Heparan sulfate regulates the number and centrosome positioning of Drosophila male germline stem cells

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ABSTRACT Stem cell division is tightly controlled via secreted signaling factors and cell adhesion molecules provided from local niche structures. Molecular mechanisms by which each niche component regulates stem cell behaviors remain to be elucidated. Here we show that heparan sulfate (HS), a class of glycosaminoglycan chains, regulates the number and asymmetric division of germline stem cells (GSCs) in the Drosophila testis. We found that GSC number is sensitive to the levels of 6-O sulfate groups on HS. Loss of 6-O sulfation also disrupted normal positioning of centrosomes, a process required for asymmetric division of GSCs. Blocking HS sulfation specifically in the niche, termed the hub, led to increased GSC numbers and mispositioning of centrosomes. The same treatment also perturbed the enrichment of Apc2, a component of the centrosome-anchoring machinery, at the hub–GSC interface. This perturbation of the centrosome-anchoring process ultimately led to an increase in the rate of spindle misorientation and symmetric GSC division. This study shows that specific HS modifications provide a novel regulatory mechanism for stem cell asymmetric division. The results also suggest that HS-mediated niche signaling acts upstream of GSC division orientation control.

INTRODUCTION
Adult stem cells have the unique potential to divide and produce both stem and differentiating daughter cells over the life of an organism (He et al., 2009). The differentiating cells produced in these divisions can go on to replace old or damaged tissue cells in response to aging, organ damage, or normal tissue homeostasis (Potten and Loeffler, 1990). A balance of production between stem and differentiating cells is of the utmost importance in ensuring that enough cells are present to carry out these processes while preventing excess cell output or cancer (Preston-Martin et al., 1990). To maintain this balance, stem cells are regulated through both cell-intrinsic and -extrinsic mechanisms (He et al., 2009). One such mechanism is asymmetric division, in which daughter cells are produced in a precisely oriented manner to maintain one stem cell in the niche while the other daughter cell moves away from it (Yamashita et al., 2010; Inaba and Yamashita, 2012).

The germline stem cell (GSC) niche in the Drosophila testis offers an excellent system for studying stem cell maintenance and asymmetric division (Yamashita and Fuller, 2008; Yuan and Yamashita, 2010). In this niche, two populations of stem cells exist: GSCs and cyst stem cells (CySCs), which are a pair of somatic cells that enwrap each GSC. Both of these stem cell populations contact the niche, a group of somatic cells called the hub. Two key processes in the GSC niche regulate self-renewal and differentiation. First, the hub secretes the ligands Unpaired (Upd) and Glass bottom boat/Decapentaplegic (bone morphogenetic proteins [BMPs]), which are necessary for the maintenance of CySCs and GSCs, respectively (de Cuevas and Matunis, 2011). Second, E-cadherin (E-cad)—mediated adhesion allows for anchoring of centrosomes at the niche interface in GSCs (Yamashita et al., 2003). This centrosome anchoring defines the future orientation of the spindle, which is perpendicular to the contact interface between each GSC and the hub, ensuring their stereotypical asymmetric division. Asymmetric division (mediated by centrosome anchoring) is a robust process in this niche, as GSC numbers must be tightly regulated to ensure renewal of the stem cell while also producing a single differentiating daughter cell,
called a gonialblast. In fact, proper anchoring of the mother centrosome in GSCs is typically essential for division to proceed (Yamashita et al., 2005, 2007). The microtubule-binding protein Adenomatous polyposis coli 2 (Apc2) is important in this process. Apc2 preferentially localizes to the GSC cortex adjacent to the hub and participates in anchoring at this interface through its interactions with the mother centrosome’s astral microtubules (Yamashita et al., 2003). Of note, disruption or loss of Apc2 results in a premitotic arrest of GSC division. However, the relationship between these two mechanisms—niche signaling and division orientation control—is poorly understood.

One class of molecules that might play key roles in the stem cell niche are the carbohydrate-modified proteins heparan sulfate proteoglycans (HSPGs). HSPGs bind a number of ligand proteins via heparan sulfate (HS) chains to regulate their distribution and signaling (Bishop et al., 2007). Such HS-dependent factors include fibroblast growth factors (FGFs), Wnts, BMPs, Hedgehog (Hh), and a Drosophila JAK/STAT ligand, Upd. Of importance, the fine structure of HS chains has a major effect on HSPG function (Nakamoto and Wojcinski, 2003; Wojcinski et al., 2001, 2006; Ai et al., 2001, 2008). Functional studies of vertebrate and Drosophila Hs6sts and Sulfs suggest that they regulate Wnt, FGF, BMP, and Hh signaling (Kamimura et al., 2001, 2006; Ai et al., 2003; Habuchi and Kimata, 2010; Kleinschmit et al., 2010, 2013; Wojcinski et al., 2011), highlighting 6-O-sulfation/desulfation as key regulatory steps for HS function.

We demonstrated that HSPGs are essential regulators of the GSC niche in the Drosophila ovary (Hayashi et al., 2009). In the female GSC niche, HSPGs expressed in the niche (cap cells) activate BMP signaling in trans in the directly contacting GSCs. This trans coreceptor activity of HSPGs can explain the contact dependence of GSC maintenance (Hayashi et al., 2009; Dejima et al., 2011). We also observed that GSCs and germ cells are substantially reduced in larval testes mutant for an essential HS biosynthetic gene, tout-velu (ttv; Hayashi et al., 2009). Although this finding indicated essential roles of HSF in the male GSC niche, the mechanism by which HSF controls GSC maintenance is unknown. In this study, we addressed three major questions regarding the role of HSF in regulation of male GSCs: 1) whether specific HS modifications play roles in the GSC niche, 2) in which cells does HSF function, and 3) whether HSF participates in asymmetric division in addition to niche signaling. Our findings suggest that HSPG-dependent signaling from the niche affects formation or function of the centrosome-anchoring complex at the hub–GSC interface and thus affects the division orientation of GSCs.

RESULTS AND DISCUSSION

Specific modifications of HS affect GSC numbers

To elucidate the mechanism by which HS regulates the male GSC niche, we asked whether HS modifications affect GSC numbers. We particularly focused on the role of the 6-O-sulfate group, a key component of the binding sites on HS for most protein ligands (Ai et al., 2003; Wojcinski et al., 2011; Kleinschmit et al., 2013).

Consistent with previous studies, wild-type testes had an average of 9–10 GSCs at 1–3 d posteclosion (Figure 1, A and D; Yamashita et al., 2005; de Cuevas and Matunis, 2011). Of interest, homozygous Hs6st-null mutants had a significantly higher number of GSCs (Figure 1, B and D), but normal hub size and structure (Figure 1E), compared with both wild-type and heterozygous control flies. Anti–Hu-li tai shao (Hts) staining of the excess germ cells in Hs6st testes showed the spherical spectrosomes characteristic of a GSC/gonialblast (Figure 1F), confirming the undifferentiated status of these cells. Conversely, null mutants for Sulfl (the enzyme removing 6-O sulfation) had GSC numbers lower than that of the wild-type strain (Figure 1, C and D). These results indicate that HS 6-O sulfation levels affect GSC numbers.

Proper HS modification is required for normal GSC centrosome positioning

One possible explanation for the observed increase in GSC number is the failure of GSC asymmetric division. If a GSC division produces two daughter cells contacting the hub, both would be maintained as GSCs. In fact, abnormalities in stem cell number frequently accompany asymmetric division defects (Yamashita et al., 2003; Lu et al., 2012). Proper centrosome positioning is critical in this process. Specifically, the apical or mother centrosome in GSCs associates with components of the centrosome-anchoring machinery, such as Baz and Apc2 (Yamashita et al., 2003; Inaba et al., 2015), which are found at regions of the cell cortex with E-cad–mediated adhesion to the hub. The daughter centrosome migrates to the opposite side of the cell for division, and thus an axis perpendicular to the hub interface is established for spindle assembly.

We asked whether 6-O sulfation affects centrosome positioning in GSCs. Because the mother centrosome is normally anchored at the niche interface, any GSC with at least one centrosome at the 90° interface with the hub was counted as “oriented,” and any GSC with neither centrosome at this interface was counted as “misoriented” (Figure 2, A–E). In wild type, mispositioning of centrosomes was observed in 14.4% of GSCs (Figure 2F). We found that Hs6st-null mutation results in a significant increase in the rate of centrosome mispositioning (34.5%) in GSCs compared with wild-type testes (Figure 2F). Thus proper 6-O sulfation is required for normal rates of oriented centrosomes in GSCs. No significant abnormality in centrosome positioning was observed in Sulfl1 mutants.

HS in the hub plays an important role in GSC number control

We next examined the cell type–specific requirement of HS in control of GSC number. To block HS biosynthesis, we used RNA interference (RNAi) knockdown of Sulfateless (Sfl), which catalyzes the first step of HS modification. Loss of sfl eliminates HS activity, and sfl-null mutants are larval lethal (Lin and Perrimon, 1999). We therefore disrupted sfl in specific cell types in the testis only at adult stages using the TARGET system, which allows temporal and spatial control of gene expression (McGuire et al., 2003).

When we induced expression of a sfl short hairpin RNAi construct (UAS-sh-sfl) in the hub using upd-Gal4, GSC number was significantly increased compared with the isogenic control strain (Figure 3A and Supplemental Figure S1, A and B). This effect was similar to the phenotype observed in Hs6st mutants (Figure 1D). However, no significant change in GSC number was caused by knockdown of sfl in the germ cell or the CySC/CyC populations (Figure 3A and Supplemental Figure S1, C–F). These results suggest that HSF expressed in the hub non–cell autonomously regulates GSC number.

We confirmed this result using the technique of germline-specific knockout (Kirchner et al., 2008; White-Cooper, 2012). In this experiment, we used another critical gene for HS biosynthesis, tout-velu (ttv), which encodes a HS copolymerase (Bellaiche et al., 1998). ttv mutants have no detectable HSF and are larval lethal (Toyoda et al., 2000). We rescued these mutants by expressing UAS-ttv in all...
hub HS is responsible for regulation of GSC number. Previous studies highlighted the importance of HS in maintenance of GSCs in both males and females (Hayashi et al., 2009). For instance, in larvae with ubiquitous ttv loss (which do not survive to adulthood), GSCs are no longer maintained in the niche, and differentiating gonialblasts are observed contacting the niche. On the other hand, here we showed that loss or down-regulation of hub-specific HS or 6-O-sulfation can result in increased GSC numbers in the male niche. One explanation is that HSPGs expressed in other cell populations, such as the CySCs, may partially rescue GSC maintenance but not hub-specific asymmetric division processes. As another possibility, reduction but not total elimination of hub HS may compromise some of its biological functions while still allowing sufficient signaling for GSC maintenance. Indeed, we did see GSC loss from the niche after long-term sfl knockdown (>12 d; unpublished data).

Hub-specific knockdown of HS perturbs normal centrosome positioning

To determine whether the increased number of GSCs caused by hub-specific HS loss is associated with abnormal orientation of GSC division, we examined centrosome positioning in upd>sh-sfl testes. We found that sfl knockdown in the hub led to a substantial increase in the rate of centrosome mispositioning (38.0%) compared with the RNAi control (17.3%; Figure 3E and Supplemental Figure S1, H and I).

Our analyses indicate that 1) hub HS is critical for normal centrosome anchoring and 2) 6-O-sulfation is an essential modification step for this function of HS. To further confirm these results, we asked whether hub-specific loss of 6-O-sulfation leads to a similar phenotype. We overexpressed a Golgi-tethered form of Sulf1 in the hub (upd>Sulf1[Golgi]), which removes 6-O-sulfate groups in a cell-autonomous manner (Kleinschmit et al., 2010). We found that the centrosome mispositioning rate in GSCs of upd>Sulf1[Golgi] (33.1%) recapitulated that of upd>sh-sfl (Figure 3E and Supplemental Figure S1J).

If HS expressed in the hub is responsible for anchoring centrosomes at the hub–GSC interface, then overexpression of HSPGs outside the niche could also disrupt centrosome positioning. To examine this idea, we expressed Dally (one of the glypican-class

somatic cells but not germ cells, using arm-Gal4. The somatic cell-specific rescued animals (ttv/ttv; arm>ttv) survived to adult stages. These animals exhibited normal overall testis morphology and niche organization, with GSC numbers slightly lower than that of the reference wild-type strain (Figures 1A and 3C and Supplemental Figure S1G) but within the normal wild-type range, as well as normal centrosome positioning (Figures 2C and 3, B and D). On the basis of these results, we concluded that hub HS is responsible for regulation of GSC number.

Previous studies highlighted the importance of HS in maintenance of GSCs in both males and females (Hayashi et al., 2009). For instance, in larvae with ubiquitous ttv loss (which do not survive to adulthood), GSCs are no longer maintained in the niche, and differentiating gonialblasts are observed contacting the niche. On the other hand, here we showed that loss or down-regulation of hub-specific HS or 6-O-sulfation can result in increased GSC numbers in the male niche. One explanation is that HSPGs expressed in other cell populations, such as the CySCs, may partially rescue GSC maintenance but not hub-specific asymmetric division processes. As another possibility, reduction but not total elimination of hub HS may compromise some of its biological functions while still allowing sufficient signaling for GSC maintenance. Indeed, we did see GSC loss from the niche after long-term sfl knockdown (>12 d; unpublished data).

FIGURE 1: HS modifications regulate GSC number. (A–C) GSCs in a single confocal section in wild-type (A), Hs6st (B), and Sulf1 (C) mutant testes. GSCs identified in these confocal sections are marked by asterisks. (D) Quantification of GSC numbers in Hs6st and Sulf1 mutants. A cumulative count of all GSCs from multiple confocal sections was tallied for each testis. There is also no significant difference in hub area in Hs6st and Sulf1.

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HSPGs in Drosophila) in the CySC/CyC population. In this experiment, we also observed a significant increase in the rate of centrosome mispositioning (32.4%; Figure 3F and Supplemental Figure S1, K and L). This result supports the idea that HS on the surface of nearby, contacting cells provides GSCs with an orientation cue. Loss of hub HS eliminates Apc2 enrichment at hub–GSC interface

To determine the mechanism for how hub HS regulates positioning of centrosomes in GSCs, we asked whether hub-specific loss of HS affects the centrosome-anchoring machinery. Previous studies identified Apc2 as a component of the cell membrane complex required for centrosome anchoring (Yamashita et al., 2003; Venkei and Yamashita, 2015; Lu et al., 2012). Apc2 anchors astral microtubules of the mother centrosome by directly or indirectly binding adherens junction components at the GSC interface with the hub, although the specific upstream partner(s) in this anchoring are not known (Inaba et al., 2010). In the absence of Apc2, centrosomes are not properly anchored and a checkpoint arrests GSCs before spindle assembly.

We examined whether hub HS affects the localization of Apc2, a representative centrosome-anchoring component. In wild type, Apc2 was detected along the cell membrane with obvious enrichment at the hub–GSC interface (Figure 4, A and A′). Testes from the RNAi control showed a similar trend (Figure 4, C and C′). This pattern of Apc2 distribution was disrupted in Hs6st and upd>sh-sfl testes. The GSCs in these testes showed a more uniformly distributed Apc2 staining along the entire cell surface, with no obvious enrichment at the hub interface (Figure 4, B, B′, D, and D′). In addition, more diffusive cortical patterns of Apc2 staining were detected, leading to broader signals near the cell membrane, as observed in testes with germline-specific perturbation of insulin signaling (Roth et al., 2012), Rac1 (Lu et al., 2012), or Lar (Srinivasan et al., 2012) activity.

FIGURE 2: Hs6st mutation perturbs centrosome positioning in GSCs. (A–E) Centrosome positioning in GSCs. Examples are shown for pairs of properly oriented (yellow arrows) and misoriented (red arrows) centrosomes. Examples are shown from Hs6st/TM6B (A, B), wild-type (C), or Hs6st (D, E) testes. (F) Quantification of misoriented centrosomes (mean ± SE). n.s not significant; ***p < 0.001. n, number of GSCs assayed. Bar, 10 μm.

To confirm these results, we quantified Apc2 distribution. We labeled GSCs with an anti-Apc2 antibody and measured the average staining intensity in the GSC cortex, specifically at the regions in contact with the hub and also the remaining GSC cortex (Figure 4E). In wild-type testes, GSCs showed a significant enrichment of Apc2 at the hub interface (Figure 4F). The quantification confirmed that both shl knockdown in the hub and Hs6st mutation caused redistribution of Apc2. Specifically, hub enrichment of Apc2 was markedly reduced in both genotypes compared with controls (Figure 4, F and G). Taken together, these results strongly suggest that HS expressed in the hub is required for normal localization and function of the centrosome-anchoring machinery.

Loss of 6-O sulfation can disrupt spindle orientation and asymmetric division

Loss of proper centrosome anchoring, and thus positioning, generally activates the centrosome orientation checkpoint (COC; Cheng et al., 2008; Inaba et al., 2010). The COC halts division of wild-type GSCs in the G2 phase of the cell cycle, before spindle assembly and mitosis (Venkei and Yamashita, 2015). To date, only a few genetic manipulations have been able to bypass the COC and result in misoriented mitotic spindles, thus increasing the rate of symmetric division of GSCs. In many of these cases, genetic manipulations of components of the COC itself were used, such as Par-1 and Baz (Yuan et al., 2012; Inaba et al., 2015). The increased number of GSCs observed in Hs6st mutants suggests that Hs6st is a novel mutant that...
A model: HSPGs participate in centrosome anchoring

HSPGs facilitate formation or stabilization of growth factor signaling complexes (Fujise et al., 2003; Akiyama et al., 2008). In the female GSC niche, HSPGs expressed in the niche cells function as trans coreceptors for niche factors, such as BMPs, to regulate the maintenance of the stem cells contacting the niche (Hayashi et al., 2009). Here we demonstrate that hub HS also affects centrosome anchoring and asymmetric division of GSCs in the testis. Our results show that hub HS affects Apc2 distribution, suggesting a link between niche signaling and the centrosome-anchoring machinery. Thus HSPGs may directly or indirectly participate in formation of a membrane complex at the niche–GSC interface, which recruits components of the centrosome-anchoring machinery to this region (Figure 5D).

The observed misorientation of spindles in animals with perturbed HS function, along with increased rates of centrosome mispositioning, indicates a failure in the asymmetric division of these stem cells. This increased rate of symmetric division likely explains the increased numbers of GSCs seen in Hs6st mutants and hub-specific knockdown of sfl. However, some questions remain, such as the mechanism by which these mutants bypass the COC and allow for GSC division despite a loss of proper centrosome anchoring.

One possibility is that HS controls localization of proteins necessary for centrosome anchoring/asymmetric cell division at specific regions of the GSC cortex, thus providing a “polarization signal.” Other studies showed HSPGs to be capable of such a mechanism in a cell-autonomous manner (Dejima et al., 2014). Here we propose that HS on the hub affects distribution of niche factor(s), such as Upd/BMP, and/or their receptor(s) by “tethering” these factors to the hub. Similar mechanisms have been shown in which HSPGs limit the diffusion of ligand proteins away from their source (Hayashi et al., 2012). Because signaling events on the membrane of stem cells at the niche should inherently provide positional information about the extracellular environment of these cells, these signals may also trigger intracellular positional cues for the division machinery. Specifically, this tethering of niche factors to hub HS leads to the formation of the niche factor signaling complex only at the hub–GSC interface, causing a polarity in the GSC membrane. Thus, in wild type, the localized formation of the signaling complex allows for anchoring of the centrosome at this interface (Figure 5D). Under conditions of whole-animal loss of 6-O sulfation or loss of sulfation in the hub, this normal sequestration of niche factors at the hub may be lost and result in an expanded range of niche signaling. HS outside the niche, such as on the surface of CySCs/GSCs, appears to allow for ectopic signaling, which can lead to bypass of the COC in a fraction of misoriented GSCs (Figure 5E). Additional study is required to determine whether expanded niche signaling and ectopic localization of other centrosome-anchoring components are detected in these mutant and knockdown animals.

MATERIALS AND METHODS

Fly strains

Detailed information for the fly strains used is given at FlyBase (http://flybase.bio.indiana.edu/), except where noted. The wild-type strain used was Canton S. Other strains used were Hs6st<ts>, a null allele of Hs6st (Kamimura et al., 2010); Sulf1<null>, a null allele of Sulf1 (Kleinschmit et al., 2010); tvt<null>, a null allele of tvt (Takei et al., 2004); unpaired (upd)-Gal4 (Halder et al., 1999); nanos (nos)-Gal4 VP16 (Van Doren et al., 1998); c587-Gal4 (Kai and Spradling, 2003); armadillo (arm)-Gal4 (Sanson et al., 1996); tubulin (tub)-Gal80<null> (McGuire et al., 2003); UAS-ttv (The et al., 1999); UAS-daily-Myc (Takeo et al., 2005); UAS-sh-sfl, a UAS short hairpin RNAi strain for
FIGURE 4: Hub HS regulates Apc2 distribution in GSCs. (A–D′) Apc2 localization (green in A–D and gray in A′–D′) in wild-type (A, A′), Hs6st mutants (B, B′), upd-Gal4 (C, C′), and upd>sh-sfl (D, D′) testes. Hub (FasIII) and germline cells (Vas) are shown in red and blue, respectively (A–D). Positions of hubs are marked by asterisks, and strong Apc2 signals are highlighted by yellow lines (A′–D′). (E–G) Quantification of Apc2 enrichment. The hub–GSC interface and the remaining GSC cortex are shown by red and blue lines, respectively (E). Hs6st mutation (F) and hub-specific knockdown of sfl (G) disrupted Apc2 localization to the interface, resulting in a significant loss of Apc2 enrichment at the hub interface (F, G). Numerical values are the mean ± SE. n, number of GSCs assayed. **p < 0.01, ***p < 0.001. Bar, 10 μm.
For the germline-specific knockout experiment, the fly stocks ttv524/CyO; arm-Gal4 and ttv524/CyO; UAS-ttv were crossed at 25°C. The resulting ttv524; arm-Gal4/UAS-ttv offspring had rescued ttv activity exclusively in the somatic cells of the animal but not the germline (Kirchner et al., 2008; White-Cooper, 2012). Although loss of ttv is larval lethal, the rescued animals survived to adult stages. By allowing for normal fly development and HS function in the somatic hub and CySC/CyC lineages, we were able to investigate the role of HS loss specifically in the germline.

Immunofluorescence staining

Immunostaining was performed as previously described (Fujise et al., 2001; Dejima et al., 2013; Salzmann et al., 2013). Briefly, samples were fixed for 30 min with 4% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.3% PBST (0.3% Triton X-100 in PBS) for 20 min (10 min, twice), and washed with 0.1% PBST (10 min, twice). They were then blocked in 5% normal goat serum in 0.1% PBST for 1 h and incubated overnight in primary

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**FIGURE 5:** Misoriented spindle in dividing Hs6st GSCs. (A–C) Spindle orientation in GSCs. Examples are shown for properly oriented (yellow arrow) and misoriented (red arrow) spindles. No misoriented spindles were observed in wild type (A), whereas a significant fraction (21.1%) of dividing Hs6st GSCs showed misoriented (B) or aberrant spindles. n = 20 (wild type) and 19 (Hs6st) dividing GSCs counted. (C) Quantification of spindle orientation/misorientation. In wild type, the rates of spindle orientation vs. misorientation were 3.4 and 0% of GSCs, respectively. In Hs6st mutants, there was no significant difference in the rate of oriented divisions (1.9%) from wild type; however, a significantly higher rate of misoriented divisions was detected (0.5%). (D, E) Model for the role of HSPGs in centrosome anchoring. HSPGs may participate in formation of a membrane complex at the niche–GSC interface, which allows for a “polarization signal” and recruits components of the centrosome-anchoring machinery to this interface (D). When HS is lost, the signaling range of niche factor(s) involved in this process is expanded, resulting in mispositioning of centrosomes and misoriented division (E). Numerical values are the mean ± SE, n, number of GSCs assayed. n.s., not significant; *p < 0.05. Bar, 10 μm.
antibodies at 4°C. Samples were again washed in 0.1% PBST (10 min, three times) and incubated with the appropriate Alexa Fluor secondary antibodies either overnight at 4°C or for 2 h at room temperature. They were then washed in 0.1% PBST (10 min, four times) before being mounted in VECTASHIELD (H-1000 or H-1200; Vector Laboratories, Burlingame, CA).

For centrosome-positioning scoring experiments, samples were fixed for 1 h with 4% formaldehyde in PBS, permeabilized for 30 min in 0.3% sodium deoxycholate plus 0.3% PBST (15 min, twice), and washed with 0.1% PBST (10 min, twice). For Apc2 staining experiments, samples were prepared according to Srinivasan et al. (2012) with some modification. Briefly, samples were fixed for 20 min with 4% formaldehyde in PBS, permeabilized for 1 h in 0.6% sodium deoxycholate plus 0.3% PBST (20 min, three times), and washed with 0.1% PBST (10 min, twice). Images were obtained using either a Zeiss 710 or a Nikon Eclipse E800 laser scanning confocal microscope.

The following primary antibodies were used: rabbit anti-Vasa (Vas; 1:500; a gift from S. Kobayashi, Okazaki Institute for Integrative Bioscience, Okazaki, Japan), chick anti-Vas (1:1000; a gift from S. Kobayashi), mouse anti-Hts (1:5; Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA), rat anti-E-cad (1:40; DSHB), rat anti-N-cad (1:20; DSHB), mouse anti-FasIII (1:200; DSHB), mouse anti–γ-tubulin (GTU-88; 1:500; Sigma-Aldrich, St. Louis, MO), rabbit anti–phospho-histone H3 (Ser-10; 1:1000; EMD Millipore, Billerica, MA), and rabbit anti-Apc2 (1:5000; a gift from M. Bienz, MRC Laboratory of Molecular Biology, Cambridge, UK). Secondary antibodies were from the Alexa Fluor series (1:500; Thermo-Fisher Scientific, Waltham, MA).

Quantification of GSC number and hub measurement
GSCs were counted as described previously (Boyle et al., 2007; Hayashi et al., 2009; Srinivasan et al., 2012). Germline cells were counted as stem cells only when directly contacting hub cells labeled with FasIII*, N-cad*, or E-Cad*. In brief, a confocal microscope was used to scan through the samples, and a cumulative count of all Vasa* germline cells contacting the hub was tallied for each testis. These numbers were then used to generate an average GSC number for each genotype (in each experiment). For most genotypes, GSC identity was also verified by spectroscope morphology (spherical Hts staining) in a subset of samples. This is a characteristic marker for GSCs and gonialblasts only, whereas a branched fusome morphology is seen by Hts staining in later stages of spermatogonial development.Briefly, while scanning through the depth of a sample, Hts staining was examined in each germline cell contacting the niche. If the Hts' organelle showed a small, spherical morphology, the germ-line cell was counted as a GSC (vs. a dumbbell-shaped or branched morphology characteristic of differentiating germline cells).

For measurement of hub area, images showing a cross section through the middle portion of each hub (with the largest cross-sectional diameter) were taken of each testis at the same magnification through the middle portion of each hub (with the largest cross-sectional diameter) were taken of each testis at the same magnification. We first obtained images of many GSCs/niches of each genotype using confocal microscopy. In obtaining these images, the focal plane was adjusted to roughly bisect the hub while also bisecting several of the contacting GSCs. The signal intensity/gain for the Apc2 channel was adjusted to achieve maximal signal without saturation (since our goal was to compare distribution, not absolute protein levels). We then used ImageJ to trace the Apc2 signal intensity at the GSC interface with the hub (“interface”; red line, Figure 4D) separately from the rest of the GSC cortex (“periphery”; blue line, Figure 4D). To ensure unbiased measurement, we first defined positions of the interface versus the periphery using the Vasa- and N-cad–stained channels from confocal images and traced a line along each of these membranes. After a switch to the Apc2 channel, the Apc2 intensity (averaged over a 5-pixel width) was measured separately along each of these lines for each GSC. The average Apc2 intensity for 1) the hub interface and 2) the hub interface added to the remaining (periphery) cortex was calculated for each GSC. The Apc2 hub enrichment was then calculated as the ratio of the average hub Apc2 intensity divided by the average cumulative Apc2 intensity minus one for each GSC. A two-tailed Student’s t test was used for determining statistical significance for centrosome mispositioning, spindle orientation, and Apc2 hub enrichment.

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