The centrosome orientation checkpoint is germline stem cell specific and operates prior to the spindle assembly checkpoint in *Drosophila* testis

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ABSTRACT

Asymmetric cell division is utilized by a broad range of cell types to generate two daughter cells with distinct cell fates. In stem cell populations asymmetric cell division is believed to be crucial for maintaining tissue homeostasis, failure of which can lead to tissue degeneration or hyperplasia/tumorigenesis. Asymmetric cell divisions also underlie cell fate diversification during development. Accordingly, the mechanisms by which asymmetric cell division is achieved have been extensively studied, although the check points that are in place to protect against potential perturbation of the process are poorly understood. *Drosophila melanogaster* male germline stem cells (GSCs) possess a checkpoint, termed the centrosome orientation checkpoint (COC), that monitors correct centrosome orientation with respect to the component cells of the niche to ensure asymmetric stem cell division. To our knowledge, the COC is the only checkpoint identified to date that specializes in monitoring the orientation of cell division in multicellular organisms. Here, by establishing colcemid-induced microtubule depolymerization as a sensitive assay, we examined the characteristics of COC activity and find that it functions uniquely in GSCs but not in their differentiating progeny. We show that the COC operates in the G2 phase of the cell cycle, independently of the spindle assembly checkpoint. This study may provide a framework for identifying and understanding similar mechanisms that might be in place in other asymmetrically dividing cell types.

KEY WORDS: Centrosome, Checkpoint, *Drosophila*, Stem cells, Asymmetric cell division, Spindle orientation

INTRODUCTION

Asymmetric cell division plays a fundamental role in the development of multicellular organisms. It is achieved either by the asymmetric segregation of fate determinants or the positioning of daughter cells into distinct microenvironments that dictate cell fates after the completion of the cell division (Inaba and Yamashita, 2012; Knoblich, 2008; Siller and Doe, 2009). In either mechanism, orientation of cell division must be coordinated with respect to the polarity of the extracellular microenvironment and/or intrinsic cell fate determinants. Many cell types are known to divide asymmetrically by orienting mitotic spindles. For example, in the first division of embryonic *Drosophila* neuroblasts, the mitotic spindle is first formed parallel to the epithelia, then rotates by 90° to divide asymmetrically (Kaltschmidt et al., 2000). In early *Caenorhabditis elegans* embryogenesis, a series of asymmetric divisions to generate distinct cellular lineages is achieved by stereotypical spindle orientation, some of which are mediated by the programmed rotation of spindles (Goldstein, 2000; Sawa, 2012). In these examples, it is predicted that cell division without the establishment of correct orientation would result in failure of asymmetric division and thus of tissue development. However, it remains unclear whether these cells have a mechanism that prevents precarious mitosis before the establishment of cell polarity.

A mechanism that ensures the correct temporal order of two potentially independent events is generally defined as a checkpoint. It is currently unknown whether many of the asymmetric cell divisions studied to date are under the regulation of checkpoints that couple cell division and polarity. We have shown that *Drosophila* male germline stem cells (GSCs) possess a checkpoint mechanism that coordinates cell polarity and cell division (Cheng et al., 2008; Inaba et al., 2010; Yuan et al., 2012). Male GSCs divide asymmetrically by orienting their mitotic spindles perpendicular to the hub cells, which constitute the major component of the stem cell niche (Yamashita et al., 2003) (Fig. 1A,B). Spindle orientation is prepared during interphase by stereotypical positioning/orientation of the centrosomes. The mother centrosome is consistently located near the hub-GSC junction, whereas the daughter centrosome migrates to the other side of the GSC, leading to spindle orientation perpendicular to the hub cells (Yamashita et al., 2007). Further evidence suggested that GSCs actively monitor centrosome orientation to ensure that the centrosomes are correctly oriented prior to mitotic entry (Cheng et al., 2008; Inaba et al., 2010; Yuan et al., 2012). This checkpoint, termed the centrosome orientation checkpoint (COC), delays mitotic entry when centrosomes are not correctly oriented. We have previously shown that Cnn, an integral component of the centrosome, and Par-1, a kinase that is widely involved in cell polarity, are essential components of the COC (Inaba et al., 2010; Yuan et al., 2012). In the absence of Cnn or Par-1, GSCs enter mitosis without correct centrosome orientation, leading to misoriented spindles and symmetric stem cell divisions. Defects in the COC, which can lead to symmetric stem cell divisions, may in turn compromise long-term tissue homeostasis due to imbalance in self-renewal and differentiation. Additionally, genes that are required for centrosome orientation but not COC have been identified: mutations in *Apc1* (*APC-like – FlyBase*) or *Apc2* result in a high frequency of centrosome misorientation, yet the effect on spindle orientation is not profound, suggesting that the COC is largely intact in these mutants (Inaba et al., 2010); although the centrosomes are highly misoriented, the asymmetric outcome of GSC divisions is not compromised.

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Thus far, the COC is the only checkpoint identified in multicellular organisms that ensures correct spindle orientation. It is known that a unicellular organism, budding yeast, possesses a spindle position checkpoint (SPOC) that ensures correct spindle positioning prior to mitotic exit, such that both mother and daughter cells inherit nuclei (Pereira and Yamashita, 2011). Considering the importance of asymmetric division in the development of multicellular organisms, it is possible that other types of asymmetrically dividing cells possess similar checkpoint mechanisms. However, such orientation checkpoint mechanisms are currently underexplored. Thus, understanding the COC might provide a framework to aid the study of universal polarity checkpoint mechanisms that ensure asymmetric cell divisions.

We have undertaken a detailed characterization of the COC in *Drosophila* male GSCs, in order to further our understanding of its nature. Here, using the microtubule (MT)-depolymerizing drug colcemid, we show that the COC is a GSC-specific cell cycle checkpoint that operates in the G2 phase of the cell cycle. Interestingly, the COC does not operate in spermatogonia (SGs) and cyst stem cells (CySCs), although both of these populations undergo cell division with oriented spindles (Cheng et al., 2011; Wilson, 2005). We further show that the COC is distinct from the spindle assembly checkpoint (SAC). Finally, we show that the Sas-4 and asl mutants, which lack centrioles/centrosomes, do not show the COC activity to arrest GSCs in G2 phase upon MT depolymerization. Taken together, these results establish the COC as a GSC-specific checkpoint mechanism that ensures oriented stem cell division independently of the SAC.

**RESULTS**

**Spindle orientation is established at distinct stages of the cell cycle in GSCs and SGs**

In the *Drosophila* testis, a group of postmitotic somatic cells, termed hub cells, constitutes the stem cell niche. GSCs and somatic stem cells, the CySCs, attach to the hub cells, which secrete the ligand Upd (Outstretched – FlyBase) to specify their stem cell identity (Fig. 1A) (Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). GSCs divide asymmetrically by orienting mitotic spindles perpendicular to the hub cells (Fig. 1B,E) (Yamashita et al., 2003). Mitotic spindle orientation in GSCs is predetermined by positioning of the mother centrosome near the hub-GSC junction in interphase (Fig. 1B-D) (Yamashita et al., 2007). Upon GSC division, one daughter cell that is displaced away from the hub initiates a differentiation program as a gonialblast (GB). The GB undergoes four rounds of mitosis with incomplete cytokinesis, producing a cluster of 16 interconnected SGs (Fig. 1A) (Fuller, 1993). The SGs are classified as 2-cell, 4-cell, 8-cell and 16-cell stage based on the number of interconnected germ cells. It is known that the metaphase spindles in SGs are oriented toward the fusome, which is a membranous structure that spans interconnected germ cells (Wilson, 2005). We confirmed spindle orientation toward the fusome in SGs (Fig. 1F,I). Detailed inspection of interphase centrosome orientation and mitotic spindle orientation in 2- to 8-cell stage SGs revealed that SGs first enter mitosis without an oriented centrosome/spindle, and then orient toward the fusome between prophase and metaphase (Fig. 1F-I), as opposed to GSCs in
which the centrosomes are already oriented in interphase (Fig. 1B-E). These results indicate that, although spindles in both GSCs and SGs are stereotypically oriented, the establishment of spindle orientation is temporally distinct in these two cell types.

**GSCs misorient centrosomes and arrest in G2 phase upon depolymerization of MTs**

Proliferating cells are known to react to the lack of a bipolar spindle by arresting in mitosis due to activation of the SAC (Musacchio and Salmon, 2007). Accordingly, in a population of non-synchronized proliferating cells, cells accumulate in mitosis over time in the presence of colcemid, an MT-depolymerizing agent. To monitor the response of GSCs and other cells in the testes to MT depolymerization, we incubated dissected testes in the absence (DMSO control) or presence of colcemid for up to 6 h. We confirmed that colcemid treatment of testes results in depolymerization of MTs, eliminating characteristic MT structures such as cytoplasmic asters and mitotic spindles (supplementary material Fig. S1A,B). This treatment did not change the number of GSCs (supplementary material Fig. S1C) or affect any gross anatomical feature of the testis during the course of our 6-h experiments. Incubation of testes with colcemid led to a gradual accumulation of mitotic cells in the GB, SG and CySC populations, presumably via activation of the SAC in these cell types (Fig. 2A-D).

After 6 h of incubation in colcemid, the frequency of mitotic arrest decreased slightly compared with 4.5 h in these populations (Fig. 2D), which is likely to reflect ‘mitotic slippage’, a phenomenon whereby SAC-arrested normal cells eventually undergo adaptation and exit from mitosis (Brito and Rieder, 2006). In stark contrast to the accumulation of mitotic cells in the GB, SG and CySC populations, the frequency of mitotic GSCs did not increase during the course of incubation with colcemid (Fig. 2A-D).

Considering that the GSC cell cycle is similar in length to those of GBs, SGs and CySCs, the lack of mitotic arrest in the GSC population is not because the cell cycle is too slow to be captured by a 6-h colcemid treatment. Two main possibilities remain to explain the lack of mitotic arrest in GSCs upon depolymerization of MTs: (1) GSCs lack SAC activity and thus do not arrest in mitosis even if the spindle is not properly formed; or (2) GSCs arrest before mitosis upon depolymerization of MTs.

First, we addressed the possibility that GSCs arrest before mitosis. Considering that centrosome orientation is mediated by MTs that anchor the mother centrosome to the hub-GSC interface (Yamashita et al., 2007), we hypothesized that colcemid treatment might lead to centrosome misorientation due to perturbation of MTs, resulting in COC-mediated cell cycle arrest. Our previous studies showed that the COC prevents mitotic entry upon centrosome misorientation (Cheng et al., 2008; Yuan et al., 2012), which might explain the lack of mitotic arrest in GSCs upon colcemid treatment. This hypothesis leads to two testable predictions: (1) colcemid treatment induces centrosome misorientation in GSCs, leading to cell cycle arrest prior to mitotic entry (i.e. in interphase); and (2) such interphase arrest would depend on the COC. Accordingly, COC-defective mutants would not arrest in interphase and would enter mitosis. We found that GSCs indeed misorient centrosomes in the presence of colcemid (Fig. 2E-G): 57% of GSCs had misoriented centrosomes after 4.5 h of colcemid treatment, whereas mock-treated controls maintained a low frequency (7%) of centrosome misorientation (Fig. 2G). Next, we monitored the localization/accumulation of Dacapo (Dap), a CDK inhibitor of the Cip/Kip family that is known to accumulate in the nucleus during the G2 phase of the cell cycle (Hong et al., 2003; Meyer et al., 2002). After colcemid treatment, the frequency of Dap-positive GSCs increased significantly (Fig. 2H-J), suggesting that GSCs arrest in G2 phase upon colcemid treatment. Importantly, the frequency of Dap-positive GBs and SGs did not change significantly (Fig. 2J), suggesting that G2 arrest in the presence of colcemid is specific to GSCs. Together, these data support the hypothesis that elimination of MTs with colcemid treatment induces centrosome misorientation, leading to cell cycle arrest in G2 phase, specifically in GSCs but not in their progeny (i.e. GBs and SGs).

**The COC is responsible for G2 arrest in GSCs upon MT depolymerization**

Having established that GSCs possess a unique cell cycle arrest point in G2 phase upon colcemid treatment, we next tested the...
second prediction that the G2 arrest is mediated by the COC. We have previously shown that Par-1 and Cnn are essential components of the COC. In the absence of either of these proteins, GSCs show a high frequency of spindle misorientation due to the lack of cell cycle arrest upon centrosome misorientation (Inaba et al., 2010; Yuan et al., 2012). If the COC is responsible for G2 arrest in colcemid-treated GSCs, abolition of the COC in these mutants would allow them to enter mitosis. Then, if GSCs possess an active SAC, such COC-deficient GSCs would arrest in metaphase due to the lack of a bipolar spindle.

Consistently, after colcemid treatment, both cnn and par-1 strong loss-of-function mutants showed a striking increase in the mitotic index of GSCs compared with control GSCs (Fig. 3). The mitotic index of par-1 or cnn mutant GSCs was similar to that of wild-type GBs and SGs (Fig. 3D,E). These results reveal two important aspects of COC and GSC cell cycle regulation. First, the COC is indeed responsible for G2 arrest of GSCs upon depolymerization of MTs. Second, GSCs possess robust SAC activity to arrest in response to MT depolymerization once in mitosis. It is important to note that metaphase arrest of GBs or SGs due to SAC was not affected by par-1 or cnn mutation in the absence of MTs (Fig. 3D,E), indicating that par-1 and cnn are not involved in the SAC.

The COC and SAC are distinct checkpoints

Upon MT depolymerization, the SAC is activated to arrest cells in mitosis (Musacchio and Salmon, 2007). In the absence of the SAC, such as in the mad2 mutant, cells do not accumulate in mitosis even in the presence of colcemid. Because both the SAC and the COC respond to the lack of MTs, we examined the potential relationship between the two checkpoints. We first examined the response of mad2 null mutant testes to colcemid treatment. The gross morphology of mad2 mutant testes was similar in the absence or presence of colcemid, with similar numbers of GSCs per testis (supplementary material Fig. S2). Neither centrosome nor spindle orientation was perturbed in mad2 mutant GSCs compared with the control (Fig. 4A).

Mitotic arrest in GBs/SGs upon colcemid treatment was significantly decreased in mad2 mutants (Fig. 4B), confirming that Mad2 is required for SAC-dependent metaphase arrest in these cells. However, both control and mad2 mutant GSCs maintained a low mitotic index in the presence of colcemid (Fig. 4B), suggesting that mad2 mutant GSCs are capable of arresting in G2 phase upon MT depolymerization. These results show that Mad2 is not involved in the COC, and indicate that the COC and the SAC are distinct checkpoint mechanisms.

par-1 mad2 double-mutant GSCs showed a lower mitotic index than par-1 single-mutant GSCs (Fig. 4C); an increase in mitotic index due to par-1 mutation in colcemid-treated GSCs was
considerably reduced by the further addition of the mad2 mutation. These results suggest that, once GSCs enter mitosis, mitotic arrest in the presence of colcemid is indeed due to SAC activity. par-1 mad2 double-mutant GBs/SGs showed mitotic indices similar to the mad2 single mutant in the presence of colcemid (Fig. 4D), consistent with the idea that par-1 is not involved in the SAC in GBs/SGs, as described above. Taken together, these data demonstrate that the COC and the SAC constitute distinct pathways to regulate the cell cycle progression of GSCs at distinct temporal points, i.e. the G2-M transition and the metaphase-anaphase transition, respectively.

**Apc1, Apc2 and Spd-2 are not required for COC-mediated GSC arrest**

Apc1 and Apc2 are required for correct centrosome orientation in GSCs, acting at the centrosome and the cell cortex, respectively (Yamashita et al., 2003). We have previously shown that mutations in Apc1 or Apc2 lead to a high frequency of centrosome misorientation, whereas spindle orientation is only mildly affected (Inaba et al., 2010). These results led us to hypothesize that Apc1 and Apc2 might contribute to the COC only to a limited extent, or that Apc1 and Apc2 might be required for the maintenance of spindle orientation once in mitosis (i.e. independent of the COC). To address these possibilities, we used colcemid treatment as a sensitive assay to examine COC activity. The mitotic indices of Apc1 and Apc2 strong hypomorphic mutant GSCs did not significantly increase after colcemid treatment compared with the control, indicating that Apc1 and Apc2 retain substantial COC activity (Fig. 5A,B). Therefore, we speculate that the reported low frequency of spindle misorientation in these mutants (Inaba et al., 2010) might be due to defective maintenance of spindle orientation in mitosis, independent of the COC.

Much like Cnn (Megraw et al., 1999), Spd-2 is an essential component of the pericentriolar material (PCM) required for γ-tubulin ring complex recruitment (Giansanti et al., 2008). Recruitment of Cnn and Spd-2 to the PCM is, in part, mutually dependent and their functions are to some extent redundant (Conduit et al., 2010; Fu and Glover, 2012; Giansanti et al., 2008). Therefore, we examined whether Spd-2 might also be involved in COC function, similar to Cnn. spd-2 mutant testes showed a high frequency of centrosome misorientation (Fig. 5C,D), suggesting that, like Cnn, Spd-2 is required for correct centrosome orientation. However, unlike the cnn mutant, which shows a high frequency of spindle misorientation due to a defective COC, the spd-2 strong loss-of-function mutant showed no sign of spindle misorientation (Fig. 5D), indicative of intact COC activity. This notion was further confirmed by colcemid treatment of spd-2 mutant testes, in which the GSCs showed no increase in mitotic index in the presence of colcemid (Fig. 5E). This clearly demonstrates that the spd-2 mutant is capable of arresting in G2 phase upon centrosome misorientation, and thus Spd-2 is not required for COC activity. Considering that Cnn and Spd-2 have partly redundant roles in recruiting PCM components, it is interesting that only the cnn mutant, but not the spd-2 mutant, showed defective COC activity.
This also shows that general dysfunction in centrosome organization is not sufficient to inactivate the COC.

The Sas-4 and asl mutants lack COC activity

The results described above suggest that COC activity is mediated through a subset of centrosomal components that are dependent on Cnn but not on Spd-2. If this were the case, complete elimination of the centrosome, by depletion of centriolar components such as Sas-4, might lead to defective COC activity via elimination of Cnn recruitment. However, we previously showed that Sas-4 mutants exhibit normal spindle orientation (Yuan et al., 2012), possibly arguing that the COC is intact. To resolve these issues, we further characterized the nature of spindle orientation and COC activity in the Sas-4 mutant.

We previously reported that GSC spindles in the strong loss-of-function Sas-4 mutant, which lacks centrosomes, are now anchored by the spectrosome at the apical pole (Yuan et al., 2012). The spectrosome is a membranous structure in GSCs from which the fusome derives. The spectrosome-dependent anchoring of the mitotic spindle is a primary mechanism to orient the spindle in female GSCs (Deng and Lin, 1997; Yuan et al., 2012). Therefore, we speculated that, in Sas-4 mutant male GSCs, a spectrosome-dependent spindle orientation mechanism is activated, supporting normal spindle orientation in a manner similar to that in female GSCs. Alternatively, it might be that par-1 and cnn mutants do not activate such a spectrosome-dependent mechanism because they still possess centrosomes. This raises the question of whether Sas-4 mutant GSCs are capable of arresting in G2 phase in the absence of MTs. To address this question, we examined the mitotic index of Sas-4 mutant testes in the presence of colcemid. We used pharate adults of the strong loss-of-function allele Sas-4s2214 (Fig. 6) and adult flies of germline-specific Sas-4 knockdown (nos-gal4>Sas-4RNAi), which eliminates most of the centrosomes from germline cells (supplementary material Fig. S3A-D). We observed that Sas-4 mutant GSCs arrested in mitosis, instead of G2 phase, similar to par-1 and cnn mutants (Fig. 6; supplementary material Fig. S3E), demonstrating that Sas-4 mutant GSCs are not capable of G2 arrest in the absence of MTs. These results argue that the correct spindle orientation observed in Sas-4 mutant GSCs is mediated by a distinct mechanism (i.e. spectrosome anchoring) rather than reflecting intact COC activity. Similar results were obtained when asl, another centriolar component, was knocked down using two independent RNAi lines, which completely abolished centrosomes judging from γ-tubulin staining (Fig. 7). These results suggest that the COC specifically operates in the presence of centrosomes, and so the elimination of centrosomes in Sas-4 and asl mutant/knockdown abolishes the COC entirely.

DISCUSSION

Stereotypical orientation of the mitotic spindle is a widely utilized mechanism to achieve asymmetric cell division. Although considerable knowledge has accumulated regarding how spindle
orientation is established, little is known about whether cells possess a mechanism that monitors successful spindle orientation. In the present study, using colcemid treatment as a sensitive assay, we investigated the nature of the COC, which is the only known orientation/polarity checkpoint in multicellular organisms, and established that: (1) the COC specifically operates in GSCs, but not differentiating germ cells (GBs and SGs); (2) the COC operates in G2 phase of the cell cycle to prevent precocious entry into mitosis upon centrosome misorientation; and (3) as a checkpoint mechanism, the COC is distinct from the SAC.

Our results show that the COC is a GSC-specific checkpoint that monitors centrosome orientation and arrests cells in G2 phase when centrosomes are not correctly oriented. It remains unclear whether the COC-mediated G2 arrest might eventually undergo adaptation to allow mitotic entry, as is the case with mitotic slippage in the SAC. It is worth noting in this context that the GSC mitotic index never increased during 6 h of colcemid treatment, whereas SGs seem to undergo mitotic slippage by 6 h of colcemid treatment. This suggests that the COC-mediated G2 arrest is relatively strong. The findings that the mad2 mutation has no effect on G2 arrest of GSCs and that par-1 and cnn mutants have no effect on mitotic arrest in GBs/SGs upon colcemid treatment strongly support the notion that the COC and SAC constitute distinct checkpoint mechanisms. Although CySCs and SGs also orient their mitotic spindles, our present study shows that spindle orientation in these cells is not under the control of the COC. However, the lack of a COC in these cell types does not exclude the possibility that distinct polarity checkpoint mechanisms are in place to ensure correct spindle orientation. If the arrest points of such checkpoints are not prior to the arrest point of the SAC (i.e. metaphase), our assay using colcemid would not reveal their presence.

Mutant analysis of multiple centrosomal components in this study revealed a selective requirement for centrosomal components in the function of the COC. Sas-4 and Cnn are crucial for COC function, whereas Spd-2 and Apc1 are not. This indicates that not all of the centrosomal proteins are involved in COC function. Conversely, not all COC components are localized to the centrosome. As shown in our previous study, an essential component of the COC, Par-1, is localized to the spectrosome, where it regulates the localization of Cyclin A to regulate mitotic entry (Yuan et al., 2012). How the information on centrosomal orientation is communicated to the spectrosome, where Par-1 and Cyclin A localize, remains to be determined. A major outstanding question in understanding the COC is how it senses the location of the centrosome with respect to the hub cells. Our previous studies have shown that the mother centrosome is anchored to the adherens junctions formed at the hub-GSC interface via MTs (Inaba et al., 2010; Yamashita et al., 2007). Therefore, it is plausible that the COC senses aspect(s) of these interactions. It awaits future investigation to understand how the association of the centrosome with the hub-GSC interface is mechanistically sensed, and how such information is integrated with the activity of COC component(s) on the spectrosome.

In summary, the present work establishes that the COC is a checkpoint mechanism that is distinct from the SAC and monitors correct centrosome orientation specifically in GSCs. We speculate that a similar mechanism might be in place in other systems that rely on asymmetric cell division.

MATERIALS AND METHODS

Fly maintenance and stocks
Fly stocks were raised in standard Bloomington medium at 25°C. The following strains were used: Apc128 (Ahmed et al., 1998), Apc2440 (McCartney et al., 1999), Apc2d5 (McCartney et al., 2001), asl-YFP (Varmark et al., 2007), cnn1(p63) (Li et al., 1998), cnn1(b221) (Megraw et al., 1999), dap-1mg (dacapo-myc) (Meyer et al., 2002), par-1(w3) and par-1(l06523) (Shulman et al., 2000), mad2(ET21605) (Li et al., 2010), Sas-pd(2214) (Basto et al., 2006), spd-2(d2-316) (Giansanti et al., 2008), nos-gal4 (Van Doren et al., 1998), Df(3R)BSC789, Df(3L)List-g24, Sas-t(DMSO14601), asl(dap-1MG00661) (FlyBase, obtained from the Bloomington Stock Center).

Colcemid treatment of testes
For depolymerization of MTs, testes were dissected and transferred to Schneider’s insect medium (Sigma). After sample collection, colcemid (Calbiochem) was added to the medium at a final concentration of 100 µM as described previously (Januschke and Gonzalez, 2010). Testes were incubated at 25°C for up to 6 h.

Immunofluorescence staining and microscopy
Immunofluorescence staining was performed as described previously (Cheng et al., 2008). Briefly, testes were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 30 min, then washed in PBS containing 0.1% Triton X-100 (PBST) for 30 min, followed by incubation with primary antibody in PBST containing 3% bovine serum albumin (BSA) at 4°C overnight. Samples were washed for 60 min (three 20-min washes) in PBST, incubated with secondary antibody in PBST with 3% BSA at 25°C for 2 h, washed as above, and mounted in VECTASHIELD with DAPI (Vector Labs). The following primary antibodies were used: mouse anti-Adducin-like (Add; Hu-li tai shao) [1:20; IB1, developed by H. D. Lipshitz, obtained from Developmental Studies Hybridoma Bank (DSHB), University of Iowa] (Ding et al., 1993), rabbit anti-DPLP (Cp309) (1:500; a kind gift of J. Raff) (Martinez-Campos et al., 2004), mouse anti-Fascin III (FasIII) (1:100; 7G10, developed by C. Goodman, obtained from DSHB) (Patel et al., 1987), mouse anti-Myc (1:100; 9E10, Sigma), rabbit anti-phosphorylated (Thr3) histone H3 (1:200; clone JY325, Upstate), rabbit anti-Spd-2 (1:25; a kind gift of M. Gatti) (Giansanti et al., 2008), mouse anti-α-tubulin 4.3 (1:50; developed by C. Walsh, obtained from DSHB) (Walsh, 1984), mouse anti-γ-tubulin (1:100; GTU-88, Sigma) and rabbit anti-Vasa (1:200; d-26, Santa Cruz Biotechnology). Alexa 488-, 568- or 647-conjugated secondary antibodies (1:200, Life Technologies) were used. Images were captured using a Leica TCS SPS confocal microscope with a 63× oil-immersion objective (NA=1.4) and processed using Fiji software (Schindelin et al., 2012).

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Competing interests
The authors declare no competing financial interests.

Author contributions
Z.G.V. conducted the experiments. Z.G.V and Y.M.Y. designed the experiments, interpreted and analyzed the data, and wrote the manuscript.

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Supplementary material
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References


Fig. S1. Effect of ex vivo colcemid treatment on adult testis. Apical tip region of mock- (A) and colcemid- (B) treated testes after 1-hour incubation with DMSO or colcemid. (A’, B’) Images in (A) and (B), respectively, merged with images of Vasa and DAPI staining. Red: Vasa. Green: α-tubulin. Blue: DAPI. Asterisks (*) indicate the hub. Scale bars: 10 μm. (C) GSC number per testis after ex vivo treatment with DMSO or colcemid for 6 hours. Error bar indicates s.d. P values were determined by two-tailed two-sample t-test.
**Fig. S2. GSC number in *mad2* mutant adult testis.** GSC number per testis from *mad2* heterozygous and homozygous mutants. Error bar represents s.d.. P values were determined by two-tailed two-sample t-test.
Fig. S3. RNAi-mediated knockdown of sas-4 in GSCs abolishes G2 arrest upon microtubule (MT) depolymerization. (A-D) Examples of apical tip in mock-treated control (A), colcemid-treated control (B), mock-treated nos-gal4>sas-4RNAi (TRiP.HMS01463) (C), and colcemid-treated nos-gal4>sas-4RNAi testes after 4.5 hours. Red: Vasa, PH3. Green: FasIII, γ-tubulin. Blue: DAPI. Asterisks (*) indicate the hub. Scale bars: 10 μm. (E) Mitotic index of germline cells from control and sas-4RNAi adult testes after 4.5 hours of mock (DMSO) or colcemid treatment. Error bars indicate s.e.m. P values were determined by two-tailed two-sample t-test.