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# Asymmetric division of cyst stem cells in *Drosophila* testis is ensured by anaphase spindle repositioning

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## SUMMARY

Many stem cells divide asymmetrically to balance self-renewal and differentiation. In *Drosophila* testes, two stem cell populations, germline stem cells (GSCs) and somatic cyst stem cells (CySCs), cohere and regulate one another. Here, we report that CySCs divide asymmetrically through repositioning the mitotic spindle around anaphase. CySC spindle repositioning requires functional centrosomes, Dynein and the actin-membrane linker Moesin. Anaphase spindle repositioning is required to achieve high-fidelity asymmetric divisions in CySCs, thus maintaining both GSC and CySC numbers. We propose that dynamic spindle repositioning allows CySCs to divide asymmetrically while accommodating the structure of the GSCs they encapsulate.

**KEY WORDS: Stem cells, Asymmetric division, Spindle repositioning, *Drosophila***

## INTRODUCTION

A balance between stem cell self-renewal and differentiation is vital for tissue homeostasis. Disruption of this balance can lead to tumorigenesis/tissue hyperplasia (owing to an excess of stem cell self-renewal) or tissue degeneration/aging (owing to an excess of differentiation). Asymmetric division is used by a variety of stem cells to manage this pivotal balance (Morrison and Kimble, 2006). In tissues containing multiple stem cell populations that interact with each other, this process is complicated by the necessity to coordinate their divisions. The *Drosophila melanogaster* testis provides an excellent model system for studying stem cell behavior in such composite tissues. At the testis apical tip, germline stem cells (GSCs) attach to the hub cells, a major component of the stem cell niche, via adherens junctions (Yamashita et al., 2003). The same niche maintains yet another type of stem cells, cyst stem cells (CySCs, also known as cyst progenitor cells), a pair of which encapsulates a GSC and functions as a niche component together with hub cells (Fig. 1A) (Leatherman and Dinardo, 2008). Unpaired (Upd) ligand secreted from the hub cells activates the JAK-STAT pathway in both GSCs and CySCs, maintaining the stem cell identity of these cells (Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). CySCs generate cyst cells (CCs), a pair of which encapsulates differentiating germ cells [gonialblast (GB), spermatogonia and spermatocytes] and is thought to provide essential signals to promote differentiation (Fuller, 1993; Kiger et al., 2000; Tran et al., 2000).

In the context of intercellular Upd-JAK-STAT signaling, GSCs divide asymmetrically by orienting their mitotic spindle perpendicular to the hub (Fig. 1B), keeping one daughter within the niche and displacing the other away from the niche (Yamashita et al., 2003). Recently, Leatherman and DiNardo demonstrated that the JAK-STAT pathway in CySCs not only specifies CySC identity, but also plays a dominant role in imparting GSC identity to encapsulated germ cells (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010). Therefore, the CySC decision between self-renewal versus differentiation is crucial to maintain the correct balance of stem and differentiating cell populations in both the germline and the somatic cyst cell lineage, which is vital to sustaining spermatogenesis. Although CySCs are thought to divide asymmetrically (Hardy et al., 1979; Lindsley and Tokuyasu, 1980), the frequency of asymmetric divisions is unclear, and the mechanisms for establishing such asymmetry are unknown.

Here, we establish that CySCs almost always divide asymmetrically, using a cellular mechanism strikingly distinct from GSCs or any other known stem cells. The mitotic spindle of CySCs forms in a random location within an irregularly shaped CySC, then repositions so one pole is close to the hub cells during or near the onset of anaphase. This spindle repositioning around anaphase requires a functional centrosome, Dynein, and a cortex-actin cytoskeleton linking protein, Moesin. We demonstrate that such stereotypical repositioning of the anaphase spindle is required for consistent asymmetric outcome of CySC division.

## MATERIALS AND METHODS

### Fly husbandry and strains

All fly stocks were raised on standard Bloomington medium at 25°C, and young flies (0 to 1-day-old adults) were used for all experiments. The following fly stocks were used: c587-Gal4 (Kai and Spradling, 2003; Manseau et al., 1997) from S. Hou (National Cancer Institute, NIH); *cnn*$^{mfs3}$ (Li et al., 1998) from T. Kaufman (Indiana University, Bloomington, IN, USA); UAS-GFP-α-tubulin (Grieder et al., 2000) from A. Spradling (Carnegie Institution, Baltimore, MD, USA); Ubi-GFP-α-tubulin (Rebollo et al., 2004), UAS-moesin-T559A-GFP (Polesello et al., 2002), UAS-moesin-T559D-GFP (Polesello et al., 2002) and Sqh-GFP (Sisson et al., 2000) from E. S. Glusman (Stanford University, CA); hs-FLP; Actin>FRT-stop-FRT>Gal4, UAS-GFP (Wang et al., 2008) from Y. Cai (Temasek Lifesciences Laboratory, Singapore); UAS-Pavarotti-GFP from A. Carpenter (University of Cambridge, UK) and D. Glover (University of Cambridge, UK) (Minestrini et al., 2002); UAS-DEFL (Oda and Tsukita, 1999) from H. Oda (JT Biohistory Research Hall, Japan);

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nanos (nos)-Gal4 (Van Doren et al., 1998), *cnn*$^{HK21}$ (Megraw et al., 1999), *apc2*$^{d40}$ (McCartney et al., 2001), *apc2*$^{\Delta s}$ (McCartney et al., 1999), *lis1*$^{k11702}$, *lis1*$^{k13209}$, UAS-Moesin-Myc (Karagiosis and Ready, 2004), UAS-Moesin-T559D-Myc (Karagiosis and Ready, 2004), UAS-Moesin-RNAi (Karagiosis and Ready, 2004), UAS-Gl-RNAi (Pilling et al., 2006) from the Bloomington *Drosophila* Stock Center.

### Immunofluorescence staining

Samples were fixed for 30-60 minutes with 4% formaldehyde in PBS, permeabilized for 30 minutes in PBST (0.1% Triton X-100 in PBS), incubated overnight at 4°C with primary antibodies, washed with PBST (20 minutes, three times), incubated overnight at 4°C with AlexaFluor-conjugated secondary antibodies (1:200, Molecular Probes) and washed again with PBST (20 min, three times). Samples were then mounted in VECTASHIELD (H-1200, Vector Laboratory) and imaged using a Leica SP5 confocal microscope. The primary antibodies included mouse anti-γ-tubulin (1:100; GTU-88, Sigma), mouse anti-Fasciclin III [1:20, developed by C. Goodman (University of California, Berkeley) and obtained from the Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Eya (1:20, developed by S. Benzer (California Institute of Technology) and obtained from DSHB), mouse anti-Myc (1:1000; 9E10, Upstate), rabbit anti-Thr 3-phosphorylated histone H3 (1:200, Upstate), goat anti-Vasa (1:100; dC-13, Santa Cruz), rabbit anti-zfh-1 (1:5000, R. Lehmann, Skirball Institute of Biomolecular Medicine, NY, USA) and rabbit anti-phosphorylated moesin (1:100, Cell Signaling). Images were processed using Adobe Photoshop.

Anaphase was determined by the morphology of mitotic chromatin (stained by Thr 3-phosphorylated histone H3), as well as cell shape and spindle (pole-to-pole) length: anaphase was determined by having two segregating chromatin masses and rounded (oval) cell shape and the onset of spindle elongation, while telophase was identified by two segregating chromatin masses and a 'peanut' cell shape constricted by the contractile ring. The CySC spindle is counted as 'associated' when one spindle pole is close to the hub-CySC interface (within 2 μm).

### Time-lapse live-imaging methods

Time-lapse live-imaging and tracking were carried out with young Ubi-GFP-α-tubulin flies as described (Cheng et al., 2008).

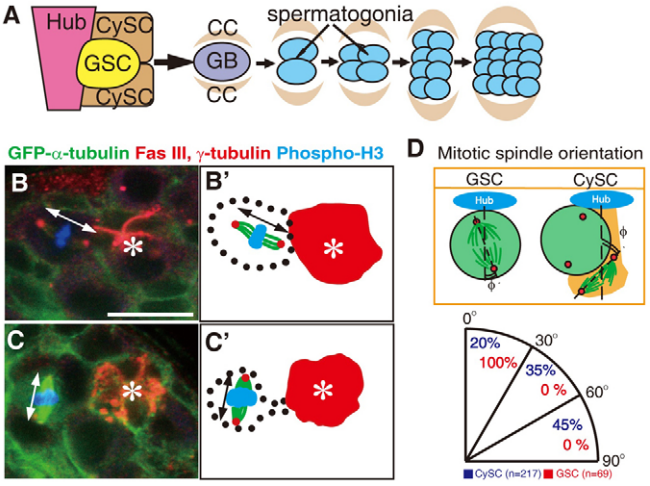


**Fig. 1. The CySC mitotic spindle is not oriented with respect to the hub.** (**A**) Schematic of *Drosophila* spermatogenesis. A germline stem cell (GSC) is encapsulated by a pair of cyst stem cells (CySCs), both of which contact the hub. The GSC daughter, known as gonialblast (GB), begins to differentiate. A pair of CySC daughters, known as cyst cells (CCs), encapsulates a developing GB and spermatogonia. (**B**,**C**) The GSC spindle is oriented nearly perpendicular to the hub (B), while the CySC spindle is not consistently oriented and is often far away from the hub (C). Spindle orientations are indicated by double-headed arrows. Asterisks indicate the hub. (**B′**,**C′**) Diagrams of images shown in B and C, respectively, showing the hub and dividing GSC or CySC. Red, Fasciclin III [hub (*)] and γ-tubulin (centrosome); blue, Thr 3-phosphorylated histone H3 (Phospho-H3) (mitotic chromatin); green, GFP-α-tubulin. Ubi-GFP-α-tubulin flies were used. Scale bar: 10 μm. (**D**) CySC spindle angle (ϕ, defined in the top panel) is random with respect to the hub. This is in striking contrast to the consistently oriented GSC spindles.

## RESULTS

### Characterization of CySC shape throughout the cell cycle

To gain insights into how CySCs divide, we first visualized individual CySCs and analyzed changes in cell shape during the cell cycle, and the cellular architecture within the context of the stem cell niche. This can be challenging as the flat CySCs intermingle with each other and around GSCs, making it difficult to identify the relationship between cells in three-dimensional tissue architecture. To overcome this problem, we activated the expression of GFP and the cell-cortex marker, Moesin, in only a subset of cells using heatshock [heatshock (hs)-FLP; Act>FRT-stop-FRT>Gal4, UAS-GFP/UAS- Moesin-Myc] (see Fig. S1 in the supplementary material). This allowed us to visualize the shape of CySCs in three-dimensional intact niche architecture. We found that, during interphase, CySCs assume a flat shape, with a thin projection reaching the hub, while the cell bodies and nuclei are displaced from the hub by round GSCs (see Fig. S1A in the supplementary material). These thin projections appear as 'thin sheets' wrapping around GSCs, rather than as 'thin stalks' observed in electron microscopic observations (Hardy et al., 1979). Thus, depending on the focal plane, CySCs appeared to have full contact with the hub (see Fig. S1A in the supplementary material, middle panel) or a thin attachment with the hub (see Fig. S1A in the supplementary material, right and left panels). The light microscopy approach also enabled us to process many samples to capture mitotic CySCs. During mitosis, CySCs tend to round up, and appear to retract their thin processes so that the entire cell body moves closer to the hub (see Fig. S1B in the supplementary material). However, this is not seen in all mitotic cells, and is thus limited to a certain period of mitosis or subset of the CySC population; indeed, some mitotic CySCs are observed to maintain thin projections (see Fig. S1C in the supplementary material). The fact that all mitotic CySCs observed ($n$>200) maintained attachment to the hub via a thin projection implies that the previously proposed transit-amplifying population of CCs (CCs that are still dividing but not attached to the hub, judged by the fact that their mitotic chromosomes appeared to be far away from the hub) (Voog et al., 2008) is very rare. This discrepancy might be explained by the fact that the thin projections are difficult to visualize unless a single CySC is marked with a membrane (or cytoplasmic) marker in the background of unmarked cells. Indeed, in our observation, some mitotic CySCs (in particular during early mitosis) appeared to be far away from the hub (see Fig. S1C in the supplementary material, for example) compared with rounded-up CySCs that had retracted their projections (see Fig. S1B in the supplementary material), reminiscent of the observation by Voog et al. (Voog et al., 2008).

### CySCs stereotypically reposition their spindles during anaphase

The ability to consistently identify CySCs allows us to examine CySC mitoses in detail. We used Ubi-GFP-α-tubulin to facilitate the analysis of mitotic spindles in GSCs and CySCs. As Ubi

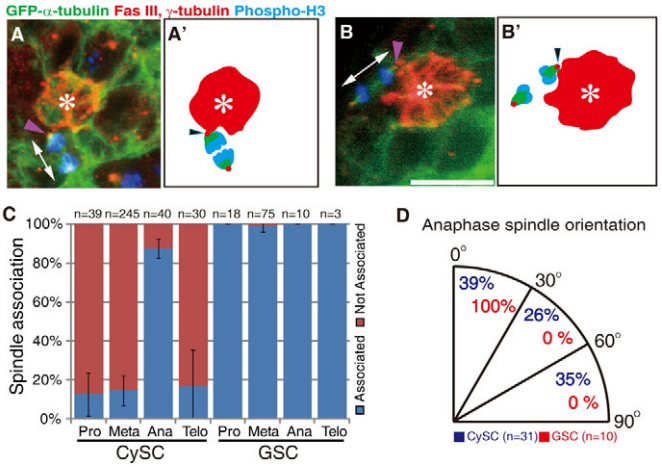


**Fig. 2. The CySC spindle is associated with the hub-CySC interface during anaphase.** (**A**,**B**) Two examples of CySC anaphase spindles (double-headed arrows) with one pole closely associated with the hub (arrowhead), but at a random angle (in A, the spindle is relatively perpendicularly oriented, while in B the spindle is almost parallel to the hub). (**A′**,**B′**) Diagrams of images shown in A and B, respectively, illustrating the hub and CySC spindles. Spindle orientations are indicated by double-headed arrows; arrowheads show proximal spindle poles associated with the hub (asterisk). Red, Fasciclin III [hub (*)] and γ-tubulin (centrosome); Blue, Thr 3-phosphorylated histone H3 (Phospho-H3) (mitotic chromatin); green, GFP-α-tubulin. Ubi-GFP-α-tubulin flies were used. Scale bar: 10 μm. (**C**) Quantification of GSC and CySC spindle association with the hub interface throughout mitosis. Pro, prophase and prometaphase; Meta, metaphase; Ana, anaphase; Telo, telophase. Error bars are s.d. (**D**) The CySC spindles do not have a consistent angle with respect to the hub, even during anaphase, when one spindle pole is closely associated with the hub.

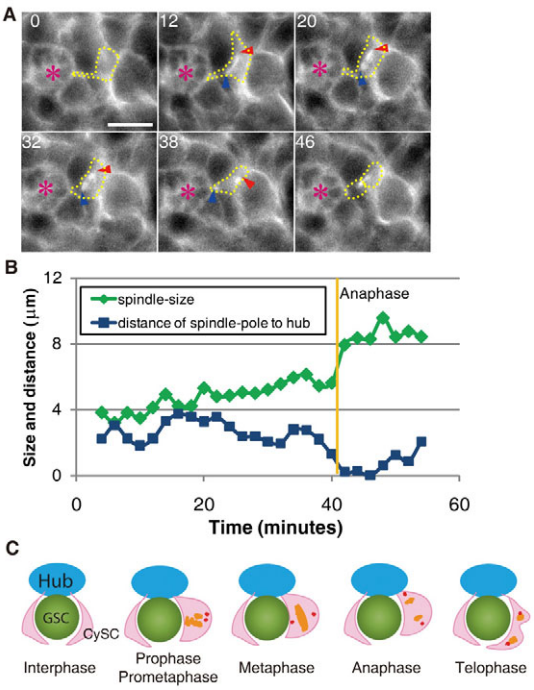


**Fig. 3. Dynamic movement and repositioning of the CySC spindle revealed by live imaging.** (**A**) Selected frames of live-imaging of CySC mitosis (see Movie 1 in the supplementary material). CySC shape is indicated with broken lines; arrowheads indicate spindle poles; hub is indicated by an asterisk. Times are in minutes. Ubi-GFP-α-tubulin flies were used. Scale bar: 10 μm. (**B**) The onset of anaphase (marked by a yellow line) coincides with the association of one spindle pole with the hub. The blue line shows proximal spindle pole position and the green line spindle length, representing the data in A. (**C**) Schematic of CySC mitosis. A CySC becomes rounder in mitosis and repositions the spindle pole (red) close to the hub during anaphase.

promoter driving GFP-α-tubulin expression is much stronger in CySCs compared with GSCs (and other germ cells), CySCs were readily distinguished from GSCs. This was confirmed by simultaneous visualization of GFP-α-tubulin and germline and/or cyst lineage markers (data not shown). In contrast to GSCs, where the mitotic spindle is oriented perpendicular to the hub throughout mitosis (Fig. 1B,D) (Yamashita et al., 2003), CySC mitotic spindles exhibit no consistent orientation (Fig. 1C,D).

Yet, we noted that, during anaphase, one pole of the CySC spindle is almost always juxtaposed with the hub-CySC interface (88%, *n*=40, Fig. 2A,B). Anaphase was easily identifiable from the fact that two separating nuclei are visible within a cell, and can be differentiated from telophase as the spindle is not fully elongated and cleavage furrow ingression is not apparent (when a membrane marker is combined in the experiments described below). The juxtaposition of one spindle pole was limited to anaphase, and no trend towards association with the hub-CySC interface was observed during the rest of mitosis (Fig. 2C), suggesting a dynamic repositioning of CySC spindles at anaphase. Time-lapse live-imaging of CySC mitosis in testes explanted into culture confirmed spindle repositioning during anaphase (Fig. 3A and see Movie 1 in the supplementary material). Although GSC spindles are consistently oriented throughout mitosis (Yamashita et al., 2003), CySC spindles were often formed far away from the hub as indicated by fixed samples. These CySC spindles are very dynamic, rocking back and forth during metaphase; then, around the onset of anaphase, one spindle pole quickly retracts to the hub-CySC interface, pulling the entire spindle near the hub (Fig. 3A,B at 38-40 minutes). In all CySC mitoses observed during live-cell imaging (*n*=10), a spindle pole always retracted to the hub-CySC interface around the metaphase-anaphase transition. This retraction of anaphase spindle poles does not appear to be simply due to changes in CySC cell shape, as rounding-up of CySCs often occurs much earlier than spindle repositioning. In spite of consistent anaphase spindle pole repositioning, the angle of the CySC anaphase spindle with respect to the hub-CySC interface was random (Fig. 2D). As a result of anaphase spindle repositioning, CySCs divide asymmetrically, with one daughter attached to the hub and the other displaced away from the hub (Fig. 3C, also see further analysis below regarding the asymmetric outcome of the division).

## Molecular requirement of anaphase spindle repositioning in CySCs

To investigate the molecular mechanisms that govern spindle repositioning in CySCs, we examined the role of several candidate genes inferred from other systems, including *Drosophila* male GSCs, *Drosophila* neuroblasts and budding yeast (Yamashita and Fuller, 2008). First, we found that normal spindle repositioning in CySCs requires *centrosomin* (*cnn*) (Fig. 4A and see Fig. S2 in the

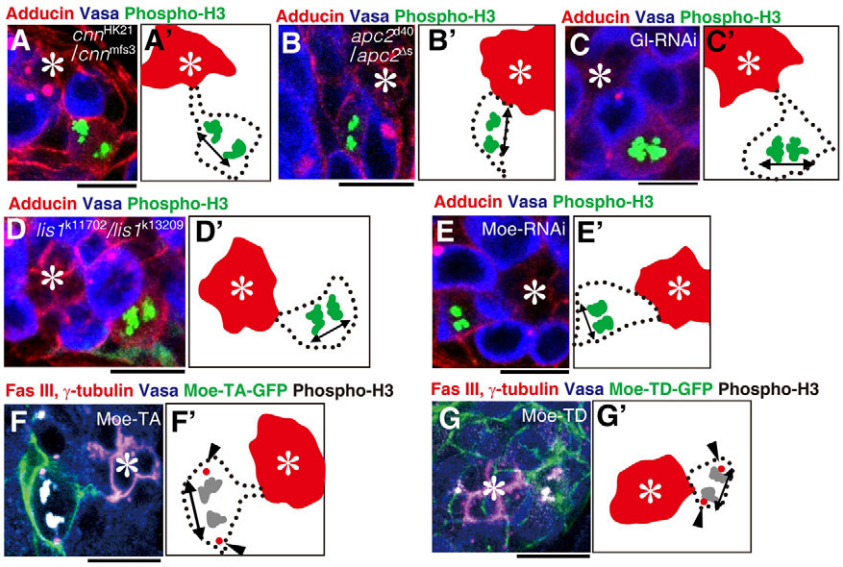


**Fig. 4. CySC spindle repositioning requires centrosome, Dynein, Myosin and Moesin.** (**A-G**) Examples of defective anaphase repositioning in CySCs from *cnn*$^{HK21}$/*cnn*$^{mfs3}$ mutant flies (A), c587-Gal4>Gl-RNAi flies (C), *lis1*$^{k11702}$/ *lis1*$^{k13209}$ mutant flies (D), c587-Gal4>Moe-RNAi flies (E), c587-Gal4>Moe-TA flies (F) and c587-Gal4>Moe-TD flies (G). Example of normal anaphase repositioning in CySCs from *apc2*$^{d40}$/*apc2*$^{Δs}$ mutant flies (B). (**A′-G′**) Diagrams of images shown in A-G, highlighting anaphase spindle and cell shape of CySCs. Hub is indicated by an asterisk; arrowheads indicate centrosomes; dotted lines outline CySC cell shape. Red in A-E indicates Adducin (cell membrane of cyst lineage and spectrosome of germline lineage); red in F,G indicates Fasciclin III [hub (*)] and γ-tubulin (centrosome); green in A-E indicates Thr 3-phosphorylated histone H3 (Phospho-H3) (mitotic chromatin); (F) green indicates Moe-TA-GFP; (G) green indicates Moe-TD-GFP; blue indicates Vasa (germ cells); white in F,G indicates Thr 3-phosphorylated histone H3 (Phospho-H3) (mitotic chromatin). Spindle orientations are indicated by double-headed arrows. Scale bar: 10 μm. (See also Fig. S2 in the supplementary material for defective spindle repositioning with their spindle poles marked.)

supplementary material), an integral component of centrosome (Heuer et al., 1995; Li and Kaufman, 1996), which is also required for GSC spindle/centrosome orientation (Yamashita et al., 2003). In the *cnn* mutant, anaphase spindle repositioning was reduced to 37 % compared with 78% in heterozygous control (Table 1). By contrast, *apc2*, a homolog of tumor suppressor Adenomatous Polyposis Coli, which is required for GSC centrosome orientation (Yamashita et al., 2003), is not required for CySC spindle repositioning (Table 1, Fig. 4B).

We next found that the motor protein dynein is also involved in CySC spindle repositioning (Table 1). Dynein is known to be located at the bud cell cortex in budding yeast, where they pull and orient the spindle pole (Huisman and Segal, 2005). In addition, it has been shown that Lis1/dynactin regulates spindle orientation in *Drosophila* neuroblasts (Siller and Doe, 2008). RNAi-mediated knockdown of Glued (Gl) (c587-gal4>UAS-Gl-RNAi) (Fig. 4C and see Fig. S2 in the supplementary material), a component of dynactin complex (Pilling et al., 2006), as well as hypomorphic mutation of Lissencephaly-1 (Lis-1) (Fig. 4D and see Fig. S2 in the supplementary material), a major regulator of dynein, significantly reduces spindle repositioning (Table 1). In budding yeast, the bud tip cortex, towards which the spindle pole is pulled, is enriched with actin-cytoskeleton and associated proteins such as Bud6 and Kar9 (Huisman and Segal, 2005). In CySCs, the hub-CySC interface, which is enriched with DE-cadherin (see Fig. S3A,B in the supplementary material) as well as with actin cytoskeleton (see Fig. S3C in the supplementary material), might serve as a cortical target site, towards which a spindle pole is pulled by dynein. It is

**Table 1. Summary of CySC spindle association during anaphase in wild type and various genotypes**

| Fly genotypes | Anaphase spindle association (%) | Anaphase number scored (testis number) | *P*-value† |
|---|---|---|---|
| Wild type | 88 | 40 (*n*=851) | |
| Control (*cnn*$^{HK21}$/+ & *cnn*$^{mfs3}$/+) | 78 | 41 (*n*=618) | |
| *cnn*$^{HK21}$/*cnn*$^{mfs3}$ | 37 | 41 (*n*=545) | *P*<0.007 |
| Control (*apc2*$^{d40}$/+ & *apc2*$^{Δs}$/+) | 84 | 19 (*n*=244) | |
| *apc2*$^{d40}$/*apc2*$^{Δs}$ | 89 | 19 (*n*=244) | *P*>0.77 |
| Control (c587-Gal4 only) | 76 | 25 (*n*=491) | |
| UAS-Gl-RNAi* | 30 | 27 (*n*=653) | *P*<0.002 |
| Control (*lis1*$^{k11702}$/+ & *lis1*$^{k13209}$/+) | 72 | 25 (*n*=626) | |
| *lis1*$^{k11702}$/*lis1*$^{k13209}$ | 15 | 20 (*n*=787) | *P*<0.03 |
| UAS-Moe-RNAi* | 18 | 61 (*n*=916) | *P*<0.0002 |
| UAS-Moe-TA* | 18 | 39 (*n*=557) | *P*<0.002 |
| UAS-Moe-TD* | 51 | 41 (*n*=623) | *P*<0.003 |

*All expressions of UAS transgenes are driven by c587-Gal4.
†*P*-value, Student's *t*-test, calculated in comparison with its corresponding control.

interesting to note that the proposed Kar9 ortholog Apc2 (Bienz, 2001) is not required for CySC spindle repositioning as described above. Gl-RNAi was specifically expressed in CySC lineage using c587-Gal4 driver (Decotto and Spradling, 2005), demonstrating the cell-autonomous requirement of Gl in anaphase spindle repositioning in CySCs. Although we cannot exclude non-cell-autonomous effects on CySC spindle repositioning (i.e. defective GSC orientation affecting the spindle repositioning of CySCs) in case of *Lis-1* and *cnn* mutants, the fact that *apc2* mutant, which is known to be defective in GSC orientation (Yamashita et al., 2003), is not defective in CySC spindle repositioning clearly demonstrates that normal CySC spindle repositioning can be maintained in the presence of GSC orientation defects and that CySC spindle repositioning defect is not explained solely by GSC orientation defects.

We also found that Moesin (Moe), a linker protein between the actin-cytoskeleton and membrane, is required for CySC spindle repositioning (Table 1). Moe is vital for cell shape changes and spindle stability during mitosis (Carreno et al., 2008; Kunda et al., 2008; Polesello and Payre, 2004). As described above, Moe localized to the entire cortex of CySCs. Moe knockdown, mediated by RNAi specifically expressed in CySC lineage (c587-gal4>UAS-Moe-RNAi), greatly reduced spindle repositioning during anaphase (76% to 18%) (Table 1, Fig. 4D; see Fig. S2 in the supplementary material), while Moe knockdown in GSCs did not affect GSC orientation (either centrosome orientation in interphase or spindle orientation in mitosis, see Fig. S4 in the supplementary material). Overexpression of dominant-negative [Moe-T559A (Moe-TA)] as well as constitutive active [Moe-T559D (Moe-TD)] forms of Moe in CySCs using c587-Gal4 driver also significantly reduced CySC spindle repositioning (Table 1, Fig. 4E,F), consistent with Moe being regulated by phosphorylation (Nakamura et al., 1995). It should be noted that Moe-RNAi in GSCs (nos-gal4>UAS-Moe-RNAi) did not result in any orientation defects in GSCs (see Fig. S4 in the supplementary material), suggesting distinct molecular requirement for orientation in GSCs and CySCs. Although Moe is required for cultured cells to change from flat interphase morphology to a rounded shape during mitosis (Carreno et al., 2008; Kunda et al., 2008), we did not observe any obvious changes in mitotic or interphase CySC shape upon expression of Moe-RNAi, Moe-TA or Moe-TD. This lack of effect may be attributable to the space allocated by GSCs and hub cells constraining the region in which CySCs reside. Or it may be due to the cellular substrata to which CySCs adhere (GSC and hub cell surfaces), as drastic changes in cell shape have been mostly observed in cultured cells grown on glass coverslips coated with artificial substrata (such as concanavalin A or a high concentration of serum) (Carreno et al., 2008; Kunda et al., 2008). Indeed, in any of the mutants that were found to be defective in anaphase spindle repositioning, cell shape was not noticeably affected during anaphase (as shown in Fig. 4) or in other mitotic phases; some cells rounded up while others did not, as observed in wild-type CySCs, suggesting that their spindle repositioning defects were not due to failures in changing the overall cell shape.

### Anaphase spindle repositioning of CySCs is required for consistent asymmetric division

We next examined the consequences of defective spindle repositioning on asymmetric CySC divisions. Unlike GSCs, in which the division outcome (asymmetric versus symmetric) is easily identifiable owing to their round shape, which allows unambiguous assessment of the positions of daughters relative to the hub (and thus their fates), identification of the daughters from CySC divisions was very challenging: owing to the irregular cell shape of CySCs and their daughters, with thin projections intermingled, it was not possible to determine which cells were immediate daughters from a CySC division and whether they were attached to the hub or not. Thus, in order to determine unambiguously the outcome of CySC division, we developed a method to identify twin daughters of a single CySC division. We generated heatshock-induced 'FLP-out' clones, in which the expression of cytosplasmic GFP and Pavarotti-GFP (Pav-GFP) is activated. Pav is a kinesin-like protein that localizes to the contractile ring and midbody (Adams et al., 1998). By adjusting the heatshock period, we were able to induce CySC clones at a low frequency, such that we could identify single CySC-derived clones in an unmarked background. Twenty-four hours post-heatshock, the twin daughters from a single cell division of CySC clones are easily identified by their connection via a Pav-GFP-positive midbody ring (Fig. 5A-C). We find that, in wild-type (or control) flies, the majority (96%, $n$=73) of CySCs divide asymmetrically: one daughter cell maintains the attachment to the hub, while the other is displaced away from the hub (Fig. 5B,D). By contrast, upon the expression of Moe-TA together with cytoplasmic GFP and Pavarotti-GFP in the FLP-out clones, asymmetric divisions are reduced to 58%; in the remainder, both daughters attach to the hub (Fig. 5C,D). Presumably as a result of such symmetric divisions, the number of CySCs and early cyst cells around the hub (counted as Zfh-1$^{+}$Eya$^{-}$ cells, see Fig. S5 in the supplementary material) increases in flies overexpressing Moe-TA (Fig. 5E). The number of GSCs also increases (Fig. 5F), probably because CySCs participate in establishing GSC identity (Leatherman and Dinardo, 2008). We could not use this method to examine asymmetric versus symmetric outcomes of CySC division after RNAi knockdown, as RNAi would not be effective within 24 hours post-heatshock, and beyond 24 hours post-heatshock additional divisions prevented identification of twin daughters from a single cell division of CySC clones. Nevertheless, we expect Moe-RNAi as well as Gl-RNAi leads to symmetric CySC divisions, as we also observe an increase in CySC and GSC numbers in Moe-RNAi or Gl-RNAi flies (Fig. 5E,F). These results demonstrate that anaphase spindle repositioning in CySCs is required for an asymmetric outcome of CySC division, and that asymmetric division is required to maintain correct stem cell numbers.

## DISCUSSION

Our findings reveal that CySCs, like GSCs, undergo stereotypical asymmetric division in the stem cell niche, but employ a different cellular mechanism to do so. To our knowledge, this is the first report of asymmetric stem cell division that consistently involves dynamic changes in cell shape and spindle repositioning specifically during mitosis. It was reported that *Drosophila* embryonic neuroblasts undergo programmed rotation of spindles to divide asymmetrically (Kaltschmidt et al., 2000), which was subsequently reported to occur only during the first division but not in the later divisions (Rebollo et al., 2009). Thus, in contrast to neuroblasts or male GSCs, which employ relatively fixed cell polarity to divide asymmetrically, CySCs constantly undergo spindle repositioning in repeated cell cycles. In addition, the male GSCs and neuroblasts maintain their round shape throughout the cell cycle, in contrast to CySCs, which undergo cell shape changes during cell cycle, involving flattening and rounding-up. Therefore, spindle repositioning in CySCs represents a new mechanism for asymmetric stem cell division.

Although the detailed molecular mechanisms by which one spindle pole is juxtaposed to the hub-CySC interface in anaphase await further investigation, the mutant analysis described here

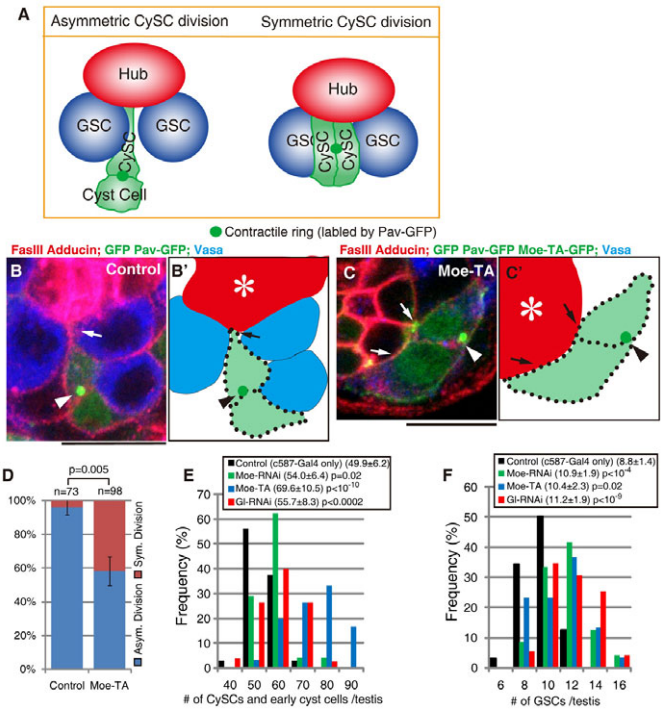


**Fig. 5. Failure in anaphase spindle repositioning leads to symmetric CySC division and increases CySC and GSC numbers.** (**A**) Experimental scheme for detecting outcome of CySC division. Expression of GFP and Pav-GFP was activated by a heatshock (hs-FLP; Actin>FRT-stop-FRT>Gal4) to induce CySC clone. Pav-GFP, which localizes to the contractile ring/midbody, allows identification of twin daughters of a single CySC division. The division outcome was analyzed 24 hours post-heatshock (when many CySCs have divided once but not more than once). (**B**,**C**) Examples of asymmetric CySC division in control flies (B) and symmetric CySC division in flies expressing Moe-TA (C). (**B′**,**C′**) Diagrams of images shown in B and C, respectively. Arrows indicate the attachment of CySCs to the hub; arrowheads indicate the contractile ring/midbody between twin daughters, demonstrating that they are indeed derived from a single CySC division; broken lines outline the cell shapes of the twin daughters. Red indicates Fasciclin III [hub (*)] and Adducin (cell membrane of cyst lineage and spectrosome of germline lineage); blue indicates Vasa (germ cells); green indicates GFP and Pav-GFP in B, and GFP, Pav-GFP and Moe-TA-GFP in C. Flies of hs-FLP; Actin>FRT-stop-FRT>Gal4, UAS-GFP/UAS-MoeTA-GFP; UAS-Pav-GFP were used, and control flies were hs-FLP; Actin>FRT-stop-FRT>Gal4, UAS-GFP; UAS-Pav-GFP. Scale bar: 10 μm. (**D**) Frequency of CySC asymmetric and symmetric divisions. CySC symmetric division increases upon Moe-TA overexpression. Error bars are s.d. *P*-values are calculated using Student's *t*-test when compared with the control. (**E**,**F**) CySC number (E) and GSC number (F) increase upon expression of Moe-TA, Moe-RNAi or Gl-RNAi in CySCs using c587-Gal4. c587-Gal4 without UAS-transgenes flies were used as control. *P*-values are calculated using Student's *t*-test when compared with the corresponding control.

illuminates basic cellular machinery for spindle repositioning that is shared by CySCs, such as dynein for spindle repositioning. However, our analysis also reveals the presence of distinct molecular mechanisms for asymmetric division between male GSCs and CySCs; while GSC requires Apc2 but not Moesin, CySCs requires Moesin but not Apc2 for their orientation. It is possible that one spindle pole maintains microtubules throughout the cell cycle running through the thin projections to the hub, which is shortened during anaphase. Alternatively, two centrosomes might be unequal in their microtubule organizing activity, as shown in male GSCs (Yamashita et al., 2007) and *Drosophila* larval neuroblasts (Rebollo et al., 2007; Rusan and Peifer, 2007), and the one with the higher activity is retracted to the hub-CySC interface. It is also possible that one of the two spindle poles is randomly chosen, depending on its location while the spindle is rocking [e.g. the first spindle pole that is captured by the cortex at hub-CySC interface becomes the 'proximal' spindle pole, without intrinsic difference(s) between two spindle poles]. In any case, CySCs would require re-establishment of spindle orientation every cell cycle, and thus we speculate that the molecular mechanism could involve more complicated regulation than those used in GSCs or larval neuroblasts that appear to maintain the polarity throughout the cell cycle.

An important subject for future investigation is how the cell cycle progression is coordinated with the anaphase spindle repositioning. The present study does not provide the exact time point of anaphase onset with respect to the spindle repositioning. (1) It is possible that spindle repositions at the very end of metaphase, which in turn triggers anaphase onset. (2) Alternatively, progression into anaphase might activate the machinery that allows spindle repositioning. In this regard, a recent study by McCarthy Campbell et al. (McCarthy Campbell et al., 2009) is particularly intriguing. They have shown that the anaphase-promoting complex is required for spindle displacement (or pulling of the spindle to the posterior of the embryo) in *C. elegans* zygotes, where spindle assembly is followed by APC activation, which in turn induces spindle displacement. Although APC activation that simultaneously triggers spindle repositioning (or displacement) and anaphase onset (sister chromatid separation) can provide timely order of multiple events, it might not provide enough time for a spindle pole to search-and-capture to link the right cortical site and spindle pole. Thus, if a similar scenario is the case for CySC spindle repositioning, it is likely that the cortex and spindle pole are already linked by microtubules before spindle repositioning, and the microtubule link shortens during the repositioning phase.

Unlike spherical GSCs, CySCs assume a flat and irregular shape, especially during interphase, which presumably allows CySCs to encapsulate GSCs and provide regulatory signals. However, such a flattened, irregular cell shape could be problematic for establishing a mitotic spindle with a consistent position and orientation. We hypothesize that the slight rounding up of cells during mitosis gives enough room for the spindle to move within CySCs, and anaphase spindle repositioning ensures appropriate asymmetric divisions. This may be analogous to asymmetric cleavage in ascidian blastomeres, where spindle pole migration is coupled with spindle rotation (Prodon et al., 2010), suggesting this mechanism is conserved across species. Although the molecular mechanisms that allow cells to round up during mitosis have been investigated intensively, the reasons why cells round up in mitosis (or why cells are flattened during interphase) are less clear. It has been speculated that such flattening allows cells to adhere to their natural substrata within the tissue. The identity of CySCs as a component of the GSC niche highlights the importance of cell flattening in interphase. CySCs must provide niche signaling to GSCs, where encapsulation of GSCs (thus flattening of CySCs) is crucial; germ cells fail to properly differentiate in mutants where CySCs fail to encapsulate GSCs (Schulz et al., 2002).

In summary, we propose that the use of different mitotic schemes by different types of stem cells may be a general mechanism whereby divisions of multiple stem cell populations are coordinated in complex tissues. The elaborate mechanisms of spindle repositioning are adapted to cooperate with the morphology

of the specific cell and its environment, thereby assuring consistent asymmetric division outcomes, even when irregularities in the cell morphology prevent consistent spindle orientation.

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**Competing interests statement**
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