Drosophila male germline stem cells do not asymmetrically segregate chromosome strands

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Summary

Adult stem cells continuously supply differentiated cells throughout the life of organisms. This increases the risk of replicative senescence or neoplastic transformation due to mutations that accumulate over many rounds of DNA replication. The immortal strand hypothesis proposes that stem cells reduce the accumulation of replication-induced mutations by retaining the older template DNA strands. Other models have also been proposed in which stem cells asymmetrically segregate chromosome strands for other reasons, such as retention of epigenetic memories. Recently, the idea has emerged that the mother centrosome, which is stereotypically retained within some asymmetrically dividing stem cells, might be utilized as a means of asymmetrically segregating chromosome strands. We have tested this hypothesis in germline stem cells (GSCs) from *Drosophila melanogaster* testis, which undergo asymmetric divisions marked by the asymmetric segregation of centrosomes and the acquisition of distinct daughter cell fates (stem cell self-renewal versus differentiation). Using 5-bromo-2-deoxyuridine labeling combined with direct visualization of GSC-gonialblast (differentiating daughter) pairs, we directly scored the outcome of chromosome strand segregation. Our data show that, in male GSCs in the *Drosophila* testis, chromosome strands are not asymmetrically segregated, despite asymmetrically segregating centrosomes. Our data demonstrate that asymmetric centrosome segregation in stem cells does not necessarily lead to asymmetric chromosome strand segregation.

Key words: Centrosome, Drosophila, Immortal strand hypothesis, Niche, Stem cells

Introduction

Adult stem cells have the ability to produce new stem cells (self-renewal) as well as differentiated progeny throughout the life of an organism (Morrison and Kimble, 2006). Given the long-term demands on self-renewing stem cells to maintain tissue homeostasis by supplying differentiated cells continuously, stem cells are probably the cell population most challenged by the risk of replicative senescence and transformation through accumulation of DNA mutations (Blasco, 2007; Rando, 2007; Ruzankina et al., 2008). How stem cells avoid the potentially deleterious effects of DNA mutations resulting from repeated cell cycles is poorly understood. The 'immortal strand hypothesis' (ISH) has been proposed as a mechanism by which adult stem cells might limit accumulation of mutations arising from errors during DNA replication. According to the ISH, adult stem cells might retain older ('immortal') DNA strands during asymmetric cell divisions, thereby excluding all replication-induced mutations into the differentiating daughters (Cairns, 1975).

This hypothesis has been intensively studied in recent years in a broad range of stem cell populations. Supporting evidence for immortal strand segregation comes from studies of cells in the small and large intestine (Potten et al., 2002; Quyn et al., 2010), neural stem cells (Fei and Huttner, 2009; Karpowicz et al., 2005), mammary epithelial cells (Smith, 2005), fibroblasts (Merok et al., 2002), skeletal muscle satellite cells (Conboy et al., 2007; Shinin et al., 2006), human lung cancer cells (Pine et al., 2010) and female germline stem cells in the *Drosophila* ovaries (Karpowicz et al., 2009). Other studies using similar techniques have failed to observe evidence for asymmetric chromosome strand segregation in mouse hematopoietic stem cells (Kiel et al., 2007), epidermal basal cells (Sotiropoulou et al., 2008), hair follicle stem cells (Waghmare et al., 2008) and neocortical precursor cells (Fei and Huttner, 2009). These results suggest that asymmetric chromosome strand segregation occurs in some cells but that this is not a general strategy used by most stem cells.

Recently, Falconer et al. observed extreme asymmetry in chromosome strand segregation in colon crypt epithelial cells. However, judging from position in the crypt, such asymmetry was observed in differentiating cells as well as in stem cells (Falconer et al., 2010), suggesting that there might be a reason(s) why a cell (not necessarily a stem cell) must segregate particular chromosome strands other than to exclude replication-induced mutations (Armakolas and Klar, 2006; Armakolas et al., 2010; Dalgaard and Klar, 2001). The authors proposed that cells asymmetrically segregate other information such as epigenetic memories (Falconer et al., 2010; Lansdorp, 2007) by asymmetric segregation of chromosome strands.

Assessing asymmetric chromosome strand segregation has been challenging in many systems. The populations that have been studied have often been heterogeneous mixtures of stem and progenitor cells, leaving ambiguity about which cells exhibit evidence of asymmetric segregation. This problem is compounded by the fact that, in most experiments, only a small percentage of cells exhibit evidence of asymmetric strand segregation, raising

questions regarding the biological significance of the observation and the extent to which it might have been influenced by technical artifacts. In most systems, it is also unclear whether stem cells divide asymmetrically, divide symmetrically, or switch between these two modes, which complicates the interpretation of DNA label segregation patterns. Finally, the fates of daughter cells have been uncertain in most studies, making it impossible to correlate asymmetries in fates with chromosome strand segregation. For these reasons, many studies that have provided evidence in support of the ISH also have alternative explanations (Lansdorp, 2007; Rando, 2007; Tajbakhsh, 2008).

The *Drosophila melanogaster* male germline stem cell (GSC) system provides an ideal model system to test the ISH, overcoming most of problems listed above. First, *Drosophila* male GSCs can be identified at single-cell resolution by combining cellular markers and tissue anatomy. At the apical tip of the testis, approximately nine GSCs physically attach, via adherens junctions (Yamashita et al., 2005; Yamashita et al., 2003), to a cluster of somatic cells called the hub, which is the major component of the stem cell niche (Kiger et al., 2001; Tulina and Matunis, 2001). Therefore, GSCs can be unambiguously identified by their attachment to the hub as well as their expression of germ cell markers such as Vasa (Hay et al., 1988; Yamashita et al., 2003) (Fig. 1A, Fig. 2A,B). Second, GSCs always divide asymmetrically by orienting the mitotic spindle perpendicular to the hub so that one daughter remains attached to the hub and maintains GSC identity, whereas the other is displaced away from the hub and becomes a differentiating gonialblast (GB) (Yamashita et al., 2003) (Fig. 1A). Because of the stereotypical mitotic spindle orientation, the fates of daughter cells (GSC versus GB) can be easily predicted during GSC anaphase and telophase, when segregation of chromosome strands can be unambiguously assessed.

We have shown that the stereotypical orientation of the spindle is determined by the precisely controlled positioning of the centrosomes during interphase. The mother centrosome normally remains adjacent to the hub and is inherited by the GSC, whereas

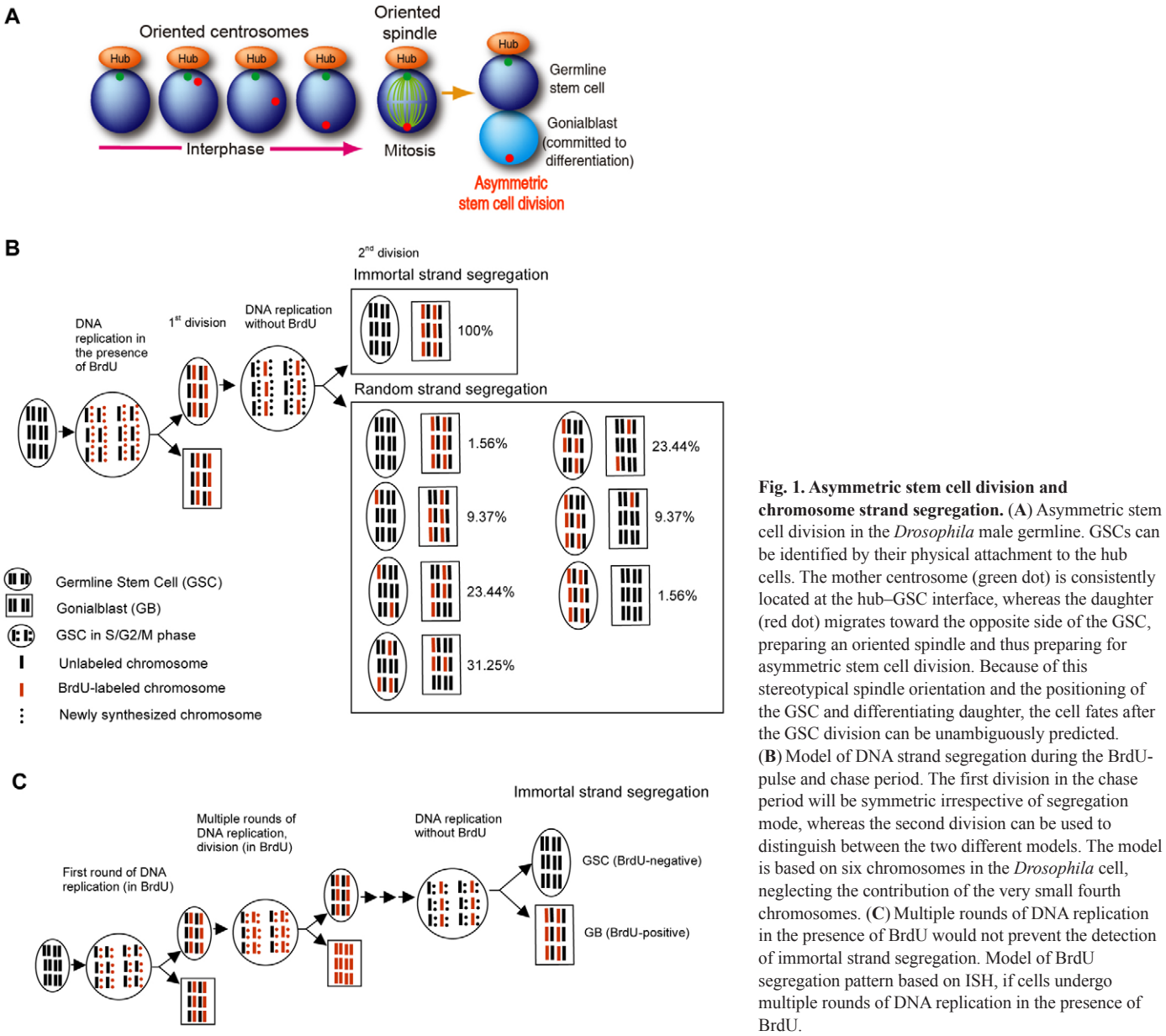

Fig. 1. Asymmetric stem cell division and chromosome strand segregation. (**A**) Asymmetric stem cell division in the *Drosophila* male germline. GSCs can be identified by their physical attachment to the hub cells. The mother centrosome (green dot) is consistently located at the hub–GSC interface, whereas the daughter (red dot) migrates toward the opposite side of the GSC, preparing an oriented spindle and thus preparing for asymmetric stem cell division. Because of this stereotypical spindle orientation and the positioning of the GSC and differentiating daughter, the cell fates after the GSC division can be unambiguously predicted. (**B**) Model of DNA strand segregation during the BrdU-pulse and chase period. The first division in the chase period will be symmetric irrespective of segregation mode, whereas the second division can be used to distinguish between the two different models. The model is based on six chromosomes in the *Drosophila* cell, neglecting the contribution of the very small fourth chromosomes. (**C**) Multiple rounds of DNA replication in the presence of BrdU would not prevent the detection of immortal strand segregation. Model of BrdU segregation pattern based on ISH, if cells undergo multiple rounds of DNA replication in the presence of BrdU.

the daughter centrosome migrates to the opposite side of the cell and is inherited by the GB (Yamashita et al., 2007) (Fig. 1A). Recently, it has been hypothesized that the asymmetric segregation of centrosomes by stem cells might be the mechanism by which chromosome strands are asymmetrically segregated. It was proposed that the mother centrosome anchors the immortal strand during repeated cell divisions, retaining the immortal strand within stem cells (Tajbakhsh and Gonzalez, 2009).

We decided to test this hypothesis in *Drosophila* male GSCs, the only system in which stem cells are known to always divide asymmetrically by the asymmetric segregation of centrosomes and in which stem cells and their daughters can be unambiguously identified. In this study, using 5-bromo-2-deoxyuridine (BrdU) pulse-labeling, combined with direct visualization of GSC–GB pairs and anaphase/telophase GSCs, we show that chromosome strands are not segregated asymmetrically in *Drosophila* male GSCs. This indicates that stem cells that divide asymmetrically and segregate their centrosomes asymmetrically do not necessarily segregate their chromosome strands asymmetrically.

Results

Establishing GSCs as a model system to test the ISH

In this study, we adopted a pulse-chase strategy to label newly synthesized DNA strands with BrdU by feeding flies BrdU and monitoring the segregation of BrdU-labeled chromosomes during the chase period (see Materials and Methods). With this strategy, the semi-conservative replication of DNA will cause BrdU to be segregated to both daughter cells in the first division during the chase period, irrespective of whether GSCs act in accordance with the ISH (Fig. 1B). If GSCs retain the immortal strands, we would expect to observe asymmetric BrdU segregation in the second division, with the GB inheriting all the BrdU-labeled, newly replicated strands (Fig. 1B, second division, immortal strand segregation). This would be true irrespective of how many times a GSC has replicated its DNA in the presence of BrdU because the immortal strands would never be labeled by BrdU and would always be retained within the GSC (Fig. 1C). By contrast, if GSCs randomly segregate their chromosome strands, BrdU would be segregated to both daughter cells in the second division (Fig. 1B, second division, random strand segregation). Importantly, asymmetric segregation of the BrdU label would sometimes be observed by chance as a result of random segregation. With random chromosome strand segregation, the BrdU label would be diluted stochastically over time (on average, by half with each round of division).

Normally, all GSCs divide regularly but asynchronously in the *Drosophila* testis. On the basis of our previous studies, it was calculated that each GSC divides approximately every 12–16 hours. About 3–4% of total GSCs are in mitosis, and each mitosis lasts about 30 minutes according to live time-lapse observation, leading to a calculated cell cycle time of 12–16 hours (Cheng et al., 2008; Yamashita et al., 2003). When GSC centrosomes were labeled by transient expression of a centriolar marker, PACT, tagged with GFP (GFP–PACT), the very first GSCs that completed the second round of centrosome duplication appeared after 12 hours. Such GSCs considerably increased at 16–18 hours, suggesting that the GSC cell cycle time (more accurately the time from G1–S transition to the next G1–S transition) exceeds 12 hours (Yamashita et al., 2007). When newly eclosed flies were fed BrdU-containing food, ~90% of GSCs were labeled after 16 hours and ~95% after 24 hours (Fig. 2A–C). This is consistent with our calculated GSC cell cycle time of 12–16 hours, considering the facts that flies might not begin feeding immediately, that GSCs do not incorporate BrdU immediately upon transfer to BrdU-containing food, and that many GSCs are in G2 (rather than G1–S) at any given time. Mitotic indices of GSCs in the presence (13.6%, 25 mitoses/184 testis) and absence (13.2%, 24 mitoses/189 testis) of BrdU were similar, showing that the BrdU feeding scheme used here did not perturb cell cycle progression. It should be noted that BrdU incorporation into GSCs plateaued at around 95%. This is presumably due to the fact that ~5% of GSCs from young flies have misoriented centrosomes, a condition that is known to delay cell cycle progression (Cheng et al., 2008). To maximize the BrdU-labeled GSC population to start the chase period, we decided to employ 24-hour feeding in subsequent experiments (Fig. 2D).

In prior studies of the ISH, it was often not possible to definitively identify daughter cells that arose from a single cell division. To overcome this problem, we strictly limited our scoring to cases where twin daughters of a stem cell division could be unambiguously identified. First, we scored BrdU segregation in GSCs in anaphase/telophase, when two segregating nuclei were visible within a single cell. However, GSCs in anaphase or telophase are extremely rare. Only 3–4% of total GSCs are in mitosis, and only ~10% of mitotic GSCs are in anaphase/telophase (i.e. only about 0.3–0.4% of total GSCs), making it challenging to obtain enough samples for statistical analysis. Therefore, we took advantage of Pavarotti–GFP (Pav–GFP), the *Drosophila* homolog of mammalian kinesin-like protein MKLP1 tagged with GFP (Minestrini et al., 2003). Pav–GFP localizes to the plus ends of microtubules during anaphase and telophase, decorating the spindle midzone (Fig. 2E) and enabling us to recognize GSCs during these periods. Pav–GFP then translocates to the contractile ring during cytokinesis and stays on the midbody ring after cytokinesis (Fig. 2F), enabling us to identify a GSC–GB pair resulting from a GSC division and to score the BrdU segregation pattern in post-mitotic (pre-abscission) cells. Because it turned out that ~50% of GSCs were still connected to GBs with the Pav–GFP-marked midbody, usage of Pav–GFP allowed us to score 100 times more cells than we otherwise could have by scoring only anaphase/telophase cells.

GSCs do not segregate their chromosome strands asymmetrically

Once we established the experimental system to test the ISH as described above, we proceeded to analyze the BrdU segregation pattern. Flies were fed with BrdU for 24 hours, followed by a chase period (fed food without BrdU for 18, 24, 36, 48, 60 or 72 hours) (Fig. 2D). By this feeding scheme, it is possible that a small population of GSCs underwent two rounds of DNA replication in the presence of BrdU. However, as mentioned above, this would not prevent us from detecting immortal strand segregation (Fig. 1C). Testes were subjected to immunofluorescence staining to detect BrdU in combination with a germ cell marker (Vasa), a hub cell marker (Fasciclin III; FasIII), and Pav–GFP. We analyzed GSC–GB pairs that were connected by the contractile ring/midbody ring as well as GSCs in anaphase and telophase, all of which are easily identifiable using Pav–GFP localization. Throughout the chase period, we observed a high frequency of GSC–GB pairs in which both cells inherited BrdU-labeled chromosome strands, until eventually most GSCs diluted out the BrdU label (Fig. 3A,E). Consistent with this result, in the majority of anaphase and telophase GSCs, BrdU was segregated to both daughter cells (Fig. 3B; 84% were symmetric, 25 anaphase/telophase GSCs scored). These data

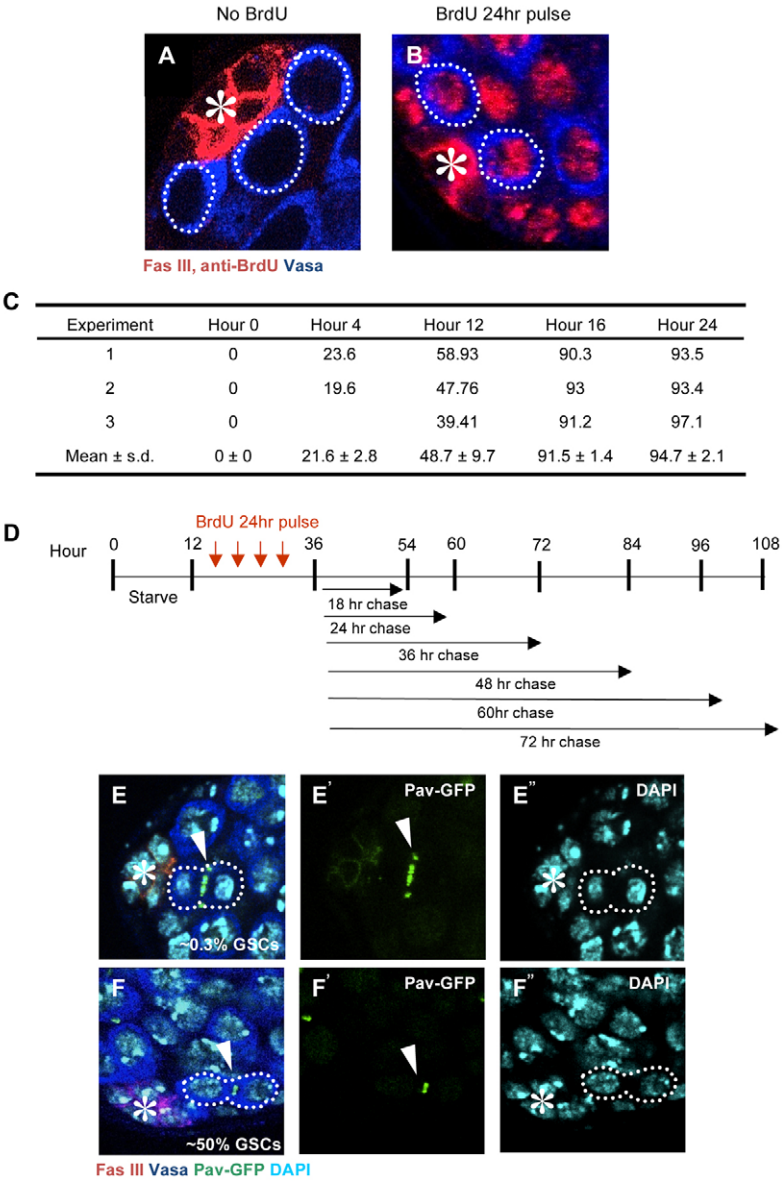

Fig. 2. Experimental schemes to address the ISH in male GSCs. (**A**,**B**) Examples of BrdU staining in GSCs (encircled by dotted lines) from flies cultured in the absence (A) or presence (B) of BrdU for 24 hours. Red, Fas III and BrdU; blue, Vasa (germ cells); * indicates the hub. (**C**) Outcome of BrdU incorporation experiment with varying pulse periods. Data is shown as the frequency (%) of BrdU-positive GSCs/total GSCs (mean ± s.d.). 300–400 cells were scored for each data point. (**D**) The experimental scheme: newly eclosed flies were starved for 12 hours, followed by a 24-hour pulse period. They were then transferred to normal media for the indicated time. (**E**,**F**) Localization of Pav–GFP in male GSCs during telophase (E) and after mitosis (F). Red, Fas III; green, Pav–GFP; blue, Vasa (germ cells); light blue, DAPI; * indicates the hub; arrowheads point to Pav–GFP-marked contractile ring/midbody (A,C) or spindle midzone (B,D).

suggest that, in male GSCs, chromosome strands do not asymmetrically segregate, despite asymmetrically segregating centrosomes.

Drosophila cells have only six large chromosomes: XX or XY chromosomes, a pair of second chromosomes, and a pair of third chromosomes, neglecting a pair of very small fourth chromosomes that constitute less than 3% of the genome (5 Mb of 180 Mb) (Adams et al., 2000; Locke and Mcdermid, 1993). This means that the probability that one cell would inherit all BrdU-labeled chromosome strands by chance would not be negligible, even if chromosome strands were randomly segregated. To quantify this probability, we performed mathematical modeling (see Materials and Methods). In a randomly segregating cell in which all six chromosomes contain a BrdU-labeled strands (i.e. during the second division in the chase period), the probability that all of the BrdU-positive chromosome strands would be inherited by the same daughter cell by chance was 3.125%, assuming that a single BrdU-positive chromosome strand is detectable (Fig. 1B). The BrdU label is diluted over successive rounds of division during the chase period so the probability that a single cell inherits all the BrdU labeled-chromosome strands is expected to increase, because each cell would contain fewer labeled chromosome strands as a result of random segregation in earlier cell cycles. In the fourth round of division during the chase period, the frequency of asymmetric BrdU segregation by chance would reach a maximum of ~50% (Fig. 4A). It should be noted that when the mathematical modeling was performed on the basis of eight chromosomes, as in Karpowicz et al. (Karpowicz et al., 2009), the outcome was similar to the outcome with six chromosomes in that cells exhibited considerable frequency of apparent asymmetric chromosome strand segregation with a peak that was delayed only by ~0.5 cell cycles compared to the modeling with six chromosomes (Fig. 4C), although the probability of asymmetric segregation in the second division was much lower (0.78125%) than in the six-chromosome modeling.

As predicted by our modeling (Fig. 1B, Fig. 4A), we observed that, indeed, some GSCs appeared to exhibit asymmetric BrdU segregation (Fig. 3C–E). However, the pattern of asymmetric segregation in these cases was random; in some cases a BrdU-

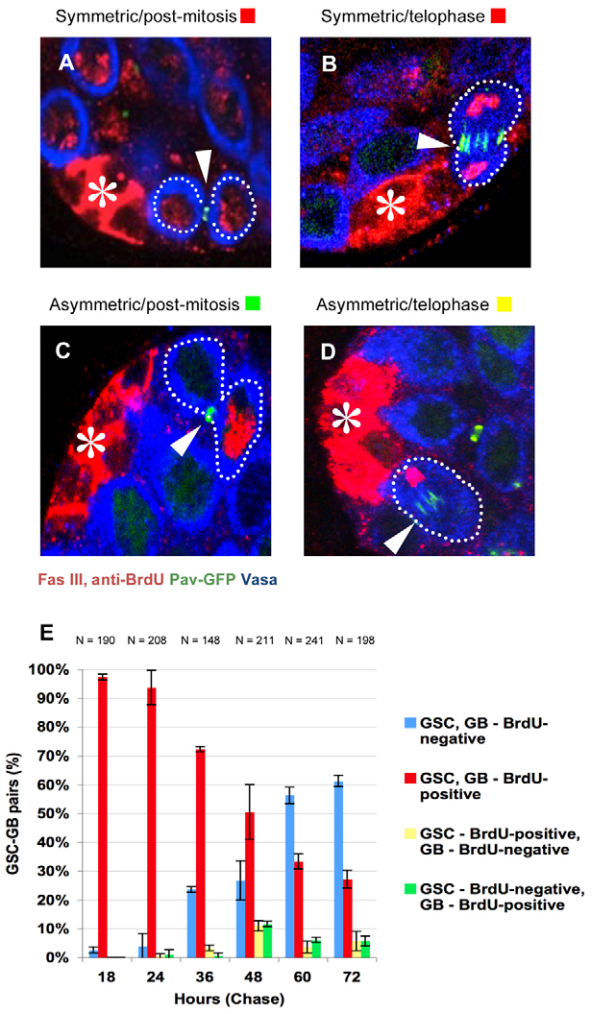

Fig. 3. Immortal DNA strands are not segregated in *Drosophila* male GSCs. (**A–D**) Examples of BrdU segregation in anaphase/telophase or post-mitotic GSCs after 24 hour pulse (BrdU) and 48 hour chase (without BrdU). (A) Symmetric BrdU segregation in a post-mitotic GSC-GB pair (encircled by dotted lines). (B) Symmetric BrdU segregation in a telophase GSC. (C) Asymmetric BrdU segregation (BrdU-negative GSC, BrdU-positive GB) in a post-mitotic GSC. (D) Asymmetric BrdU segregation (BrdU-positive GSC, BrdU-negative GB) in an anaphase GSC. Red, Fas III and BrdU; green, Pav–GFP; blue, Vasa (germ cells); * indicates the hub; arrowheads point to Pav–GFP-marked contractile ring/midbody (A,C) or spindle midzone (B,D). (**E**) Summary of BrdU segregation during the chase period. *N*, number of GSC–GB pairs or anaphase/telophase GSCs scored. Error bars show s.d. of least three independent experiments.

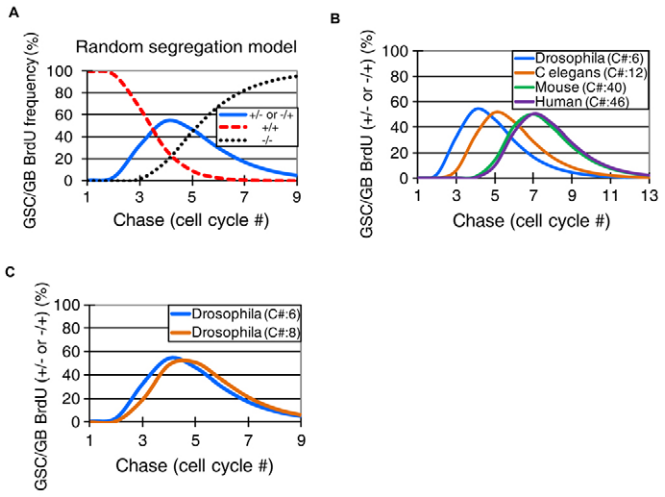

Fig. 4. Model of BrdU segregation pattern during the chase period, based on the random segregation model. (**A**) Simulation of BrdU segregation pattern in cells with six chromosomes (*Drosophila*). Apparent asymmetric segregation reaches ~50% in the fourth cell cycle, as a result of random segregation. (**B**) Simulated frequencies of asymmetric BrdU segregation in cells with different number of chromosomes. C# indicates the number of chromosomes per cell. (**C**) Simulation of BrdU segregation pattern comparing cells with six chromosomes to those with eight chromosomes, to consider the contribution of the fourth pair of chromosomes.

negative GSC was connected with a BrdU-positive GB (Fig. 3C), and in other cases a BrdU-positive GSC was paired with BrdU-negative GB (Fig. 3D). This is inconsistent with the ISH and suggests that such asymmetry is a consequence of random segregation of BrdU labeling. As predicted, as the chase period proceeded, we observed a higher incidence of asymmetric BrdU segregation, again random with respect to the cell (GSC or GB) that inherited the BrdU label (~20% of total GSC–GB pairs at 48 hours of chase, Fig. 3E). This frequency of asymmetric BrdU segregation (~20%) was lower than would be expected by chance after four rounds of division (~50%). This might be due to sister chromatid exchange between BrdU-positive and BrdU-negative chromosome strands, which would cause a mixing and redistribution of BrdU-labeled chromatin to both strands.

We have shown that GSCs can be generated via dedifferentiation of spermatogonia (Cheng et al., 2008). If this occurred during the time course of our experiments, GSCs with their immortal strand labeled with BrdU could have been generated, possibly interfering with our interpretation of the data. If this was the case and if GSCs followed the ISH, GSCs derived from a dedifferentiation process would retain BrdU-labeled strands for multiple cell cycles (theoretically forever). However, BrdU label was completely diluted out by 120 hours of chase period (0% BrdU-positive GSCs, 187 GSCs scored), suggesting that any GSCs (whether derived from dedifferentiation or not) do not retain BrdU-labeled chromosome strands. Taken together, these data demonstrate that male GSCs do not retain template DNA strands, as predicted by the ISH and other models of non-random chromosome strand segregation.

Discussion

Our results demonstrate that chromosome strands are not segregated asymmetrically in *Drosophila* male GSCs. We employed direct visualization of segregating chromosomes in dividing and post-mitotic GSCs rather than by inferring chromosome strand segregation patterns based on the kinetics of BrdU dilution. This is the first study to test the ISH using direct visualization of DNA label segregation in a stem cell population that can be definitively identified and that is known to divide asymmetrically.

Our study illuminates a few crucial pitfalls that can be encountered when addressing the ISH. For example, we observed a high incidence of asymmetric BrdU segregation as the chase period increased. This is predicted to occur as GSCs dilute BrdU-labeled chromosome strands in the previous cycles as a result of random segregation, increasing the probability that remaining BrdU-labeled chromosome strands are ‘co-segregated’ into one

cell by chance. This highlights the value of using two distinct DNA labels (such as IdU and CldU) (Conboy et al., 2007; Kiel et al., 2007) to identify cells that have divided twice (but not more). This is particularly important when the system contains heterogeneous cells with varying cell cycle times: some cells might undergo more cell cycles (and thus have higher chance of asymmetric segregation of DNA label) than others at the time of sampling.

Although mouse and human cells have many more chromosomes (40 and 46, respectively) than *Drosophila* cells (six major chromosomes and two small chromosomes), these cells, if segregating chromosome strands randomly, would need less than three cell cycles ($46/2^3<6$) to dilute the BrdU label to the point of being comparable with *Drosophila* cells. Mouse and human cells could, therefore, display some asymmetric label segregation during the chasing period despite random chromosome strand segregation (Fig. 4B).

Our study also illustrates the importance of identifying cell fate after cell division. We commonly observed asymmetric segregation of BrdU after ~48 hours of chase; however, because we could definitively distinguish stem cells from differentiating cells, we were able to confirm that the segregation was random with respect to cell identity (i.e. GSC and GB were equally likely to inherit the BrdU-labeled DNA). In other studies that lacked definitive markers of cell identity, the cells that inherited the non-labeled strands (or labeled strands, depending on the methods of labeling) might have been assumed to be stem cells, and such results might have been interpreted to support the ISH. The randomness observed in our study indicates that GSCs do not use asymmetric strand segregation as a mechanism to protect the stem cell genome.

In recent years, the finding that some stem cell populations preferentially retain mother centrosomes during division (Wang et al., 2009; Yamashita et al., 2007) raised the possibility that this could provide a mechanism for the retention of template DNA strands (Tajbakhsh and Gonzalez, 2009). However, our present study clearly demonstrates that this is not necessarily the case. That is, in male GSCs that consistently asymmetrically segregate the mother centrosome, the chromosome strands are randomly segregated. It remains possible that centrosomes are asymmetrically segregated to segregate fate determinants such as protein and RNA (Fuentealba et al., 2008; Lambert and Nagy, 2002) or other factors such as damaged proteins (Rujano et al., 2006). It is also important to note that *Drosophila* female GSCs have been reported to non-randomly segregate DNA strands (Karpowicz et al., 2009), even though centrosomes are not stereotypically segregated in these cells (Stevens et al., 2007). Thus, it remains possible that chromosome strands are asymmetrically segregated in some cells, but stem cells that asymmetrically segregate centrosomes do not necessarily asymmetrically segregate chromosome strands.

Materials and Methods

Fly husbandry, strains and BrdU feeding

All fly stocks were raised on the Bloomington Standard Media at 25°C unless otherwise noted. Young adult Ubi-Pavarotti–GFP (Minestrini et al., 2003) flies were used. For BrdU labeling, day-0 adult Ubi-Pavarotti–GFP flies were fed BrdU-containing food (1 mg/ml final concentration, apple juice and 0.7% agar). To facilitate feeding upon transfer to BrdU-containing food, we first starved flies in vials with water and 0.7% agar for ~12 hours. The BrdU-fed flies were either dissected or transferred to normal food for chase experiments.

To accurately interpret the data, we calculated the approximate time that BrdU was retained in the body of the flies after BrdU administration was discontinued, because retained BrdU might be incorporated into the newly replicating DNA strands during the chase period and complicate interpretation of the results. When flies were fed with BrdU-containing food for ~12 hours and then administered normal food (without BrdU) for 2 or 4 hours, the percentage of BrdU-positive GSCs did not increase during the chase period (48.7±9.7% at 12 hours, 48.9±2.2% at 14 hours, and 51.7±5.9% at 16 hours), demonstrating that BrdU is not retained in the body for more than 2 hours at such high levels that it could be incorporated into replicating DNA.

Immunofluorescent 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), treated with DNaseI in 1× DNaseI buffer (Invitrogen), incubated with anti-BrdU antibody for 2 hours, and incubated with primary antibodies overnight at 4°C. Samples were then washed with PBST (20 minutes, three times), incubated overnight at 4°C with Alexa-Fluor-546 and -647 conjugated secondary antibodies (1:200; Molecular Probes), and washed again with PBST (20 minutes, three times). Samples were then mounted in VECTASHIELD (H-1200, Vector Laboratory) and imaged using a Leica SP5 confocal microscope. The following primary antibodies were used: mouse anti-fasciclin III (1:20; developed by C. Goodman and obtained from the Developmental Studies Hybridoma Bank), rabbit anti-threonine 3-phosphorylated histone H3 (1:200; Upstate), goat anti-Vasa (1:100; dC-13, Santa Cruz Biotechnology), rabbit anti-Vasa (1:100; d-260, Santa Cruz Biotechnology), and mouse anti-BrdU (1: 200; BU-33, Sigma).

Simulation based on a random segregation model

Although *Drosophila melanogaster* diploid cells have eight chromosomes, the simulation of a random segregation model on male GSC division was performed with six BrdU-detectable chromosomes because the fourth chromosome pair is negligible due to their small size. To simulate BrdU detainment in a GSC at cell cycle number N during the chase period, we used $P_{0,N}$ to represent the probability of a GSC containing zero BrdU-positive chromosomes, $P_{1,N}$ to represent the probability of a GSC containing one BrdU-positive chromosome, and so on, up to $P_{6,N}$ (the probability of GSC containing all six BrdU-positive chromosomes). After one division at cell cycle number N+1, the probability of a GSC containing k BrdU-positive chromosomes can be denoted as $P_{k,N+1}$, which can be calculated on the basis of the assumption that each individual chromosome segregates independently from one another:

$$p_{k,N+1}=\sum_{m=0}^{6}\frac{1}{2^m}\binom{m}{k}p_{m,N} \quad (1)$$

where

$$\binom{m}{k}=\frac{m!}{k!(m-k)!} \quad (2)$$

is the binomial coefficient. Therefore, the probability of GSC/GB BrdU segregation at cell cycle number N+1 can be calculated as follows:

$$P_{-/-,N+1}=p_{0,N}\ , \quad (3)$$

$$P_{-/+,N+1}=\sum_{m=1}^{6}\frac{1}{2^m}\binom{m}{0}p_{m,N}\ , \quad (4)$$

$$P_{+/-,N+1}=\sum_{m=1}^{6}\frac{1}{2^m}\binom{m}{m}p_{m,N}\ , \quad (5)$$

$$P_{+/+,N+1}=\sum_{m=1}^{6}\sum_{k=1}^{m-1}\frac{1}{2^m}\binom{m}{k}p_{m,N}\ , \quad (6)$$

because

$$\binom{m}{0}=\binom{m}{m}=1\ , \quad P_{-/+,N+1} \quad (7)$$

equals

$$P_{+/-,N+1}\ . \quad (8)$$

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