Centrosome-dependent asymmetric inheritance of the midbody ring in Drosophila germline stem cell division

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ABSTRACT Many stem cells, including Drosophila germline stem cells (GSCs), divide asymmetrically, producing one stem cell and one differentiating daughter. Cytokinesis is often asymmetric, in that only one daughter cell inherits the midbody ring (MR) upon completion of abscission even in apparently symmetrically dividing cells. However, whether the asymmetry in cytokinesis correlates with cell fate or has functional relevance has been poorly explored. Here we show that the MR is asymmetrically segregated during GSC divisions in a centrosome age–dependent manner: male GSCs, which inherit the mother centrosome, exclude the MR, whereas female GSCs, which we here show inherit the daughter centrosome, inherit the MR. We further show that stem cell identity correlates with the mode of MR inheritance. Together our data suggest that the MR does not inherently dictate stem cell identity, although its stereotypical inheritance is under the control of stemness and potentially provides a platform for asymmetric segregation of certain factors.

INTRODUCTION Asymmetric stem cell division is critical for tissue homeostasis by balancing the production of stem cells and differentiating daughters (Morrison and Kimble, 2006). The centrosome has become increasingly recognized as playing key roles in asymmetric stem cell division (Yamashita et al., 2003, 2007; Rebollo et al., 2007; Rusan and Peifer, 2007; Cheng et al., 2008; Wang et al., 2009). As a microtubule-organizing center (MTOC), the centrosome position within the cell can dictate the orientation of cell division, often leading to asymmetric stem cell division. The centrosome also plays critical roles in cytokinesis (Piel et al., 2000, 2001; Doxsey et al., 2005; Gromley et al., 2005; Goss and Toomre, 2008; Pohl and Jentsch, 2008; Prekeris and Gould, 2008). In some cell types, cytokinesis has been shown to be asymmetric, in that abscission occurs on only one side of the midbody and the other side inherits the midbody ring (MR; Gromley et al., 2005). In other cases, the MR was reported to be released into the extracellular space (Dubreuil et al., 2007). Kuo et al. (2011) reported that cells containing the mother centrosome inherit the MR upon abscission. They also showed that pluripotent stem cells (embryonic stem cells and induced pluripotent stem cells) and cancer cells tend to accumulate the MRs, probably correlating with their inheritance of the mother centrosome, leading them to propose that MR inheritance may play a role in stem cell identity (Kuo et al., 2011). In contrast, Ettinger et al. (2011) reported that stem cells are characterized by a high capacity of MR release into the extracellular space. Therefore it is unclear whether the MR carries any information relevant to stem cell behavior and how MR fate is determined, possibly depending on cell type.

Drosophila male and female germline stem cells (GSCs) divide asymmetrically to produce one stem cell and one differentiating cell. In the Drosophila testis, GSCs attach to somatic hub cells, which, together with cyst stem cells (CySCs), create a signaling microenvironment—the niche—to specify GSC identity (Figure 1A; Fuller and Spradling, 2007; Yamashita et al., 2010). Similarly, in the Drosophila ovary, GSCs attach to cap cells, which form the niche...
CySCs and CCs in the testis. Proliferate or move along with the developing germ cells. However, exist in the germarium that closely associate with the GSCs and filaments (TFs) provide niche signals to GSCs. Escort cells (not shown) cap cells, orienting the mitotic spindle. Cap cells and terminal in female GSCs, the spectrosome (red circle) is located close to the Although the centrosomes (asterisks) are not stereotypically oriented; the mother centrosome (black asterisk) migrates toward the opposite side of the GSC. GSCs contain the spectrosome (red circle), which assumes a spherical morphology, whereas differentiating spermatogonia (SG) contain the fusome (red line), which is branched and runs through the ring canals. GSCs are encapsulated by a pair of CySCs. GBs and SG are encapsulated by a pair of CCs, progeny of CySCs. (B) In the germarium in the Drosophila ovary, GSCs attach to the cap cells, whereas their daughters, CBs, are displaced away from the cap cells. Although the centrosomes (asterisks) are not stereotypically oriented in female GSCs, the spectrosome (red circle) is located close to the cap cells, orienting the mitotic spindle. Cap cells and terminal filaments (TFs) provide niche signals to GSCs. Escort cells (not shown) exist in the germarium that closely associate with the GSCs and developing germ cells. Unlike CySCs, they do not normally proliferate or move along with the developing germ cells. However, they provide supportive signals for germ cell development, similar to CySCs and CCs in the testis.

**RESULTS**

**The MR is inherited by the differentiating daughter during male GSC division**

To examine MR inheritance during male GSC division, we used Pavarotti–green fluorescent protein (GFP; Minestrini et al., 2002) to visualize the MR. Pavarotti is a homologue of MKLP1, a kinesin-like protein that is required for cytokinesis (Adams et al., 1998; Minestrini et al., 2002, 2003). Consistent with the reported localization of Pavarotti in other cell types, Pavarotti-GFP was observed at the spindle midzone, the MR, and ring canals, the structures that connect differentiating spermatogonia and spermatoocytes (Figure 2A). The spectrosome, a germline-specific membranous organelle marked by Hts/adducin-like (Add), was observed to run through the MR (Figure 2A), similar to previous observations in females (Deng and Lin, 1997; de Cuevas and Spradling, 1998). As in female GSCs, cytokinesis in male GSCs is prolonged, and final resolution of the cytokinesis takes place in the G2 phase of the next cell cycle (Sheng and Matunis, 2011). As a result, GSCs stay connected to the gonialblasts (GBs), the differentiating daughter of GSCs, for ~50% of the cell cycle time. Of interest, when cytokinesis was complete, the MR was inherited by the GBs (>92%; n > 200 GSC-GB pairs; Figure 2B). We limited our analysis to cases in which the pairing of GSCs and GBs was evident by the presence of a thin thread of spectrosome material (positive for Add) connecting the GSCs and GBs. As a result of asymmetric cytokinesis, GBs containing the MR were frequently observed, even after clear separation of GSCs and GBs (Figure 2A, arrow). These observations are distinct from findings in mammalian cells, in which it was proposed that the stem cells inherit and accumulate MRs (Kuo et al., 2011). GBs in the subsequent mitosis (to become two-cell spermatogonia) never contained MR remnants (Figure 2C), suggesting that GBs somehow dispose of the MR before mitosis. Surprisingly, we found that the MR is released from GBs into somatic cells (CySCs or cyst cells [CCs]; Figure 2D). The MR found in CySCs/CCs is indeed derived from GBs rather than from somatic cells, based on the following observations: 1) the Pavarotti–GFP–marked MR was found in CySCs/CCs even when Pavarotti–GFP is expressed only in the germline (using nos-gal4 driver; Figure 2D), and 2) the MRs in CySCs/CCs are still associated with the spectrosome material, suggesting that they must have derived from the germline (Figure 2D, inset). In addition, by changing the focal plane of the microscope, we confirmed that such MRs were not part of germ cells that exist outside of the focal plane. Because MRs were not internalized into the GB, it is likely that CySCs/CCs engulf the MR from the surface of GBs after the completion of abscission. In addition, we never observed CySCs/CCs containing multiple MR remnants, suggesting that the CySCs/CCs somehow remove MRs; indeed, we often observed that the MRs in CySCs/ CCs were associated with lysosomes (Supplemental Figure S1). Of 126 testes observed, 109 had MR remnants in CySCs/CCs, 49% of which were associated with lysosomes. The MR was found in either CySCs (58%) or CCs (42%), suggesting that MR ingestion was not related to the identity of CySCs or CCs. This observation is reminiscent of mouse neural stem cells, in which the MR is released into the extracellular space (Dubreuil et al., 2007; Ettinger et al., 2011). Our observation also showed that even when the MR was eventually released into the extracellular space, the initial abscission was stereotypically asymmetric as to which cell inherits the MR. On the basis of these findings, we conclude that cytokinesis in male GSCs is asymmetric: the MR is almost always inherited by GBs and is eventually released into somatic CySCs/CCs, where it is degraded (Figure 2E).
The MR is inherited by GSCs in the female germline

We next examined MR inheritance in female GSCs. Surprisingly, cytokinesis in female GSCs is also asymmetric in the opposite manner: the MR is almost always inherited by GSCs (92%; n = 61 GSC-cystoblast [CB] pairs; Figure 3A). Immediately after cytokinesis, the MR was observed between GSCs and CBs (Figure 3A). The female spectrosome is known to display dynamic morphological changes during the cell cycle (Deng and Lin, 1997; de Cuevas and Spradling, 1998; Hsu et al., 2008) and eventually splits between GSCs and CBs, when about two-thirds of the spectrosome material is inherited by GSCs. When the spectrosome is split between GSCs and CBs, the MR is almost always inherited by GSCs associated with the GSC-inherited spectrosome (Figure 3B). MRs appear to be degraded within the spectrosome of GSCs, as a small Pav-positive structure (often retaining MR morphology) was observed in the spectrosome of GSCs (Figure 3C).

We conducted time-lapse live observation of ovaries from Pav-GFP flies to complement our observation using fixed samples. Whereas fixed samples stained for multiple cellular markers may correlate the spatial relationship between the MR and spectrosome, they do not unambiguously tell the temporal order of MR movement and inheritance. Live observation could add clarity to the temporal order of MR inheritance. Live observation of ovaries to track MR inheritance proved to be challenging: the MR typically stayed in the middle of GSCs and CBs for a long time (often >10 h), and maintaining the focal plane to visualize the MR throughout this period was often impossible. Tracking a small structure such as the MR requires flattening of the sample to some extent, limiting the duration of live culture. Yet we obtained four cases in which the MR was clearly inherited by the GSC or CB. In three of four cases, the MR was inherited by GSCs, consistent with our result obtained from fixed samples (Figure 3D and Supplemental Movie S1). In other cases (n = 15), the MR stayed between GSCs and CBs until the end of the imaging (typically 10–16 h). The cause may be that MR inheritance takes a long time and/or the culture condition compromised cell cycle progression. Yet, in four cases of such movies, we observed that the MR gradually became small without being inherited by GSCs or CBs (Figure 3E and Supplemental Movie S2). Because we observed small MRs between GSCs and CBs even in fixed samples, this likely reflects MR behavior in vivo. Observed variations in the timing of MR inheritance might indicate that MR inheritance is not synchronized with other cell cycle-dependent events, such as changes in spectrosome morphology. However, the MR is clearly degraded by the following mitosis, because we never observed MR remnants in mitotic cells. It should be noted that the scoring of MR inheritance during female GSC mitosis was limited to GSC-CB pairs in which the directionality of MR inheritance was clear. Therefore we conclude that MR is predominantly inherited by GSCs when the inheritance is asymmetric. However, from our data, it cannot be conclusively determined whether all MRs are eventually inherited by GSCs (or CBs) or some MRs may be resolved at the site of cytokinesis.

Previously, it was reported that the MR, detected by staining with anti-anillin antibody, was resolved at the site of cytokinesis instead of being incorporated into the GSC spectrosome (de Cuevas and Spradling, 1998). Although we observed cases where the MR becomes small at the site of cytokinesis, our results suggest that the MR is frequently inherited by GSCs. To reconcile these potentially conflicting observations, we stained the germaria that express Pav-GFP with anti-anillin antibody. We noted that the signal intensity ratio (signal on the MR/signal in the nucleus) was much higher with the Pav-GFP marker than with anti-anillin staining (Supplemental Figure S2), possibly because Pav-GFP was overexpressed and/or because of differences in their endogenous localization. As a result, the MR was more prominently visualized with Pav-GFP than with anti-anillin antibody. Particularly after abscission, when the MR was incorporated into the GSCs, the strong nuclear staining of the anillin antisera integrated into the GSC spectrosome (de Cuevas and Spradling, 1998). Although we observed cases where the MR becomes small at the site of cytokinesis, we never observed MR remnants in mitotic cells. It should be noted that the scoring of MR inheritance during female GSC mitosis was limited to GSC-CB pairs in which the directionality of MR inheritance was clear. Therefore we conclude that MR is predominantly inherited by GSCs when the inheritance is asymmetric. However, from our data, it cannot be conclusively determined whether all MRs are eventually inherited by GSCs (or CBs) or some MRs may be resolved at the site of cytokinesis.

The MR inheritance pattern depends on a functional centrosome

Because the centrosome plays a key role in abscission in mammalian cells (Piel et al., 2000, 2001; Gromley et al., 2005; Lee et al., 2008;...
loss-of-function allele; Basto et al., 2006), which does not contain any centriole, also showed randomization of MR inheritance during male GSC division (Supplemental Figure S3), suggesting that the stereotypical inheritance of the MR indeed depends on the function of the centrosome.

In contrast to male GSCs, it has been reported that asymmetric division of female GSCs relies on the spectrosome but not on a functional centrosome (Deng and Lin, 1997; Stevens et al., 2007). A more recent study suggested that centrosomes are oriented toward the GSC–cap cell interface throughout the cell cycle, and such centrosome positioning plays a role in asymmetric division of female GSCs (Lu et al., 2012). We found that MR inheritance was randomized in cnn-mutant female GSCs and the MR was inherited...

Kuo et al., 2011), we tested whether a functional centrosome is required for stereotypical MR inheritance. Indeed, we found that MR inheritance was randomized in male GSCs mutant for centrosomin (cnn), a core component of pericentriolar material required for the majority of centrosome functions (Megraw et al., 1999; 2001; Vaizel-Ohayon and Schejter, 1999): in 47% of cytokineses (n = 106 GSC-GB pairs in which the directionality of MR inheritance is clear), the MR was inherited by GSCs instead of GBs in cnn-mutant animals (either in animals homozygous for the strong loss-of-function allele cnn\textsuperscript{HK21}, which has a stop codon mutation after 105 amino acids out of a total of >1000 amino acids, or in animals transheterozygous for cnn\textsuperscript{HK21} and cnn\textsuperscript{mfs3}, which has a 190–amino acid truncation at the C-terminus; Figure 4, A–C). Furthermore, the dsas-4 mutant (dsas-4\textsuperscript{S2214}, a loss-of-function allele; Basto et al., 2006), which does not contain any centriole, also showed randomization of MR inheritance during male GSC division (Supplemental Figure S3), suggesting that the stereotypical inheritance of the MR indeed depends on the function of the centrosome.

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FIGURE 4: A functional centrosome is required for stereotypical MR inheritance in male and female GSCs. (A, B) Examples of a testis apical tip from control (cnn<sup>mkg2</sup>/+) and cnn<sup>mkg2</sup> / cnn<sup>mfs3</sup> mutant flies stained for FasIII and Add (red), Vasa (blue), and Pav-GFP (green). In control flies (A), the MR was segregated to the GBs, whereas the MR was frequently segregated to GSCs in the cnn<sup>mkg2</sup> / cnn<sup>mfs3</sup> mutant (B). Brackets indicate GSC-GB pairs. The asterisk indicates hub cells. Bar, 10 μm. (C) Frequency of MR inheritance by GSCs or GBs in cnn<sup>mkg2</sup> / cnn<sup>mfs3</sup>, cnn<sup>mfg2</sup> / cnn<sup>mfs3</sup>, or control flies. p value of Student’s t test (two tailed). N, number of GSC-GB pairs scored. (D, E) Examples of germaria from control (cnn<sup>mkg2</sup>/+) and cnn<sup>mfg2</sup> / cnn<sup>mfs3</sup> mutant flies stained for FasIII and Add (red), Vasa (blue), and Pav-GFP (green). In control flies (D), the MR was segregated to GSCs, whereas the MR was frequently segregated to CBs in the cnn<sup>mfg2</sup> / cnn<sup>mfs3</sup> mutant (E). The asterisk indicates cap cells.

Female GSCs inherit the daughter centrosome

Intrigued by the opposite pattern of MR inheritance (by the differentiating cell in the testis and the GSCs in the ovary) and requirement of cnn in MR inheritance in female GSCs, we decided to characterize centrosome positioning during the female GSC cell cycle in more detail. Under our experimental and culture conditions, we found that centrosomes were not oriented in 57.5% of female GSCs (n = 332 GSCs). However, when we examined the centrosome orientation in relation to the spectrosomal morphology, which corresponds to cell cycle stage (Hsu et al., 2008), it became clear that one centrosome closely associates with the spectrosome (or GSC–cap cell interface) as cells approach mitosis (Figure 5). This nicely bridges two previous reports that one centrosome is associated close to the cap cells in mitosis (Deng and Lin, 1997) but not in interphase (Stevens et al., 2007). As shown in Figure 5, A–D, centrosomes were mostly unoriented during earlier phases of the cell cycle, but in the “round-fusome” stage, which corresponds to the G2 phase of the cell cycle (Hsu et al., 2008), one centrosome was closely associated with the apical side (with either the apically localized spectrosome or GSC–cap cell interface; 68.7 ± 12.7%, n = 141 “round-stage” GSCs; Figure 5, B and D). The centrosome–spectrosome association subsequently reached 100% in mitosis (Figure 5, C and D), as reported previously (Deng and Lin, 1997). These data demonstrate that female GSCs also orient their centrosomes with respect to the niche but during a limited period of the cell cycle (i.e., G2 to mitosis). We do not know where the discrepancy arises between the report by Stevens et al. (2007), which is similar to our observation, and the report by Lu et al. (2012). The cause may be differences in cell cycle distribution under different culture conditions. Lu et al. used a very protein-rich diet (Lu et al., 2012; C. Ferguson, personal communication), whereas we did not supplement our regular fly culture medium with additional dry yeast. We showed that, in male GSCs, the diet influences centrosome orientation, leading to regulation of GSC division frequency, depending on the availability of nutrients (Roth et al., 2012). A similar regulation might be operating in female GSCs.

Januschk et al. (2011) reported Drosophila centrobin (Cnb) as the first molecular marker that distinguishes mother and daughter centrioles in Drosophila. Using this marker and live observation (Conduit and Raff, 2010; Januschk et al., 2011), it was shown that the larval neuroblasts inherit the daughter centrosome upon division. When we scored female GSCs with apparently oriented centrosomes in which only one of the two centrosomes was marked with Cnb-YFP, we found a strong bias toward the Cnb-yellow fluorescent protein (YFP)–labeled daughter centrosome being close to the cap cells/spectrosome (70%; n = 54, Figure 5, E and F). The frequency of daughter centrosomes associated with the cap cells/spectrosome is lower than that of mother centrosomes associated with the hub cells in male GSCs (>90%; Yamashita et al., 2007). This might be because of the contribution of female GSCs in early phases: at this time, ~20% of GSCs have “apparently oriented” centrosomes (Figure 5D), but these cells might not actually be oriented yet, and either the mother or daughter centrosome may be randomly located near the cap cells. To test this idea, we combined Cnb-YFP and stained for the spectrosome and centrosome to score GSCs exclusively at the “round stage,” which corresponds to late G2 phase of the cell cycle (Hsu et al., 2008). This approach proved to be very challenging because at the “round stage,” many GSCs had two Cnb-YFP–positive centrosomes, presumably due to the maturation of both centrosomes (i.e., both centrosomes are composed of mother and daughter centrioles): of 585 GSCs at the round stage, only 66 cells had one Cnb-positive and one Cnb-negative centrosome, with the remainder containing two Cnb-positive centrosomes. Of these 66 cells, 31 had centrosomes that were not oriented. Among the remaining 35 cells, however, the Cnb-marked...
daughter centrosome was associated with the cap cells in 30 cells (86 ± 4.2%; Figure 5G). The strong tendency toward association of the daughter centrosome with the cap cells/spectrosmes during late stages of the cell cycle suggests that the daughter centrosome is inherited by female GSCs. In the testes, almost all GSCs had two Cnb-YFP–positive centrosomes, likely because centrosome splitting occurs after centrosome duplication, and it was not possible to distinguish mother and daughter centrosomes using Cnb-YFP (Llamazares and Gonzalez, personal communication and unpublished data). We concluded that the daughter centrosome is preferentially inherited by the female GSCs, similar to the situation in Drosophila neuroblasts. These results also reveal a strong correlation between centrosome age and MR inheritance: male GSCs inherit the mother centrosome (Yamashita et al., 2007) and exclude the MR, whereas female GSCs inherit the daughter centrosome and inherit the MR. In other words, in both male and female GSCs, the cell that inherits the daughter centrosome inherits the MR as well. Of interest, this pattern is opposite to the previous observation of (Kuo et al., 2011), in which stem cells were shown to inherit the mother centrosome and MR. This finding may suggest that the MR inheritance pattern and
ectopic expression of niche factors, which leads to symmetric stem cell divisions, on the MR inheritance pattern. Unpaired (Upd) is secreted from hub cells to specify male GSC identity, and overexpression of Upd in the germline is known to lead to GSC tumors (Kiger et al., 2001; Tulina and Matunis, 2001). The MR inheritance pattern was scored upon overexpression of Upd in the germline (nos-gal4>UAS-Upd; Figure 6, A and B). We focused on GSCs that were attached to the hub because GSC tumors outside the normal niche do not have a reference point (landmark) to address the pattern of MR inheritance. On overexpression of Upd, the frequency of GSCs that had completed the abscission (and thus are not connected with the GBs) decreased mildly but significantly compared with controls (31% in Upd-expressing testes vs. 45% in control testes, \( p = 2.4 \times 10^{-5} \) by chi-squared test), concomitant with an increase in the frequency of GSCs that are still connected to GBs (61% in Upd-expressing testes vs. 49% in control testes, \( p = 2.4 \times 10^{-5} \) by chi-squared test). This finding may suggest that GSC cytokinesis is stalled, possibly due to a problem in deciding on which side of the MR abscission should occur. Consistent with this idea, we also frequently observed GSC-GB pairs in which the MR was apparently pinched from both sides or stuck in the middle upon expression of Upd (Figure 6A). Such instances were very rare in wild-type or control testes (2% of total GSCs, \( n = 451 \) GSCs) but increased to 8% (\( n = 598 \) GSCs) in Upd-expressing testes (Figure 6B). We speculate that in Upd-expressing testes, GSC division yields two GSCs, both of which activate the program to pinch off the MR. We also speculate that the similar (albeit low frequency) observation in wild-type/control testes might reflect a low frequency of symmetric GSC divisions, as reported recently (Sheng and Matunis, 2011). Together these results indicate that Upd, and presumably stem cell fate, regulates the abscission site during GSC divisions.

The MR inheritance pattern is influenced by stem cell factors

What is the biological relevance of asymmetric MR inheritance during stem cell division? To address whether asymmetric MR inheritance correlates with cell fate, we examined the effect of its relationship to centrosome inheritance are cell type–specific (or species-specific) phenomena.

**FIGURE 6:** MR inheritance correlates with stem cell fate. (A) An example of an MR that is apparently pinched off from both sides upon GSC division in a Upd-expressing testis (nos-gal4>UAS-Upd). Green, Pav-GFP; red, Fas III and Add; blue, Vasa. The asterisk indicates hub cells. Bar, 10 μm. (B) The MR inheritance pattern in control vs. Upd-expressing testes. (C) An example of MR inherited by the cell that is displaced away from the niche (i.e., the cell at the position of the cystoblast; c587-gal4>UAS-Dpp, raised at 18°C, shifted to 29°C for 7 d). Arrowheads indicate the site of abscission. Note that Dpp expression only caused a mild increase in the GSC number under our experimental conditions. (D) The MR inheritance pattern in control vs. Dpp-expressing ovary. Only GSC-CB pairs in which the direction of MR inheritance was clear were scored.
DISCUSSION
Here we show that the MR is inherited asymmetrically during GSC divisions in the Drosophila germline and that this correlates with centrosome age and depends on a functional centrosome. Of interest, inheritance of the MR by the cell containing the daughter centrosome is opposite to a recent observation in mammalian cells (Kuo et al., 2011). Further studies are required to determine whether the asymmetrically inherited MR, or factors associated with it, regulates stem cell behavior, and whether this regulation occurs in a species- or cell type–dependent manner. Of importance, mutations that randomize MR inheritance (cnn and dsas-4) do not drastically modulate stem cell identity, and cnn and dsas-4 mutants show apparently normal progression of differentiation regarding the cell fate (Yamashita et al., 2003; Stevens et al., 2007). Furthermore, the MR is inherited by the differentiating daughter in the male germline, whereas it is inherited by the stem cell in the female germline. Therefore it is unlikely that the MR harbors an inherent fate determinant. However, it is tempting to speculate that certain fate determinants “hitchhike” the MR in certain cell types, taking advantage of its stereotypical inheritance. In addition, it is possible that the MR regulates an aspect of stem cell behavior rather than identity per se; for example, the MR could regulate the rate of stem cell division. The fact that we never see multiple MRs in a single cell (GSC or CySC) may indicate that removal of the MR is a prerequisite of cell cycle progression into the next cell cycle. Moreover, the MR that is transferred from the GB to the CySC/CC might function as a messenger to coordinate the division frequency between GSCs and CySCs (Inaba et al., 2011).

The reports by Kuo et al. (2011) and Ettinger et al. (2011) are seemingly contradictory in that the former reported that stem cells are characterized by the accumulation of MRs, whereas the latter reported that they are characterized by the high capacity for MR release into the extracellular space. Our study using male and female GSCs demonstrates that MR fates are highly stereotypical yet strikingly distinct, depending on the cell type. This finding indicates that each cell type handles MRs with its own elaborate cellular program. The reason why MR must be handled in such an elaborate manner awaits future investigation. Nonetheless, our study reveals that a basic cellular asymmetry such as MR inheritance correlates with asymmetry during stem cell division.

MATERIALS AND METHODS
Fly husbandry and strains
All fly stocks were raised in standard Bloomington medium at 25°C. The following fly stocks were used: nos-gal4 (Van Doren et al., 1999), cnnHG27 (Megraw et al., 2001), and UAS-dpp (obtained from the Bloomington Drosophila Stock Center, Bloomington, IN); Ubi-Pavarotti-GFP and UAS-Pavarotti-GFP (Minestrini et al., 2002; obtained from David Glover, University of Cambridge); cnnmfs3 (Megraw et al., 1999; obtained from Thom Kaufman, Indiana University); dsas-452214 (Basto et al., 2006; obtained from the Bloomington Stock Center); and UAS-Upld (Zeidler et al., 1999), Asl(asterless)-YFP (Varmark et al., 2007), and Ubi-Cnb-YFP (Januschke et al., 2011; obtained from Cayetano Gonzalez, IRB Barcelona). To assess MR inheritance in cnn mutants, a cnnHG27/CyO; Ubi-Pav-GFP/TM3 fly stock was generated, which was subsequently crossed with cnnmfs3/CyO to obtain transheterozygous mutant flies (cnnHG27/cnnmfs3; Ubi-Pav-GFP/+), as well as control siblings (cnn/CyO; Ubi-Pav-GFP/+).

Immunofluorescence microscopy
Samples were fixed for 30–60 min with 4% formaldehyde in phosphate-buffered saline (PBS) and permeabilized for 30 min in PBST (0.1% Triton X-100 in PBS). Samples were then incubated overnight at 4°C with primary antibodies, washed three times with PBST for 20 min, incubated overnight at 4°C with Alexa Fluor–conjugated secondary antibodies (1:200; Molecular Probes, Eugene, OR), and washed again with PBST (three times for 20 min). For lysosome staining, testes were dissected into PBS, incubated with LysoTracker (conjugated with Alexa 594; Invitrogen, Carlsbad, CA) for 30 min, and fixed with 4% formaldehyde for 30 min, followed by a standard immunofluorescence staining procedure as described. Stained samples were mounted in Vectashield (H-1200; Vector Laboratories, Burlingame, CA). The primary antibodies used were mouse anti-fasciclin III (1:20; developed by C. Goodman, University of California, San Francisco, and obtained from the Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA); mouse antialdadin-like (1:20; developed by H. D. Lipshitz, University of Toronto, and obtained from the DSHB); goat anti-Vasa (1:100; cdc13; Santa Cruz Biotechnology); rabbit anti-Vasa (1:100; Santa Cruz Biotechnology); rat anti-Vasa (1:40; developed by Allan Spradling, Carnegie Institution, and obtained from the DSHB); rabbit anti-Spd-2 (1:100; a gift from Maurizio Gatti, Sapienza University of Rome; Giannanti et al., 2008); and rabbit anti-anillin antibody (1:1300; a gift from Christine Field, Harvard University; Field and Alberts, 1995). Images were taken using a Leica TCS SP5 confocal microscope with a 63× oil immersion objective (numerical aperture, 1.4) and processed using Photoshop software (Adobe).

Time-lapse live-imaging methods
Newly eclosed Pav-GFP flies were dissected inside Drosophila culture medium containing Schneider’s Drosophila medium and 10% fetal bovine serum. The ovaries were placed inside a sterile glass-bottom chamber covered with a gas-permeable membrane and were mounted on a three-axis, computer-controlled piezoelectric stage and imaged using an inverted microscope equipped with an electron multiplier cooled charge-coupled device camera. Image sequences were acquired every 600 s. The supplemental movies were generated using ImageJ software (National Institutes of Health, Bethesda, MD).

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