue bars) and the number of germ cells with STAT92E immunoreactivity (purple bars) in the gonad collected at various times. (F) Early L1 gonad immunostained for phospho-STAT92E and Sox100B (green), DN-Cadherin (blue), and Vasa (red).

2006). Finally, to confirm that STAT92E activity is restricted to hub-proximal germ cells, we examined phosphorylated STAT92E (P-STAT92E) protein with respect to the newly coalesced hub marked by DN-Cadherin stain (DN-Cad; Le Bras and Van Doren, 2006). In early first-instar larvae (~25 h AEL, Fig. 1F), P-STAT92E was also restricted to hub-proximal germ cells.

To determine whether this subset of germ cells was taking on GSC identity, we examined the expression of additional adult male GSC markers during early gonad development. In adult testes the *escargot* (*esg*) enhancer trap, M5-4, is expressed in hub cells, GSCs and their daughter gonialblasts (Gonczy and DiNardo, 1996; Terry et al., 2006). We therefore examined M5-4 expression at the time of STAT92E restriction. In mid-stage 17 gonads (~20 h; Supplementary Fig. 1A), we find that M5-4 is expressed strongly in somatic hub cells at the anterior tip of the gonad, and weakly in male germ cells throughout the gonad. However, in early to mid first-instar larva (26–30 h AEL; Supplementary Fig. 1B), M5-4 expression began to be restricted in hub-proximal germ cells in a subset of gonads and this expression pattern became more prominent as development progressed (Supplementary Fig. 1C). Similar observations were made with *esg* protein-Trap lines (Buszczak et al., 2007; Kelso et al., 2004) (data not shown). Thus, both STAT92E and several additional reporters that mark adult GSCs display restriction to hub-proximal germ cells at the embryo to larval transition. The slight delay in reporter restriction compared to STAT92E is likely due to perdurance of the β-gal and GFP transgenes. Together, these data suggest that hub-proximal germ cells behave as function GSCs by the end of embryogenesis. We, therefore, tested this hypothesis through functional assays.



Hub-proximal germ cells form polarized cell adhesions at the end of embryogenesis

Asymmetric GSC divisions in adult testes are oriented by polarized adhesions between GSCs and hub cells. Formation of adhesions between developing germ cells and the hub would be expected to occur at the time of GSC establishment. Expression of the homophilic cell adhesion molecule, DE-cadherin (DE-Cad) is highly enriched at the adult GSC/hub interface (Yamashita et al., 2003). The tumor suppressor protein, Adenomatous Polyposis Coli 2 (APC2) also localizes to this interface, and is required to orient GSC divisions with respect to the hub (Yamashita et al., 2003). We therefore examined DE-Cad and APC2 localization at the time of hub formation. Localization of DE-Cad was assessed by expressing a GFP-tagged E-Cad (E-cad::GFP) specifically in the germline. E-Cad::GFP concentrated at the germ cell/SGP interface throughout the gonad in early stage 17 embryonic gonads (~17 h AEL; Fig. 2A). At this time, hub precursor cells are present, but the hub has not compacted into a tight cell cluster that expresses adult hub cell markers (Le Bras and Van Doren, 2006). However, after hub compaction (~22 h AEL), E-Cad::GFP was less concentrated at the germ cell/SGP interface, and became slightly enriched at the germ cell–hub interface in some hub-proximal germ cells (Fig. 2B). This localization was apparent in most hub-proximal germ cells by the 1st instar larval stage (Fig. 2C). Similar observations were made when we examined APC2 localization in late stage 17 gonads (Figs. 2D, E); although the subtle enrichment of APC2 at the hub is less striking than that observed in adult testes (Yamashita et al., 2003).

To further investigate the polarized nature of hub-proximal germ cells, we expressed the actin-binding domain of Moesin fused to GFP (GMA) in developing germ cells. Like E-Cad and APC2, GMA is concentrated at the GSC/hub interface in adult testes (Sheng et al., in press) and at all cell–cell contacts. For germ cells localized to the anterior portion of the gonad, we compared GFP intensity at the hub-

Fig. 2. E-cadherin and APC2 enrich at the hub/germ cell interface at the embryo to larval transition. (A–C) *nos-Gal4/UAS-Ecadherin::GFP* embryos immunostained for GFP (red), FasIII (green), and counterstained for DAPI (blue). (A'–C') GFP channels shown alone, with yellow arrows indicating regions of E-Cadherin enrichment. (A, A') Early stage 17 male gonad with E-Cadherin present at the cell membrane of germ cells (arrow). (B, B') late stage 17/early 1st instar and (C, C') late 1st instar larvae showing E-Cadherin concentrated at the hub/anterior germ cell interface in some germ cells (arrows). (D, E) Stage 16 and late stage 17 embryos immunostained for APC2 (green) and Vasa (red) with hub outlined. (D', E') APC2 channel shown alone APC2 is not polarized in stage 16 gonads (D, D'), but becomes slightly enriched at the hub/germ cell interface in late stage 17 embryos (E, E' arrow). Anterior is left, and scale bars indicate 10 μM (A–C) or 15 μM (D, E).

proximal membrane versus the hub-distal membrane. Shortly after gonad coalescence, GFP intensity was roughly equivalent on the proximal and distal portions of germ cell membranes (Supplementary Fig. 2A), indicating that there was no preferential enrichment of GMA within the germ cells. However, at the end of embryonic development (21–26 h AEL; Supplementary Fig. 2B), GFP intensity at the proximal region was approximately 2-fold higher than that at the distal region, indicating an enrichment of GMA at the hub/anterior germ cell interface. In contrast, GMA became enriched at cell–cell contacts similar to that observed in adult testes in posterior germ cells, but showed no preference in polarization with respect to the hub (Supplementary Fig. 2B). Thus, hub-proximal germ cells form polarized cell adhesions with the hub by the end of embryonic gonad development.

Hub-proximal germ cells display oriented divisions by the end of embryogenesis

A defining characteristic of a functional stem cell is its ability to self-renew. Adult GSCs in the *Drosophila* testis make divisions that are oriented perpendicular to the hub, such that one daughter cell remains attached to the hub and retains GSC identity, while the other is displaced one cell-diameter away and differentiates (Yamashita et al., 2003). During this process, centrosome duplication occurs at the GSC/hub interface. Subsequently, the daughter centrosome migrates to the hub-distal pole to become partitioned to the daughter gonialblast following mitosis (Yamashita et al., 2007). Thus, oriented division ensures that every GSC division produces one GSC and one gonialblast.

To determine when oriented cell division is first observed after gonad coalescence, we examined the division plane of male germ cells using either γ-Tubulin (γ-Tub) or Centrosomin (Cnn) to mark centrosome location. Dividing germ cells were further identified by the presence of the mitotic marker Phospho-Histone H3 (PH3). The plane of cell division was examined with respect to the A–P axis of the gonad. After hub formation, the division plane was assayed with respect to hub location. Dividing germ cells in different regions of the gonad were then scored as perpendicular, intermediate or parallel, depending on their orientation of division (see Materials and methods for details).

Prior to hub formation, germ cells in all regions of the gonad exhibited random orientation of division (Figs. 3A–C). In contrast, after hub formation, germ cells in the anterior/hub-proximal region divided perpendicular to the hub (Figs. 3E, F χ^2-test $p<0.0001$). Germ cells in middle and posterior regions away from the hub showed random division orientation. The onset of oriented divisions was gradual, beginning in mid-stage 17 embryos (Supplementary Fig. 3). This correlates exactly with onset of hub formation (Le Bras and Van Doren, 2006). Together, this suggests that PGCs in newly coalesced gonads initially undergo symmetric divisions that serve to expand the PGC pool. As the hub develops, however, hub-proximal germ cells gradually adopt the same orientation as self-renewing adult GSCs, indicating onset of GSC behavior.

Hub-proximal germ cells give rise to differentiating spermatogonia in the posterior gonad

If germ cells associated with the hub are truly behaving as GSCs, then these cells should remain undifferentiated, while their progeny should enter spermatogenesis. To investigate differentiation in germ cells of the early larval gonad, we examined cytoplasmic expression of the Bag-of-marbles protein (Bam-C), which accumulates in early spermatogonia but is shut off as spermatogenesis proceeds (McKearin and Ohlstein, 1995). At the time of activated STAT92E restriction (20–24 h AEL), Bam-C expression was not detected in male germ cells (Fig. 4A), and this was also true in early first-instar larval gonads.

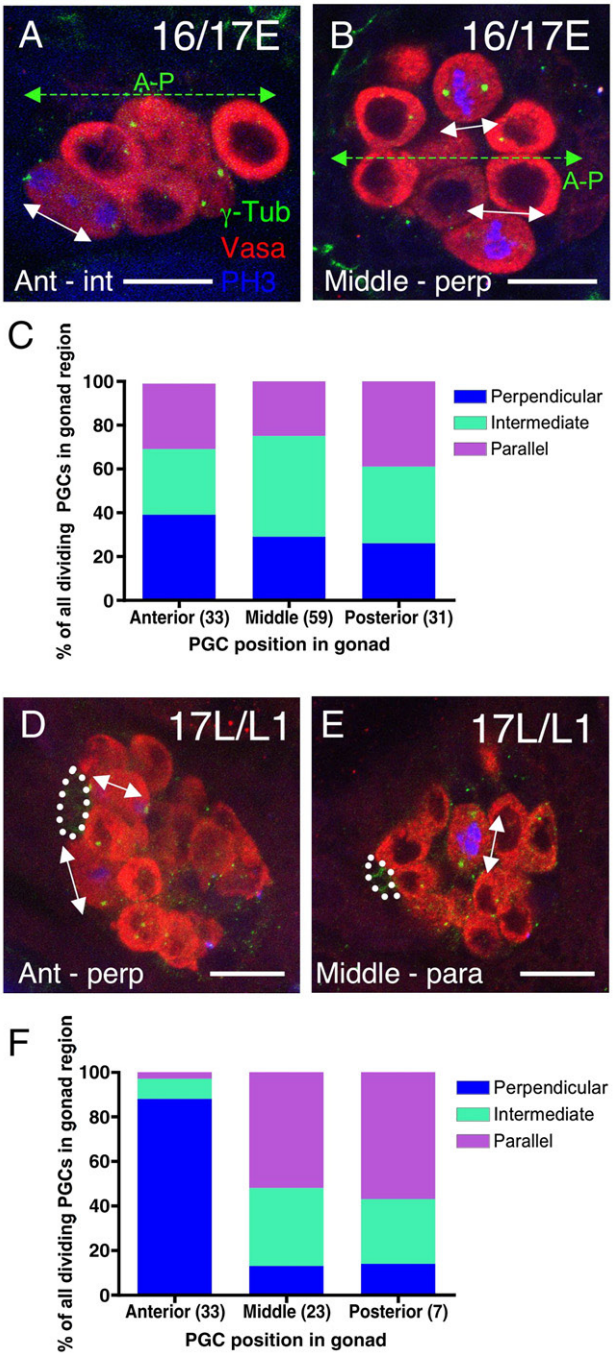


Fig. 3. Anterior germ cells orient their divisions with respect to the hub at the end of embryogenesis. w^{1118} embryos/larva co-immunostained for γ-tubulin (green), FasIII (green), Vasa (red), and PH3 (blue) to mark orientation of dividing germ cells with respect to the A–P axis of the gonad (see Materials and methods). Green dashed lines denote the A–P axis with anterior to the left. White lines denote germ cell division orientation. (A–C) Stage 16/early stage 17 embryos have equal proportions of germ cells with divisions oriented perpendicular (blue), intermediate (green), and parallel (purple) to the A–P axis throughout the gonad (see graph; panel C). Representative immunostains showing (A) an anteriorly localized germ cell with intermediate division orientation, and (B) two posterior germ cells dividing with perpendicular orientation. (D–F) Late stage 17 and early L1 embryos display germ cells with the majority of divisions oriented perpendicular to the hub (outlined) in anterior regions, while divisions are randomly oriented in all other regions (see graph; panel F). Representative immunostains showing (D) two anterior germ cells making perpendicular divisions, or (E) one germ cell in the middle of the gonad with its division plane oriented parallel to the hub. Scale bars indicate 10 μM.

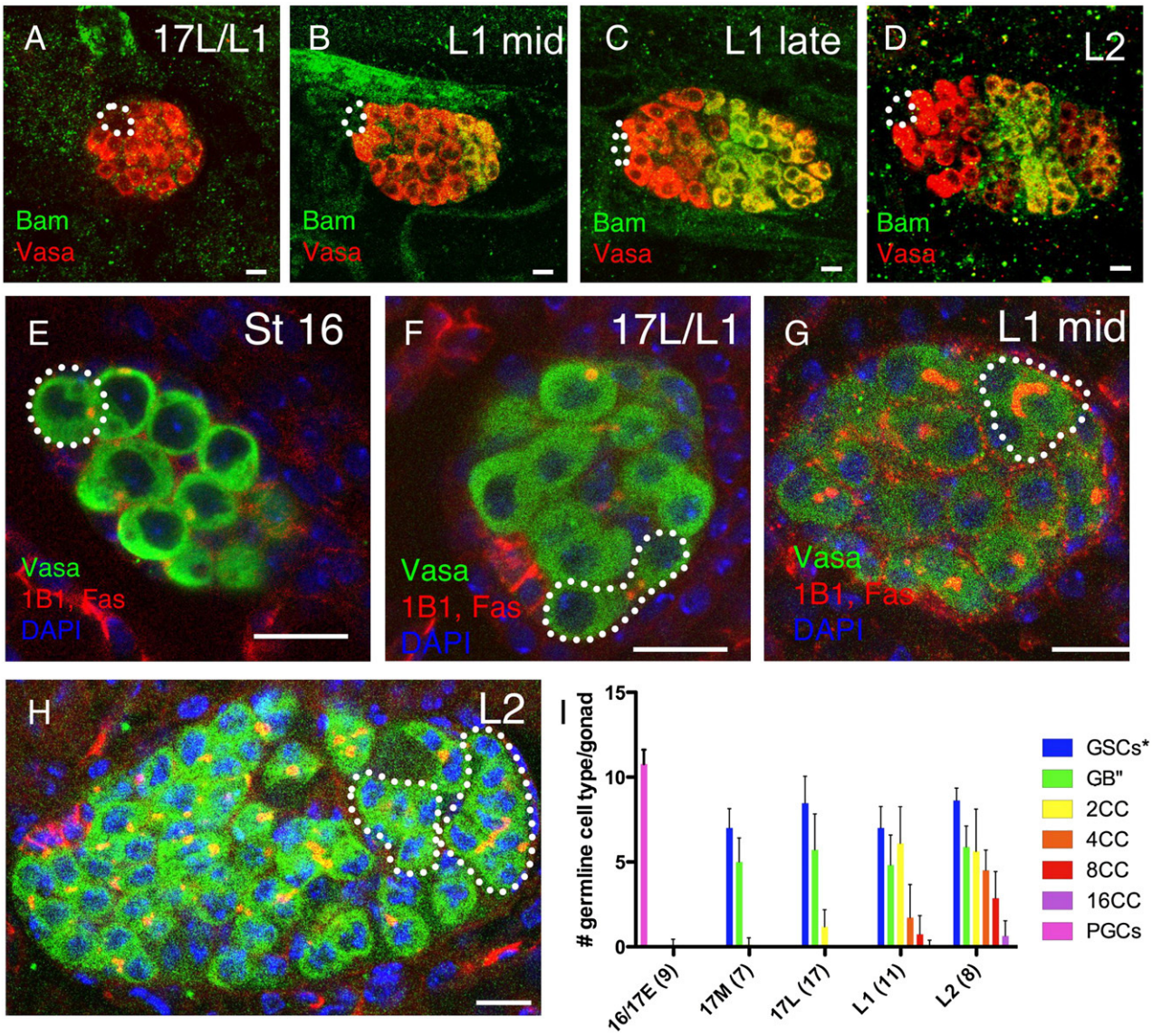


Fig. 4. Spermatogonial differentiation is observed in posterior germ cells shortly after onset of asymmetric germ cell division at the hub. (A–D) w^{1118} larva immunostained for BAM (green) and Vasa (red). Anterior to the left and presumptive hub outlined. (A) Male stage 17/early 1st instar larval gonad lacking BAM expression. (B,C) Mid and late larval 1st instar testes with BAM expressed in posterior germ cells. (D) 2nd larval instar testes showing BAM expression in germ cells a few cell diameters away from the hub, but absent in posterior germ cells. (E–I) w^{1118} embryos/larva immunostained for Vasa (green), FasIII (red), 1B1 (red), and counterstained with DAPI (blue). (E) Stage 16 embryo with all germ cells (example outlined) displaying spherical fusomes. (F) Late stage 17 embryo with germ cells showing spherical fusomes, and bar-like fusomes (outlined) connected to a daughter cell. (G) Mid 1st instar larval testes displaying spherical and bar-like fusomes in hub-proximal germ cells, but elongated fusomes (outlined) in posterior germ cells. (H) Mid 2nd instar larval testes with spherical and bar-like fusomes in hub-proximal germ cells, but elongated and branched fusomes (outlined) in posterior germ cells. (I) Graph showing the gradual appearance of differentiated gonial cyst stages over time (see Materials and methods) without the depletion of undifferentiated germline cell types. Note: (*) Once a hub is detected via FasIII immunostain, all germ cells adjacent to the hub are denoted GSCs, (") while germ cells not adjacent to the hub, either single, or attached to a GSC, are denoted as GBs. Scale bar indicates 10 μm.

However, by mid L1 (~28 h AEL), Bam-C was regularly detected in posterior (hub-distal) germ cells (Fig. 4B) but remained absent in hub-proximal germ cells. This expression pattern persisted throughout development (Figs. 4C, D), except that the distal-most germ cells eventually lost Bam-C expression by the second larval instar (~48 h AEL), suggesting passage into later stages of spermatogenesis. Thus, germ cells further away from the hub gradually begin to differentiate, while hub-proximal germ cells remain undifferentiated.

To determine the developmental stage of germ cells in the early gonad, we examined spectrosome/fusome morphology. The spectrosome is a germline-specific organelle composed of membrane cytoskeletal proteins and vesicles (reviewed in Dubielecka et al., 2003) required for male germ cell differentiation (Wilson, 2005). PGCs and GSCs have a spherical spectrosome that is segregated completely between the two daughter cells after division. Differentiating spermatogonia, on the other hand, have spectrosomes that elongate and branch to form a fusome that interconnects each cell of the spermatogonial cyst. Shortly after gonad coalescence, only spherical fusomes were observed in the germline (Fig. 4E; Lin and Spradling, 1995). At the embryo to larval transition, single germ cells had spherical spectrosomes, while germ cells connected to a single daughter had bar-shaped fusomes (Fig. 4F). By mid L1, however, elongated fusomes between 4-cell spermatogonia were observed in posterior germ cells (Fig. 4G), and branched fusomes between 8- and 16-cell spermatogonia were apparent by L2 (Fig. 4H). Meanwhile, anterior germ cells always retained spherical or bar-shaped spectrosomes. Thus, these data suggest that posterior germ cells differentiate, while hub-proximal germ cells do not. Further support for this interpretation is provided by the observation that larger, presumably stabilized ring canals, marked by Anillin (Field and Alberts, 1995),

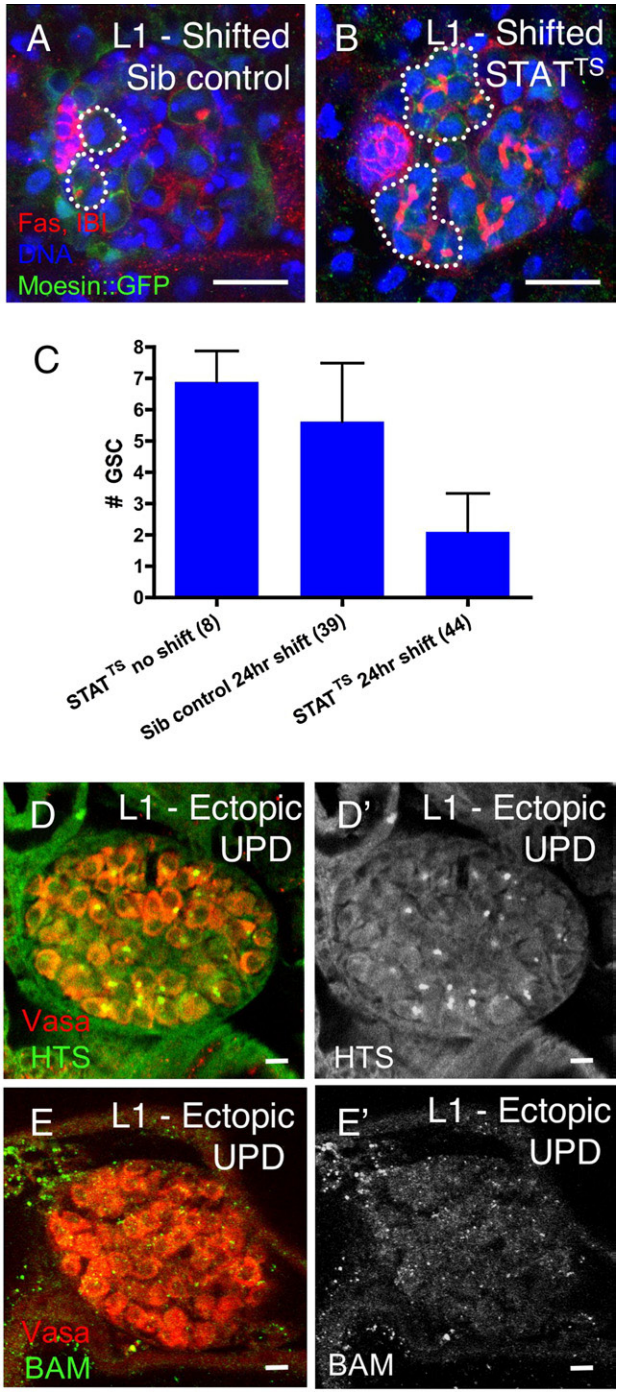


Fig. 5. Jak–STAT activation is required for GSC maintenance and promotes germ cell self-renewal. (A, B) *Nos::GMA;;STAT^{TS}* and sib control embryos/larvae immunostained for GFP (green) to detect germ cells and determine genotype (see Materials and methods), FasIII (red), 1B1 (red), and counterstained with DAPI (blue). Samples collected after 24 h growth at non-permissive temperature (see Materials and methods). (A) 1st instar larval testes from sibling control showing GSCs with spherical spectrosomes adjacent to the hub. (B) *STAT^{TS}* mutant larval 1st instar testes with spermatogonial cells containing branched fusomes near the hub. (C) Quantitative analyses reveal a marked decrease in the average number of GSCs in *STAT^{TS}* mutant testes grown at non-permissive temperature compared to sibling controls ($p<0.0001$). (D, E) *Nos-Gal4/UAS-Upd* larvae immunostained for Vasa (red) and either HTS (D, green) or Bam (E, green) to detect spermatogonial differentiation. Individual HTS (D') and BAM (E') channels show for clarity. (D, D') Late 1st instar larval testes lacking presence of branched fusomes. (E, E') Late 1st instar larval testes lacking BAM expression. Scale bar is indicates 10 μm (D,E).

co-localize with branched fusomes (Supplementary Fig. 4D), while only smaller, presumably transient, ring canals were observed in spherical fusomes in stage 17 and early L1 gonads (Supplementary Fig. 4B, C).

The above data show that germ cells begin to differentiate by the mid-L1 stage of development (~28 h AEL). If the hub-proximal germ cells are truly GSCs, they should divide to produce both GSCs and differentiating daughter cells (spermatogonia). Since direct lineage analysis is not feasible at this stage of development, we investigated this question by quantifying the different classes of germ cells and differentiating cysts in the gonads. If the hub-proximal germ cells are functional GSCs that give rise to both GSCs and gonialblasts, we would expect that the pools of both GSCs and early gonialblasts would be replenished, even as the number of differentiating cysts continually increases. We found that the number of hub-proximal germ cells (GSCs) and individual germ cells (gonialblasts) remained constant, even though the total number of differentiating cysts (containing from 2–16 cells) increased dramatically (Fig. 4I). This indicates that the oriented divisions observed in the hub-proximal germ cells are likely to be true self-renewing divisions, where some daughter cells maintain the hub-proximal GSC population, while others differentiate and enter spermatogenesis.

Jak–STAT signaling mediates male GSC establishment

The Jak–STAT pathway regulates GSC maintenance in the adult testis, but it is not known whether the same or different pathway(s) regulate the initial establishment of this adult GSC population during development. To assess the role of Jak–STAT signaling in male GSC establishment, we tested whether STAT92E activation is necessary for the PGC to GSC transition. During early embryogenesis, *stat* regulates a number of processes including embryonic patterning and germ cell migration (Arbouzova and Zeidler, 2006), so it was necessary to conditionally remove STAT92E in order to bypass these earlier requirements in development. Because generation of germline clones is not feasible at this early stage of development, we used a temperature-sensitive allelic combination of STAT92E (STAT^{TS} = STAT^{F}/STAT^{mrl}) (Brawley and Matunis, 2004) (Baksa et al., 2002) to determine if STAT92E is required for the onset of GSC divisions. By shifting embryos to the non-permissive temperature after gonad coalescence it was possible to prevent perturbation of earlier stages of development (see Materials and methods for details). At the non-permissive temperature, control germ cells were indistinguishable from wild-type, having 5.6 ± 1.9 ($n = 39$) single GSCs localized immediately adjacent to the hub with spherical spectrosomes (Figs. 5A, C). In contrast, the number of single GSCs in STAT^{TS} larvae were significantly reduced to 2.1 ± 1.2 ($n = 44$, $p<0.0001$, Figs. 5B, C). The majority of germ cells docked at the hub were 4-, or 8-cell spermatogonia with branched fusomes (Fig. 5B), a phenotype never observed in wild-type controls. Since spermatogonial cysts containing more than two germ cells are not observed until mid-larval first-instar in wild-type testes (Fig. 4), the observation that the majority of germ cells have developed into late stage spermatogonia in STAT^{TS} testes indicates that germ cells never gained the capacity to undergo self-renewing stem cell divisions, but instead directly entered differentiation. Thus, STAT92E activation appears to be necessary to promote male GSC establishment.

We also assessed whether ectopic STAT92E activation could prevent onset of spermatogonial differentiation in developing testes. We find that over-expression of the Jak–STAT activating ligand, UPD, in gonads resulted in STAT92E accumulation in germ cells throughout the gonad (Supplementary Fig. 5A). The majority of these cells were single germ cells characteristic of GSCs. Furthermore, differentiation markers such as the presence of elongated fusomes (Fig. 5D) and Bam-C expression (Fig. 5E) were never observed. Finally, as an assay for induction of male GSC-specific gene expression, induction of the male germline stem cell-specific LacZ enhancer trap, *male germline*

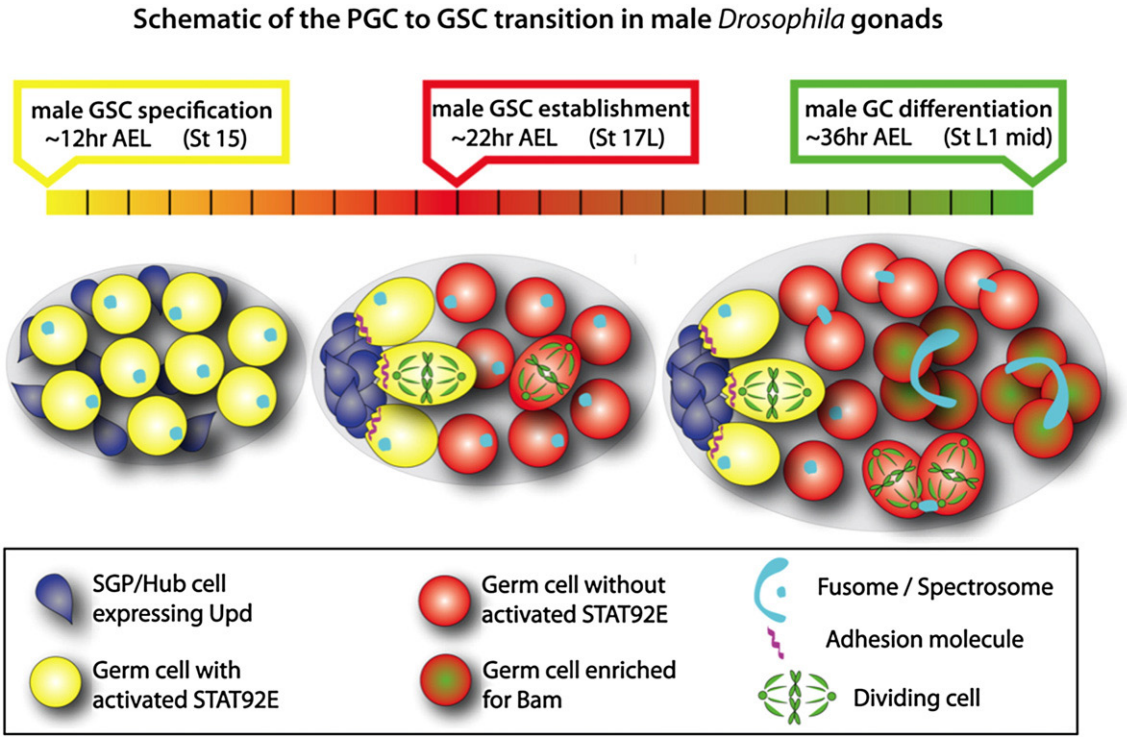


Fig. 6. Schematic of the PGC to GSC transition during *Drosophila* testes development. (Left gonad) Jak–STAT signaling specifies male GSC identity in all germ cells after gonad coalescence. (Middle gonad) As hub formation occurs toward the end of embryogenesis, restriction of Jak–STAT activity to hub-proximal germ cells establishes GSCs, which are adherent to the hub and divide perpendicularly. Germ cells at the posterior of the gonad have low levels of Jak–STAT activation and initiate differentiation by the mid first-instar larval stage (right gonad). SGPs expressing Upd are dark blue; germ cells with activated STAT92E are yellow; germ cells without activated STAT92E are red; germ cells enriched for Bam are red with green centers, fusomes and spectrosomes are aqua, adhesion molecules are pink, and mitotic spindles are green. Notches in scale represent hours during embryogenesis.

marker-1 (*mgm1*) (Staab et al., 1996), was detected in germ cells throughout gonads after ectopic UPD expression (Supplementary Fig. 5B). Thus, ectopic Jak–STAT activation in developing testes appears sufficient both to prevent onset of germ cell differentiation and to promote onset of GSC-specific behavior. As recent data suggests that intrinsic activation of Jak–STAT signaling in the germline is not sufficient for their self-renewal (Leatherman and Dinardo, 2008), it will be interesting to determine whether ectopic Jak–STAT activation represses onset of spermatogenic differentiation by acting on nascent GSCs, developing cyst progenitor cells, or both. Regardless, these findings, along with our analyses of STAT92E protein accumulation and *upd* expression (Fig. 1), as well as our observation that STAT92E is required for onset of self-renewing GSC divisions (Figs. 5A–C), implicate the Jak–STAT pathway as key mediator of male GSC establishment during late embryonic and early larval gonad development.

Discussion

Maintenance of stem cell populations is controlled by signals from the surrounding microenvironment (Morrison and Spradling, 2008). Relatively little is known, however, about how stem cell populations are established during development. In this study, we have shown that PGCs in developing *Drosophila* testes transition into functional GSCs by the end of embryogenesis (Fig. 6). These newly-formed GSCs are localized immediately adjacent to the hub, form polarized cell adhesions with the hub, and undergo oriented divisions that produce steady-state numbers of differentiating spermatogonia localized to the posterior of the developing gonad. The transition of male PGCs to functional GSCs correlates with hub formation and restricted activation of the Jak–STAT signaling pathway to hub-proximal germ cells. Together, these data indicate that a functional male GSC niche develops almost immediately after onset of gonad morphogenesis, and that similar mechanisms regulating GSC maintenance and behavior in adult testes are likely to control the PGC to GSC transition in developing gonads. These data have implications for our general understanding of germline development, regulation of germ cell sex determination, as well as mechanisms controlling stem cell maintenance and establishment in other tissues and organisms.

Pathways regulating Drosophila GSC establishment

We find that Jak–STAT signaling is both necessary for male GSC maintenance in early male larvae, and sufficient to prevent spermatogenic differentiation at this same developmental stage. As STAT92E activation has previously been shown to promote male germ cell sex determination during gonad coalescence (Wawersik et al., 2005), this suggests a model (Fig. 6) by which the newly coalesced gonad initially serves as a signaling center that acts to specify male germline sexual identity, and to expand the pool of potential GSCs through symmetric cell divisions. As hub formation proceeds, however, this signaling center gradually becomes restricted to the anterior tip of the embryonic gonad and promotes maintenance of male GSCs at the hub through differential cell adhesion and restricted activation of the Jak–STAT pathway. Whether the Jak–STAT pathway has a direct role in promoting onset of self-renewing GSC divisions, perhaps by regulating formation of GSC–hub adhesions, or whether it plays a more passive role by simply preventing PGC differentiation remains to be determined. Regardless, these data suggest that the coordinated regulation of Jak–STAT signaling and hub morphogenesis play a critical role in the regulation of functional GSC establishment.

A similar situation occurs in the ovary, where Dpp signaling maintains adult GSCs (Xie and Spradling, 1998), PGC differentiation is actively repressed by Dpp signaling in larvae (Gilboa and Lehmann, 2004), such that GSC establishment in developing ovaries is not

observed until the larval to pupal transition (~96 h AEL) (Bhat and Schedl, 1997; Zhu and Xie, 2003). Dpp pathway inhibition causes precocious differentiation of PGCs in third instar larval gonads, indicating that the same GSC maintenance factors in the adult ovary function during development. Finally, terminal filament cells are generated and organized during late larval stages, but it is not until cap cell differentiation during pupation that GSCs are specified in the anterior of the gonad and Bam-expressing germ cells are found at the posterior of the gonad. Thus, the ovarian GSC niche also appears to form simultaneously as PGCs transition to GSCs. As differential timing of stem cell establishment may be a conserved feature of germline development (reviewed in Casper and Van Doren, 2006; Wawersik, 2006), this suggests that the processes of GSC establishment and germ cell sex determination may be integrally linked.

Excess PGCs may contribute to stem cell quality control

The tight correlation between hub formation and restriction of STAT92E activation suggests that STAT92E restriction is controlled by limited access to signals emanating from hub precursor cells as the hub coalesces. Further studies are required to rigorously determine the fate of germ cells that do not maintain STAT92E activation and are not physically associated with the newly-formed niche. Interestingly, experiments involving transplantation and subsequent tracking of GFP-marked PGCs show that GSCs in the male can be established from PGCs that were located either anteriorly or posteriorly in the gonad before hub formation (Asaoka and Lin, 2004). As our data indicates that hub-proximal germ cells become GSCs, we postulate that there may be movement of PGCs or even competition for niche occupancy within the gonad during stem cell establishment. Live-imaging of germ cells in the coalesced gonad would be helpful at elucidating whether cells change position.

Perhaps specifying an excess of potential GSCs bypasses the need for GSCs to be generated through other means, such as dedifferentiation (Brawley and Matunis, 2004), as GSCs generated by gonial cell dedifferentiation may not be as robust as those derived embryonically. Recently, it has been shown that male *Drosophila* GSCs generated from dedifferentiation occasionally have defects in cell-cycle progression and centrosome positioning (Cheng et al., 2008). There may also be an evolutionary benefit for the organism to contain as many of the original GSCs as possible at the onset of spermatogenesis. In the *Drosophila* ovary, GSCs that are mutant for the differentiation factor Bam out-compete wild-type GSCs for niche occupancy by having a greater amount of E-Cadherin-mediated contact with the cap cells (Jin et al., 2008). Thus, male *Drosophila* PGCs may also be inherently heterogeneous, and a similar mechanism may exist in the male gonad where germ cells most strongly contacting the hub are preferentially maintained as GSCs.

Endowing the developing organ with an excess of potential stem cells may be a conserved feature of many organisms. In mice, the entire mouse germline is derived from a small fraction of PGCs selected from a larger pool originating in the fetus (Ueno et al., 2009). Spermatogenesis begins around postnatal day 3 (P3), with the migration of PGCs to the basement membrane of the seminiferous tubule (Bellve, 1998). It is debated exactly how and when these cells transition into functional GSCs. However, lineage analysis experiments show that the first round of spermatogenesis does not necessarily derive from spermatogonial stem cells, termed undifferentiated spermatogonia (Yoshida et al., 2006). Instead, some of the precursors of spermatogonia, called gonocytes, are able to directly differentiate into maturing spermatogonia without first being established as undifferentiated spermatogonia. How these gonocytes are directed to differentiation versus becoming spermatogonial stem cells may be related to where the gonocytes resided in the seminiferous tubule.

Comparison to other stem cell niches

Ovaries and testes in adult *Drosophila* are highly polarized and display clearly defined niches that control GSC behavior. Other stem cell systems, however, are not as well defined. Recent work in mice suggests that undifferentiated spermatogonia reside on the basement membrane of the seminiferous tubule and preferentially localize to niches near vascular branch-points (Oatley and Brinster, 2008; Yoshida et al., 2007). Signals emanating from this niche are hypothesized to balance GSC maintenance and differentiation. However, the exact cellular architecture of the mammalian spermatogonial stem cell niche is not known. During development, the mammalian spermatogonial stem cell niche appears to be highly dynamic; arising as an indirect consequence of vascular branch formation and remodeling (Yoshida et al., 2007). The developing *Drosophila* male GSC niche may, therefore, provide a more tractable model for its mammalian counterpart.

A comparison between *Drosophila* germline stem cell establishment and the development of other stem cell systems has also begun to reveal common themes that may broadly apply to initiation of asymmetric cell division. In the developing *Drosophila* nervous system, asymmetric division of neural stem cells, or neuroblasts, is controlled by apical localization of an evolutionary conserved PAR protein complex that is required for orientation of the mitotic spindle away from the stem cell niche (Chia et al., 2008; Siegrist and Doe, 2006). A similar PAR complex is also required for oriented division of epithelial stem cells at the onset of stratification during mouse skin development (Lechler and Fuchs, 2005). In both systems, this protein complex promotes positioning of one microtubule organizing center, or centrosome, near the presumed stem cell-niche interface. Linkage to integrin- and/or cadherin-based cell adhesions at this interface is critical for asymmetric PAR complex positioning and mitotic spindle orientation. While the PAR complex has not been shown to mediate asymmetric division of *Drosophila* GSCs, our observation that formation of polarized cell adhesions between male GSCs and the developing niche correlates with APC2 and centrosome recruitment to the GSC–hub interface, suggests that similar mechanisms may also regulate onset of asymmetric GSC divisions. Thus, adhesion-mediated centrosome anchoring near the stem cell-niche interface may be a common mechanism for stem cell establishment. It is therefore, likely, that insights gained through analysis of *Drosophila* GSC establishment will lead to significant insight into general mechanisms by which stem cell populations transition between symmetric and asymmetric divisions during development, tissue regeneration, niche engraftment and cancer progression. A general understanding of how progenitor cells initiate asymmetric stem cell divisions may also lead to the development of techniques aimed at long-term culture of progenitor cells with therapeutic potential.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.07.031.

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