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The germline stem cells of *Drosophila melanogaster* partition DNA non-randomly

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Abstract

The Immortal Strand Hypothesis proposes that asymmetrically dividing stem cells cosegregate chromatids to retain ancestral DNA templates. Using both pulse-chase and label retention assays, we show that non-random partitioning of DNA occurs in germline stem cells (GSCs) in the *Drosophila* ovary as these divide asymmetrically to generate a new GSC and a differentiating cystoblast. This process is disrupted when GSCs are forced to differentiate through the overexpression of Bag of Marbles, a factor that impels the terminal differentiation of cystoblasts. When Decapentaplegic, a ligand which maintains the undifferentiated state of GSCs, is expressed ectopically the non-random partitioning of DNA is similarly disrupted. Our data suggest asymmetric chromatid segregation is coupled to mechanisms specifying cellular differentiation via asymmetric stem cell division.

Keywords: Stem cells; Chromosomes; Asymmetric division

Introduction

During mitosis, dividing cells segregate their replicated chromatids into each daughter to ensure the inheritance of the complete genome. This repeated replication of DNA presents a problem to a long-term dividing cell such as a stem cell (SC). If segregation is random, and DNA is copied from a previous copy, replication errors will accumulate in frequently dividing SCs and their progeny. The Immortal Strand Hypothesis (ISH) (Cairns, 1975) proposes that DNA is segregated non-randomly between recipient daughter cells, as a means through which SCs might lower their mutation load (Cairns, 2002). According to the ISH, asymmetrically dividing SCs cosegregate chromatids in order to retain ancestral DNA templates in the SC daughter (Fig. 1A). Given that DNA replication is semi-conservative, such chromosomes are distinguished because they contain one ancestral strand associated with a newer strand from the preceding round of DNA synthesis. This asymmetry in DNA molecule inheritance between daughter cells might also segregate differences in chromatin architecture to retain sequence fidelity and enzyme accessibility (Jablonka and Jablonka, 1982a, b)

Abbreviations: Bam, Bag of marbles; BrdU, 5-bromo-2-deoxyuridine; CldU, 5-chloro-2-deoxyuridine; Dpp, decapentaplegic; GSC, germline stem cell; HTS, Hu Li Tai Shao; IdU, 5-iodo-2-deoxyuridine; ISH, immortal strand hypothesis; PGC, primordial germ cell; SC, stem cell.

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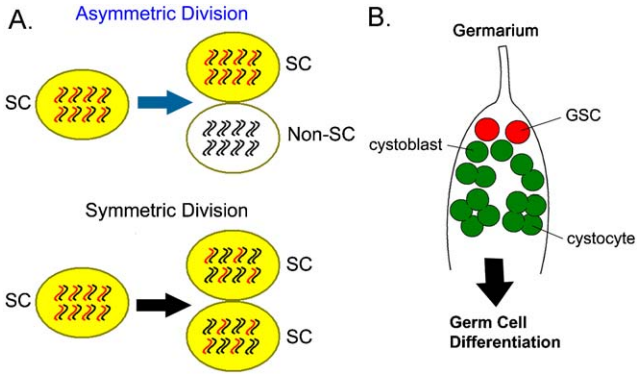


Fig. 1. The immortal strand hypothesis: (A) When SCs divide asymmetrically (blue), producing a daughter SC and a daughter non-SC, chromatids containing ancestral DNA templates (indicated in red) are segregated to SCs. DNA is replicated semi-conservatively, thus chromosomes contain newly synthesized strands (indicated in black) associated with ancestral template strands. When SCs divide symmetrically (black), chromosome segregation is random. (B) Schematic of the germarium, the region in which GSCs (red), cystoblasts (green single cells) and cystocytes (green clusters) reside. Note that the GSCs are the germ cells that occupy positions adjacent to the tip of the germarium. More differentiated cystocytes are further down from this area, forming cysts with 16 nuclei that mature into follicles.

for genes conferring pluripotency to SCs, and might allow non-SCs to adopt a novel chromatin architecture. Hence, the ISH also provides an attractive single-factor explanation for an epigenetic genome maintenance of the self-renewing SC and the concomitant differentiation of its non-SC offspring.

There is some data to suggest the separation of older and newer chromosomes following DNA replication in vitro (Karpowicz et al., 2005; Lark et al., 1966; Merok et al., 2002) as well as in vivo (Potten et al., 1978, 2002; Smith, 2005; Shinin et al., 2006; Lark, 1967) and that it is coupled to the differentiation of the non-stem cell counterparts (Conboy et al., 2007). Though these studies are notable, cells which demonstrated asymmetric DNA segregation were not identified unequivocally as asymmetrically dividing SCs. As well, there are a significant number of studies that have failed to support the ISH both in SCs and other cell types as well (Neff and Burke, 1991; Kuroki and Murakami, 1989; Ito and McGhee, 1987; Kiel et al., 2007; Waghmare et al., 2008; Sotiropoulou et al., 2008). Thus, the ISH remains an active and controversial subject of research (Tajbakhsh, 2008).

The ovary of the fruit fly, *Drosophila melanogaster*, contains germaria with a germline stem cell (GSC) population that can be identified unambiguously (Ohlstein et al., 2004). Each germarium is known to possess either 2 or 3 SCs (Fig. 1B), that divide asymmetrically to give rise to daughter SCs and cystoblasts. The cystoblast progeny of GSCs undergo a further four divisions to produce a cyst containing 16 nuclei, which matures into a follicle, and which develops into a single egg (King, 1970). This asymmetric division of GSCs to produce a GSC and cystoblast daughter continues throughout the lifetime of the female fly. Here we demonstrate that asymmetric segregation of DNA occurs in the dividing GSCs. We show that this process ceases when differentiation is molecularly perturbed and that, unlike GSCs, the differentiated progeny of GSCs segregate DNA randomly.

Materials and methods

Fly stocks and dissection

Wild-type, w^{1118}, *c*587-*Gal*4; *UAS-Dpp*, *w*; *P*[*hsp*70-*bam*]11*d* and *P*[*hsp*70-*bam*]18*d* stocks were maintained at 25 °C. For retention experiments, BrdU stock (25 mg/ml in 40% EtOH) was applied to medium at a final concentration of 0.2 mg/ml. For pulse-chase experiments, female prepupae were selected and maintained at 25 °C on apple plates. Heat shock was performed one day prior to injections as described (Ohlstein and McKearin, 1997). Pupae were fixed to slides using double-sided scotch tape and injected at 3 days pupation with 1.0 mM BrdU (Sigma) dissolved in Ringer's buffer (pH 6.9), or with 1.0 mM BrdU (Sigma) together with 100 mM BrdU thymidine (Sigma) dissolved in Ringer's buffer (pH 6.9). Injections were done using 25° ground capillary needles directly into the abdomen of the pupa. Subsets of BrdU-injected pupae were injected 24 h following BrdU infusion, with 100 mM BrdU thymidine (Sigma) dissolved in Ringer's (pH 6.9). Pupae were maintained at 25 °C, ovaries were dissected in 10 mM phosphate-buffered saline (PBS) and fixed 12 min at room temperature with 5% formaldehyde diluted in PBS (Roche). Following fixation, ovaries were washed three times with PBS + 1.0% Triton X-100 (Sigma) and triturated using an 1c.c. syringe and 30G1/2 tip (Becton-Dickenson) to dissociate ovaries.

Immunostaining

The following antibodies were used: (1) rat monoclonal anti-BrdU Bu1/75 (Abcam, 1:500), (2) mouse monoclonal anti-pan-histone (Chemicon, 1:500), (3) rabbit polyclonal anti-VASA (courtesy of Paul Lasko, 1:2000), (4) mouse monoclonal anti-HTS 1B1 (courtesy of Howard Lipshitz, 1:1). Secondary 568 or 633 nm cross-adsorbed Alexa Fluor antibodies (Molecular Probes, 1:300) were used excepting BrdU secondary stain which was amplified using biotin-conjugated antibodies (Jackson, 1:250) followed by streptavidin-DTAF

(Jackson, 1:300). Because the anti-HTS antibody is used at such high concentration, this was applied in only a subset of analyses. Samples were washed four times with PBS + 1.0% Triton X-100 and blocked with 5.0% normal goat serum (Sigma) or 5.0% normal donkey serum + 0.1% bovine serum albumin (Sigma) prior to addition of each antibody. Nuclei were counterstained with 5.0 μM Sytox Orange (Molecular Probes). BrdU staining specificity was confirmed by staining negative control samples not exposed to BrdU. In such control germaria, unexposed to BrdU, background fluorescence was approximately tenfold lower than fluorescence emitted by germaria exposed to BrdU for 24 h.

Microscopy and analysis

Samples were mounted on glass slides using Gold-mount (Molecular Probes). Germaria were visualized and photographed under confocal microscopy, using a Plan-Apochromat 100 × /1.40 oil-immersion lens objective on an LSM510 (Carl Zeiss). Confocal sections of <1 μm thickness were taken every ~2 μm spanning the entire germarium. Detection settings were kept constant when comparing 24 h BrdU versus 24 h BrdU + thymidine injection controls, otherwise these were adjusted as needed. Quantification of fluorescence in each raw confocal section was done using ImageJ software. Confocal sections were examined to locate the largest section of each cell's nucleus, and these were outlined to determine fluorescence emitted by that cell. In control experiments, fluorescence emitted in all confocal sections for one nucleus were determined and averaged to compare with fluorescence emitted in the largest and central nuclear section. Graphs shown depict means and standard error of the mean for the average nuclear BrdU signal normalized to GSCs, calculated for each individual germarium. Statistical analysis was carried out using Graphpad Prism 4.0. In most cases, comparison between normalized GSC and cyst nuclei quantifications was carried out by *t*-tests, with comparisons between multiple groups (Fig. 3G and H; and Supplementary Fig. 1C) carried out by ANOVA with Dunnett post-test as required. Comparison between quantifications done by VASA versus HTS staining was carried out using an unpaired *t*-test. For figures, photos were processed using Adobe Photoshop 6.0 software.

Results

Adult GSCS partition DNA strands asymmetrically

GSCs have been estimated to cycle once every 24 h based on the number of eggs that are produced daily by one germarium (Lin and Spradling, 1993). Each GSC is itself the founder of other germ cells that reside in the germarium: one cystoblast, one bi-nucleated cyst, and three additional cysts of 4, 8 and 16 nuclei (King, 1970). Importantly, GSCs divide only asymmetrically with only the cell next to the cap cells of the germarium persisting as a GSC. The halogenated thymidine analog, 5-bromo-2-deoxyuridine (BrdU), was applied to label DNA strands in these germ cells. Animals were dissected immediately following eclosion (the developmental timepoint when the fly emerges from its pupal case), to avoid the effects of nutrition on GSC and germ cell division rates.

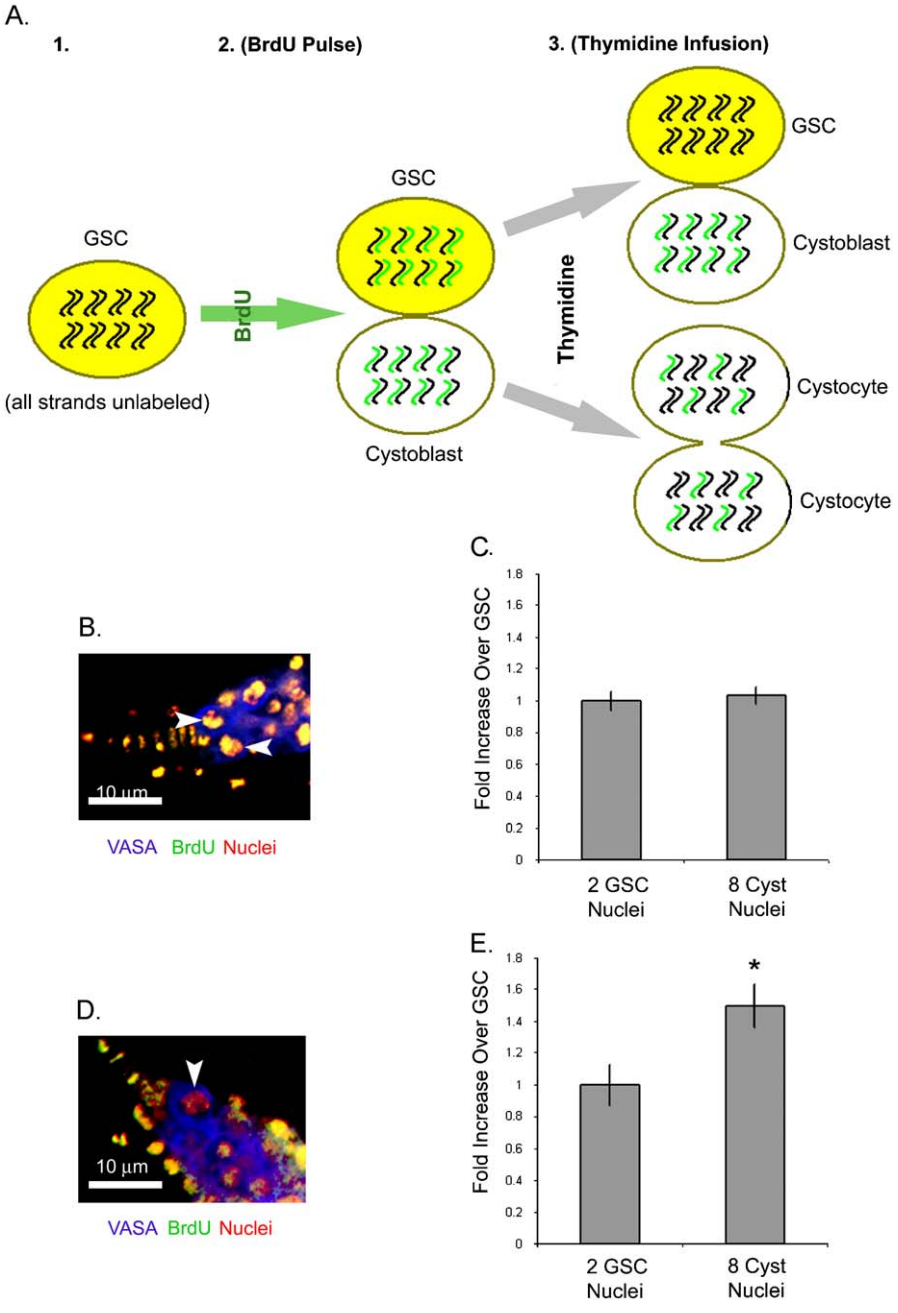
If ancestral DNA strands are unlabeled, the ISH predicts that BrdU will be transiently incorporated and subsequently cleared in asymmetrically dividing GSCs as opposed to their cystoblast and cyst cell progeny (Fig. 2A). Following one cell division in BrdU, both daughter cells would contain 4 pairs of chromosomes, each chromosome half-unlabeled with the original unlabeled DNA template strand and half-labeled with the new BrdU(+) synthesized strand (Fig. 2A). All germ cells would be thus evenly labeled for BrdU. However after a second division, this time during a thymidine chase, BrdU signal would be lowered (Fig. 2A). Although non-GSCs would also lower their BrdU signal during their symmetric expansionary divisions, these would still be quantifiably more BrdU positive if cosegregation of BrdU-containing templates occurred in GSCs but not the adjacent non-GSCs.

Of course it is possible that the uneven distribution of BrdU-labeled strands might result from chance which can be estimated straightforwardly. During mitosis, replicated sister chromatids are segregated to opposite poles, but for each pair of chromosomes, one of each of the two homologs is segregated to the same poles. There are four pairs of homologs in the fly cell. The frequency of labeled chromatids of the two different homologs being segregated to the same pole is 50% for each segregation event. Therefore the probability of chance segregation of all labeled chromatids into one pole is 6.25% ($1/2^n$ for $n = 4$ pairs of homologs) and it is more likely that random segregation would label GSCs and non-GSC germ cells equally for BrdU. Thus, it is predicted that GSCs would lose BrdU more rapidly than cystoblasts or cyst nuclei only one division after uptake according to the ISH.

It has been shown that germaria containing 2 GSCs, contain an average of 10.7 cysts (Pan et al., 2007). Cysts are spawned from the one-celled cystoblast and expand to reach 2-, 4-, 8- and 16-nucleated cysts. If the ratio of GSC/cysts is 1:5, this alone implies that the division rate of GSCs and their progeny is similar. It was necessary to confirm that GSCs and their progeny divided at equal rates. BrdU was injected into 3-day pupae for a 4-h pulse, and the proportion of labeled GSC versus all other germ cells were determined to be 22.5 ± 6.8% and

19.3 ± 4.5%, respectively ($n = 20$ germaria sampled). The equivalence between germ cells after 4 h exposure ($p > 0.05$) suggested these cells divide at a similar rate, as any discrepancies in cell cycle between GSCs and non-GSCs should be manifested as a difference in the proportion of labeled cells which had entered S-phase. We further confirmed this by staining germaria for phosphorylated histone 3, a marker of mitosis, and similarly found no significant differences between the proportion of GSCs and germ cells labeled (data not shown).

BrdU was again injected into pupae, and germaria were recovered 24 h afterwards. In females, all germline cells of the germarium, including GSCs, cystoblasts and all cyst nuclei were strongly positive for BrdU (Fig. 2B). This showed that BrdU persists for at least 24 h in the pupal body. If BrdU was cleared efficiently from the pupa, a shorter exposure of BrdU would reduce the number of labeled GSCs, which cycle about once daily. We quantified the fluorescence emitted by BrdU(+) nuclei in confocal sections through the centre of these nuclei, and found that within each germarium,



the signals emitted by GSCs, cystoblasts and cyst nuclei were equivalent at 24 h (Fig. 2C). These results were calculated by comparing BrdU signals emitted by 10 germ cells closest to the terminal filament in one individual germarium. Cells further down from these cells were not included in this analysis. The average signals from the 2 GSCs relative to those emitted by the next 8 germ cells (closest to the terminal filament) were obtained (see Supplementary Fig. 3A) and then ratios from each germarium were averaged together.

It was also noted that the somatic cells in the germarium, for instance the terminal filament nuclei, were labeled for BrdU at these timepoints. While previous studies have failed to observe an S-phase in these cells (Margolis and Spradling, 1995), these authors had exposed such cells to BrdU for only one hour. Because our assay exposes these cells to BrdU for 24 h, this suggests that somatic cells undergo slow turnover or endoreplication in adulthood.

We next examined the GSCs of the male testes, to see if these would be amenable to the same analysis. The cell divisions timing of male GSCs is not as easily inferred as that of females, which lay eggs and hence allow inferences of cell turnover in the female GSC founder cells to be drawn. Following a 24-h pulse of BrdU, only a subset (1–4 nuclei) of male GSCs out of the approximately 10 present were labeled, indicating that the cells did not divide once every 24 h (data not shown). It was not possible to test the ISH in the male GSCs using the strategy outlined above.

Female *Drosophila* pupae were injected with BrdU but, following a 24-h exposure, the same pupae were injected with thymidine at 100-fold higher concentration than the BrdU. Twenty-four hours following this thymidine infusion, germaria were dissected and BrdU was visualized (Fig. 2D). We again calculated the fluorescence emitted by cystoblasts and cystocyte nuclei relative to the average fluorescence emitted by 2 GSCs in that same germarium (see Supplementary Fig. 3C). Interestingly, GSCs now demonstrated significantly lower signal than non-GSCs (Fig. 2E) ($n = 35$ germaria sampled). Nearly all GSCs had lower fluorescence than their daughter cells (45/52 germaria) and, in 26/52 germaria there was a $>50\%$ lower fluorescence in the GSCs relative to their neighbouring daughter cells. This high incidence of signal asymmetry observed between SC and non-SCs is not a stochastic distribution.

Incomplete BrdU chase and differences in nuclear packaging offer alternative interpretations to the asymmetric segregation of DNA strands. In particular, the incomplete loss of BrdU signal in GSCs, subsequent to thymidine chase, did not seem to support complete ancestral DNA cosegregation as predicted by the ISH. A series of control experiments were undertaken to validate the fluorescence quantification outlined above (see Supplementary Figs. 1, 3 and 4). These controls showed that the BrdU signal measured approximately reflected the amount of BrdU present in the nuclei. The co-administration of BrdU and thymidine also confirmed that it was possible to chase the residual BrdU injected in the pupae. These controls also showed that the nuclear size and staining is not appreciably different between GSCs and the 8 closest non-GSCs, allowing comparisons to be drawn between these different cell types. Collectively, our data show that the BrdU signal is lowered in GSCs relative to non-GSCs one round of DNA replication after its initial incorporation. These findings suggest labeled DNA is partitioned non-randomly in GSCs and non-GSCs.

Fig. 2. Chromatids are segregated asymmetrically in adult GSCs: (A) Schematic showing the BrdU pulse-chase strategy. (1) The GSC (yellow) possesses four pairs of chromosomes (double strands). Initially, all strands of DNA are unlabeled for BrdU (black). (2) Following the first division, newly synthesized DNA strands were copied from unlabeled strands during DNA replication in the presence of BrdU (green). (3) Thymidine is now infused and outcompetes residual BrdU as the daughters complete a second round of DNA replication prior to the next cell division (new strands are marked in black). The GSC daughter (yellow) now shows BrdU loss relative to the non-GSC daughter (white) which retains highly BrdU-labeled DNA strands. Note that symmetric divisions shown at right (white cystoblast cells) result in a dilution but not outright depletion of BrdU label. The ISH predicts that if all four cells were compared after 2 cell divisions, the GSC (yellow) would emit reduced BrdU signal relative to the average in its non-GSC cell progeny (white). (B) Confocal section of a germarium dissected 24 h following BrdU injection (see Supplementary Fig. 1A for unmerged image). The merged image shows germ cells stained for VASA (blue), and labeled for BrdU (green). Arrows indicate GSCs, counterstained nuclei are in red. (C) All germ cells at 24 h following BrdU infusion emit equivalent fluorescence. Graph shows signal emitted by 8 closest germ cell progeny (''Cyst Nuclei'' includes cystoblast and cystocytes) as normalized to stem cell founders. There are no significant differences between GSCs and their progeny at this timepoint ($p > 0.05$). Quantifications were established from three separate experiments ($n = 18$ germaria sampled in total). (D) Confocal section of a germarium dissected 48 h following BrdU injection and 24 h following thymidine chase (see Supplementary Fig. 1B for unmerged image). The merged image shows a lower signal in GSCs (arrow) as compared to the 24-h timepoint in (B). VASA is shown in blue, BrdU in green, nuclear counterstain in red. (E) Following thymidine chase, GSCs emit significantly lower BrdU signal than cystoblasts or cystocytes. Graph shows increase in signal emitted by germ cell progeny as normalized to stem cell founders. Asterisks indicate that BrdU signal was found to be significantly lower in GSCs than their differentiated progeny at this timepoint ($t = 5.421$, $df = 34$, $p < 0.05$). Quantifications were established from three separate experiments, in which signal emitted by 8 non-GSCs is normalized to 2 GSCs for each germarium ($n = 35$ germaria sampled in total).

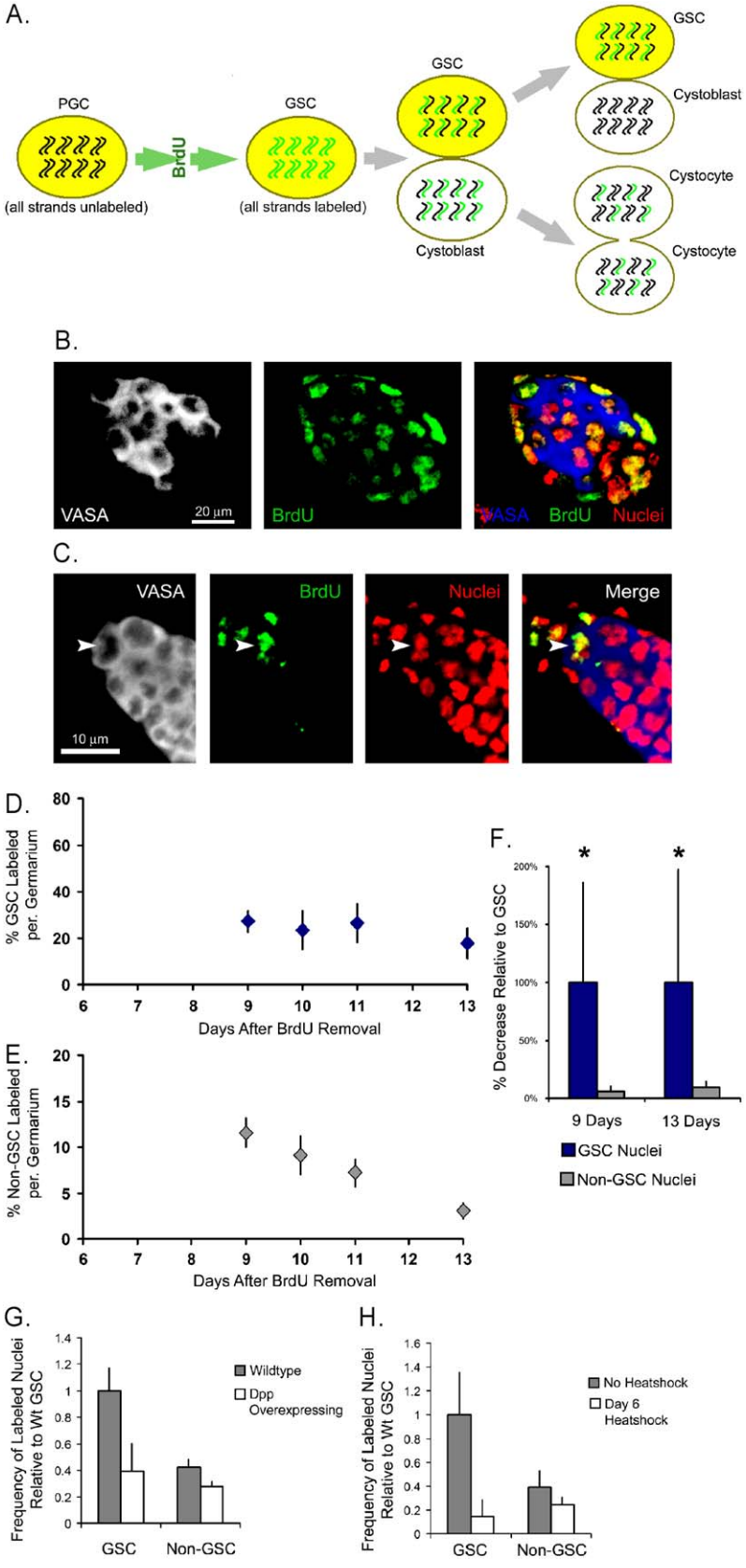
Adult GSCS retain BRDU following exposure at larval stages

An implication from the ISH is that the same DNA strands should be retained in the GSCs over long periods of time. If immortal strands were labeled, the ISH predicts they would be retained and BrdU signal would persist in GSCs as opposed to their progeny (Fig. 3A). The embryonic gonad contains about ~12 primordial germ cells (PGCs) which expand to a population of ~100 by the middle of the third instar (Gilboa and Lehmann, 2006). Not all PGCs will give rise to GSCs. If GSCs were specified between embryonic and the end of larval development from PGCs, expansionary symmetric divisions of PGCs in BrdU would label their DNA. This would result in detectable retention of BrdU-labeled DNA in the future adult GSCs.

Wild-type larvae were raised in BrdU medium up to the 2nd instar. Longer exposure resulted in high pupal mortality, presumably due to BrdU toxicity. If larvae were moved into BrdU-free medium at the 2nd instar, this resulted in approximately 50% lethality. At the 2nd instar, the population of PGCs is ~60 which represents roughly two population doublings (Gilboa and Lehmann, 2006). Larval ovaries examined at this stage (Fig. 3B) had incorporated BrdU in $53.6 \pm 9.4\%$ of their germ cells, and these ovaries contained 28.3 ± 8.1 germ cells ($n = 5$ ovaries sampled). This result suggested some heterogeneity in cell cycle exists in the larval PGCs, consistent with other published findings (Asaoka and Lin, 2004).

BrdU-exposed larvae were allowed to complete pupation and were collected at various timepoints 9–13 days after exposure to BrdU. Both female and male flies were collected in equal numbers, suggesting there is no sex-specific susceptibility to BrdU toxicity. We focused on examining only female GSCs rather than male GSCs, which were previously noted to divide non-synchronously with a doubling time of >24 h. Female GSC-labeled germaria were found at all timepoints between 9 and 13 days after BrdU exposure (Fig. 3C). The number of labeled GSCs was between 17 and 29% per germarium from day 9 up to day 13 following larval exposure (no significant differences at any timepoint, $F_{3,83} = 0.4118$) (Fig. 3D). In contrast to GSCs, labeling frequency of non-SC germ cells significantly declined over this period of time ($F_{3,83} = 3.956$) (Fig. 3E). The BrdU signal emitted by labeled GSCs was significantly higher than that of non-GSCs at the 9 day timepoint and the 13 day timepoint (Fig. 3F). We confirmed these observations using a second method (HTS staining) to identify GSCs, and found that at the 10 day timepoint $32.1 \pm 8.6\%$ of GSCs were labeled per germarium ($n = 14$ germaria sampled) similar to the previous results (compare to 10 day timepoint in Fig. 3D). A 2.7% daily loss of GSCs also has been observed in germaria (Ward et al., 2006) which might account for the slightly lower, albeit non-significant, number of

Fig. 3. Chromatids are cosegregated during asymmetric GSC divisions, following BrdU exposure during symmetric PGC divisions. (A) Schematic showing BrdU retention strategy. The PGC (yellow) possesses four pairs of chromosomes (double strands). All strands in the embryo are unlabeled (black). Following exposure to BrdU, at most all strands will become labeled (green) during multiple symmetric divisions. As the cell begins to divide asymmetrically in the absence of BrdU, GSCs retain labeled chromosomes more frequently than non-GSCs. The ISH predicts an initially labeled GSC (yellow) will retain BrdU signal which is successively diluted in non-GSCs. (B) Confocal section of 2nd instar ovary at the timepoint when larvae are transferred from BrdU-containing medium to fresh BrdU-free medium. All PGCs are marked with VASA (blue in the merged image), BrdU in green, and counterstained nuclei are in red. (C) Confocal section of an adult germarium 10 days following BrdU removal. VASA staining is shown in blue in the merged image, BrdU in green. The arrow marks the position of the GSC, nuclei are counterstained in red. Note the strong BrdU signal in the GSC even at this late timepoint. (D, E) Proportion of labeled GSCs (D) and non-GSCs (E) per germarium at 9 ($n = 55$ germaria), 10 ($n = 15$ germaria), 11 ($n = 15$ germaria) and 13 days ($n = 14$ germaria) following BrdU removal. There is a slight but non-significant decrease in the frequency of labeled GSCs (~10%) between day 9 and day 13. (F) Quantification of BrdU signal emitted from GSCs versus closest 10 non-GSCs in germaria. Graph shows the percentage decrease in signal emitted by germ cell progeny relative to stem cell founders. Asterisks indicate that BrdU signal is significantly higher in GSC nuclei at 9 days ($t = 46.37$, df = 6, $p < 0.05$) as well as at 13 days ($t = 27.98$, df = 4, $p < 0.05$). Quantifications were established from germaria containing BrdU-positive GSCs ($n = 7$ germaria sampled at 9 day timepoint, and $n = 5$ germaria sampled at 13 day timepoint). Error bars for GSCs are high because we include all BrdU(+) and BrdU(−) germ cells in these analyses, even though only 50% of PGCs are labeled at the 2nd larval instar. (G) Results of the BrdU retention experiment in the *c*587-*Gal*4; *UAS-Dpp* line. Proportion of BrdU(+) nuclei at GSC positions as compared to GSC-like nuclei in the Dpp-overexpressing germarium at the 9 day timepoint ($n = 14$ germaria). "GSC" refers to nuclei of cells at GSC position, and "non-GSC" refers to other GSC-like nuclei, although all are undifferentiated. Frequency of labeled germ cells was normalized relative to that of wild-type GSCs at day 9. There are no differences in BrdU retention between wild-type non-GSCs and any germ cells in the Dpp-overexpressing germarium ($p > 0.05$). (H) Results of the BrdU retention experiment in the *P*[*hsp*70-*bam*]18*d* line. BrdU(+) germ cell frequency with and without heat shock. "GSC" refers to nuclei of cells closest to GSC position, and "non-GSC" refers to other germ cell nuclei, although all are differentiating. Frequencies of labeled cells were normalized relative to that of non-heat-shocked control GSCs. Upon heat shock, administered at day 6 following BrdU removal, BrdU(+) nuclei at GSC position decrease to the same levels as all non-GSCs ($p > 0.05$).


A.
PGC
(all strands unlabeled)
BrdU
GSC
(all strands labeled)
GSC
Cystoblast
GSC
Cystoblast
Cystocyte
Cystocyte
B.
VASA
20 μm
BrdU
VASA BrdU Nuclei
C.
VASA
BrdU
Nuclei
Merge
10 μm
D.
% GSC Labeled per. Germarium
Days After BrdU Removal
E.
% Non-GSC Labeled per. Germarium
Days After BrdU Removal
F.
% Decrease Relative to GSC
9 Days
13 Days
GSC Nuclei
Non-GSC Nuclei
G.
Frequency of Labeled Nuclei Relative to Wt GSC
Wildtype
Dpp Overexpressing
GSC
Non-GSC
H.
Frequency of Labeled Nuclei Relative to Wt GSC
No Heatshock
Day 6 Heatshock
GSC
Non-GSC

marked GSCs at later stages (compare 9–13 day timepoint in Fig. 3D).

Next, the thymidine analog CldU (5-chloro-2-deoxyuridine) was applied up to the 2nd instar. Larvae were then moved into CldU-free media, and at day 9 an injection of IdU (5-iodo-2-deoxyuridine) was carried out. Female germaria were recovered 24 h after injection. Not all cells were labeled for both analogs, indicating the specificity of the antibodies used to detect them. The ability to distinguish between these analogs revealed that 60% of CldU-retaining GSCs ($17.9 \pm 4.6\%$) colabeled with IdU. This showed that analog-retaining GSCs are not quiescent, but continue to enter S-phase and divide. Moreover, the BrdU injections carried out above further show that the presence of BrdU does not cause the senescence of GSCs and other germ cells.

BrdU or CldU label is retained in GSCs following only two symmetric divisions of PGCs in the presence of analog, and potentially >9 asymmetric divisions of GSCs in the absence of analog. Given that BrdU uptake is incomplete at the stage when 2nd instar larvae are removed from BrdU-containing media, it is surprising that labeled ancestral strands are retained in these dividing cells.

BrdU retention is not observed when GSCs divide asymmetrically

GSCs switch to an asymmetric mode of division during early pupation (Wieschaus and Szabad, 1979) as the GSC niche is established during this time (Zhu and Xie, 2003). Since the last population doubling of PGCs in BrdU during the 3rd instar to the early pupal stage was not assayed in the BrdU retention experiment, we placed larvae reared in normal medium into BrdU-containing medium at the 2nd instar. Flies were collected as above, and it was again noted that approximately 50% of the animals died. Germaria from the surviving flies were examined at 9 days following exposure. Unlike our prior results, only $5.0 \pm 5.0\%$ of GSCs retained label in this assay ($n = 10$ germaria sampled). Either GSCs did not divide symmetrically to take up BrdU at these late larval timepoints, or one symmetric division in the presence of BrdU was insufficient to result in the retention of BrdU at later timepoints.

Asymmetric DNA partitioning is coupled to asymmetric GSC divisions

GSCs are maintained in an undifferentiated state by the BMP family member *Decapentaplegic* (Dpp) (Xie and Spradling, 1998) released by cap cells. Dpp signalling causes the phosphorylation of *Mothers against Dpp* in GSCs which, in turn, blocks the transcription of genes involved in germ cell differentiation. The over-expression of Dpp is thus thought to maintain germline cells in an undifferentiated SC-like state (Kai et al., 2005).

We sought to test BrdU retention in lines which overexpress Dpp. The germaria of these flies develop into large cysts of undifferentiated and continuously proliferating germline cells. *c*587-*Gal*4 X *UAS-Dpp* transgenic stocks were raised in BrdU-containing medium to the middle of the 2nd instar, transferred to BrdU-free medium, and recovered 9 days following BrdU exposure. It was noted that no significant differences existed between the percentage of BrdU(+) cells at the GSC position and GSC-like cells elsewhere in the Dpp-overexpressing germarium (Fig. 3G) suggesting that in conditions of high ectopic Dpp, strand segregation is random ($n = 14$ germaria sampled). Moreover, the percentages of labeled GSC-like cells in the *c*587-*Gal*4 X *UAS-Dpp* germaria were the same as non-GSCs in wild-type germaria (data not shown), suggesting that the average probability of any symmetrically dividing germ cell to retain BrdU following exposure is 8–13% at 9 days.

Next, BrdU/thymidine pulse-chase injections were undertaken on pupae overexpressing Dpp. Twenty-four hours following BrdU injection all Dpp-overexpressing germline cells were equivalently labeled ($n = 4$ germaria sampled; data not shown). Interestingly, 24 h following thymidine infusion the germaria of these mutants showed the similar BrdU signal among the GSCs versus the 8 closest germline cells (Supplementary Fig. 2A). Quantification confirmed that the equivalence present among germ cells at 24 h BrdU pulse, persisted following 24 h thymidine chase (Supplementary Fig. 2B) ($n = 9$ germaria sampled). These data are consistent with the results obtained using BrdU retention assays in the Dpp-overexpressing lines, and both suggest that asymmetry in chromatid segregation is dependant on the presence of the localized, cell-extrinsic Dpp signalling pathway.

Asymmetric DNA partitioning occurs in GSCs but not other germline cells

Expression of *Bag of Marbles* (Bam) protein is suppressed by Dpp signalling in GSCs and Bam is required for differentiation in cystoblast daughters exiting the stem cell niche (Chen and McKearin, 2003; Ohlstein et al., 2000). Thus the overexpression of Bam by heat shock has been shown to empty the germarium and GSC niche by forcing all germline cells to differentiate via symmetric divisions (Ohlstein and McKearin, 1997). We predicted that differentiating

cystoblasts and cystocytes would not segregate chromatids asymmetrically as opposed to GSCs.

Template strand segregation was tested using the BrdU retention assay in flies containing heat-inducible Bam transgenes. *P*[*hsp*70-*bam*]18*d* larvae were raised on BrdU as above, and heat shocked two days prior to examination at day 9 following BrdU removal Fig. 3(H). While non-heat shocked *P*[*hsp*70-*bam*]18*d* demonstrated identical frequencies of BrdU(+) GSCs versus non-GSCs as the wild-type strain (data not shown), heat shock reduced the frequency of labeled cells at the GSC position to the same level as labeled germ cells anywhere in the germarium Fig. 3(H).

This result was further examined using the BrdU pulse-chase assay. Pupae were heat shocked 24 h prior to BrdU injection, and were injected in a state where GSCs and their progeny were differentiating under the control of ectopic Bam expression. Twenty-four hours post BrdU injection, all germ cells from *P*[*hsp*70-*bam*]11*d* flies ($n = 5$ germaria sampled) and *P*[*hsp*70-*bam*]18*d* ($n = 5$ germaria sampled) flies were equally labeled for BrdU (data not shown). However following 24 h thymidine chase, again no asymmetry in BrdU signal was observed in the *P*[*hsp*70-*bam*]11*d* mutant germaria ($n = 7$ germaria sampled) (Supplementary Fig. 2C and D) nor in the *P*[*hsp*70-*bam*]18*d* strain germaria ($n = 6$ germaria sampled) (Supplementary Fig. 2E). Thus, taken together, our results indicate that asymmetric chromatid partitioning does not occur in differentiating germ cells, but only in asymmetrically dividing GSCs. This suggests the cosegregation of labeled DNA strands may be a unique characteristic of GSCs in this system.

Discussion

We find evidence to support the non-random segregation of ancestral DNA in the dividing GSCs of *Drosophila melanogaster*. Two separate lines of evidence, a pulse-chase strategy carried out during adulthood and a retention strategy carried out during larval development to adulthood, were unable to falsify the ISH. However, we note that the results obtained in these experiments are not wholly consistent with the strong predictions established from the ISH.

In the pulse-chase experiments, GSCs, cystoblasts and cystocytes take up equivalent amounts of BrdU during 24 h. Following a 24-h thymidine chase, BrdU is preferentially lost in GSCs but not other germ cells. If DNA is segregated randomly, differences between GSCs and their daughter cells should not be observed among the germ cells of the *Drosophila* germarium. However, this difference is predicted by the ISH because GSCs divide asymmetrically in the pupa, therefore incorporating and shedding BrdU during successive divisions. Conversely, the BrdU retention strategy demonstrates a striking retention of labeled DNA strands in the GSC lineage when BrdU is administered at early larval stages. This is consistent with the ISH because expansionary PGC divisions in BrdU cause nascent GSCs to select BrdU-labeled strands as ancestral strands, to be retained during later asymmetric divisions when BrdU is no longer present.

Strictly speaking, the ISH presupposes that all ancestral strand-bearing chromosomes are cosegregated. However, given that our results do not show a complete segregation of BrdU signal, it remains possible that only a subset of the 4 pairs of chromosomes is selectively retained in GSCs (Fig. 4). Such a scenario is reminiscent of selective strand retention observed in yeast, where particular strands have been shown to confer distinct properties between daughter cells (Klar, 1990; Dalgaard and Klar, 2001). Thus our results, while inconsistent with random DNA strand segregation, are unable to resolve between the ISH and other proposed hypotheses of selective non-random chromatid segregation (Armakolas and Klar, 2006; Lansdorp, 2007).

The late larval ovary is thought to develop a niche similar to that of the adult (Gilboa and Lehmann, 2004) which implies that GSCs divide asymmetrically at later

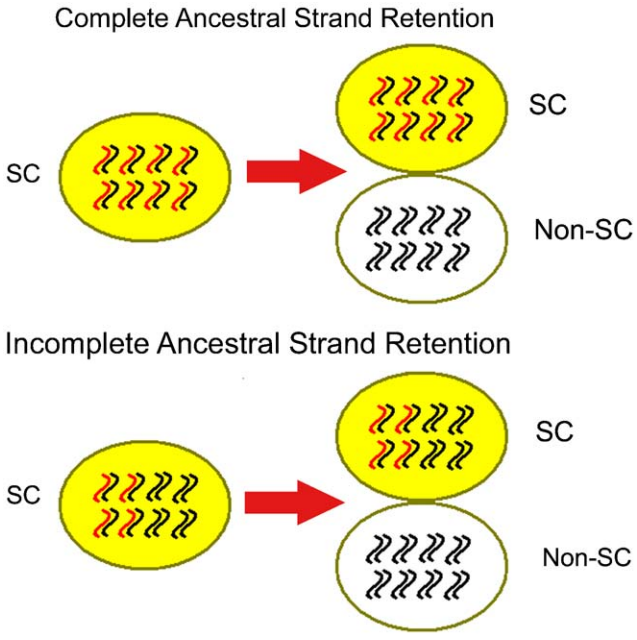


Fig. 4. Two models of chromatid cosegregation. (A) Complete chromatid cosegregation as accounted for by the ISH. Schematic shows retention of one ancestral strand of every chromosome (red) being retained in the SC daughter but not the non-SC daughter. (B) Incomplete ancestral strand retention as an alternative to the ISH. In this model, not all ancestral strands are retained, only those particular to that cell type. Shown is a hypothetical case where four ancestral strands (red) are retained in the SC – a possibility which cannot be excluded in the present study.

larval stages. Our examination of germaria when larvae are placed on BrdU during the 2nd to 3rd instar, suggests that GSCs do not retain BrdU when exposed at this time. These data suggest wild-type asymmetrically-dividing GSCs retain BrdU preferentially, if and only if it is administered during their symmetric expansionary divisions.

The incorporation of BrdU into DNA has been shown to affect the physical nature of DNA strands resulting in possible alterations in protein-DNA binding (David et al., 1974), increases in the radiosensitivity of DNA (Dewey et al., 1966) and increases in sister chromatid exchange (Taguchi and Shiraishi, 1989). In all germaria examined in our studies, animals were viable and germline cells and follicles (of non-mutants) appeared normal. However, we note the toxicity of this analog when larvae are raised in it for extended periods. Increased DNA strand exchange or increased DNA repair cannot explain our results as these would only reduce rather then magnify the differences we report between GSCs and their progeny. If additional exchanges between BrdU-labeled and unlabeled DNA occur, these offer another reason why the loss of BrdU signal is incomplete in GSCs in the BrdU pulse-chase experiments and why not all GSCs are found labeled in the retention experiments.

Female GSCs are maintained in an undifferentiated state by proximity to Dpp signalling sources (Xie and Spradling, 1998; Chen and McKearin, 2003). Yet there are several lines of evidence to think that the divisions of GSCs are intrinsically asymmetric as well. Localization of the DE-cadherin protein (Song et al., 2002) between GSCs and cap cells promotes GSC contact with regions high in Dpp and this protein is then unevenly partitioned between GSC daughters. Male GSCs segregate new and old centrosomes (Yamashita et al., 2007) to the gonialblast and GSC, respectively, and *Drosophila* orthologues of adenomatous polyposis coli tumour suppressor protein tether microtubules to the cadherin complex that maintains the fixed spindle orientation of GSCs (Yamashita et al., 2003). This strongly suggests the divisions of these cells are inherently asymmetric, although their differentiated progeny have been shown to replace GSCs under certain conditions (Brawley and Matunis, 2004). We did not test the ISH in the male germline as the cell cycle of male GSCs was not as well established as that of female GSCs. While old centrosome retention does not seem to occur in female GSCs (Stevens et al., 2007), these do segregate spectrosome (de Cuevas and Spradling, 1998) organelles unevenly between GSC and cystoblast daughter cells; and the plane of GSC division is, in part, directed by asymmetric segregation of the spectrosome and its associated protein, HTS (Deng and Lin, 1997). Thus female germline cell divisions do possess some aspects of cellular asymmetry, and the non-random segregation of DNA is another example of asymmetric partitioning of molecules in these cells.

It is important to note that the partitioning of intracellular components does not fully commit a non-SC daughter to differentiate, given reports that early SC progeny are competent to revert into SCs by dedifferentiation mechanisms (Brawley and Matunis, 2004; Kai and Spradling, 2004). Such findings show that asymmetric partitioning of the spectrosome and its associated proteins, HTS and Bam do not invariably determine cystoblast fate (de Cuevas and Spradling, 1998; Deng and Lin, 1997). It is possible that in contexts where SC-progeny dedifferentiation is observed (Brawley and Matunis, 2004), intrinsic cell division asymmetry is restored and that intrinsic partitioning of components is reset. It is also possible that proximity to sources of ligands, such as Dpp, polarizes GSCs in order to carry out the asymmetric segregation of organelles and molecules. Hence, ancestral DNA template retention might not irreversibly commit GSC fate, but the reselection of ancestral strands may occur during the reversion of germ cell progeny into GSCs.

Studies on mouse cells (Karpowicz et al., 2005; Lark et al., 1966; Merok et al., 2002; Potten et al., 1978, 2002; Smith, 2005; Shinin et al., 2006; Conboy et al., 2007), molluscs (Tomasovic and Mix, 1974), fungi (Rosenberger and Kessel, 1968) and plants (Lark, 1967) show that chromatid cosegregation may occur in a wide variety of organisms. Our findings that insect GSCs demonstrate non-random chromatid segregation *in vivo*, adds to this diversity. *Caenorhabditis elegans* has been shown to not retain ancestral DNA strands (Ito and McGhee, 1987; Crittenden et al., 2006) and recent findings suggest that mouse blood SCs do not retain BrdU (Kiel et al., 2007) nor do mouse epidermal SCs (Waghmare et al., 2008; Sotiropoulou et al., 2008). This suggests that chromatid cosegregation is not a universal SC characteristic, and that genomic DNA is maintained by other means in these cells. It is notable, however, that relative label retention in SCs versus non-SCs was observed in two of these studies (Crittenden et al., 2006; Kiel et al., 2007) which means it is possible that non-random DNA segregation has been overlooked. Furthermore, mammary SCs retain ancestral strands under varying physiological conditions which hints at the importance of this process (Booth and Smith, 2006; Booth et al., 2008).

It has been proposed that during DNA replication, leading and lagging strands might be distinguished to enable their non-random segregation (Lew et al., 2008), a phenomenon that has been recently observed in *Escherichia coli* (White et al., 2008). In mouse cells, differentiation programmes have been correlated with non-random segregation of sister homologues (Armakolas and Klar, 2006). Such findings are similar to those observed in yeast (Dalgaard and Klar, 2001), in

the sense that both occur during phases of cellular differentiation mediated by cell division asymmetry. Similarly, we find that when differentiation via self-renewing asymmetric division does not occur, non-random chromatid segregation is abolished. In line with these reports, we suggest that asymmetric DNA segregation may be a mechanism to promote or repress genes expressed by particular chromosomes, the presence of which is involved in the generation of discrete cell types.

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Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2009.03.001.

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