LETTER

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# Chromosome-specific nonrandom sister chromatid segregation during stem-cell division

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**Adult stem cells undergo asymmetric cell division to self-renew and give rise to differentiated cells that comprise mature tissue[1]. Sister chromatids may be distinguished and segregated nonrandomly in asymmetrically dividing stem cells[2], although the underlying mechanism and the purpose it may serve remain elusive. Here we develop the CO-FISH (chromosome orientation fluorescence *in situ* hybridization) technique[3] with single-chromosome resolution and show that sister chromatids of X and Y chromosomes, but not autosomes, are segregated nonrandomly during asymmetric divisions of *Drosophila* male germline stem cells. This provides the first direct evidence, to our knowledge, that two sister chromatids containing identical genetic information can be distinguished and segregated nonrandomly during asymmetric stem-cell divisions. We further show that the centrosome, SUN–KASH nuclear envelope proteins and *Dnmt2* (also known as *Mt2*) are required for nonrandom sister chromatid segregation. Our data indicate that the information on X and Y chromosomes that enables nonrandom segregation is primed during gametogenesis in the parents. Moreover, we show that sister chromatid segregation is randomized in germline stem cell overproliferation and dedifferentiated germline stem cells. We propose that nonrandom sister chromatid segregation may serve to transmit distinct information carried on two sister chromatids to the daughters of asymmetrically dividing stem cells.**

The *Drosophila* male germline stem cell (GSC) system is an excellent model system for the study of asymmetric stem cell division. GSCs can be identified at single-cell resolution at the apical tip of the testis, where they attach to a cluster of somatic hub cells, a major component of the stem-cell niche[4]. GSCs divide asymmetrically by orienting the mitotic spindle perpendicular to the hub[5]. We showed previously that the mother centrosome is inherited by the GSCs[6].

We adapted the CO-FISH (chromosome orientation fluorescence *in situ* hybridization) protocol, which allows strand-specific identification of sister chromatids[3], combined with chromosome-specific probes[7] (Fig. 1a). Using this method, we identified the sister chromatids of each chromosome in GSCs and their differentiating daughter gonialblasts (Fig. 1b and Supplementary Fig. 1). We found that sister chromatids of the Y chromosome are inherited with a strong bias during GSC division: In approximately 85% of cases, GSCs inherited the sister chromatid of the Y chromosome, whose template strand contains the $(GTATT)_6$ satellite (and thus hybridizes to the Cy3-$(AATAC)_6$ probe), and gonialblasts inherited the sister chromatid whose template contains the $(AATAC)_6$ sequence (and thus hybridizes to the Cy5-$(GTATT)_6$ probe; Fig. 1c, d). Using X-chromosome-specific probes, we found that the X chromosome shows a similar bias (Fig. 1e, f). Essentially the same results were obtained when the Cy5 probe for the X chromosome was replaced with a probe that is not complementary to the Cy3-labelled probe (Supplementary Fig. 2). Although both X and Y chromosomes show a similar bias in segregation (approximately 85:15), we found that the two chromosomes segregate independently of each other (Fig. 1g–i) (see Methods for details).

Two major scenarios can explain the observed bias of approximately 85:15. In the first scenario, approximately 85% of GSCs inherit the 'red strand' (that is, the sister chromatid containing the template strand that hybridizes to Cy3 probes) with near 100% accuracy, whereas approximately 15% of GSCs inherit the 'blue strand' with near 100% accuracy. This would indicate that GSCs maintain particular strands of the X and Y chromosomes forever ('immortal strands'). In the second scenario, each GSC inherits the 'red strand' with 85% probability and the 'blue strand' with 15% probability at each division. In this case, GSCs do not retain immortal strands; instead, the 'template strands' switch approximately once in every seven divisions (15% ≈ 1/6.7). To distinguish between these possibilities, we conducted a long-pulse experiment where flies were continuously exposed to 5-bromodeoxyuridine-containing medium (see Supplementary Fig. 3 for details). The results of this experiment clearly supported the second scenario.

In contrast to X and Y chromosomes, we found that the autosomes (chromosomes 2 and 3) do not show biased segregation (~50:50; Fig. 2). Consistent with previous reports that homologous chromosomes are paired, even in non-meiotic cells in *Drosophila*[8], we observed that two autosome signals corresponding to homologous chromosomes were always juxtaposed to each other (Fig. 2a–d). In spite of the lack of biased segregation with regard to which strands are inherited by GSCs, cells always inherited two Cy3 signals or two Cy5 signals, the mechanism and significance of which remain elusive. It should be noted that the repeat sequences used as probes for chromosome 2 and 3 also exist on the Y chromosome[9], yielding a third 'lone' signal in addition to the paired autosome signals. The identity of the lone signal was confirmed by combining autosome probes and a Y chromosome probe, 488-$(AATAC)_6$. The Y chromosome signal was always close to the lone signal (Fig. 2e, f). Importantly, the Y chromosome detected as a lone signal showed biased segregation, despite the fact that the paired autosome signals showed a random segregation pattern in the same set of samples (Fig. 2g). This result further confirms our observation that sister chromatids of the Y chromosome are segregated nonrandomly.

Although many studies have reported biased sister chromatid segregation, the genes responsible for biased segregation have never been described. We found that *centrosomin* (*cnn*), a core component of the pericentriolar material[10], SUN domain protein KOI[11], and KASH domain protein KLAR[12] are required for biased sister chromatid segregation (Fig. 3, Supplementary Table 1). It is well established that the LINC (linker of nucleoskeleton and cytoskeleton) complex, composed of SUN- and KASH-domain proteins, tethers the nucleus to cytoskeletal components (such as microtubules, which in turn connect to the centrosome) via the nuclear envelope[13]. Thus, we speculate that specific sister chromatids are tethered to the mother centrosome of the GSC that is consistently located at the hub–GSC junction (see Fig. 4e).

We further found that sister chromatid segregation of X and Y chromosomes was randomized in *dnmt2* mutants (Supplementary Table 2a and Supplementary Fig. 4). Although some studies indicated that DNMT2 has DNA methyltransferase activity[14,15], other studies showed that it functions as an RNA methyltransferase[16] and that DNA methylation is barely detectable in the *Drosophila* genome[17]. Therefore, the mechanism by which DNMT2 participates in nonrandom sister chromatid

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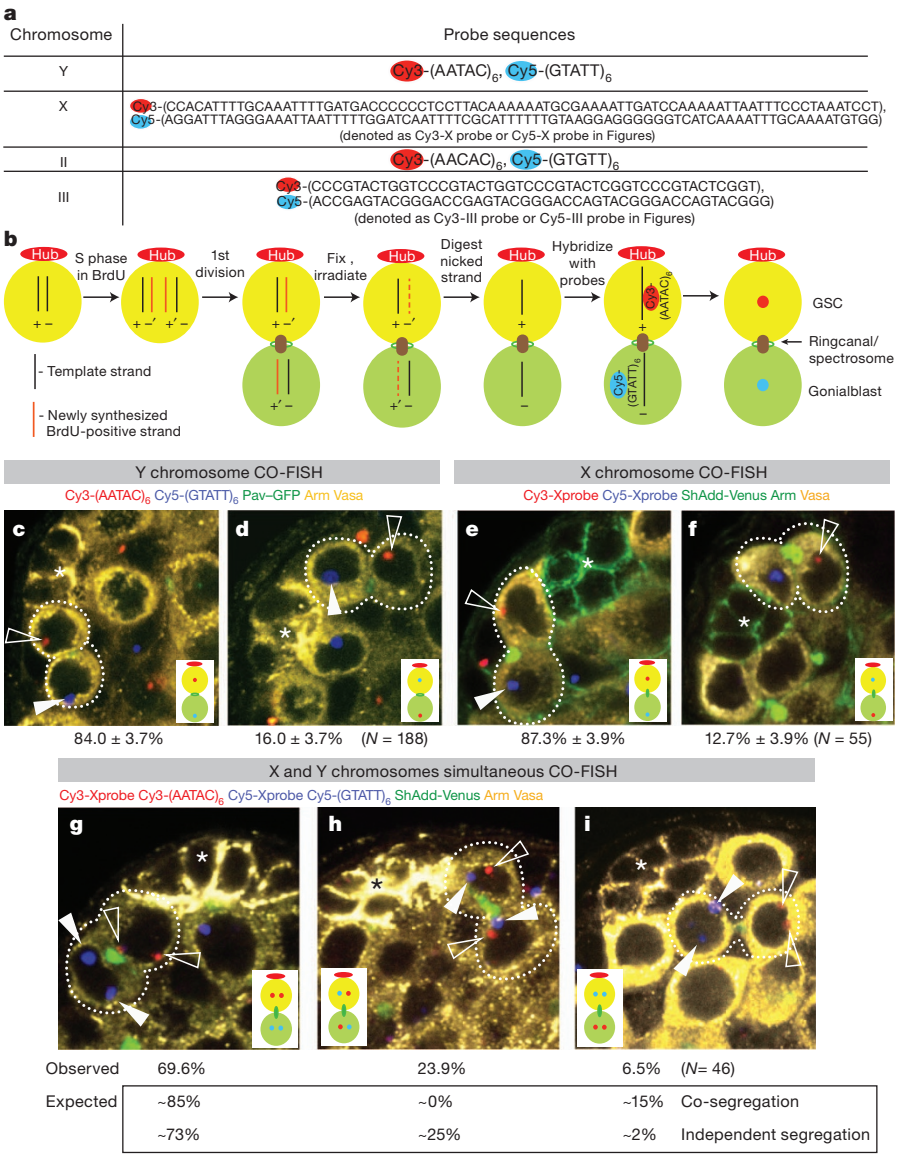


**Figure 1 | Nonrandom segregation of Y and X chromosome strands during GSC divisions.** **a**, Chromosome-specific probes used in this study. **b**, Schematic diagram of the CO-FISH procedure. Cy3- and Cy5-labelled probes for the Y chromosome are shown as an example. Green fluorescent protein-labelled PAVAROTTI (PAV–GFP)[27] (midbody/ring canal), SH–ADD–Venus[28] or anti-ADD antibody (spectrosome) was used to identify GSC–gonialblast pairs. **c**–**i**, Representative images of CO-FISH results using Y chromosome probes (**c**, **d**), X chromosome probes (**e**, **f**), and both X and Y probes (**g**–**i**). Expected segregation patterns based on co-segregation versus random segregation are shown at the bottom of **g**, **h** and **i**. In all figures the Cy5 signal is indicated by solid arrowheads and the Cy3 signal by open arrowheads. An asterisk marks the position of the hub. *N*, number of GSC–gonialblast pairs scored. Data are presented as mean ± standard deviation.

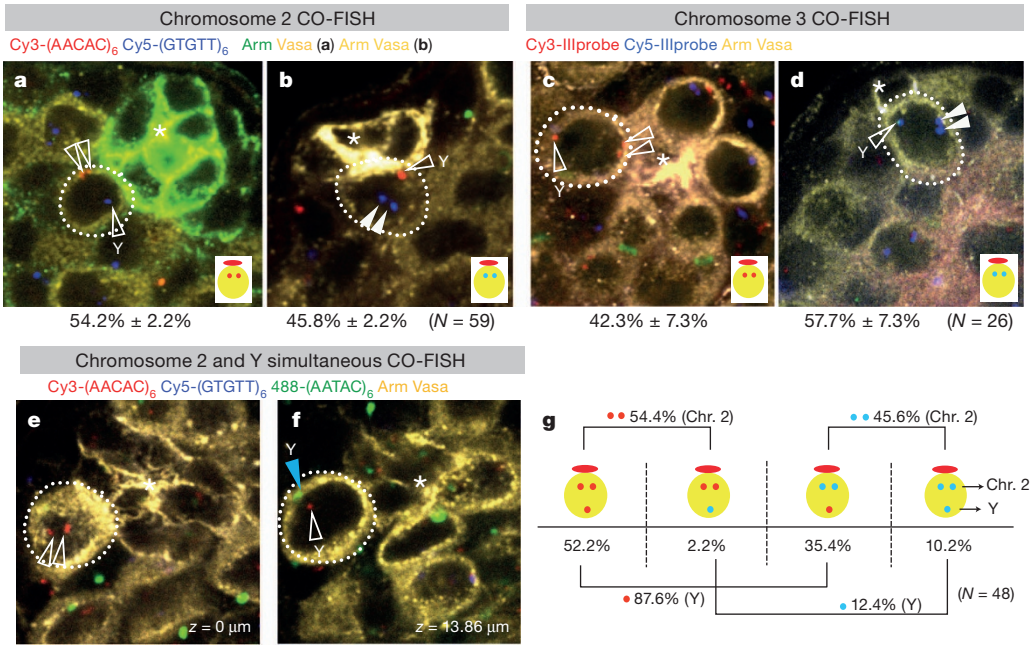


**Figure 2 | Autosomes are randomly segregated during GSC divisions.** **a**–**d**, Representative images of CO-FISH results using chromosome 2 probes (**a**, **b**), and chromosome 3 probes (**c**, **d**). Lone signals that correspond to the Y chromosome are marked with 'Y'. *N*, number of GSCs scored. An asterisk marks the position of the hub. **e**, **f**, A representative image showing that the lone signal of the $(AACAC)_6$ probe (open arrowheads) is close to the $(AATAC)_6$ signal (blue arrowhead). **g**, Summary of scoring results using chromosome 2 probes. Paired signals segregate randomly (Cy3-Cy3:Cy5-Cy5 = 54.4:45.6), whereas lone signals segregate nonrandomly. (Cy3:Cy5 = 87.6:12.4). $(AACAC)_6$ and $(AATAC)_6$ sequences are on the same strand of the *Drosophila* Y chromosome.

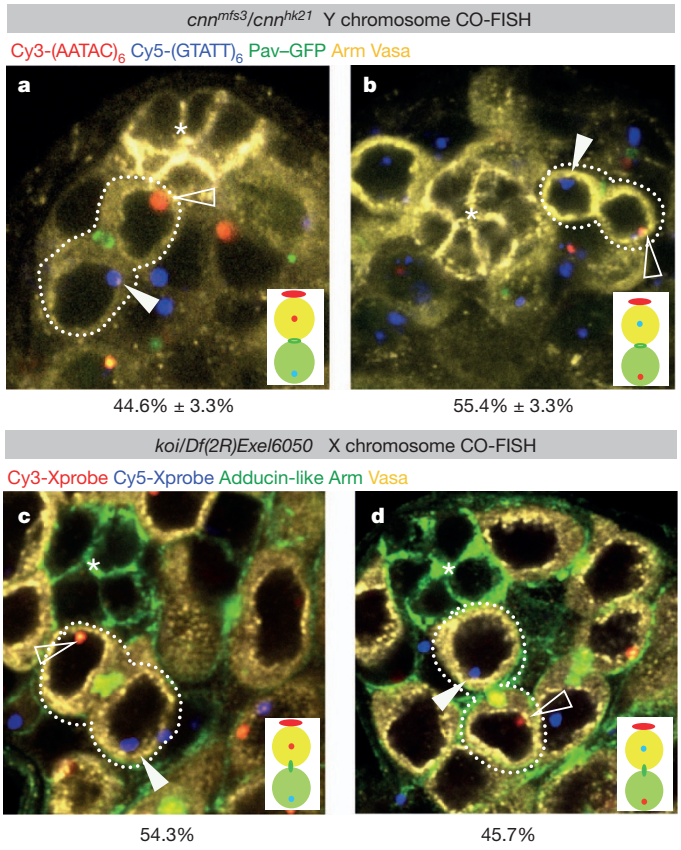


**Figure 3 | *cnn*, *koi* and *klar* are required for nonrandom sister chromatid segregation.** **a**, **b**, Representative images of Y chromosome CO-FISH in *cnn* mutant. Open arrowheads indicate the Cy3-$(AATAC)_6$ probe; closed arrowheads indicate the Cy5-$(GTATT)_6$ probe; asterisk indicates the hub. **c**, **d**, Representative images of X chromosome CO-FISH in *koi* mutant. Open arrowheads indicate the Cy3-X probe; closed arrowheads indicate the Cy5-X probe; asterisk indicates the hub.

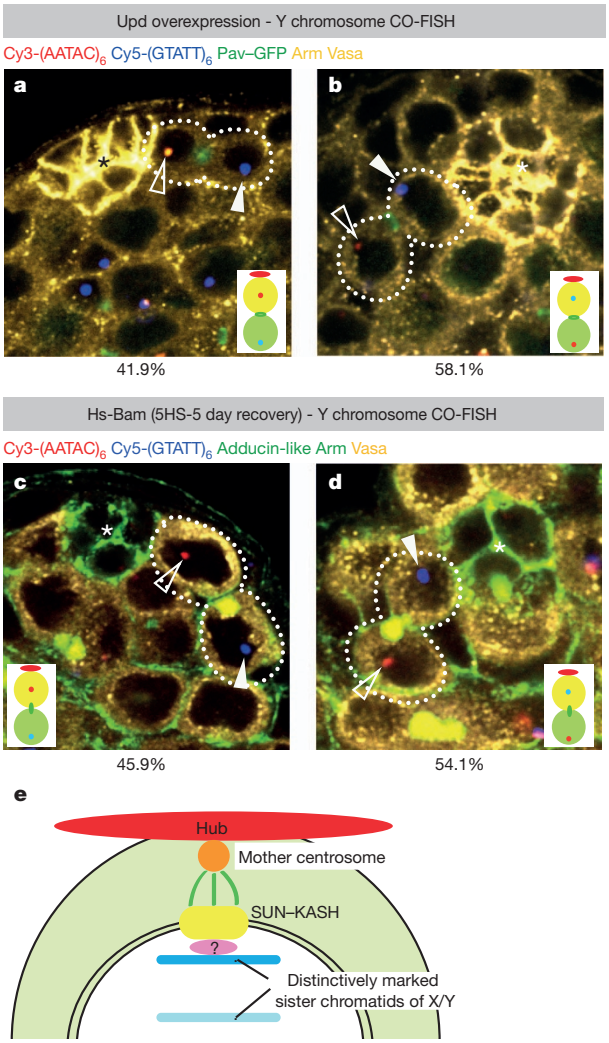


**Figure 4 | Nonrandom segregation of Y and X chromosomes is disrupted in *upd*-overexpressing testes and dedifferentiated stem cells.** **a**, **b**, Representative images of CO-FISH using the Y probe upon overexpression of UPD (nos-gal4> UAS-UPD). For this experiment we limited our analysis to GSCs juxtaposed to hub cells, because GSCs located away from the hub do not have a spatial reference point for assessment of the sister chromatid segregation pattern. *N*, number of GSC-gonialblast pairs scored. An asterisk marks the position of the hub. **c**, **d**, Representative images of CO-FISH using the Y probe in dedifferentiated GSCs. Differentiation was induced by heat-shock treatment of hs-Bam flies followed by a 5-day recovery period[29]. **e**, Model of nonrandom sister chromatid segregation (see text for details).

segregation remains elusive. However, our analysis, using various crossing schemes (crosses of homozygous mother/father with heterozygous father/mother), indicates that DNMT2 confers heritable, DNA sequence-independent information on the X and Y chromosomes during gametogenesis in the parents, leading to nonrandom sister chromatid segregation of X and Y chromosomes in the GSCs of the progeny (Supplementary Table 2b). For example, in GSCs from flies that are genetically heterozygous ($dnmt2^{+/-}$), where the X chromosome is inherited from a mutant mother ($dnmt2^{-/-}$) and the Y chromosome from a heterozygous father ($dnmt2^{+/-}$), X chromosome segregation was randomized, whereas Y chromosome segregation remained nonrandom. These results suggest the striking possibility that the information that enables nonrandom sister chromatid segregation of X and Y chromosomes in adult stem cells is primed during gametogenesis in the parents, transmitted to the zygote on single X and Y chromosomes, and maintained through many cell divisions during embryogenesis and adult tissue homeostasis.

We found that sister chromatid segregation of X and Y chromosomes is randomized in GSC overproliferation induced by ectopic expression of UPD (also known as OS; Fig. 4a, b and Supplementary Table 3). UPD is a signalling ligand that is normally expressed exclusively in hub cells and activates the JAK-STAT pathway in GSCs and cyst stem cells to specify stem cell identity[4]. This finding indicates that nonrandom sister chromatid segregation is under the control of stem cell identity. However, it is unlikely that nonrandom sister chromatid segregation determines GSC identity, because the mutants defective in nonrandom segregation described above (*cnn*, *koi*, *klar*, *dnmt2*) do not show GSC overproliferation or depletion.

We also found that sister chromatid segregation is randomized in dedifferentiated GSCs (Fig. 4c, d and Supplementary Table 3). Partially differentiated germ cells can revert back to GSC identity to replenish the stem-cell pool[18,19]. Although these dedifferentiated GSCs are apparently functional because they can produce differentiating spermatogonia and reconstitute spermatogenesis[18,20], they did not recover nonrandom sister chromatid segregation. This result may indicate that the information on X and Y chromosomes that allows nonrandom sister chromatid segregation is lost upon commitment to differentiation as a gonialblast. Consistent with our earlier observation that dedifferentiation increases during ageing[20], we found that nonrandom sister chromatid segregation was compromised during ageing (at day 30, 63:37 for the X chromosome ($N = 35$) and 68:32 for the Y chromosome ($N = 28$)).

This study provides the first evidence that adult stem cells can distinguish two sister chromatids, and further points to a model in which sister chromatids are distinctly recognized, leading to anchorage of particular strands to the mother centrosome through the SUN–KASH proteins

(Fig. 4e). Our data also indicate that nonrandom sister chromatid segregation does not necessarily mean that they are immortal[21].

At present it is not clear why X and Y chromosomes segregate nonrandomly. Considering the data presented in this study, we favour the possibility that certain epigenetic information is transmitted distinctively to GSCs and gonialblasts. Indeed, X and Y chromosomes are subject to various forms of epigenetic regulation, such as dosage compensation[22] and male-specific meiotic sex chromosome inactivation[23]. In addition, *Stellate*, a repetitive sequence that encodes a polypeptide known to reduce fertility, and Suppressor of *Stellate* (*Su(Ste)*), the Piwi-interacting RNA (piRNA) that suppresses *Stellate* expression, are located on the X and Y chromosomes, respectively[24,25]. Intriguingly, we observed that *Stellate* is derepressed in mutants of *cnn*, *dnmt2*, *koi* and *klar* (Supplementary Fig. 5), although determination of whether derepression of *Stellate* is due to a failure in nonrandom sister chromatid segregation awaits future investigation. Not surprisingly, we found that the mutants in which *Stellate* is derepressed show reduced fertility (Supplementary Fig. 6).

Recently, it was shown that old versus new histones segregate asymmetrically during GSC divisions[26]. Our study demonstrates that GSCs do not segregate old (immortal) DNA strands. Thus, the relationship between biased sister chromatid segregation and histone segregation remains elusive. In summary, our study presents the first evidence of chromosome-specific nonrandom sister chromatid segregation in adult stem cells and provides mechanistic insights into how cells segregate sister chromatids nonrandomly.

## METHODS SUMMARY

For CO-FISH combined with immunofluorescence staining, newly eclosed flies (unless otherwise noted) were fed with 5-bromodeoxyuridine for ~10 h, followed by a period in non-5-bromodeoxyuridine medium (~10 h). The testes were then immunostained as described previously[20]. Subsequently, testes were irradiated with ultraviolet light, followed by treatment with exonuclease III. Then, CO-FISH probes were hybridized to detect template strands.

**Full Methods** and any associated references are available in the online version of the paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** S.Y. conceived the project and developed the single-chromosome CO-FISH protocol for *Drosophila* cells. S.Y. and Y.M.Y. designed and conducted experiments, interpreted the data, and wrote the manuscript.

**Author Information** Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.Y. (swathi@umich.edu) and Y.M.Y. (yukikomy@umich.edu).

## METHODS

**Fly husbandry.** All fly stocks were raised on Bloomington Standard Media at 25 °C unless otherwise noted. The following fly stocks were used: Ubi–Pavarotti–GFP, SH–adducin–Venus, *cnn*$^{mfs3}$/CyO, *cnn*$^{HK21}$/CyO, *koi*$^{HRKO80.w}$, Df(2R)Exel6050/CyO, *klar*$^{1}$, Df(3L)emc-E12, P(EP)Mt2$^{G3429}$ (denoted *dnmt2*$^{G3429}$ in the text), *dnmt2*$^{499}$, *dnmt2*$^{149}$, Df(2L)ED775/CyO, hs-Bam, UAS-UPD/CyO, and nos-gal4. These stocks are described in FlyBase.

**Combined immunofluorescence staining and CO-FISH.** Newly eclosed adult flies (day 0) were fed food containing 5-bromodeoxyuridine (950 µl 100% apple juice, 7 µg agar, and 50 µl 100 mg ml$^{-1}$ 5-bromodeoxyuridine solution in a 1:1 mixture of acetone and DMSO) for approximately 10 h. After the feeding period, flies were transferred to regular fly food for approximately 10 h. Because the average GSC cell cycle length is 12 h, most GSCs undergo a single S phase followed by mitosis during our feeding procedure. GSCs that have undergone more or less than one S phase or mitosis were excluded from our analysis by limiting scoring to GSC–gonialblast pairs that have complementary CO-FISH signals in the GSC and gonialblast (that is, red signal in one cell, blue signal in the other). All possible scenarios are explained in Supplementary Fig. 1. Samples were dissected in 1× PBS, fixed for 30–60 min with 4% formaldehyde in PBS, permeabilized for at least 1 h in PBST (0.1% Triton X-100 in PBS) and incubated with primary antibodies overnight at 4 °C. Samples were then washed with PBST (20 min, three times), incubated overnight at 4 °C with Alexa Fluor-conjugated secondary antibodies (1:200; Molecular Probes), and washed again with PBST (20 min, three times). Samples were fixed for 10 min with 4% formaldehyde followed by three washes in PBST for 5 min each. Samples were then treated with RNase A (2 mg ml$^{-1}$ in water) for 10 min at 37 °C, washed with PBST for 5 min, and stained with 100 µl Hoechst 33258 (Sigma Aldrich) at 2 µg ml$^{-1}$ for 15 min at room temperature. The samples were then rinsed with 2× SSC, transferred to a tray, and irradiated with ultraviolet light in a UV Stratalinker 1800 (calculated dose, 5400 J m$^{-2}$). Nicked 5-bromodeoxyuridine strands were digested with exonuclease III (New England Biolabs) at 3 U µl$^{-1}$ in buffer supplied by the manufacturer (50 mM Tris-HCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol (DTT), pH 8.0) at 37 °C for 10 min. Samples were rinsed once with PBST for 5 min and then fixed in 4% formaldehyde in PBS for 2 min and washed three times for 5 min each in PBST. To allow gradual transition into 50% formamide/2× SSC, samples were incubated sequentially for a minimum of 10 min each in 20% formamide/2× SSC, 40% formamide/2× SSC, and 50% formamide/2× SSC. The hybridization mixture consisted of 50% formamide, 2× SSC, 10% dextran sulphate, 0.5 µg ml$^{-1}$ Cy3-labelled probe, and 0.5 µg ml$^{-1}$ Cy-5-labelled probe. Fluorescence-labelled probes were obtained from Integrated DNA Technologies. The hybridization solution was added to the samples and hybridization was carried out at 37 °C overnight. Using non-complementary pairs of probes for the X chromosome, we detected a similar bias in segregation pattern (Supplementary Fig. 2), excluding the possibility that annealing of complementary probes interferes with correct hybridization between the probes and the target sequences. Autosome probes were denatured in hybridization solution at 65 °C for 3 min before hybridization. The samples were never heat-denatured. As a critical control, hub cells, which are predominantly quiescent and, thus, do not incorporate 5-bromodeoxyuridine, did not show any CO-FISH signal (evident in all images).

Following hybridization, samples were washed once in 50% formamide/2× SSC, once in 25% formamide/2× SSC, and three times in 2× SSC. Samples were then mounted in VECTASHIELD (H-1200, Vector Laboratories) and images were recorded using a Leica TCS SP5 confocal microscope with a 63 × oil immersion objective (numerical aperture = 1.4) and processed using Adobe Photoshop software. The primary antibodies used were rabbit anti-Vasa (1:200; Santa Cruz Biotechnology), mouse anti-Adducin-like (1:20; developed by H. D. Lipshitz and obtained from the Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Armadillo (1:20; developed by Eric Wieschaus and obtained from DSHB), rabbit anti-Stellate (1:1,000, a gift of P. Zamore[30]). The secondary antibodies used were Alexa Fluor 594- and 488-conjugated secondary antibodies (1:200; Molecular Probes).

**CO-FISH with both X and Y probes.** The X and Y probes were labelled such that GSCs retain the Cy3 signal in ~85% of cases. If segregation of X and Y chromosomes is correlated, the probability that a GSC inherits two Cy3 signals will be approximately 85%, and that of inheriting two Cy5 signals will be approximately 15%, whereas there will be few instances where a GSC inherits one Cy3 and one Cy5 signal. In contrast, if the X and Y chromosomes segregate asymmetrically independently of each other, the probability of GSCs inheriting two Cy3 signals will be 72% (85% × 85%), that of inheriting two Cy5 signals will be 2% (15% × 15%), and that of inheriting one Cy3 and one Cy5 signal will be 26% (85% × 15% × 2).

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