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UNDERSTANDING THE MEIOTIC ROLES OF SISTERS UNBOUND IN DROSOPHILA MELANOGASTER

Badri Krishnan
University of Tennessee - Knoxville, bkrishn1@vols.utk.edu

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Bruce D. McKee, Major Professor

We have read this dissertation and recommend its acceptance:

Ranjan Ganguly, Mariano Labrador, Hong Guo, Igor Jouline

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

UNDERSTANDING THE MEIOTIC ROLES OF SISTERS UNBOUND IN DROSOPHILA MELANOGASTER

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DEDICATION

To my mother Shanthi Krishnan and my father Raghavan Krishnan. They were a constant source of support and encouragement throughout my PhD. I could not have been in a position to even pursue my PhD without the great education, training and environment that they provided me.

To my wife Megha for all the love, support and understanding

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ABSTRACT

During meiosis, cohesin is required for sister chromatid cohesion and for formation of chromosome cores. Multiple processes including chromosome segregation, recombination and synaptonemal complex (SC) are dependent on cohesin. Cohesin complex consists of two SMC subunits- SMC1, SMC3 and two non-SMC subunits RAD21/REC8 in meiosis and SA. But in *Drosophila*, non-SMC subunits have not been shown to be required for cohesion. We have identified a gene *sisters unbound*, which along with previously identified *ord* and *solo*, form a group of three genes (*sos*) which do not have any sequence similarity to cohesins but performs functions demonstrated by cohesins.

Proper chromosome segregation requires that homologs are connected by chiasmata during meiosis I and that sister centromeres are mono-oriented at meiosis I and bi-oriented at anaphase II. For both these functions cohesion is necessary. Cohesins are also required for proper assembly axial elements/lateral elements (AE/LE), SC, inhibiting sister chromatid exchange (SCE) and recombination. SUNN is required for all these functions and it localizes to chromosomes in a pattern similar to cohesion proteins ORD, SOLO and cohesin subunit SMC1 and is mutually interdependent on SOLO, SMC1 for localization. Bioinformatics analysis suggests that SUNN is a structural homolog of SA. Based on functional and structural similarity to cohesin complex components we predict that SUNN is a part of the *Drosophila* meiotic cohesin complex.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER III: SISTERS UNBOUND IS REQUIRED FOR RECOMBINATION, SYNAPSIS, HOMOLOG BIAS AND DOUBLE STRAND BREAK REPAIR IN DROSOPHILA FEMALES

CHAPTER I: GENERAL INTRODUCTION

Meiosis: An Essential Biological Process

Meiosis is a cell division process that leads to the production of haploid gametes from diploid precursor cell. Haploid gametes are produced by two continuous rounds of chromosome segregation without an intervening round of DNA replication. Gametes (sperm and egg) are necessary for sexual reproduction. Each diploid precursor cell has two sets of chromosomes – one set from the mother and the other from the father (homolog set). A haploid gamete receives one complete set of chromosomes and when gametes fertilize they create a diploid zygote with similar number of chromosomes as the parent cell. Accurate meiosis is essential for producing gametes with the complete haploid complement of chromosomes and aneuploid gametes (gametes with too many or too few chromosomes) lead to aneuploid zygotes and embryos (Petronczki et al. 2003).

In humans, aneuploid embryos are often miscarried or develop genetic disorders if they survive. The most common surviving aneuploid zygotes are trisomic (three copies of a chromosome are found instead of two). Down's syndrome (21^{st} chromosome trisomy) and Klinefelter's syndrome (XXY, XXX, XYY) are two examples of viable trisomic disorders and 45 X monosomy is a viable monosomic disorder (one copy of a chromosome is present instead of two) found in humans. Most trisomies and monosomies are caused by fertilization of gametes containing incorrect chromosome number produced as a result of mis-segregation events during meiosis (HASSOLD and HUNT 2001).

Female age at the time of pregnancy determines the probability of occurrence of aneuploidy. The causes for increased incidence of age-related aneuploidy are less

availability of healthy oocytes, inability to terminate eggs with incorrect chromosome number and inability of older oocytes to undergo proper chromosome segregation due to improper chromosome alignment or abnormally placed connections between homologs (HASSOLD and HUNT 2001; HUNT and HASSOLD 2008). Trisomies were found in 35% of pregnancies in women older than 42 and therefore understanding the mechanism of meiotic chromosome segregation and the factors that influence it has huge biomedical significance.

Chromosome Segregation during Meiosis and Role of Cohesins

Prior to meiosis each chromosome consists of a single DNA duplex (a chromatid). During pre-meiotic S phase, DNA replication yields chromosomes that consist of two identical (sister) chromatids that remain aligned and connected throughout meiosis (referred to as cohesion). A multi-protein complex called cohesin is loaded all along the chromosomes prior to DNA replication and establishes cohesion between sister chromatids during S phase (Figure 1-1). Meiosis is divided into two sub-stages- meiosis I and II -- and chromosome segregation occurs at anaphase of each sub-stage. At anaphase I, homologs segregate towards opposite poles such that each cell gets one homolog chromosome set containing a mixture of both paternal or maternal chromosomes. This is called reductional segregation because the chromosome number per nuclei/cell is reduced by half at this stage. Anaphase II segregation is mitosis-like in that sister chromatids segregate to opposite poles. This is called equational segregation.

Cohesin is a conserved complex that functions in both mitosis and meiosis and consists of four major subunits. The SMC1 and SMC3 (Structural Maintenance of

Chromosomes 1 and 3) subunits form long rigid intra-molecular, anti-parallel (hairpin) coiled coil domains flanked by globular “hinge” and ATP (Nucleotide Binding Domain-NBD) binding domains at opposite ends. SMC1 and SMC3 associate with each other at their hinge domains and with the C-terminal and N-terminal domains, respectively, of the “α-kleisin” subunit, either SCC1/MDC1 (in yeast, RAD21 in other eukaryotes) in mitotic cohesion complexes, or REC8 (a paralog of SCC1/RAD21) in most meiotic cohesion complexes. These interactions generate a closed tripartite ring-like structure that is thought to encircle chromatids and provide cohesion. The fourth subunit, SCC3 (Stromalin or SA in higher eukaryotes), is not part of the ring and does not interact directly with SMC subunits but binds to the SCC1/RAD21/REC8 subunit and is required for cohesion. This ring cohesin structure is resolved when the SCC1/MCD1/REC8 kleisin subunit is cleaved by Separase during anaphase. This cleavage releases cohesion and triggers chromosome segregation (LEE and ORR-WEAVER 2001; NASMYTH and HAERING 2009).

Sister chromatid cohesion is required for both the reductional and equational meiotic chromosome segregations. During the early stages of meiosis I, crossingover (DNA exchange) takes place between homologous chromatids (i.e., one chromatid from the maternal homolog crosses over with one chromatid from the paternal homolog). Each such crossover creates a stable connection site (known as a chiasma) between homologs because cohesin complexes, which are abundant on chromosome arms, connect each of the crossover chromatids to both of the non-crossover sister chromatids (see Figure 1-1). In most eukaryotes, each chromosome pair normally experiences at least one crossover and therefore has at least one chiasma to keep the

homologs connected while they align on the meiosis I spindle. At anaphase I, arm cohesin is removed by the Separase-mediated cleavage of cohesin rings connecting sister chromatid arms. This resolves chiasmata and triggers segregation of homologs. Cohesin is also present at the centromeric regions, where it is essential to maintain cohesion between sister chromatids while they align on the meiosis II spindle. These centromere cohesins are not cleaved at anaphase I and persist until anaphase II when a second round of Separase activity cleaves centromere cohesion and triggers sister chromatid separation. Therefore, two-step removal of cohesin dictates the meiotic chromosome segregation pattern. At anaphase I, centromeric cohesins are protected by a conserved family of protein called Shugoshins, which includes *Drosophila* MEI-S332 (WATANABE 2005). Shugoshins are centromeric proteins that associate with PP2A (Protein Phosphatase 2A) which is necessary for the dephosphorylation of CK1 mediated phosphorylation of REC8 (phosphorylation of REC8 makes it susceptible to Separase mediated cleavage). The absence of phosphorylated-REC8 at the centromeres protects cohesin from Separase mediated cleavage. But Shugoshins are either removed or inactivated from the centromere post anaphase I and therefore cannot protect the centromeric cohesins from Separase-mediated cleavage at anaphase II (KERREBROCK *et al.* 1992; WATANABE 2005).

Chiasmate homolog connections form the basis for reductional segregation in most but not all eukaryotes. In *Drosophila* and other Dipteran males and in Lepidopteran females, crossing-over does not occur but homologs are attached and segregate normally at anaphase I. In *Drosophila*, connections between homologs are provided until anaphase I by a conjunction complex which consists of two proteins-

Figure 1-1: Major events during meiotic chromosome segregation. Pre-meiotic S phase: Only one chromatid is present per chromosome. S phase: Sister chromatid is synthesized by DNA replication. Sister chromatids of a chromosome are held together by cohesins (blue and red rings). Early prophase I: Chromosome condensation, homolog pairing and recombination is initiated. Mid prophase I: Synaptonemal complex (yellow connectors) is completely constructed between homologs. Exchange of DNA occurs between paired homologs. DNA exchange is completed by the end of prophase I and synaptonemal complex is disassembled but the homologs are still connected by chiasma. Metaphase I: Sister centromeres are mono-oriented towards the same pole and homologs are mono-oriented towards opposite poles. Microtubules from opposite poles attach to homolog kinetochores. Anaphase I: Arm cohesins are destroyed and chiasma are resolved and homologs are pulled towards opposite poles by microtubules (reductional segregation). Metaphase II: Sister centromeres are oriented towards opposite poles. Anaphase II: Cohesins near the centromeres are destroyed and sister chromatids segregate towards opposite poles. After chromosomes division, nuclear membrane is re-formed and cytokinesis occurs which leads to the production of four diploid cells.

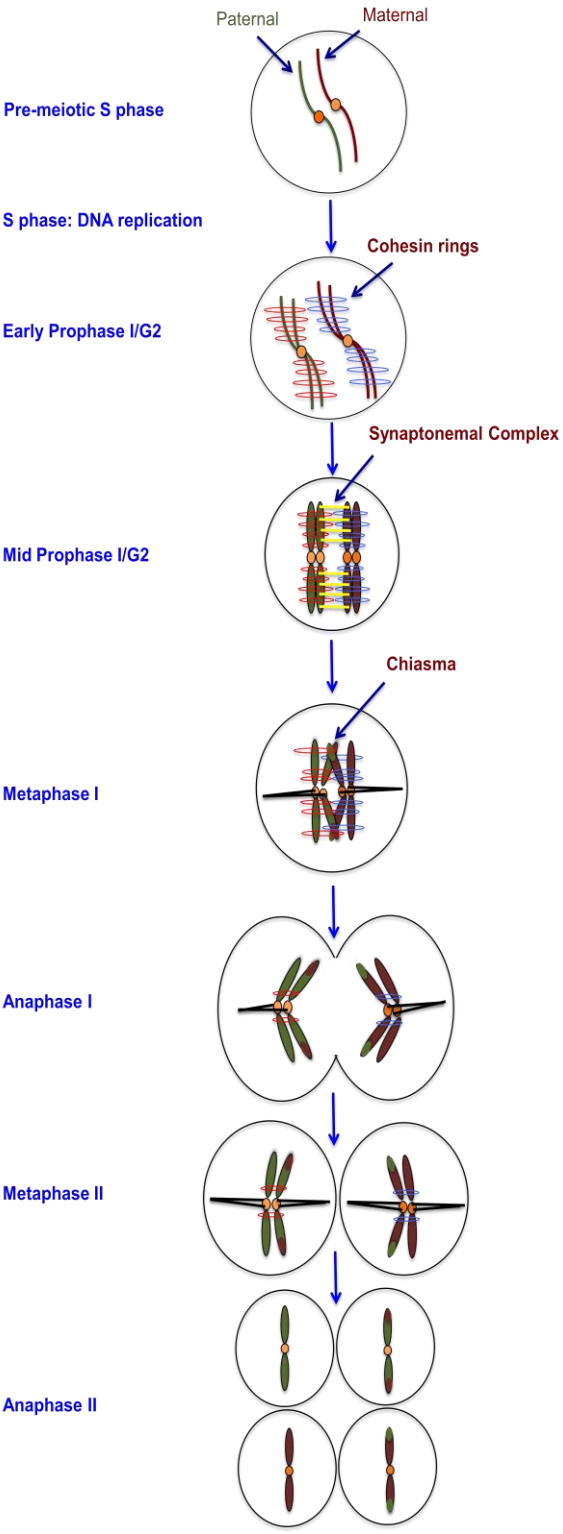


Figure 1-1. Continued

Stromalin in Meiosis (SNM) and Modifier of Mdg4 in Meiosis (MNM) (THOMAS *et al.* 2005).

In *Drosophila melanogaster*, the composition of the meiotic cohesin complex is not understood. There are three main reasons for this: 1) No known REC8 homolog which is required for cohesion has been found 2) Mitotic cohesin subunits such as RAD21 and SA have not been shown to be required for meiotic cohesion (URBAN *et al.* 2014) 3) Role of SMC subunits in cohesion has not been directly assayed. In light of this there exist two proteins ORD and SOLO which do not have any sequence homology to known cohesins and is required for cohesion and other associated roles such as recombination and synaptonemal complex (SC) stability during meiosis (MASON 1976; WEBBER *et al.* 2004; YAN *et al.* 2010; YAN and MCKEE 2013). Therefore, an unsolved puzzle in *Drosophila* is the composition and structure of the meiotic cohesin complex.

Major events during prophase I

Synaptonemal complex formation

During early prophase I (leptotene) in most organisms sister chromatids start condensing along their longitudinal axes and form prominent linear structures called axial elements (AEs). The core components (so called “chromosome cores”) of AEs are cohesins that connect the axes of the sister chromatids and assemble into continuous axial structures. AEs also contain additional non-cohesin proteins that are typically dependent on the cohesin cores for their recruitment and assembly. AE/LEs are discussed in more detail below. During zygotene and pachytene, the AEs of homologous chromosomes align and evolve into the lateral elements (LEs) of synaptonemal complexes (SCs). SCs are tripartite structures, in which the two LEs and

a parallel central element (CE) are connected all along their length by multiple transverse filaments (TFs) (LEE and ORR-WEAVER 2001; PAGE and HAWLEY 2004). SC does not form all at once. During zygotene, short stretches of SC appear at initiation sites. SC subsequently spreads until, during the pachytene stage, it extends from one end of chromosome axis to the other. At the end of pachytene, SC is disassembled from chromosome arms, but chiasmata persist and keep homologs joined together until anaphase I.

In *Drosophila* females, there are some significant deviations from this standard pattern. In particular, a distinct zygotene stage is absent and no unpaired AEs have been observed. Both LE and SC formation are initiated at zygotene, when a few patches of the LE protein C(2)M and the TF protein C(3)G are observed on chromosome arms. C(3)G (but not C(2)M) also accumulates at centromere clusters during zygotene where it colocalizes with patches of the SMC cohesins and the ORD and SOLO cohesion proteins. In early pachytene these initiation sites are extended and by end of pachytene SC forms thread-like structures along chromosome arms and homologs are associated tightly along their entire length with the help of SC (ZICKLER and KLECKNER 1998; PAGE and HAWLEY 2004; LAKE and HAWLEY 2012).

SC proteins have been identified and analyzed in many eukaryotes. TF proteins (Zip1 in *S. cerevisiae*, C(3)G in *Drosophila melanogaster*, SYCP1 in mammals, Syp1, Syp2 in *C. elegans*, Syn1 in *A. thaliana*) are coiled-coil proteins that localize to the homolog interface and align perpendicular to the LEs and CE; their absence leads to failure of homolog synapsis. CE proteins (SYCE1, SYCE2, SYCE3 and Tex12 in mammals; Corona and Corolla in *Drosophila*) have been identified by their distinctive

central localization pattern (COSTA *et al.* 2005; PAGE *et al.* 2008; DAVIES *et al.* 2012; COLLINS *et al.* 2014). Both meiotic cohesins (e.g. SMC1, SMC3, REC8 in numerous eukaryotes) and non-cohesin AE/LE components (Hop1, Red1 in *S. cerevisiae*, Him 3 in *C.elegans*, Asy1 in *A. thaliana*, SYCP3, SYCP2 in mammals) have been specifically localized along chromosome axes. In general, formation of chromosome cores appears to be a prerequisite for proper assembly of AE/LEs and SC central elements and for chromosome synapsis to occur. In the absence of *smc3* or *rec8* in *S. cerevisiae*, RED1 (an AE protein) and ZIP1 (a TF protein) do not assemble properly on chromosome arms (KLEIN *et al.* 1999). In mouse, the SYCP2 and SYCP3 AE proteins do not assemble properly in cohesin mutants (LLANO *et al.* 2012). In *C. elegans*, the AE protein HIM3 is unable to assemble properly on chromosome arms in *rec8* mutants (PASIERBEK *et al.* 2001). In maize, assembly of the Asy1/Hop1 AE protein is improper in mutants of *afd1* the a *rec8* homolog (GOLUBOVSKAYA *et al.* 2006).

In *Drosophila*, a number of candidate LE proteins have been identified but in some cases, definitive localization data are lacking. In general, the absence of unpaired AEs in Drosophila makes it impossible to tell by light microscope analysis alone whether an SC protein localizes to the axes or to the central region. Moreover, even for definite or probable LE proteins, it has not yet been possible to definitively sort them into core and non-core components. The best-studied LE protein, C(2)M, assembles into continuous linear structures and has been shown by ultrastructural analysis to localize to two parallel tracks that flank a single track of the TF protein C(3)G. In the absence of C(2)M, the SC TF protein C(3)G does form some patches on chromosomes, including at centromeres, but is unable to extend from its inititation sites at centromeres and

chromosome arms and form thread-like structures (MANHEIM and MCKIM 2003; TANNETI *et al.* 2011). Since C(2)M is an α-kleisin, it could be a component of the cohesin cores, but this is uncertain because the role of C(2)M in arm cohesion remains unclear. Several other proteins assemble into continuous ribbon-liike structures during pachytene and are postulated to be components of or associated with LEs rather than SC central regions on the basis of their molecular identities and/or genetically identified functions but have not been localized ultrastructurally. These include SMC1 and SMC3, which are presumed to define the cohesin cores, and Nipped-B, an adherin (cohesin loading complex) component. Absence of SMC1 or SMC3 completely blocks chromosome localization of C(2)M and C(3)G, suggesting complete failure of axis assembly and synpasis. The cohesion proteins ORD and SOLO also localize continuously to SCs in pachytene oocytes and are presumed to be associated with LEs on the basis of their role in cohesion and their spatial/temporal localization patterns, Like SMC1 and SMC3 but unlike C(3)G, they localize to chromosomes prior to SC initiation and to chromosomes of nurse cells in meiotic cysts in which SCs are absent. However, the phenotypes of *ord* and *solo* mutants are much milder than those of *smc1* or *smc3* mutants. In light microscope analyses, SCs appear to form normally but fragment and disassemble prematurely. In the EM, however, the SCs that form in ord mutants appear quite abnormal, often appearing to lack distinct LEs. Moreover, neither ord nor solo is required for stable localization of SMC1 or SMC3 to chromosome arms at any stage. Thus, ORD and SOLO appear to be LE components and have a role in SC assembly and stability but cannot be clearly assigned to the cores. Their precise roles in SC formation remain to be determined.

Homolog pairing

The main purpose of SC is to ensure that homologs are intimately paired and this is necessary for the occurrence of recombination. In most organisms like *S. cerevisiae*, mouse and plants, homologs establish initial connections with each other during early prophase I; these are called axial associations. Axial associations are formed as a result of DNA exchange at initial DNA double strand break sites and these associations act as nucleation centers for SC formation. Complete SC formation leads to tight pairing up of homologs in these organisms (ROEDER 1997; PAGE and HAWLEY 2004). In *Drosophila*, homologs enter meiosis already paired and it is hypothesized that it is a continuation of pre-meiotic somatic pairing in the *Drosophila* germline. In *Drosophila* males, recombination and SC formation does not occur but homologs are paired till mid-prophase I. In a study using 14 euchromatic lacO insertions, it was found that homologs are paired >95% of time in spermatocytes in early prophase I and at mid-prophase I (S3) they loose pairing at these loci. In pre-meiotic 2,4,8 cell cysts the level of pairing is less than that of early prophase I but high pairing among the euchromatic loci is achieved by the time 16 cell cyst enters meiosis. High percentage of homolog pairing is also observed at heterochromatic loci during early prophase I of meiosis after which it is lost. Centromeres of homologs are also paired in spermatocytes during early prophase I and only 3-4 centromeric foci are observed (as assessed by immunostaining using CID (centromere identifier) antibody) but by mid-prophase I homologous centromeres separate and upto eight centromeric foci are observed at prometaphase I (Figure 2-2) (VAZQUEZ *et al.* 2002; MCKEE 2004; TSAI *et al.* 2011).

In *Drosophila* females, homologs are unpaired in germline stem cells at tested heterochromatic and euchromatic loci except for a 359 bp repeat region at the X chromosome pericentromere. These loci start to pair up gradually as mitotic divisions occur and 2, 4, 8 and 16 cell cysts are formed. As a result, when the 16 cell cyst enters meiosis all sites are paired. In *Drosophila* females, centromeres of oocytes/pro-oocytes are together and appear as a single large foci. This is called centromeric clustering. Centromeric clustering is hypothesized to be necessary for SC loading on to chromosomes and mutations of *ord* and *solo* disrupt centromeric clustering in *Drosophila* oocytes/pro-oocytes (TANNETI *et al.* 2011; JOYCE *et al.* 2013; YAN and MCKEE 2013).

Double Strand Breaks and Crossing over

Homologous recombination is an integral part of meiosis I in most eukaryotes and is required for formation of chiasmata. Double strand breaks (DSB) are necessary for the initiation of recombination. A highly conserved enzyme belonging to DNA topoisomerase family (Topo VIA) called Spo11 (identified in *S. cerevisiae*, Rec12 *is S. pombe*, mei-w68 in *Drosophila*) creates DNA DSBs by a trans-esterification reaction (ROEDER 1995; KEENEY 2008). DSBs are resected by 5'-3' exonucleases which expose two staggered single stranded 3' tails. An exposed 3' end of the duplex invades the DNA duplex (chromatid) of its homologous non-sister partner and causes the displacement of a D-loop to the strand with the other exposed 3'tail. This is called strand invasion and it is performed by a conserved bacterial RecA homologs Rad51, Rad55, Dmc1 and Rad57 in *S. cerevisiae*. The 3' single strand ends are extended and repaired based on the complementary sequence of the intact DNA duplex that it invades and are

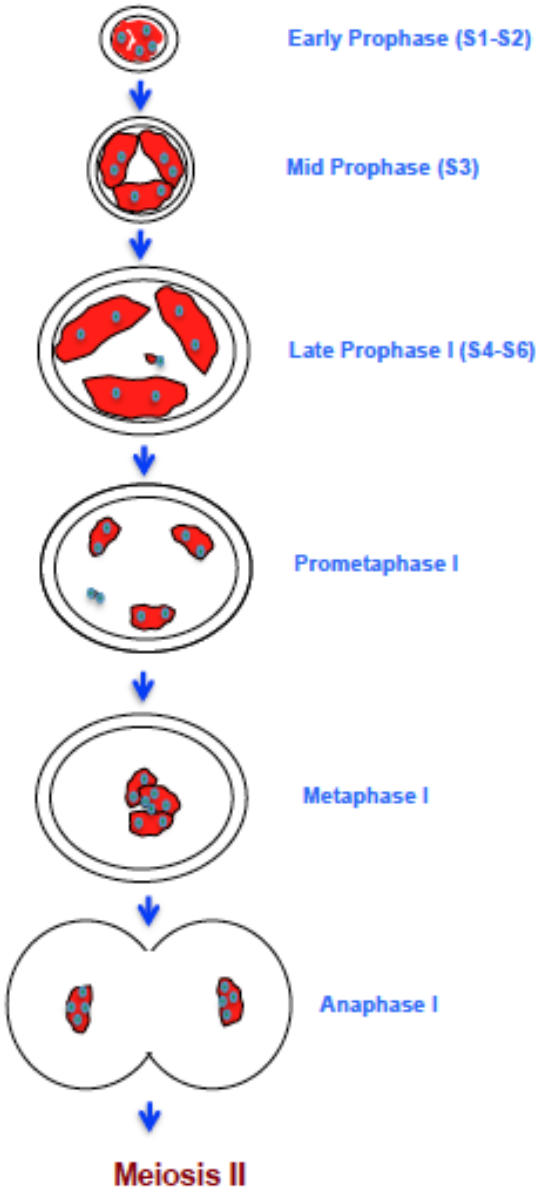


Figure 1-2: Chromosome behavior during meiosis I in *Drosophila* spermatocytes: Red regions depict chromosome. Blue dots represent centromeres. Prophase I consists of S1-S6, which is followed by prometaphase I, metaphase I and anaphase I.

ultimately joined by ligation to the resected 5’ ends of the parent strands from which they broke off. Strand invasion, D-loop formation and homologous DNA repair creates two junctions consisting of intersecting DNA duplexes from a homolog pair. This is called Double Holliday Junction (DHJ) and it is resolved to produce a crossover product.

The function and consequence of recombination is to ensure exchange of DNA between homolog pairs (which usually differ slightly in DNA sequence) and generate chromosomal variation in the population. In addition to promoting DNA sequence variation in the population, meiotic homolog exchange is also essential for making stable connections between homologs which is required for accurate homolog segregation during meiosis. There is strong evidence from yeast and *Drosophila* that homologs are preferential repair templates in meiosis and sister chromatid exchange (SCE) is actively suppressed. This preference for repair using homologs rather than sister strands is called homolog bias. In *S. cerevisiae*, AE/LE components Hop1 and Red1 and Mek1 kinase, are required for homolog bias and in *Drosophila*, two proteins ORD, which is a LE component and SOLO, a probable LE component are required for homolog bias (WEBBER *et al.* 2004; YAN and MCKEE 2013).

In *S. cerevisiae* and mouse, DSB formation is also essential for homolog pairing and synapsis. However, in *Drosophila*, synapsis occurs normally in the absence of DSBs (MCKIM *et al.* 2002). This may be because homologs are paired as they enter meiosis in *Drosophila* which aids in synapsis, whereas in *S.cerevisiae* the formation of axial associations during early prophase I are dependent on DSB creation and a complex consisting of zip proteins (PAGE and HAWLEY 2004).

In cohesin mutants recombination is reduced significantly. In *S. cerevisiae* reduction in homologous recombination is observed in *smc3* and *rec8* mutants (KLEIN *et al.* 1999). In *S. pombe, rec8* and *rec11* mutants reduce recombination. In *Drosophila*, direct assay of recombination has not been performed for SMC cohesin subunits but severe reduction in homologous recombination is observed for cohesion proteins ORD and SOLO mutants (MASON 1976; YAN and MCKEE 2013). A reduction in recombination is also accompanied by loss of homolog bias during recombination and increased levels of sister chromatid exchange (SCE) in *S. cerevisiae smc3*, *rec8* mutants and *ord solo* mutants in *Drosophila*.

Role of cohesion proteins in *Drosophila* and other organisms

Cohesin performs multiple functions during meiosis. The role of cohesin in recombination, chromosome core assembly and SC formation has been described above. The major function of cohesin is to provide sister chromatid cohesion and the absence of cohesins causes chromosome mis-segregation/non-disjunction. In *S. cerevisae*, both *rec8* and *smc3* mutants cause random chromosome segregation at anaphase I and II. In *S. pombe, rec8* mutants exhibit a mitosis like equational segregation at anaphase I (KLEIN *et al.* 1999; WATANABE and NURSE 1999). In *Drosophila,* genetic tests have shown that *ord* and *solo* cause both sister chromatid and homolog non-disjunction during meiosis in both sexes. Analysis of cohesion at centromeres has revealed that it is disrupted in these mutants (MIYAZAKI and ORR-WEAVER 1992; BICKEL *et al.* 1997; BALICKY *et al.* 2002; YAN *et al.* 2010; TAKEO *et al.* 2011; YAN and MCKEE 2013). Due to unavailability of viable mutants genetic tests have not been carried out for SMC subunit mutants and therefore their role in chromosome

segregation has not been directly assayed and is implied through studies on *solo* and *ord*. The other cohesin proteins like C(2)M, RAD21, and SA are not required for chromosome segregation or cohesion during meiosis in *Drosophila* (MANHEIM and MCKIM 2003; HEIDMANN *et al.* 2004; URBAN *et al.* 2014). The various roles of cohesin subunits and cohesin associated/interacting proteins in *Drosophila* are depicted in Table 1-1.

Therefore, both ORD and SOLO are hypothesized to be a substitute for non-SMC subunits in some cohesin complexes. In addition there has to be another complex which is not required for cohesion but for forming SC. This is because C(2)M, RAD21 and SA all are required for SC formation.

Cell Cycle and Meiosis

Cell cycle controllers regulate meiosis through control of Separase activity and Separase activity depends on the binding of its inhibitor ligand Securin. Spindle Assembly Checkpoint (SAC) controls activity of Anaphase Promoting Complex (APC/C), which is a ubiquitin ligase that tags ubiquitin on to Securin and targets it for degradation. Cell cycle regulators (Cdc kinase) determine if the cell is ready for chromosome segregation by deciding to destroy Securin and activating Separase. Activated Separase cleaves the cohesin kleisin subunit REC8 in *S*. *cerevsiae* and other eukaryotes (MARSTON and AMON 2004; NASMYTH and HAERING 2009). In *Drosophila*, no REC8 has been found but it is hypothesized that REC8 substitutes like SOLO/ORD is cleaved to remove cohesin ring from chromosomes. In mitosis, cell cycle regulators control chromosome segregation by different methods in *S. cerevisiae* and in humans (vertebrates). In yeast, polo-like-kinase directed phophorylation of the cohesin kleisin

subunit SCC1 makes it susceptible to proteolytic cleavage by Separase. Even in the absence of Separase activation, a phosphorylated SCC1 subunit causes a slow dissociation of cohesins from the chromosome. After ensuring bi-orientation of homologous chromosomes, APC/C gets activated by Cdc20 in *S. cerevisiae*. Activated APC/C ubiquitinates Securin and cleaves it and removes cohesins from the arms and the centromeres. The other method for chromosome segregation control is found in humans (and possibly in other eukaryotes such as *Drosophila*) and involves two-step cohesin removal: the arm cohesin is released by a Separase independent prophase pathway and the centormeric cohesin is released by a Separase dependent pathway. The arm cohesins are released by the phosphorylation of SA and SCC1 by polo-like-kinase which is also controlled by cell checkpoint (MARSTON and AMON 2004; MILLER *et al.* 2013).

In meiosis, a separate mechanism prevents the phosphorylation of the centromeric cohesins and is responsible for ensuring two-step chromosome segregation. This protection to phosphorylation is provided by shugoshins and PP2A. Bub1 targets PP2A to the centromeres, which recruits shugoshin to the centromeres. This complex protects the centromeric cohesins from CK1 mediated phosphorylation.

By anaphase I, the activation of SAC, Cdc20 and APC causes destruction of Securin and activation of Separase. Activated Separase cleaves REC8 subunit and releases arm cohesins. Generally, shugoshin is removed after anaphase I or is inactivated by cell cycle components or by the tension generated across centromeres and therefore centromeric cohesins are not protected against phosphorylation or

Table 1-1: Meiotic functions of known *Drosophila* cohesin proteins, cohesin subunit homologs and other proteins that interacts with known cohesins.

	Centromere Cohesion	Centromere Clustering	SC Form-ation	SC Stability	Non-Disjunc-tion	Double Strand Break Repair
SMC1	ND*	Lost	No	-	ND	Normal
Cap (SMC3)	ND	Lost	No	-	ND	Normal
C(2)M	Normal	Lost	No	-	Yes (Mild)	Normal
Rad21	Normal	Lost	ND	Unstable	ND	ND
SA	ND	ND	ND	ND	ND	ND
Nipped-B	Normal	Normal	Yes	Unstable	No	ND
ORD	Lost	Lost	Yes	Unstable	Yes (Severe)	Normal
SOLO	Lost	Lost	Yes	Unstable	Yes (Severe)	Delayed

*Not Determined

Separase mediated cleavage at anaphase II (WATANABE 2005; CLIFT and MARSTON 2011; MILLER *et al.* 2013).

Mono-orientation during Meiosis

In order to ensure that chromosomes follow the typical meiotic segregation pattern where homologs segregate towards opposite poles (sister chromatids segregate towards the same pole) at anaphase I and sister chromatids segregate towards opposite poles at anaphase II, orientation patterns have to be established at the centromeric region prior to segregation. Sister centromeres are mono-oriented to travel towards the same pole at anaphase I (and homologs to opposite poles) and at anaphase II sister centromeres are bi-oriented to move to opposite poles of the cell (PETRONCZKI *et al.* 2003). Mono-orientation of sister centormeres is unique to meiosis I and is different from what is observed during meiosis II and mitosis. In *S. cerevisiae*, a kinetochore associated protein called monopolin is required to ensure that the sister kinetochores are mono-oriented towards the same pole (TOTH *et al.* 2000). In *S. pombe*, Moa1 helps in the mono-orientation of sister centromeres at meiosis I by ensuring proper REC8 localization to the centromeres. Loss of REC8 causes mono-orientation defects even in the presence of Moa1 (WATANABE 2004; YOKOBAYASHI and WATANABE 2005). Studies in *S. pombe* have shown that cohesion ensures sister chromatid mono-orientation by holding heterochromatic region around centromeres and inner centromeric regions of sister chromatids. This is required for side-by-side arrangement of sister kinetochores, which ensures that microtubules emanating from the same pole attach to sister kinetochores. But at anaphase I the inner centromeric cohesion are selectively removed by Separase mediated cleavage of cohesins but cohesion at

heterochromatic regions are retained. This causes the disruption of side-by-side arrangement although the sister chromatids are connected due to cohesion at heterochromatic region at centromeres (SAKUNO *et al.* 2009). Therefore, at anaphase II bi-orientation of sister centromeres occur and the sister chromatids are segregated towards opposite poles when cohesion at heterochromatic regions are removed.

In *Arabidopsis, Drosophila* and other higher eukaryotes, no monopolin has been found but cohesion is essential for mono-orientation. REC8 and SCC3 in *Arabidopsis* are necessary for proper reductional segregation at anaphase I (CHELYSHEVA *et al.* 2005). Mutation of cohesion genes such as *solo* and *ord* in *Drosophila* has been shown to cause defects in mono-orientation at meiosis I. In *solo* mutants, mitosis-like equational segregation is observed twice as frequently than reductional segregation at anaphase I (BALICKY *et al.* 2002; YAN *et al.* 2010).

Kinetochores and Chromosome Segregation

Kinetochores are multi-protein complexes, which are assembled at the centromeres (*S. cerevisiae*), larger centromeric region (*S. pombe* and *Drosophila*) and sometimes throughout the chromosome. Kinetochores are essential for chromosome segregation because they act as point of contact through which microtubules contact chromosomes. Kinetochores get assembled over a layer of histone H3 variant- CENP-A, which are present on either sides of the chromosome long axis. Kinetochore geometry is essential to ensure that they are connected to the microtubules from the correct pole. Kinetochore geometry is different in mitosis and meiosis because in mitosis kinetochores are arranged in a back-to-back configuration, which allows them to attach to microtubules coming from opposite poles, whereas in meiosis kinetochores

are arranged in a side-by-side configuration, which causes them to attach to microtubules from the same pole. This is the crucial factor, which leads to the segregation of sister centromeres to the same pole. At meiosis II, the side-by side arrangement of kinetochores is converted into a back-to-back arrangement and sister chromatids segregate towards opposite poles (HAUF and WATANABE 2004).

In addition to kinetochore configuration, aurora-B kinase localization pattern at the centromeres plays a major role in ensuring mono-orientation during meiosis I and bi-orientation during meiosis II and mitosis. During meiosis II and mitosis, aurora-B is localized to the inner side of centromeres sandwiched between sister centromeres and this ensures that kinetochore are bi-oriented. Tension generated by microtubules attached to kinetochores would pull away outer kinetochore components to which microtubules are attached from aurora-B concentrated region at the centromeres. However, if enough pull is not generated by a microtubule-kineotchore attachment that would make it proximal to the aurora-B concentrated region, which then will phosphorylate the kineotochore components and sever its attachment with the microtubule. In meiosis I, side-by-side arrangement of kinetochores and the chiasmate attachment of homologs ensures that aurora-B is places underneath the paired kinetochores and therefore aurora-B would be farthest from its kinetochore substrates if the sister kinetochores are attached to the microtubules emanating from the same pole and are pulled towards the same pole (MARSTON and AMON 2004; MILLER *et al.* 2013).

Spermatogenesis in *Drosophila* males

Spermatocyte formation begins at the testis tip where germline stem cell divides by mitosis to produce 2, 4, 8 cell cysts. A final round of mitosis in 8 cell cyst produces

16 cell cyst which synchronously enters meiosis. *Drosophila* male meiosis is divided into multiple stages- prophase I consists of six stages called S1, S2, S3, S4, S5 and S6. This is followed by pro-metaphase I, metaphase I. and anaphase I. Following anaphase I and telophase I, meiosis II starts which is divided into prometaphase II, metaphase II and finally anaphase II. A 16 cell cyst entering meiosis will produce 64 haploid spermatids after the completion of meiotic divisions. Chromosome decondensation occurs and volume of the nucleus increases as prophase I progresses. The chromosomes separate out into 3 territories each representing a homolog pair (X-Y, 2nd and 3rd). The fourth chromosome is small and its territory is not visible till prometaphase I. Following completion of prophase I, chromosome territories start to condense and congress towards the cell center and at metaphase I a single mass of condensed chromosome is seen at the cell center. At anaphase I, two equal sized DNA territories are seen separating towards opposite poles (Figure 1-2). In telophase I there is brief decompaction of the chromosomes but by prometaphase I chromosome again condense and by metaphase I all chromosome territories congress at the center of the cell and then separate towards opposite poles at anaphase II (CENCI *et al.* 1994).

Oogenesis in *Drosophila*

In *Drosophila*, development of oocyte takes place in structures called ovarioles. Each female contains two pairs of ovaries and each ovariole contains 12-13 ovarioles. An ovariole is divided into two parts - germarium and vitellarium (Figure 1-3). The germarium is transluscent and is present at the tip of the ovariole and the rest of the ovariole consists of the vitellarium, which appears opaque under light microscope due to the presence of yolk. The ovariole consists of a factory line oocytes present at various

stages of meiosis and development. In region 2A of germarium, multiple pro-oocytes are present per cyst (as identified by C(3)G linear structures in atleast 3-4 nuclei) and are in zygotene and early pachytene stage of meiosis. By the time cyst is in region 2B, only one or two nuclei have complete C(3)G and ORB staining (Figure 1-3) but by region 3 oocyte is determined and only one nuclei has complete C(3)G structure and ORB staining. Region 3 consists of mid-pachytene oocyte and pachytene continues till stage 6 of the vitellarium. Out of the 16 cells in the cyst only one cell develops into an oocyte and and the rest of the 15 cells develop into nurse cells. In the vitellarium the nurse develop becomes polyploid by undergoing mitotic endocycle. The oocyte arrests at prophase I of meiosis and stays at that stage and due to unknown reason this arrest is released and then oocyte cyst enters prometaphase I and metaphase I where again oocytes arrests. This is similar to arrest points in mouse and *C.elegans* (TROUNSON and GOSDEN 2003; VON STETINA and ORR-WEAVER 2011; LAKE and HAWLEY 2012).

Two major events occur during late prophase I and metaphase I in the ovariole and these stages correspond to stage 13 and stage 14 of the vitellairum. Karyosome formation (Chromosome condense into a mass), which initiates at stage 3-4 of the vitiellarium, is complete by stage 12. This is followed by nuclear envelope breakdown (NEBD) and at stage 13 and nurse cells start to disintegrate by stage 14, all nurse cells disintegrates and the oocyte arrests. The release of the metaphase I arrest and completion of meiosis is caused due to egg laying process (due to pressure of going through oviduct and rehydration).

Figure 1-3: Structure of *Drosophila* Ovariole and Major events during oogenesis: (A) Ovariole Structure and meiotic events: The ovariole is divided into two parts: germarium and vitellarium. Vitellarium is divided multiple stages from stage 2-14. (B) Germarium is divided into region1, region 2A, region 2B and region 3 (stage 1). The various stages in the germarium and vitellarium can be roughly assigned to various meiotic stages. Germaria consists of pre-meiotic and early-mid prophase I oocyte stages, whereas the vitellarium consists from middle -late prophase I to metaphase I (stage 14). (A) After this stage, egg containing the oocyte exits the female body and is laid. Major meiotic events occurs in the vitellarium. Stage 6 marks the end of pachytene and by that time the SC is disassembled. Stage 14 is metaphase I and chromosome are aligned at the metaphase plate by this stage. By stage 14, all nurse cells have died and egg develops dorsal appendages. (B) Germaria and Stage2. In region 1 of germaria, germline stem cell divides to create a cyst cell which divides by mitosis four times to create a 16 cell cyst. 16 cell synchronously enters meiosis prophase I in region 2. Synaptonemal complex formation is initiated in Zygotene (earliest 16 cell cyst in region 2A) in multiple cyst cell nuclei and as the cyst progresses down the germarium into region 2B and region 3 synaptonemal complex is only restricted to one nuclei (as observed by C(3)G staining) (green linear structures). Orange staining represents ORB staining which is first seen at region 2B and is restricted to a single cell which is the oocyte by region 3.

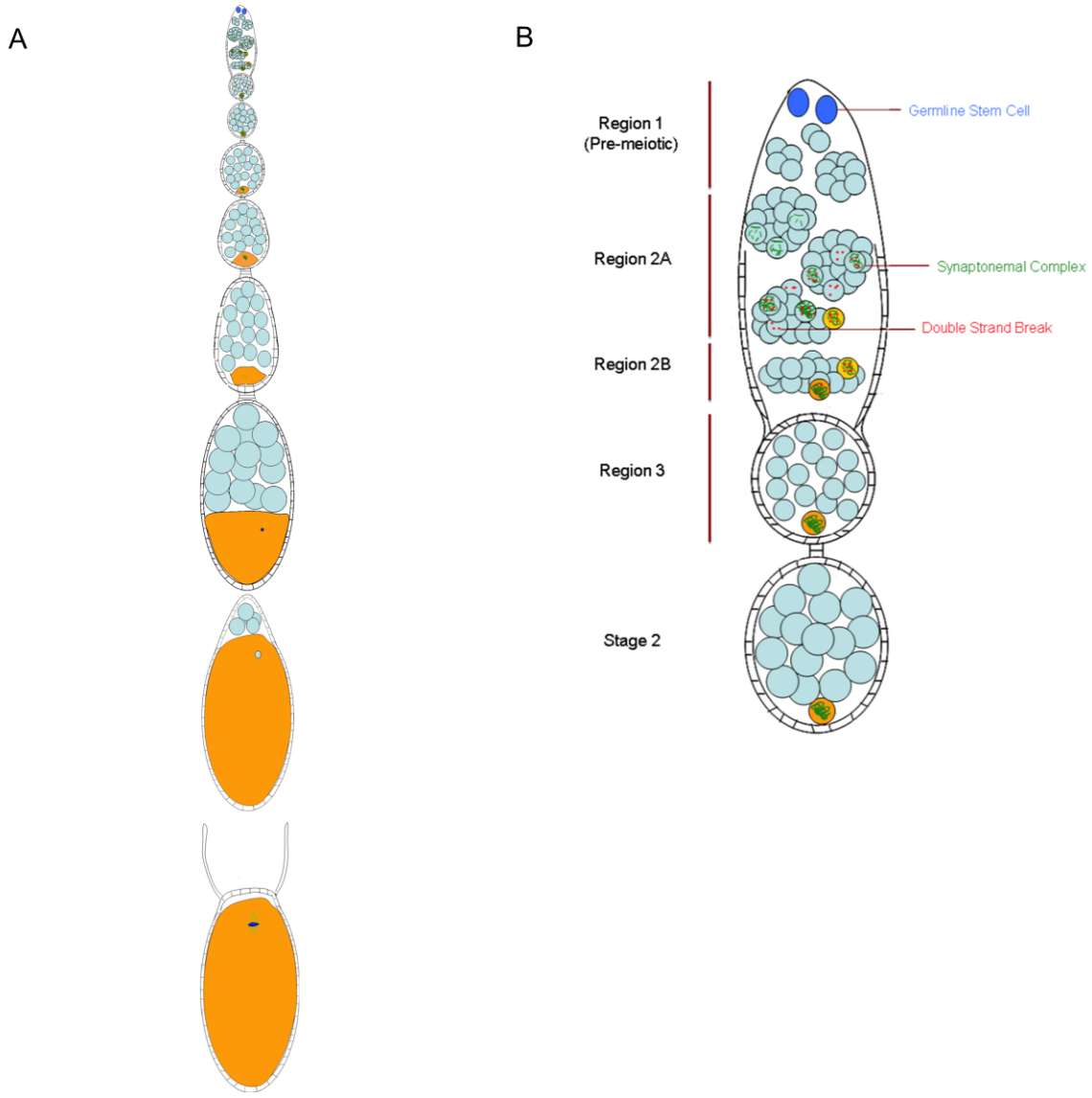


Figure 1-3. Continued

Non-disjunction

Non-disjunction (mis-segregation event) is a phenomenon in which chromosomes do not separate properly during meiosis or mitosis. Non-disjunction is harmful for the health of a cell because it leads to incorrect number and combination of chromosomes in daughter cells. Non-disjunction during meiosis causes various genetic disorders. Non-disjunction during meiosis is caused due to many factors, which includes cohesion defect along sister chromatid arms and the centromeres, spindle defects and centrosomal defects. The most common example of non-disjunction due to defective centrosomal arrangement is in dTOPORS mutants where a multipolar spindle causes chromosomes to show meiosis I non-disjunction similar to Figure 1-4A (MATSUI *et al.* 2011).

But the major cause of non-disjunction is improper attachments between homologs and sister chromatids, which prevent generation of correct chromosome orientation patterns and tension in meiosis I and II. Non-disjunction can occur due to loss of homolog attachment during meiosis I, which prevents the homologous chromosome bi-orientation (Fig 1-4). This usually leads to a segregation pattern at anaphase I which is either 4:0 (homolog pair travel to the same pole and none travel to the other pole) or the normal looking segregation of 2:2 (homologs segregate towards opposite poles). Both these segregation patterns have chance of occurring in a situation where homologous chromosome attachment is compromised as in *snm* and *mnm* mutants (THOMAS *et al.* 2005) (Figure 1-4A). The other kind of mis-segregation is caused due to loss of centromeric cohesion during anaphase I or shortly after it. This is usually observed in *mei-s332* mutants. The loss of MEI-S332 from the centromeres

leaves the centromeric cohesins susceptible to cleavage at anaphase I and they are removed at the same time as arm cohesins are removed. Therefore, sister chromatids are unconnected and they segregate improperly at anaphase II. The loss of centromeric cohesion during anaphase I causes very little anaphase I segregation defects but causes sister chromatid segregation error at anaphase II due to inability of the sister centormeres to bi-orient themselves and segregate to opposite poles (Figure 1-4B).

Both meiosis I and meiosis II NDJ is observed in *ord* and *solo* mutants in both males and female (Figure 1-4D). Both these genes are necessary for cohesion between sister chroamtids and in the absence of these proteins, cohesion is lost both along chromosome arms and centromeres. This is essential for generating bi-orientation of homologous chromosome towards opposite poles and mono-orientation of sister centromeres towards the same pole at meiosis I. This would also lead to unattached sister chromatids at meiosis II. Therefore, a random chromosome segregation (like Figure 1-4C) through both anaphase I and anaphase II is expected and is probably the case in *Drosophila* females (where in normal situation chiasma is formed which requires sister chromatid cohesion). But in *Drosophila* males, at anaphase I, a 2:2 equational segregation pattern is observed but no 4:0 segregation or very few 3:1 segregation is observed. This is because in *Drosophila* males SNM-MNM conjunction complex performs the role of chiasma and holds homologs together even in the absence of sister chromatid cohesion and this is hypothesized to generate a 2:2 segregation pattern. This hypothesis of ours is confirmed in *solo snm* double mutants where we do see random chromosome segregation (2:2, 4:0, 3:1 at anaphase I) at both segregation events (Figure 1-4D)

Figure 1-4: Different types of improper chromosome segregation observed during meiosis. Homolog pair is shown where each homolog is assigned its own color. Yellow and blue line depicts intact connections between sister chromatids and homologs respectively. (A) Meiosis I Non-Disjunction. Homolog pair segregates towards the same pole at meiosis I. At meiosis II, sister chromatids segregate equationally. Only Non-disjunction event is shown. The other possibility would look like normal chromosome segregation based on just chance separation of homologs away from each other. Chromosome entaglement between homologs would always result in both homologs traveling towards the same pole. (B) Meiosis II Non-Disjunction. Chromosome segregates reductionally at meiosis I (Homologs move towards opposite poles). At meiosis II, sister chromatids fails to segregate towards opposite poles (left panel). (C) Random segregation. Chromatids behave independently and segregate in any possible combination at meiosis I and II. Some of the commonly occurring sperms as a result of random segregation are shown above. (D) Premature Sister Chromatid Separation. At meiosis I, chromosome segregation occurs randomly but more commonly a 2:2 segregation pattern (two chromatids, either sisters or non-sisters segregate towards the same pole) is seen. Rarely, three or four chromatids segregate towards the same pole. Mostly, chromatids select their partner randomly and either segregates with their sister (reductional segregation) or with chromatid from their homolog partner (equational segregation).

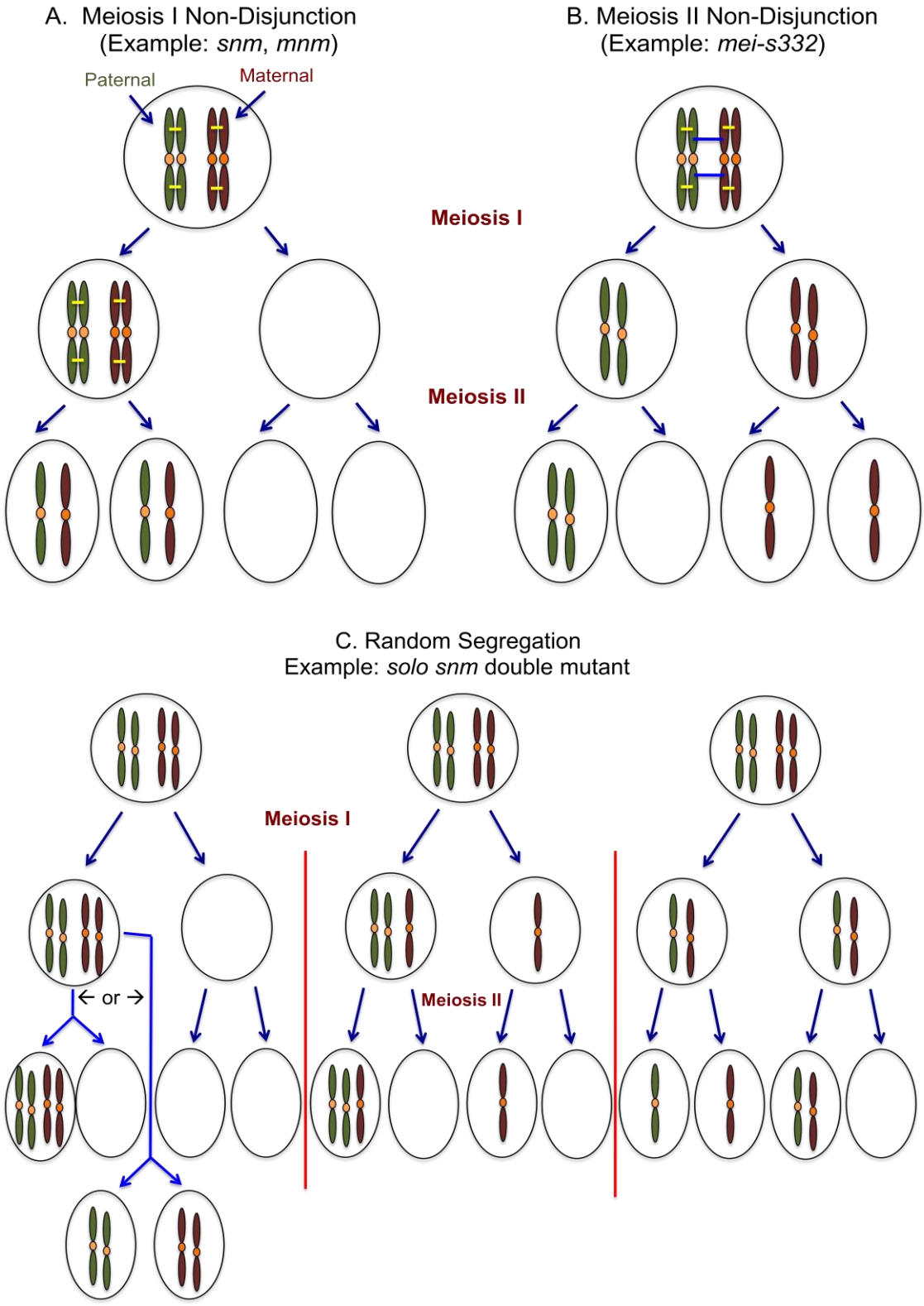


Figure 1-4. Continued

D. Premature Sister Chromatid Separation (PSCS)
(Example: *solo, ord, sun* mutant)

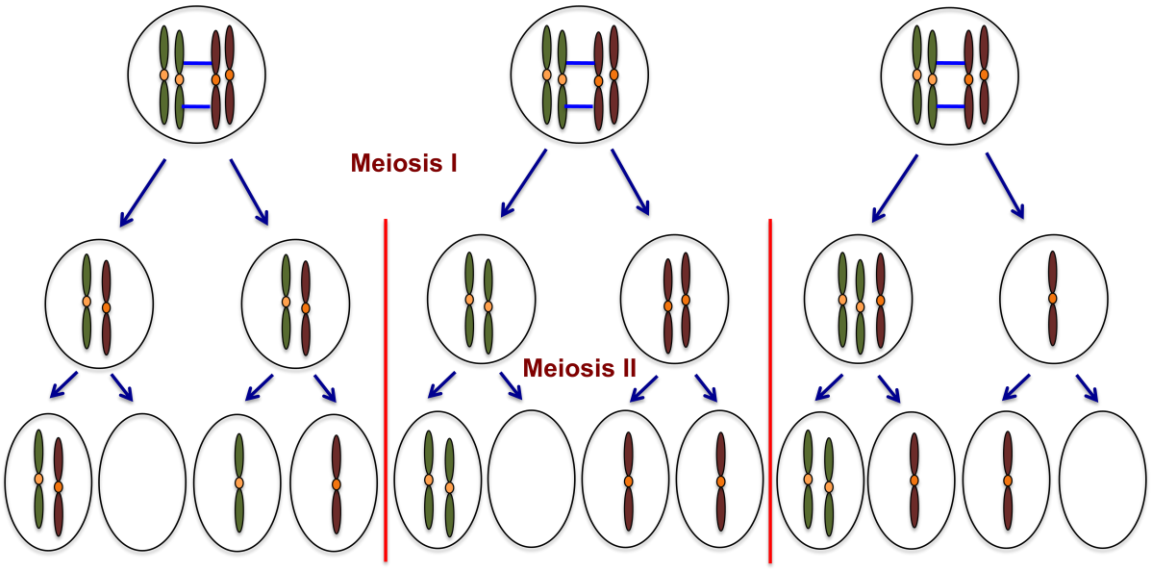


Figure 1-4. Continued

REFERENCES

BALICKY, E. M., M. W. ENDRES, C. LAI and S. E. BICKEL, 2002 Meiotic cohesion requires accumulation of ORD on chromosomes before condensation. Mol Biol Cell **13:** 3890-3900.

BICKEL, S. E., D. W. WYMAN and T. L. ORR-WEAVER, 1997 Mutational analysis of the Drosophila sister-chromatid cohesion protein ORD and its role in the maintenance of centromeric cohesion. Genetics **146:** 1319-1331.

CENCI, G., S. BONACCORSI, C. PISANO, F. VERNI and M. GATTI, 1994 Chromatin and microtubule organization during premeiotic, meiotic and early postmeiotic stages of Drosophila melanogaster spermatogenesis. J Cell Sci **107 (Pt 12):** 3521-3534.

CHELYSHEVA, L., S. DIALLO, D. VEZON, G. GENDROT, N. VRIELYNCK *et al.*, 2005 AtREC8 and AtSCC3 are essential to the monopolar orientation of the kinetochores during meiosis. J Cell Sci **118:** 4621-4632.

CLIFT, D., and A. L. MARSTON, 2011 The role of shugoshin in meiotic chromosome segregation. Cytogenet Genome Res **133:** 234-242.

COLLINS, K. A., J. R. UNRUH, B. D. SLAUGHTER, Z. YU, C. M. LAKE *et al.*, 2014 Corolla is a novel protein that contributes to the architecture of the synaptonemal complex of Drosophila. Genetics **198:** 219-228.

COSTA, Y., R. SPEED, R. OLLINGER, M. ALSHEIMER, C. A. SEMPLE *et al.*, 2005 Two novel proteins recruited by synaptonemal complex protein 1 (SYCP1) are at the centre of meiosis. J Cell Sci **118:** 2755-2762.

DAVIES, O. R., J. D. MAMAN and L. PELLEGRINI, 2012 Structural analysis of the human SYCE2-TEX12 complex provides molecular insights into synaptonemal complex assembly. Open Biol **2:** 120099.

GOLUBOVSKAYA, I. N., O. HAMANT, L. TIMOFEJEVA, C. J. WANG, D. BRAUN *et al.*, 2006 Alleles of afd1 dissect REC8 functions during meiotic prophase I. J Cell Sci **119:** 3306-3315.

HASSOLD, T., and P. HUNT, 2001 To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet **2:** 280-291.

HAUF, S., and Y. WATANABE, 2004 Kinetochore orientation in mitosis and meiosis. Cell **119:** 317-327.

HEIDMANN, D., S. HORN, S. HEIDMANN, A. SCHLEIFFER, K. NASMYTH *et al.*, 2004 The Drosophila meiotic kleisin C(2)M functions before the meiotic divisions. Chromosoma **113:** 177-187.

HUNT, P. A., and T. J. HASSOLD, 2008 Human female meiosis: what makes a good egg go bad? Trends Genet **24:** 86-93.

JOYCE, E. F., N. APOSTOLOPOULOS, B. J. BELIVEAU and C. T. WU, 2013 Germline progenitors escape the widespread phenomenon of homolog pairing during Drosophila development. PLoS Genet **9:** e1004013.

KEENEY, S., 2008 Spo11 and the Formation of DNA Double-Strand Breaks in Meiosis. Genome Dyn Stab **2:** 81-123.

KERREBROCK, A. W., W. Y. MIYAZAKI, D. BIRNBY and T. L. ORR-WEAVER, 1992 The Drosophila mei-S332 gene promotes sister-chromatid cohesion in meiosis following kinetochore differentiation. Genetics **130:** 827-841.

KLEIN, F., P. MAHR, M. GALOVA, S. B. BUONOMO, C. MICHAELIS *et al.*, 1999 A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell **98:** 91-103.

LAKE, C. M., and R. S. HAWLEY, 2012 The molecular control of meiotic chromosomal behavior: events in early meiotic prophase in Drosophila oocytes. Annu Rev Physiol **74:** 425-451.

LEE, J. Y., and T. L. ORR-WEAVER, 2001 The molecular basis of sister-chromatid cohesion. Annu Rev Cell Dev Biol **17:** 753-777.

LLANO, E., Y. HERRAN, I. GARCIA-TUNON, C. GUTIERREZ-CABALLERO, E. DE ALAVA *et al.*, 2012 Meiotic cohesin complexes are essential for the formation of the axial element in mice. J Cell Biol **197:** 877-885.

MANHEIM, E. A., and K. S. MCKIM, 2003 The Synaptonemal complex component C(2)M regulates meiotic crossing over in Drosophila. Curr Biol **13:** 276-285.

MARSTON, A. L., and A. AMON, 2004 Meiosis: cell-cycle controls shuffle and deal. Nat Rev Mol Cell Biol **5:** 983-997.

MASON, J. M., 1976 Orientation disruptor (ord): a recombination-defective and disjunction-defective meiotic mutant in Drosophila melanogaster. Genetics **84:** 545-572.

MATSUI, M., K. C. SHARMA, C. COOKE, B. T. WAKIMOTO, M. RASOOL *et al.*, 2011 Nuclear structure and chromosome segregation in Drosophila male meiosis depend on the ubiquitin ligase dTopors. Genetics **189:** 779-793.

MCKEE, B. D., 2004 Homologous pairing and chromosome dynamics in meiosis and mitosis. Biochim Biophys Acta **1677:** 165-180.

MCKIM, K. S., J. K. JANG and E. A. MANHEIM, 2002 Meiotic recombination and chromosome segregation in Drosophila females. Annu Rev Genet **36:** 205-232.

MILLER, M. P., A. AMON and E. UNAL, 2013 Meiosis I: when chromosomes undergo extreme makeover. Curr Opin Cell Biol **25:** 687-696.

MIYAZAKI, W. Y., and T. L. ORR-WEAVER, 1992 Sister-chromatid misbehavior in Drosophila ord mutants. Genetics **132:** 1047-1061.

NASMYTH, K., and C. H. HAERING, 2009 Cohesin: its roles and mechanisms. Annu Rev Genet **43:** 525-558.

PAGE, S. L., and R. S. HAWLEY, 2004 The genetics and molecular biology of the synaptonemal complex. Annu Rev Cell Dev Biol **20:** 525-558.

PAGE, S. L., R. S. KHETANI, C. M. LAKE, R. J. NIELSEN, J. K. JEFFRESS *et al.*, 2008 Corona is required for higher-order assembly of transverse filaments into full-length synaptonemal complex in Drosophila oocytes. PLoS Genet **4:** e1000194.

PASIERBEK, P., M. JANTSCH, M. MELCHER, A. SCHLEIFFER, D. SCHWEIZER *et al.*, 2001 A Caenorhabditis elegans cohesion protein with functions in meiotic chromosome pairing and disjunction. Genes Dev **15:** 1349-1360.

PETRONCZKI, M., M. F. SIOMOS and K. NASMYTH, 2003 Un menage a quatre: the molecular biology of chromosome segregation in meiosis. Cell **112:** 423-440.

ROEDER, G. S., 1995 Sex and the single cell: meiosis in yeast. Proc Natl Acad Sci U S A **92:** 10450-10456.

ROEDER, G. S., 1997 Meiotic chromosomes: it takes two to tango. Genes Dev **11:** 2600-2621.

SAKUNO, T., K. TADA and Y. WATANABE, 2009 Kinetochore geometry defined by cohesion within the centromere. Nature **458:** 852-858.

TAKEO, S., C. M. LAKE, E. MORAIS-DE-SA, C. E. SUNKEL and R. S. HAWLEY, 2011 Synaptonemal complex-dependent centromeric clustering and the initiation of synapsis in Drosophila oocytes. Curr Biol **21:** 1845-1851.

TANNETI, N. S., K. LANDY, E. F. JOYCE and K. S. MCKIM, 2011 A pathway for synapsis initiation during zygotene in Drosophila oocytes. Curr Biol **21:** 1852-1857.

THOMAS, S. E., M. SOLTANI-BEJNOOD, P. ROTH, R. DORN, J. M. LOGSDON, JR. *et al.*, 2005 Identification of two proteins required for conjunction and regular segregation of achiasmate homologs in Drosophila male meiosis. Cell **123:** 555-568.

TOTH, A., K. P. RABITSCH, M. GALOVA, A. SCHLEIFFER, S. B. BUONOMO *et al.*, 2000 Functional genomics identifies monopolin: a kinetochore protein required for segregation of homologs during meiosis i. Cell **103:** 1155-1168.

TROUNSON, A., and R. G. GOSDEN, 2003 *Biology and pathology of the oocyte : its role in fertility and reproductive medicine*. Cambridge University Press, Cambridge, UK ; New York.

TSAI, J. H., R. YAN and B. D. MCKEE, 2011 Homolog pairing and sister chromatid cohesion in heterochromatin in Drosophila male meiosis I. Chromosoma **120:** 335-351.

URBAN, E., S. NAGARKAR-JAISWAL, C. F. LEHNER and S. K. HEIDMANN, 2014 The Cohesin Subunit Rad21 Is Required for Synaptonemal Complex Maintenance, but Not Sister Chromatid Cohesion, during Drosophila Female Meiosis. PLoS Genet **10:** e1004540.

VAZQUEZ, J., A. S. BELMONT and J. W. SEDAT, 2002 The dynamics of homologous chromosome pairing during male Drosophila meiosis. Curr Biol **12:** 1473-1483.

VON STETINA, J. R., and T. L. ORR-WEAVER, 2011 Developmental control of oocyte maturation and egg activation in metazoan models. Cold Spring Harb Perspect Biol **3:** a005553.

WATANABE, Y., 2004 Modifying sister chromatid cohesion for meiosis. J Cell Sci **117:** 4017-4023.

WATANABE, Y., 2005 Shugoshin: guardian spirit at the centromere. Curr Opin Cell Biol **17:** 590-595.

WATANABE, Y., and P. NURSE, 1999 Cohesin Rec8 is required for reductional chromosome segregation at meiosis. Nature **400:** 461-464.

WEBBER, H. A., L. HOWARD and S. E. BICKEL, 2004 The cohesion protein ORD is required for homologue bias during meiotic recombination. J Cell Biol **164:** 819-829.

YAN, R., and B. D. MCKEE, 2013 The Cohesion Protein SOLO Associates with SMC1 and Is Required for Synapsis, Recombination, Homolog Bias and Cohesion and Pairing of Centromeres in Drosophila Meiosis. PLoS Genet **9:** e1003637.

YAN, R., S. E. THOMAS, J. H. TSAI, Y. YAMADA and B. D. MCKEE, 2010 SOLO: a meiotic protein required for centromere cohesion, coorientation, and SMC1 localization in Drosophila melanogaster. J Cell Biol **188:** 335-349.

YOKOBAYASHI, S., and Y. WATANABE, 2005 The kinetochore protein Moa1 enables cohesion-mediated monopolar attachment at meiosis I. Cell **123:** 803-817.

ZICKLER, D., and N. KLECKNER, 1998 The leptotene-zygotene transition of meiosis. Annu Rev Genet **32:** 619-697.

CHAPTER II: SISTERS UNBOUND IS A NOVEL PROTEIN REQUIRED FOR MEIOTIC CENTROMERIC COHESION IN DROOSPHILA MELANOGASTER

This chapter is a modified version of the manuscript and has been accepted by the journal *Genetics* for publication and is in press now.

Badri Krishnan's primary contributions were: Identified *sisters unbound (sunn)*, investigated some genetic phenotypes of *sunn* mutants, performed cytological studies to understand function of *sunn*, cloned *sisters unbound* and determined sequence of its untranslated regions, created transgenic flies containing fluorescent tagged constructs of *sisters unbound*, analyzed *sisters unbound's* localization patterns in wildtype and cohesion gene mutants, wrote the manuscript draft and made all the figures and tables. Bioinformatic analysis of *sisters unbound* structure was performed by Dr. Igor Zhulin.

ABSTRACT

Regular meiotic chromosome segregation requires sister centromeres to mono-orient (orient to the same pole) during the first meiotic division (meiosis I) when homologous chromosomes segregate, and to bi-orient (orient to opposite poles) during the second meiotic division (meiosis II) when sister chromatids segregate. Both orientation patterns require cohesion between sister centromeres, which is established during meiotic DNA replication and persists until anaphase of meiosis II. Meiotic cohesion is mediated by a conserved four-protein complex called cohesin that includes two Structural Maintenance of Chromosomes (SMC) subunits (SMC1 and SMC3) and two non-SMC subunits. In *Drosophila melanogaster,* however, the meiotic cohesion apparatus has not been fully characterized and the non-SMC subunits have not been identified. We have identified a novel *Drosophila* gene called *sisters unbound (sunn)*, which is required for stable sister chromatid cohesion throughout meiosis. *sunn* mutations disrupt centromere cohesion during prophase I and cause high frequencies of nondisjunction (NDJ) at both meiotic divisions in both sexes. SUNN co-localizes at centromeres with the cohesion proteins SMC1 and SOLO in both sexes and is necessary for the recruitment of both proteins to centromeres. Although SUNN lacks sequence homology to cohesins, bioinformatic analysis indicates that SUNN may be a structural homolog of the non-SMC cohesin subunit Stromalin (SA), suggesting that SUNN may serve as a meiosis-specific cohesin subunit. In conclusion, our data show that SUNN is an essential meiosis-specific *Drosophila* cohesion protein.

INTRODUCTION

Meiosis is a specialized cell division that generates haploid gametes from diploid precursor cells and is essential for sexual reproduction. Segregation of chromosomes during meiosis occurs in two stages called meiosis I and meiosis II that follow a single round of DNA replication. During meiosis I, homologs pair and orient towards opposite poles of the spindle (bi-orient) with sister centromeres oriented towards the same pole (mono-oriented). As a result, homologous chromosomes segregate to opposite poles at the onset of anaphase I in a reductional segregation pattern. In meiosis II, as in mitosis, the sister centromeres are bi-oriented and sister chromatids segregate to opposite poles at the onset of anaphase II, a pattern referred to as equational segregation (PAGE and HAWLEY 2003; PETRONCZKI *et al.* 2003).

In most eukaryotes, pairing of homologs during meiosis I is facilitated and reinforced by synapsis and recombination. Synapsis involves formation of elaborate zipper-like structures, called synaptonemal complexes (SCs), which hold homologs tightly together during prophase I. SCs are composed of the tightly paired sister chromatid axes of the two homologs, known as axial elements (AEs) before synapsis or as lateral elements (LEs) after synapsis, cross-linked by multiple transverse filament (TF) proteins. Synapsis initiates at a limited number of discrete sites of homolog alignment during zygotene and subsequently spreads until SCs form continuous chromosome-length structures during pachytene (PAGE and HAWLEY 2004). Most synapsis initiation sites appear to be in the euchromatin but in some eukaryotes, including yeast, several plant species, and female Drosophila, synapsis also initiates at centromeres and is preceded by homologous and/or non-homologous pairing of

centromeres (KHETANI and BICKEL 2007; STEWART and DAWSON 2008; TAKEO *et al.* 2011; TANNETI *et al.* 2011). Recombination overlaps temporally with synapsis and involves programmed formation and repair of double strand-breaks, resulting in high levels of exchange (crossing over) between homologous chromatids. After SC disassembly at the end of pachytene, homolog crossovers, stabilized by cohesion between sister chromatid arms, serve as stable interhomolog linkers known as chiasmata (PAGE and HAWLEY 2003; KLECKNER 2006). Some eukaryotes achieve stable homolog pairing and regular homolog segregation during meiosis I without SCs or recombination. In *Drosophila* males, which lack meiotic recombination, stable homolog connections are provided by a male-specific "homolog conjunction complex" that serves as a functional substitute for chiasmata and is removed at anaphase I (THOMAS *et al.* 2005).

Proper chromosome segregation at both meiotic divisions, as well as in mitosis, requires cohesion between sister chromatids provided by conserved four-protein complexes called cohesins. The mitotic cohesin complex is composed of SMC1, SMC3, SCC1/MCD1/RAD21 (henceforth called RAD21) and SCC3/Stromalin/SA (henceforth called SA). SMC1, SMC3 and RAD21 form a tripartite ring structure that is thought to embrace the newly formed sister chromatids during S phase. Cleavage of the RAD21 subunit by the conserved protease Separase at anaphase releases cohesion and allows sister chromatids to segregate to the poles. SA is an all α-helical protein that binds to RAD21. It is essential for cohesion but its precise role in cohesion remains unclear. Meiotic cohesins are similar in composition to mitotic cohesin but frequently contain one or more paralogous meiosis-specific subunits that replace their mitotic counterparts.

Most such paralogs are restricted to fairly narrow taxonomic lineages and have specialized functions, but REC8 replaces RAD21 in most meiotic cohesins and is required for nearly all meiotic chromosome interactions in most eukaryotes (LEE and ORR-WEAVER 2001; NASMYTH 2001; NASMYTH and HAERING 2009).

In meiosis I, cohesin is abundant all along the chromosome axes but arm and centromere cohesion play distinct roles in meiosis. During meiosis I, arm cohesion stabilizes the chiasmata that provide resistance to poleward forces required for homologs to bi-orient on the meiosis I spindle. Release of arm cohesion at the onset of anaphase I, by Separase-mediated cleavage of REC8, destabilizes chiasmata and serves as the triggering event for homolog segregation (PETRONCZKI *et al.* 2003; NASMYTH and HAERING 2009). Arm cohesins also play important roles in synapsis and recombination during meiosis I although it remains unclear to what extent those roles are related to arm cohesion (NASMYTH 2001; BRAR *et al.* 2009; NASMYTH and HAERING 2009)**.** Cohesion between sister centromeres enables sister chromatids to bi-orient on the meiosis II spindle and is preserved until a second round of Separase activation at anaphase II cleaves centromeric cohesins and triggers sister chromatid separation. Preservation of centromeric cohesins during anaphase I is mediated by Shugoshins, which are centromere proteins that inhibit Separase cleavage of cohesin (WATANABE 2005; CLIFT and MARSTON 2011).

Centromere cohesion is also required during meiosis I, to enable sister centromeres to mono-orient. Mono-orientation is thought to require a side-by-side alignment of sister centromeres (rather than the back-to-back alignment characteristic of mitosis or meiosis II) enabling them to form a functionally single kinetochore that binds

microtubules from only one pole (HAUF and WATANABE 2004). In S. pombe, this specialized centromere orientation entails establishing cohesion within the kinetochore-forming centromere core domain and requires both REC8 cohesin and a specialized meiosis-specific centromere protein called Moa1 (WATANABE and NURSE 1999; YOKOBAYASHI and WATANABE 2005). In *S. cerevisiae*, both cohesin and a meiosis I-specific centromere complex called Monopolin are required for regular mono-orientation (TOTH *et al.* 2000). In several higher eukaryotes, mutations in *rec8* or other cohesion genes have also been found to disrupt mono-orientation (KLEIN *et al.* 1999; PASIERBEK *et al.* 2001; CAI *et al.* 2003; WANG *et al.* 2003; CHELYSHEVA *et al.* 2005; GOLUBOVSKAYA *et al.* 2006; SEVERSON *et al.* 2009). However, no specific mono-orientation factors have been identified in higher eukaryotes and the mechanism of mono-orientation remains unclear.

Drosophila has been a major model for meiotic studies for more than a century. However, insight into the mechanism and roles of cohesion in *Drosophila* meiosis has been hampered by limited data on the composition of the meiotic cohesion apparatus. Recent findings have pointed to meiotic roles for the cohesin SMC proteins. SMC1 localizes to centromeres during meiosis in both sexes and persists on centromeres until anaphase II in male meiosis (KHETANI and BICKEL 2007; YAN *et al.* 2010). Both SMC1 and SMC3 localize to LEs in female prophase I and loss of either protein completely ablates formation of LEs and SCs (KHETANI and BICKEL 2007; TANNETI *et al.* 2011; YAN and MCKEE 2013). However, as yet there is only indirect evidence for roles of SMC1 and SMC3 in arm or centromere cohesion. Moreover, the non-SMC subunits of meiotic cohesins in *Drosophila* remain unidentified. Neither RAD21 nor SA has been reported to

localize to meiotic chromosomes at any stage and no meiotic phenotypes have been reported for either gene. In addition, unlike all other characterized eukaryotes, the *Drosophila* genome lacks a true *rec8* homolog. The *Drosophila* genome does encode a meiosis-specific RAD21 homolog, C(2)M, that localizes to LEs and is required for synapsis, SC formation and normal levels of recombination (MANHEIM and MCKIM 2003). However, C(2)M does not form centromeric foci and is not required for either centromere or arm cohesion during the meiotic division stages in female meiosis , or for any aspect of male meiosis (MANHEIM and MCKIM 2003; HEIDMANN *et al.* 2004) .

Curiously, the best-characterized meiotic cohesion genes in *Drosophila* are two genes with no apparent homology to any of the cohesins: *orientation disruptor* (*ord)* and *sisters on the loose* (*solo)* (BICKEL *et al.* 1996; YAN *et al.* 2010). Mutations in both genes cause premature loss of sister centromere cohesion, accompanied by absence of centromeric SMC1 foci, leading to very high frequencies of both homolog and sister chromatid non-disjunction (NDJ) (MIYAZAKI and ORR-WEAVER 1992; BICKEL *et al.* 1997; BALICKY *et al.* 2002; YAN *et al.* 2010). Both ORD and SOLO co-localize with SMC1 on centromeres in both sexes and persist there until anaphase II in male meiosis, disappearing simultaneously with SMC1 (BALICKY *et al.* 2002; KHETANI and BICKEL 2007; YAN *et al.* 2010). Consistent with a cohesin-related role, SOLO was recently shown to reciprocally co-immunoprecipitate with SMC1 from ovary extracts (YAN and MCKEE 2013). These findings have led to suggestions that, despite their lack of sequence homology to cohesins, ORD and SOLO might be functional homologs of REC8 (KHETANI and BICKEL 2007; YAN *et al.* 2010; YAN and MCKEE 2013).

Here we describe a third *Drosophila*-specific, meiosis-specific cohesion gene, *sunn (sisters unbound),* with properties remarkably similar to those of *ord* and *solo*. *sunn* mutations cause high levels of both meiosis I and meiosis II NDJ in both sexes. In male meiosis, SUNN localizes primarily to centromeres until anaphase II and is required for centromeric cohesion, for mono-orientation of sister centromeres and for stable centromere recruitment of SMC1 and SOLO. In female meiosis, SUNN also localizes to centromeres during prophase I and is required for centromere pairing and cohesion during pachytene. These data identify SUNN as a major component of the meiotic cohesion apparatus in *Drosophila*. Although no sequence homologs of SUNN were identified in genome searches, structure-based bioinformatic analysis revealed similarity between SUNN and the *Drosophila* cohesin subunit Stromalin (SA), suggesting SUNN's possible role as a meiosis-specific cohesin subunit.

MATERIALS AND METHODS

Fly stocks, and *Drosophila* culturing: *sunn* mutations were obtained from the Zuker-3 (Z3) collection of EMS mutagenized third chromosomes (KOUNDAKJIAN *et al.* 2004). The Z3- lines used in this study were identified in a screen for loss of paternal 4th chromosomes (WAKIMOTO *et al.* 2004). *Dp (1:1) sc^{v1}* was obtained from Dr. Kim McKim (Rutgers University). All of the chromosome 3 deficiency stocks and compound chromosome stocks used in the crosses were obtained from the Bloomington Stock Center at Indiana University. Details about markers and special chromosomes can be found in Flybase and Bloomington stock center webpage (http://flystocks.bio.indiana.edu/).

Flies were cultured at 22^{o}C on a food mix containing cornmeal, malt, corn syrup, yeast and propionic acid (antifungal agent) and crosses were set and maintained at 22^{o}C. Progeny from the cross were scored between 14 and 21 days after the cross was set.

Measuring NDJ: The methods for measuring male NDJ are explained in the Results section and in the legends to Tables 1 and 2. To measure female NDJ, *Dp(1;1)sc^{v1}, y .y^{+}/y; sunn/Df* females were crossed singly to 2 males of the genotype *YSX.YL*, *In(1)EN, y B/Y*. Regular segregation yields B females and B^{+} males. Progeny from diplo-X and nullo-X non-disjunctional eggs are B^{+} females and y B males, respectively. The B^{+} daughters carry two maternal X chromatids and were classified as resulting from sister chromatid NDJ if they were y (yellow body). *Dp(1;1)sc^{v1}* has a duplication of the tip of the X chromosome on the right arm that carries the dominant *y^{+}* marker. Both X chromosomes have recessive *y* alleles at the native locus near the tip of

XL. There is no recombination between the duplicated y^+ allele and the X centromere so a NDJ female lacking both copies of the y^+ allele is expected to carry two sister centromeres.

Mapping and identification of *sunn* mutations: Mapping of *sunn* alleles was performed by deficiency complementation against the 3rd chromosome deficiency kit (Bloomington Stock Center, Indiana University) using the X-Y NDJ phenotype. *sunn* location was narrowed down to a critical region, 68C8-68D6, on chromosome arm 3L using the following chromosome deficiencies (deleted region in parenthesis) – *Df(3L)vin6* (68C8-69A5), *Df(3L)vin2* (67F2-68D6), *Df(3L)vin3* (68C5-68E4), *Df(3L)vin5* (68A2-69A1), *Df(3L)vin4* (68B1-68F6), *Df(3L)vin7* (68C8-69B5), *Df(3L)ED4470* (68A6-68E1) and *Df(3L)BK9* (68E2-69A1). Exons of candidate genes from the critical region were amplified by PCR using the genomic DNA of *sunn* mutants and sequenced (Cycle Sequencing Kit, Life Technologies) to identify SNPs (Single Nucleotide Polymorphisms). All three *sunn* alleles exhibited mutations in exons of CG32088 that were predicted to alter the protein sequence (Fig. 5).

Generation of *sunn* cDNA clone and UAS-SUNN::Venus transgene: *sunn* cDNA was amplified from total ovary RNA of *y w* (*yellow white*) females using Superscript^R III Reverse Transcriptase (Invitrogen) and Pfx polymerase (Invitrogen). Total RNA for reverse transcription was extracted using TRI-reagent (Sigma-Aldrich) and treated with DNase I (Invitrogen) before reverse transcription. *sunn* cDNA was amplified in two overlapping fragments, the first fragment stretching from the first exon to the sixth exon and the second fragment extending from the sixth exon to the tenth exon using Pfx polymerase (Invitrogen) and the following primers:

First Fragment- Forward: ATGGAATTTGTAAGCGCCATTTCGA, Reverse: CAT CACACTCTGCTACTGAGTCAA; Second fragment- Forward: GAATTGAGCCTT ATTGCTGCGCAA, Reverse:ATCAGTTAGATCTGTTGTATTATGAATAGTTTT AATCT. The two fragments were cloned separately into pJet 1.2/Blunt vector (Fermentas) using CloneJET™ PCR cloning kit (Fermentas) then ligated together into a pJet 1.2/Blunt vector using restriction sites common to the overlapping fragments. The cDNA was then transferred to pENTR4 (Invitrogen) and recombined into the Gateway P-element vector pPWV 1094 (*Drosophila* Genomics Resource Center), which contains a C-terminal Venus tag and UAS sequences, using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen). The resulting construct was transformed into w^{1118} flies by BestGene Inc.

Determining the 5' and 3' ends of Sunn mRNA using RACE: Total RNA was extracted from ovaries of *y w* (yellow white) females using TRI-reagent (Sigma Aldrich) and treated with DNase I (Invitrogen). RACE was performed using FirstChoice™ RLM-RACE kit (Ambion Inc). The length of the 5' UTR determined by 5' RLM-RACE was 69 bp. The 5'UTR of CG32088 shown in Flybase is 72bp, longer by 3bp at the 5' end when compared to the sequence we determined. The 3'UTR determined by 3'RACE was found to be 75 bp long and expected to have the features that should be present in a 3'UTR of the mRNA and the surrounding DNA sequence: a consensus polyadenylation sequence 10-25bp upstream of the mRNA cleavage site and a conserved element located within 30bp, downstream of the cleavage site (RETELSKA *et al.* 2006). The putative polyadenylation sites, AGUAAA and UAUAAA, are located 23bp and 32bp upstream of the cleavage site, respectively, and a U-rich downstream element is positioned 21bp downstream of the cleavage site. The 3'UTR for CG32088 shown in

Flybase is 105 bp long, but it lacks essential features of a 3'UTR. Primer sequences used for 5' RLM RACE and 3'RACE are available upon request.

Generation of SMC1::Venus transgene: *smc1* was PCR amplified from a *smc1* cDNA clone using the following primers: Forward- CACCATGACCGAAGAGGACGACG; Reverse-TTACGTGTCCTCGAA CGTTGTC. The product was cloned into pENTR/D-TOPO vector (Invitrogen) and the entry clone was recombined with Gateway P element vector pPVW (1093) (*Drosophila* Genomics Research Center) using Gateway^{R} LR Clonase^{TM} II Enzyme Mix (Invitrogen). This vector contains an N terminal Venus tag and UAS. The construct was transformed into w^{1118} flies (Best Gene Inc.).

Testis immunostaining: Testes were dissected and fixed according to (CENCI *et al.* 1994). Immunostaining was performed using the protocol described in (BONACCORSI 2000) with modifications. Testes were dissected in 1X Phosphate buffered saline (PBS) (137 mM Nacl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4) and covered with Sigmacote (Sigma Aldrich) treated cover slips and frozen in liquid nitrogen. Cover slips were removed and slides were immersed in -20^{0}C ethanol for 20 minutes, followed by 10 minutes in PBS solution containing 4% formaldehyde. Slides were washed twice with PBT (PBS with 0.2% TritonX-100) and blocked with 1% BSA-PBT solution. Primary antibodies were diluted in 1% BSA-PBT solution and secondary antibodies were diluted in PBT solution. Primary antibody incubations were done for 12-16 hours at 4^{0}C and secondary antibody incubations were done for 1 hour at room temperature. Antibody incubations were followed by PBT washes and finally DAPI stain was incubated for 20 minutes followed by PBS washes and slide mounting using Vectashield (Vector laboratories, CA). For identifying centromere cohesion phenotypes, a rabbit anti-CID

primary antibody (Active Motif) and Alexa Fluor 555, a donkey anti-rabbit IgG secondary antibody (H+L, Invitrogen) were used at 1:1000 dilutions. For the Venus::SMC1 and GFP-LacI localization experiments, native fluorescence was used to detect the tagged proteins. Slides were prepared according to the above protocol without the antibody staining steps. For the anti-tubulin/DAPI experiment, immunostaining was performed according to (THOMAS *et al.* 2005) using FITC-conjugated monoclonal anti-tubulin antibody (Sigma) at a 1:150 dilution. Meiosis I and meiosis II cells were discriminated on the basis of number of cells per cyst (16 or 32, respectively) and size of DAPI-stained masses. The criteria for meiosis I and II substages are described in (CENCI *et al.* 1994).

FISH (Fluorescent in situ hybridization): FISH experiments were performed according to (BALICKY *et al.* 2002) with modification (THOMAS *et al.* 2005). The 359bp satellite-repeat probe was amplified by PCR according to (THOMAS *et al.* 2005) and labeled using the Fluorescein-High Prime kit (Roche). The AATAC repeat probe was synthesized as a single-stranded oligonucleotide (IDT Biophysics) and labeled with Alexa Fluor 546 (Invitrogen) using terminal deoxynucleotidyl transferase (Promega).

Ovary immunostaining: Virgin females were placed in a food vial with yeast paste and males. After 2 days, their ovaries were dissected, fixed and stained using the protocol described in (PAGE and HAWLEY 2003). Slides were mounted using Prolong Gold Antifade reagent (Invitrogen). To determine centromeric clustering and cohesion phenotypes, rabbit anti-CID (Active Motif) and mouse anti-C(3)G (Scott Hawley, Stowers Institute For Medical Research) primary antibodies were used at 1:1000 dilutions. Alexa-Fluor 488 donkey anti-rabbit IgG (H+L, Invitrogen) and Alexa-Fluor 555

donkey anti-mouse IgG (H+L, Invitrogen) secondary antibodies were used at 1:1000 dilutions. For the CID spot counts, C(3)G positive cells in germaria and stage 2 were identified as oocytes/pro-oocytes. CID foci were counted to be part of an oocyte if they were within the C(3)G stained and DAPI stained boundary of the cell. (Note: C(3)G is a transverse filament protein that provides a useful marker of the SC). For quantification, non-overlapping CID foci were counted as separate spots. To determine SMC1 localization to centromeres, guinea pig anti-SMC1 (Sharon Bickel, Dartmouth University) and rabbit anti-CID (Active Motif) primary antibodies were used at 1:2000 and 1:1000 dilutions respectively. Alexa fluor 488 goat anti-guinea pig IgG (H+L, Invitrogen) and Alexa fluor 647 donkey anti-rabbit IgG (H+L, Invitrogen) were used as secondary antibodies at 1:1000 dilutions. Classification of oocyte stages was done according to (MATTHIES 2000).

Microscopy: All micrographs were obtained using an Axioplan microscope (ZEISS), which is equipped with a HBO 100 W mercury lamp**.** This microscope is fitted with a high-resolution charge-coupled device camera (Roper Industries). Metamorph software (Universal Imaging Corporation) was used to acquire pictures, pseudocolor them and merge them together**.** For all immunostaining and FISH images, Z-series pictures were taken, deconvolved and merged/stacked using sum algorithm. Images and figures were prepared using Adobe Photoshop (CS2), Adobe Illustrator and Microsoft Powerpoint.

RESULTS

Mutation of *sunn* causes homologous and sister chromatid NDJ in both male and female meiosis: Three alleles of *sunn* were identified in a screen of the Zuker-3 collection of EMS-treated third chromosomes for mutants that showed increased rates of fourth chromosome loss in male meiosis (KOUNDAKJIAN *et al.* 2004; WAKIMOTO *et al.* 2004). Males hemizygous for each *sunn* allele and which carried a genetically marked Y chromosome ($B^{S}Yy^{+}$) were tested for X and Y chromosome NDJ in crosses to chromosomally normal females. Progeny from XY and nullo-XY (O) sperm were recovered at frequencies of 42-45% in all three *sunn* mutants compared to less than 0.2% in wild-type (WT) controls (Table 2-1). A similar NDJ frequency was obtained in homozygous $sunn^{Z3\text{-}5839}$ males (Table 2-1). Taken together, these data suggest, but do not prove, that all three *sunn* alleles are genetic null alleles.

The results in Table 2-2 show that *sunn* mutations cause high frequencies of homolog NDJ but do not address whether *sunn* mutations also cause sister chromatid NDJ. The diagnostic sperm class for NDJ of X sister chromatids is XX sperm which yield inviable XXX progeny in crosses to chromosomally normal females. To detect XX sperm and compare the frequencies of homolog and sister chromatid NDJ, *sunn* males were crossed to females carrying an attached-X chromosome (*C(1)RM/O)* which produce only diplo-X and nullo-X eggs in roughly equal proportions. In such crosses, all major sperm classes, including the XX, XY and nullo-XY (O) NDJ classes, yield viable progeny in combination with one of the egg classes (see Table 2-2 legend for detailed explanation). Males hemizygous for the three *sunn* alleles produced XX, XY and O

Table 2-1: Sex chromosome NDJ in *sunn* mutant males

	Sperm Genotype					
Paternal Genotype	X	Y	XY	O	n[a]	%NDJ[b]
sunn$^{Z3\text{-}1956}$/*Df*^{c}	337	353	177	337	1204	42.7
sunn$^{Z3\text{-}5839}$/*Df*^{c}	388	416	181	419	1404	42.7
sunn$^{Z3\text{-}4085}$/*Df*^{c}	344	353	158	400	1255	44.5
sunn$^{Z3\text{-}5839}$/*sunn*$^{Z3\text{-}5839}$	286	266	92	311	955	42.2
Total *sunn*	**1355**	**1388**	**608**	**1467**	**4818**	**43.1**
Gamete Freq.(%)	**28.1**	**28.8**	**12.6**	**30.4**	-	-
Df^{c}/+ (WT)	803	735	0	2	1540	0.1

w/*B*^{S}*Yy*$^{+}$ males with the indicated third chromosome genotype for *sunn* were each crossed to 2 *y w* females. The dominant *B*^{S} marker causes Bar eyes and was used to determine whether progeny inherited the Y chromosome. [a]total number of progeny scored. [b]%NDJ = 100 x (XY+ O)/n. [c]*Df (3L) ED4470.*

Table 2-2: Sister chromatid versus homolog NDJ in *sunn* mutant males

Paternal Genotype	Sperm Genotype	X	Y	XY	XX	O	n[a]	%NDJ[b]	%sis[c]
	Progeny Phenotype	w B^+ ♂	su-w^a B^S ♀	w B^S ♂	w B^+ ♀	su-w^a B^+ ♀			
sunn$^{Z3\text{-}5839}$/*Df*		388	234	270	64	265	1221	54.3	32.2
sunn$^{Z3\text{-}1956}$/*Df*		366	265	223	56	279	1189	51.6	33.4
sunn$^{Z3\text{-}4085}$/*Df*		256	188	138	50	264	896	56.0	42.0
Total *sunn*		**1010**	**687**	**631**	**170**	**808**	**3306**	**53.8**	**35.0**
Gamete Freq. (%)		**30.6**	**20.8**	**19.1**	**5.1**	**24.4**	-	-	-
Df/+ (WT)		730	489	0	1	1	1221	0.3	1

w/*B*^{S}*Yy*$^+$ males with the indicated third chromosome genotype for *sunn* were each crossed to 2 *C(1)RM*, *y*^{2} *su (w*^{a}*) w*^{a} /*O* females. These females produce only diplo-X and nullo-X eggs and permit recovery of viable progeny derived exclusively from sister chromatid NDJ sperm (XX), XY homolog NDJ sperm and nullo-XY (O) sperm (which result from both types of NDJ). The diplo-X eggs yield viable progeny when fertilized by Y or nullo-XY (O) sperm. These progeny exhibit a suppressed white-apricot (light brown) eye color (su-w^a) caused by the *su (w*^{a}) and *w*^{a} alleles on *C(1)RM*. The nullo-X eggs yield viable progeny when fertilized by X, XY, XX or XXY sperm. These progeny all have white eyes because of the null *w* allele carried on the paternal X chromosome. Progeny were classified by sperm genotype as described above in column labels. [a]total

Table 2-2. Continued

number of progeny scored. [b]% NDJ = 100 x ((2 x XX) + XY + O)/ n. [c]% sister chromatid NDJ = 100 x (2 x XX)/ ((2 x XX) + XY).

Notes: 1) Progeny with one or two copies of $B^{S}Yy^{+}$ cannot be discriminated, so some progeny scored as derived from Y or XY sperm could have been YY or XYY. 2) In the %NDJ and %sis formulae, the XX sperm-derived progeny are doubled to account for the YY-sperm derived progeny which cannot be discriminated from regular Y sperm-derived progeny and are poorly viable. 3) Two, seven and two progeny derived from XXY non-disjunctional sperm were recovered from $sunn^{Z3\text{-}5839}$/*Df*, $sunn^{Z3\text{-}1956}$/*Df* and $sunn^{Z3\text{-}4085}$/*Df* hemizygotes, respectively (not shown in table).

non-disjunctional sperm at average frequencies of 5.1 %, 19.1% and 24.4 % respectively (Table 2-2), indicating that *sunn* mutations cause NDJ of both homologous and sister chromatids. The average NDJ frequency was 53.8% and the average relative frequency of sister chromatid NDJ out of total NDJ was 35%. In this assay, as in the previous one, differences among the three alleles were minor and insignificant. These results are consistent with random sex chromatid assortment through both meiotic divisions, as might result from loss of sister chromatid cohesion prior to meiosis I. Similar NDJ frequencies and patterns have been reported for null alleles of *ord* and *solo* (MIYAZAKI and ORR-WEAVER 1992; BICKEL *et al.* 1997; YAN *et al.* 2010). Mutation of *sunn* also causes high frequencies of both sister chromatid and homologous chromosome NDJ of the autosomal second (Table 2-3) and fourth chromosomes (data not shown).

To determine whether *sunn* mutations also cause sex chromosome NDJ in female meiosis, *sunn* hemizygous females were crossed with males carrying a dominant *Bar (B)* mutation on their X chromosomes. The regular progeny from this cross are B females and B^+ males; the NDJ progeny are B^+ females and B males. The results showed that 56.9% of progeny from *sunn* females resulted from X-X NDJ compared to 0.1% in sibling WT control females (Table 2-4). Analysis of centromere-linked markers revealed that 25.4% of the B^+ females carried two maternal sister chromatids and the remainder carried two maternal homologous chromatids, indicating that both homologous and sister chromatids non-disjoin in *sunn* females (Table 2-4). Thus, like *ord* and *solo*, *sunn* is required for proper chromosome segregation in both meiotic divisions in both sexes (MIYAZAKI and ORR-WEAVER 1992; BICKEL *et al.* 1997; YAN *et al.* 2010; YAN and MCKEE 2013).

Table 2-3: Second chromosome NDJ in *sunn* mutant males

Sperm Genotype	NDJ type	Ova genotype	Progeny Phenotype	No. of progeny Obtained
+/+ and bw/+	Sister+Homologue	O	WT	432
O	Sister+Homologue	2^2 b pr	b pr	543
bw/bw	Sister	O	bw	72
Total	**-**	**-**	**-**	**1047**

+/Y; bw/+; *sunn*$^{Z3\text{-}5839/\ Z3\text{-}1956}$ males were crossed with *C(2)EN, b pr* females in vials containing two males and four females each. In total, 76 males were tested, and they produced 1047 progeny. The average number of progeny produced per male was 13.8. *C(2)EN b pr* females carry two copies of each arm of chromosome 2 attached to a single centromere and produce only diplo-2 (2^2 b pr) and nullo-2 (O) eggs. Fertilization of diplo-2 or nullo-2 eggs with sperm containing a single copy of chromosome 2 causes the production of inviable monosomic or trisomic embryos. However, paternal NDJ yields diplo-2 or nullo-2 sperm that can generate viable embryos. Thus, the level of second chromosome NDJ in males is proportional to the number of progeny produced per male. Parent males have bw/+ second chromosome genotype, so the presence of bw/bw progeny indicates the occurrence of sister chromatid NDJ. The following formula was used to calculate % sister chromatid NDJ – 100 x 2 (bw progeny) / (bw + WT progeny). % sister chromatid NDJ = 28.6. Note: None of the WT males tested produced any progeny.

Table 2-4: X chromosome NDJ in *sunn* mutant females

Maternal Genotype	Progeny types: DJ[a] B♀	DJ[a] B$^+$♂	NDJ[b] y$^+$ B$^+$♀	NDJ(Sis)[b,c] y B$^+$♀	NDJ[b] y B♂	n[d]	%NDJ[e]	%sis[f]	P/F[g]
sunn$^{Z3\text{-}5839}$/*Df*	219	198	146	19	124	706	58.1	23.0	11.6
sunn$^{Z3\text{-}4085}$/*Df*	264	174	136	19	123	716	55.9	24.5	10.9
sunn$^{Z3\text{-}1956}$/*Df*	268	149	123	21	128	689	56.6	29.2	11.9
Total *sunn*	**751**	**521**	**405**	**59**	**375**	**2111**	**56.9**	**25.4**	**-**
Gamete Freq.	**35.6**	**24.7**	**19.2**	**2.8**	**17.8**	-	-	-	-
Df/+	1033	879	0	0	1	1913	0.1	NA[h]	79.7

Dp(1;1)sc^{v1}*, y .y*$^+$*/y* females with the above third chromosome genotypes were crossed with 2 *YSX.YL, In(1)EN, y B/Y* males. [a]DJ: progeny from normal (disjunctional) eggs. [b]NDJ: progeny from NDJ eggs. The B+ daughters result from diplo-X eggs and the y B sons from nullo-X eggs. [c]NDJ(Sis): The y B+ daughters derive from diplo-X eggs carrying two sister chromatids lacking the *y*$^+$ centromere marker, so represent sister chromatid NDJ only. The other two NDJ categories reflect a mix of sister chromatid and homolog NDJ. [d]n: total number of progeny counted. [e]%NDJ = 100 x 2 (NDJ)/ (n + NDJ). [f]%sis = % sister chromatid NDJ = 2 x (y B$^+$♀)/ (y B$^+$♀ + y$^+$ B$^+$♀). [g]P/F (Progeny/Female) = Average number of progeny a single female produces when crossed to two males. [h]Not Applicable.

***sunn* mutations disrupt sister chromatid cohesion during male meiosis:** For an in-depth study of the NDJ mechanism in *sunn* mutants, we surveyed chromosome and nuclear morphology throughout male meiosis by staining spermatocytes with DAPI to label chromosomes and with a α-tubulin antibody to label spindles. WT male meiosis I occurs synchronously in interconnected cysts of 16 primary spermatocytes derived from a single germline stem cell. Although axial elements and synaptonemal complexes are absent and the chromosomes are decondensed, *Drosophila* spermatocytes traverse a series of prophase I substages, labeled S1-S6, during which the chromosomes undergo distinctive changes, the most prominent of which are the separation of the four bivalents into distinct nuclear territories near the end of stage S2 and their condensation during stage S6 to form four compact and roughly spherical bivalents. The bivalents then congress during prometaphase I to form a tight metaphase I bundle and segregate reductionally at anaphase I to form daughter nuclei with equal staining intensity. After a brief prophase II, meiosis II univalents recondense, congress and segregate equationally at anaphase II, yielding cysts of 64 spermatids with round nuclei of uniform size (CENCI *et al.* 1994).

Despite the high rates of meiosis I NDJ in genetic crosses, DAPI-stained *sunn* spermatocytes appeared remarkably normal during meiosis I (Figure 2-1A). As in WT, three large DAPI-stained territories, corresponding to the X-Y, 2nd and 3rd chromosome bivalents, were present throughout mid- and late-prophase I, indicating that homolog pairing and territory formation are intact in *sunn* mutants. Sometimes, a small fourth territory is observed in WT and *sunn* mutants which corresponds to 4th chromosome bivalent. The territories condensed into compact “blobs” by prometaphase I, congressed

Figure 2-1: Chromosome segregation during meiosis in spermatocytes of *sunn* mutants. (A) and (B) Immunostaining was performed on WT and *sunn* (*sunn*$^{Z3\text{-}5839}$*/Df*) mutant spermatocytes using anti-α-Tubulin antibody conjugated with FITC (Fluorescein isothiocyanate) to visualize the spindle and DAPI to visualize DNA. PM I stands for prometaphase I, M I stands for metaphase I, A I stands for anaphase I, A II stands for anaphase II, M II stands for metaphase II. (A) Chromosome territory formation at S5 and PM I is normal in *sunn* mutants. At A I, roughly equal DAPI masses were observed at opposite poles in both *sunn* mutants and WT. (B) Metaphase II congression is defective and anaphase II segregation is unequal in *sunn* mutants. (C) Aceto-orcein staining of spermatocytes from *sunn* mutants revealed the presence of DNA territory extrusions (red arrows) in prometaphase I and metaphase I cells, which are diagnostic of loose packing of bivalent territories and loss of cohesion between sister chromatids. Aceto-orcein staining was performed according to (BONACCORSI 2000). Scale bars = 5 μM.

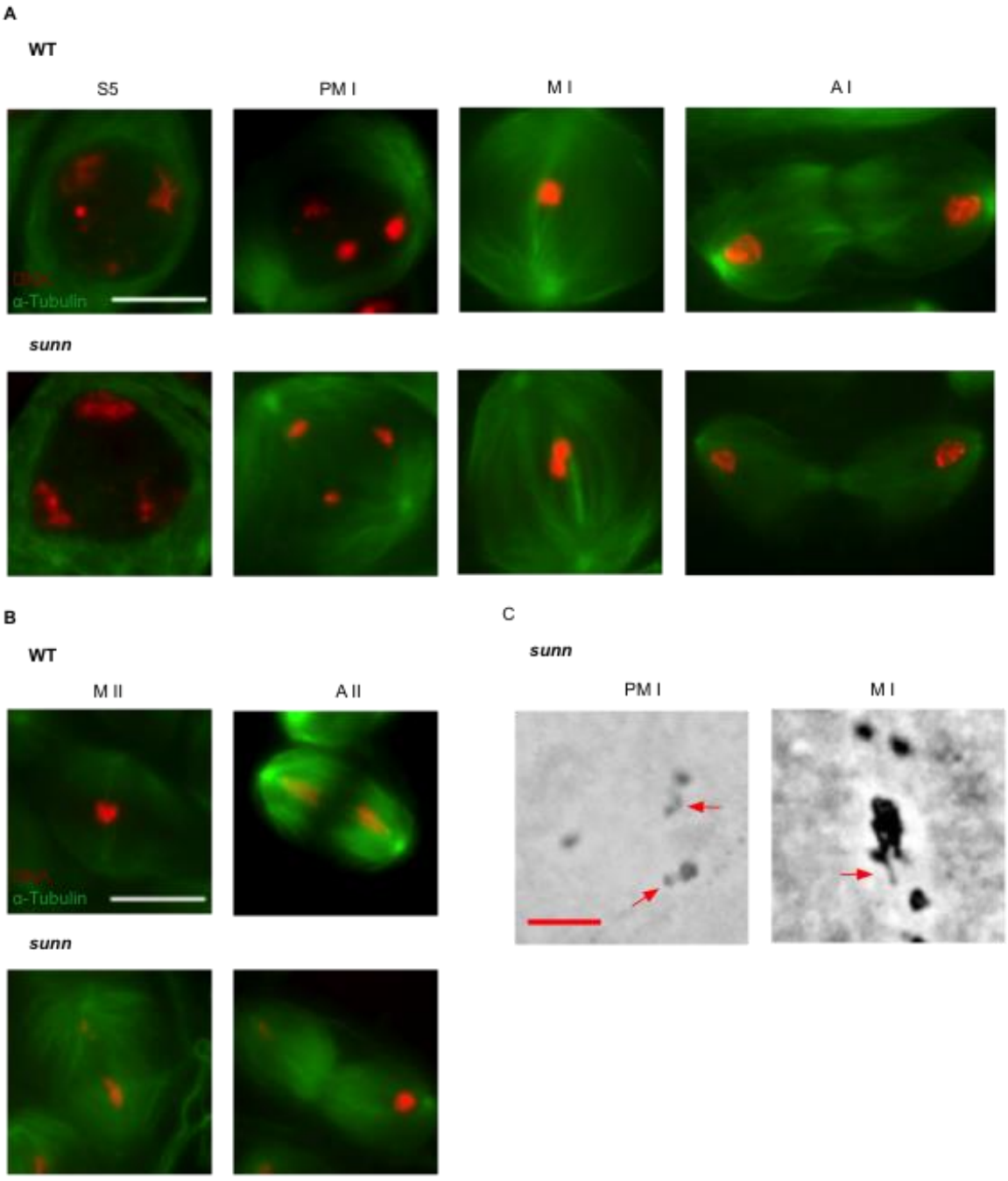


Figure 2-1. Continued

normally and segregated at anaphase I to form daughter nuclei that in most cases appeared to contain roughly equal amounts of chromatin. However, prematurely separated sister chromatids were common during and after anaphase I, and meiosis II was chaotic in *sunn* mutants. We frequently observed single chromatids during prometaphase II and metaphase II, defective metaphase II congression, and unequal segregation at anaphase II (Figure 2-1B). In light of the genetic evidence for high rates of both meiosis I and meiosis II NDJ, the absence of gross abnormalities during meiosis I would have been surprising had the same anomaly not been previously observed in *ord* and *solo* mutants (MIYAZAKI and ORR-WEAVER 1992; BICKEL *et al.* 1997; YAN *et al.* 2010). As in those cases, abnormalities in prometaphase I and metaphase I bivalent morphology consistent with premature loss of sister chromatid cohesion -- loose chromatid packing, extruded single kinetochore regions and, occasionally, fully separated chromatids -- were seen in acetic-orcein preparations of mutant chromosomes, presumably because of harsher fixation procedures than are normally used in DAPI staining (Figure 2-1C). These observations suggested that although homologs remain paired throughout meiosis I in *sunn* mutants, defects in sister chromatid cohesion might underlie the abnormal segregation patterns.

To examine sister chromatid cohesion directly, we immunostained *sunn* and WT spermatocytes with an antibody against the centromere marker CID (Centromere Identifier) (BLOWER and KARPEN 2001) (Figure 2-2). The cohesion status of sister centromeres was determined by counting the number of discrete CID spots per nucleus (Table 2-5). When homologous centromeres are unpaired but sister centromere cohesion is intact, as is generally the case after stage S3, spermatocytes are expected

to show maxima of 8 CID spots during meiosis I and 4 CID spots during meiosis II. Consistent with previous results (VAZQUEZ *et al.* 2002; YAN *et al.* 2010), WT spermatocytes rarely exhibited more than 8 CID spots per nucleus during meiosis I (mean CID spot numbers of 6.1-7.2 from stage S4 through metaphase I) or more than 4 CID spots during meiosis II. *sunn* mutants did not differ from WT in early prophase I but began to diverge from WT by stage S4 when 34% of spermatocytes showed more than 8 CID spots. By late prophase I (stages S5 and S6) and throughout the division stages, more than 90% of *sunn* spermatocytes showed more than 8 spots, with a mean of ~11-12 spots per spermatocyte (Table 2-5). 14-16 CID spots were seen in a substantial fraction of *sunn* spermatocytes at prometaphase I and metaphase I, indicating that *sunn* function is required for cohesion of all eight *Drosophila* chromosomes. In meiosis II, 82% of *sunn* spermatocytes showed more than 4 CID spots. Thus the data shows that *sunn* mutants begin losing centromere cohesion by stage S4 and exhibit extensive cohesion loss by stage S5, long before chromosomes begin orienting on the meiosis I spindle.

***sunn* mutants disrupt sister centromere mono-orientation:** The absence of cohesion between most sister centromere pairs during prometaphase I might impair sister centromere mono-orientation and thereby disrupt reductional segregation. To track the segregation of the X and Y chromatids at anaphase I, we performed Fluorescent-in-situ-hybridization (FISH) using probes which bind to the 359bp satellite repeats in the pericentromeric region of the X chromosome and to a block of AATAC satellite repeats in the long arm of the Y chromosome. Signals were scored both during anaphase I and metaphase II. In WT, as expected, only reductional segregations (XX-

Figure 2-2: Sister centromere cohesion is lost during prophase I in *sunn* spermatocytes. Immunostaining was performed using anti-CID antibody, which marks centromeres (green). DNA was stained with DAPI (red). PM I = Prometaphase I, M I = Metaphase I and M II = Metaphase II. (A) In WT (*Df*/+) spermatocytes, spot numbers never exceeded 8 during meiosis I or 4 during meiosis II. Representative images of S3, S4, S5/S6, PM I, M I and M II show 6, 6, 7, 8, 8 and 4 CID spots respectively. (B) In *sunn (sunn$^{Z3\text{-}5839}$/Df)* spermatocytes, CID spot numbers exceeded 8 in most meiosis I spermatocytes from stage S4 onwards and exceeded 4 in most meiosis II spermatocytes. Representative images of S4, S5/S6, PM I, M I and M II stages show 14, 12, 15, 14 and 7 spots respectively. S3 scale bars apply to S4, PM I, M I, M II. Scale bars = 5 μM. See Table 2-4 for quantification. *Df=Df(3L)ED4470*

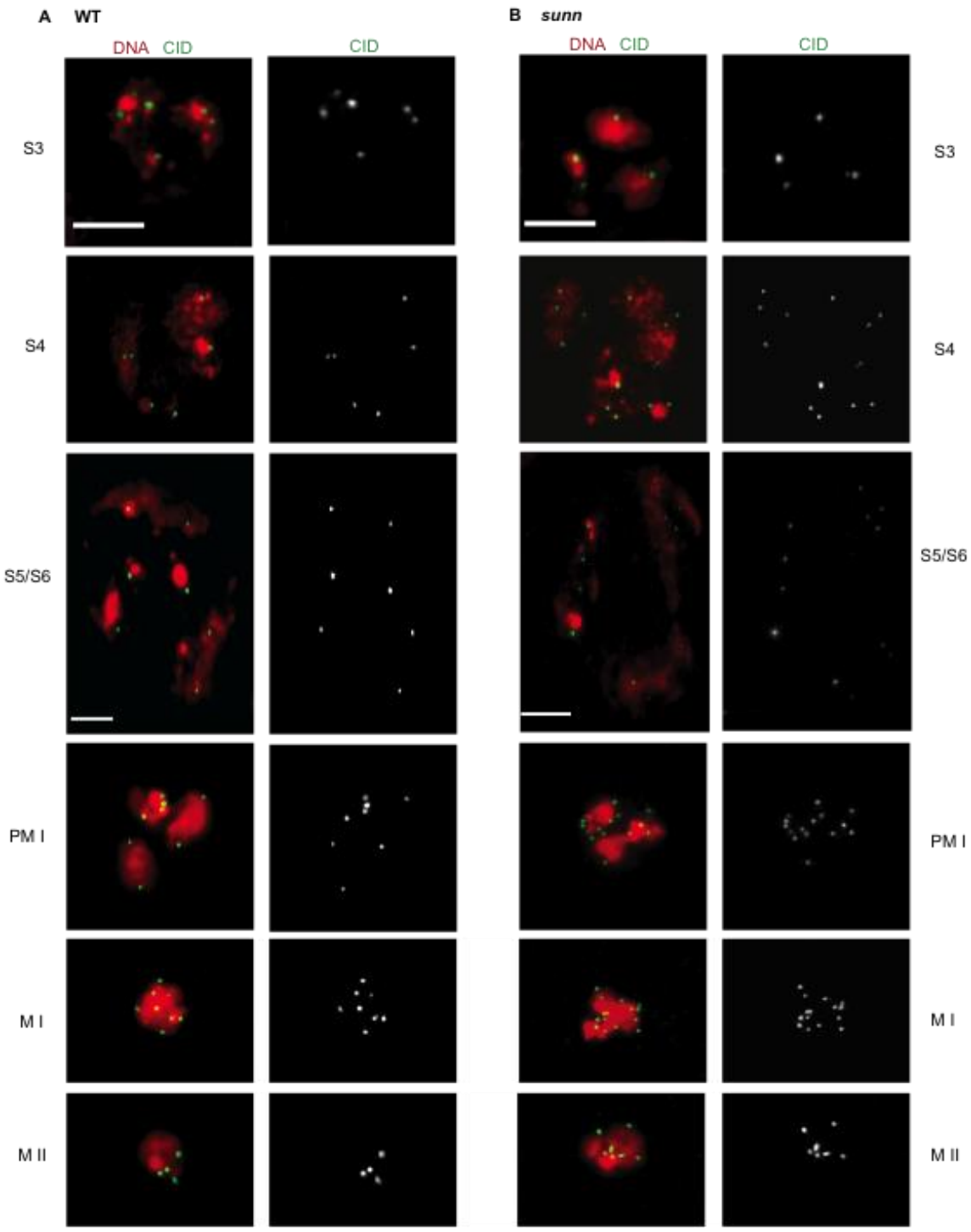


Figure 2-2. Continued

Table 2-5: Quantification of CID spots in *sunn* mutant spermatocytes.

A Meiosis I stages

	sunn[a]			WT[b]		
	≤ 8 spots	>8 spots	Mean spot#	≤ 8 spots	> 8 spots	Mean spot#
S1	75 (100)	0	3.96	71 (100)	0	3.31
S2	55 (100)	0	3.82	60 (100)	0	3.14
S3	96 (98.97)	1 (1.03)	4.12	53 (100)	0	3.96
S4	60 (65.9)	31 (34.1)	7.62	58 (98.25)	1 (1.75)	6.08
S5, S6	9 (6.2)	138 (93.8)	10.41	134 (99.3)	1 (0.7)	6.92
PM I[c]	5 (3.9)	122 (96.1)	10.75	68 (100)	0	6.89
M I[d]	1 (9)	10 (91)	12.20	11 (100)	0	7.23

B Meiosis II Stage

	sunn[a]			WT[b]		
	≤ 4 spots	> 4 spots	Mean spot#	≤ 4 spots	> 4 spots	Mean spot#
M II[e]	8 (17.4)	38 (82.6)	6.22	79 (93)	6 (7)	3.80

Number in parentheses indicates percentage values calculated from the total number of nuclei scored at each spermatocyte stage. [a]*sunn*$^{Z3\text{-}5839}$/ *Df.* [b]*Df*/+. [c]PM I- Prometaphase I. [d]M I- Metaphase I. [e]M II- Metaphase II

YY) were observed, as shown by a complete absence of anaphase I poles or metaphase II nuclei with both X and Y signals or with no signals (Figure 2-3A and Table 2-6).

The segregation pattern in *sunn* mutants was completely different. Only 31% of the 553 *sunn* poles/nuclei scored exhibited the reductional segregation pattern. Most (60%) of the *sunn* poles/nuclei exhibited one X signal and one Y signal, reflecting an XY-XY equational segregation pattern (Fig. 2-3B; Table 2-5). The remaining 9% of *sunn* nuclei exhibited either 3 signals (2 X and 1 Y or vice versa) or 1 signal (either X or Y, reflecting unbalanced XXY-Y or XYY-X segregations (Table 2-5). No completely unbalanced (XXYY-O) segregations were observed. Absence of sister centromere cohesion in *sunn* mutants was also evident in the FISH data. Unlike in WT in which the two X signals were usually fused or overlapping (due to cohesion of pericentric regions), sister X signals in *sunn* nuclei were usually separate even when they co-segregated (Figure 2-4B). The presence of two separate AATAC (Y chromosome arm) spots in most WT spermatocytes reflects the fact that arm cohesion is lost early in male meiosis – by stage S3 (VAZQUEZ *et al.* 2002; YAN *et al.* 2010)). The complete absence of sister chromatid cohesion in *sunn* mutants was also apparent in many pro-metaphase II nuclei in which sister 359bp or AATAC signals were present in separate DAPI-stained masses (Fig. 2-4B). In conclusion, *sunn* mutations perturb the segregation pattern of the X and Y chromosomes at anaphase I, prematurely eliminating sister centromere cohesion, thereby disrupting sister centromere mono-orientation.

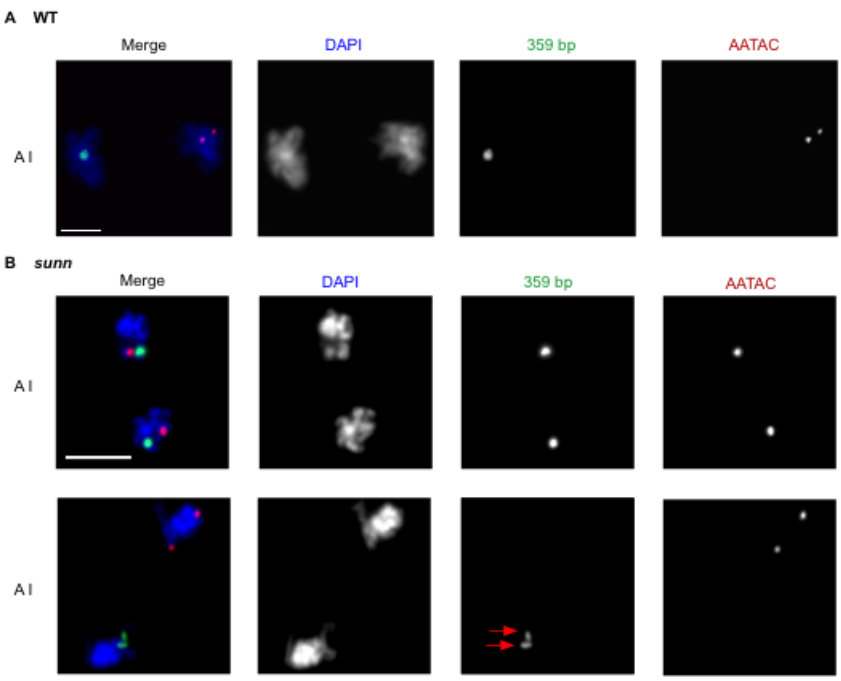


Figure 2-3: X and Y chromatids segregate both equationally and reductionally at meiosis I in *sunn* spermatocytes. FISH was performed using probes for the 359 bp repeats on the X chromosome (green) and a block of AATAC repeats on the Y chromosome (red). DNA was stained with DAPI (blue). A I = Anaphase I. (A) Meiosis I segregation is exclusively reductional in WT (*Df*/+) spermatocytes. Representative image of a reductional anaphase I segregation. The single 359 bp spot reflects maintenance of cohesion at and near the X centromere. Scale bar = 3 μM (B) Both reductional and equational segregation occur at meiosis I in *sunn* (*sunn*$^{Z3\text{-}5839}$/*Df*) spermatocytes. Representative images at anaphase I showing normal reductional segregation (bottom panel) and abnormal equational segregation (top panel). The two pericentromerically located 359 bp signal spots are separated in *sunn* mutants (red arrows), reflecting premature loss of X centromere cohesion. Scale bar = 5 μM. See Table 5 for quantification.

Table 2-6: Quantification of X-Y chromatid segregation patterns in *sunn* mutant spermatocytes

Chromatid pattern	*sunn*[a]	WT[b]
Anaphase I		
XX/YY	47 (30%)	62 (100%)
XY/XY	95 (61%)	0
XXY/Y	9 (6%)	0
XYY/X	5 (3%)	0
Total	**156 (100%)**	**62 (100%)**
Prometaphase II		
XX or YY	78 (32%)	121 (100%)
XY	143 (59%)	0
XXY or Y	11 (5%)	0
XYY or X	9 (4%)	0
XXYY or O	0	0
Total	**241 (100%)**	**121 (100%)**

X and Y chromatids were identified by FISH using probes for the 359bp and AATAC loci as described in legend of Fig. 2 and Materials and Methods. [a]*sunn*$^{Z3\text{-}5839}$/ *Df*. [b]*Df*/+.

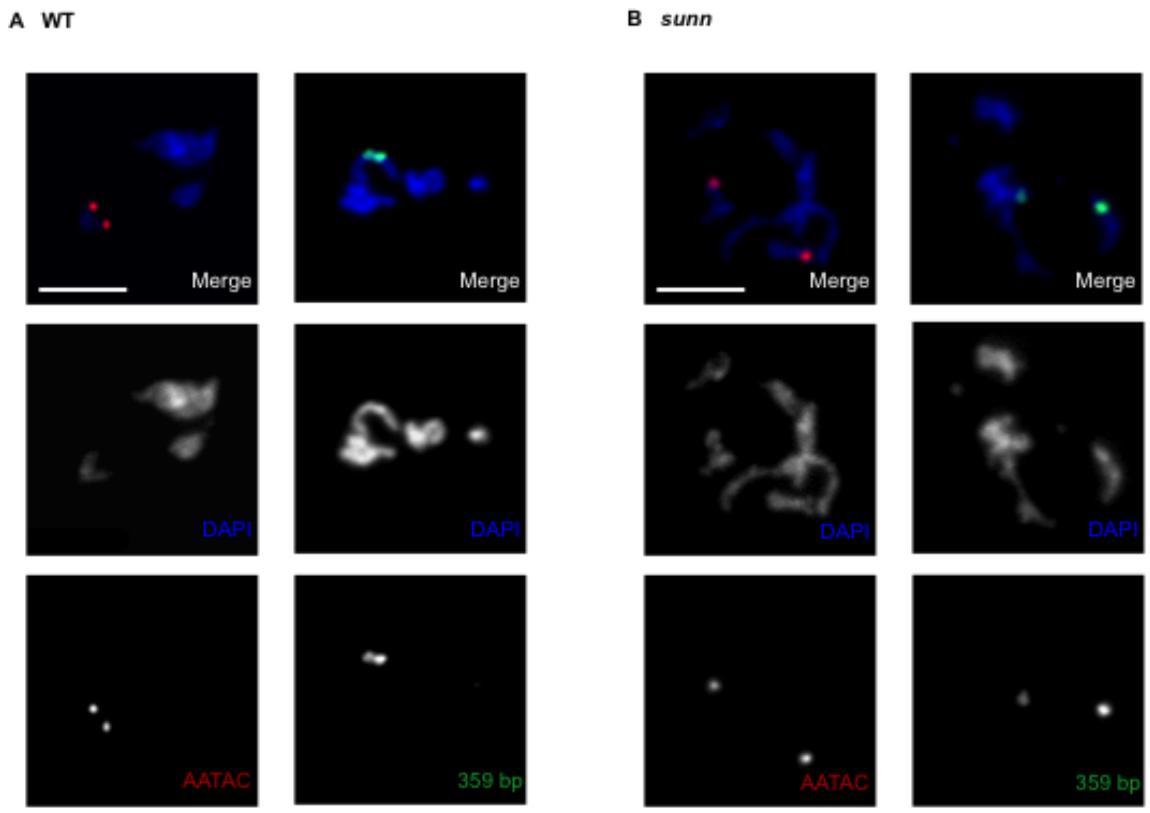
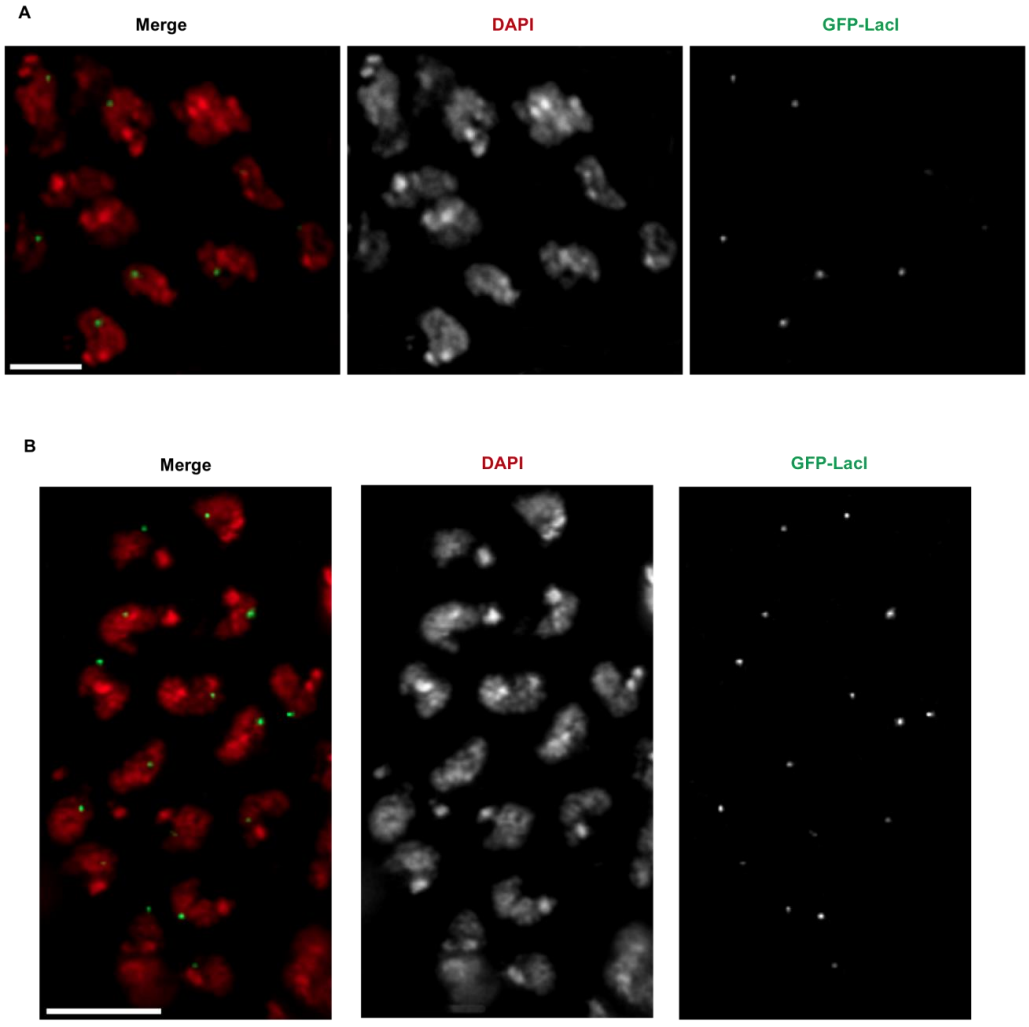


Figure 2-4: Sister chromatid separation in prometaphase II spermatocytes of *sunn* mutants. X and Y chromatids were identified by the presence of 359 bp (green) and AATAC (red) probes respectively. (A) Sister chromatid cohesion is maintained in WT (*Df*/+). In two prometaphase II spermatocytes bearing a Y chromosome (left panel) or an X chromo- some (right panel), both AATAC signals and both 359 bp signals are situated in the same chromosome territory. (B) Premature sister chromatid separation in *sunn* (*sunn*$^{Z3\text{-}5839}$/*Df*) spermatocytes. In two prometaphase II spermatocytes bearing two separated Y chromatids (left panel) or two separated X chromatids (right panel), the two AATAC signals and the two 359 bp signals are located in completely separate chromosome territories. See Table 2-5 for quantification. Scale bars = 5 μM

***sunn* is not required for mitotic segregation or for arm cohesion**: In WT male meiosis, arm cohesion is established in meiotic S phase but, unlike centromere cohesion, it is lost during mid-prophase I (late S2/S3). Consequently, when a single chromosome arm site is labeled by FISH or the GFP-LacI/ *lac*O assay, only one spot is observed during early prophase I but two separate (sister) spots are generally observed at later stages of meiosis I (VAZQUEZ *et al.* 2002; YAN *et al.* 2010). Thus the effect of a mutation on arm cohesion can be assayed by counting spots (one versus two) during early prophase I. We examined arm cohesion in *sunn* spermatocytes by labeling a heterozygous *lac*O array inserted in the euchromatin of chromosome 2 with GFP-LacI expressed under control of the *hsp83* promoter. In both WT and *sunn* mutants, a single spot was observed in the great majority of stage S1 and S2 spermatocytes, indicating that *sunn* is not required for arm cohesion in early prophase I (Figure 2-5). Similar results were reported for *solo* mutants (YAN *et al.* 2010). In later stages of meiosis I (when arm cohesion is lost), the number of spots in single-locus arm labeling experiments provides a reliable measure of chromatid copy number, useful to diagnose aneuploidy due to mitotic NDJ. The Y chromatids are particularly useful for such studies because they are normally present in two copies but can be absent altogether (XO) or present in four copies (XYY) without blocking spermatocyte development. Thus, in the FISH analysis reported above (Figure 2-3 and Table 2-6), mitotic NDJ of the Y chromosome in *sunn* spermatogonia would be expected to generate XYY or XO spermatocytes exhibiting four or no AATAC signals, respectively by late prophase I. However, 100% of the anaphase I *sunn* spermatocytes (N=156) reported in Table 2-5 showed two AATAC spots (sometimes at opposite poles, sometimes at the same pole)

Figure 2-5: Arm cohesion is not affected in *sunn* mutants. Single GFP foci were observed in nuclei from DAPI-stained spermatogonial 8-cell cysts (A) and stage S1 meiotic 16-cell cysts (B) from *sunn* $^{Z3\text{-}5839}$*/Df* males heterozygous for a second chromosome insertion of a 256-mer lacO array and expressing GFP-Lac I under control of the *hsp83* promoter (VAZQUEZ *et al.* 2002). Nuclei exhibit one or two foci depending on whether sister chromatid arms are together or apart, respectively. Scale bars represent 5uM in (A), 10 uM in (B). In eight cell cysts (A) and S1 16 cell cyst (B), *sunn* mutants mostly display a single GFP-Lac I focus. (C) Quantification of GFP-Lac I foci in *sunn* and WT spermatogonia and S1 and S2 stage spermatocytes.



C

Genotype	Stage	1 spot	2 spot	Total
sunn				
	Spermatogonia + S1	263 (92.6)	19 (6.7)	282
	S2	38 (100)	0	38
WT				
	Spermatogonia + S1	29 (87.9)	4 (12.1)	33
	S2	15 (100)	0	15

Figure 2-5. Continued

as did 100% of prometaphase I and metaphase I *sunn* spermatocytes (N=68, data not shown). These results strongly suggest that there is no significant mitotic NDJ in *sunn* mutants.

***sunn* mutations disrupt centromere clustering, pairing and cohesion in female meiosis:** To determine if centromere cohesion is also lost prematurely in female meiosis, CID spot numbers were scored in pro-oocytes and oocytes from $sunn^{Z3\text{-}5839}/Df$ females and WT sibling controls. Analysis of CID spot numbers in *sunn* oocytes was also of interest because of recent evidence for clustering of centromeres throughout prophase I in WT female meiosis and for the dependence of that clustering on *ord* and *solo* as well as on the genes encoding SC components--*c(3)G* and *cona* and kinetochore components--*cenp-c* and *cal-1* (KHETANI and BICKEL 2007; TAKEO *et al.* 2011; TANNETI *et al.* 2011; UNHAVAITHAYA and ORR-WEAVER 2013; YAN and MCKEE 2013). In female *Drosophila*, meiosis occurs in ovaries, which contain 10-30 ovarioles, each consisting of linear arrays of oocytes of increasing developmental age from stem cells to metaphase I-arrested oocytes.

Meiosis initiates in the germarium, the anterior-most compartment of each ovariole. Region 1, at the anterior end of the germarium, contains stem cells and pre-meiotic cysts undergoing mitotic amplification. Regions 2A, 2B and 3 of the germarium contain 16-cell cysts in the zygotene (region 2A) or pachytene (regions 2A, 2B and 3) stages of meiosis. Meiosis initiates and SCs begin forming in up to 4 pro-oocytes in each cyst in region 2A but by region 3, only a single oocyte retains SC. The other 15 germ cells in each cyst develop as polyploid nurse cells that support the oocyte during its development. The maturing cysts leave the germarium and continue developing in

the vitellarium. SC is disassembled in vitellarial stages 5-7, marking the end of pachytene (McKim *et al.* 2002; Lake and Hawley 2012). The oocyte subsequently enters an arrested late prophase I state termed the karyosome, in which the chromosomes are highly compact. Nuclear envelope breakdown in stage 12 is followed by prometaphase I and metaphase I in stages 13 and 14.

In agreement with reports above, we found 1-3 CID foci in nearly all (~90-97%) nuclei in WT pro-oocytes/oocytes in germarial regions 2A, 2B and 3 nuclei (average of 2.1-2.6 CID foci/nucleus), indicative of pairing and clustering of centromeres. Clustering was also present in stage 2 of WT vitellaria (Figure 2-6A, Table 2-7). However, in *sunn* mutants, 1-3 CID foci were observed in only 11.5%, 0%, 5% and 0% of nuclei in regions 2A, 2B, 3, and stage 2, respectively, indicating an absence of centromere clustering during prophase I. Most *sunn* pro-oocytes in regions 2A (89%) and 2B (85%) exhibited 4-8 CID spots, with means of 5.1 and 7 spots per nucleus, respectively, indicating that pairing of homologous centromeres was also compromised, somewhat more completely in region 2B than in region 2A. Cohesion was intact in region 2A since no pro-oocytes with more than 8 CID spots were observed. However, more than 8 CID spots were observed in 16% of region 2B pro-oocytes, 50% of region 3 oocytes and 56% of stage 2 oocytes in *sunn* mutants (Figure 2-6B and Table 2-7). Thus, cohesion begins deteriorating by region 2B and is compromised by region 3 in *sunn* mutants. As in male meiosis, this cohesion loss occurs long before centromeres must orient on the meiosis I spindle and thus provides a likely explanation for the high levels of meiosis I NDJ.

SUNN is a novel protein produced from the CG32088 locus: Using deficiency complementation and candidate gene sequence analysis, *sunn* was

Figure 2-6: Centromeric clustering is disrupted in *sunn* mutant females. Whole-mount ovaries were immunostained with anti-CID and anti-C(3)G which serve as markers for centromeres and SCs, respectively (BLOWER and KARPEN 2001; PAGE and HAWLEY 2001). (A) Centromeres are paired and clustered in WT (*Df /TM3*) oocytes. Pro-oocytes/oocytes showed one to three CID foci throughout the germarium in regions 2A, 2B and 3 and in the vitellarium at stage 2. Representative oocyte/pro-oocyte images all show 2 large CID foci in region 2A, region 2B, region 3 and stage 2. (B) Loss of centromere clustering, pairing and cohesion in *sunn* (*sunn*$^{Z3\text{-}5839}$*/Df*) oocytes. Pro-oocytes/oocytes averaged more than 4 CID spots throughout pachytene. Representative images show 5, 8, 10 and 13 CID foci in region 2A, region 2B, region 3 and stage 2, respectively. Scale bars = 5 μM. See Table 6 for quantification.

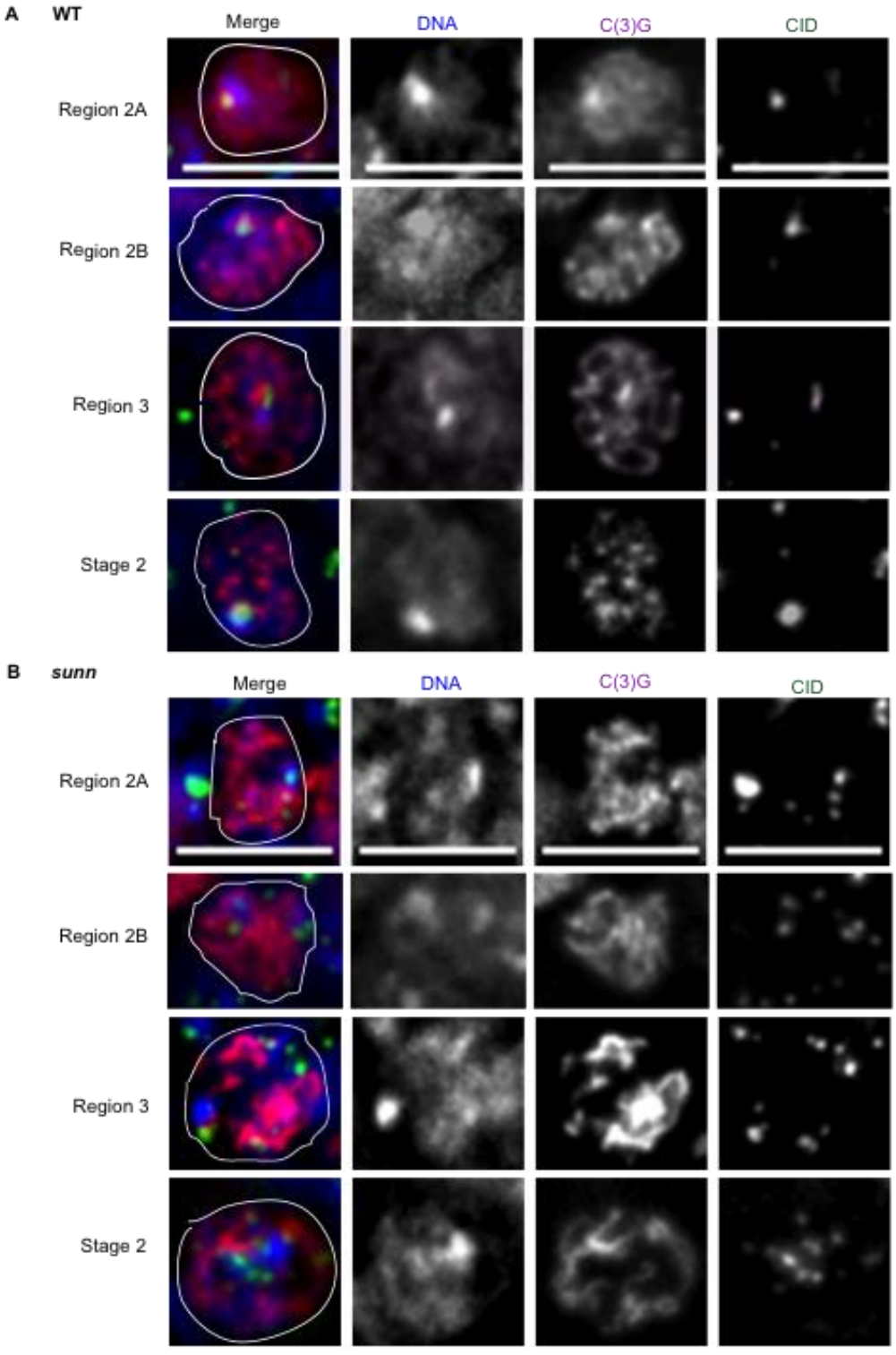


Figure 2-6. Continued

Table 2-7: Quantification of CID spots in WT and *sunn* mutant oocytes/pro-oocytes

	Oocyte Stages			
Genotype	Region 2A	Region 2B	Region 3	Stage 2
WT[a]	2.14 ± 0.91 (N=21)	2.19 ± 0.79 (N=36)	2.6 ± 1.07 (N=10)	3 ± 1.12 (N=12)
< 4 CID (%)	95.2	97.2	90	75
≥ 4, ≤ 8 CID (%)	4.8	2.8	10	25
> 8 CID (%)	0	0	0	0
sunn[b]	5.15 ± 1.43 (N=26)	6.96 ± 1.96 (N=32)	8.5 ± 2.8 (N=20)	8.8 ± 2.6 (N=9)
< 4 CID (%)	11.5	0	5	0
≥ 4, ≤ 8 CID (%)	88.5	84.4	45	44.4
> 8 CID (%)	0	15.6	50	55.6

Table entries in the "WT" and "*sunn*" lines are mean CID spots per oocyte with standard deviations. N indicates the total number of oocytes counted in the indicated region of the ovariole. [a]*Df/ TM3*. [b]$sunn^{Z3\text{-}5839}$*/Df*

mapped to the CG32088 locus in region 68D3 of chromosome arm 3L. We note that a gene named *mei(3)M20* that exhibited mutant phenotypes similar to those of *sunn* was previously reported and mapped to the 68C8-11;69A4-5 interval (HIRAI *et al.* 2004). Complementation analysis will be required to determine whether *sunn* and *mei(3)M20* are allelic. Genomic DNA sequencing of CG32088 exons from the three alleles of *sunn* revealed single mutations in each line: a nonsense mutation predicted to truncate the protein 132 amino acids from the C-terminus (Z3-1956); a mis-sense mutation predicted to substitute arginine for a conserved glycine (G170) (Z3-4085); and a 8 bp deletion (Z3-5839) which creates a frameshift that leads to a predicted in-frame stop codon near the middle of the coding sequence (Figure 2-7). As no full-length cDNAs for *sunn* were available, a cDNA was obtained by reverse transcription and PCR from ovary RNA using primers designed on the basis of genomic sequence. The resulting *sunn* cDNA consists of 10 exons and contains a predicted coding sequence of 2856 bp corresponding to a protein 952 amino acids in length (Figure 2-7). Rapid –Amplification-of-cDNA-Ends (RACE) revealed short 5' and 3' UTRs, 69 bp and 75 bp in length, respectively, that contained no potential alternative start or stop codons.

To verify that CG32088 corresponds to *sunn*, we cloned the full-length cDNA-derived coding sequence of *sunn* (without its 5' and 3' UTRs) into a UAS (Upstream Activator Sequence) vector in frame with C-terminal Venus, (enhanced yellow fluorescent protein (eYFP). Transgenic insertions of UAS-SUNN::Venus were generated and found to complement the meiotic NDJ phenotypes of *sunn* mutants in both sexes when expressed under control of the germline specific driver *nos-GAL4::VP16* (Tables S2 and S3). In addition to verifying the identity of *sunn* and CG32088, these results

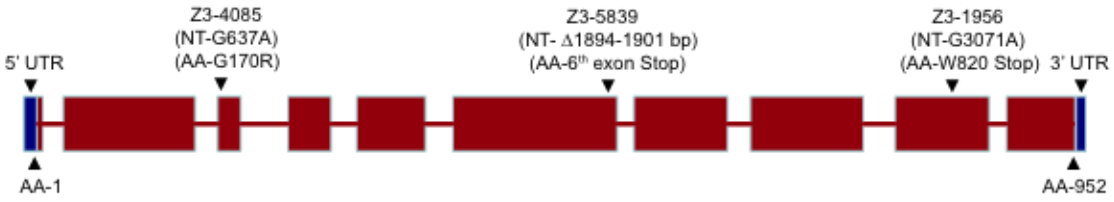


Figure 2-7: Structure of *sunn* gene. Gene structure of *sunn* and location and identity of *sunn* mutations. Sequencing of ovary cDNAs showed that there are 10 exons in *sunn*. The red boxes above show the predicted coding sequence of *sunn* and the blue boxes show the UTRs. The length of the predicted coding sequence of *sunn* is 2856 bp and the lengths of the 5' and 3'UTRs are 69 bp and 75 bp respectively. The locations and identities of the sequenced *sunn* mutations discussed in the text are shown above. NT denotes genomic nucleotide position (introns included) from the start of the translation unit (position 1 is the first nucleotide of the predicted initiator AUG) and AA depicts the respective predicted amino acid numbers.

show that SUNN::Venus functions similarly to wild-type SUNN protein and that the *sunn* cDNA sequence used for the construct likely represents the true *sunn* coding region.

SUNN exhibits structural similarity to the cohesin protein SA. To identify homologs of SUNN, the complete amino acid sequence of SUNN was used to search the protein sequence database using the BLASTp tool offered by FlyBase (http://flybase.org/blast/). We found single SUNN homologs in all of the sequenced species of *Drosophila.* No homologs of SUNN were found in other eukaryotes and no conserved domains were identified by searching a Conserved Domain database at NCBI. However, structure-based searches, described in detail in the Supplementary Information, proved more informative. In particular, the fold-recognition/threading programs MUSTER and I-TASSER revealed statistically significant similarity of SUNN to multiple templates, all of which belong to the HEAT repeat protein family (Table 2-8 and 2-9) (WU and ZHANG 2008; ROY *et al.* 2010). HEAT repeats are conserved domains that form all-α superhelices and are involved in protein-protein interactions. They are particularly abundant in chromosomal proteins involved in cohesion and condensation, including the cohesin cofactors Nipped-B and Pds5 and the condensin subunits Cap-G and Cap-D2. The cohesin subunit Stromalin (SA) also exhibits weak similarity to HEAT repeats (NEUWALD and HIRANO 2000; ANDRADE *et al.* 2001; NASMYTH and HAERING 2009; HIRANO 2012). Detailed comparisons of the MUSTER and I-TASSER analyses revealed a stronger similarity of SUNN to SA than to the other *Drosophila* chromosomal HEAT repeat proteins.

Table 2-8: UASp-SUNN::Venus rescues the *sunn* mutant NDJ phenotype in *Drosophila* males

Male Genotype	X	Y	XY	O	n[a]	%NDJ[b]
UASp-SUNN::Venus; $\underline{sunn^{Z3\text{-}5839}}$ / *Df* [c], *nos-GAL4::VP16*	718	565	0	6	**1289**	0.46
$\underline{sunn^{Z3\text{-}5839}}$ / *Df*, *nos-GAL4::VP16*	137	127	24	265	**553**	52.2
$\underline{sunn^{Z3\text{-}5839}}$ or *Df*, *nos-GAL4::VP16* / +	586	447	1	4	**1038**	0.48

w/B^{S}*Yy*$^{+}$ males with the indicated second and third chromosome genotypes were each crossed to 2 *y w* females. *nos-GAL4::VP16* drives expression of the UASp construct in germline cells. [a]total number of progeny scored. [b]%NDJ = 100 x (XY+ O)/n. [c]*Df (3L) ED4470.* Note: Although this cross does not specifically assay for sister chromatid NDJ, the O sperm class results from both homolog and sister chromatid NDJ. Thus, the near absence of progeny from O sperm in line 1 indicates that the UASp-SUNN::Venus transgene suppresses sister chromatid as well as homolog NDJ. In addition, a cross of the transgene males to *C(1)RM* females yielded no progeny from XX sperm (n=294), the class of sperm diagnostic of sister chromatid NDJ (data not shown).

Table 2-9: UASp-SUNN::Venus rescues *sunn* mutant NDJ phenotype in *Drosophila* females

Female Genotype	X	XX	O	n[a]	[b]%NDJ
UASp-SUNN::Venus ; $\underline{sunn^{Z3\text{-}5839}}$ / *Df*, *nos-GAL4::VP16*	593	10	5	**608**	4.9
$\underline{sunn^{Z3\text{-}5839}}$ / *Df*, *nos-GAL4::VP16*	12	10	4	**26**	70
$\underline{sunn^{Z3\text{-}5839}}$ or $\underline{Df, nos\text{-}GAL4::VP16}$ / +	269	0	0	**269**	0

w/+ females with the indicated second and third chromosome genotype were each crossed to 2 *YSX.YL, In(1)EN, y B* males. [a]total number of progeny scored. [b]%NDJ = 100 x 2 (XX + O)/ (n + XX + O)).

In the MUSTER analysis five of the top six matches for SA were also among the top six matches for SUNN (Table 2-10). Similarly, eight of the top ten matches for SUNN and SA in the I-TASSER analysis overlapped, and six of these common templates overlapped with the top common templates hit by SUNN and SA in MUSTER (Table 2-11). Although some of the top-matching templates for the other *Drosophila* chromosomal HEAT repeat proteins, Nipped-B, Pds5, Cap-G and Cap-D2, overlapped with the templates matched by SUNN, the overlap was much less extensive than for SA. For example, in the MUSTER analysis, the highest-scoring and 2nd-highest scoring templates for Cap-G, Cap-D2, Nipped-B and Pds5 were not among the 10 best matches for SUNN (Table 2-10). Taken together, these results suggest that SUNN is a distant member of the HEAT-repeat family and exhibits stronger structurally similarity to the cohesin subunit SA than to other *Drosophila* chromosomal HEAT-repeat proteins.

SUNN co-localizes with CID during meiosis: Immunostaining of spermatocytes expressing SUNN::Venus with anti-CID antibody revealed bright Venus foci that co-localized with CID spots (Figs. 2-8A & 2-9). These centromeric SUNN::Venus foci were present at all stages of meiosis through metaphase II but were absent at later stages. The SUNN::Venus foci sometimes showed small extensions, suggesting that SUNN localizes to heterochromatic domains that extend beyond the centromeres. However, no localization of SUNN::Venus to chromosome arms in male germ cells was detected at any stage. SUNN::Venus also co-localized with CID in pre-meiotic eight-cell cysts but these centromeric signals were weaker than in spermatocytes. In addition, SUNN::Venus formed large foci and bright smears outside the DNA in pre-meiotic 8-cell cysts (Figure 2-9A).

Table 2-10: Threading/fold recognition (Z scores) results of hits generated by SUNN, Stromalin (SA) and some *Drosophila* chromosomal HEAT repeat proteins using MUSTER

PDB ID	Protein	SUNN	SA	Pds5	Nipped-B	CapG	CapD2
1wa5C	CSE1P	**8.333**	**8.711**	10.255	-	**10.343**	10.313
3m1iC	Exportin-1	**8.116**	**8.839**	**11.174**	7.733	9.980	**11.181**
1qgkA	Importin-β	**7.995**	**10.060**	**10.933**	7.637	**11.106**	**11.310**
3ea5B	Importin-β1	**7.969**	**9.981**	**11.422**	-	**11.083**	**11.386**
4fgvA	Exportin 1	**7.918**	-	10.350	7.416	9.901	10.350
2x1gF	Importin-13	**7.914**	**9.278**	10.838	-	9.904	10.727
1u6gC	TIP120	-	**8.912**	**12.815**	**7.945**	**11.406**	**12.807**
3icqT	Exportin-T	7.480	8.494	-	-	-	-
2x19B	Importin-13	7.389	8.596	10.140	-	-	10.092
3a6pA	Exportin-5	7.386	-	-	-	-	-
3gjxA	Exportin-1	7.156	-	-	-	9.648	-
1b3uA	PP2A	-	8.258	-	-	-	-
3nowA	UNC45	-	8.221	-	-	-	-
3w3tA	Importin-β3	-	-	**12.645**	**8.222**	**12.213**	**12.645**
1qbkB	Karyopherin-β2	-	-	-	**7.759**	-	-

Table 2-10. Continued

PDB ID	Protein	SUNN	SA	Pds5	Nipped-B	CapG	CapD2
4c0oA	Transportin 3	-	-	**11.585**	**8.295**	**10.977**	**11.596**
1vw1A	TcdA1	-	-	-	**8.405**	-	-
4acqA	Macroglobulin	-	-	-	**7.984**	-	-
4jspB	mTOR	-	-	-	7.580	-	-

Bold Z scores represent the top six PDB templates matched by the proteins tested. Above depicted proteins were derived from the following organisms: 1wa5C: Exportin CSE1P (*Saccharomyces cerevisiae*). 3m1iC: Exportin-1 (*Saccharomyces cerevisiae*). 1qgkA: Importin-β (Human). 3ea5B: Importin-β1 subunit (*Saccharomyces cerevisiae*). 4fgvA: Exportin 1 (*Chaetomium thermophilum*). 2x1gF: Importin-13 (*Drosophila melanogaster*). 1u6gC: Cand1/TIP120 (Human). 3icqT: Exportin-T (*Schizosaccahromyces pombe*). 2x19B: Importin-13 (Human). 3a6pA: Exportin-5 (Human). 3gjxA: Exportin-1 (Mouse). 1b3uA: PP2A (Human). 3nowA: UNC45 (*Drosophila melanogaster*). 3w3tA: Importin subunit β3 (*Saccharomyces cerevisiae*) (Kap121p). 1qbkB: Karyopherin-β2 (Human). 4c0oA: Transportin 3 (Human). 1vw1A: TcdA1 (*Photorhabdus luminescens*). 4acqA: α-2-Macroglobulin (Human). 4jspB: (Human Serine/threonine-protein kinase mTOR).

Table 2-11: Threading/fold recognition results (TM scores) for SA (NP_477268.2) and SUNN (CG32088, NP_729739.3) protein sequences by I-TASSER.

PDB ID	Protein	I-TASSER (TM scores)	
		SA	SUNN
4c0oA	Transportin 3	0.538	0.875
2x19B	**Importin-13**	**0.517**	**0.750**
1wa5C	**Exportin CSE1P**	**0.542**	**0.687**
1qgkA	**Importin-β**	**0.601**	**0.606**
3nbyA	Exportin-1	0.494	0.710
3icqT	**Exportin-T**	**0.519**	**0.663**
2x1gF	**Importin-13**	**0.479**	**0.701**
2bptA*	**Importin-β1**	**0.570**	**0.595**
1w3tA	Importin-β3	0.816	-
4fgvA	Exportin-1	-	0.710
3m1iC	Exportin-1	-	0.697
4fddA	Transportin-1	0.541	-

The top 10 templates hit by SUNN and SA using I-TASSER (ROY *et al.* 2010) are shown ordered by total TM scores. Templates also hit by both SUNN and SA in the MUSTER analysis are highlighted in bold. PDB templates. 1 qgkA: Importin-β (Human). 2x1gF: Importin-13 (*Drosophila melanogaster*). 1wa5C: Exportin CSE1P (*Saccharomyces cerevisiae*). 2x19B: Importin-13 (*Saccharomyces cerevisiae*). 3icqT: Exportin-T

Table 2-11. Continued

(*Schizosaccharomyces pombe*). 4c0oA: Transportin 3 (Human). 2bptA: Importin-β1 subunit (*Saccharomyces cerevisiae*). 4fgvA: Exportin-1 *(Chaetomium thermophilum).* 3nbyA: Exportin-1 (Mouse). 3m1iC: Exportin-1 (*Saccharomyces cerevisiae*). 3w3tA: Importin subunit β3 (*Saccharomyces cerevisiae*) (Kap121p). 4fddA: Transportin-1 (Human). *2bptA represents the same protein chain as 3ea5B in Table 7.

Figure 2-8: Co-localization of SUNN::Venus with CID. (A) SUNN and CID co-localize in male meiosis. Spermatocytes from *sunn$^{Z3-5839}$/Df, nos-GAL4::VP16* males carrying *UASp-SUNN::Venus* were immunostained with anti-CID antibody. SUNN::Venus forms bright spots which co-localize with CID spots. At S1 stage, SUNN::Venus also shows diffuse signals and large foci which do not localize with CID and are present predominantly on the nuclear membrane and outside the nucleus in the cytoplasm. In representative images, SUNN::Venus and CID both form 3-4 CID spots, 8 spots and 4 spots at S1, PM I and M II respectively. SUNN::Venus signals are absent at A II (Anaphase II). (B) Colocalization of SUNN::Venus and CID in female germ cells. Immunostaining was performed using anti-CID antibody on whole-mount ovaries from *UASp-SUNN::Venus; sunn$^{Z3-5839}$/Df, nos-GAL4::VP16* females. SUNN::Venus foci were observed in germ cells in all regions of the germarium including region 1, and they co-localized with CID spots. SUNN::Venus expression was absent from the follicle cells due to germ cell-specific expression directed by the nos-GAL4 driver. Arrow shows enlarged germ cell used in the inset. Scale bars = 5 μM.

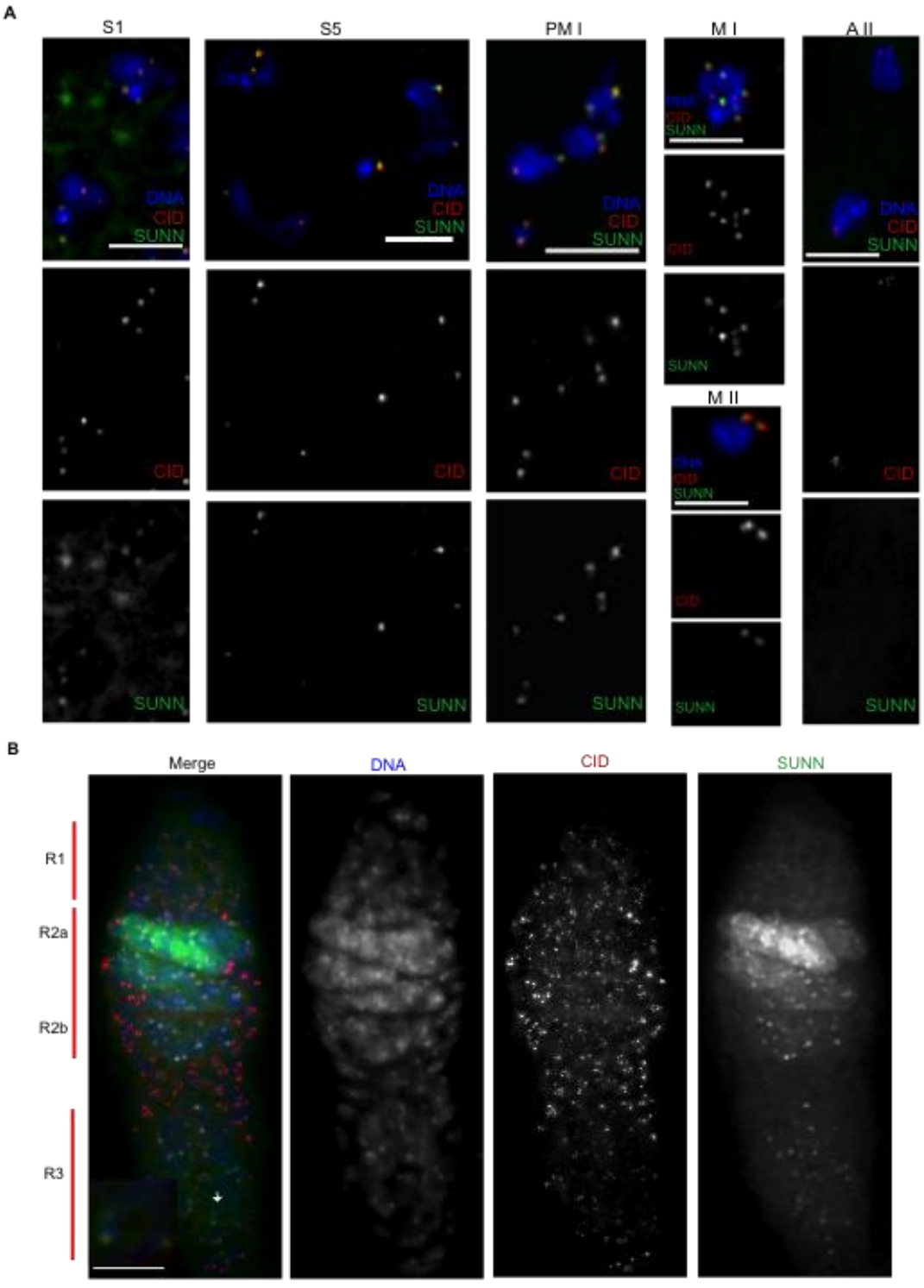


Figure 2-8. Continued

Figure 2-9: SUNN co-localizes with CID in male germ cells. 8 cell spermatogonial (mitotic) cysts (A) and 16-cell stage S1 (meiotic) spermatocyte cysts (B) from w/$B^{s}Yy^{+}$; *UASp-SUNN::Venus*; $sunn^{Z3\text{-}5839}$ */Df, nos-GAL4::VP16* males were stained with anti-CID antibody and DAPI. Co-localizing SUNN::Venus and CID spots are indicated with red and white arrows respectively. The level of SUNN::Venus co-localizing with CID is higher at the sixteen-cell stage when compared to eight-cell stage. SUNN::Venus also localizes to the cytoplasm and between cells in the cyst during two-cell cyst, four-cell cyst stage, (not shown) and eight-cell cyst stages. This localization decreases in 16 cell cysts and is inconspicuous by S2. In pre-16 cell cysts, very large SUNN foci are also observed which do not co-localize with CID and are present on DNA periphery. The functional relevance of this localization is not clear. Scale bars = 10 μM.

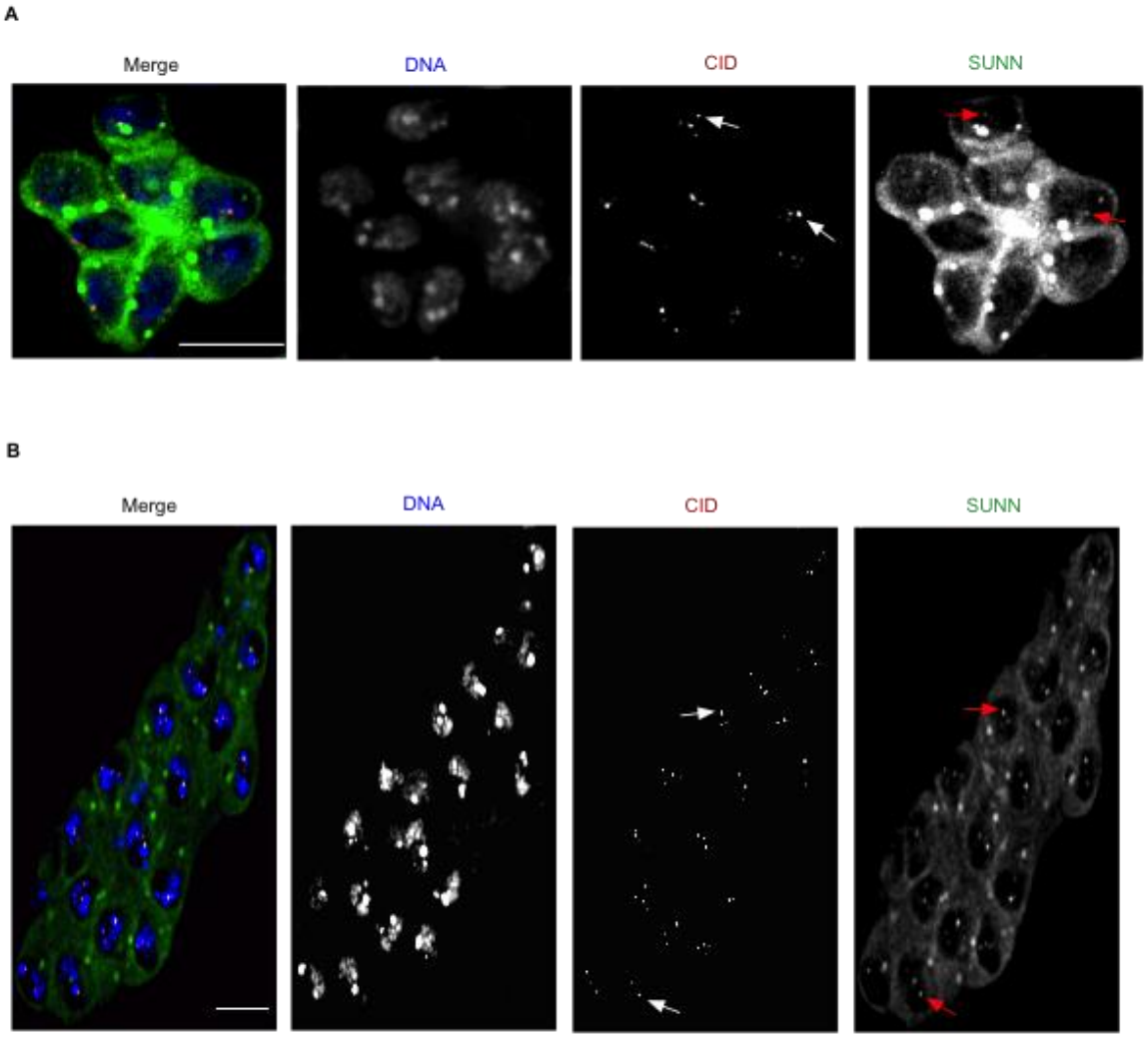


Figure 2-9. Continued

These non-chromosomal staining patterns were still present but greatly attenuated in the earliest 16-cell cysts, and were not seen in later stages (Figs. 2-8 & 2-9B). The significance of this non-chromosomal staining is unknown; the possibility that it is an artifact of ectopic expression cannot be excluded. We conclude that SUNN localizes to centromeric regions prior to the beginning of male meiosis and persists at centromeres through metaphase II but is removed by anaphase II.

To evaluate the localization pattern of SUNN in female meiosis, we immunostained ovaries expressing SUNN::Venus with anti-CID. Discrete SUNN::Venus foci that co-localized with CID foci were observed in the germ cells, but not the somatic follicle cells, in the proximal half of region 1, where 4- and 8-cell pre-meiotic gonial cysts reside, throughout regions 2A, 2B and 3 of the germarium (Figure 2-8B) and in the vitellarium at least up to stage 3 (not shown). In addition to the centromeric foci, SUNN::Venus also exhibited diffuse localization in female germ cells, particularly in regions 2A and 2B (Figure 2-8B). The nature of this non-centromeric localization could not be ascertained from the whole-mount squash preparations and remains under investigation. SUNN's localization to the centromeres in both sexes could explain the phenotypes of NDJ, centromeric cohesion and centromeric clustering defects associated with *sunn* mutants

Mutual co-dependence of SUNN, SOLO and SMC1 centromere foci: The cohesion proteins SMC1, ORD and SOLO also localize to centromeric regions in spermatocytes and SMC1 centromeric foci have been shown to depend on both *ord* and *solo* (BALICKY *et al.* 2002; THOMAS *et al.* 2005; KHETANI and BICKEL 2007; YAN *et al.* 2010). To test for dependence of SMC1 centromeric foci on *sunn*, we expressed

Venus::SMC1 using the nos-GAL4::VP16 driver in both WT and *sunn* mutant backgrounds. Although bright Venus::SMC1 foci were observed throughout WT meiosis, no Venus::SMC1 foci were seen at any stage of meiosis in *sunn* spermatocytes (Figure 2-10A). In WT females, SMC1 forms bright foci at the centromeres and it localizes to the chromosome arms in oocytes/pro-oocytes. However, in *sunn* mutants SMC1 is absent from the centromeres but is still present (although weakly) on the chromosome arms (Figure 2-10B). Thus stable centromere localization of SMC1 requires wild-type function of *sunn*.

To investigate whether SUNN localization depends on *solo*, SUNN::Venus was expressed using the nos-GAL4::VP16 driver in both WT and *solo* mutant backgrounds. Although SUNN::Venus foci that colocalized with CID were readily observed throughout meiosis in WT spermatocytes, no SUNN::Venus foci were observed at any stage of meiosis in *solo* spermatocytes. Pre-meiotic SUNN-Venus foci were also absent in *solo* spermatocytes (Figure 2-10C). We conclude that localization of SUNN to centromere regions requires wild-type *solo* function.

To determine whether SOLO localization requires *sunn* function, we expressed Venus::SOLO in WT and *sunn* mutant backgrounds in male meiosis. Venus::SOLO foci are visible on chromosomes in WT males throughout meiosis, but no Venus::SOLO foci were detected at any stage of meiosis in *sunn* mutants (data not shown). Thus, SOLO and SUNN foci are reciprocally co-dependent. This pattern is consistent with SUNN and SOLO participating in the same cohesion complex.

Figure 2-10: Interactions among SUNN, SMC1 and SOLO. (A) SMC1 foci in spermatocyte nuclei require *sunn* function. Venus::SMC1 expressed under the control of *nos-GAL4::VP16* formed DNA-associated foci in WT but not *sunn* (*sunn*$^{Z3\text{-}5839}$*/Df*) spermatocytes. (B) SMC1 localization to centromeres in pro-oocytes/oocytes requires *sunn*. Whole mount ovaries where immunostained with anti-SMC1 and anti-CID antibodies. In WT, SMC1 forms centromeric foci (white arrows) and localizes to chromosome arms whereas in *sunn* (*sunn*$^{Z3\text{-}5839}$*/Df*), SMC1 is lost from the centromeres (red arrows) observed as gaps in C(3)G staining but is weakly present on chromosome arms. R2b = Region 2b. R3 = Region 3 (C) Centromeric SUNN::Venus foci in spermatocyte nuclei require *solo* function. Spermatocytes expressing SUNN::Venus under control of *nos-GAL4::VP16* were immunostained with anti-CID antibody. DNA-associated SUNN::Venus foci co-localized with CID in WT spermatogonia (8-cell stage) and spermatocytes (S1 and S4 stages) but were absent at the same stages in *solo* (*Df(2L)A267/solo*$^{Z2\text{-}0198}$) spermatogonia and spermatocytes. Scale bars = 5 μM.

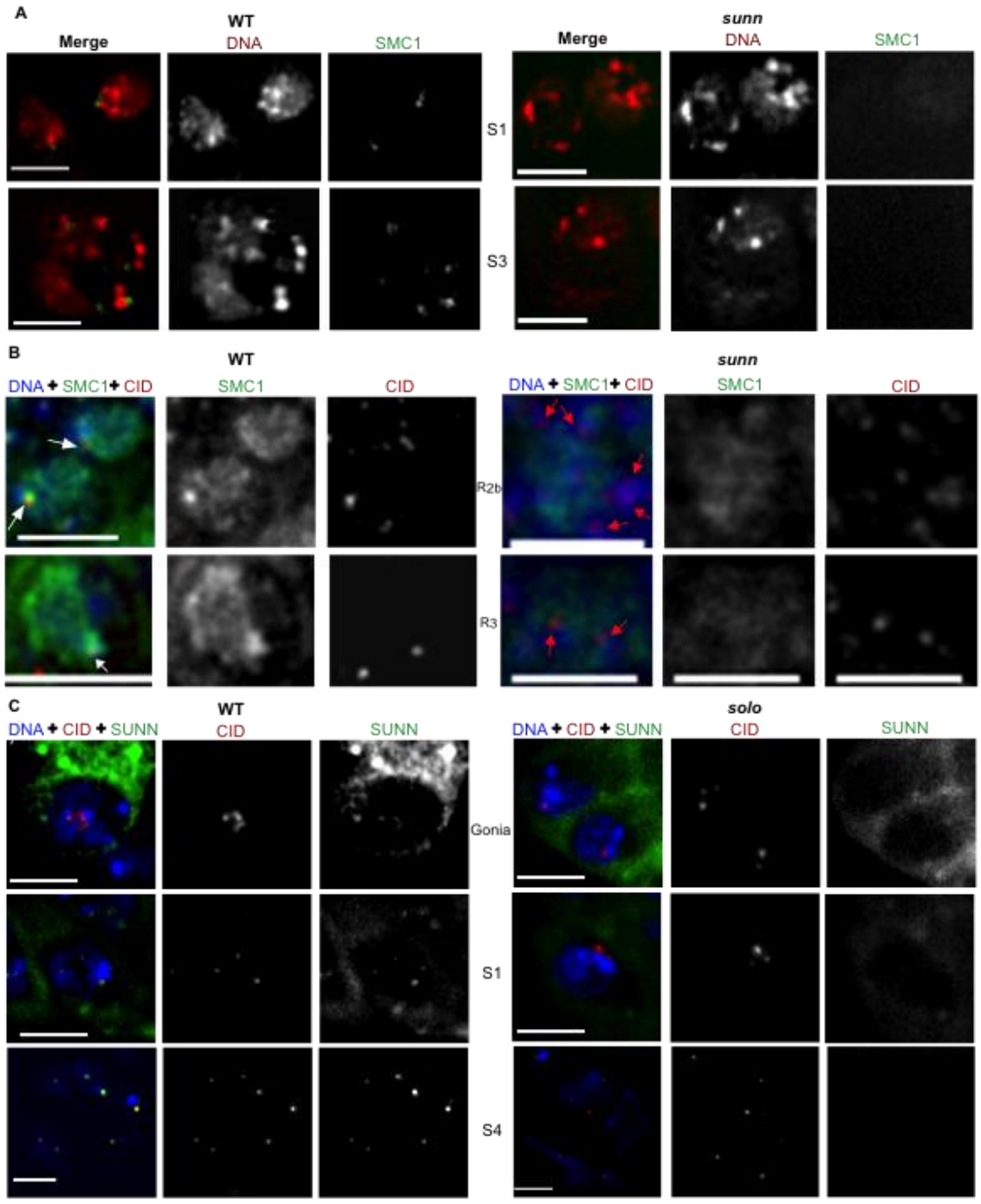


Figure 2-10. Continued

DISCUSSION

SUNN is a *Drosophila*-specific meiotic cohesion protein: Several components and/or regulators of the meiotic cohesion machinery in *Drosophila* have been identified but critical questions about meiotic cohesion remain unanswered. Chief among these are the composition of meiotic cohesin(s) and the role(s) of cohesion factors in pairing, synapsis and recombination. The core cohesin subunits SMC1 and SMC3 are required for SC formation and have been implicated in centromere cohesion (KHETANI and BICKEL 2007; YAN *et al.* 2010; TANNETI *et al.* 2011; YAN and MCKEE 2013). The non-SMC components of meiotic cohesin remain uncharacterized. Meiotic roles of the mitotic non-SMC subunits RAD21 and SA have yet to be demonstrated, and meiosis-specific paralogous substitutes, such as the highly conserved SCC1/RAD21 paralog REC8, have not been found.

Heretofore, the best-characterized meiotic cohesion factors are two meiosis-specific proteins, ORD and SOLO, not found outside of the genus *Drosophila* but required for all aspects of meiotic cohesion. This report adds a third protein, SUNN, to this group of *Drosophila*-specific meiotic cohesion factors. Like fluorescently tagged versions of ORD and SOLO, SUNN::Venus localizes to centromeres of pre-meiotic gonial chromosomes (most clearly in 8-cell cysts) and meiotic chromosomes in both sexes. The disappearance of SUNN from spermatocyte centromeres at anaphase II is similar to timing of ORD and SOLO removal and coincident with the disappearance of SMC1. Like *solo* and *ord,* mutations in *sunn* abolish SMC1 centromere foci and disrupt centromere cohesion during prophase I, well in advance of prometaphase I when sister centromeres would normally mono-orient. The result is high frequencies of meiosis I

and meiosis II NDJ in both sexes, as previously described for *ord* and *solo* mutants (MIYAZAKI and ORR-WEAVER 1992; BICKEL *et al.* 1997; YAN *et al.* 2010; YAN and MCKEE 2013). The similarities among the phenotypes and localization patterns are striking and suggest that ORD, SOLO and SUNN play closely related roles in meiotic cohesion and cohesion-related processes.

SUNN is required for centromere clustering and pairing: Both homologous and non-homologous centromere pairing have been described during meiosis in yeast, plants and *Drosophila* (STEWART and DAWSON 2008). In *Drosophila* female meiosis, centromeres typically aggregate into one to three clusters throughout prophase I from zygotene until NEBD. This phenomenon is meiosis-specific since it is not observed in pre-meiotic gonia or in nurse cells. Centromere clustering requires the SC proteins C(3)G and CONA, the cohesion proteins ORD and SOLO (KHETANI and BICKEL 2007; TAKEO *et al.* 2011; TANNETI *et al.* 2011; YAN and MCKEE 2013) and the centromere proteins CENP-C and CAL-1 (UNHAVAITHAYA and ORR-WEAVER 2013). Our data show that SUNN is also required for centromere clustering. The frequency of *sunn* oocytes with fewer than 4 CID spots (the hallmark of clustering) was less than 12% in region 2A and 5% or less in all later stages. Moreover, the great majority of oocytes in all stages exhibited more than 4 CID spots, indicating a substantial disruption of homologous centromere pairing as well (Table 6). It remains to be determined whether pairing and clustering of centromeres are somehow consequences of centromere cohesion or whether they reflect separate functions of SUNN.

SUNN is required for centromere cohesion in both male and female meiosis: Sister centromeres normally remain tightly cohesive throughout meiosis until

anaphase II, when the release of centromere cohesion triggers sister chromatid segregation. Sister centromere cohesion underlies not only the proper bipolar orientation of sister kinetochores during meiosis II but also their mono-orientation during meiosis I. Mutations in *ord* and *solo* were previously shown to disrupt centromere cohesion prior to prometaphase I in both sexes (BALICKY *et al.* 2002; BICKEL *et al.* 2002; YAN *et al.* 2010; TAKEO *et al.* 2011; TANNETI *et al.* 2011; YAN and MCKEE 2013). In this paper, we have shown that *sunn* mutations have similar effects. In spermatocytes, FISH analysis revealed substantial loss of cohesion at the pericentromeric X chromosome 359bp repeat locus by prometaphase I, consistent with similar observations in *ord* and *solo* mutants (BALICKY *et al.* 2002; YAN *et al.* 2010). CID spot counts showed that although centromere cohesion remains intact throughout stages S1-S3 of prophase I in *sunn* mutants, it begins deteriorating by stage S4 and is largely absent by stage S5-S6. Results of CID spot counts for *solo* mutants were similar except that no cohesion loss was detected until stage S5.

Results of CID spot counts in *sunn* oocytes were similar. No region 2A pro-oocytes with more than 8 CID spots were seen, indicating that sister centromere cohesion remained intact in early pachytene. However, by region 2B, 16% of pro-oocytes exhibited more than 8 CID spots and by region 3 and stage 2, at least half of oocytes did. Again, the progressive loss of cohesion during pachytene broadly parallels results of similar studies in *ord* and *solo* mutants. However, no pro-oocytes/oocytes with more than 8 spots were observed in any region of the germarium in those mutants. Oocytes with more than 8 spots were present in vitellarial stages 5-7 in *ord* and *solo* mutants (TAKEO *et al.* 2011; YAN and MCKEE 2013). Thus cohesion is compromised at

an earlier stage of pachytene in *sunn* oocytes than in *ord* or *solo* oocytes, paralleling the difference in timing of cohesion loss between *sunn* and *solo* spermatocytes.

Three conclusions seem warranted. First, SUNN, SOLO and ORD work together during prophase I to maintain centromere cohesion in both sexes. In the absence of any of the three proteins, centromere cohesion is completely lost by the onset of prometaphase I. In light of the shared phenotype of loss of centromeric SMC1 foci in *ord, solo* and *sunn* mutants, it seems likely that this cohesion pathway is mediated by a cohesin complex, although this inference remains to be verified. Second, since centromere cohesion is intact during early prophase in both spermatocytes and oocytes in all three groups of mutants, there must be at least one additional centromere cohesion mechanism that does not require ORD, SOLO or SUNN. This mechanism may be independent of cohesin as well although the possibility that below-detection levels of SMC cohesins remain at centromeres in the absence of these proteins cannot be excluded. The nature of this alternative mechanism (or mechanisms) remains to be elucidated. Third, since there is an earlier onset of centromeric cohesion loss in *sunn* mutants than in *ord* or *solo* mutants in both male and female meiosis, SUNN may play a minor role in SOLO/ORD-independent early-prophase cohesion. Although we cannot completely exclude the possibility that the earlier onset of cohesion loss in *sunn* mutants resulted from a background genotype effect, we think this explanation is unlikely as the *sunn* and *solo* alleles used in these studies were derived on the same strain background.

SUNN is required for sister centromere mono-orientation: Reductional segregation during meiosis I requires sister centromeres to mono-orient so that

homologous centromeres can reliably bi-orient. Mono-orientation requires that sister centromeres form a functionally single kineotchore by adopting a “side-by-side” configuration instead of a “back-to-back” configuration which is characteristic of meiosis II and mitosis (HAUF and WATANABE 2004; YOKOBAYASHI and WATANABE 2005). How sister centromeres achieve this unique orientation is poorly understood but genetic studies in several model eukaryotes have pinpointed sister centromere cohesion as a necessary prerequisite for mono-orientation. Mutation of cohesin genes including *smc3*, *rec8 and scc3/sa* in budding yeast, fission yeast, *Arabidopsis* and *C. elegans* have been found to disrupt mono-orientation and cause chaotic and/or equational meiosis I segregation (KLEIN *et al.* 1999; WATANABE and NURSE 1999; PASIERBEK *et al.* 2001; WANG *et al.* 2003; CHELYSHEVA *et al.* 2005; GOLUBOVSKAYA *et al.* 2006; SEVERSON *et al.* 2009).

In *Drosophila*, no cohesins have been shown directly to be required for mono-orientation, but the detailed FISH analyses of meiosis I segregation reported previously for *solo* mutants (YAN *et al.* 2010) and herein for *sunn* mutants show that the products of these essential centromere cohesion genes are also essential for mono-orientation, at least in male meiosis. The simplest interpretation is that the mono-orientation defect is a consequence of the cohesion defect, although the possibility that SUNN or SOLO has an independent role in mono-orientation cannot be excluded. The fact that SMC1 centromere foci are absent in *sunn* mutants, as in *ord* and *solo* mutants, is consistent with the idea that mono-orientation in *Drosophila* requires cohesin (again presumably derivative of its role in cohesion), but direct proof of this inference is lacking as yet. The data available so far also do not address the question of whether the known cohesion

factors are sufficient for mono-orientation. It would not be surprising if additional factors were needed since the same proteins mediate cohesion during both meiosis I and II but mono-orientation is restricted to meiosis I. Specific mono-orientation factors have been identified in both *S. cerevisiae* and *S. pombe* but no such factors have been identified as yet in higher eukaryotes (TOTH *et al.* 2000; YOKOBAYASHI and WATANABE 2005).

Is SUNN also required for mono-orientation during meiosis I in female *Drosophila*? Since we did not conduct cytological analysis of meiosis I segregation in females, our data do not provide direct evidence on this point. In principle, the observed combination of homolog and sister chromatid NDJ in the cross experiments could be explained without invoking any mono-orientation defects. The homolog NDJ products could result solely from dyad-dyad NDJ due to a failure of arm cohesion during meiosis I, and the sister chromatid NDJ products could result solely from meiosis II NDJ. However, we think this explanation is unlikely, mainly because of our data showing that centromere cohesion is extensively compromised during prophase I. It is difficult to see how prematurely separated sister centromeres could mono-orient on the meiosis I spindle. Although we cannot rule out the possibility that the dissociated sister centromeres somehow reassociate by prometaphase I, no such reassociation was seen in a FISH-based analysis of X chromatid segregation in *ord* females, which exhibit very similar centromere cohesion loss and chromatid mis-segregation phenotypes as *sunn* females. Instead, X chromatids often appeared fully separate after NEBD and segregated chaotically (BICKEL *et al.* 2002). Thus, we suggest that meiosis I mis-segregation in both sexes in *sunn* mutants is likely due mainly to the premature loss of centromere cohesion and the resulting failure of sister centromeres to mono-orient.

Balanced versus unbalanced segregations in the absence of cohesion:

Our FISH analyses showed that in more than 90% of sex chromosome bivalents in both *solo* (YAN *et al.* 2010) and *sunn* (this manuscript, Fig. 2 & Table 5) males, the chromatids segregate in numerical balance (two towards each pole) at anaphase I even though the cross data indicate very high rates of meiosis I NDJ. Our FISH data show that the explanation for the high meiosis I NDJ is random sister chromatid partner choice, which results in a 1:2 ratio of reductional to equational sister chromatid segregation. This "random 2x2" segregation pattern requires the homolog conjunction complex since *snm* mutations in a *solo* mutant background result in completely random segregation (Yan et al 2010). Might the random 2x2 mechanism also apply to meiosis I segregation in *sunn* females? Unfortunately, cross data are not informative on this point because the predicted ratio of sister versus homolog NDJ products among XX eggs is identical (1:2) whether segregation is completely random or random 2x2, and the unbalanced segregation products (XXX and XXXX) which are critical to distinguishing which mechanism is operative cannot be recovered. Nevertheless, we favor the fully random model in females for two reasons. First, the homolog conjunction complex that is essential for the random 2x2 mechanism in male meiosis is absent in female meiosis (THOMAS *et al.* 2005). Although females also have a robust achiasmate segregation mechanism (HAWLEY *et al.* 1992), it bears little mechanistic resemblance to that in males. Second, the FISH analysis of meiotic segregation of X chromatids in *ord* females mentioned above found no indication of orderly segregation (BICKEL *et al.* 2002). It seems likely, then, that the random 2x2 mechanism is male-specific and that the uncohesive chromatids in *sunn* females segregate fully randomly during both

meiotic divisions. It will be important to test this prediction experimentally. The mechanism underlying random 2x2 segregation in male meiosis also remains to be investigated.

What role does SUNN play in cohesion?: Several of the findings summarized above are consistent with SUNN functioning as a component of a cohesion-providing complex along with SOLO and ORD. One possibility is that all three proteins are subunits, along with SMC1 and SMC3, of a specialized meiotic cohesin complex, perhaps replacing either or both of the mitotic non-SMC subunits RAD21 and SA, neither of which has been shown as yet to have a role in meiosis. This idea is supported by several lines of evidence. First, mutations in *ord, solo* and *sunn* abolish detectable centromeric foci of SMC1 at all stages in both male and female meiosis (KHETANI and BICKEL 2007; YAN *et al.* 2010; TANNETI *et al.* 2011; YAN and MCKEE 2013). Second, centromeric foci of SOLO require both *ord* and *sunn* function and centromeric foci of SUNN require *solo* function, suggesting reciprocal co-dependence of the three proteins (YAN *et al.* 2010). Third, survival of centromeric foci of SMC1, ORD and SOLO beyond metaphase I in male meiosis depends on *mei-S332* (BALICKY 2005; YAN *et al.* 2010), the *Drosophila* Shugoshin homolog. We expect that SUNN centromere foci will prove to be similarly dependent on *mei-S332*, although this remains to be shown. Fourth, SOLO interacts physically with SMC1 in co-immunoprecipation assays from ovarian extracts (YAN and MCKEE 2013) and with both SMC1 and SMC3 in yeast two-hybrid analysis (Q Ma and BD McKee, unpublished data). At minimum, these data indicate very strong interactions of ORD, SOLO and SUNN with each other and with the SMC cohesins and are consistent with roles as cohesin components. This idea does not exclude the

possibility of other meiotic cohesin complexes, perhaps involving the mitotic non-SMC subunits and/or C(2)M. Multiple meiosis-specific cohesin complexes have been demonstrated in several higher eukaryotic systems (NASMYTH and HAERING 2009; SEVERSON *et al.* 2009; LLANO *et al.* 2012).

The bioinformatic analysis of SUNN presented above is of interest in light of these considerations. Although no homologs of SUNN were identified in sequence- or profile-based searches, fold recognition and structural analysis indicated that SUNN may be a homolog of the cohesin protein SA. Although this line of reasoning is inconclusive, it suggests that the shared roles of SA and SUNN in sister chromatid cohesion may have a basis in a shared overall structure and raises the possibility that SUNN might serve as a meiosis-specific substitute for SA in some meiotic cohesin complexes. It will clearly be important to establish the role, if any, of SA in meiotic cohesion. More detailed biochemical and genetic studies of SUNN and its partners will be required to resolve the precise functions of these intriguing proteins in cohesion.

REFERENCES

ANDRADE, M. A., C. PETOSA, S. I. O'DONOGHUE, C. W. MULLER and P. BORK, 2001 Comparison of ARM and HEAT protein repeats. J Mol Biol **309:** 1-18.

BALICKY, E. M., 2005 Regulation of Chromosome Segregation by ORD and dRING During Drosophila Meiosis, pp. 201 in *Biological Sciences*. Dartmouth College, Hanover, New Hampshire.

BALICKY, E. M., M. W. ENDRES, C. LAI and S. E. BICKEL, 2002 Meiotic cohesion requires accumulation of ORD on chromosomes before condensation. Mol Biol Cell **13:** 3890-3900.

BICKEL, S. E., T. L. ORR-WEAVER and E. M. BALICKY, 2002 The sister-chromatid cohesion protein ORD is required for chiasma maintenance in Drosophila oocytes. Curr Biol **12:** 925-929.

BICKEL, S. E., D. W. WYMAN, W. Y. MIYAZAKI, D. P. MOORE and T. L. ORR-WEAVER, 1996 Identification of ORD, a Drosophila protein essential for sister chromatid cohesion. EMBO J **15:** 1451-1459.

BICKEL, S. E., D. W. WYMAN and T. L. ORR-WEAVER, 1997 Mutational analysis of the Drosophila sister-chromatid cohesion protein ORD and its role in the maintenance of centromeric cohesion. Genetics **146:** 1319-1331.

BLOWER, M. D., and G. H. KARPEN, 2001 The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. Nat Cell Biol **3:** 730-739.

BONACCORSI, S. G., M.G.; CENCI, G.; AND GATTI, M., 2000 *Cytological Analysis of Spermatocyte Growth and Male Meiosis in Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

BRAR, G. A., A. HOCHWAGEN, L. S. EE and A. AMON, 2009 The multiple roles of cohesin in meiotic chromosome morphogenesis and pairing. Mol Biol Cell **20:** 1030-1047.

CAI, X., F. DONG, R. E. EDELMANN and C. A. MAKAROFF, 2003 The Arabidopsis SYN1 cohesin protein is required for sister chromatid arm cohesion and homologous chromosome pairing. J Cell Sci **116:** 2999-3007.

CENCI, G., S. BONACCORSI, C. PISANO, F. VERNI and M. GATTI, 1994 Chromatin and microtubule organization during premeiotic, meiotic and early postmeiotic stages of Drosophila melanogaster spermatogenesis. J Cell Sci **107 (Pt 12):** 3521-3534.

CHELYSHEVA, L., S. DIALLO, D. VEZON, G. GENDROT, N. VRIELYNCK *et al.*, 2005 AtREC8 and AtSCC3 are essential to the monopolar orientation of the kinetochores during meiosis. J Cell Sci **118:** 4621-4632.

CLIFT, D., and A. L. MARSTON, 2011 The role of shugoshin in meiotic chromosome segregation. Cytogenet Genome Res **133:** 234-242.

DERNBURG, A. F., J. W. SEDAT and R. S. HAWLEY, 1996 Direct evidence of a role for heterochromatin in meiotic chromosome segregation. Cell **86:** 135-146.

GERTON, J. L., and R. S. HAWLEY, 2005 Homologous chromosome interactions in meiosis: diversity amidst conservation. Nat Rev Genet **6:** 477-487.

GOLUBOVSKAYA, I. N., O. HAMANT, L. TIMOFEJEVA, C. J. WANG, D. BRAUN *et al.*, 2006 Alleles of afd1 dissect REC8 functions during meiotic prophase I. J Cell Sci **119:** 3306-3315.

HASSOLD, T., and P. HUNT, 2001 To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet **2:** 280-291.

HAUF, S., and Y. WATANABE, 2004 Kinetochore orientation in mitosis and meiosis. Cell **119:** 317-327.

HAWLEY, R. S., H. IRICK, A. E. ZITRON, D. A. HADDOX, A. LOHE *et al.*, 1992 There are two mechanisms of achiasmate segregation in Drosophila females, one of which requires heterochromatic homology. Dev Genet **13:** 440-467.

HAWLEY, R. S., K. S. MCKIM and T. ARBEL, 1993 Meiotic segregation in Drosophila melanogaster females: molecules, mechanisms, and myths. Annu Rev Genet **27:** 281-317.

HEIDMANN, D., S. HORN, S. HEIDMANN, A. SCHLEIFFER, K. NASMYTH *et al.*, 2004 The Drosophila meiotic kleisin C(2)M functions before the meiotic divisions. Chromosoma **113:** 177-187.

HIRAI, K., S. TOYOHIRA, T. OHSAKO and M. T. YAMAMOTO, 2004 Isolation and cytogenetic characterization of male meiotic mutants of Drosophila melanogaster. Genetics **166:** 1795-1806.

HIRANO, T., 2012 Condensins: universal organizers of chromosomes with diverse functions. Genes Dev **26:** 1659-1678.

HIROSE, Y., R. SUZUKI, T. OHBA, Y. HINOHARA, H. MATSUHARA *et al.*, 2011 Chiasmata promote monopolar attachment of sister chromatids and their co-segregation toward the proper pole during meiosis I. PLoS Genet **7:** e1001329.

KHETANI, R. S., and S. E. BICKEL, 2007 Regulation of meiotic cohesion and chromosome core morphogenesis during pachytene in Drosophila oocytes. J Cell Sci **120:** 3123-3137.

KLECKNER, N., 2006 Chiasma formation: chromatin/axis interplay and the role(s) of the synaptonemal complex. Chromosoma **115:** 175-194.

KLEIN, F., P. MAHR, M. GALOVA, S. B. BUONOMO, C. MICHAELIS *et al.*, 1999 A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell **98:** 91-103.

KOUNDAKJIAN, E. J., D. M. COWAN, R. W. HARDY and A. H. BECKER, 2004 The Zuker collection: a resource for the analysis of autosomal gene function in Drosophila melanogaster. Genetics **167:** 203-206.

LAKE, C. M., and R. S. HAWLEY, 2012 The molecular control of meiotic chromosomal behavior: events in early meiotic prophase in Drosophila oocytes. Annu Rev Physiol **74:** 425-451.

LEE, J. Y., and T. L. ORR-WEAVER, 2001 The molecular basis of sister-chromatid cohesion. Annu Rev Cell Dev Biol **17:** 753-777.

LLANO, E., Y. HERRAN, I. GARCIA-TUNON, C. GUTIERREZ-CABALLERO, E. DE ALAVA *et al.*, 2012 Meiotic cohesin complexes are essential for the formation of the axial element in mice. J Cell Biol **197:** 877-885.

MANHEIM, E. A., and K. S. MCKIM, 2003 The Synaptonemal complex component C(2)M regulates meiotic crossing over in Drosophila. Curr Biol **13:** 276-285.

MASON, J. M., 1976 Orientation disruptor (ord): a recombination-defective and disjunction-defective meiotic mutant in Drosophila melanogaster. Genetics **84:** 545-572.

MATTHIES, H. J. G. C., M; SAINT, R.B; NAMBA, R; HAWLEY, R.S, 2000 Analysis of Meiosis of Fixed and Live Oocytes by Light Microscopy, pp. 67-85 in *Drosophila Protocols*, edited by W. A. SULLIVAN, M; HAWLEY, R.S. Cold Spring Harbor Laboratory Press, New York.

McKee, B. D., 2004 Homologous pairing and chromosome dynamics in meiosis and mitosis. Biochim Biophys Acta **1677:** 165-180.
McKim, K. S., J. K. Jang and E. A. Manheim, 2002 Meiotic recombination and chromosome segregation in Drosophila females. Annu Rev Genet **36:** 205-232.
Miller, M. P., A. Amon and E. Unal, 2013 Meiosis I: when chromosomes undergo extreme makeover. Curr Opin Cell Biol **25:** 687-696.
Miyazaki, W. Y., and T. L. Orr-Weaver, 1992 Sister-chromatid misbehavior in Drosophila ord mutants. Genetics **132:** 1047-1061.
Nasmyth, K., 2001 Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. Annu Rev Genet **35:** 673-745.
Nasmyth, K., and C. H. Haering, 2009 Cohesin: its roles and mechanisms. Annu Rev Genet **43:** 525-558.
Neuwald, A. F., and T. Hirano, 2000 HEAT repeats associated with condensins, cohesins, and other complexes involved in chromosome-related functions. Genome Res **10:** 1445-1452.
Page, S. L., and R. S. Hawley, 2003 Chromosome choreography: the meiotic ballet. Science **301:** 785-789.
Page, S. L., and R. S. Hawley, 2004 The genetics and molecular biology of the synaptonemal complex. Annu Rev Cell Dev Biol **20:** 525-558.
Pasierbek, P., M. Jantsch, M. Melcher, A. Schleiffer, D. Schweizer *et al.*, 2001 A Caenorhabditis elegans cohesion protein with functions in meiotic chromosome pairing and disjunction. Genes Dev **15:** 1349-1360.
Petronczki, M., M. F. Siomos and K. Nasmyth, 2003 Un menage a quatre: the molecular biology of chromosome segregation in meiosis. Cell **112:** 423-440.
Retelska, D., C. Iseli, P. Bucher, C. V. Jongeneel and F. Naef, 2006 Similarities and differences of polyadenylation signals in human and fly. BMC Genomics **7:** 176.
Roy, A., A. Kucukural and Y. Zhang, 2010 I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc **5:** 725-738.
Severson, A. F., L. Ling, V. van Zuylen and B. J. Meyer, 2009 The axial element protein HTP-3 promotes cohesin loading and meiotic axis assembly in C. elegans to implement the meiotic program of chromosome segregation. Genes Dev **23:** 1763-1778.
Stewart, M. N., and D. S. Dawson, 2008 Changing partners: moving from non-homologous to homologous centromere pairing in meiosis. Trends Genet **24:** 564-573.
Takeo, S., C. M. Lake, E. Morais-de-Sa, C. E. Sunkel and R. S. Hawley, 2011 Synaptonemal complex-dependent centromeric clustering and the initiation of synapsis in Drosophila oocytes. Curr Biol **21:** 1845-1851.
Tanneti, N. S., K. Landy, E. F. Joyce and K. S. McKim, 2011 A pathway for synapsis initiation during zygotene in Drosophila oocytes. Curr Biol **21:** 1852-1857.
Thomas, S. E., M. Soltani-Bejnood, P. Roth, R. Dorn, J. M. Logsdon, Jr. *et al.*, 2005 Identification of two proteins required for conjunction and regular segregation of achiasmate homologs in Drosophila male meiosis. Cell **123:** 555-568.
Toth, A., K. P. Rabitsch, M. Galova, A. Schleiffer, S. B. Buonomo *et al.*, 2000 Functional genomics identifies monopolin: a kinetochore protein required for segregation of homologs during meiosis i. Cell **103:** 1155-1168.

TROUNSON, A., and R. G. GOSDEN, 2003 *Biology and pathology of the oocyte : its role in fertility and reproductive medicine*. Cambridge University Press, Cambridge, UK ; New York.
UNHAVAITHAYA, Y., and T. L. ORR-WEAVER, 2013 Centromere proteins CENP-C and CAL1 functionally interact in meiosis for centromere clustering, pairing, and chromosome segregation. Proc Natl Acad Sci U S A **110:** 19878-19883.
VAZQUEZ, J., A. S. BELMONT and J. W. SEDAT, 2002 The dynamics of homologous chromosome pairing during male Drosophila meiosis. Curr Biol **12:** 1473-1483.
VON STETINA, J. R., and T. L. ORR-WEAVER, 2011 Developmental control of oocyte maturation and egg activation in metazoan models. Cold Spring Harb Perspect Biol **3:** a005553.
WAKIMOTO, B. T., D. L. LINDSLEY and C. HERRERA, 2004 Toward a comprehensive genetic analysis of male fertility in Drosophila melanogaster. Genetics **167:** 207-216.
WANG, F., J. YODER, I. ANTOSHECHKIN and M. HAN, 2003 Caenorhabditis elegans EVL-14/PDS-5 and SCC-3 are essential for sister chromatid cohesion in meiosis and mitosis. Mol Cell Biol **23:** 7698-7707.
WATANABE, Y., 2004 Modifying sister chromatid cohesion for meiosis. J Cell Sci **117:** 4017-4023.
WATANABE, Y., 2005 Shugoshin: guardian spirit at the centromere. Curr Opin Cell Biol **17:** 590-595.
WATANABE, Y., and P. NURSE, 1999 Cohesin Rec8 is required for reductional chromosome segregation at meiosis. Nature **400:** 461-464.
WEBBER, H. A., L. HOWARD and S. E. BICKEL, 2004 The cohesion protein ORD is required for homologue bias during meiotic recombination. J Cell Biol **164:** 819-829.
WU, S., and Y. ZHANG, 2008 MUSTER: Improving protein sequence profile-profile alignments by using multiple sources of structure information. Proteins **72:** 547-556.
YAN, R., and B. D. MCKEE, 2013 The Cohesion Protein SOLO Associates with SMC1 and Is Required for Synapsis, Recombination, Homolog Bias and Cohesion and Pairing of Centromeres in Drosophila Meiosis. PLoS Genet **9:** e1003637.
YAN, R., S. E. THOMAS, J. H. TSAI, Y. YAMADA and B. D. MCKEE, 2010 SOLO: a meiotic protein required for centromere cohesion, coorientation, and SMC1 localization in Drosophila melanogaster. J Cell Biol **188:** 335-349.
YOKOBAYASHI, S., and Y. WATANABE, 2005 The kinetochore protein Moa1 enables cohesion-mediated monopolar attachment at meiosis I. Cell **123:** 803-817.
ZICKLER, D., and N. KLECKNER, 1998 The leptotene-zygotene transition of meiosis. Annu Rev Genet **32:** 619-697.

CHAPTER III: SISTERS UNBOUND IS REQUIRED FOR RECOMBINATION, SYNAPSIS, HOMOLOG BIAS AND DOUBLE STRAND BREAK REPAIR IN DROSOPHILA FEMALES

This part is to be submitted for publication. Badri Krishnan did all experiments, wrote the manuscript draft, made all figures and tables.

ABSTRACT

Sister chromatid cohesion is essential for proper chromosome segregation during meiosis. Cohesion is mediated by a multi-protein complex called cohesin which entraps sister chromatids together until the end of meiosis. *sisters unbound* (*sunn*) encodes a *Drosophila*-specific cohesion protein, which is structurally similar to cohesin complex component Stromalin (SA), but lacks sequence homology to any known cohesins. In this article we report that *sunn* mutants are defective in homologous recombination during *Drosophila* female meiosis. *sunn* mutants have unstable SC which disintegrates prematurely by mid-pachytene. *sunn* mutants also show increased levels of sister chromatid exchange (SCE) and exhibit delay in repair of double strand breaks during meiosis.

Drosophila cohesion genes *ord* and *solo* are also associated with reduced recombination, increased SCE levels and unstable SC. SUNN localizes to the centromeres and we now report that like ORD, SOLO and SMC1, it localizes to chromosome arms in nurse cells and in oocytes where it co-localizes with C(3)G. Previously, we had shown that localization of SMC1 to centromeres and its stable association to chromosome arms in oocytes requires *sunn* function. We conclude that SUNN plays an essential role in recombination and for forming proper chromosome core during meiosis. Based on strong similarities of function and localization of ORD, SMC1, SOLO and SUNN we suggest that they are part of the same complex that in addition to centromeric cohesion plays these other essential roles during female meiosis.

INTRODUCTION

Haploid gametes are required for sexual reproduction and a specialized cell division process called meiosis generates them. Meiosis is divided into two stages- meiosis I and meiosis II and chromosome segregation occurs at sub-stages anaphase I and anaphase II respectively. Prior to meiosis the diploid precursor cell undergoes DNA replication at S phase but once a diploid cell enters meiosis no DNA replication occurs. For proper chromosome segregation to occur during meiosis it is essential that sister chromatids of a chromosome are joined together by the cohesin complex. Meiotic cohesin complex consists of two SMC subunits (Structural Maintenance of Chromosomes)-SMC1 and SMC3 and two non-SMC subunits- REC 8 and Stromalin (SA) (SCC3 in *S. cerevisiae*) (LEE and ORR-WEAVER 2001; NASMYTH and HAERING 2009). In *Drosophila,* in addition to the known cohesin subunits, a group of three proteins ORD, SOLO and SUNN (SOS) exist, which do not share sequence homology to any cohesins but are required for sister chromatid cohesion. It is hypothesized that ORD and SOLO are meiotic substitutes of REC8 and SUNN is a meiotic substitute of SA. (KHETANI and BICKEL 2007; YAN and MCKEE 2013; KRISHNAN *et al.* 2014).

At anaphase I, homologs segregate towards opposite poles, this is called reductional segregation and at anaphase II sister chromatids segregate towards opposite poles, which is called equational segregation. Two chromosome segregation events without an intervening DNA replication round leads to halving of chromosome number and each gamete gets only one complete homolog chromosome set. For this segregation pattern to occur, two conditions are essential: 1) It is essential that proper orientation patterns are established at the sister kinetochores/centromeres. During

meiosis I, sister centromeres are mono-oriented towards the same pole (they are attached to the microtubules emanating from the same pole) which ensures that both sister chromatids are pulled towards the same pole at anaphase I. In meiosis II, sister centromeres are oriented towards opposite poles and at anaphase II they segregate towards opposite poles of the cell (PETRONCZKI *et al.* 2003). Specialized proteins such as Mam1/Monopolin in *S. cerevisiae* is required to ensure that sister kinetochores are oriented towards the same pole at meiosis I (TOTH *et al.* 2000). In *S.* pombe, a special protein Moa1 along with cohesin subunit REC8 are necessary to ensure that sister kinetochores are mono-oriented (YOKOBAYASHI and WATANABE 2005). However, in higher organisms no specialized proteins necessary for mono-orientation has been identified yet, but cohesin components such as REC8 are required for this function. 2) It is also required that homologs are joined by chiasmata, which are produced as a result of crossing over and are formed at DNA exchange sites between homologs. Chiasmata are held together by sister chromatid cohesion distal to the exchange point and without cohesion chiasmata would not form. Chiasmata ensure that appropriate tension is created by microtubules and homologs are pulled towards opposite sides by microtubules attached to sister centromeres (LEE and ORR-WEAVER 2001; MANHEIM and MCKIM 2003; PAGE and HAWLEY 2004; LAKE and HAWLEY 2012). Chiasmata also provides a back-up mechanism to eliminate any bi-oriented sister kinetochores before or during anaphase I. Chiasmata does this by generating strong tension in the right direction and eliminate/eclipse any weak tension generated by incorrectly oriented sister centromeres (bi-oriented) during meiosis I (HIROSE *et al.* 2011).

Faithful chromosome segregation of achiasmate homologs also occurs in *Drosophila* males and females. In *Drosophila* males, which lack meiotic recombination and chiasmata altogether, two proteins, Stromalin in Meiosis (SNM) and Modifier of Mdg4 in Meiosis (MNM), ensure that homologs are held together until anaphase I (THOMAS *et al.* 2005). In the case of 4th chromosome and X chromosome in *Drosophila* females, achiasmate segregation occurs greater than 99% and approximately 5% of time respectively. The mechanism for achiasmate segregation involves pairing at heterochromatic sites (HAWLEY *et al.* 1993).

Removal of cohesins triggers chromosome segregation during meiosis and cohesins are removed at two stages. At anaphase I, arm cohesins are removed by the cleavage of REC8 kleisin subunit by Separase. This leads to resolution of chiasma and segregation of homologs towards opposite poles. Prior to Separase cleavage at anaphase I, REC8 is phosphorylated by CK1 and this makes it susceptible for Separase cleavage. At anaphase I centromeric cohesins are protected by Shugoshins which by ensuring dephosphorylation of centromeric REC8 ensures protection against Separase. At anaphase II, Shugoshins are either removed or inactivated from the centromeres and therefore the centromeric REC8 are cleaved and sister chromatids segregate towards opposite poles (WATANABE 2004; WATANABE 2005; CLIFT and MARSTON 2011).

Apart from playing an essential role in the formation of chiasmata and for establishing correct orientation patterns of kinetochores/centromeres, cohesins are required for proper assembly of SC and axial elements/lateral elements (AE/LE). At the beginning of meiosis (leptotene), AE forms chromosome axis around which sister chromatids are organized and when AE becomes a part of SC it is called LE. SC is a

structure, which is formed at the interface of homologs during prophase I of meiosis and it is required for holding homologs together for recombination/crossing over. Apart from AE/LE along chromosome axis, SC consists of transverse element (TE) and central element (CE), which connects AE/LE of homologs (PAGE and HAWLEY 2004). In *S. cerevisiae, rec8*, *smc3* cohesin mutants show improper assembly of AE component RED1 and SC TE component ZIP1 along chromosome arms (KLEIN *et al.* 1999). In maize, mutation of *Afd1* gene causes unstable association of AE Hop1 with chromosome arms (GOLUBOVSKAYA *et al.* 2006). In *C*. *elegans*, HIM-3 AE/LE protein does not assemble properly in *rec8* depleted cells (PASIERBEK *et al.* 2001). In mouse, AE components SYCP 2, 3 co-localizes with cohesins during meiosis and their assembly along chromosome arms is impaired in cohesin mutants (LLANO *et al.* 2012). In *Drosophila*, assembly of LE component C(2)M is impaired severely and no SC formation occurs when cohesin subunits *smc1* and *smc3* are mutated (TANNETI *et al.* 2011). Genes required for cohesion such as *ord* and *solo* forms unstable SC, which disassembles prematurely in prophase I (WEBBER *et al.* 2004; YAN and MCKEE 2013). In most organisms, cohesin subunits (including ORD and SOLO in *Drosophila*) localize to chromosome arms and co-localize with AE/LE elements and SC components.

An essential process during meiosis is formation and repair of double strand breaks (DSB). Initiation of recombination requires formation DSBs and in *S. cerevisiae* and other organisms it is also required for synapsis. DSBs are formed by a conserved topoisomerase enzyme Spo11 and DSBs are repaired by a process of homologous recombination where the homolog partner is used as template to fill in DNA sequences at the break point. In order to generate chromosomal variation it is necessary that the

homolog partner be chosen as a template for DSB repair. In *S. cerevisiae* a pathway involving AE/LE proteins RED1, HOP1 and MEK1 ensures homolog partner is used for DSB repair (Homolog Bias) and prevents sister chromatid exchange (SCE) (ROEDER 1995; ROEDER 1997). Other proteins such as ORD in *Drosophila*, HIM-3 in *C. elegans* and SYCP3, SYCP2 in mouse are also required for homolog bias and prevent SCE. Co-incidentally all the above proteins are also AE/LE proteins (WEBBER *et al.* 2004; LI *et al.* 2011).

In *Drosophila*, SC initiates at zygotene and DSBs appear after the initiation of SC. In *Drosophila*, mutation of SC components such as *c(3)g* reduces DSB formation but SC LE component *c(2)m* mutation does not affect either the formation or repair of DSBs. Mutants of *mei-W68 (spo11)* and *mei-P22* which abolish DSB formation do not affect SC formation, but they do severely reduce crossing over. In *Drosophila melanogaster*, SC is required for crossing-over/recombination and recombination-defective mutants including *c(3)G, c(2)M, ord* and *solo* form unstable or no SC. In both *ord* and *solo* mutants, SC (as indicated by formation of ribbon-like C(3)G structures) is unstable and disintegrates prematurely. C(2)M, a *Drosophila* α-kleisin and REC8/RAD21 ortholog, is a SC LE component and *c(2)m* mutants never form thread-like structures but show centromeric and patchy localization of C(3)G (MASON 1976; MANHEIM and MCKIM 2003; HEIDMANN *et al.* 2004; WEBBER *et al.* 2004; YAN and MCKEE 2013). This difference in SC phenotype could be because *c(2)m* and *ord* are involved in different pathways of SC initiation and formation. C(2)M is required for SC initiation at a subset of euchromatic sites and for elongation of these accumulations into thread-like structures. ORD is required for centromeric loading of SC components and initiation and

elongation of SC at few euchromatic sites. Together both these pathways ensure formation of complete and stable SC (TANNETI *et al.* 2011).

The phenotypes of SC and recombination do not correlate perfectly. Despite having different appearance of C(3)G in *c(2)m vs solo-ord* mutants they all show similar reduction in homologous recombination. Protein localization experiments have shown that all three proteins form thread like structures which co-localizes with both C(3)G and SMC1. But unlike C(2)M, SOLO and ORD form thread-like structures that co-localize with SMC1 in nurse cells (nurse cells). Also, C(2)M is essential for the formation of proper SC but its mutation does not affect the localization of SMC1 or ORD to chromosome arms. With respect to SMC1, *ord* mutations disrupt SMC1 linear structure on chromosome arms but do not abolish its localization to arms during meiosis. In *solo* and *sunn* mutants diffuse SMC1 staining is observed on chromosome arms (MANHEIM and MCKIM 2003; WEBBER *et al.* 2004; KHETANI and BICKEL 2007; YAN and MCKEE 2013; KRISHNAN *et al.* 2014)

We had identified a novel cohesion gene *sisters unbound* (*sunn*) in *Drosophila*, which is required for centromeric cohesion and clustering in female meiotic prophase I onwards. Its mutation causes high frequency of homolog and sister chromatid NDJ in females. SUNN localizes to centromeres in oocytes and nurse cells at least until stage 3. Bioinformatic analysis showed that SUNN is a SA structural homolog and genetically interacts with both SOLO and SMC1. We hypothesized that SUNN provides cohesion by being a meiotic substitute of SA in meiotic cohesin complex in *Drosophila*. We have characterized additional functions of SUNN which suggests that it is an essential component of chromosome core. *sunn* mutation causes reduction in recombination

frequency and premature disintegration of SC by mid-pachytene. In addition, its mutation causes slight delay in DSB repair and SCE. Like SMC1, ORD and SOLO, SUNN localizes to chromosome arms in both oocytes and nurse cells. We hypothesize based on its shared functions and localization of SUNN, SMC1, SOLO, ORD that they form a complex and function at the chromosome cores.

MATERIALS AND METHODS

Female recombination test: X chromosome recombination test- *Dp(1;1)sc^{v1}, y pn cv m f.y^{+}/y; sunn/Df* females were crossed singly to 2 males of the genotype *YSX.YL, In(1)EN, y B/Y,* which produces attached-XY and Y sperm. Diplo-X and nullo-X non-disjunctional eggs that were produced during female meiosis were identified by the recovery of B^{+} females and y B males respectively. Normal eggs yielded B females and B^{+} males. B^{+} male progeny were used for measuring the recombination frequencies between the X chromosome markers. Recombinants were scored based on the appearance of the X chromosome markers in the B^{+} male progeny. To calculate recombination frequency, recombinant B^{+} male progeny were divided by total number of B^{+} males.

2nd Chromosome recombination test: *+/+; cn bw/+; sunn/Df* females were singly crossed to two males of the genotype *+/y; cn bw/cn bw; +/+.* Eye color was used to identify recombinants and calculate recombination frequency across *cn-bw* interval. *cn-bw* when present in homozygous configuration would cause the eye color to be white. Amongst the progeny, recombinants were either *cn* (cinnabar eye) or *bw* (brown eye) and non-recombinants were *cn bw* (white eye) and +/+ (Normal eye). To calculate recombination frequency across *cn-bw* interval, the number of recombinant progeny recovered were divided by total number of progeny. In this assay, non-disjunctional eggs do not produce viable progeny, therefore all progeny recovered were derived from normal eggs.

Ring X/Rod X chromosome recovery assay: In females a combination of ring X chromosome (*R(1)2 y^{1} w^{1} f^{1})* and a normal rod X chromosome *(y)* was introduced in a hemizygous *sunn* background. *R(1)2 y^{1} w^{1} f^{1}/y; +/+; sunn/Df* females were crossed

singly to two males of the genotype $y^1\ w^1\ f^1/Y;$ +/+; +/+ and the progeny were counted for recovery or ring X chromosome and rod X chromosome. WT sibling control, *R(1)2* $y^1\ w^1\ f^1/y;$ +/+; *Df*/+ were also crossed to $y^1\ w^1\ f^1/Yy^+;$ +/+; +/+ males. Ring X chromosome is a special chromosome, which is joined at its end to form a ring. Ring chromosome has three associated markers $y^1\ w^1\ f^1$ and these were used to track it. We used both y^1 (yellow body) w^1 (white eye) to identify ring chromosome since they are centromere linked and do not undergo frequent recombination. Presence of ring X chromosome in male progeny produces white eyed male (*R(1)2* $y^1\ w^1\ f^1\ /Yy^+$) with normal body color and sometimes forked (f^1) bristles (sometimes f^1 loci undergoes recombination and is lost from the ring chromosome). Presence of ring X chromosome in female progeny produces yellow bodied (y^1), white eyed females (w^1) (with sometimes forked bristles) (*R(1)2* $y^1\ w^1\ f^1\ /y^1\ w^1\ f^1$). $y^1\ w^1\ f^1$ markers were used to track rod chromosome. Rod chromosome containing male progeny have the following genotype -$y^1\ w^1\ f^1\ /Yy^+$ and show normal body, eye color and bristles and rod chromosome containing female progeny would have the following genotype- $y^1\ w^1\ f^1$ /y and show yellow body, normal eye and bristles.

Ovary immunostaining: In order to fatten ovaries for immunostaining, virgin females were placed in a food vial with yeast paste and males. After 2 days, their ovaries were dissected, fixed and stained. To determine the SC phenotype of *sunn* mutants, ovaries were fixed and stained using the Buffer A protocol described in (McKim *et al.* 2009). Rabbit anti-C(3)G (Lily Lab) (1:300 dilution) and mouse anti-ORB (6H4 and 4H8, Developmental Studies Hybridoma Bank (DSHB)) (1:250 dilution) primary antibodies were used. Alexa-Fluor 488 donkey anti-rabbit IgG (H+L, Invitrogen) and

Alexa-Fluor 647nm donkey anti-mouse IgG (H+L, Invitrogen) secondary antibodies were used at 1:1000 dilutions. Slides were mounted using Prolong Gold Slowfade reagent (Invitrogen). For investigating double strand break phenotype in *sunn* mutants, ovaries were dissected, fixed and stained using the protocol described in (PAGE and HAWLEY 2001). Rabbit anti-γH2Av (Rockland) (1:5000 dilution) and mouse anti-ORB (1:250 dilution) primary antibodies were used. Secondary antibodies Alexa-Fluor 488 donkey anti-rabbit IgG (H+L, Invitrogen) and Alexa-Fluor 555 donkey anti-mouse IgG (H+L, Invitrogen) were used at 1:1000 dilutions. Slides were mounted using Prolong Gold Antifade reagent (Invitrogen).

Chromosome Spread and Immunostaining: Flies expressing SUNN::Venus in ovaries were utilized for making chromosome spreads. The expression of SUNN::Venus was induced by nos-GAL4::VP16. Before dissection, virgin females with the above genotype were placed in a food vial with yeast paste and a few males. After 2 days the females were removed, their ovaries were dissected in 1X PBS. Chromosome spreads of *Drosophila* ovaries were prepared according to protocol described in (WEBBER *et al.* 2004). We used rabbit anti-GFP (to stain SUNN::Venus) and mouse anti-C(3)G primary antibodies at 1:1000 dilutions. Alexa Fluor 488 donkey anti-rabbit IgG (H+L, Invitrogen) and Alexa Fluor 555 anti-mouse IgG (H+L, Invitrogen) secondary antibodies were used at 1:1000 dilutions.

Microscopy: Images were obtained using an Axioplan microscope (ZEISS) with a 100x lens, which is equipped with a HBO 100 W mercury lamp**.** This microscope is fitted with a high-resolution charge-coupled device camera (Roper Industries). Metamorph software (Universal Imaging Corporation) was used to acquire pictures,

pseudocolor them and merge them together**.** Z-series pictures were taken, deconvolved and merged/stacked using sum algorithm. To determine SC phenotype of *sunn* mutants, images were obtained using Leica TCS SP8 microscope with a 63x lens. Leica LAS AF Lite software was utilized to acquire Z series images, pseudocolor them and generate maximum projections. All final images and figures were prepared using Adobe Photoshop (CS2), Adobe Illustrator and Microsoft Powerpoint.

RESULTS

***sunn* mutants reduce recombination/crossing over frequency:** We tested *sunn* mutants for recombination across four marker intervals- *pn-cv, cv-m, m-f* and *f-y*$^{+}$ on the X chromosome and across *cn-bw* marker interval on the 2nd chromosome. Recombination frequency for X chromosome markers were tested in females hemizygous for each of the three alleles of *sunn (sunn/Df)* and in WT sibling control (*Df*/+). All three alleles of *sunn* showed severe reduction in recombination frequency across all X chromosome marker intervals. *sunn* alleles showed an average ~ 5.5 fold reduction of recombination frequency across *pn-y*$^{+}$ interval. An average map length of ~13 cM was measured for *sunn* mutants across this interval, which constitutes nearly the entire length of the X chromosome, compared to a map length of ~ 68 cM for WT sibling controls (Table 3-1). Variation in recombination frequency reduction was observed for the four marker intervals, with the greatest reduction (~10 fold reduction) observed in the *m-f* interval and least reduction (~ 3 fold reduction) observed in *cv-m* interval. This variation could be due to the overall small number of recombinants recovered in *sunn* mutants for these intervals. This reduction in recombination frequency across *pn-y*$^{+}$ is similar to what has been observed for some *ord* null alleles (~6.1 fold reduction) and in *solo* alleles which show an average 4.6 fold reduction when compared to its WT sibling controls (MASON 1976; MIYAZAKI and ORR-WEAVER 1992; BICKEL *et al.* 1997; YAN and MCKEE 2013).

We also observed a reduction in recombination frequency across the *cn-bw* interval, which encompasses most of the right arm of the 2nd chromosome. In

Table 3-1: X chromosome recombination frequency in *sunn* mutant females

	Map length of X chromosome intervals (in centi morgans (cM))					
Female Genotype	*pn-cv*	*cv-m*	*m-f*	*f-y*$^{+}$	*pn-y*$^{+}$	n[a]
sunn$^{Z3\text{-}5839}$*/Df*	3.8	3.5	1.2	3.9	12.4	259
sunn$^{Z3\text{-}4085}$*/Df*	3.9	3.9	2.8	1.1	11.7	178
sunn$^{Z3\text{-}1956}$*/Df*	1.8	8.4	2.1	1.0	13.3	191
sunn average	3.2	5.3	2.0	2.0	12.5	628
% Change	17.7	35.6	9.8	13.2	18.3	-
Df[b] *or sunn/+*	18.0	14.9	20.4	15.1	68.4	616

Dp(1;1)sc^{v1}*, y pn cv m f.y*$^{+}$*/y* females with the above third chromosome genotype were crossed with 2 *YSX.YL, In(1)EN, y B/Y males.* The *B*$^{+}$ males were used for scoring recombination and calculating recombination frequencies. [a]n is the total number of *B*$^{+}$male progeny scored. [b]*Df = Df(3L)ED4470.* Recombination events that occurred in the various X chromosome marker intervals were scored and frequencies were calculated.

sunn$^{Z3\text{-}5839}$/*Df* hemizygous females, a map length of 4.2 cM was measured and in WT sibling controls (*Df*/+) ~41 cM was observed (Table 3-2). This reduction in recombination frequency is 10 fold and a similar reduction in recombination frequency is observed across *cn-bw* interval in *solo* alleles (~ 8 fold reduction) and in null *ord* alleles (MASON 1976; YAN and MCKEE 2013). Therefore, we conclude that *sunn* is required for proper sex chromosome and autosomal recombination.

***sunn* is required for homolog bias during recombination:** During recombination double strand breaks are formed on the chromosomes and are repaired by a process which utilizes one of the chromatids of the homolog partner as a repair template. This process leads to exchange of genetic information/DNA between slightly varying homolog partners. However, if the sister chromatid is utilized as a template for double strand break repair then no such genetic information exchange would take place since both sister chromatid strands of chromosome are identical. Therefore, a reduction in recombination frequency could be observed due to a tendency to utilize sister chromatid strand for DSB repair. In order to determine if there is increased sister chromatid exchange in *sunn* mutants, we tested *R(1)2 y*^{1} *w*^{1} *f*^{1} /+; +/+; *sunn*/Df females for the transmission of the ring chromosome (*R(1)2 y*^{1} *w*^{1} *f*^{1}). A single crossover between sister strands of a ring X chromosome would produce a ring shaped dicentric chromosome, which would not be transmitted whereas recombination between sister strands of a normal rod X chromosome would not form a dicentric chromosome and they can be transmitted normally. Therefore, a sister chromatid exchange would cause an increased appearance of progeny which inherit the rod X chromosome when compared to ring X chromosome (Figure 3-1).

Table 3-2: Second chromosome recombination in *sunn* mutants

Female Genotype	Map length (cM) cn bw	n[a]	P/F[b]
sunn$^{Z3\text{-}5839}$*/Df*	4.2 (10.1)	185	7.5
Df or *sunn*$^{Z3\text{-}5839}$/+	41.6	178	59

Recombination frequency was calculated by counting the recombinants obtained in *cn-bw* interval. *cn-bw* markers are present right arm of 2nd chromosome and both are recessive markers. Females of the following genotypes- *+/+; cn bw/+; sunn*$^{Z3\text{-}5839}$*/Df* (for *sunn* mutant), +/+; cn bw/+; Df or *sunn*$^{Z3\text{-}5839}$ (For WT control) were crossed individually to two males of the following genotype: *+/y; cn bw/cn bw; +/+.* Recombination events in parental females would produce recombinant progeny with cinnabar (*cn*) eyes or brown (*bw*) eyes and non-recombinants would produce white eyed (*cn bw/cn bw*) or red eyed progeny (cn + or bw +). [a]Total progeny scores; [b]P/F= Progeny obtained per female

In females hemizygous for *sunn* alleles, an average ring X/rod X recovery ratio of 0.24 was calculated whereas in WT females (Df/+) the ratio was 0.7 (Table 3-3). The low ratio in *sunn* mutants demonstrates that ring X chromosomes undergo sister chromatid exchange and as a result they are not transmitted to the progeny in equal numbers as the rod X chromosome. However, theoretically in WT females a ring X/rod X ratio of 1 should be observed, but we obtain a ratio of 0.7, which we attribute to background sister chromatid exchange activity. This shows that *sunn* is required for homolog bias and in its absence sister chromatid exchange pathway is preferred. This increase in sister chromatid exchange is also observed for cohesion genes *ord* and *solo* mutants, which show a ring X/rod X ratio of 0.2-0.4 and ~0.35 respectively (WEBBER *et al.* 2004; YAN and MCKEE 2013).

***sunn* affects SC stability in oocytes:** In *Drosophila*, SC is essential for homologous recombination and mutants of SC components such as C(3)G and C(2)M cause severe reduction in recombination between homologs (PAGE and HAWLEY 2001; MANHEIM and MCKIM 2003). Since *sunn* mutants exhibit reduced recombination frequency, we examined SC assembly and stability in females hemizygous for two *sunn* alleles by visualizing SC transverse filament (TF) C(3)G form thread-like structures. SC are assembled normally and no structural defects were detected in region 2A cyst in *sunn* mutants. However in both *sunn* alleles region 2A showed ~2.3 complete C(3)G nuclei/cyst which is marginally less than ~3.2 complete C(3)G nulei/cyst observed in WT control (Table 3-4). In region 2B, ~65% oocytes showed fragmented SC in $sunn^{Z3\text{-}5839}/Df$ and ~14% oocytes in $sunn^{Z3\text{-}1956}/Df$. But by region 3 both alleles of *sunn* showed extensive fragmentation and absence of SC.

Figure 3-1: Outcomes of Ring X/Rod X chromosome recovery test. Three possibilities exist when one X homolog is a ring chromosome and the other is a rod X chromosome. If there is no recombination then after meiotic chromosome segregation equal number of ring X and rod X chromosome containing gametes would be produced as they both will be transmitted with equal probability. (A). But if there is recombination between sister chromatids (like in *solo*, *ord* mutants), then it would give rise to gametes containing only rod X chromosomes because recombination between sister chromatids of a ring X chromosome would produce a dicentric chromosome (containing two centromeres) which would not be transmitted (B). If recombination occurs normally between homologs then equal number of ring X and rod X chromosome containing gametes would be produced (C). A dicentric rod chromosome is indeed produced which is not transmitted into gametes.

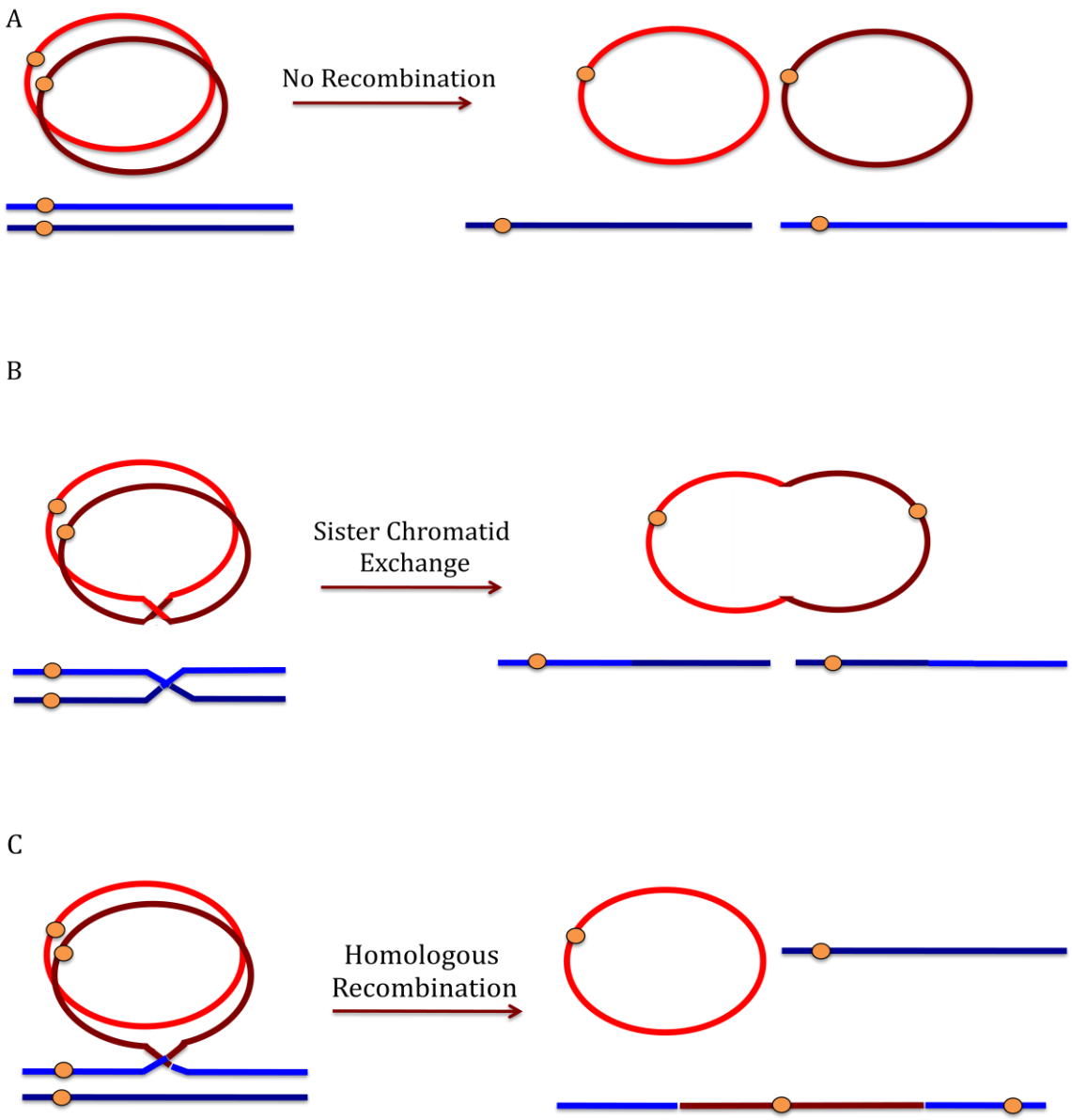


Figure 3-1. Continued

Table 3-3: Ring X chromosome/Rod X chromosome recovery ratio.

Female Genotype	Ring X Progeny	Rod X Progeny	Ring X/Rod X
R(1)2 y^1 w^1 f^1/y^1; +/+; Df/+	495	705	0.70
R(1)2 y^1 w^1 f^1/y^1; +/+; $sunn^{Z3\text{-}4085}$/Df	97	426	0.23
R(1)2 y^1 w^1 f^1/y^1; +/+; $sunn^{Z3\text{-}5839}$/Df	33	130	0.25

R(1)2 y^1 w^1 f^1/y^1 females with the above first, second and third chromosome genotypes were crossed individually to two males of *y w f/ $B^S$$Y$$y^+$* genotype.

Ring X and Rod X chromosome were identified by the presence of associated markers (See materials and methods).

Progeny obtained from disjunctional eggs only were counted to calculate Ring X/Rod X ratio.

100% of oocytes in *sunn*$^{Z3\text{-}5839}$/*Df* and ~83% of oocytes in *sunn*$^{Z3\text{-}1956}$/*Df* showed fragmented or no SC. Similar disintegration of SC is seen in ~ 70-75% oocytes in stage 2 in both alleles (Figure 3-2 and Table 3-4). In contrast and as expected, WT control had normal SC in oocytes in region 2A, 2B and 3 (Figure 3-2 and Table 3-4).

The SC phenotype observed for *sunn* is similar to what is observed in *ord* mutants, where majority of oocytes show SC disintegration by region 3 but it is less severe than *solo* mutants where oocytes show significant SC disintegration as early as region 2A. We think that the disintegration of SC in *sunn* mutants is not on account of delayed development of germarium cysts since we only find a slight decrease in region 2A cysts/germarium (1.7 cysts/germarium) in *sunn* mutants vs wildtype control (2.4 cysts/germarium). In early region 2A cysts of wildtype cysts, we observed C(3)G patches localizing at the centromeres and at a few places on chromosome arms, but no threads are detected at this stage (Figure 3-2D). This C(3)G pattern is exhibited by zygotene stage oocytes when SC formation is initiated. We do not find such zygotene SC pattern in region 2A cysts of *sunn* mutants. We directly observe the appearance of thread like structures in region 2A cysts (Figure 3-2 D). Based on our results we conclude that *sunn* is required for SC stability and this phenotype manifests itself as meiotic prophase progresses in the germaria.

***sunn* mutation leads to a delay in DSB repair in oocytes:** Mutants of *mei-W68* and *mei-P22*, which abolish DSB formation in *Drosophila*, severely reduce homologous recombination. It is necessary for recombination that DSBs are not only formed properly at the appropriate time but are repaired at the appropriate time.

Figure 3-2: SC in *sunn* mutant females: To visualize SC, immunostaining was performed using anti-C(3)G, anti-CID and anti-ORB antibody. CID was used to identify centromeres at zygotene stage (D). ORB was used to identify oocytes in the germarium. (A) [a]WT(*w)* (wildtype control). In WT, we observed normal thread like C(3)G in region 2B, region 3 and stage 2 oocytes. (B) [b]*sunn*$^{Z3\text{-}5839}$*/Df (3L)ED4470* and (C) [c]*sunn*$^{Z3\text{-}1956}$*/Df (3L)ED4470* showed mostly normal C(3)G staining in region 2B but by region 3 fragmented, spotty and sometimes no C(3)G are seen in majority of oocytes. Similar fragmented or no C(3)G is seen in stage 2 oocytes in *sunn*^{b} and *sunn*^{c} whereas normal C(3)G staining in oocytes is observed in WT. See Table 3-4 for quantification. (D) In WT zygotene cysts, we observed C(3)G localization at the centromeres (red arrows) and multiple small patches of C(3)G. We never found such zygotene cysts in *sunn*^{b}, but nuclei which most closely represented late zygotene/or early pachytene stage showed thread like C(3)G structures (white arrow) and no C(3)G localization at centromeres

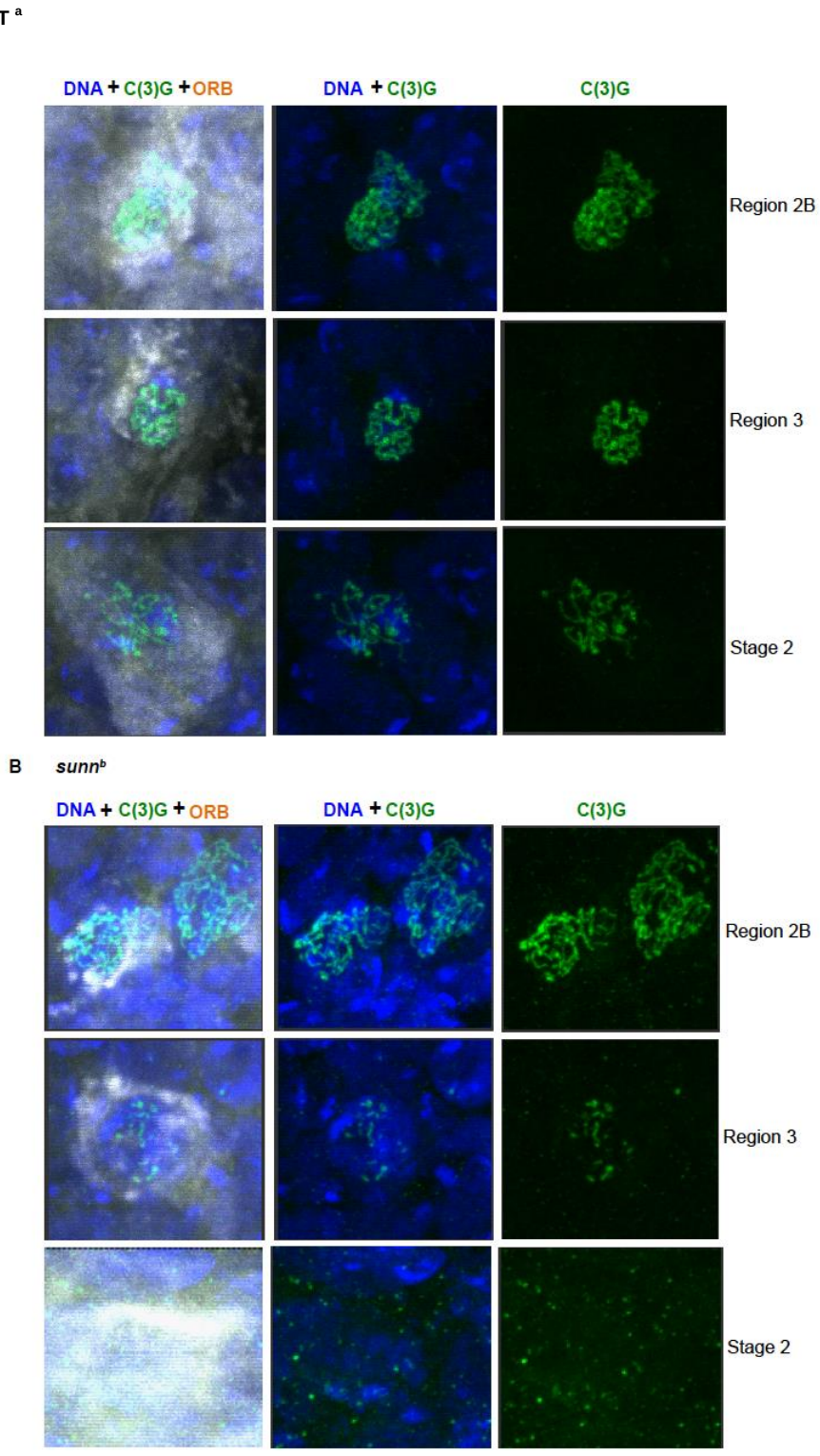


Figure 3-2. Continued

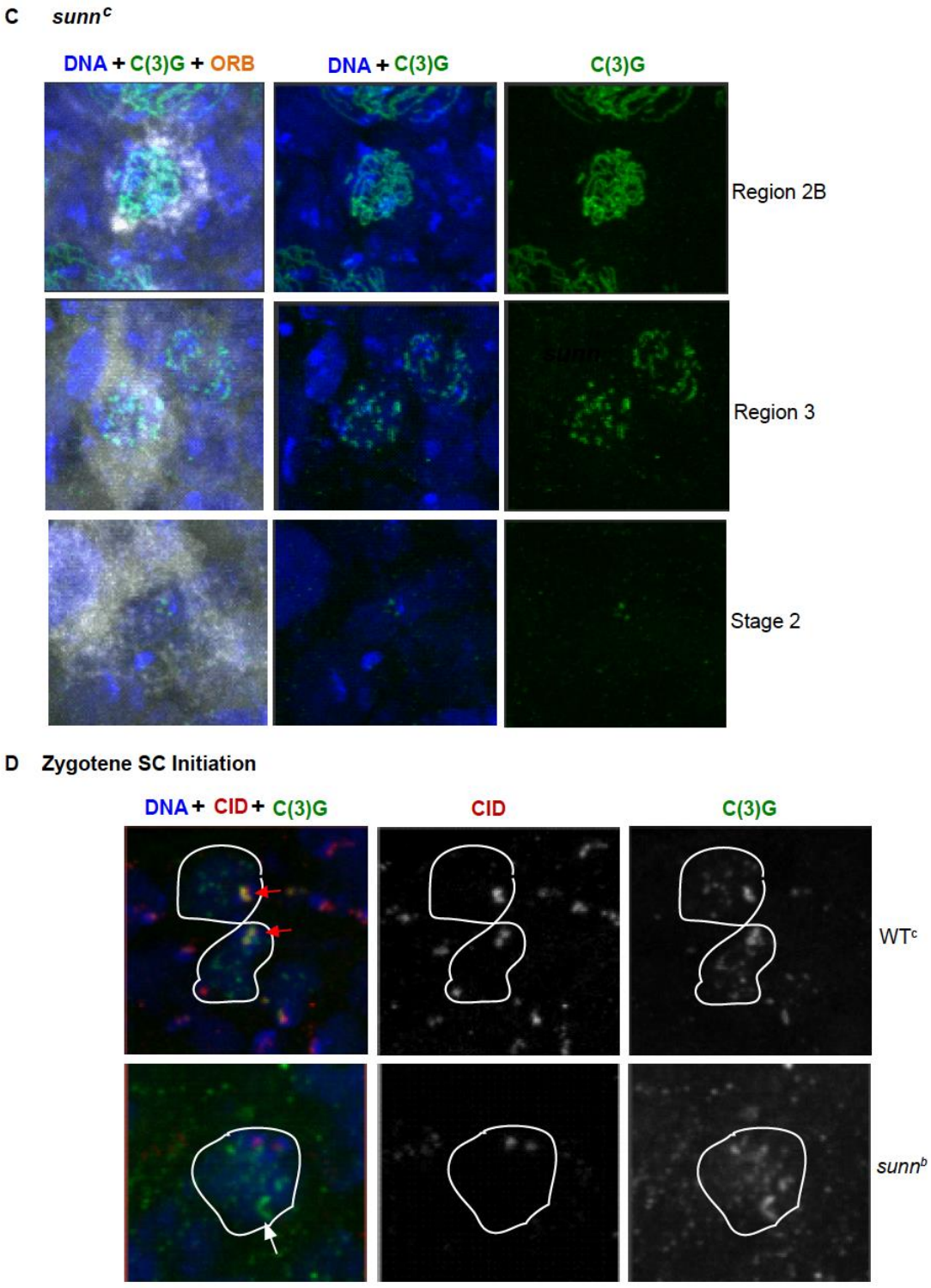


Figure 3-2. Continued

Table 3-4: Quantification of SC phenotype in *sunn* mutants

SC Phenotype	*sunn*[a]	*sunn*[b]	WT[c]
Region 2A (Total)	**25**	**50**	**30**
Complete	22 (88)	50 (100)	30 (100)
Incomplete/Fragmented	3 (12)	0	0
Spotty/None	0	0	0
Region 2B (Total)	**26**	**14**	**9**
Complete	9 (34.6)	12 (85.7)	8 (89)
Incomplete/Fragmented	17 (65.4)	2 (14.3)	1 (11)
Spotty/None	0	0	0
Region 3 (Total)	**17**	**12**	**19**
Complete	0	2 (16.7)	17 (89.5)
Incomplete/Fragmented	8 (47)	10 (83.3)	2 (10.5)
Spotty/None	9 (53)	0	0
Stage 2 (Total)	**13**	**12**	**20**
Complete	3 (23)	4 (33.3)	18 (90)
Incomplete/Fragmented	3 (23)	5 (41.7)	2 (10)
Spotty/None	7 (53.8)	3 (25)	0

Table entries are the numbers and percentages (parentheses) of oocyte/pro-oocytes with complete, fragmented, spotty and no C(3)G staining in various stages of meiotic

Table 3-4. Continued

prophase. Table entries in the Region 2B and Region 3 and Stage 2 lines represent the total nuclei scored for that stage.

Note: there were somewhat fewer pro-oocytes with complete C(3)G staining in region 2 in the *sunn* mutants relative to the wildtype controls. In *sunn*^{a}, 2A and 2B cysts averaged 2.3 and 2.16 complete C(3)G positive cells per cyst, respectively. In *sunn*^{b}, 2A and 2B cysts averaged 2.27 and 1.15 complete C(3)G positive cells per cyst, respectively. In 2A and 2B cysts wildtype sibling controls averaged 3.1 and 2 complete C(3)G positive cells per cyst respectively. Also, *sunn*^{a} and *sunn*^{b} mutants showed an average of 1.63 and 1.83 region 2A cysts/germarium respectively compared to 2.4 region 2A cysts/ germarium in wildtype sibling controls respectively. [a]*sunn*$^{Z3\text{-}5839}$*/Df (3L) ED4470,* [b]*sunn*$^{Z3\text{-}1956}$*/Df (3L) ED4470,* [c]WT(*w)*

In *spnA*, *spnB* and *okr* mutants in *Drosophila*, both DSB repair and crossing over is impaired (McKim *et al.* 2002). Since *sunn* mutants show reduced recombination frequency, we investigated if *sunn* mutants are defective in DSB formation and repair. For this purpose, we utilized anti- γH2AV antibody which recognizes phosphorylated *Drosophila* H2Av and it is a useful marker for identifying DSBs during meiotic prophase. Immunostaining using anti- γH2AV antibody and anti–ORB antibody was performed on ovaries from *sunn* mutants and WT. In the WT controls, DSB formation in oocytes/pro-oocytes in region 2A and 2B (Figure 3-3) occurred normally. An average of 7.1 and 6.5 γH2Av foci were seen in regions 2A and 2B respectively (Table 3-5). As expected, by region 3 no γH2AV foci could be seen as all DSBs are usually repaired by this stage. In *sunn* mutants, we found that DSBs are formed in region 2A and region 2B (Fig. 1B), but they show higher number of average H2Av foci per oocyte/pro-oocyte (8.3 and 9.6 foci in region 2A and 2B respectively) at both of these stages compared to WT. We also observed DSB foci in region 3 and stage 2. However, region 3 and stage 2 oocytes show an average of 3.8 and 2.1 γH2AV foci/oocyte respectively, whereas in WT no foci are seen in these stages (Table 3-5). This shows that DSB repair occurs on schedule in *sunn* mutants but due to unknown reason complete repair is not achieved by region 3.

When investigated for DSB foci, both *solo* and *ord* show different results. *solo* mutants do not completely repair their DSB foci by region 3 and as high as 6.8 γH2AV are observed but these are completely repaired and none are present in stage 2. *ord* mutants, do not show DSB foci in region 3 therefore in these mutants DSBs are repaired on schedule. The significance for the delay in DSB repair is not understood but in cohesin mutants in *S. cerevisiae* DSBs are not repaired properly (Klein *et al.* 1999).

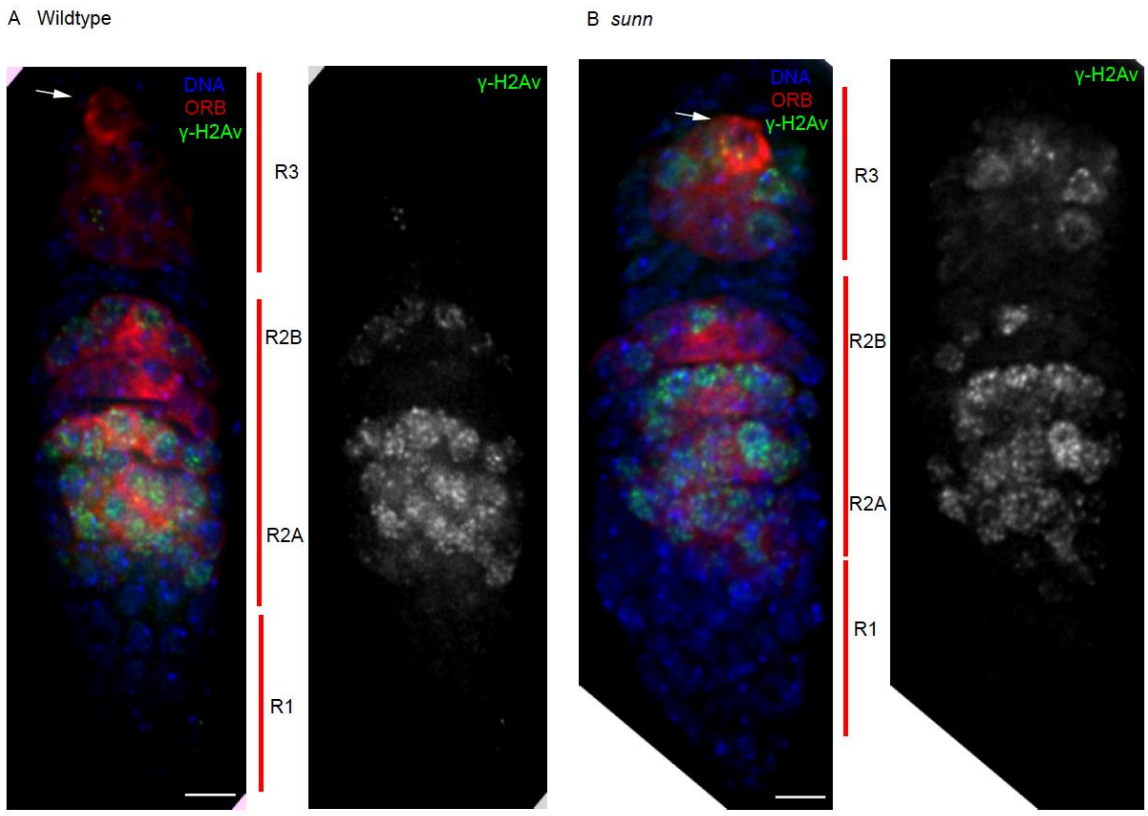


Figure 3-3: DSBs in *Drosophila* germarium in WT and *sunn* mutants. Immunostaining was performed using anti-γ-H2Av and anti-ORB antibody. (A) WT= Wildtype control (*Df/TM3, Sb*). γ-H2Av foci were present in pro-oocytes and oocytes (identified by ORB staining) in regions 2A, 2B. In region 3, no γ-H2Av foci were seen (white arrow) in WT controls. (B) *sunn* = *sunn* $^{Z3\text{-}5839}$*/Df (3L) ED4470*. Pro-ocytes and oocytes in regions 2A, 2B and 3 showed multiple γ-H2Av foci (white arrow). By region 3, γ-H2Av foci are reduced significantly but do not disappear completely (See Table 3-5 for quantification).

Table 3-5: γ-H2AV foci in WT and *sunn* mutant oocytes

	Oocyte Stage			
Genotype	Region 2A	Region 2B	Region 3	Stage 2
WT[a]	7.1 (N=15)	6.5 (N=13)	0.5 (N=8)	0.43 (N=7)
sunn[b]	8.3 (N=9)	9.6 (N=18)	3.8 (N=11)	2.1 (N=9)

Table entries in the "WT" and "*sunn*" lines are mean γ-H2AV foci per oocyte/pro-oocyte. N indicates the total number of oocytes /pro-oocytes counted in the indicated region of the ovariole. [a]*Df/ TM3, Sb*. [b]$sunn^{Z3\text{-}5839}$/*Df*

The extent of consequence of DSB repair delay in *sunn* mutant on the recombination phenotype is not understood.

SUNN co-localizes with C(3)G in oocytes: SUNN localizes to the centromeres in spermatocytes and oocytes. Centromeric localization is observed until metaphase I in spermatocytes and at least until stage 3 in oocytes (KRISHNAN *et al.* 2014). SUNN also exhibits diffuse DNA localization in germ cells throughout the germarium in whole mount preparations. To further evaluate this pattern, chromosome spreads of germ cells from germaria were immunostained with anti-C(3)G and anti-GFP antibodies. In these spreads, SUNN exhibited localization to chromosome arms both in C(3)G-positive cells, which are oocytes or pro-oocytes, and in C(3)G-negative which are nurse cells. In C(3)G-positive cells, in addition to bright foci that likely represent centromere-region staining, SUNN::Venus exhibited linear structures which co-localized with C(3)G except in a few small stretches (Figure 3-4A, B). Similar localization patterns were previously reported for ORD and SOLO cohesion proteins and the SC lateral elements. This localization pattern is consistent with the evidence presented above for roles of SUNN in synapsis and recombination in females during meiosis (KHETANI and BICKEL 2007; YAN and MCKEE 2013) and are thought to represent staining along chromosome arms.

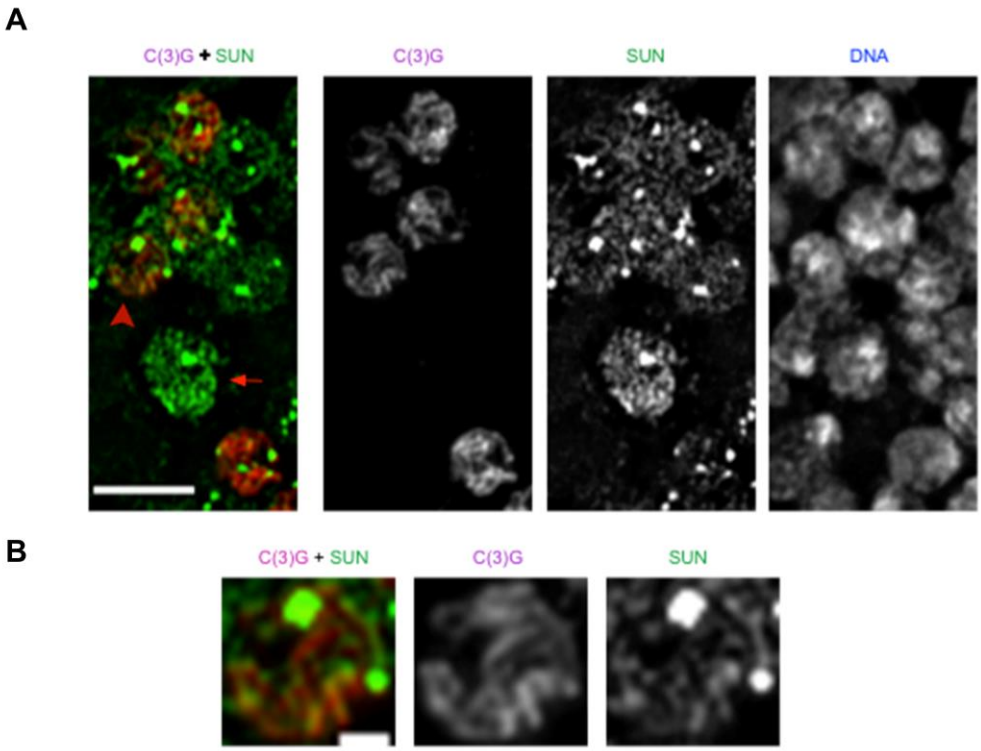


Figure 3-4: Co-localization of SUNN::Venus and C(3)G in pro-oocytes/oocytes. and nurse cells. Chromosome spread of germarium from *UASp-SUNN::Venus; sun* $^{Z3\text{-}5839}$ */Df, nos-GAL4::VP16* females immunostained with anti-GFP and anti-C(3)G antibodies. (A) SUNN:Venus formed linear structures in both C(3)G-positive pro-oocytes/oocytes, where it co-localized with C(3)G linear structures (red arrowhead) and C(3)G negative cells, likely nurse cells (red arrow). (C) SUN linear structures co-localizes with C(3)G linear structures. (B) Magnified image of pro-oocyte/oocyte (red arrowhead) shows SUNN:Venus forming linear thread like structures and large foci. These structures mostly co-localize with C(3)G, barring few stretches. Large foci likely represents centromeric SUNN:Venus, which also co-localizes with C(3)G. (A) and (B) scale bars = 5 μM, (C) scale bar = 1 μM.

DISCUSSION

SUNN co-localizes with C(3)G and is required for SC stability: SUNN localizes to chromosome arms and forms thread-like structures in oocytes and nurse cells and in oocytes SUNN co-localizes with C(3)G. This localization of SUNN to chromosome arms in both oocytes and nurse cells is similar to ORD, SOLO and SMC1 localization (KHETANI and BICKEL 2007; YAN and MCKEE 2013). This pattern might reflect the SUNN's role at chromosome core in nurse cells and oocytes. Partial evidence for this is that in *sunn* mutants, SMC1 does not localize to the centromeres and forms weak diffused structures on chromosome arms in oocytes (KRISHNAN *et al.* 2014). Similarly, in the absence of *sunn*, SOLO does not localize to the centromeres in both spermatocytes and oocytes (data not shown). The arm localization of SOLO is greatly diminished in *sunn* mutants (data not shown) but chromosome spreads of oocytes of *sunn* mutants needs to be performed to detect the extent of this loss from chromosome arms. SMC1 is a cohesin and possibly a part of the chromosome core, therefore it is necessary for proper assembly of AE/LE. Chromosome core is formed along sister chromatid axis during prophase I upon which AE/LE assemble of the SC assemble. Significant weakening of SMC1 from chromosome arms in *sunn* mutants shows it requires SUNN's function to stabilize chromosome core. Also, SUNN's similar localization pattern as SMC1 suggests that they co-localize and reside together at chromosome arms. Whether they directly interact and form a complex needs to be determined by performing immuno-precipitation experiments. More evidence of SUNN as a chromosome core component could be determined by examining co-localization of

SUNN with SMC1, ORD and SOLO and testing its arm localization in *smc1*, *solo*, *ord* mutants.

Additional evidence of SUNN's role as a chromosome core component comes from its requirement for stable SC formation. In *sunn* mutants, SC disintegrates prematurely by region 3 (in 65-75% of oocytes), which corresponds to mid-pachytene. Most region 2A oocytes in *sunn* mutants show normal SC. Although formation of SC appears normal and linear structures of C(3)G are obtained for region 2A and 2B, no zygotene like stage was isolated for *sunn* mutants. We directly observe appearance of thread-like structures in region 2A cysts (Figure 3-2D). This is also observed in *ord* mutants, where zygotene stage oocytes are not found (TANNETI *et al.* 2011). In addition, *sunn* mutants are defective in centromeric clustering, which is hypothesized to be required for chromosomal loading of SC components and SC initiation. These findings show that although normal SC linear structures are seen in *sunn* mutants there are processes which occur at initial stages of SC formation in WT but are defective in *sunn* mutants. Also, in *sunn* mutants, there is reduced association of C(3)G at centromeres (data not shown) in region 2A and throughout the germaria. Even though *ord* mutants show normal looking SC structures in immunofluorescence experiments at region 2A and 2B, transmission electron micrographs revealed that *ord* mutants never form proper SC (WEBBER *et al.* 2004). Since *sunn* shows similar timing of SC disassembly and all the minor SC defects described above, it is possible that despite showing normal SC structures in our experiments, *sunn* mutants have fundamentally flawed SC structure to begin with which falls apart as prophase progresses. Improper chromosome core in *sunn* mutants might be a reason for aberrant SC assembly.

The appearance of thread like SC structures could be due to C(2)M which is required for SC initiation at a subset of euchromatic sites on chromosome arms. In *sunn's* absence, C(2)M might not be able to initiate SC formation at the centromeres and on other euchromatic sites but are able complete SC formation from its initiation sites. If this is true then like *ord*, *sunn* is a component of a SC pathway that with help of *c(2)m* constructs normal SC. If this is true then a *sunn c(2)m* double mutant should abolish all SC formation. This is what we observe in *sunn c(2)m* double mutants, where only poly-complexes of C(3)G are observed and no centromeric localization or thread-like structures of C(3)G are seen (Figure 3-6).

***sunn* is required for homolog bias:** In *S. cerevisiae*, AE/LE components RED1, HOP1 is required to ensure homolog bias during meiotic DSB repair. In *Drosophila*, cohesin interacting proteins ORD and SOLO are strong AE/LE candidates and are required for homolog bias during meiosis (WEBBER *et al.* 2004; YAN and MCKEE 2013). It is hypothesized that normal AE/LE would not only promote homolog bias pathway but also inhibit pathway that would lead to SCE. The consequence of SCE is that enough crossover products are not produced and chiasmata are not formed. Homolog bias ensures that after DSB formation the nascent 3' end invades a DNA strand in its homolog partner and not its own sister DNA strand. This is important because 1) Strand invasion to homolog partner ensures that it will be repaired based on the sequence of its homolog and this might help incorporate any sequence variation. 2) Sister chromatid cohesion proximal to DNA exchange sites forms chiasma which joins them and helps homologs segregate properly at anaphase I.

Figure 3-5: SC phenotype in *sunn c(2)m* double mutant and *sunn c(2)m ord* triple mutant: To visualize SC, immunostaining was performed using anti-C(3)G and anti-CID antibody. (A) $^{a}sunn^{Z3\text{-}5839}/Df\ (3L)ED4470$, $^{e}c(2)m^{EP2115}/c(2)m^{EP2115}$. C(3)G in *sunn c(2)m* double mutants do not form thread-like structures on the DNA but instead forms poly-complexes around DNA territory (red arrows). (B) $^{a}sunn^{Z3\text{-}5839}/Df\ (3L)ED4470$, $^{e}c(2)m^{EP2115}/c(2)m^{EP2115}\ ^{f}ord^{10}/ord^{10}$ Similar poly-complexes are found in *ord sunn c(2)m* triple mutants and elongated poly-complexes which are formed in the triple mutants that are localized to DNA territory periphery. (C) $^{e}c(2)m$. C(3)G in c(2)m mutants co-localizes with CID at the centromeres and forms few patches on the DNA. No thread-like structures are found. (D) $^{e}ord^{10}/^{e}ord^{10}$ C(3)G forms poly-complexes (red arrows) and does not form thread like structures. Few foci could be seen localizing to DNA and not on to the DNA periphery, the significance of this localization is not known. Similar DNA localization is also observed for *sun c(2)m* double mutants and *sun c(2)m ord* triple mutants.

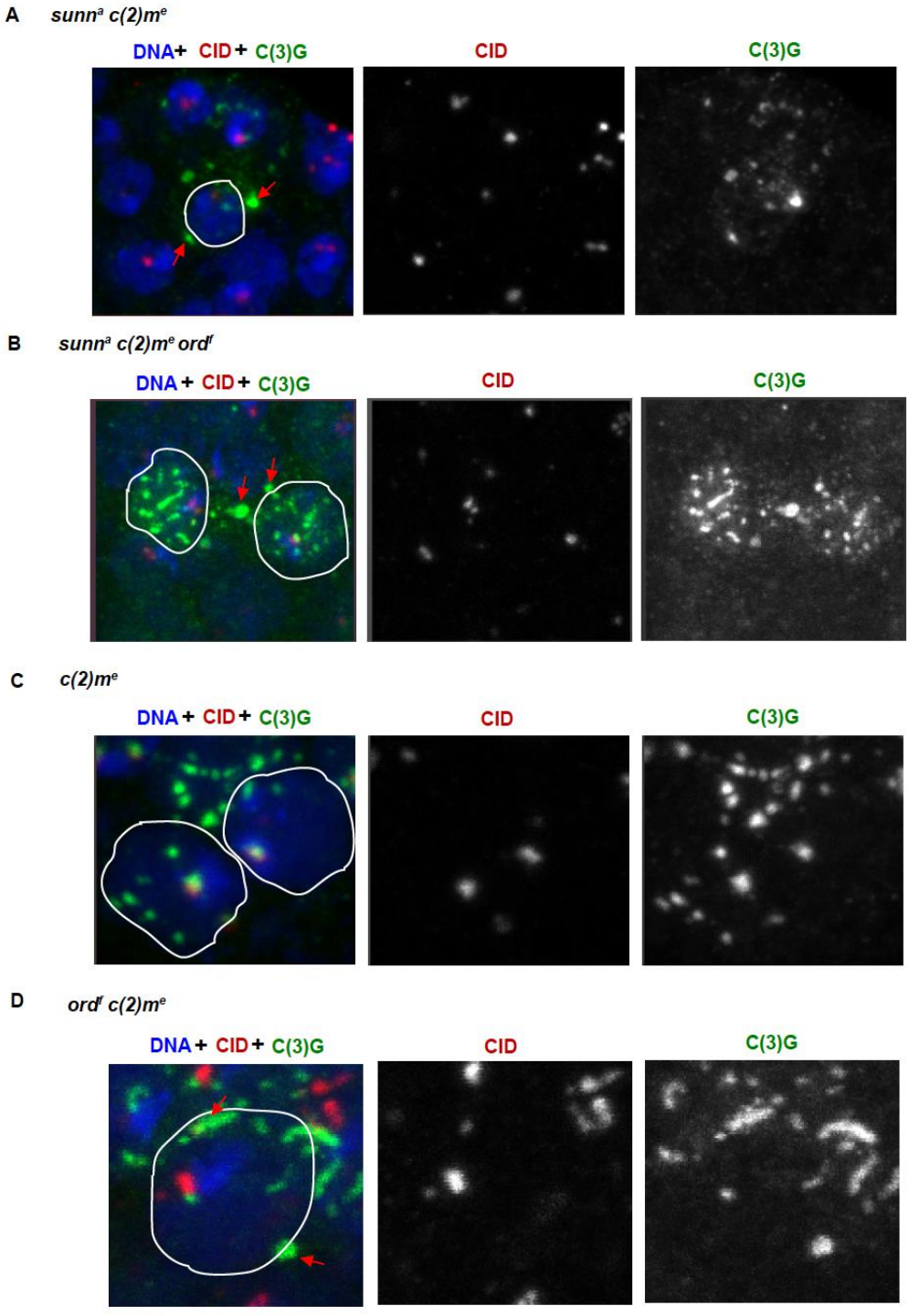


Figure 3-5. Continued

Our Ring X chromosome assay determined that in *sunn* mutants homolog bias is reduced and SCE is elevated compared to WT controls (Table 3-3). This also shows that SUNN is an essential part of the chromosome axis and provides additional evidence of it being a AE/LE component. The lack of chiasmata as a consequence of elevated SCE could also contribute to the extensive homolog NDJ observed during meiosis in *sunn* mutant females (KRISHNAN *et al.* 2014).

***sunn* mutation causes delay in DSB repair:** DSBs in *sunn* mutants are not repaired completely until after stage 2 in the ovariole, which represents mid to late pachytene. This delay in DSB repair could be due to aberrant chromosomal axis and inefficient recombination. Since SC disintegrates prematurely in *sunn* mutants it is possible that all DSBs are not repaired properly by homologous recombination and repair. However, since SCE could provide an alternative pathway for DSB repair, and since *sunn* mutants derepress the SCE pathway, it is somewhat surprising that some DSBs remain unrepaired. Apart from *solo* mutants, which show a transient delay in DSB repair, genes such as *c(2)m* and *ord* do not affect DSB repair. However, in other organisms such as *S. cerevisiae*, DSBs are not repaired properly in cohesin - *rec8* and *smc3* mutants (KLEIN *et al.* 1999; NASMYTH and HAERING 2009).

***sunn* is required for homologous recombination:** *sunn* mutation is required for sex chromosome and autosomal homologous recombination. The average *sunn* mutant recombination frequency is 5.5 times lower than WT controls. The reasons for reduced homologous recombination could be multiple and it is not clear how much part do these processes individually play in the outcome of the recombination phenotype. The inability of *sunn* mutants to form proper SC could lead to reduced homologous

recombination frequency. Evidence for this comes from *ord*, *solo* and *c(2)m* mutants all of which show defective SC and reduced recombination frequency. Lack of SC would prevent the homologs from being held close together so that they can complete the repair process properly. In order to prove this it would be necessary to see if synapsis is disrupted and homologs are separated at euchromatic and heterochromatic sites. In order to do this, FISH using euchromatic and heterochromatic probes in *sunn* mutants has to be performed.

In addition to defective SC, increased SCE could also explain reduced crossing over observed in *sunn* mutants. *ord* and *solo* but not *c(2)m* mutants shows elevated SCE. This defect is also seen in other organisms in *red1* and *hop1*, which disrupt AE/LE formation (SCHWACHA and KLECKNER 1994; SCHWACHA and KLECKNER 1997). Therefore, the inability of *sunn* mutants to form proper chromosome cores could lead to reduction in recombination as DSBs getting repaired by SCE would not produce any chromosomal variation and crossovers. Why despite C(2)M being a component of LE, *c(2)m* mutants do not cause increased SCE is not known.

How does SUNN function at chromosome cores? Bioinformatic analysis showed that SUNN is a structural homolog of SA. In our model, SUNN substitutes SA in the meiotic cohesin complex, which consists of SMC1, SMC3, ORD/SOLO. SUNN localizes to centromeres in spermatocytes and oocytes/pro-oocytes and provides cohesion by directly by being a part of the cohesin complex or indirectly through its ability to affect localization of cohesion proteins. We also observed that SUNN localizes to chromosome arms in both oocytes/pro-oocytes and nurse cells. Therefore, in addition to SUNN's role at centromeres it is possible that SUNN by being a part of cohesion

complex at the chromosome cores performs multiple functions. Direct proof of this would require immunoprecipitation experiments to prove that SUNN physically interacts with other *Drosophila* cohesion proteins (SMC1, ORD, SOLO) at chromosome cores as well as centromeres.

Does SUNN play a role in arm cohesion? In *Drosophila*, so far no cohesion gene mutants have shown defects in arm cohesion. This has been tested at few loci for *ord* and *solo* and none of them shows any cohesion defect at the tested loci. This is puzzling because both disrupt centromeric cohesion and localize to chromosome arms. Therefore, arm cohesion are more difficult to disrupt and might be dependent on SMC1, which does localize to chromosome arms (although diffused) in *sunn*, *solo* and *ord* mutants. We wanted to test if arm cohesion is disrupted in *sunn* mutants. To test this we performed FISH using X chromosome heterochromatic probe 359bp and our initial studies showed that *sunn* mutants do not affect cohesion at that locus (data not shown). However, it would be interesting to see if cohesion is lost at euchromatic loci in *sunn* mutants or in double-triple mutants of the cohesion genes.

What role does SUNN play at chromosome cores? Based on localization of SUNN to chromosome arms in oocytes and nurse cells and its role in SC stability and homolog bias, it is possible that SUNN is a chromosome core component. This is strengthened by the observation that *sunn* is required for proper localization of chromosome core component SMC1 on chromosome arms. Based on shared function and localization pattern, we hypothesize that at chromosome cores SMC1, ORD, SOLO and SUNN are in the same pathway or complex and C(2)M is not in this complex. Evidence for this also comes from the difference between their SC phenotypes and

localization. C(2)M only localizes to oocytes where SC formation occurs. This qualifies C(2)M as a AE/LE component only in oocytes or pro-oocytes. Proof that any of the above complexes exist will require detailed biochemical studies.

REFERENCES

BICKEL, S. E., D. W. WYMAN and T. L. ORR-WEAVER, 1997 Mutational analysis of the Drosophila sister-chromatid cohesion protein ORD and its role in the maintenance of centromeric cohesion. Genetics **146:** 1319-1331.

CLIFT, D., and A. L. MARSTON, 2011 The role of shugoshin in meiotic chromosome segregation. Cytogenet Genome Res **133:** 234-242.

GOLUBOVSKAYA, I. N., O. HAMANT, L. TIMOFEJEVA, C. J. WANG, D. BRAUN *et al.*, 2006 Alleles of afd1 dissect REC8 functions during meiotic prophase I. J Cell Sci **119:** 3306-3315.

HAWLEY, R. S., K. S. MCKIM and T. ARBEL, 1993 Meiotic segregation in Drosophila melanogaster females: molecules, mechanisms, and myths. Annu Rev Genet **27:** 281-317.

HEIDMANN, D., S. HORN, S. HEIDMANN, A. SCHLEIFFER, K. NASMYTH *et al.*, 2004 The Drosophila meiotic kleisin C(2)M functions before the meiotic divisions. Chromosoma **113:** 177-187.

HIROSE, Y., R. SUZUKI, T. OHBA, Y. HINOHARA, H. MATSUHARA *et al.*, 2011 Chiasmata promote monopolar attachment of sister chromatids and their co-segregation toward the proper pole during meiosis I. PLoS Genet **7:** e1001329.

KHETANI, R. S., and S. E. BICKEL, 2007 Regulation of meiotic cohesion and chromosome core morphogenesis during pachytene in Drosophila oocytes. J Cell Sci **120:** 3123-3137.

KLEIN, F., P. MAHR, M. GALOVA, S. B. BUONOMO, C. MICHAELIS *et al.*, 1999 A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell **98:** 91-103.

KRISHNAN, B., S. E. THOMAS, R. YAN, H. YAMADA, I. B. ZHULIN *et al.*, 2014 Sisters Unbound Is Required for Meiotic Centromeric Cohesion in Drosophila melanogaster. Genetics.

LAKE, C. M., and R. S. HAWLEY, 2012 The molecular control of meiotic chromosomal behavior: events in early meiotic prophase in Drosophila oocytes. Annu Rev Physiol **74:** 425-451.

LEE, J. Y., and T. L. ORR-WEAVER, 2001 The molecular basis of sister-chromatid cohesion. Annu Rev Cell Dev Biol **17:** 753-777.

LI, X. C., E. BOLCUN-FILAS and J. C. SCHIMENTI, 2011 Genetic evidence that synaptonemal complex axial elements govern recombination pathway choice in mice. Genetics **189:** 71-82.

LLANO, E., Y. HERRAN, I. GARCIA-TUNON, C. GUTIERREZ-CABALLERO, E. DE ALAVA *et al.*, 2012 Meiotic cohesin complexes are essential for the formation of the axial element in mice. J Cell Biol **197:** 877-885.

MANHEIM, E. A., and K. S. MCKIM, 2003 The Synaptonemal complex component C(2)M regulates meiotic crossing over in Drosophila. Curr Biol **13:** 276-285.

MASON, J. M., 1976 Orientation disruptor (ord): a recombination-defective and disjunction-defective meiotic mutant in Drosophila melanogaster. Genetics **84:** 545-572.

MCKIM, K. S., J. K. JANG and E. A. MANHEIM, 2002 Meiotic recombination and chromosome segregation in Drosophila females. Annu Rev Genet **36:** 205-232.

MCKIM, K. S., E. F. JOYCE and J. K. JANG, 2009 Cytological analysis of meiosis in fixed Drosophila ovaries. Methods Mol Biol **558:** 197-216.

MIYAZAKI, W. Y., and T. L. ORR-WEAVER, 1992 Sister-chromatid misbehavior in Drosophila ord mutants. Genetics **132:** 1047-1061.
NASMYTH, K., and C. H. HAERING, 2009 Cohesin: its roles and mechanisms. Annu Rev Genet **43:** 525-558.
PAGE, S. L., and R. S. HAWLEY, 2001 c(3)G encodes a Drosophila synaptonemal complex protein. Genes Dev **15:** 3130-3143.
PAGE, S. L., and R. S. HAWLEY, 2004 The genetics and molecular biology of the synaptonemal complex. Annu Rev Cell Dev Biol **20:** 525-558.
PASIERBEK, P., M. JANTSCH, M. MELCHER, A. SCHLEIFFER, D. SCHWEIZER *et al.*, 2001 A Caenorhabditis elegans cohesion protein with functions in meiotic chromosome pairing and disjunction. Genes Dev **15:** 1349-1360.
PETRONCZKI, M., M. F. SIOMOS and K. NASMYTH, 2003 Un menage a quatre: the molecular biology of chromosome segregation in meiosis. Cell **112:** 423-440.
ROEDER, G. S., 1995 Sex and the single cell: meiosis in yeast. Proc Natl Acad Sci U S A **92:** 10450-10456.
ROEDER, G. S., 1997 Meiotic chromosomes: it takes two to tango. Genes Dev **11:** 2600-2621.
SCHWACHA, A., and N. KLECKNER, 1994 Identification of joint molecules that form frequently between homologs but rarely between sister chromatids during yeast meiosis. Cell **76:** 51-63.
SCHWACHA, A., and N. KLECKNER, 1997 Interhomolog bias during meiotic recombination: meiotic functions promote a highly differentiated interhomolog-only pathway. Cell **90:** 1123-1135.
TANNETI, N. S., K. LANDY, E. F. JOYCE and K. S. MCKIM, 2011 A pathway for synapsis initiation during zygotene in Drosophila oocytes. Curr Biol **21:** 1852-1857.
THOMAS, S. E., M. SOLTANI-BEJNOOD, P. ROTH, R. DORN, J. M. LOGSDON, JR. *et al.*, 2005 Identification of two proteins required for conjunction and regular segregation of achiasmate homologs in Drosophila male meiosis. Cell **123:** 555-568.
TOTH, A., K. P. RABITSCH, M. GALOVA, A. SCHLEIFFER, S. B. BUONOMO *et al.*, 2000 Functional genomics identifies monopolin: a kinetochore protein required for segregation of homologs during meiosis i. Cell **103:** 1155-1168.
WATANABE, Y., 2004 Modifying sister chromatid cohesion for meiosis. J Cell Sci **117:** 4017-4023.
WATANABE, Y., 2005 Shugoshin: guardian spirit at the centromere. Curr Opin Cell Biol **17:** 590-595.
WEBBER, H. A., L. HOWARD and S. E. BICKEL, 2004 The cohesion protein ORD is required for homologue bias during meiotic recombination. J Cell Biol **164:** 819-829.
YAN, R., and B. D. MCKEE, 2013 The Cohesion Protein SOLO Associates with SMC1 and Is Required for Synapsis, Recombination, Homolog Bias and Cohesion and Pairing of Centromeres in Drosophila Meiosis. PLoS Genet **9:** e1003637.
YOKOBAYASHI, S., and Y. WATANABE, 2005 The kinetochore protein Moa1 enables cohesion-mediated monopolar attachment at meiosis I. Cell **123:** 803-817.

CHAPTER IV: FUTURE DIRECTIONS

FUTURE DIRECTIONS

Chapter II and III characterizes a novel cohesion gene in *Drosophila melanogaster* called *sister unbound* (*sunn*) that is required for various functions during *Drosophila* meiosis. Many of the functions exhibited by SUNN are similar to what has been characterized for ORD and SOLO, which are two other *Drosophila* specific cohesion proteins. Similarity of functions and mutual interdependence of SOLO, ORD and SUNN (SOS) has led us to hypothesize that they are involved in the same complex. A cohesin complex is formed of SMC subunits (SMC1/SMC3) and non-SMC subunits (RAD21/REC8 in meiosis and SA). In light of no evidence for non-SMC1 subunits RAD21 and SA in cohesion and absence of REC8 homolog in *Drosophila*, SOS proteins are hypothesized to fill in this gap.

The only direct proof of such a complex comes from co-immunoprecipitation studies, which shows SOLO and SMC1 physically interacts (YAN and MCKEE 2013). Therefore, in order to characterize the SOS-cohesin complex it is necessary to perform mass spectrometry analysis each of SUNN, SOLO, ORD and SMC1 and identify interacting proteins. If they do interact with each other it is necessary to confirm these interactions with immuno-precipitation experiments. The nature of interactions that we find between these proteins would provide insight into how SOS and cohesins form a complex. It will answer questions such as: 1) Do SOS proteins exist in the same complex and if they do what position do they occupy in the cohesin model? 2) Do SOS proteins also form a SMC independent complex?

Since SOS-cohesin complex is necessary for both cohesion and other associated roles at chromosome cores (such as recombination, SC stability) it makes

this complex different from the other possible complex, which is necessary for proper SC formation and recombination and is not required for cohesion. This complex could include proteins such as C(2)M, RAD21, SA and cohesin SMC subunits. In order to confirm this hypothesis interaction studies using mass spectrometry, immuno-precipitation has to be performed to prove they interact and form a complex.

The other essential question is does SUNN play a role any other role that what has been identified. SUNN localizes to the centromeres and in pre-meiotic four cell, eight cell and sixteen cysts in spermatocytes it forms as bright blobs at DNA periphery and localizes diffusedly at the nuclear membranes (KRISHNAN *et al.* 2014). This could be an artifact of SUNN over-expression, but our bioinformatic analysis suggests that it has structural similarities with exportin class of proteins, which has functions at the nuclear membrane. Our mutant analysis has been unable to pick up any possible role of SUNN at the nuclear envelope or surroundings. In order to confirm if the localization of SUNN in pre-meiotic cysts is real, we want clone the native promoter of *sunn*. Currently, we are in the process of amplifying a region 2500 bp upstream of the *sunn* start codon. We will clone this region upstream of Sunn and Venus cDNA and create transgenic lines with this construct. If we find the localization is true, then we would check if SUNN localization is disrupted in mutants of proteins involved in known exportin/importin mutants.

The other question is to see the mechanism by which arm cohesion is provided in oocytes during meiosis? All cohesion proteins SMC1, ORD, SOLO and SUNN localize to chromosome arms and are hypothesized to provide cohesion at the arms. Cohesion at chromosome arms are required for formation of chiasma. *ord* and *solo* has

been investigated in a limited way in this regard and arm cohesion has not been found to be disrupted in these mutants. We want to perform a comprehensive analysis of arm cohesion by utilizing FISH (Fluorescent in situ Hybridization) using multiple heterochromatic and euchromatic probes in double, triple mutant backgrounds of the above cohesion proteins. It is possible that the residual presence of SMC1 on chromosome arms in all these mutants are responsible for the arm cohesion therefore we would like to test arm cohesion in *sunn smc1* double mutants. In order to think about this we need to perform extensive double and triple mutant studies.

REFERENCES

KRISHNAN, B., S. E. THOMAS, R. YAN, H. YAMADA, I. B. ZHULIN *et al.*, 2014 Sisters Unbound Is Required for Meiotic Centromeric Cohesion in Drosophila melanogaster. Genetics.

YAN, R., and B. D. MCKEE, 2013 The Cohesion Protein SOLO Associates with SMC1 and Is Required for Synapsis, Recombination, Homolog Bias and Cohesion and Pairing of Centromeres in Drosophila Meiosis. PLoS Genet **9:** e1003637.

VITA

Badri Krishnan was born in Chennai, Tamil Nadu state, India in 1984. He graduated with Bachelor of Science in Biochemistry (Honors) in 2006 from University of Delhi and then went on to pursue and graduate with a Master of Science in Biomedical Sciences in 2008 from University of Delhi. He began his graduate study at the University of Tennessee, Knoxville and defended his dissertation in 2014.