Non-Random Sister Chromatid Segregation During Stem Cell Division in *Drosophila Melanogaster* Testis

by

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Dedication

This thesis is dedicated to my incredibly wonderful husband, Pramod, and my beautiful and precious children Siddharth, and Medha. Thank you for your love, support, patience, and numerous sacrifices throughout my academic career.

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List of Abbreviations

15N: Heavy Nitrogen isotope

Add-GFP: Adducin-GFP

APC2: Ademomatous polyposis coli 2

Bam: Bag-of-marbles

BrdU: 5-bromo-2'-deoxyuridine

CldU: Chlorodeoxyuridine

Cnn: Centrosomin

CO-FISH: Chromosome oriented fluorescence in-situ hybridization

Cy3: Cyanine 3 dye (excitation peak: 550 nm, emission peak: 570 nm)

Cy5: Cyanine 5 dye (excitation peak: 650 nm, emission peak: 670 nm)

CySC: Cyst stem cell

DE: *Drosophila* epithelial

DNA: Deoxyribonucleic acid

Dnmt2: DNA methyltransferase 2

Dpp: Decapentaplegic

DSas-4: *Drosophila* spindle assembly abnormal 4

ES: Embryonic stem

FasIII: Fasciclin III

G1-phase: Gap 1-phase

G2-phase: Gap 2-phase

GB: Gonialblast

Gbb: Glass bottom boat

GSC: Germline stem cell

Hop: hopscotch

Hs-bam: Heat shock-bam

IdU: Iododeoxyuridine

ISH: Immortal strand hypothesis

JAK-STAT: Janus kinase–signal transducer and activator of transcription

KASH-domain: Klarsicht/ANC-1/Syne-1 homology-domain

Klar: Klarsicht

Koi: Klaroid

Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5

LINC: Linker of nucleoskeleton and cytoskeleton

M-phase: Mitosis-phase

MKLP1: Mammalian kinesin-like protein 1

MSP-300: Muscle-specific protein 300

PACT: Pericentrin-AKAP-450 centrosomal targeting

Pav: Pavarotti

PBS: Phosphate buffer saline

PBST: Phosphate buffer saline-tween

PCM: Pericentriolar material

PCR: Polymerase chain reaction

piRNA: Piwi-interacting RNA

RNA: Ribonucleic acid

S-phase: Synthesis-phase

Spag4: Sperm-associated antigen 4

SSC: Saline sodium citrate

SSH: Silent sister hypothesis

Su(Ste): Supressor of stellate

SUN-domain: Sad1 and UNC-84 homology domain

TGF-beta: Transforming growth factor beta

tRNA: Transfer RNA

Upd- Unpaired

Zfh-1: Zinc finger-homeodomain transcription factor 1

Abstract

Non-random Sister Chromatid Segregation During Stem Cell Division in *Drosophila Melanogaster* Testis

Adult stem cells undergo asymmetric cell division to self-renew and to produce differentiated cells throughout the life of an organism. 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 all the older template DNA strands. In addition, other models have also been proposed in which stem cells non-randomly segregate only a subset of chromosomes for different reasons, such as retention of epigenetic memories. However, the mechanism and the biological relevance of these chromosome asymmetries remain elusive. This is primarily due to the lack of model systems in which chromosome asymmetries can be assessed in the context of other asymmetries, such as cell fate.

The *Drosophila melanogaster* testis is one of the few well-characterized model systems that enable a detailed study of the regulation of stem cells. To elaborate, unlike many other model systems *Drosophila* male germline stem cells (GSCs) can be unambiguously identified at single-cell resolution. Further, GSCs divide asymmetrically giving rise to a stem cell and a differentiating cell, which can be readily identified in vivo enabling unambiguous identification of both

asymmetric stem cell division and any other potential asymmetries such as nonrandom sister chromatid segregation.

In this thesis, I describe work where I first showed that the bulk of chromosomes are not segregated asymmetrically in dividing *Drosophila* GSCs, suggesting that GSCs do not retain all the older template DNA strands to maintain their genomic integrity. However, these initial results did not exclude the possibility that GSCs might be non-randomly segregating individual chromosomes. In order to unambiguously study the segregation patterns of individual chromosomes, I adapted the CO-FISH (chromosome orientation fluorescence in situ hybridization) protocol, which allows strand-specific identification of sister chromatids. Using this method, I found that sister chromatids of X and Y chromosomes, but not autosomes, are segregated non-randomly during asymmetric divisions of GSCs. These results provide the first direct evidence that sister chromatids of certain chromosomes can be distinguished and segregated non-randomly during asymmetric stem cell divisions. Further, in this work I also showed that centrosomal proteins, nuclear envelope proteins, and methyltransferase are all required for non-random sister chromatid segregation of X and Y chromosomes. This study establishes the first genetically tractable experimental model system to study chromosome strand segregation pattern with unprecedented resolution during cell division. Finally, this work suggests that non-random sister chromatid segregation in asymmetrically dividing stem cells is potentially an evolutionarily conserved mechanism that is critical for diversification of cell fates—thus establishing a new

paradigm for understanding stem cell regulation.

Chapter 1

Introduction and Outline

1.1 An overview of stem cell asymmetric division

Stem cells are essential contributors to tissue development, homeostasis, and repair. Throughout the life of an organism, stem cells are required to proliferate and supply differentiated but short-lived cells such as blood, skin, intestinal epithelium, and sperm cells (Morrison and Kimble, 2006). The main function of adult stem cells is to generate identical copies of themselves (self-renewal) as well as to produce various differentiated cells that give rise to tissue (Morrison and Kimble, 2006). Many stem cells are known to achieve this function through asymmetric cell division—*i.e.,* one of the daughter cells adopts the fate of its mother, whereas the other adopts a more committed fate (Morrison and Spradling, 2008). *Drosophila melanogaster* has emerged as an extremely powerful and tractable model system for identifying and analyzing complex behavior of stem cells. Specifically, the gonad of *Drosophila melanogaster* is an excellent system to study mechanisms regulating stem cell function as it is one of the very few systems where stem cells are easily identifiable and their division is well characterized (Davies and Fuller, 2008; Yamashita et al., 2010). *Drosophila* model systems have played key instructional roles in understanding of many mammalian stem cell systems (Losick et al., 2011) due to the striking similarities

between invertebrate and vertebrate stem cell systems.

Studies in several model systems have suggested two distinct mechanisms by which asymmetric cell division is achieved. The first mechanism involves extrinsic fate determinants provided by the stem cell niche (the microenvironment that instructs stem cell identity) and relies on the asymmetric placement of daughter cells within or outside of the niche (Knoblich, 2008). Alternatively, the second mechanism involves intrinsic fate determinants and relies on the asymmetric partitioning of fate determinants to the daughter cells (Knoblich, 2008). In addition to these asymmetries, which are clearly related to their function and fate, a series of studies have suggested other types of asymmetries during stem cell division. These include non-random segregation of sister chromatids (Charville and Rando, 2013; Yennek and Tajbakhsh, 2013), midbody (Ettinger et al., 2011), and protein aggregates (Rujano et al., 2006). However, the relevance of these asymmetries is yet to be established.

In recent times, non-random sister chromatid segregation has been studied extensively in many stem cell model systems (Yamashita, 2013b; Yennek and Tajbakhsh, 2013). It has been suggested that the sister chromatids might be distinguished and segregated non-randomly in certain asymmetrically dividing stem cells (Yamashita, 2013b; Yennek and Tajbakhsh, 2013). During the cell cycle, cells replicate their genome and subsequently segregate sister chromatids into the two daughter cells. DNA replication is generally a very precise process and the replicated copy is expected to be an exact copy of the original template. However, one of the strands acts as the template for the other, thus replication-

induced errors only occur on the newly synthesized strand, while the template strand remains unchanged. Further, since DNA methylation and histone modifications do not necessarily occur simultaneously with the DNA replication, sister chromatids can be different from each other in their epigenetic marks. These mechanisms can potentially impose asymmetries in genome and/or epigenome during cell divisions.

1.2 Models of non-random sister chromatid segregation during stem cell division

Non-random sister chromatid segregation during stem cell division has been intensely studied in recent years in a broad range of stem cell populations. There are two major models of non-random sister chromatid segregation – Immortal Strand Hypothesis (ISH) and Silent Sister Hypothesis (SSH). ISH proposes that stem cells retain older template DNA strands of all the chromosomes to limit replication-induced errors (Cairns, 1975; Cairns, 2006). This idea is termed the Immortal Strand Hypothesis (ISH) since stem cells would inherit the template strand for many cell cycles (essentially forever), making the template strand 'immortal' (Figure 1.1A). However, the validity of this hypothesis remains untested, as there is only limited data to compare the number of mutations in stem cells and differentiated cells (Rossi et al., 2007). Moreover, retaining older template strands would not prevent non-replication-based mutations that can be caused by other naturally occurring DNA damaging events, such as environmental factors and cellular stresses. Although this hypothesis has been widely studied,

the interpretation of some of these studies remains controversial, owing to the complexities of the different model systems employed and the differences in techniques used (Yamashita, 2013a; Yennek and Tajbakhsh, 2013).

Silent Sister Hypothesis (SSH) proposes that stem cells might non-randomly segregate sister chromatids of only a subset of chromosomes, perhaps to retain epigenetic memories (Klar, 1994; Klar, 2007; Lansdorp, 2007)(Figure 1.1B). However, it remains a mystery as to how distinct epigenetic information is placed on two sister chromatids during replication (or perhaps during the subsequent G2 phase) and how information is segregated during division leading to distinct cell fates. For example, it is well known that haploid fission yeast controls mating type by differentially marking only one DNA strand at the mating-type locus *mat1*, thereby producing non-equivalent sister chromatids of chromosome II (the *mat1* locus is on chromosome II) (Klar, 1987). This study provides a clear example of where the difference between sister chromatids correlates with fate determination. Recently, it has been suggested that homologs of chromosome 7 are non-randomly segregated during mouse Embryonic Stem (ES) cell division (Armakolas and Klar, 2006), where the maternal sister chromatid which contains the Watson strand as a template always co-segregated with the paternal sister chromatid that contains the Watson strand as a template. The relationship between the segregation pattern of chromosome 7 and cell fates has not been addressed. However, more recent work using the chromosome-oriented fluorescence in situ hybridization (CO-FISH) method (see below) did not confirm this observation (Sauer et al., 2013). Some of the difficulties involved in answering

these questions can be overcome by using simpler model systems and more sophisticated techniques as described next.

1.3 Challenges in addressing sister chromatid segregation

Addressing sister chromatid segregation patterns in a stem cell population can be very challenging due to multiple factors (Yamashita, 2013b). First, stem cell populations available for experiments are often a heterogeneous mixture of stem and differentiating cells and cellular markers to specifically identify stem cells are often lacking. Second, direct evidence regarding the mode of stem cell divisions (symmetric versus asymmetric) over extended cell generations is limited. Third, it is challenging to unequivocally identify daughter cells after cell division in most model systems. As explained later in this chapter and in further detail in Chapters 2 and 3, *Drosophila* male germline stem cells (GSC) provide an ideal model system to study sister chromatid segregation overcoming all of the problems listed above. Brielfy, 1) GSCs can be identified unambiguously at single-cell resolution, 2) GSC division is always asymmetric (Sheng and Matunis, 2011; Yamashita et al., 2003), 3) Pairs consisting of stem cells and differentiating cells can be easily identified in the GSC system, and, finally, 4) GSC cell cycle characteristics, such as cell cycle length and S-phase duration, are well established.

In addition to the complexities of the model systems, previous studies have been limited by the low resolution of the techniques used to detect different sister chromatid segregation patterns. Below, I briefly describe these techniques and discuss their strengths and limitations.

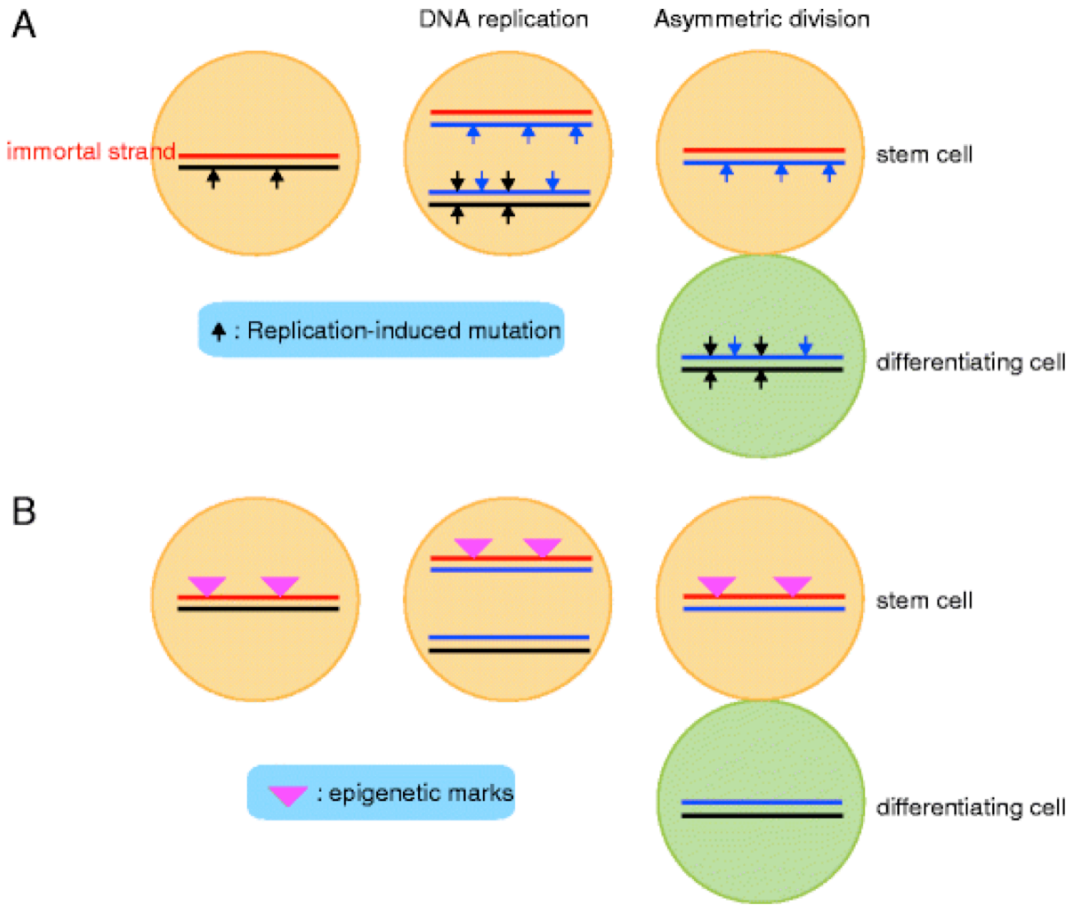


Figure 1.1 Hypotheses that explain nonrandom sister chromatid segregation. A) The immortal strand hypothesis proposes that the template copy of the sister chromatids with fewer replication-induced mutations may be retained in the stem cells. **B)** An alternative hypothesis to explain nonrandom sister chromatid segregation. Distinct epigenetic information is transmitted to daughter cells through nonrandom sister chromatid segregation (Images from (Yamashita, 2013b))

1.3.1 Pulse-Chase assay

In a majority of studies, nucleotide analogs such as 5-bromo-2'-deoxyuridine (BrdU) have been used to label and distinguish two sister chromatids. In a pulse–chase experiment, nucleotide analogs are used to label the newly synthesized DNA strands during asymmetric division of stem cells, and the

segregation of BrdU-labeled chromatids is monitored during the chase period (Figure 1.2A). If stem cells were indeed segregating all the template DNA strands, the differentiated cell would inherit all the BrdU-labeled chromatids. In contrast, if sister chromatids were randomly segregated, both the stem cell and the differentiating daughter cell would inherit BrdU-labeled chromatids (Yadlapalli et al., 2011). Alternatively, BrdU can also be administered for extended periods to label the nascent DNA when stem cells or their precursors are dividing symmetrically, so that immortal template strands, if they exist, will be labeled. In this scenario, if cells follow the ISH model, the labeled DNA will continue to be inherited by stem cells despite undergoing many rounds of cell divisions during the label-free chase period (Figure 1.2B). Such DNA-labeling experiments, which support the ISH results, have been reported in small and large intestinal cells (Potten et al., 2002; Quyn et al., 2010), neural stem cells (Karpowicz et al., 2005), skeletal muscle satellite cells (Conboy et al., 2007; Shinin et al., 2006), mammary epithelial cells (Smith, 2005) and others. However, experiments using the same technique which involve for instance mouse hematopoietic stem cells (Kiel et al., 2007), epidermal basal cells (Sotiropoulou et al., 2008), hair follicle stem cells (Waghmare et al., 2008), neocortical precursor cells (Fei and Huttner, 2009) showed that stem cells randomly segregated the template strands. It should be noted that because BrdU is incorporated into the newly synthesized DNA of all the chromosomes, one can only address non-random sister chromatid segregation that applies to the whole set of the chromosomes. The non-random segregation of a small subset of chromosomes cannot be detected by this method.

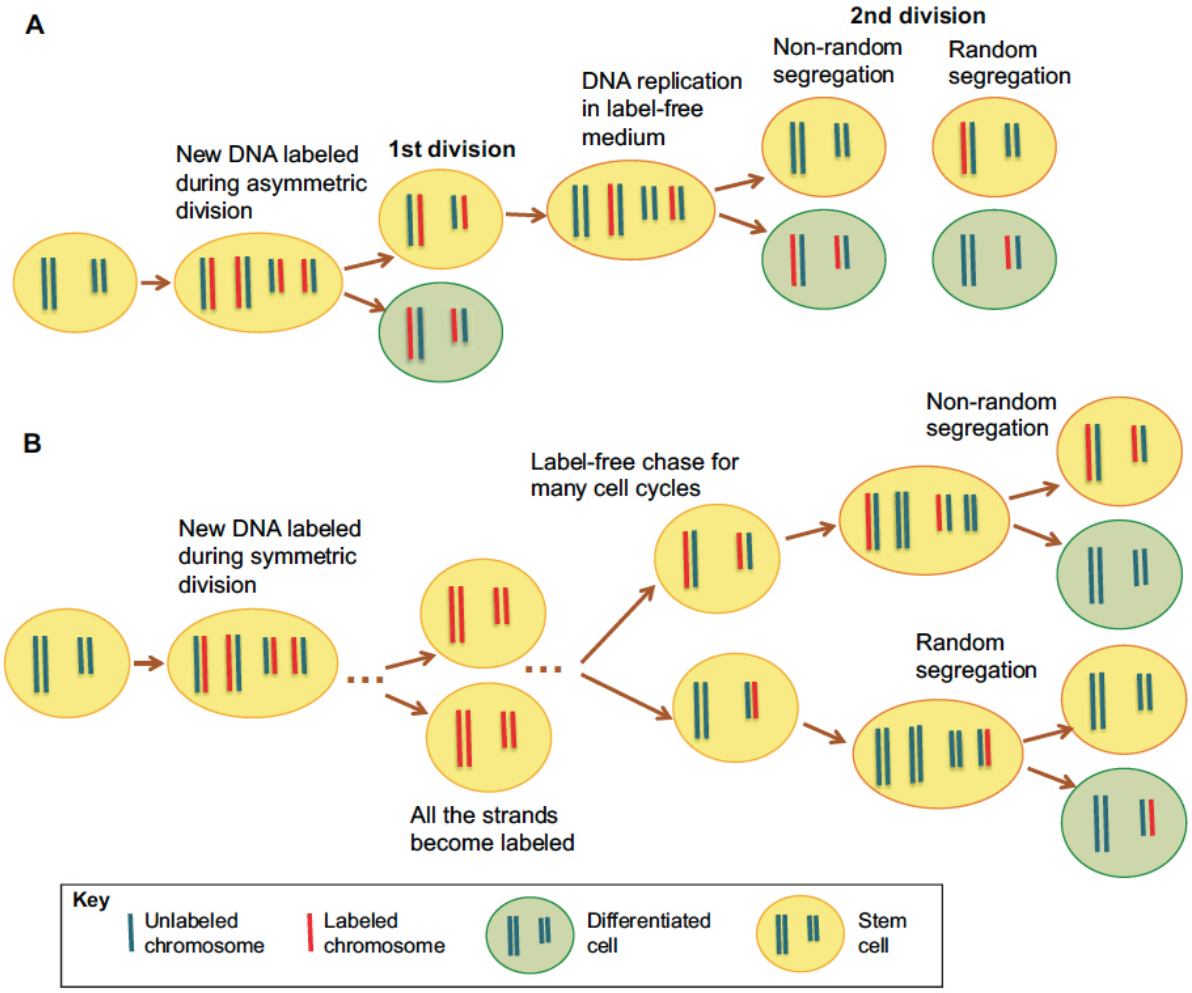


Figure 1.2 Pulse–chase assay to study sister chromatid segregation during stem cell division. A) In an asymmetrically dividing stem cell, new DNA strands are labeled with a nucleotide analog during the S phase of one cell cycle. Inheritance of all the label by the differentiating cell in the second cell cycle would indicate that stem cells inherit 'immortal' strands. If the label is inherited symmetrically, it would suggest the cells do not follow the ISH model. **B)** All stem cell DNA strands are labeled through administration of nucleotide analogs over multiple generations when the stem cells or their precursors are dividing symmetrically. If immortal template strands exist, they would become labeled. During the following label-free chase period, stem cells would retain the labeled strands even after many cell divisions if they follow the ISH model. However, if they do not, the label would be quickly diluted and lost from the stem cells.

1.3.2 Multi-isotope imaging mass spectrometry

Multi-isotope imaging mass spectrometry (MIMS) is a novel technique to

image stable isotopes (such as (15)N-thymidine) in cells with a new type of secondary ion mass spectrometer (Steinhauser et al., 2012). This method is essentially the same as the use of nucleotide analogs because the isotope would label all the chromosomes. However, this has a strong advantage over the use of nucleotide analogs, because isotopes are non-toxic and their concentration within the cell can be precisely measured by mass spectrometry. Using this technique, Steinhauser and colleagues showed that stem cells in the mouse small intestine do not follow the ISH model (Steinhauser et al., 2012). Specifically, they administered (15)N-thymidine to mice from gestation until post-natal week 8, but found no (15)N label retention by dividing small intestinal crypt cells after a four-week chase. Additionally, when they administered (15)N-thymidine pulse-chase to adult mice, they observed that proliferating crypt cells dilute the (15)N label, consistent with random strand segregation.

1.3.3 Strand-seq technique

A novel sequencing technique, called strand-seq, which sequences only the parental template strands of all the chromosomes from single cells has recently been developed to study sister chromatid segregation during cell division (Falconer et al., 2012). Briefly, in this technique newly synthesized strands are labeled with BrdU, the genome is fragmented and a paired-end library is constructed. Prior to PCR amplification, the nascent BrdU-labeled strand is nicked so that it is not amplified during the subsequent PCR step. As a result, only the original intact template strand is selectively amplified, resulting in directional library

fragments. The library fragments are then sequenced and the resulting paired short sequencing reads are used to identify the original template strands that have been inherited from the parental cell. (Figure 1.3)

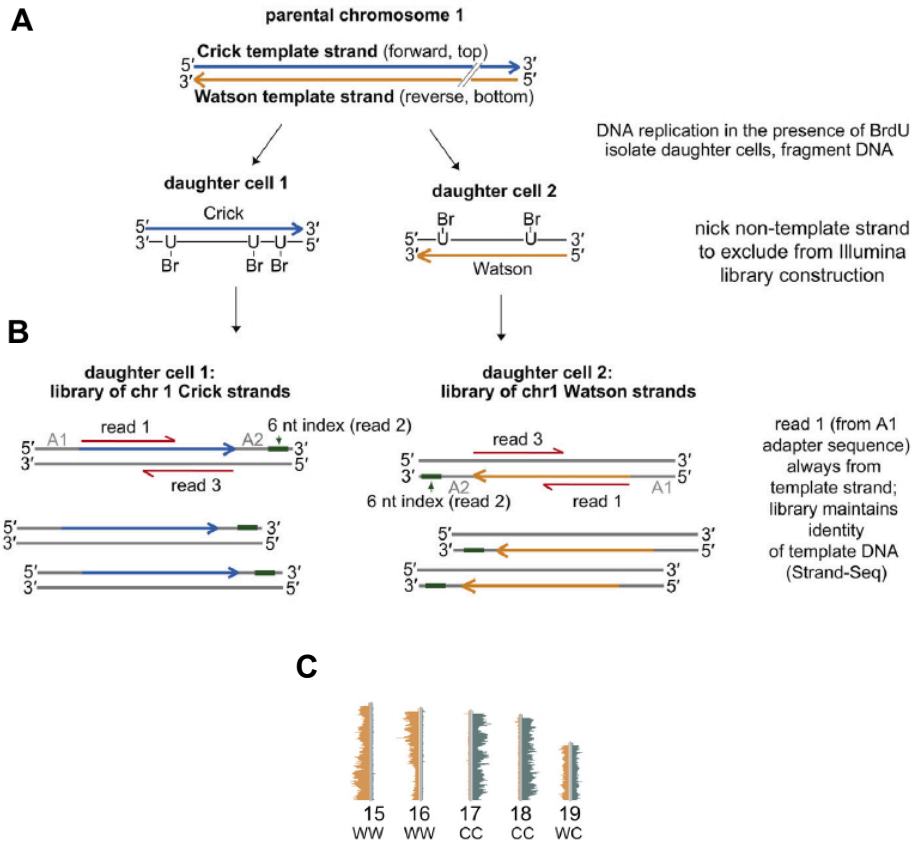


Figure 1.3 Strand-seq technique to study sister chromatid segregation in single cells A) When DNA is replicated in the presence of BrdU, only the newly-formed DNA (black lines) is substituted with BrdU while the original template strands remain un-substituted. Nicks are created at the sites of BrdU incorporation. A modified Illumina library construction protocol can be used to exclude the nicked BrdU-positive strands from the final amplification step. **B)** The resultant library fragments maintain the genomic directionality of the DNA strands such that read 1 of a paired-end read is always from the template strand while read 3 is from the complementary strand (gray). These reads can be aligned to the reference genome to clearly show the template strands that were inherited by that cell for each chromosome. **C)** The output for a single mouse embryonic stem cell library clearly shows the Watson and Crick template strands that were inherited by that cell for chromosomes 15-19. (Images from (Falconer et al., 2012).)

This technique allows the detection of non-random sister chromatid segregation at single chromosome resolution, without being limited by the availability of suitable

probe sequences (a requirement for CO-FISH technique, described below). Using this technique, it has been shown that in mouse embryonic stem (ES) cells, chromosome 7 is randomly segregated (Falconer et al., 2012).

1.3.4 Chromosome-Oriented Fluorescence In-situ Hybridization

Chromosome-Oriented Fluorescence In-situ Hybridization (COFISH) technique allows the unambiguous identification of the two sister chromatids – one which contains the Watson strand as a template and the other which contains the Crick strand as a template (Bailey et al., 2004; Falconer et al., 2010). In the CO-FISH protocol, cells are allowed to replicate once in the presence of BrdU. Following BrdU incorporation, cells complete mitosis in a BrdU-free medium, such that the sister chromatids are segregated into stem cell and daughter cell. The BrdU-containing strands are removed by treatment with ultraviolet irradiation and exonuclease III. The remaining template strands can be identified using differentially labeled strand-specific probes (Figure 1.4). This technique has been used to follow the segregation patterns of sister chromatids in mouse colon epithelial cells in vivo. The authors observed significant non-random sister chromatid segregation in the differentiating cells but not in the Lgr5+ stem cells in the colon crypts (Falconer et al., 2010).

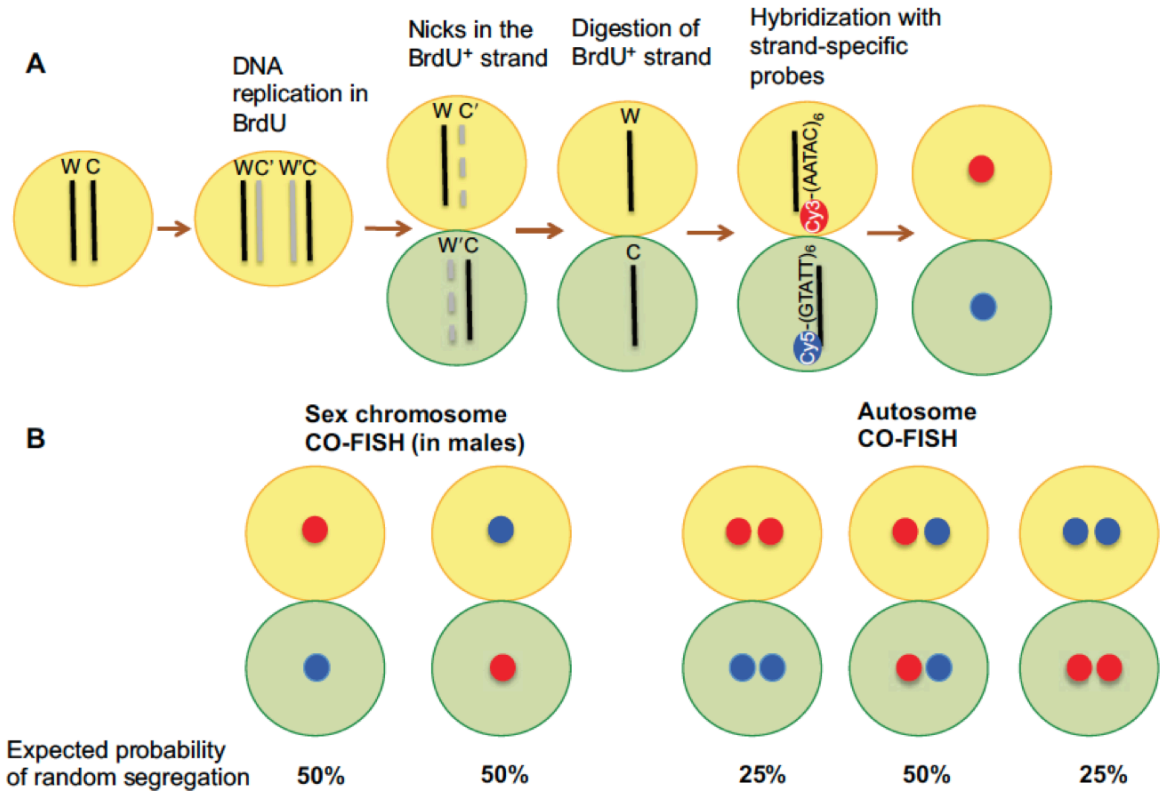


Figure 1.4 CO-FISH to identify sister chromatids of individual chromosomes. **A)** Schematic illustration of the CO-FISH protocol. Cy3–$(AATAC)_6$ and Cy5–$(GTATT)_6$ probes which are used to identify the sister chromatids of *Drosophila* Y chromosome are shown as an example. Upon DNA replication in the presence of BrdU, only newly synthesized strands will contain BrdU. After fixation and irradiation with UV, the BrdU-containing strands specifically will be nicked. Treatment with exonuclease III is used to remove the nicked strand, leaving the template strand intact. Upon hybridization of the CO-FISH probe, sister chromatids that contain $(AATAC)_6$ as a template versus $(GTATT)_6$ as a template can be distinguished. C, Crick strand and W, Watson strand are shown in black; C' and W' indicate newly synthesized strands after replication shown in grey. Green, differentiated cells; yellow, stem cells. **B)** Expected probabilities of CO-FISH signal pattern based on random segregation. In the case of sex chromosomes, if there is no bias in sister chromatid segregation, we expect that a stem cell inherits a red (Cy3-based) signal and a differentiated cell a blue (Cy5- based) signal in 50% of the cases, and the opposite, i.e. a blue signal in the stem cell and a red signal in the differentiated cell, in the other 50%. For autosomes, two signals per cell (originating from paternal and maternal chromosomes) are expected. If there is no bias or coordination between homologous chromosomes, stem cells would inherit either two red signals, a blue and a red signal or two blue signals with the probability of 25%, 50%, 25%, respectively. A skewed pattern would suggest the presence of biased segregation (see Figure 3.12).

Another study applied CO-FISH to mouse muscle satellite stem cells and reported that stem cells retain the template strands with a strong bias, thus supporting the ISH model (Rocheteau et al., 2012). It should be noted that these studies used probes that are targeted to centromeric and telomeric repeats that are present on all the chromosomes, and thus it was impossible to detect any non-random sister chromatid segregation that applies to only a small subset of the chromosomes.

1.4 Description of the *Drosophila melanogaster* male germline stem cell system

I used *Drosophila melanogaster* male germline stem cells (GSCs) as the model system in this work as it is one of the few well-characterized model systems that enable a detailed study of the regulation of stem cells. Here, I briefly describe the system architecture, and signaling pathways. At the apical tip of the testis, approximately 8–10 GSCs lie in a rosette around a cluster of post-mitotic support cells called hub cells, which represents a major component of the GSC niche (Davies and Fuller, 2008; Yamashita et al., 2010). *Drosophila* male GSCs always divide asymmetrically by keeping one daughter attached to the hub which retains stem cell identity and displacing the other away from the hub which starts differentiation (Gonialblast (GB)) (Yamashita et al., 2003)(Figure 1.4). GBs further undergo four mitotic divisions with incomplete cytokinesis, producing clusters of 16 interconnected spermatogonia, which produce spermatocytes that then commit to meiosis and ultimately differentiate into sperm. The testicular niche also contains a second stem cell type called cyst stem cells (CySCs). The function of CySCs,

together with the hub cells, is to create a niche for GSCs (Voog et al., 2008) (Figure 1.5). A pair of CySCs encapsulates a GSC and provides essential signals for GSC identity (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010). Similar to GSCs, CySCs also divide asymmetrically to generate a continuous supply of non-mitotic somatic support cells called cyst cells, which encapsulate and escort differentiating germ cells (Cheng et al., 2011). A pair of cyst cells envelops each GB and its progeny, providing signals that mediate differentiation. GSCs and CySCs are physically attached to the hub cells by adherens junctions consisting of *Drosophila* epithelial (DE)-cadherin and *β*-catenin/Armadillo, which are concentrated at the cell cortex adjacent to the hub (Yamashita et al., 2003). Indeed, cell adhesion between GSCs and hub cells, as well as between CySCs and hub cells, has been demonstrated to be required for stem cell maintenance (Voog et al., 2008).

1.4.1 Signaling pathways in GSC niche

The hub supports self-renewal of GSCs and CySCs by secreting a short-range signaling ligand, unpaired (Upd), which activates the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway in neighboring cells (Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). GSCs and CySCs mutant for *stat92E (STAT)* or *hop (JAK;* encoded by the *hopscotch* gene) lose their ability to self-renew and instead differentiate. In addition, overexpression of Upd in early germ cells (GSCs or spermatogonia) or in early somatic cells (CySCs or cyst cells) causes overproliferation of

undifferentiated, stem-like cells and results in tumor formation.

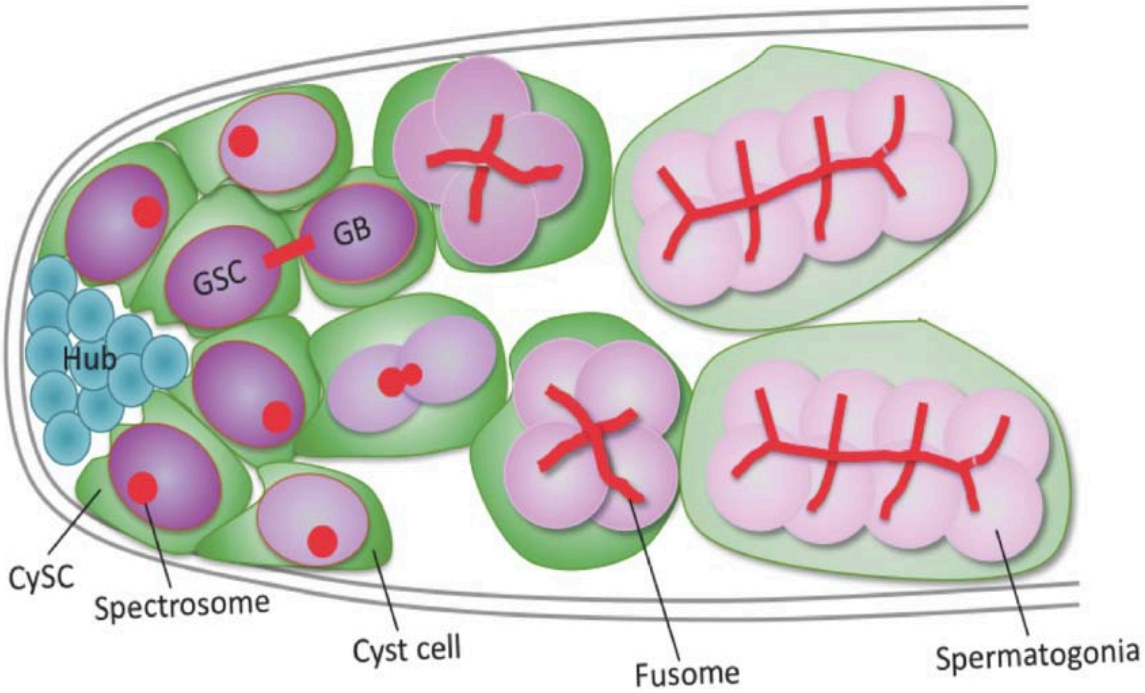


Figure 1.5 *Drosophila* male germline stem cell system. At the apical tip of the testis, germline stem cells (GSCs), and cyst stem cells (CySCs) are physically attached to the hub cells via an DE-cadherin-based adherens junction. GSCs divide asymmetrically where one of the daughters maintains stem cell identity and the other initiates differentiation as a gonialblast (GB). GBs further undergo four synchronous divisions with incomplete cytokinesis, producing clusters of 16 interconnected spermatogonia, which give rise to spermatocytes and ultimately to sperm. A pair of CySCs encapsulates a GSC and provides essential signals for GSC identity. CySCs divide asymmetrically to self-renew and produce somatic support cells called cyst cells. A pair of cyst cells envelop each GB and its progeny, providing signals mediating differentiation.

Currently, the direct downstream targets of the JAK–STAT pathway that specify GSC identity are not well studied, although candidate genes have been described through microarray analysis (Terry et al., 2006). Recent studies have demonstrated that the transcriptional repressor *zinc finger-homeodomain transcription factor 1* (*zfh-1*) is a critical downstream target of the JAK–STAT pathway in CySCs (Leatherman and Dinardo, 2008). Strikingly, overexpression of

Zfh-1 or forced expression of constitutively active JAK in CySCs resulted in overproliferation of not only CySCs but also GSCs. In contrast, ectopic expression of an active form of JAK tyrosine kinase in the germline did not cause massive proliferation of GSCs or CySCs (Leatherman and Dinardo, 2008). Furthermore, GSCs mutant for STAT can be maintained as long as CySCs have active Zfh-1, demonstrating CySCs as a critical component of the GSC niche (Leatherman and Dinardo, 2010). GSCs were suggested to require STAT activity only to correctly orient toward and to adhere to the hub cells. Zfh-1 appears to instruct GSC self-renewal via TGF-β-dpp/gbb signaling (Leatherman and Dinardo, 2010). Decapentaplegic (dpp) and glass bottom boat (gbb) are normally expressed in hub cells and CySCs, which ultimately lead to shutoff of Bam (Bag-of-marbles, a master regulator of differentiation) in germline, contributing to GSC self-renewal (Kawase et al., 2004; Chen et al., 2003; McKearin et al., 1995). However, interestingly, overexpression of dpp does not cause GSC tumors but leads to spermatogonial overproliferation (Kawase et al., 2004; Schulz et al., 2004; Shivdasani and Ingham, 2003), implying that there is an additional factor(s) downstream of Zfh-1 that function with TGF-β signaling to confer GSC identity.

1.4.2 Orientation of the spindle by the positioning of centrosomes

GSCs divide asymmetrically by orienting the mitotic spindle perpendicular to the hub–GSC interface. Adherens junctions concentrated at the GSC cortex adjacent to the hub, along with ademomatous polyposis coli 2 (APC2), provide a polarity cue toward which GSCs orient throughout the cell cycle (Inaba et al.,

2010; Yamashita et al., 2003). In G1, the single centrosome in each GSC localizes near the cell cortex where the cell attaches to the hub. When the duplicated centrosomes are separated, one of the centrosomes stays next to the hub while the other migrates to the opposite side of the cell (Figure 1.6). This stereotyped position of the centrosomes in turn orients the mitotic spindle perpendicular to the GSC–hub interface, leading to asymmetric division. Interestingly, the mother centrosome normally remains adjacent to the hub and is inherited by the GSC, whereas the newly duplicated centrosome migrates to the opposite side of the cell and is inherited by the GB (Yamashita et al., 2007) (Figure 1.5). This suggests that male GSCs retain the original (very old) centriole for a long time probably from the time the GSC population first arose during development. Indeed, centrosomes marked by a transient expression of a centriolar marker green fluorescent protein-pericentrin/AKAP450 (GFP-PACT) during early development were retained in GSCs even in adult stage (Yamashita et al., 2007).

The higher capacity of the mother centrosome to anchor astral microtubules may be the underlying cellular mechanism by which GSCs inherit the mother centrosome during division. In GSCs, centrosomes are separated unusually early, right after duplication, rather than at the G2/M transition: the mother centrosome appears to retain robust astral microtubules throughout the cell cycle, whereas the daughter centrosome migrating to the opposite side of the cell has few associated astral microtubules until late in G2, near the onset of mitosis. Consistently, positions of mother and daughter centrosomes as well as spindle orientation were randomized in GSCs mutant for *centrosomin (cnn),* which have severely impaired

astral microtubules as a result of defective pericentriolar material (Megraw and Kaufman, 2000; Megraw et al., 2002; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). In Chapters 3 and 4, I discuss the role of mother centrosome in non-random sister chromatid segregation during GSC division.

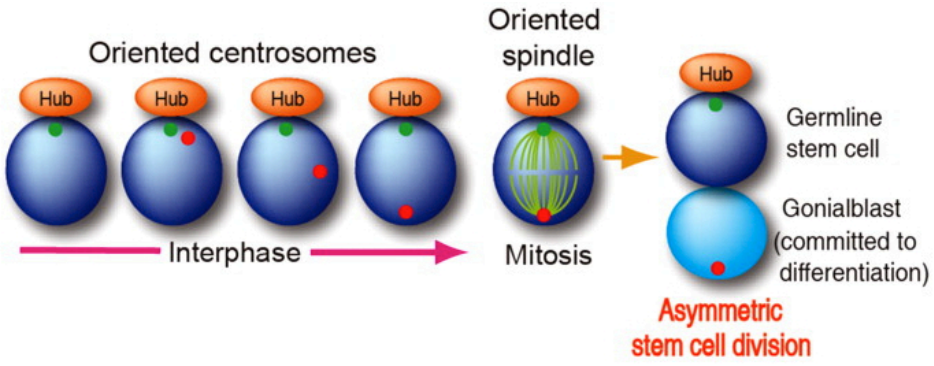


Figure 1.6 Asymmetric cell division in *Drosophila* male germline stem cells. GSCs always orient their spindle perpendicular to the hub. This stereotypical orientation of mitotic spindle is prepared by the precisely controlled positioning of the centrosomes during interphase. Specifically, the mother centrosome (red) normally remains adjacent to the hub and is inherited by the GSC, where as the daughter centrosome (purple) migrates to the opposite side of the cell and is inherited by the gonialblast (GB)

The above results suggest that male GSCs have adopted cellular mechanisms that maintain stereotypical centrosome position and orient the mitotic spindle to tightly regulate the asymmetric outcome of stem cell divisions within the niche. Interestingly, a recent study demonstrated that mutants of *DSas-4,* a core component of centriole, normally orient mitotic spindle in male GSCs, despite the complete lack of centrioles (and thus centrosomes) (Riparbelli and Callaini, 2011). It has also been recently shown that the spectrosome is located at the apical side of the GSC anchoring the spindle pole in *DSas-4* mutant male GSCs (Yuan H et al., 2012), which is reminiscent of spindle orientation mechanism in female GSCs

(Deng and Lin, 1997), suggesting that a parallel mechanism might compensate the loss of the centrosomes during asymmetric stem cell division.

1.4.3 Effect of aging on centrosome and spindle orientation

A decrease in stem cell number or activity may lead to tissue degeneration associated with age and disease. Indeed, age-dependent decrease in *Upd* expression in the hub has been reported to contribute to GSC loss with advanced age (Boyle et al., 2007). Stem cell intrinsic and extrinsic changes appear to be general characteristics of stem cell aging, as is observed in mammalian stem cells (Conboy et al., 2003; Molofsky et al., 2006; Rossi et al., 2005). In addition, changes in stem cell orientation with respect to the niche, which precedes the decrease in GSC number, contribute to the decline in spermatogenesis: before the decrease in GSC number becomes significant, GSCs already slow down their proliferation due to increased centrosome mis-orientation (Cheng et al., 2008). GSCs containing mis-oriented centrosomes accumulate progressively with age and these GSCs are arrested or delayed in the cell cycle and do not undergo mitosis. As a result, as *Drosophila* males age, a significant fraction of GSCs becomes arrested. Strikingly, this cell cycle arrest appears to be transient and GSCs re-enter the cell cycle upon correction of centrosome orientation. The latter implies that a novel checkpoint mechanism exists that blocks progression into mitosis unless a centrosome is properly situated next to the attachment to the hub.

Remarkably, many of the mis-oriented GSCs originate from the de-differentiation of spermatogonia, a mechanism thought to be responsible for

maintaining the stem cell population over extended periods of time (Brawley and Matunis, 2004; Kai and Spradling, 2004). Throughout *Drosophila* adulthood, individual GSCs are lost at a certain rate (Wallenfang et al., 2006; Xie and Spradling, 1998). De-differentiation of partially differentiated spermatogonia to replace lost stem cells may be especially important in the male germline, because mis-oriented spindles, or symmetric stem cell division, are rarely observed in wild-type GSCs. Such de-differentiated GSCs show a high incidence of mis-oriented centrosomes and undergo cell cycle arrest until proper centrosome orientation toward the hub is reestablished, increasing the average cell cycle length of GSCs, even if none of them are permanently arrested (*i.e.,* quiescent). This observation might be correlated to the fact that germ cells that commit to differentiation do not inherit the 'very old' centrosome and that de-differentiated GSCs have lost their 'very old' centrosome once they have committed to differentiation (Cheng et al., 2008). In Chapters 3 and 4, I revisit this issue of whether there are any differences between original GSCs and de-differentiated GSCs.

1.5 Outline of the thesis

This thesis explores asymmetries in sister chromatid segregation patterns during GSC division to elucidate the mechanisms and biological relevance of non-random sister chromatid segregation.

In Chapter 2, I describe my studies of sister chromatid segregation during *Drosophila* male GSC division wherein I used the pulse-chase strategy to follow the segregation of old *vs.* new strands through multiple rounds of GSC division.

Specifically, I used BrdU to label newly synthesized strands and followed the segregation of BrdU-labeled chromatids during the label-free chase period. During the chase period, I observed that in a majority of cases (95% at 24 hour chase period), the label was equally distributed to stem cell - daughter pairs until the label is finally diluted to undetectable levels. This finding strongly argues against the immortal strand hypothesis in male GSCs (Yadlapalli et al., 2011).

In Chapter 3, I describe my follow-up work where I adapted the CO-FISH technique combined with chromosome-specific probes to study sister chromatid segregation at single chromosome resolution. Using this method, I found that sister chromatids of sex chromosomes, but not autosomes, are non-randomly segregated during GSC divisions (Yadlapalli and Yamashita, 2013). These results provide the first direct evidence that sister chromatids of certain chromosomes (not the entire genome) can be distinguished and segregated non-randomly during asymmetric stem cell divisions. Such chromosome-specific non-random segregation cannot be detected by pulse-chase experiments using BrdU, raising the possibility that chromosome-specific non-random segregation might have remained unresolved in many systems studied to date. Later on, I also discuss my findings that shed light on the complex mechanisms involved in this fascinating process of non-random sister chromatid segregation. This study offers the first genetically tractable experimental model system to study chromosome strand segregation pattern at single chromosome strand resolution. These studies may open up an exciting new venue of research to understand stem cell self-renewal/differentiation through asymmetric chromosome segregation.

This chapter is revised from the content published as:

Yadlapalli S, Yamashita YM (2013) DNA asymmetry in stem cells – immortal or mortal?, Journal of Cell Science. 2013 Sep 15;126(Pt 18):4069-4076

Yadlapalli S, Yamashita YM (2012) Spindle positioning in the stem cell niche. Wiley Interdiscip Rev Dev Biol. 2012 Mar-Apr;1(2):215-30

Chapter 2

Drosophila Male Germline Stem Cells do not Follow the Immortal Strand Model

This chapter presents the content published as:

Yadlapalli S, Cheng J, Yamashita YM. (2011) Drosophila male germline stem cells do not asymmetrically segregate chromosome strands. Journal of Cell Science 2011 Mar 15;124 (Pt 6):933-9

2.1 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 male GSCs in the *Drosophila* testis do not follow the immortal strand model despite asymmetrically segregating centrosomes.

2.2 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 (Cairns, 1975). 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.

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 (Karpowicz et al., 2005), mammary epithelial cells (Smith, 2005), fibroblasts (Merok et al., 2002), skeletal muscle satellite cells (Conboy et al., 2007), 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 (Falconer et al., 2010). However, judging from position in the crypt, such asymmetry was observed in differentiating cells as well as in stem cells, 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). The authors proposed that cells asymmetrically segregate

other information such as epigenetic memories by asymmetric segregation of chromosome strands (Falconer et al., 2010; Lansdorp, 2007).

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; Yennek and Tajbakhsh, 2013).

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., 2003; Yamashita et al., 2010), 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) (Figure 2.3A,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). 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 the daughter centrosome migrates to the opposite side of the cell and is inherited by the GB (Figure 1.5) (Yamashita et al., 2007). 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 as they 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 *Drosophila* male GSCs do not follow the immortal strand model.

2.3 Results

2.3.1 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 (Figure 2.1). 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 (Figure 2.1, 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 (Figure 2.2). In contrast, if GSCs randomly segregate their chromosome strands, BrdU would be segregated to both daughter cells in the second division (Figure 2.1, second division, random strand segregation).

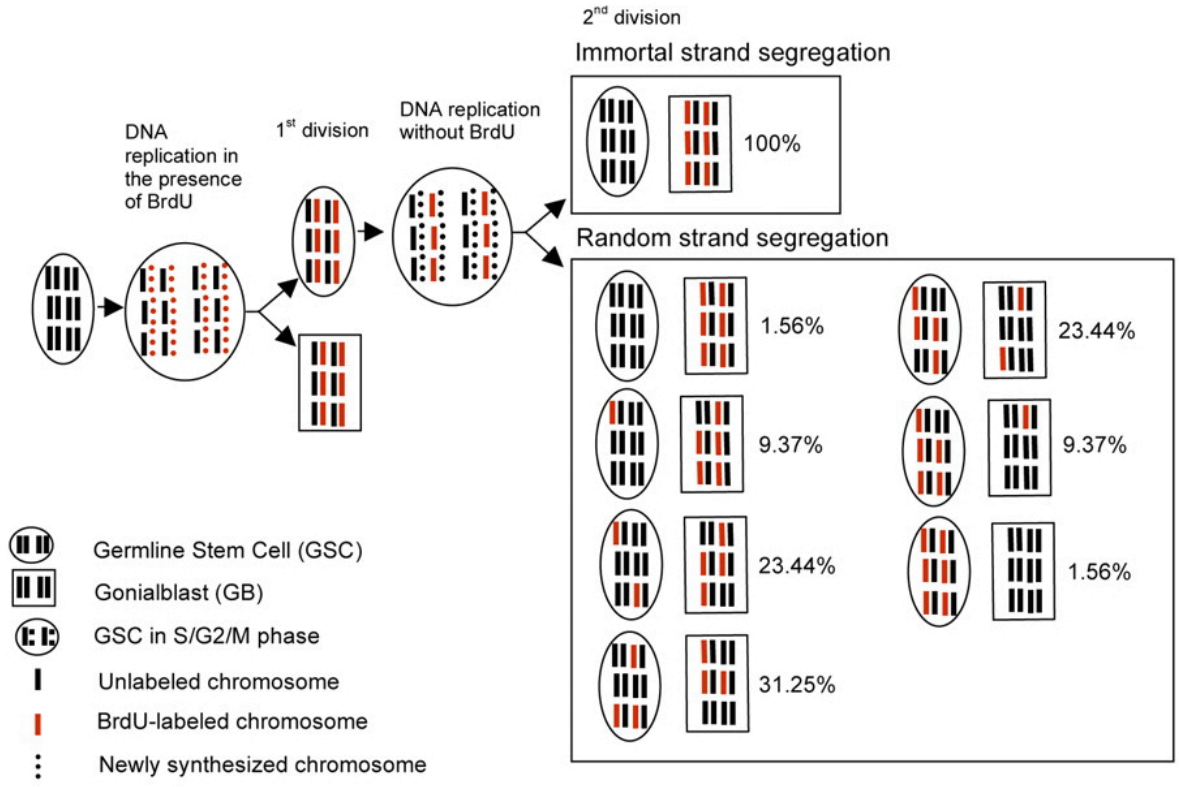


Figure 2.1 Model of DNA strand segregation during the BrdU-pulse and chase period. The first division in the chase period will be symmetric irrespective of segregation mode, whereas the second division can be used to distinguish between the two different models. The model is based on six chromosomes in the *Drosophila* cell, neglecting the contribution of the very small fourth chromosomes.

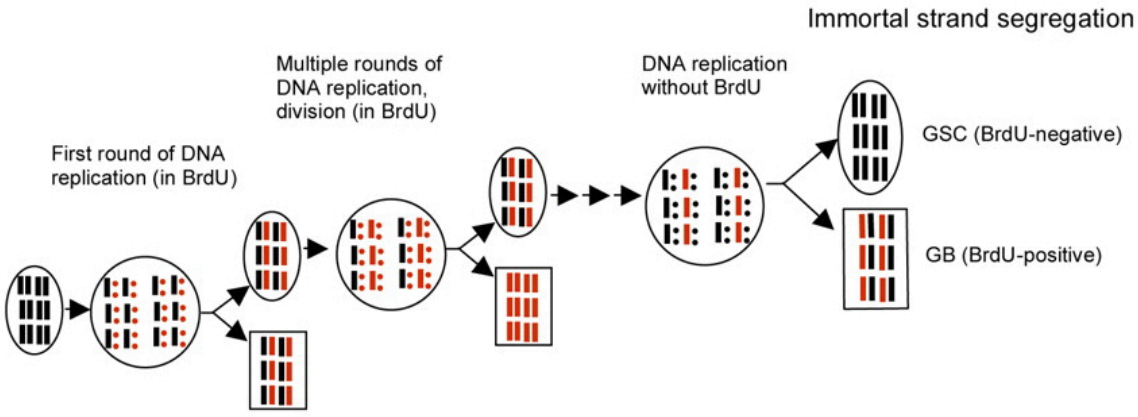


Figure 2.2 Model of BrdU segregation pattern based on ISH, if cells undergo multiple rounds of DNA replication in the presence of BrdU. Multiple rounds of DNA replication in the presence of BrdU would not prevent the detection of immortal strand segregation.

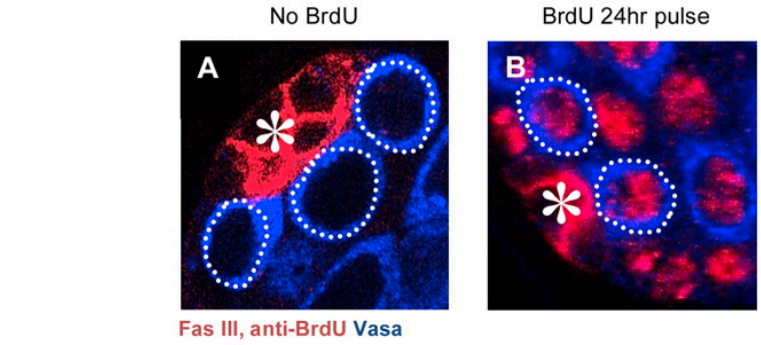
Importantly, asymmetric segregation of the BrdU label would sometimes be observed by chance as a result of random segregation. With random chromosome strand segregation, the BrdU label would be diluted stochastically over time (on average, by half with each round of division).

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 (Figure 2.3 A-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 mis-oriented 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 (Figure 2.3D).

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 (Figure 2.5A) 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 (Figure 2.5B), 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.



C

Experiment	Hour 0	Hour 4	Hour 12	Hour 16	Hour 24
1	0	23.6	58.93	90.3	93.5
2	0	19.6	47.76	93	93.4
3	0		39.41	91.2	97.1
Mean ± s.d.	0 ± 0	21.6 ± 2.8	48.7 ± 9.7	91.5 ± 1.4	94.7 ± 2.1

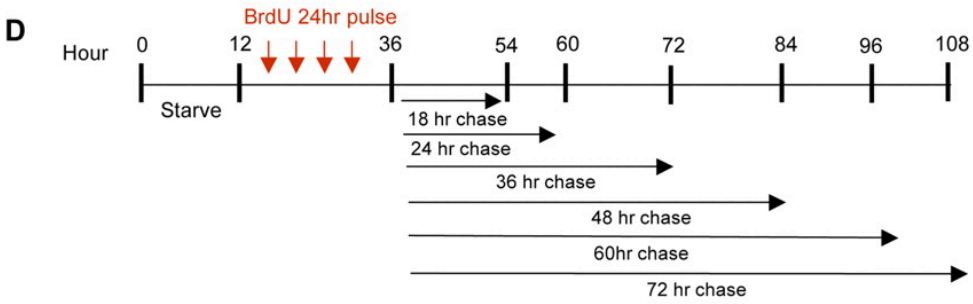


Figure 2.3 Experimental schemes to address the ISH in male GSCs. A,B) Examples of BrdU staining in GSCs (encircled by dotted lines) from flies cultured in the absence (A) or presence (B) of BrdU for 24 hours. Red, Fas III and BrdU; blue, Vasa (germ cells); * indicates the hub. **C)** Outcome of BrdU 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.

2.3.2 GSCs do not follow the immortal strand model

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) (Figure 2.3D). 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 (Figure 2.2). 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 (Figure 2.5A, Figure 2.6). Consistent with this result, in the majority of anaphase and telophase GSCs, BrdU was segregated to both daughter cells (Figure 2.5B; 84% were symmetric, 25 anaphase/telophase GSCs scored). These data suggest that, male GSCs do not follow the immortal strand model.

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).

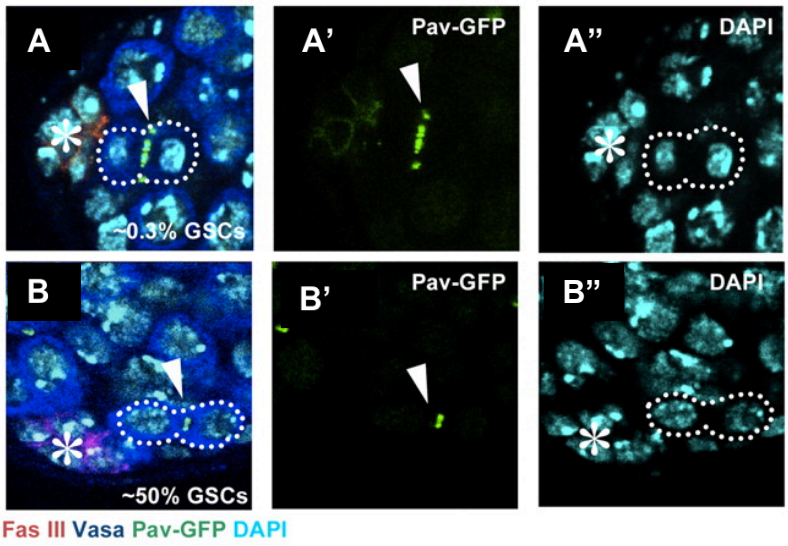


Figure 2.4 Pavarotti-GFP to identify GSC-GB pairs. A.B) Localization of Pav–GFP in male GSCs during telophase (A) and after mitosis (B). Red, Fas III; green, Pav–GFP; blue, Vasa (germ cells); light blue, DAPI; * indicates the hub; arrowheads point to Pav–GFP-marked contractile ring/midbody.

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 (Figure 2.1). 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% (Figure 2.7A). 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 (Figure 2.7C), 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 (Figure 2.1, Figure 2.7A), we observed that, indeed, some GSCs appeared to exhibit asymmetric BrdU segregation (Figure 2.5C,D, Figure 2.6). However, the pattern of asymmetric segregation in these cases was random; in some cases a BrdU-negative GSC was connected with a BrdU-positive GB (Figure 2.5C), and in other cases a BrdU-positive GSC was paired with BrdU-negative GB (Figure 2.5D). 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, Figure 2.6). 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.

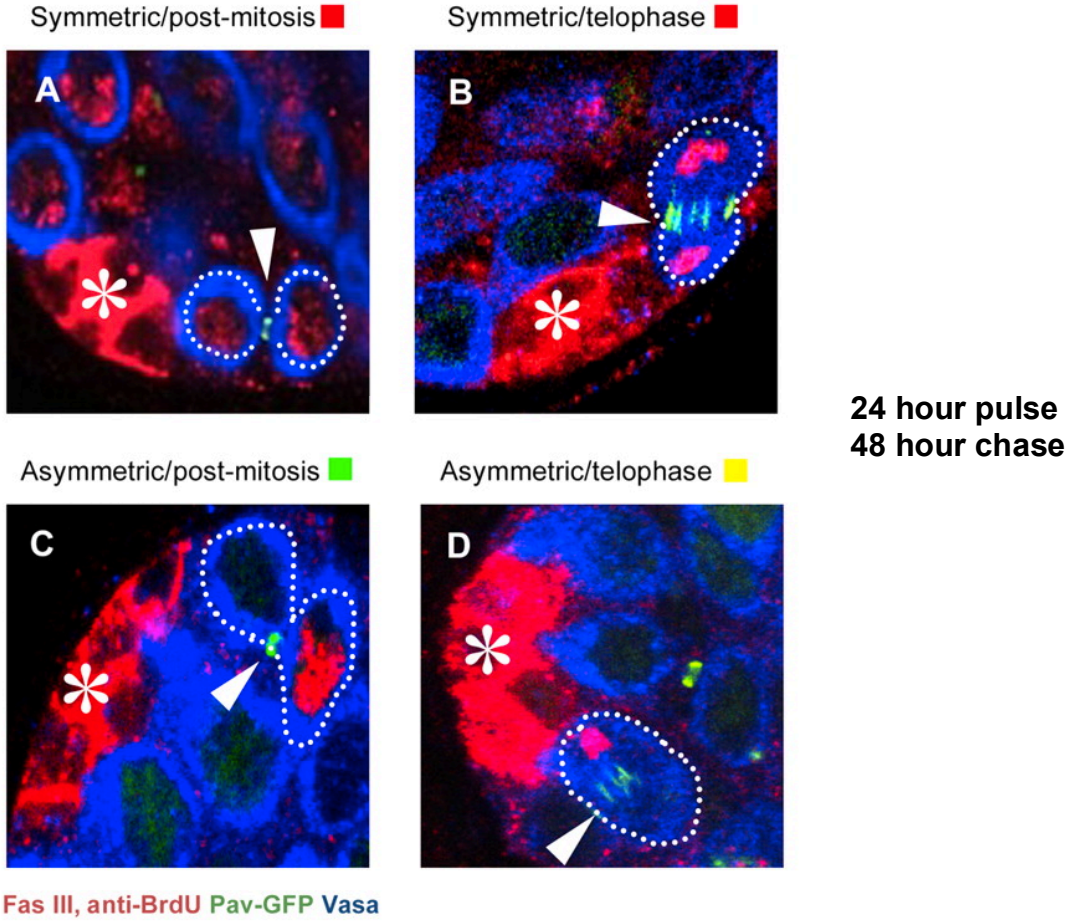


Figure 2.5 *Drosophila* male GSCs do not follow the immortal strand model. A–D) Examples of BrdU segregation in anaphase/telophase or post-mitotic GSCs after 24 hour pulse (BrdU) and 48 hour chase (without BrdU). (A) Symmetric BrdU segregation in a post-mitotic GSC-GB pair (encircled by dotted lines). (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).

We have shown that GSCs can be generated via de-differentiation 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 de-differentiation 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 de-differentiation 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.

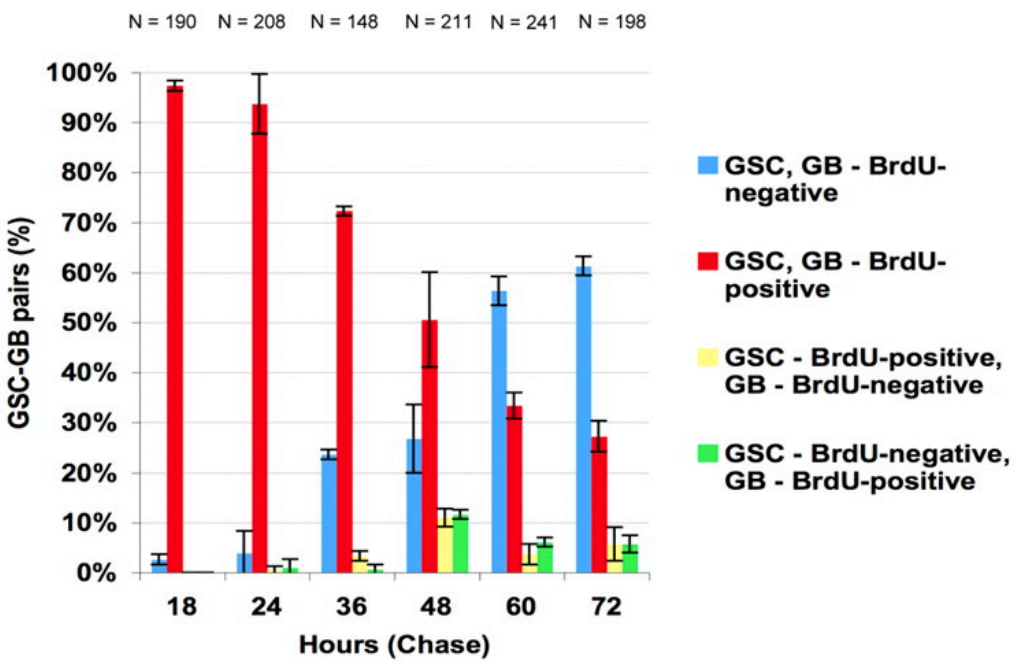


Figure 2.6 Summary of BrdU segregation pattern during chase period. Through out the chase period, majority of GSC-GB pairs showed symmetric BrdU label distribution. As predicted by the modeling, the percentage of asymmetric looking pairs increases with time, reaches a peak around 48 hrs and then decreases until BrdU label eventually dilutes out. N, number of GSC-GB pairs/anaphase-telophase GSCs scored.

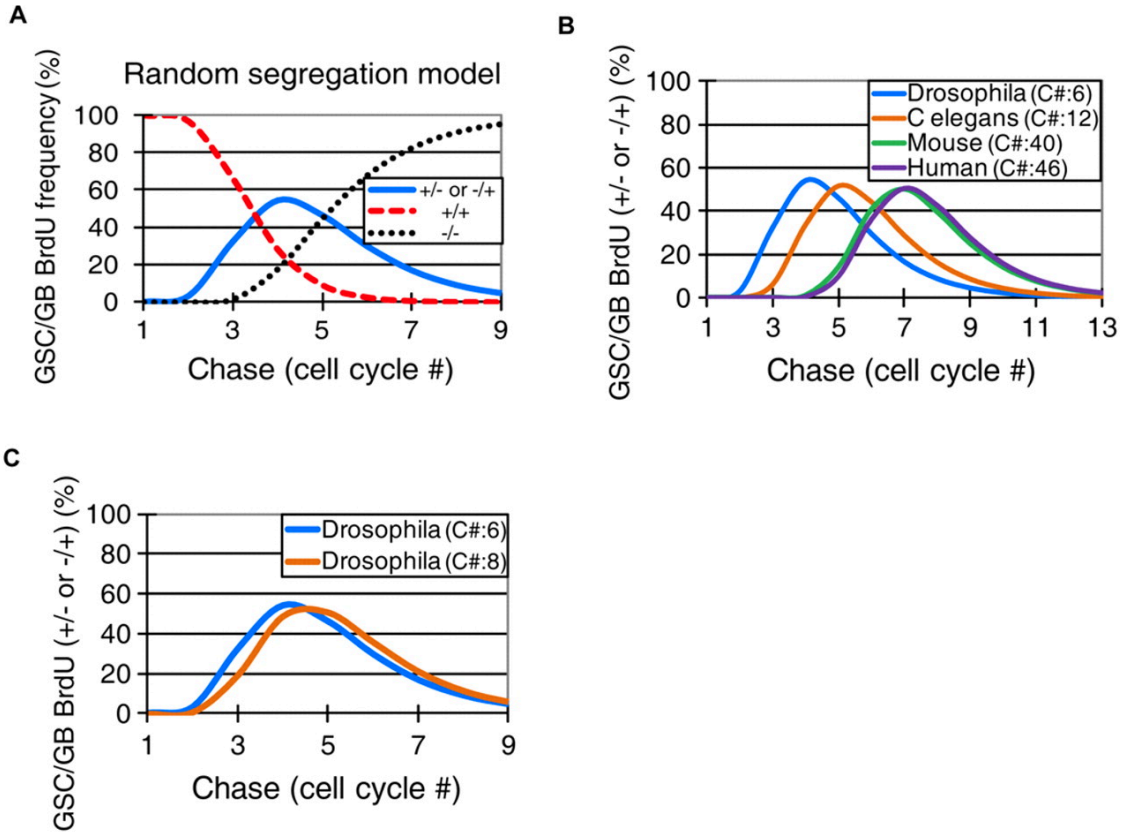


Figure 2.7 Model of BrdU segregation pattern during the chase period, based on the random segregation model. A) Simulation of BrdU segregation pattern in cells with six chromosomes (*Drosophila*). Apparent asymmetric segregation reaches ~50% in the fourth cell cycle, as a result of random segregation. **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.

2.4 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 (Figure 2.7B).

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). 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.

2.5 Materials and Methods

2.5.1 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.

2.5.2 Immunofluorescence staining

Samples were fixed for 30–60 minutes with 4% formaldehyde in PBS, permeabilized for 30 minutes in PBST (0.1% Triton X-100 in PBS), 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).

2.5.3 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, since the 4th 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 based on the assumption that each individual chromosome segregates independently from one another.

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

where $\binom{m}{k} = \frac{m!}{k!(m-k)!}$ is the binomial coefficient. Therefore, the probability of GSC/GB BrdU segregation at cell cycle number *N+1* can be calculated as follows:

$$P_{-/-,N+1} = p_{0,N}$$

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

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

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

Because $\binom{m}{0} = \binom{m}{m} = 1$, $P_{-/+,N+1}$ equals $P_{+/-,N+1}$.

2.6 Acknowledgements

We thank Adelaide Carpenter and David Glover (University of Cambridge, Cambridge, UK), the Bloomington Stock Center, and the Developmental Studies Hybridoma Bank for reagents. We also thank Sean Morrison and Shenghui He (University of Michigan, Ann Arbor, MI), and the Yamashita laboratory members for discussion and comments on the manuscript. This research was supported by

the Searle Scholar Program and NIH R01GM086481-01 (to Y.M.Y.).

Chapter 3

Chromosome-specific Non-random Sister Chromatid Segregation in *Drosophila* Male Germline Stem Cells

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3.1 Summary

Adult stem cells undergo asymmetric cell division to self-renew and give rise to differentiated cells that comprise mature tissue. We developed the CO-FISH (chromosome orientation fluorescence in situ hybridization) technique with single chromosome resolution and show that sister chromatids of X and Y chromosomes, but not autosomes, are segregated non-randomly during asymmetric divisions of *Drosophila* male germline stem cells (GSCs). This provides the first direct evidence that sister chromatids of certain chromosomes can be distinguished and segregated non-randomly during asymmetric stem cell divisions. We further show that the centrosome, SUN-KASH nuclear envelope proteins, and *Dnmt2* are required for non-random sister chromatid segregation. Moreover, we show that sister chromatid segregation is randomized in GSC overproliferation and de-differentiated GSCs. We propose that non-random sister

chromatid segregation may serve to transmit distinctive information carried on two sister chromatids in asymmetrically dividing stem cells.

3.2 Introduction

Adult stem cells from diverse systems are known to divide asymmetrically to produce one stem cell and one differentiating cell, maintaining tissue homeostasis (Morrison and Kimble, 2006). 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. GSCs divide asymmetrically by orienting the mitotic spindle perpendicular to the hub; the daughter that remains attached to the hub retains GSC identity, whereas the daughter that is displaced from the hub starts differentiation as a gonialblast (GB) (Yamashita et al., 2003).

The immortal strand hypothesis proposes that stem cells retain a template copy of genomic DNA to avoid replication-induced mutations(Cairns, 1975; Potten et al., 2002; Rando, 2007). It was also proposed that certain cells may segregate sister chromatids non-randomly to transmit distinct epigenetic information(Falconer et al., 2010; Lansdorp, 2007). However, it remains unclear how sister chromatids are distinguished and segregated non-randomly and what purpose non-random sister chromatid segregation may serve.

3.3 Results

3.3.1 Sister chromatids of X and Y chromosomes are segregated non-randomly during *Drosophila* male GSC divisions.

To examine the pattern of sister chromatid segregation at single chromosome resolution, we adapted the CO-FISH (chromosome orientation fluorescence in situ hybridization) protocol, which allows strand-specific identification of sister chromatids (Bailey et al., 2004; Falconer et al., 2010) combined with chromosome-specific probes that are available for *Drosophila* chromosomes (Abad et al., 1992; Brutlag et al., 1978; Carmena et al., 1993; Lohe et al., 1993; Makunin et al., 1999) (Figure 3.1a). In our CO-FISH protocol, cells in testes are allowed to replicate once in the presence of BrdU; as a result, each sister chromatid contains a BrdU-negative template strand and a BrdU-positive newly synthesized strand (Figure 3.1b). Following BrdU incorporation, cells are allowed to complete mitosis in BrdU-free media, such that the sister chromatids are segregated into the GSC-GB pair. Based on previous studies that determined the GSC cycle length to be 12–16 hours (Yadlapalli et al., 2011), we fed flies with BrdU for approximately 10 hours, followed by a period in non-BrdU media (~10 hours). The testes are then dissected, fixed, and treated with ultraviolet (UV) irradiation, which creates nicks preferentially on the BrdU-containing strands(Cecchini et al., 2005). When these cells are treated with exonuclease III, only the template strand is left intact, whereas the BrdU-containing strands are removed (Figure 3.1b). The remaining template strands can be identified using differentially labelled CO-FISH probes, e.g., Cy3-$(AATAC)_6$ and Cy5-$(GTATT)_6$ for

the Y chromosome (Figure 3.1a). With these probes, it can be determined which cell inherited which sister chromatid.

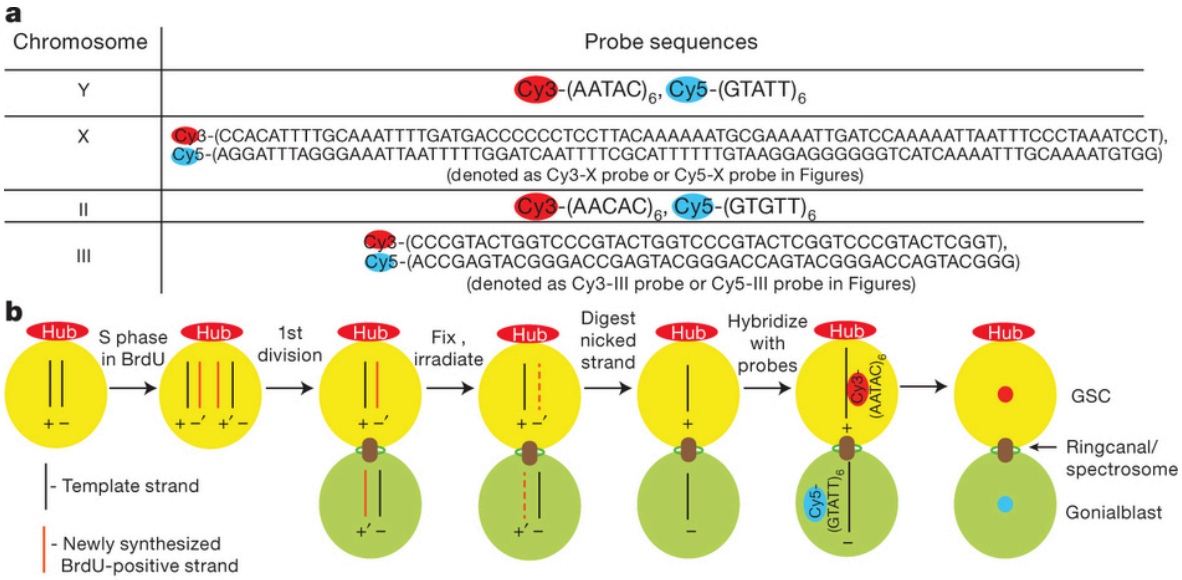


Figure 3.1 CO-FISH using *Drosophila* probes. a) Chromosome-specific probes used in this study. **b)** Schematic diagram of the CO-FISH procedure. Cy3- and Cy5-labelled probes for the Y chromosome are shown as an example. Green fluorescent protein-labelled PAVAROTTI (PAV–GFP) (midbody/ring canal), SH–ADD–Venus or anti-ADD antibody (spectrosome) was used to identify GSC–gonialblast pairs.

Because GSC divisions are not synchronized in *in vivo* experiments, there is variation in the number of S phases and mitoses that each GSC undergoes. However, only GSCs that have undergone one S phase and one mitosis are relevant to analysis. Therefore, to exclude irrelevant GSC-GB pairs, we limited scoring to GSC-GB pairs that contain complementary CO-FISH signals (e.g., GSC with red signal and GB with blue, or *vice versa*). The major probable scenarios are summarized in Figure 3.2. For example, if GSCs have undergone S phase in the presence of BrdU, but have not undergone mitosis, complementary CO-FISH signals will appear in one cell (Figure 3.2a, b) and will be excluded from analysis.

If GSCs did not undergo DNA replication during the BrdU labelling period, both sister chromatids will be intact after exonuclease III treatment, yielding no CO-FISH signal. Throughout this study GSC-GB pairs were identified either by Pav-GFP (Minestrini et al., 2002), which labels the ring canal between the GSC and GB, or Adducin-like antibody/ShAdd-Venus (Petrella et al., 2007), which labels the spectrosome formed between the GSC and GB.

Using this method, we examined the pattern of sister chromatid segregation during GSC divisions, and found that sister chromatids of the Y chromosome are inherited with a strong bias during GSC division: in ~85% of cases, the GSC inherited the sister chromatid of the Y chromosome that contains the $(GTATT)_6$ satellite sequence as a template (and thus hybridizes to the Cy3-$(AATAC)_6$ probe). As a result, we observed red signal (Cy3-$(AATAC)_6$) in GSCs and blue signal (Cy5-$(GTATT)_6$) in GBs in approximately 85% of the GSC-GB pairs (Figure 3.4a, b). Using X chromosome-specific probes, we found that the X chromosome shows a similar biased segregation pattern (Figure 3.4c, d). Essentially the same results were obtained when the Cy5 probe for the X chromosome was replaced with a probe consisting of a second unique X chromosome sequence that is not complementary to the Cy3-labelled probe (Figure 3.5). Importantly, non-random sister chromatid segregation was observed even at day 5 (~82%:18%; N>30 GSC-GB pairs for X and Y chromosome CO-FISH), a similar level of bias as that observed at day 0, when most of experiments in this study were carried out. This result suggests that GSCs have a strong tendency to inherit a particular sister chromatid.

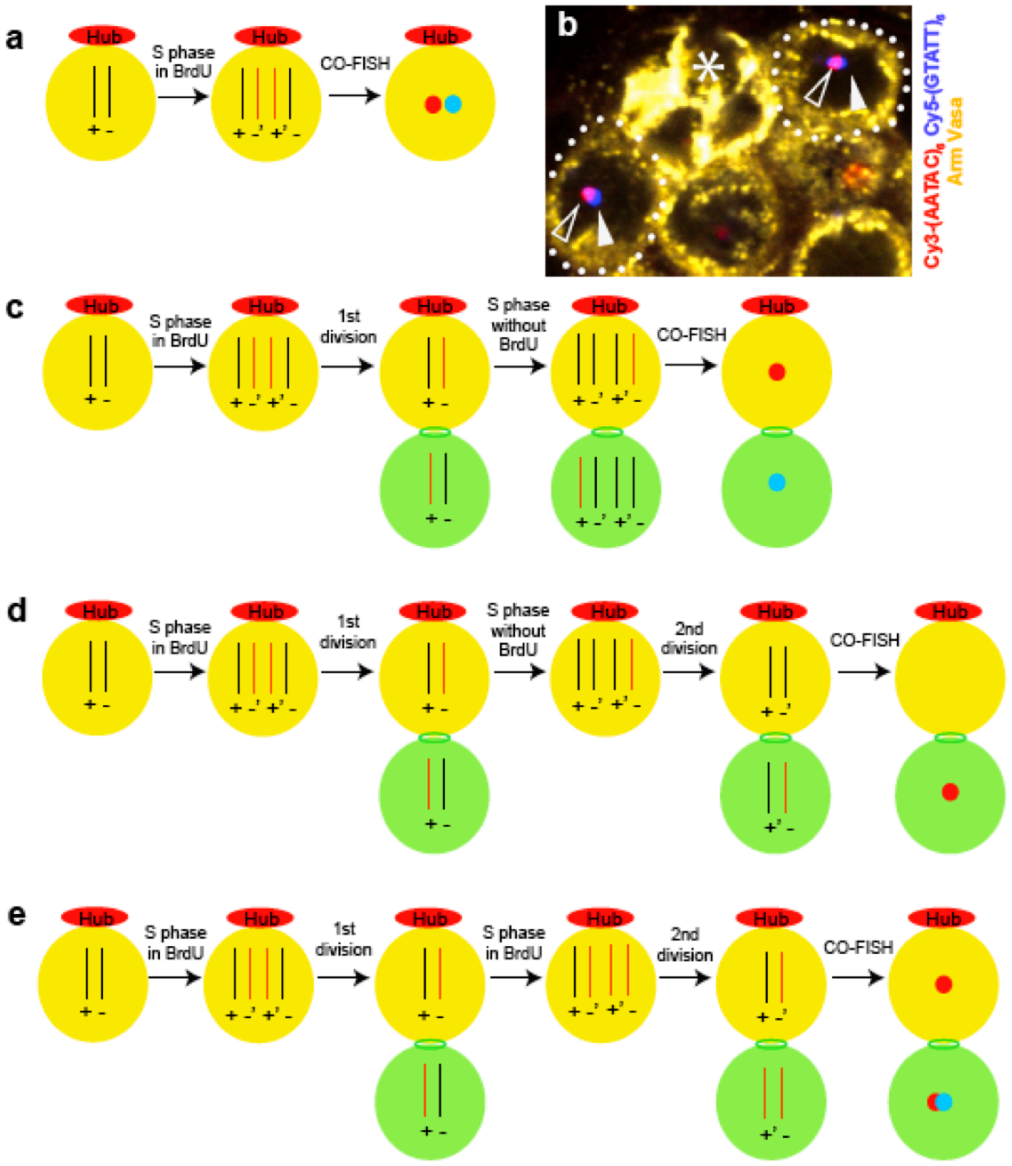


Figure 3.2 Cell cycle progression during BrdU-feeding and chase period and CO-FISH outcomes. a) S phase (+BrdU) with no mitosis results in two complementary CO-FISH signals in a GSC. **b)** An example of CO-FISH image resulting from condition described in a). **c)** S phase (+BrdU)➔mitosis➔S (no BrdU)➔ mitosis results in complementary CO-FISH signals in the GSC and GB. **d)** S phase (+BrdU)➔mitosis➔S phase (–BrdU)➔ mitosis results in GSC or GB lacking signal. **e)** S phase (+BrdU)➔mitosis➔S phase (+BrdU)➔mitosis results in one cell containing a single signal and the other cell containing overlapping signals (see Figure 3.3 for explanation). Only the case described in c) is relevant to the analysis. After optimizing the BrdU pulse-chase period (see main text), we did not see many cases of e), which is the result of too long a pulse period. BrdU-positive strands are represented by red lines, and BrdU-negative strands by black lines.

Two major possible scenarios can explain this. In the first scenario, approximately 85% of GSCs inherit the “red strand” (i.e. the sister chromatid 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 immortal strands of the X and Y chromosomes. 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, sister chromatids of the X and Y chromosomes in GSCs are not immortal, and “template strands” are switched 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 BrdU-containing media. The results of this experiment (see Figure 3.6, Figure 3.7 for details) clearly indicate that although sister chromatids of X and Y chromosomes are segregated with a strong bias, they are not immortal.

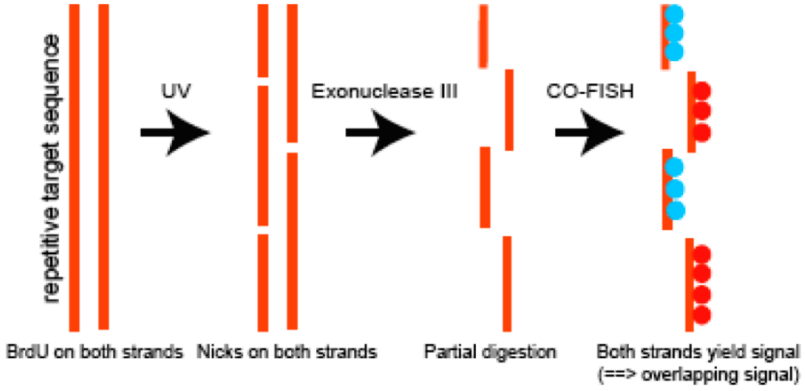


Figure 3.3 CO-FISH with two BrdU-positive sister chromatids. Exonuclease III uses double-stranded DNA as a substrate to remove a nicked strand, yielding single-stranded DNA. Single-stranded DNA is not a good substrate for exonuclease III, and remains undigested, even if nicked. Therefore, the CO-FISH procedure starting with BrdU+/+ chromatids results in partial digestion of each strand and overlapping CO-FISH signals.

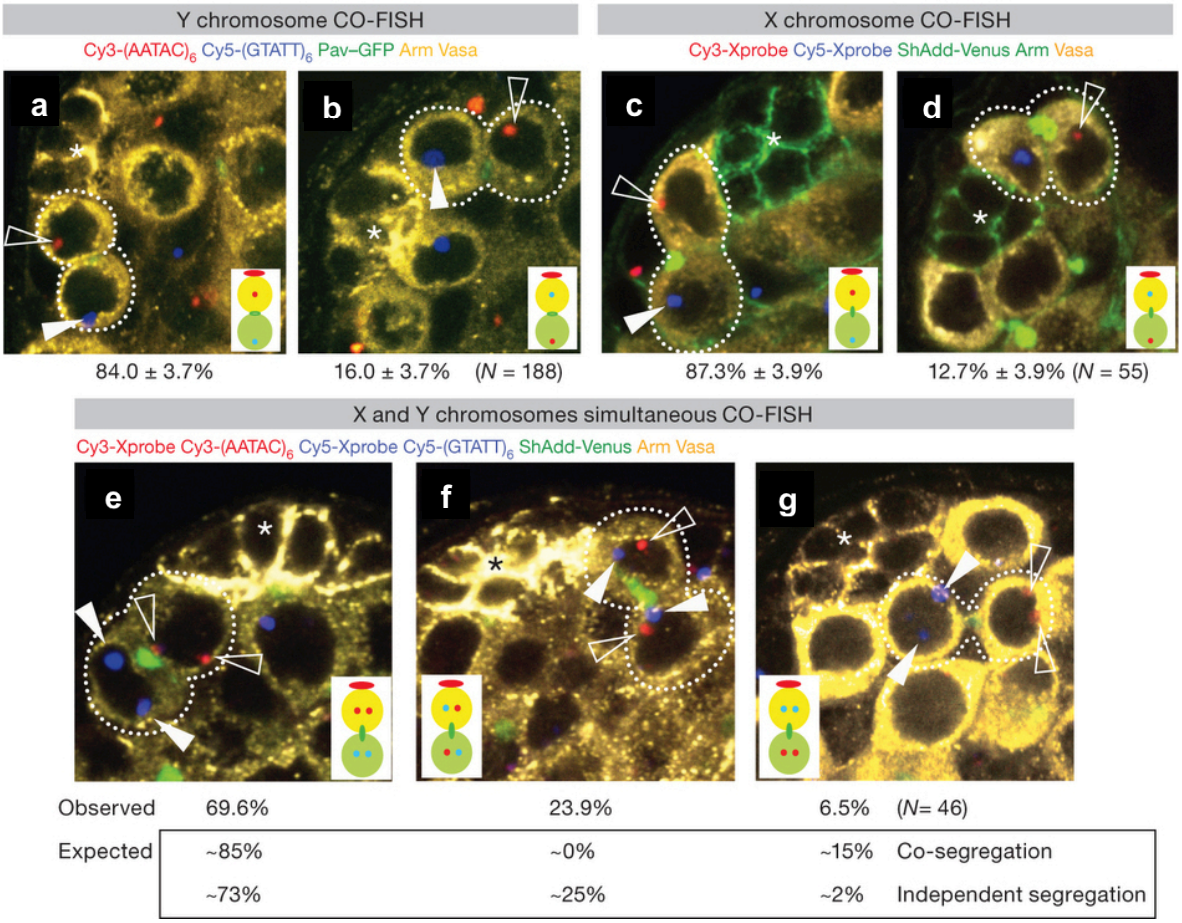


Figure 3.4 Non-random segregation of Y and X chromosome strands during GSC divisions. a,b) Representative images of CO-FISH outcome using Y chromosome probes. The strand that hybridizes to Cy3-$(AATAC)_6$ probe is preferentially inherited by GSCs. Green, Pav-GFP. In all figures the Cy5 signal is marked with solid arrowheads and the Cy3 signal with open arrowheads. (*) Hub. N, number of GSC-GB pairs scored. Data are shown as mean ± standard deviation. **c,d)** Representative images of CO-FISH outcome using X chromosome probes. The strand that hybridizes to the Cy3-X probe is preferentially inherited by GSCs. **e-g)** Representative images of CO-FISH outcome using X and Y probes simultaneously. Expected segregation patterns based on co-segregation *vs.* random segregation are shown in the lower panel.

Since both X and Y chromosomes show a similar bias in segregation (approximately 85:15), it is possible that they are co-segregated. To address this, we performed CO-FISH experiments using X and Y probes simultaneously. The X and Y probes were labelled in such a way 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 ~85% and that of inheriting two Cy5 signals will be ~15%, whereas there will be few instances where a GSC inherits one Cy3 and one Cy5 signal. In contrast, if X and Y chromosomes segregate independently, the probability of GSCs inheriting two Cy3 signals will be 72% (85% x 85%), that of two Cy5 signals will be 2% (15% × 15%), and that of one Cy3 and one Cy5 signal will be 26% (85% × 15% × 2). In our experiments, the observed segregation pattern was very similar to the latter scenario (Figure 3.4e, f, g), suggesting that X and Y chromosomes are segregated independently.

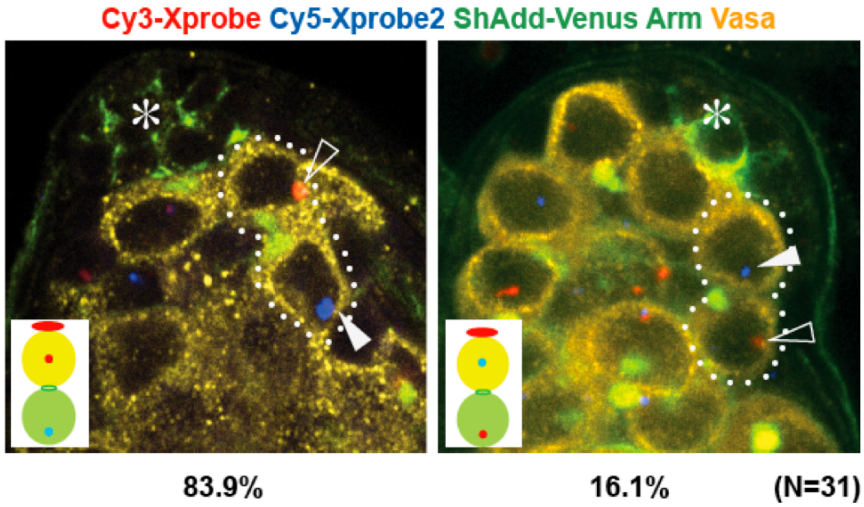


Figure 3.5 CO-FISH using non-complementary probes for the X chromosome. CO-FISH analysis was conducted using non-complementary probes, which do not anneal to each other. A second Cy5 probe (Cy5-Xprobe2: Cy5-TTATTTGATGACCGAAATTTGGAAAAACAGACTCTGCAAAAAAGTGGATA TTTACAAA CGAAATTTTCGTTATAACTTGG) was used in combination with the Cy3-X probe shown in Figure 3.1a. This combination of non-complementary probes yielded a similar pattern of biased segregation. These results exclude the possibility that annealing of complementary probes causes experimental artifacts. Open arrowheads indicate Cy3-Xprobe; closed arrowheads indicate Cy5-Xprobe2.

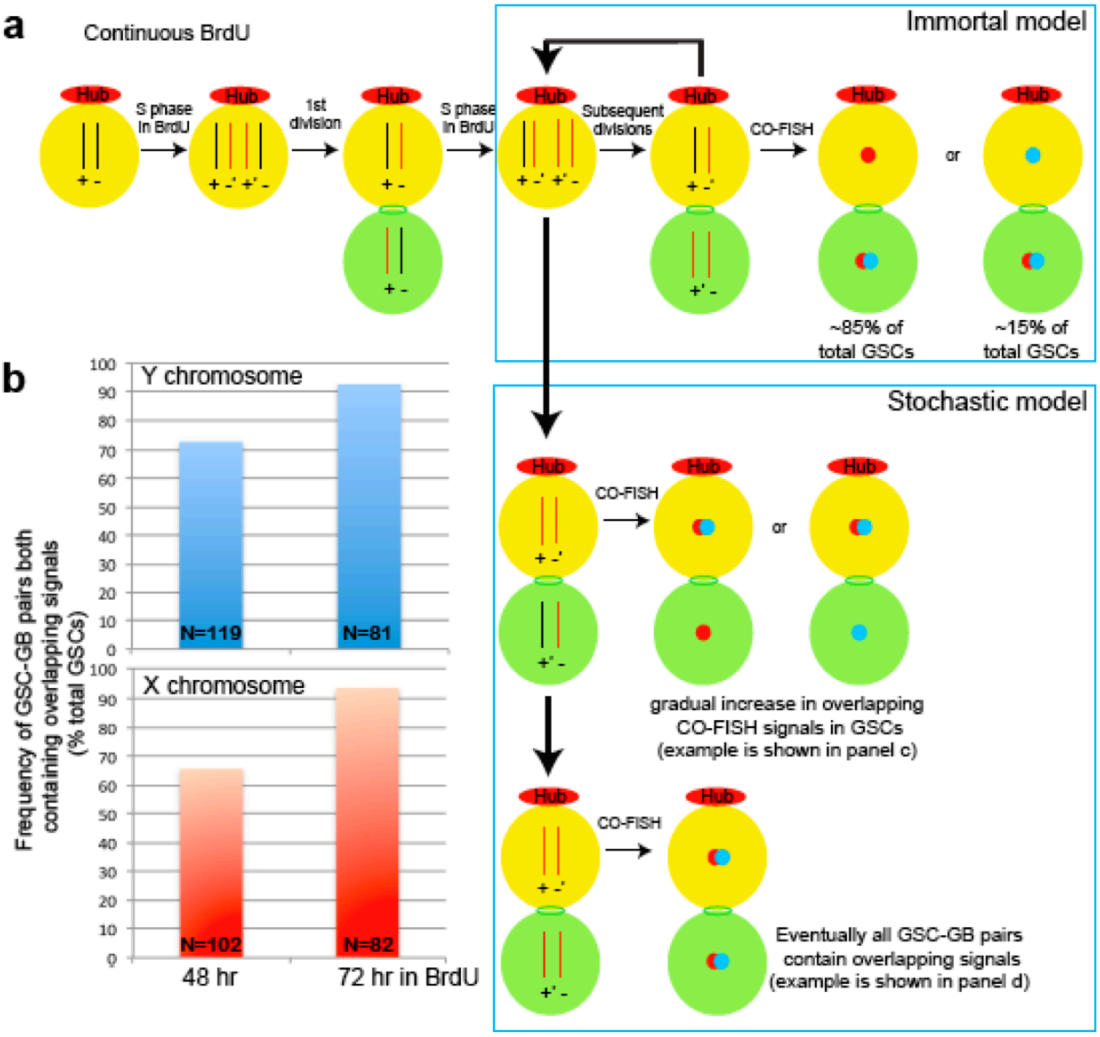


Figure 3.6 X and Y chromosome strands are not immortal. a) If GSCs maintain immortal X and Y strands, those strands would remain BrdU-negative irrespective of the number of cell cycles the GSCs undergo in the presence of BrdU ("continuous BrdU"). Such BrdU+/- strands would yield a single CO-FISH signal (red signal in ~85% of GSCs and blue signal in ~15% of GSCs). BrdU+/+ strands would yield overlapping, red/blue signals (see Figure 3.7). In contrast, if GSCs do not maintain immortal X and Y strands, and switch strands stochastically, GSCs would eventually lose the BrdU-negative strand. As a result, GSCs would increasingly contain overlapping CO-FISH signals. BrdU-negative DNA strands are represented by black lines and BrdU-positive DNA strands by red lines. **b)** Frequency of GSC-GB pairs, in which both contain overlapping signals, increases during continuous BrdU incorporation, and approaches 100% after 72 hours of BrdU feeding. This demonstrates that the X and Y chromosomes are not immortal, and favors the possibility that certain strands of X and Y are stochastically inherited with a strong bias.

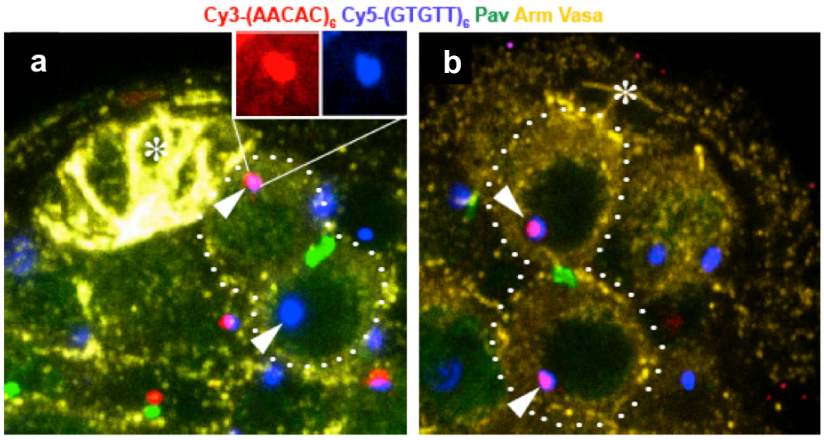


Figure 3.7 Long-pulse CO-FISH Y images. a) An example of Y chromosome CO-FISH of a GSC-GB pair in which the original, BrdU-negative strand (with a single CO-FISH signal) is lost from the GSC and inherited by the GB. After 48 hours of BrdU feeding, 19% (N=32 total GSC-GB pairs in which one cell contains a single signal and the other contains overlapping signals) showed this pattern. Inset: overlapping signals of Cy3 and Cy5 probes in the GSC are shown in separate channels. Arrowheads indicate single and overlapping signals; asterisk indicates hub. Similar results were obtained for X chromosome CO-FISH after 48 hours of BrdU feeding; 26% (N=35) of GSC-GB pairs showed a single CO-FISH signal in the GB and overlapping signals in the GSC. **b)** An example of Y chromosome CO-FISH in a GSC-GB pair, in which both cells contain overlapping signals, indicating that these cells have lost the original, BrdU-negative strand. After 72 hours of BrdU feeding, 93% (N=81 total GSC-GB pairs) showed this pattern. Arrowheads indicate overlapping signals; asterisk indicates hub.

3.3.2 Autosomes segregate randomly during GSC divisions.

In contrast to X and Y chromosomes, there are two homologs of the autosomes in male cells. Thus, we expect to see two CO-FISH signals in each cell after performing the CO-FISH procedure as described above. The CO-FISH signals from autosome probes were always juxtaposed (Figure 3.8a-d), consistent with previous reports that homologous chromosomes are paired even in non-meiotic cells in *Drosophila (Fung et al., 1998)*. We observed that autosomes (chromosomes II and III) did not show a biased segregation pattern in GSC-GB pairs (Figure 3.8a-d). These results indicate that the autosomes segregate

randomly with respect to which copy goes to the GSCs. Again, this is consistent with our previous study showing that *Drosophila* male GSCs do not retain an immortal strand for all chromosomes (Yadlapalli et al., 2011).

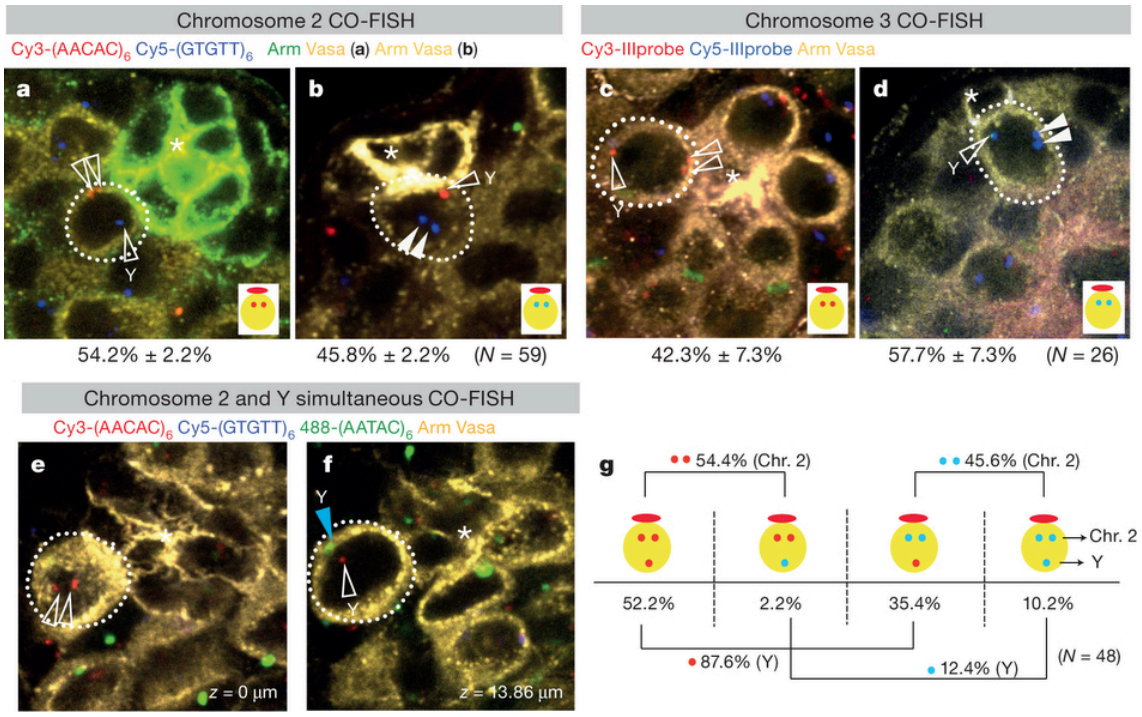


Figure 3.8 Autosomes are randomly segregated during GSC divisions. a–d, Representative images of CO-FISH results using chromosome 2 probes (**a**, **b**), and chromosome 3 probes (**c**, **d**). Lone signals that correspond to the Y chromosome are marked with 'Y'. *N*, number of GSCs scored. **e**, **f**, 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.

However, we did observe an interesting pattern: GSCs always inherited two Cy3 signals or two Cy5 signals, but we never observed a Cy3 and a Cy5 signal in a GSC. It should be noted that the repeat sequences used as probes for chromosome II and III also exist in the Y chromosome (Makunin et al., 1999).

Therefore, we observed a third “lone” signal in addition to the paired autosome signals (Figure 3.8a-d). The identity of the lone signal was confirmed as the chromosome by combining autosome probes, for example Cy3-$(AACAC)_6$/Cy5-$(GTGTT)_6$ for chromosome II, and a Y chromosome probe, 488-$(AATAC)_6$. In this case, we observed that the 488-$(AATAC)_6$ signal was always close to the lone Cy3-$(AACAC)_6$ signal, whereas the paired Cy3-$(AACAC)_6$ signals did not associate with 488-$(AATAC)_6$ (Figure 3.8e, e’). The *Drosophila* genome sequence indicates that the $(AACAC)_6$ and $(AATAC)_6$ sequences are on the same strand, and we observed that the lone signal labelled with the Cy3-$(AACAC)_6$ probe was frequently inherited by GSCs (~87%, Figure 3.8f), irrespective of the segregation pattern of the paired autosomal CO-FISH signals. This result further confirms our earlier observation that sister chromatids of the Y chromosome are segregated non-randomly.

3.3.3 The centrosome and SUN-KASH domain proteins are required for non-random sister chromatid segregation.

We have previously shown that the mother centrosome is inherited by the GSCs (Yamashita et al., 2007), leading to speculation that the mother centrosome might anchor the immortal DNA strands (Tajbakhsh, 2008; Tajbakhsh and Gonzalez, 2009). To investigate the role of the centrosome in non-random sister chromatid segregation, we examined mutants for *centrosomin (cnn*), a core component of the pericentriolar material (PCM) that we have shown to be required for stereotypical centrosome segregation and spindle orientation in GSCs

(Yamashita et al., 2003; Yamashita et al., 2007). We found that segregation of both X and Y chromosomes is randomized in the *cnn* mutant (Figure 3.9, Table 3.1).

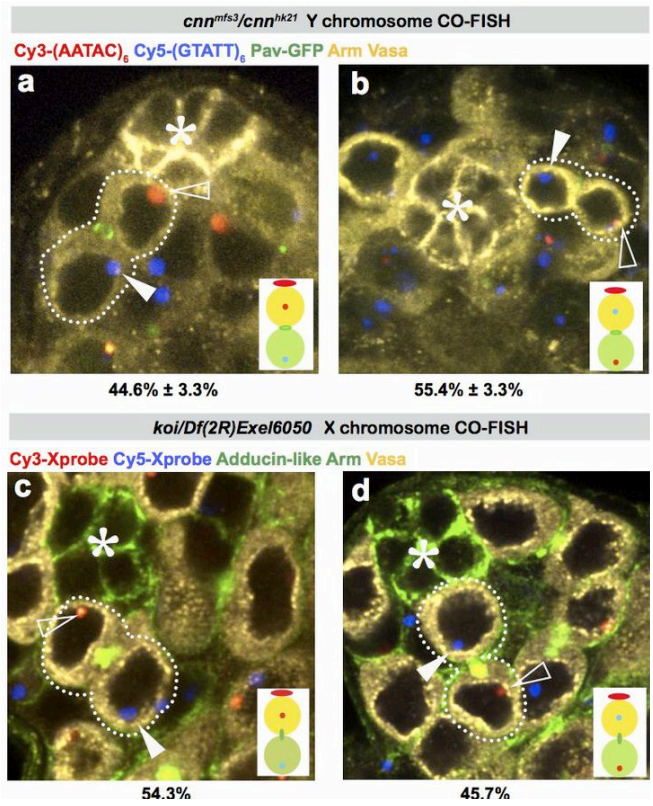
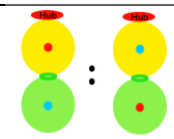


Figure 3.9 *cnn*, *koi,* and *klar* are required for non-random sister chromatid segregation. a, b) Representative images of Y chromosome CO-FISH in *cnn* mutant. Open arrowheads indicate the Cy3-$(AATAC)_6$ probe; closed arrowheads indicate the Cy5-$(GTATT)_6$ probe; asterisk indicates hub. **c, d)** 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 hub.

However, it is unlikely that the mother centrosome directly anchors specific sister chromatids of the X and Y chromosomes throughout the cell cycle, because the nuclear envelope separates the chromosomes from the centrosomes during interphase in eukaryotic cells. It is well established that the LINC (linker of nucleoskeleton and cytoskeleton) complex composed of SUN- and KASH- domain

proteins tethers the nucleus to the cytoskeleton via the nuclear envelope (Razafsky and Hodzic, 2009).

Table 3.1 Summary of sister chromatid segregation pattern in *cnn, koi and klar* mutants

Genotype	Outcome 	
	Y chromosome	X chromosome
cnn^{mfs3}/ *cnn*^{HK21}	45:55 (n=92)	49:51 (n=94)
cnn^{mfs3}/+, *cnn*^{HK21}/+,	85:15 (n=93)	83:17 (n=30)
koi$^{HRKO80.w}$/ *Df(2R)Exel6050*	59:41 (n=59)	54:46 (n=35)
koi$^{HRKO80.w}$/+, *Df(2R)Exel6050*/+	86:14 (n=56)	86:14 (n=36)
Klar^{1}/ *Df(3L)emc-E12*	50:50 (n=38)	54:46 (n=39)
Klar^{1}/ +	85:15 (n=41)	85:15 (n=34)

n, number of GSC-GB pairs scored.

Interactions between the centrosome and chromatin via the LINC complex are known to play critical roles in various biological processes such as meiotic homologous pairing and protection of nuclear structures from the shearing force of microtubule-based motors. In *Drosophila*, two SUN-domain proteins, Klaroid (Koi) and Spag4, and two KASH-domain proteins, Klarsicht (Klar) and Msp-300, have been identified (Kracklauer et al., 2007; Kracklauer et al., 2010; Mosley-Bishop et al., 1999; Patterson et al., 2004; Xie and Fischer, 2008). Although neither *koi* nor *klar* genes are essential for viability or fertility, sister chromatid segregation of X and Y chromosomes was randomized in *koi* and *klar* mutants (Figure 3.9, Table 3.1). Since Spag4 is known to be specifically required in later spermiogenesis (Kracklauer et al., 2010) and Msp-300 is known to connect the nuclear envelope to

the actin cytoskeleton, rather than microtubules (Xie and Fischer, 2008), we did not study mutants of these genes. These data demonstrate that the centrosome and the SUN-KASH domain proteins are required for asymmetric segregation of X and Y chromosomes, probably via anchorage of the sister chromatids to the mother centrosome through the nuclear envelope (Figure 3.12).

3.3.4 *Dnmt2* is required for non-random sister chromatid segregation.

The above results suggest that GSCs have the ability to distinguish two sister chromatids that are supposedly identical products of a precise DNA replication process. How do cells distinguish between the two sister chromatids? We found that sister chromatid segregation of X and Y chromosome was randomized in *dnmt2* mutants (Figure 3.10, Table 3.2). *Dnmt2* is the only gene in the *Drosophila* genome that encodes a potential DNA methyltransferase, although it has been suggested that the gene product is an RNA methyltransferase.

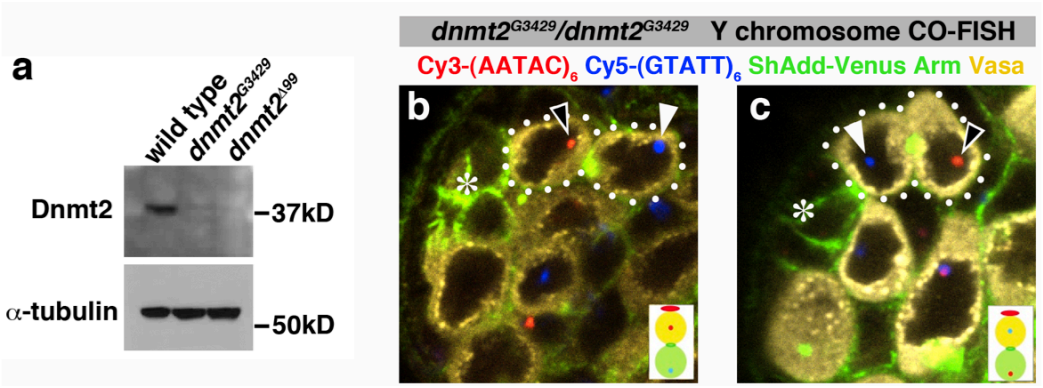


Figure 3.10 *Dnmt2* is required for non-random sister chromatid segregation. **a)** Characterization of *dnmt2*^{G3429} allele. Western blotting using anti-Dnmt2 antibody demonstrates that *dnmt2*^{G3429} is a protein null allele, similar to *dnmt2*^{D99}, a known null allele. **b, c)** Representative images of CO-FISH analysis of *dnmt2*^{G3429} mutant testes. Open arrowheads indicate Cy3-(AATAC)$_6$ signal and closed arrowheads indicate Cy5-(GTATT)$_6$ signal; asterisk indicates hub.

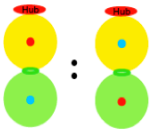
Further analysis using various cross schemes (crosses of homozygous mother/father with heterozygous father/mother) revealed that *Dnmt2* is required in the gametes of the parents and continuously required in the zygote (Table 3.2). For example, non-random sister chromatid segregation of the X chromosome relies on the gene function of *Dnmt2* in the mother (who provides the original X chromosome to the individual). Conversely, non-random sister chromatid segregation of the Y chromosome relies on the gene function of *Dnmt2* in the father (who provides the original Y chromosome). Importantly, the segregation pattern of X was not affected even when the father was a homozygous mutant (*dnmt2/dnmt2*) and segregation of Y was randomized. Likewise, the segregation of Y was not affected when the mother was a homozygous mutant (*dnmt2/dnmt2*) and segregation of X was randomized (Table 3.2). These results suggest the striking possibility that the epigenetic information that allows non-random sister chromatid segregation in adult stem cells is primed during gametogenesis in the parents and maintained through many cell divisions during embryogenesis and adult tissue homeostasis.

3.3.5 Non-random sister chromatid segregation is perturbed in GSC overproliferation.

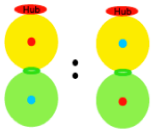
To gain insights into whether non-random sister chromatid segregation is controlled by stem cell identity, we investigated whether sister chromatid segregation is affected in GSC overproliferation induced by the ectopic expression of Upd.

Table 3.2 Summary of sister chromatid segregation pattern in *dnmt2* mutants and progeny.

a. Pattern of sister chromatid segregation in *dnmt2* mutants.

Genotype	Outcome 	
	Y chromosome	X chromosome
$dnmt2^{G3429}$/ $dnmt2^{G3429}$	49:51* (n=41)	56:44 (n=43)
$dnmt2^{G3429}$/*Df(2L)ED775*	53:47 (n=36)	51:49 (n=41)
$dnmt2^{D99}$/ $dnmt2^{D99}$	49:51 (n=45)	46:54 (n=41)
$dnmt2^{147}$/ $dnmt2^{147}$	46:54 (n=35)	59:41 (n=46)
$dnmt2^{G3429}$/ $dnmt2^{D99}$	56:44 (n=39)	59:41 (n=44)
$dnmt2^{G3429}$/ $dnmt2^{147}$	54:46 (n=37)	42:58 (n=41)

b. Pattern of sister chromatid segregation in progeny of *dnmt2* mutants.

Maternal genotype	Paternal genotype	Progeny genotype	Outcome 	
			Y chromosome	X chromosome
$dnmt2^{G3429}$/ $dnmt2^{G3429}$	$dnmt2^{G3429}$/ +	$dnmt2^{G3429}$/ $dnmt2^{G3429}$	55:45 (n=40)	61:39 (n=33)
		$dnmt2^{G3429}$/ +	81:19 (n=32)	45:55 (n=33)
$dnmt2^{G3429}$/ +	$dnmt2^{G3429}$/ $dnmt2^{G3429}$	$dnmt2^{G3429}$/ $dnmt2^{G3429}$	42:58 (n=52)	51:49 (n=35)
		$dnmt2^{G3429}$/ +	47:53 (n=32)	82:18 (n=34)
$dnmt2^{G3429}$/ +	$dnmt2^{G3429}$/ +	$dnmt2^{G3429}$/ $dnmt2^{G3429}$	55:45 (n=40)	53:47 (n=30)
		$dnmt2^{G3429}$/ +	82:18 (n=28)	83:17 (n=35)
$dnmt2^{D99}$/ $dnmt2^{D99}$	$dnmt2^{D99}$/ +	$dnmt2^{D99}$/ $dnmt2^{D99}$	51:49 (n=37)	46:54 (n=35)
		$dnmt2^{D99}$/ +	82:18 (n=39)	51:49 (n=39)
$dnmt2^{D99}$/ +	$dnmt2^{D99}$/ $dnmt2^{D99}$	$dnmt2^{D99}$/ $dnmt2^{D99}$	48:52 (n=46)	50:50 (n=36)
		$dnmt2^{D99}$/ +	38:62 (n=37)	81:19 (n=36)

*, Values represent percent segregation of Cy3-labelled strand to GSC: percent segregation of Cy3-labelled strand to GB.

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(Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). We examined the mode of sister chromatid segregation in GSCs upon ectopic expression of 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. We observed that segregation of both X and Y chromosomes is randomized in Upd-expressing testis (Figure 3.11a, b, Table 3.3), suggesting that non-random sister chromatid segregation is under the control of stem cell identity. However, it is unlikely that non-random sister chromatid segregation determines GSC identity, because the mutants defective in non-random segregation described above (*cnn, koi, klar, dnmt2*) do not exhibit GSC overproliferation or depletion.

3.3.6 De-differentiated GSCs do not recover non-random sister chromatid segregation.

Partially differentiated germ cells can revert back to GSC identity to replenish the stem cell pool (Brawley and Matunis, 2004; Kai and Spradling, 2004). These de-differentiated GSCs are apparently functional as stem cells since they can produce differentiating spermatogonia and can reconstitute spermatogenesis (Brawley and Matunis, 2004; Cheng et al., 2008). When we induced de-differentiation by transient expression of *Bam*, the master regulator of differentiation, followed by a recovery period as described previously (Sheng et al.,

2009), the de-differentiated GSCs displayed random sister chromatid segregation (Figure 3.11c, d, Table 3.3). Furthermore, we found that non-random sister chromatid segregation was compromised during aging [at day 30, 63:37 for the X chromosome (N=35) and 68:32 for the Y chromosome (N=28)], consistent with our previous report that de-differentiation increases during aging (Cheng et al., 2008). This result suggests that de-differentiated GSCs do not re-establish non-random sister chromatid segregation.

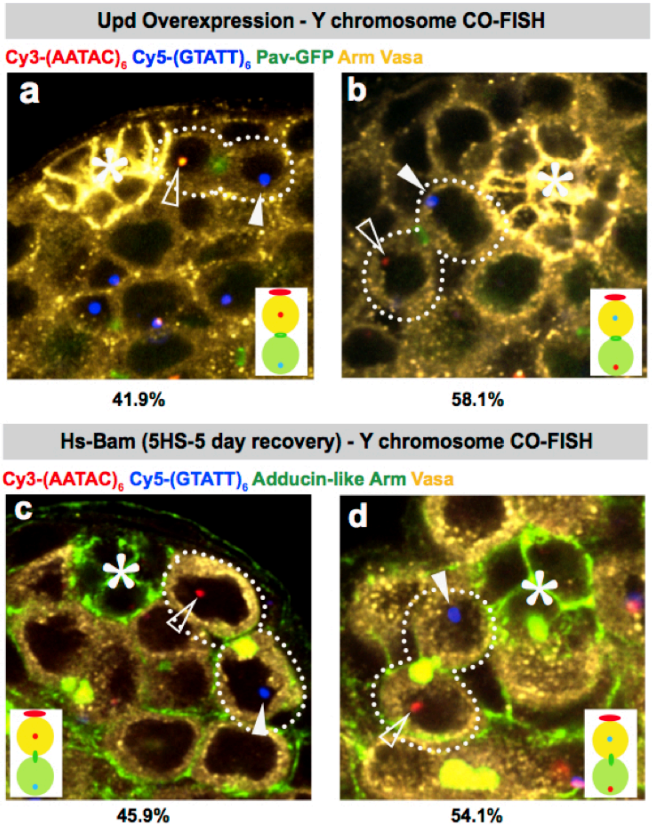
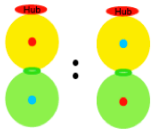


Figure 3.11 Non-random segregation of Y and X chromosomes is disrupted in *upd* overexpression testes and de-differentiated stem cells. a, b) Representative images of CO-FISH using the Y probe upon overexpression of Upd (nos-gal4>UAS-Upd). N, number of GSC-GB pairs scored. (*) Hub. **c, d)** Representative images of CO-FISH using the Y probe in de-differentiated GSCs. Differentiation was induced by heat shock treatment of *hs-Bam* flies followed by a 5-day recovery period.

Table 3.3 Summary of sister chromatid segregation pattern upon Upd overexpression and de-differentiation

Genotype	Outcome 	
	Y chromosome	X chromosome
nos-gal4>Upd	42:58 (n=43)	56:44 (n=32)
*Upd control**	83:17 (n=36)	83:17 (n=36)
*hs-bam***	46:54 (n=111)	51:49 (n=47)

*: cross siblings of nos-gal4>Upd that do not express Upd (either nos-gal4 only or UAS-Upd only) were used as control
**; *hs-bam* flies were subjected to 5 heatshocks (30min for 5 times within 2.5 days), followed by 5 days recovery.
n, number of GSC-GB pairs scored.

3.4 Discussion

This study provides the first evidence that adult stem cells can distinguish two sister chromatids, which are supposedly exact copies of each other, and segregate them non-randomly to self-renewing *vs.* differentiating cells. We identified molecular components required for non-random sister chromatid segregation. Our data point to a model in which sister chromatids are distinctively recognized, leading to anchorage of particular strands to the mother centrosome through the SUN-KASH proteins (Figure 3.12). It is currently unknown how *Dnmt2* participates in distinguishing two sister chromatids. Whereas some studies indicate that *Dnmt2* has DNA methyltransferase activity (Kunert et al., 2003; Phalke et al., 2009), other studies show that it functions as an RNA methyltransferase(Schaefer et al., 2010) and that DNA methylation is detectable only at a very low level in the *Drosophila* genome (Zemach et al., 2010). Yet, our data clearly suggest that *Dnmt2* functions during gametogenesis in the parents to

confer information on the X and Y chromosomes that is inheritable through many cell divisions, leading to non-random sister chromatid segregation in the GSCs of the offspring.

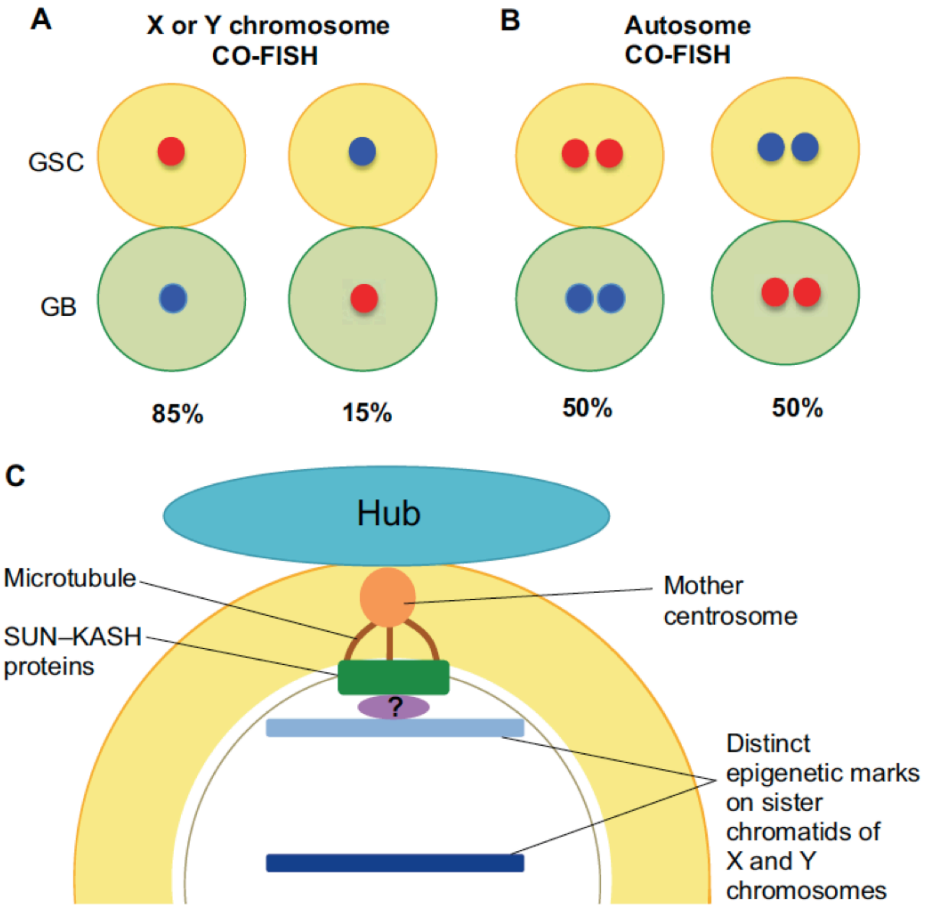


Figure 3.12 Model of non-random sister chromatid segregation of X and Y chromosomes during GSC division. A) Sister chromatid segregation pattern of X and Y chromosomes examined by CO-FISH. GSCs inherit the red (Cy3-based) signal in a majority of the cases, suggesting that GSCs inherit particular sister chromatids of X and Y chromosomes with a striking bias (85%:15%). **B)** Sister chromatid segregation pattern of the autosomes observed in GSCs. GSCs inherit two Cy3 signals or two Cy5 signals with equal probability, but never a Cy3 and a Cy5 signal, suggesting the existence of a certain type of bias. The CO-FISH experiments using the chromosome II probe and chromosome III probe showed the same trend. **C)** Model of non-random sister chromatid segregation of X and Y chromosomes. Sister chromatids might be distinctly recognized by the SUN–KASH components of the LINC complex, resulting in the anchorage of particular DNA strands to the mother centrosome that is mediated by microtubule–LINC

interactions.

At present it is not clear why X and Y chromosomes are segregated non-randomly. It is unlikely that non-random sister chromatid segregation serves to protect the “immortal strand” to avoid replication-induced mutations, because X and Y chromosomes are segregated in a stochastic manner and lose the template strand with approximately 15% probability during each division. Furthermore, the autosomes are apparently segregated randomly. Therefore, we favour the possibility that certain epigenetic information is transmitted distinctively to GSCs and GBs. Indeed, many processes involving X and Y chromosomes, such as dosage compensation (Conrad and Akhtar, 2011) and male-specific meiotic sex chromosome inactivation (Hense et al., 2007), are subject to epigenetic regulation. In addition, *Stellate*, a repetitive sequence that encodes a polypeptide whose expression is known to reduce fertility, as well as *Suppressor of Stellate* [*Su(Ste)*], the piRNA that suppresses *Stellate* expression, are known to be located on the X and Y chromosomes, respectively (Aravin et al., 2001; Tulin et al., 1997). Intriguingly, we found that *Stellate* is de-repressed in mutants of *cnn, dnmt2, koi*, and *klar* (Figure 3.13). Although determination of whether de-repression of *Stellate* is due to a failure in non-random sister chromatid segregation awaits future investigation, the shared outcome of *Stellate* de-repression in mutants that are otherwise unrelated suggests that non-random sister chromatid segregation may be responsible for suppression of *Stellate*. Not surprisingly, we found that the mutants in which *Stellate* is de-repressed show reduced fertility (Figure 3.14).

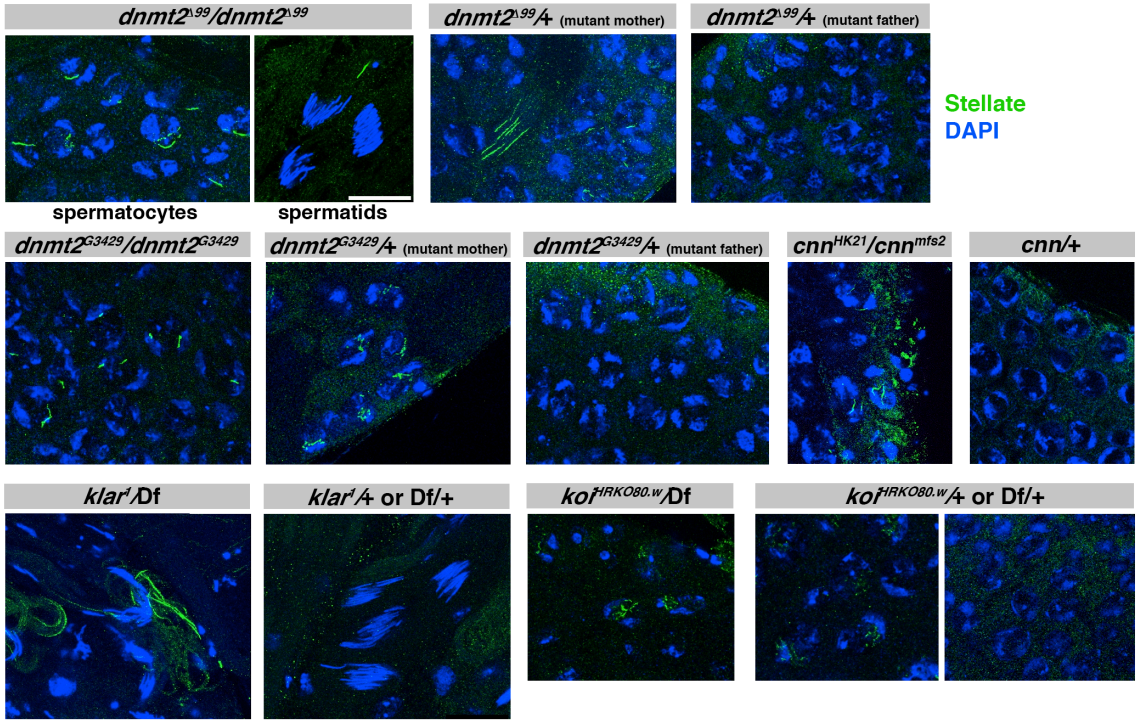


Figure 3.13 *Stellate* is de-repressed in mutants defective in non-random sister chromatid segregation. Representative images of *Stellate* expression in the indicated genotypes are shown. Green, anti-Stellate; blue, DAPI. *Stellate* expression was observed in spermatocyte and/or spermatid stages. In the *koi* mutant, weak *Stellate* expression was sometimes observed in the heterozygous control (albeit at lower frequency and expression level). Bar, 25μm. It is worth noting that *Stellate* was de-repressed in *dnmt2* heterozygous animals that have a mutant female mother (but not those with a mutant male father), suggesting that the X chromosome (which harbors the *Stellate* gene locus) but not the Y chromosome (which harbors the *Su(Ste)* gene locus) is important for suppression of *Stellate*.

It was previously shown that production of non-equivalent sister chromatids as a result of directionality of the DNA replication forks at the *mat1* gene locus underlies mating-type switching in fission yeast(Dalgaard and Klar, 1999). It was also reported that mouse chromosome 7 is non-randomly segregated in embryonic stem cells and endoderm cells (Armakolas and Klar, 2006). Combined with these findings, the present study strongly indicates that non-random segregation of sister chromatid is a mechanism that is widely utilized by diverse systems. Recently, it

was shown that old *vs.* new histones are segregated asymmetrically during GSC divisions (Tran et al., 2012). 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 non-random sister chromatid segregation in adult stem cells and provides mechanistic insights into how cells segregate sister chromatids non-randomly.

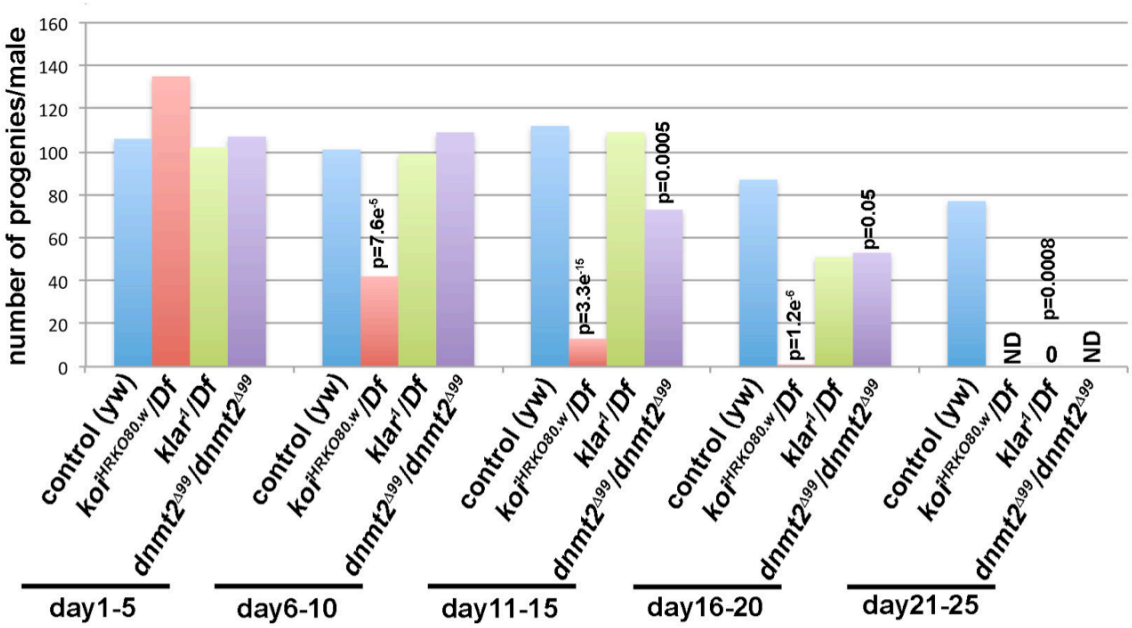


Figure 3.14 *koi, klar*, and *dnmt2* mutants show reduced fertility. A single virgin male was crossed with three virgin *yw* females. Every 5 days, the male was transferred to a new vial with three new virgin *yw* females. The number of adult flies eclosed from each vial was scored. P-value is shown for statistically significant data points (compared to age-matched control). yw is shown as control but cross sibling controls also showed similar trend. ND: not determined (since statistically significant reduction in fertility was observed in earlier time period). The *cnn* mutant was not tested, because it is known to be sterile due to a defect in cytokinesis during meiotic divisions.

3.5 Materials and Methods

3.5.1 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*^{D99}, *dnmt2*^{149}, Df(2L)ED775/CyO, *hs-Bam*, UAS-Upd/CyO, and nos-gal4. These stocks are described in FlyBase.

3.5.2 Combined immunofluorescence staining and CO-FISH

Newly eclosed adult flies (day 0) were fed food containing BrdU (950 µl 100% apple juice, 7 µg agar, and 50 µl 100 mg/ml BrdU solution in a 1:1 mixture of acetone and DMSO) for ~10 hours. After the feeding period, flies were transferred to regular fly food for ~8 hours. Because the average cell cycle length of GSCs is 12 hours, 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-GB pairs that have complementary CO-FISH signals in the GSC and GB (*i.e.,* red signal in one cell, blue signal in the other). Samples were dissected in 1X PBS, fixed for 30-60 min with 4% formaldehyde in PBS, permeabilized for at least 1 hour 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 RNaseA (2 mg/ml 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 for 15 min at room temperature. The samples were then rinsed with 2X SSC, transferred to a tray, and irradiated with ultraviolet light in a UV Stratalinker 1800 (calculated dose 5400 J/m^2). Nicked BrdU strands were digested with 100 µl exonuclease III (New England Biolabs) at 3 U/µl 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/2X SSC, samples were incubated for a minimum of 10 min each in 20% formamide/2X SSC, 40% formamide/2X SSC, and finally in 50% formamide/2X SSC. The hybridization mixture consisted of 50% formamide, 2X SSC, 10% dextran sulfate, 0.5 µg/ml Cy3-labelled probe, and 0.5 µg/ml Cy-5 labeled 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 (Figure 3.5) 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 prior

to hybridization. The samples were never heat-denatured. As a critical control, hub cells, which are predominantly quiescent and, thus, do not incorporate BrdU, did not show any CO-FISH signal (evident in all images). Following hybridization, samples were washed once in 50% formamide/2X SSC, once in 25% formamide/2X SSC and finally three times with 2X SSC. Samples were then mounted in VECTASHIELD (H-1200, Vector Laboratory) and images were recorded using a Leica TCS SP5 confocal microscope with a 63× oil immersion objective (NA=1.4) and processed using Adobe Photoshop software. The following primary antibodies were used: 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 (1:1000, a generous gift of Phillip Zamore(Forstemann et al., 2005)). The secondary antibodies used were Alexa Fluor 594- and 488-conjugated secondary antibodies (1:200; Molecular Probes).

3.6 Acknowledgements

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Academic Affairs) (to YMY) and AHA (12PRE9630000) and NIH (1F31HD071727-01) (to SY). YMY is supported by the MacArthur Foundation.

Chapter 4

Conclusions and future directions

The primary insight from this work is that sister chromatids, which are genetically exact copies of each other, are segregated non-randomly during asymmetric stem cell division. This key insight—enabled by my experiments performed at a single sister chromatid resolution for the first time—implies that potentially all cells might be employing non-random sister chromatid segregation during asymmetric cell division to diversify cell fates. In this chapter, I will first summarize the results from my studies, and then I will discuss the molecular mechanisms and biological relevance of non-random sister chromatid segregation. Finally, I will describe possible future directions.

In the first study, I have combined cell biological analysis of BrdU-labeled sister chromatids and mathematical modeling, and concluded that *Drosophila melanogaster* male GSCs are randomly segregating the template strands. (Yadlapalli et al., 2011). Throughout the label-free chase period, I observed that BrdU label was equally distributed to GSC–GB pairs in a majority of cases (95% of the cases at 24 hour chase period), until BrdU is finally diluted to undetectable levels (Figure 4.1). This suggests that GSCs do not retain the immortal strands for the entire genome. However, these results did not exclude the possibility that GSCs might be non-randomly segregating sister chromatids of only a subset of

chromosomes.

In the follow-up study, I adapted the CO-FISH technique and showed that sister chromatids of X and Y chromosomes, but not autosomes, are non-randomly segregated during GSC divisions (Yadlapalli and Yamashita, 2013). In this study, I used chromosome-specific probes that are available for *Drosophila* to study sister chromatid segregation at single chromosome resolution.

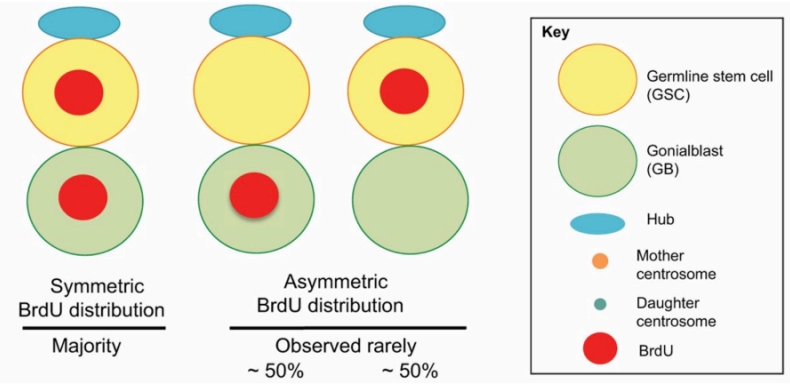

Figure 4.1 *Drosophila melanogaster* male GSCs do not follow immortal strand model. During the chase period, majority of GSC-GB pairs (95% of the cases at 24 hour chase period) show symmetric distribution of BrdU label. In a very few cases, BrdU label appeared to be asymmetrically segregated to either GSC or GB. However, even in such cases, it was apparently random as to which cell inherited the BrdU label.

For instance, I used a Cy3-labeled $(AATAC)_6$ probe (red) and the complementary Cy5-labeled $(GTATT)_6$ probe (blue) to examine the sister chromatid segregation pattern of Y chromosome, because these sequences were repeated uniquely on the Y chromosome (Bonaccorsi and Lohe, 1991). Using this method, I found that in 85% of GSC divisions, GSCs inherited the sister chromatid of the Y chromosome that contains $(GTATT)_6$ repeats as a template (and thus hybridize to the Cy3-$(AATAC)_6$ probes) (Yadlapalli and Yamashita, 2013) (Figure 4.2A). A similar trend (of 85:15) was observed for X chromosome

segregation. Despite the comparable segregation bias for both X and Y chromosomes, X and Y chromosomes are not co-segregated, suggesting that sister chromatids of X and Y chromosomes are segregated independently of each other. Interestingly, in spite of the strong bias, I have shown that X and Y chromosome template strands in GSCs are not 'immortal'; instead, each GSC appears to switch the template strands once in approximately seven cell divisions on average. This type of non-random sister chromatid segregation is novel in that both chromosomes (X and Y) show a bias in sister chromatid segregation with respect to the cell fate (i.e. stem cell and differentiating cell). Furthermore, this provides a clear example of biased segregation that does not lead to retention of the immortal strand.

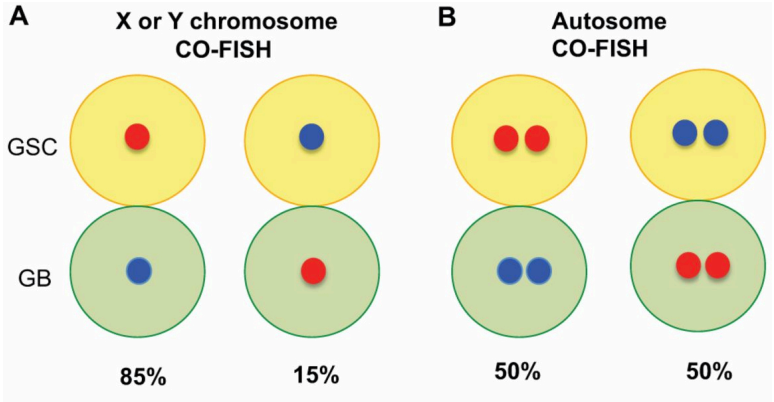

Figure 4.2 Non-random sister chromatid segregation of X and Y chromosomes during GSC division. A) Sister chromatid segregation pattern of X and Y chromosomes examined by CO-FISH. GSCs inherit the red (Cy3-based) signal in a majority of the cases, suggesting that GSCs inherit particular sister chromatids of X and Y chromosomes with a striking bias (85%:15%). **B)** Sister chromatid segregation pattern of the autosomes observed in GSCs. GSCs inherit two Cy3 signals or two Cy5 signals with equal probability, but never a Cy3 and a Cy5 signal, suggesting the existence of a certain type of bias.

Using CO-FISH with autosome probes, I noticed that GSCs always

inherited either two Cy3 signals or two Cy5 signals, but never a Cy3 and a Cy5 signal. If the paternal and maternal chromosomes behave independently, one would expect to observe a distribution of Cy3–Cy3:Cy3–Cy5:Cy5–Cy5 that equals to 25%:50%:25% (1:2:1) (Figure 1.3B). However, I observed a distribution pattern of 50%:0%:50% (or 1:0:1) (Figure 4.2B) Although it was random with regard to which signals (either Cy3–Cy3 or Cy5–Cy5) are inherited by GSCs, this pattern is clearly distinct from numbers that would be expected from a 'truly random' segregation pattern, suggesting the existence of certain bias. This pattern is similar to the sister chromatid segregation pattern previously reported in *Drosophila* (Beumer et al., 1998) and mouse ES cells (Armakolas and Klar, 2006). Future investigation is required to determine whether these coordinated segregations are related with regard to their biological significance and/or underlying molecular mechanisms.

4.1 Molecular mechanisms of non-random sister chromatid segregation

There have been many hypotheses regarding how and for what reasons a cell might non-randomly segregate sister chromatids (Lew et al., 2008; Tajbakhsh and Gonzalez, 2009). However, the cellular machinery responsible for nonrandom sister chromatid segregation remained elusive. My recent work provided insight into how cells might mechanistically carry out non-random sister chromatid segregation (Yadlapalli and Yamashita, 2013). In this study, I showed that the centrosomal component *cnn*, nuclear envelope components (i.e. SUN–KASH-domain proteins) and *Dnmt2* are required for the non-random segregation of the

sister chromatids of X and Y chromosomes. Although much has yet to be learned to fully elucidate the mechanisms that allow non-random sister chromatid segregation of X and Y chromosomes, the genes that are required for nonrandom sister chromatid segregation allow us to propose the following model. First, the mother centrosome is anchored to the adherens junctions at the hub–GSC interface throughout the cell cycle (Yamashita et al., 2007). Through its association with microtubules, the mother centrosome is linked to the SUN–KASH-domain proteins located on the nuclear envelope that form the linker of nucleoskeleton and cytoskeleton (LINC) complex (Razafsky and Hodzic, 2009). The LINC complex might associate only with a particular sister chromatid to allow biased segregation (Hiraoka and Dernburg, 2009; Razafsky and Hodzic, 2009).

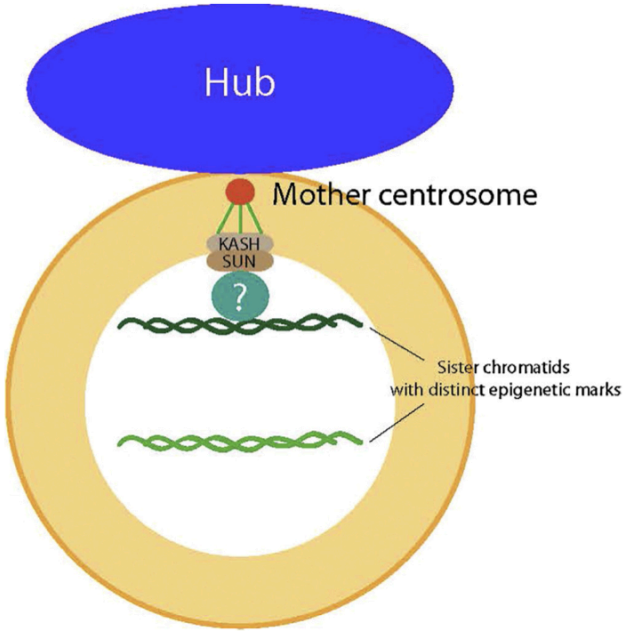

Figure 4.3 Model for non-random sister chromatid segregation. Molecular machinery that enables non-random sister chromatid segregation. Based on the requirement for *cnn*, *koi*, *klar*, and *dnmt2*, we propose that specific sister chromatids (specified by *Dnmt2*-dependent modification) of X and Y chromosomes are anchored to the SUN-KASH domain proteins, which, in turn, interact with the mother centrosome, leading to non-random sister chromatid segregation.

I hypothesize that chromosomal components (such as centromeres or other regions) and associated proteins (such as kinetochore proteins or other chromatin-associated proteins) are distinct between the sister chromatids (Thorpe et al., 2009), thereby allowing for the selective capture of a particular sister chromatid by the mother centrosome (Figure 4.3).

4.1.1 Role of centrosome in non-random sister chromatid segregation

Centrosomes play a fundamental role in chromosome segregation in general as they form spindles that pull chromosomes into two daughter cells. Our laboratory has previously shown that the mother centrosome is consistently inherited by stem cells during asymmetric GSC division (Yamashita et al., 2007). This stereotypic centrosome inheritance is shown to require *centrosomin (cnn)*, a major component of pericentriolar material (Megraw et al., 1999), which is thought to connect the mother centrosome to the GSC-hub interface. In my recent study, I observed that in the *cnn* mutant, sister chromatids of X and Y chromosomes are randomly segregated, even though GSCs segregated chromosomes equally into two daughter cells without causing obvious genomic instability. This data implies that in the *cnn* mutant, there is a specific problem in distinguishing two sister chromatids of X and Y chromosomes during stem cell division. It is tempting to speculate that the two sister chromatids are distinct in their ability to organize/bind kinetochore microtubules (Maiato et al. 2004), and such sister kinetochores are captured by mother *vs.* daughter centrosomes that

have been shown to differ in microtubule-organizing activity (Yamashita et al., 2007).

4.1.2 Role of nuclear envelope proteins in non-random sister chromatid segregation

It is unlikely that specific sister chromatids are directly anchored to the mother centrosome throughout the cell cycle, because the nuclear envelope separates the chromosomes from the centrosomes in interphase of eukaryotic cells. Is it well established that Linker of Nucleoskeleton and Cytoskeleton (LINC) complex consisting of SUN-KASH domain proteins on the nuclear envelope mediate the interactions between the cytoplasm and nucleus (Razafsky and Hodzic, 2009). KASH domain proteins are known to be on the outer nuclear membrane and interact with cytoskeleton components such as microtubules and actin filaments. Conversely, SUN domain proteins, which directly bind to KASH domain proteins, localize on the inner nuclear membrane and connect to chromatin(Hiraoka and Dernburg 2009; Razafsky and Hodzic 2009) (Figure 4.4). Such linkage between the cytoskeleton and chromosomes via the LINC complex is known to be required for multiple processes, such as meiotic homologous pairing and protecting the nucleus from the shearing force of cytoskeletons (Hiraoka and Dernburg 2009; Razafsky and Hodzic 2009). Thus, the requirement of SUN-KASH proteins in nonrandom sister chromatid segregation suggests that particular strands of X and Y chromosomes are anchored to the mother centrosome through the nuclear envelope during interphase.

4.1.3 Role of epigenetics in non-random sister chromatid segregation

How can the centrosome and LINC complex capture a particular sister chromatid when both of them have identical genetic information? I hypothesize that two sister chromatids have distinct epigenetic marks, which in turn is utilized to build a platform for distinct capture by the mother *vs.* daughter centrosomes. In this regard, the requirement of *Dnmt2* in non-random sister chromatid segregation is intriguing.

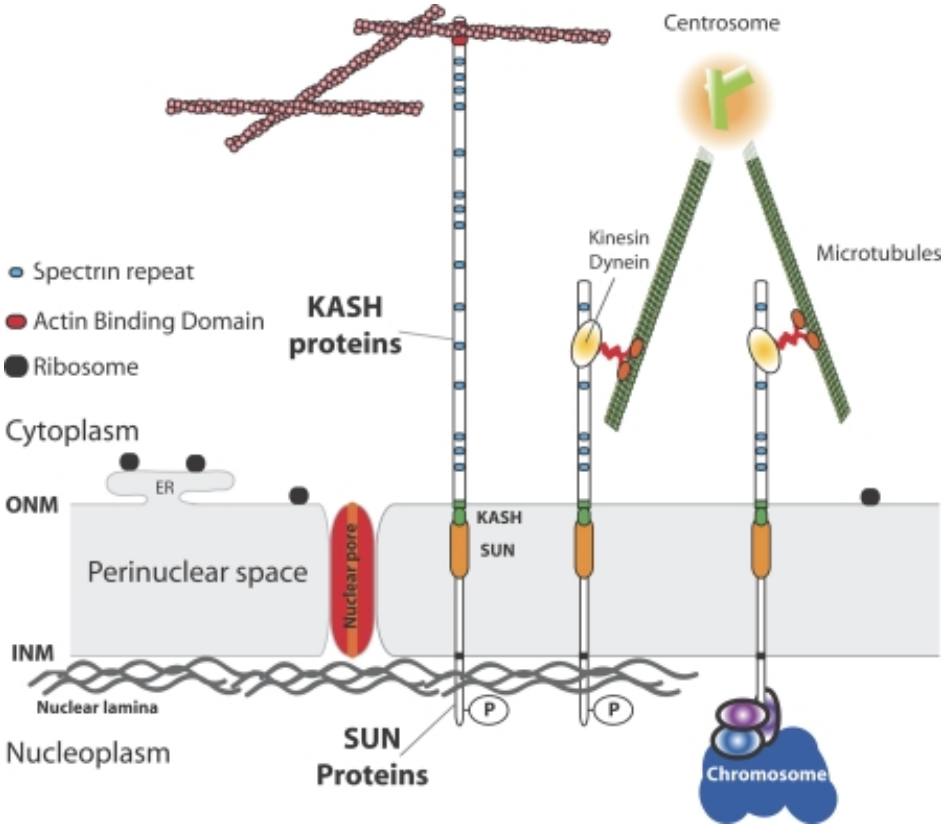

Figure 4.4 Function of Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. In the perinuclear space, evolutionarily conserved SUN (orange oval) and KASH (green) domain-containing proteins physically connects the nuclear lamina to essential cytoskeletal elements such as the actin and microtubule networks. SUN-KASH interactions play essential roles in nuclear migration or anchorage at specific locations within cells. (Image from Razafsky and Hodzic 2009)

Dnmt2 is the sole gene in the *Drosophila* genome that encodes a potential DNA methyltransferase (Kunert et al., 2003). However, the function of *Dnmt2* in *Drosophila* is highly controversial; some studies suggested that it methylates DNA (Kunert et al., 2003; Marhold et al., 2004; Schaefer et al., 2008; Phalke et al., 2009), while other studies have suggested that it only functions as a tRNA methyltransferase (Goll et al., 2006; Schaefer et al., 2010) and that DNA methylation is barely detectable in *Drosophila* (Raddatz et al., 2013; Zemach et al., 2010). However, bisulfite sequencing studies, which have supported the view that *Drosophila* lacks DNA methylation (Raddatz et al., 2013; Zemach et al., 2010), have a few caveats. First, such studies might not be able to detect cell type-specific methylation, especially if it is rare or only exist in adult tissues, as the authors used *Drosophila* embryos as starting material. Second, bisulfite sequencing method cannot detect DNA methylation unless cytosines at particular positions are methylated across many cells (typically at least ~50% of 1000-2000 reads). Due to these uncertainties regarding the molecular function of *Dnmt2*, it is still unclear how *Dnmt2* is involved in the non-random sister chromatid segregation of X and Y chromosomes. However, it is clear that *Dnmt2* confers “epigenetic” (non-genetic) information on the X and Y chromosomes starting from the gametogenesis of parents. To elaborate, we observed that non-random sister chromatid segregation of the Y chromosome specifically relies on the gene function of *Dnmt2* in the father (who provides the original Y chromosome to the individual) and zygotic expression of *Dnmt2*. Similarly, non-random segregation of X chromosome is only dependent on the gene function of *Dnmt2* in the mother.

Importantly, the segregation pattern of Y was not affected even when the mother was a homozygous mutant (*dnmt2/dnmt2*) and segregation of X was randomized, consistent with our previous observation that X and Y chromosomes are indeed segregated independently. These results point to the striking possibility that the very first X and Y chromosomes that are transmitted from the parents to the zygote contain the essential information that allows non-random sister chromatid segregation in the GSCs of the progeny.

Recently, it was shown that male GSCs segregate old *vs.* newly synthesized histones non-randomly. Specifically, the 'old' pool of histone H3 was retained in the stem cells, while the newly synthesized histones were segregated to the differentiating daughter cells (Tran et al. 2012). Interestingly, the histone variant H3.3 was distributed symmetrically during GSC division. The authors of this study hypothesized that these different pools ('old' vs. 'new') could carry information that can distinguish sister chromatids. The relationship between non-random sister chromatid segregation and histone segregation remains to be investigated.

4.2 Is non-random sister chromatid segregation important for stem cell identity?

It is clear from my study that non-random sister chromatid segregation does not confer stem cell identity, because mutants that randomize sister chromatid segregation do not show any defects in GSC identity (either GSC loss or overproliferation). Nevertheless, we found that non-random sister chromatid

segregation was compromised in two conditions where GSC identity is affected (Yadlapalli and Yamashita, 2013). First, when the stemness factor Upd is overexpressed resulting in GSC overproliferation (Kiger et al., 2001; Tulina and Matunis, 2001), GSCs no longer show biased sister chromatid segregation of X and Y chromosomes. It has been shown previously that when Upd is overexpressed, GSCs no longer divide asymmetrically, and both daughters from a GSC division retain stem cell identity (Tran et al., 2012). In such cases, these daughter cells might not be able to control which side of the dividing cell a certain sister chromatid should be segregated. Alternatively, it is possible that both sister chromatids retain GSC-specific epigenetic information with Upd overexpression, and it does not matter which strand goes to which cell.

Second, we found that sister chromatid segregation is randomized in de-differentiated GSCs. It has been shown that partially differentiated spermatogonia can revert back to stem cell identity (Brawley and Matunis, 2004; Kai and Spradling, 2004). Although de-differentiated GSCs can apparently function normally, producing differentiating cells to reconstitute spermatogenesis, randomized sister chromatid segregation in these cells may indicate that de-differentiated GSCs have some defects that have not been detected thus far. Our results imply that the sister chromatid that remains in the GSC contains specific information, whereas the copy transmitted to the differentiating daughter lacks such information, explaining why de-differentiated GSCs cannot regain non-random segregation—that information is lost forever. Alternatively, the lack of proper centrosome orientation or the original mother centrosome might explain

randomized sister chromatid segregation, since it has been shown that de-differentiated GSCs cannot correctly orient the centrosomes (presumably because they have lost the original mother centrosome) (Cheng et al., 2008). Here, it is interesting to note that *koi*, *klar* and *dnmt2* mutants which are defective in non-random sister chromatid segregation do not show increased centrosome mis-orientation (our unpublished results). This clearly indicates that the centrosome is not the only factor that determines sister chromatid segregation; instead, sister chromatids themselves contain information dictating the segregation pattern.

4.2.1 Are de-differentiated GSCs as good as original GSCs?

My recent study not only provided insights into how biased sister chromatid segregation might be achieved, but also improved our understanding of the potential differences between native *vs.* de-differentiated GSCs. Thus far, the only reported difference between native *vs.* de-differentiated GSCs is their centrosome orientation: native GSCs maintain stereotypical centrosome orientation toward the hub cells, whereas de-differentiated GSCs have mis-oriented centrosomes (Cheng et al., 2008). Due to centrosome mis-orientation, de-differentiated GSCs have a lower division rate compared to native GSCs (Cheng et al., 2008). Other than these differences, de-differentiated GSCs have been thought to function “perfectly” as GSCs. However, the randomized sister chromatid segregation in de-differentiated GSCs raises a possibility that de-differentiated GSCs might be fundamentally different from native GSCs. A careful characterization of de-differentiated GSCs will therefore be necessary to determine whether there are

any functional differences between the de-differentiated and original GSCs. I propose in my future work to investigate the expression of *stellate* (a polypeptide that is de-repressed in mutants in which X and Y chromatid segregation is randomized) in de-differentiated GSCs and also to examine whether there is any effect on the fertility (explained later in Section 4.5.4).

4.3 Biological relevance of non-random sister chromatid segregation

At present it is not clear why X and Y chromosomes are segregated non-randomly. It is unlikely that non-random sister chromatid segregation serves to protect the "immortal strand" to avoid replication-induced mutations as our data suggests that GSCs do not retain the original template strands of X and Y chromosomes forever. We favor the possibility that certain epigenetic information is transmitted distinctively to GSCs and GBs, particularly considering the involvement of *Dnmt2* in this process. Indeed, X and Y chromosomes are subjected to many epigenetic regulations, such as dosage compensation (Park and Kuroda, 2001; Gelbart and Kuroda, 2009; Conrad and Akhtar, 2011) and male-specific meiotic sex chromosome inactivation (Hense et al., 2007), although the extent to which sex chromosomes are subject to this type of regulation in *Drosophila* male germ cells remains to be elucidated. Additionally, it is known that *Drosophila* Y chromosome is highly heterochromatic and contains only a few known genes required for spermatogenesis, such as axonemal dynein (Piergentili and Mencarelli, 2008). It is known that precocious expression of these genes is toxic to non-spermatid cells, and gene expression must be tightly suppressed,

except during late spermatogenesis (likely including in GSCs). Moreover, transposons and polypeptide repeats such as, *Gypsy* and *Stellate*, as well as the piRNAs that suppress their expression, are known to be located on the X and Y chromosomes (Malone et al., 2009). These are some of the examples of potential epigenetic regulation specific to X and Y chromosomes. Intriguingly, I found that non-random sister chromatid segregation of X and Y chromosomes might be involved in the suppression of *stellate* and its corresponding piRNA expression in *Drosophila* testis (Yadlapalli and Yamashita, 2013). *Stellate*, which encodes polypeptides and whose de-repression is known to reduce fertility, is located on the X chromosome, and the piRNAs that suppress *stellate* expression [*Su(ste)*] are located on the Y chromosome (Aravin et al., 2001; Tulin et al., 1997). I found that *stellate* is de-repressed in all the mutants that are defective in non-random sister chromatid segregation. Although my current data do not provide direct evidence that randomized sister chromatid segregation is the underlying molecular reason for de-repressed *stellate* expression, these data raise the intriguing possibility that non-random sister chromatid segregation might serve to transmit an epigenetically modified copy of *Su(ste)* and/ or *stellate*, thereby contributing to suppression of *stellate*. To obtain a definitive answer to this question, it is important to determine whether the *stellate* or *Su(ste)* loci have any distinct epigenetic marks, which are segregated asymmetrically during GSC division.

4.4 Summary

In summary, my study presents the first evidence of chromosome-specific non-random sister chromatid segregation in adult stem cells and provides mechanistic insight into how cells distinguish sister chromatids and segregate them non-randomly. This mechanism may be utilized in many other systems to transmit distinct epigenetic information. We have only just started to understand how cells might be able to distinguish sister chromatids, and segregate them in a nonrandom manner. It will be interesting to see whether the molecular mechanisms involved in the non-random sister chromatid segregation of X and Y chromosomes in *Drosophila* male GSCs (involving *cnn*, SUN–KASH proteins and methyltransferase) have similar roles in other systems. What purpose the non-random segregation of sister chromatids might be serving remains very much an open question. Our recent study provides a tantalizing clue that non-random sister chromatid segregation might be involved in regulating repression of repetitive elements. We foresee exciting research in the future that will help us to improve our understanding of how and why stem cells non-randomly segregate their sister chromatids.

4.5 Future Directions

4.5.1 Identify chromosomal sequences on X and Y chromosomes that are required for non-random sister chromatid segregation

Our study suggested that particular template strands of X and Y chromosomes might be anchored to the nuclear envelope, which links to the

mother centrosome (Yadlapalli and Yamashita, 2013). We hypothesize that specific DNA sequences on X and Y chromosomes interact with the SUN domain proteins to allow for the anchorage of template DNA strands to the nuclear envelope. To address this question, we can use publicly available chromosomal duplication kits in which a portion of X chromosome is duplicated onto 3rd chromosome (Figure 4.5). We can then conduct CO-FISH on GSCs from such flies with a 3rd chromosome probe to see whether the presence of a specific X chromosome region allows non-random segregation of the sister chromatids of 3rd chromosome. In our recent study with autosomes, we noticed that sister chromatids of paternal and maternal homologs of 3rd chromosome are coordinated, *i.e.,* maternal sister chromatid which contains the Watson strand as a template always co-segregated with the paternal sister chromatid that contains the Watson strand as a template. However, it was random as to whether GSCs retain sister chromatids with Watson template strands or Crick template strands. If we conduct CO-FISH on 3rd chromosome duplication flies and detect any bias with which GSCs inherit Watson template strands *vs.* Crick template strands, then we can conclude that the particular sequence of X, which is duplicated, is required for the non-random sister chromatid segregation of X chromosome. Once we identified the sequences on X chromosome, we can further confirm their functionality by removing these sequences from X chromosome and/or adding them to autosomes and testing the segregation patterns of the altered chromosomes.

Dp(1;3)DC420	XHet:2721;81472	h26--h32;h26--h32;65B2
Dp(1;3)DC421	XHet:45803;147046	h26--h32;h26--h32;65B2
Dp(1;3)DC422	XHet:135373;?	h26--h32;h26--h32;65B2
Dp(1;3)DC423	XHet:153883;?	h26--h32;h26--h32;65B2

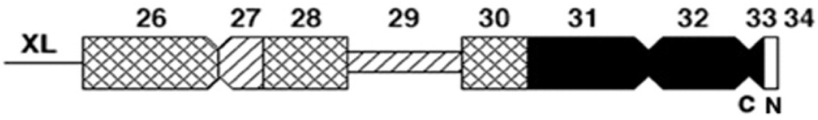

Figure 4.5 Duplication of X chromosome heterochromatic region on 3rd chromosome. Fly strains available publicly from Bloomington stock center where the BAC clones of heterochromatic region of X chromosome was inserted into an attP docking site on chromosome arm 3L. Cytological map showing the heterochromatic and peri-centromeric regions of X chromosome is shown on the bottom.

Another parallel approach that we can take is to identify unique sequences on X and Y chromosomes by using bioinformatics analyses and testing whether they play any role in non-random sister chromatid segregation. It is reasonable to assume that the sequences on X and Y chromosomes that are required for non-random sister chromatid segregation, if they exist, are unique to sex chromosomes as autosomes are shown to be randomly segregated (Yadlapalli and Yamashita, 2013). To test whether these unique sequences play any role in non-random sister chromatid segregation, we can use similar approaches as described above, for example, we can delete the sequences from sex chromosomes or add the sequences to autosomes and observe sister chromatid segregation pattern.

4.5.2 Identify proteins that interact with the specific DNA sequences

Once we identify DNA sequences that are responsible for non-random sister chromatid segregation, we can isolate proteins that bind to these sequences and also potentially physically interact with the SUN domain proteins (Figure 4.6).

Finding the adaptor proteins will be crucial to improving our understanding of how particular template strands are anchored to the nuclear envelope. We can gain significant insights into the biological relevance of the phenomenon of non-random sister chromatid segregation by examining the mutants of these DNA binding proteins: while mutants of *Dnmt2*, centrosomes, and the LINC complex have a broad range of phenotypes, mutants of adaptor proteins that link X/Y chromosomes and Koi protein might exhibit phenotype(s) specifically associated with randomized sister chromatid segregation.

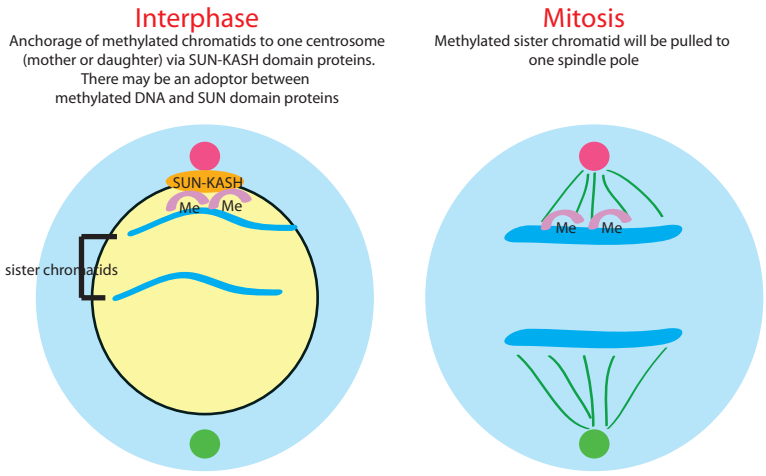

Figure 4.6 Adaptor proteins that link X/Y chromosomes to SUN domain proteins. Only one sister chromatid is methylated ("Me") at certain genomic loci, which is recognized by an adopter protein (pink crescent) that links the chromosome to the centrosome (mother or daughter) via the LINC complex (SUN-KASH domain proteins). This helps the mother (or daughter) centrosome retain the methylated copy of the sister chromatid in mitosis.

4.5.3 Investigate the role of *Dnmt2* in non-random chromatid segregation

In our recent study, we have shown that methyltransferase enzyme, *Dnmt2,* is required for non-random sister chromatid segregation of X and Y chromosomes

(Yadlapalli and Yamashita, 2013). There has been a lot of debate in the field regarding the function of *Dnmt2*, whether it methylates DNA or tRNA (Goll et al., 2006; Phalke et al., 2009). Therefore, it will be interesting in future studies to explore the identity of the signal generated by *Dnmt2* and the mechanism by which this signal affects the mitotic spindle to carry out nonrandom chromosome segregation.

Another interesting question that we would like to address is whether *Dnmt2* is continuously required in the zygotic stage and/or adult stage to maintain the non-random sister chromatid segregation. Our recent study suggested that *Dnmt2* is required during development for non-random sister chromatid segregation in adult GSCs. To test whether *Dnmt2* is required continuously during the adult stage too, we can examine sister chromatid segregation in *Dnmt2* RNAi mutants where *Dnmt2* is knocked down during adult stage.

It will be interesting to test whether there is a transgenerational effect on sister chromatid segregation. In heterozygous animals (*Dnmt2*−/+), where non-random Y chromosome segregation is compromised due to having a mutant father (*Dnmt2*−/−) (Table 3.2), what is the chromosome segregation in their progeny like? Half of their sperm carry the wild-type *Dnmt2* gene, but their Y chromosomes came from GSCs that do not segregate the Y chromosome non-randomly. Can they transmit the “correct” information on Y chromosomes, such that their progeny (“grandsons” of the original mutant male) segregate the Y chromosome non-randomly? Alternatively, the Y chromosome may never be able to re-establish the correct information. A related fascinating question is what is the nature of the

information that is transmitted from the parents to the progeny that is required for non-random sister chromatid segregation in the progeny.

4.5.4 Examine the relationship between non-random sister chromatid segregation and suppression of transposons

In our recent study, we have shown that *stellate*, a polypeptide repeat, is de-repressed in mutants where sister chromatid segregation of X and Y chromosomes is randomized (Yadlapalli and Yamashita, 2013). This data does not provide a clear answer whether *stellate* de-repression is indeed caused by non-random sister chromatid segregation. To further investigate the relationship between non-random sister chromatid segregation and suppression of transposons, we would like to examine the expression of other transposons that are located on X and Y chromosomes in the mutants. Specifically, we would like to examine the expression pattern of transposon, *gypsy,* which is present on Y chromosome and is usually suppressed by the piRNA gene *flamenco* located on the X chromosome (Aravin et al., 2001).

We are also interested in examining the expression of *stellate* in other cases where non-random sister chromatid segregation is compromised, for example, in GSCs from aged animals and in de-differentiated GSCs. These two conditions might be related as we have shown previously that the number of de-differentiated GSCs increases as the animal ages (Cheng et al., 2008). In any case, it is well established that fertility decreases as the fly ages (Cheng et al., 2008), so it will be interesting to look at *stellate* expression in such flies.

We do not yet know if randomized sister chromatid segregation is indeed causing de-repression of *stellate* or whether de-repression is a byproduct of some other defect (pleiotropic effect of mutants). To unambiguously answer this question, we need to examine mutants of adaptor proteins, which might exhibit phenotype(s) specifically associated with randomized sister chromatid segregation (see section 4.5.2 for more details). Another interesting line of investigation is to examine the correlation between the timing of *Dnmt2* requirement for asymmetric chromosome segregation and transposon suppression.

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