

Studies on the mechanism of homolog pairing in *Drosophila* male meiosis

**A Dissertation Presented for the
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DEDICATION

This dissertation is dedicated to my parents, Kuei-Feng Tsai and Chin-Sui Lai, for their love and care that have seen me through all these years of studying away from home. I would also like to dedicate this work to my lovely wife, Jui-Min Lee, for all the support she provided.

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ABSTRACT

Drosophila male is an example of achiasmatic meiosis which lacks crossingover and chiasmata during meiosis. Previous studies showed that homologous pairing of both euchromatin and centromeres is lost during middle prophase I, however, homologs are still connected as they form bivalents. The X-Y pair utilizes a specific repeated sequence within the heterochromatic ribosomal DNA blocks as a pairing site. No pairing sites have yet been identified for the autosomes. To search for such sites, we utilized probes specifically targeting heterochromatin regions to assay pairing sequences and behavior in meiosis by fluorescence *in situ* hybridization (FISH). We found that the fourth homologs pair at the heterochromatic region 61 and associate with the X chromosome throughout prophase I. The pairing of the fourth homologs is disrupted in the homolog conjunction complex mutants. Conversely, six tested heterochromatic regions of the major autosomes (second and third chromosomes) have proved to be largely unpaired after early prophase I. This suggests that pairing mechanism of the major autosomes may differ from the sex and fourth chromosomes; stable connections between major autosomal homologs might occur at different sites along chromosomes in different cells by analogy to chiasmata. Moreover, FISH analysis also revealed two distinct patterns of sister chromatid cohesion in heterochromatin: regions with stable cohesion and regions lacking cohesion, suggesting that sister chromatid cohesion is incomplete within heterochromatin but with preferential sites in male meiosis.

Modifier of Mdg4 in Meiosis (MNM) and Stromalin in Meiosis (SNM) are components of homolog conjunction complex and essential for homolog pairing and segregation in male meiosis. Using yeast two-hybrid assay and co-immunoprecipitation, we showed that the MNM and SNM interact with each other. Specifically, the BTB domain of MNM is responsible for the interaction with SNM, whereas FLYWCH domain of MNM is crucial for this interaction but does not directly interact with SNM. Additionally, point mutation analysis revealed that L9K replacement of the BTB domain weakened the MNM-SNM interaction and caused high frequencies of chromosome nondisjunction.

In conclusion, these results provide a biochemical basis for the mechanism of homolog pairing and support the role of homolog conjunction complex in male meiosis.

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CHAPTER 1

HOMOLOGOUS PAIRING AND THE ROLE OF PAIRING CENTERS IN MEIOSIS

A version of this chapter is modified from the manuscript that has been accepted by *Journal of Cell Science* and is in press now. Jui-He Tsai was responsible for writing the manuscript draft.

ABSTRACT

Homologous pairing establishes the foundation for accurate reductional segregation during meiosis I in sexual organisms. This chapter summarizes recent progress in our understanding of homologous pairing in meiosis, and will focus on the characteristics and mechanisms of specialized chromosome sites, called pairing centers (PCs), in *Caenorhabditis elegans* and *Drosophila melanogaster*. In *C. elegans*, each chromosome contains a single PC that stabilizes chromosome pairing and initiates synapsis of homologous chromosomes. Specific zinc-finger proteins recruited to PCs link chromosomes to nuclear envelope proteins – and through them to the microtubule cytoskeleton – thereby stimulating chromosome movements in early prophase, which are thought to be important for homolog sorting. This mechanism appears to be a variant of the ‘telomere bouquet’ process, in which telomeres cluster on the nuclear envelope, connect chromosomes through nuclear envelope proteins to the cytoskeleton and lead chromosome movements that promote homologous synapsis. In *Drosophila* males, which undergo meiosis without recombination, pairing of the largely non-homologous X and Y chromosomes occurs at specific repetitive sequences in the ribosomal DNA. Although no other clear examples of PC-based pairing mechanisms have been described, there is evidence for special roles of telomeres and centromeres in aspects of chromosome pairing, synapsis and segregation; these roles are in some cases similar to those of PCs.

INTRODUCTION

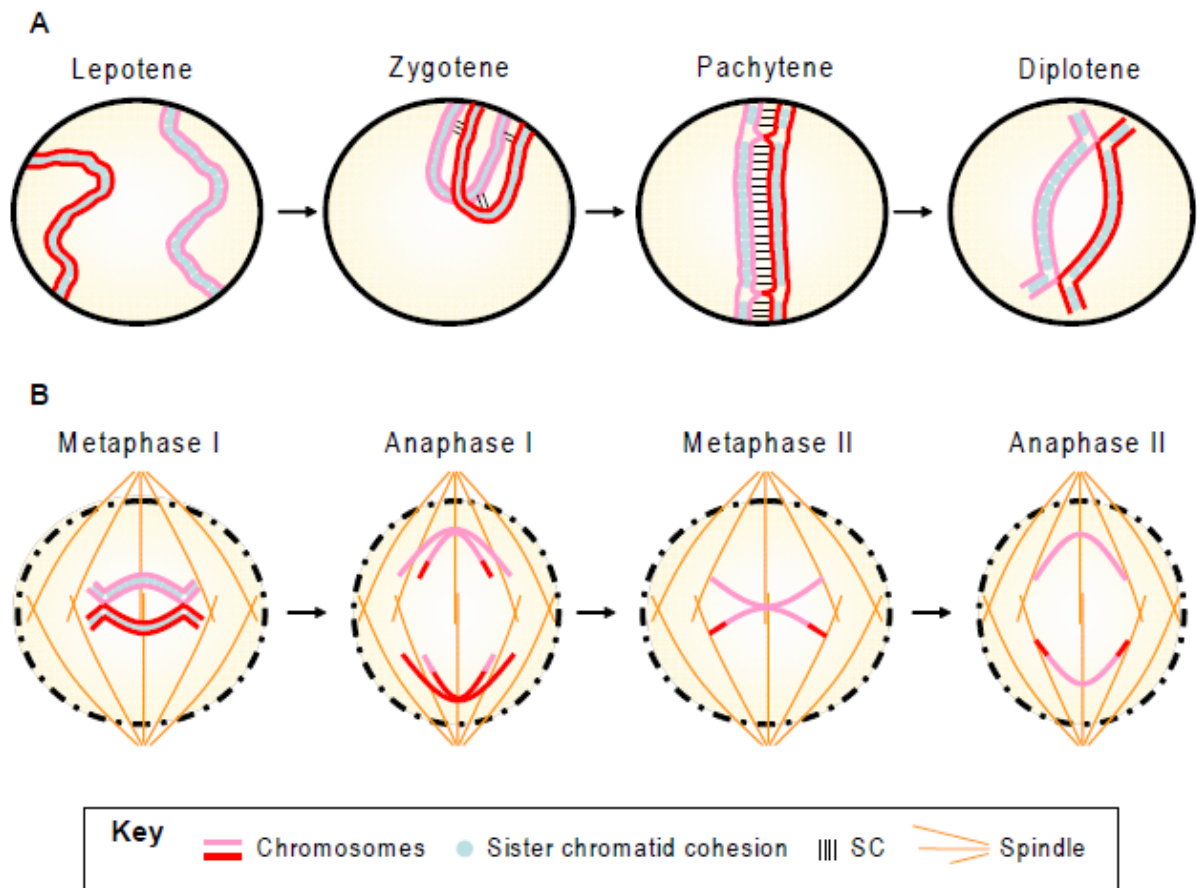
Meiosis comprises one round of DNA replication followed by two nuclear divisions, meiosis I and meiosis II (Kleckner 1996). Meiosis I and II are both divided into five phases: prophase, prometaphase, metaphase, anaphase and telophase. Prophase I is the first stage in meiosis and initiates when diploid cells enter meiosis. It is subdivided into the stages leptotene, zygotene, pachytene, diplotene and diakinesis on the basis of the morphology of chromosomes and the association of homologous chromosomes during synapsis. Several events occur during prophase I, including DNA double-strand break (DSB) formation and repair, crossover formation, homologous chromosome pairing, synapsis and chromosome condensation. Nuclear envelope breakdown marks the start of prometaphase I. Meanwhile, homologous centromeres attach to the microtubules emanating from the spindle poles. The paired homologs (bivalents) are arranged on the equatorial plate at metaphase I. Segregation of homologs to opposite poles initiates at anaphase I with the resolution of chiasmata, and the formation of two daughter cells at telophase I concludes meiosis I. Meiosis II, an equational division that does not reduce chromosome number, is a mitosis-like division. During prophase II, sister chromatids condense again. The nuclear membrane breaks down at prometaphase II; sister chromatids align at the metaphase plate during metaphase II and then separate at anaphase II. The process ends with telophase II producing four haploid cells containing half the original number of chromosomes.

During meiosis, accurate segregation of homologous chromosomes relies on pairing of homologs to form so-called bivalents that interact with the meiotic spindle

as a unit, enabling homologous centromeres to orient to opposite poles (Fig. 1-1). In most eukaryotes, the formation of bivalents requires both homologous recombination and synapsis. Meiotic recombination is initiated by the induction of DSBs on chromosomes by the widely conserved topoisomerase-like protein, sporulation-specific protein 11 (SPO11). The DSBs are resected from 5' to 3' by the RAD50–MRE11–XRS2 complex to generate ~300-nucleotide-long 3' single-stranded tails. Then, RecA family proteins, which are essential for the repair and maintenance of DNA, target the ends of the DSBs to form filaments and catalyze strand-invasion reactions to find a repair template (Pawlowski and Cande 2005). The DSB repair process leads to gene conversion (the copying of genetic information from the repair template into the DSB-bearing homolog) and to the formation of one of two types of products, either crossovers or non-crossovers. Crossovers result from reciprocal exchange between homologous chromosomes and appear as chiasmata, whereas non-crossovers are without reciprocal exchange (Borner et al. 2004). Chiasmata are thought to be the cytological manifestations of crossovers and a chiasma will arise for every crossover.

Figure 1-1 General model of homologous pairing in meiosis.

One pair of homologous chromosomes is shown in red and pink lines, whereas pairs of sister chromatids are shown in the same color. (A) Before entering meiosis, unpaired homologous chromosomes are distributed randomly within the nucleus. At leptotene, telomeres have attached randomly along the nuclear envelope. Initially, chromosomes search for homologous sequences. This, at first, leads to an approximate parallel alignment of chromosomes. After chromosomes are aligned through bouquet formation, synapsis (the association of chromosomes) initiates at zygotene. During pachytene, high levels of homologue alignment are achieved along the entire length, to produce a mature bivalent with fully synapsed chromosomes. Paired homologs recombine with each other during zygotene and pachytene. The SC is disassembled at diplotene, when recombination is completed. Chromosomes then condense further during the diakinesis stage. (B) At metaphase I, paired homologous chromosomes line up on the metaphase plate. Segregation of homologous chromosomes occurs at anaphase I. Only one pair of sister chromatids is shown in meiosis II. Sister chromatids align on the center plate at metaphase II and segregate to opposite poles at anaphase II.



Synapsis involves the formation of the SC, an elaborate zipperlike structure that connects two aligned homologous chromosomes along their entire length. After homologs recognize each other, synapsis enhances and stabilizes these initial associations by connecting homologous chromosomes until the SC is disassembled at diplotene, when the chromosomes are joined only by chiasmata and sister chromatid cohesion. In general, the SC structure is conserved among diverse organisms, although the sequence similarity between the protein components is fairly low. The SC comprises two lateral elements that flank the chromatin, a single central element that is midway between the lateral elements, and a large number of individual transverse filaments that lie perpendicular to the long axis of the complex and act to connect the lateral elements with the central element (Page and Hawley 2004). The components of the SC structure are crucial for synapsis. Synapsis normally occurs between homologous chromosomes; however, the formation of the SC between nonhomologous chromosomes, so called non-homologous synapsis, can occur. The processes involved in initial homolog pairing appear to be independent of synapsis. For example, mutations in the *C. elegans* gene *syp-1*, which encodes an SC structure component, disrupt synapsis but the homologs still align locally during early meiosis in these mutants (MacQueen et al. 2002). Furthermore, homolog juxtaposition in yeast is unaffected by the absence of ZIP1, a component of the central region of SC (Peoples et al. 2002).

During the formation of bivalents, homologs usually enter meiosis unpaired and 'search' for homologous sequences during leptotene (Roeder 1997; McKee 2004). Chromosome synapsis initiates during zygotene and then extends from

these initiation sites so that, by pachytene, homologous chromosome axes are fully aligned and synapsed (Page and Hawley 2004). Once recombination is completed, the synaptonemal complex (SC) is disassembled, but homologs remain connected along their arms, through sister chromatid cohesion, and at discrete sites known as chiasmata until anaphase I (Carpenter 1994). Chiasmata, in conjunction with sister chromatid cohesion, enable homologs to orient to opposite poles on the meiosis I spindle (Fig. 1-1).

Although synapsis and meiotic double-strand breaks (DSBs) are required for homologous pairing in most organisms, both DSB-independent and synapsis-independent meiotic segregation pathways have also been described (Zickler 2006). In *Bombyx mori* (domesticated silkworm) females, synapsis occurs without crossovers and a modified form of the SC substitutes for chiasmata (von Wettstein et al. 1984). In *Drosophila* females, which utilize chiasmata to connect their three large chromosome pairs, pairing and segregation of the small fourth chromosomes proceeds without crossovers or chiasmata (Hawley and Theurkauf 1993). By contrast, all four chromosome pairs in *Drosophila* males form stable bivalents in the absence of recombination, chiasmata or a SC (McKee 1996).

Comprehensive and detailed studies in yeast and other model eukaryotes have revealed much detail on the mechanisms of synapsis and recombination, and these topics are summarized in many excellent reviews (Roeder 1997; Page and Hawley 2004; San Filippo et al. 2008; Inagaki et al. 2010). In this chapter, we focus on ‘pairing’, the still largely mysterious process by which homologs find each other

and form initial connections, and we will pay particular attention to the roles of pairing centers (PCs) in this process.

HOMOLOGOUS PAIRING AND THE TELOMERE BOUQUET

Before homologous chromosomes recombine and form a bivalent, they must find each other within the cell nucleus. In most organisms, the initiation of homologous pairing occurs at numerous sites along chromosomes by a mechanism that still remains unclear. These early interactions are then stabilized only at sites where there is good flanking homology between chromosomes. In many organisms, this sorting and stabilizing process appears to be promoted by a meiosis-specific organization of chromosomes called the 'bouquet configuration', which is initiated by a clustering of telomeres on the inner nuclear envelope. The bouquet appears to facilitate homologous recognition and alignment by concentrating chromosomes within a limited region of the nuclear volume, thus enabling chromosome movements that promote the identification of homologs, perhaps by the DNA DSB repair process (Hiraoka 1998; Scherthan 2001; Harper et al. 2004). These movements are facilitated by the attachment of telomeres to nuclear envelope proteins that contain Sad1 and Unc-84 (SUN) and Klarsicht, ANC-1 and Syne-1 homology (KASH) domains. The SUN–KASH bridge interacts with specific elements of the cytoskeleton, such as dynein and kinesin, and provides a connection to cytoskeletal forces for moving chromosomes (Fridkin et al. 2009). An extreme example is observed in *Schizosaccharomyces pombe* in which a tight

bouquet forms near the spindle pole body in early prophase I, which drags the whole nucleus back and forth several times within the cell, forming elongated horsetail nuclei (Chikashige et al. 1994; Scherthan et al. 1994). Studies in live yeast cells, in which specific loci on the chromosome arms are labeled or when GFP-tagged Rap1, a telomere-associated protein, is used to label telomeres, have shown that oscillatory chromosome movements promote alignment of homologous chromosomes in early meiotic prophase (Ding et al. 2004; Trelles-Stricken et al. 2005). By contrast, mutants that are defective in bouquet formation, such as *taz1* (for telomere associated in *Schizosaccharomyces*) and *bqt2* (for telomere bouquet protein 2) mutants in *S. pombe* (Cooper et al. 1998; Davis and Smith 2006) and *pam1* (for *plural abnormalities of meiosis 1*) mutants in maize (Golubovskaya et al., 2002), exhibit a reduction in homologous pairing. These findings support the notion that bouquet formation facilitates homologous recognition and pairing.

INDUCTION OF PAIRING AT SPECIALIZED PAIRING CENTERS

Most organisms appear to use the type of pairing pathway described above, in which the telomere-led bouquet configuration facilitates presynaptic alignment, with the alignment stabilized by a combination of DSB repair and synapsis (Fig. 1-1). However, an alternative method for initiating chromosome pairing, which involves specialized pairing sites, has been described in both *Drosophila* and *C. elegans*.

Pairing centers in *C. elegans*

The existence of specialized pairing sites in *C. elegans* was initially inferred from the effects of reciprocal translocations – chromosome rearrangements involving the exchange of chromosome segments between two non-homologous chromosomes (Fig. 1-2A) – on the frequency of recombination. In individuals that are heterozygous for such a translocation, recombination is severely suppressed to one side of each translocation breakpoint but is elevated on the other side of the breakpoint (Rosenbluth and Baillie 1981; McKim et al. 1988; McKim et al. 1993). Similar behavior has also been reported for other types of rearrangements, such as deletions and duplications (Herman and Kari 1989; McKim et al. 1993; Villeneuve 1994). For example, duplications of the right end of the X chromosome rarely recombine with the homologous region of the normal X chromosome, whereas duplications of the left end of the X chromosome engage in recombination frequently (Herman and Kari 1989). These findings suggest that the homologous pairing capacity (i.e. information enabling homologous chromosomes to pair and recombine) is restricted to one end of each chromosome, and this observation has led to the mapping of homolog recognition regions (HRRs) or PCs (the term we will use in this chapter) near one end of each chromosome. Recent findings have verified the notion that autonomous homologous pairing capacity is restricted to one end of each chromosome. In translocation heterozygotes, all chromosomes synapse as bivalents even though two of the pairs are therefore homologously synapsed only over part of their lengths. In these mismatched pairs, the ends of chromosomes that contain the PC synapse homologously, whereas the non-PC

ends synapse non-homologously (Fig. 1-2B) (MacQueen et al. 2005). PCs have crucial roles in the homolog-pairing pathway, which is supported by the observation that deletion of both copies of a PC from homologs severely disrupts their recombination and segregation (Villeneuve, 1994; MacQueen et al. 2005).

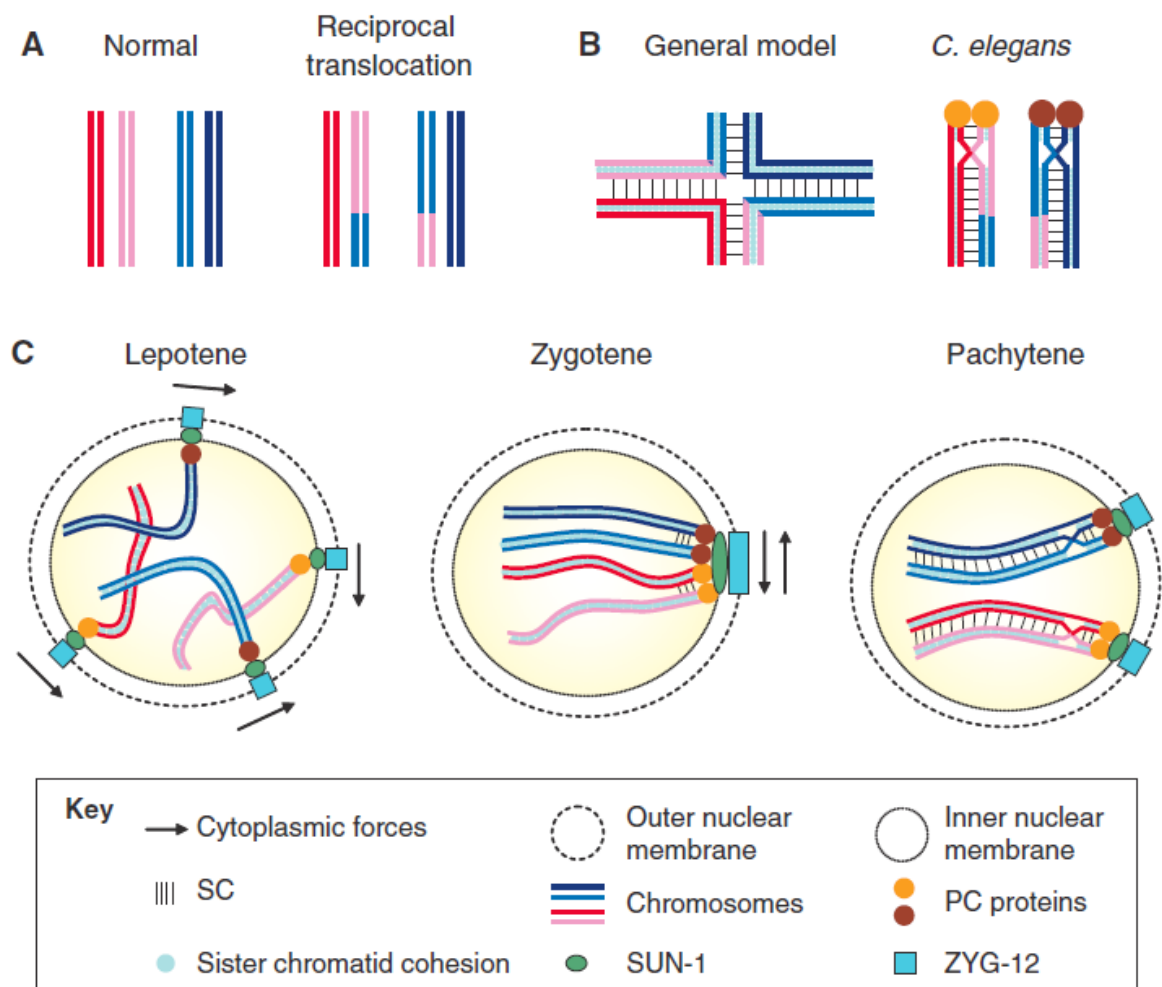
Detailed analyses have demonstrated two roles for PCs. First, they act locally to stabilize homolog alignment in a synapsis-independent manner (MacQueen et al. 2002; MacQueen et al. 2005). In the absence of synapsis (i.e. in animals depleted of essential SC components) transient pairing occurs at all tested chromosome sites during leptotene and zygotene. The ends of all chromosomes that contain the PC remain paired throughout prophase I, whereas sites that are distant from PCs are largely unpaired by mid-pachytene. This suggests that PCs function to locally stabilize an earlier chromosome-wide pairing process and, indeed, deleting PCs does eliminate this preferential stabilization. A second role of PCs is to initiate synapsis, a process that, once initiated, is largely homology independent. These roles are apparently independent of each other as synapsis occurs even in PC-deletion heterozygotes, in which the PC lacks a pairing partner (MacQueen et al. 2005).

Figure 1-2. Reciprocal translocation and homologous pairing model in *C. elegans*.

One Two pairs of homologous chromosomes are shown. Similar colors (i.e. blue and light blue and red and pink) indicate homologous chromosomes. Pairs of sister chromatids are shown in the same color.

(A) A reciprocal translocation is a type of chromosome rearrangement that involves the exchange of chromosome segments between two non-homologous chromosomes. (B) If all segments of chromosomes have autonomous pairing capacity and synapsis initiation activity, synapsis in translocation heterozygotes would be predicted to result in a quadrivalent configuration. In *C. elegans* reciprocal translocations, PCs are able to drive synapsis between two chromosomes as bivalents, even if some chromosomal regions are non-homologous (different colors). Recombination is suppressed in the non-homologous synapsed regions. (C) At leptotene, PC proteins are recruited to PCs. PCs are anchored to the nuclear envelope through interaction of PC proteins and the complex of the inner nuclear membrane protein SUN-1 and outer nuclear membrane protein ZYG-12. Chromosome ends are moved by cytoskeletal forces transmitted through the SUN-1–ZYG-12 bridge. Ongoing movement during the leptotene to zygotene stages brings multiple chromosome ends together into SUN-1-containing patches. Non-homologous chromosomes normally separate quickly. When the homologous chromosomes are found, cytoplasmic forces

oppose the association between homologous PCs resulting in tension, which triggers synapsis initiation. Once synapsis is initiated, homologous connections are cemented by the formation of the SC at zygotene and pachytene. At post-pachytene stages, the connections between homologs become dependent on chiasmata and not on the presence or absence of PCs, or their cognate proteins.



PC proteins and target sites *C. elegans*

Each PC is bound by one of four zinc-finger proteins, HIM-8, ZIM-1, ZIM-2 and ZIM-3, which are encoded in a single gene cluster. Two of these proteins bind in a chromosome-specific manner; HIM-8 binds to the PC on the X chromosome and ZIM-2 binds to the PC on chromosome V. The other two proteins bind to PCs on two different chromosomes – ZIM-1 to both the chromosome II and III PCs, and ZIM-3 to the PCs on chromosomes I and IV. Mutations in the genes that encode these proteins result in the expected chromosome-specific phenotypes. For example, *him-8* mutations disrupt X chromosome pairing, recombination and segregation but do not affect the meiotic behavior of autosomes. Interestingly, the phenotypes of *him-8* mutations are subtly, but consistently, more severe than the phenotypes that result from the deletion of the X chromosome PC, suggesting that HIM-8 acts at other sites in addition to the PC (Phillips et al. 2005; Phillips and Dernburg 2006).

Specific, but similar, target sequences for each of the zinc-finger proteins have recently been identified and found to be enriched in the PC regions of the appropriate chromosomes. These sequences are repeats of varying length and spacing that all have similar 12- bp core sequences, which have been shown to recruit the cognate zinc-finger proteins to their specific chromosomal target sites (Phillips et al. 2009). For example, deletion of the X chromosome PC abrogates recruitment of HIM-8 to the X chromosome. Insertion of arrays of target sequences onto a PC-deficient X chromosome restores HIM-8 recruitment and PC function

(Phillips et al. 2009). It remains unclear whether the target sequences have any function other than recruitment of the zinc-finger proteins.

Once the zinc-finger proteins are recruited to PCs, the resulting protein–PC complexes attach to the nuclear envelope by interacting with the SUN-domain-containing protein SUN-1 and the KASHdomain- containing protein zygote defective protein 12 (ZYG-12) to form a bridge spanning the nuclear envelope. This mechanism is very similar to that mediated by the telomere bouquet and is generally considered to be a variant of it (Penkner et al., 2009; Sato et al., 2009). SUN-1 is required for movement of chromosome ends and forms dynamic aggregates at the sites of PC attachment to the nuclear envelope. ZYG-12 is necessary to localize dynein, a cytoskeletal motor protein, to the nuclear envelope. Subsequently, the PC–SUN-1–ZYG-12 complex moves chromosomes along the nuclear envelope using dynein-dependent microtubule forces (Fig. 1-2C). This movement is thought to facilitate homologous recognition and synapsis during early prophase. Besides mediating chromosome movements, SUN-1 and ZYG-12 also cooperate to inhibit initiation of synapsis between transiently associated non-homologous chromosomes (Sato et al. 2009). However, how homology is assessed is still an open question. It has been proposed that dynein is required for SC polymerization. When dynein exerts forces that oppose the association of homologous PCs, the resulting tension might induce a mechanochemical signal through SUN-1 and ZYG-12 that leads to the initiation of synapsis (Sato et al. 2009). Baudrimont and colleagues (Baudrimont et al. 2010) have characterized the dynamic movements of SUN-1–GFP aggregates – the equivalent of chromosomal

attachment plaques – and demonstrated that multiple chromosome ends are brought together as SUN-1 foci fuse into SUN-1 patches. When homologous chromosomes encounter each other, sufficient affinity between them is generated to resist the cytoplasmic forces, so that synapsis can then be initiated, whereas cytoplasmic forces rapidly separate non-homologous chromosomes (Fig. 1-2C). The movement of chromosome ends through these patches continues until all of the homologous chromosomes have paired (Baudrimont et al. 2010).

The X–Y pairing site in *Drosophila*

In *Drosophila* the X and Y chromosomes share homology for the ribosomal RNA genes (the genes encoding 18S, 5.8S, 2S and 28S rRNAs; also known as rDNA), but are otherwise non-homologous. The rRNA genes are present in tandem arrays of 200–250 copies in the heterochromatin (genetically inactive chromatin) of the X chromosome and near the base of the short arm of the Y chromosome. In male meiosis, deletion of most of the proximal X chromosome heterochromatin, including the rRNA genes, results in a failure of X–Y pairing and high levels of X–Y nondisjunction (McKee 1996). Insertions of transgenes that contain single complete rRNA genes on such X chromosomes substantially restore X–Y pairing and segregation, indicating that the rDNA functions as the X–Y pairing site (McKee and Karpen 1990). Mapping studies have revealed that the pairing activity resides in 240-bp sequences that are found in tandemly repeated arrays of six to ten copies upstream of each rDNA transcription unit (Fig. 1-3A) (McKee et al. 1992). rDNA

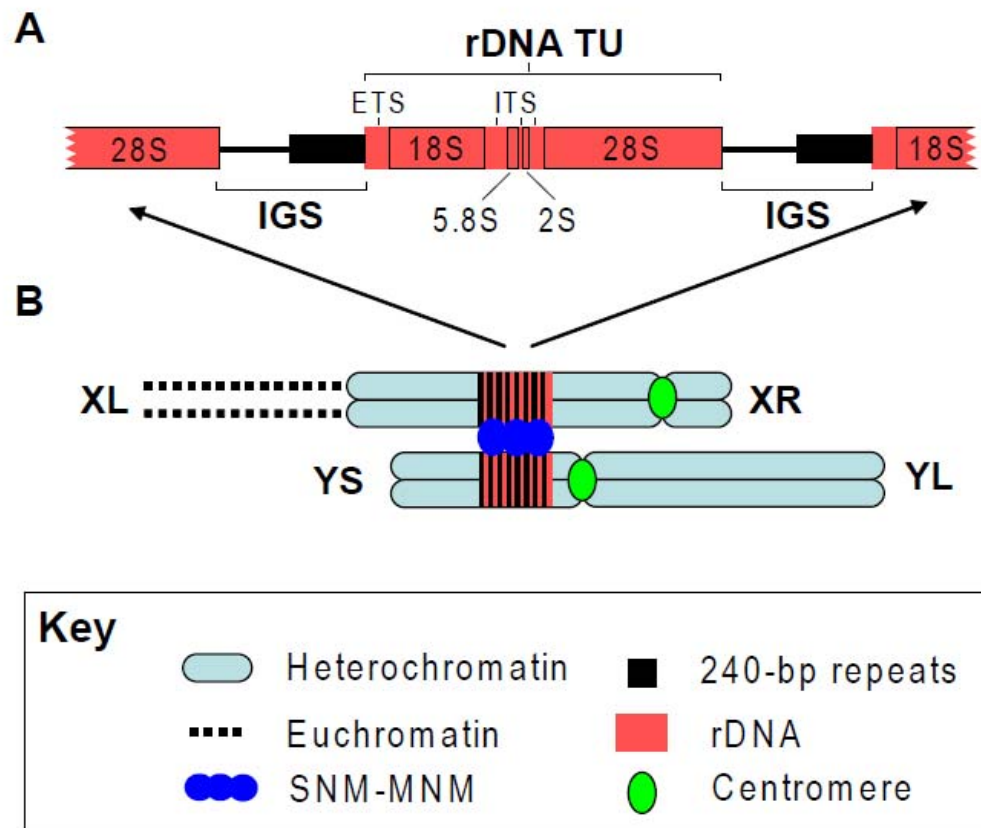
transgenes that include arrays of these 240-bp repeats restore pairing of rDNA-deficient X chromosomes, whereas rDNA transgenes lacking these repeats do not (McKee 1996). Thus, the X–Y pairing site comprises the 240-bp rDNA repeats.

Pairing proteins in *Drosophila* males

The X–Y pairing site is bound by the two proteins Stromalin in Meiosis [SNM; also known as Stromalin-2 (SA-2)] and Modifier of Mdg4 in Meiosis [MNM or Mod(mdg4)56.3], which are required for stable homolog pairing and segregation in male, but not female, meiosis (Thomas et al. 2005). Both proteins localize to chromosomes throughout meiosis I until they suddenly disappear at anaphase I, coincident with homolog segregation (Thomas et al. 2005). Thus, these proteins appear to substitute for chiasmata in supporting the association between homologs. Throughout meiosis I, SNM and MNM colocalize with each other and with the 240-bp repeats on the X–Y chromosome pair (Fig. 1-3B) (Thomas et al. 2005). Moreover, localization of SNM and MNM to the X chromosome is lost when the rDNA genes are deleted, but restored when transgenic 240-bp repeat arrays are inserted (Thomas et al. 2005; Thomas and McKee 2007). These findings indicate that the 240-bp repeats function to recruit the SNM–MNM complex to the sex chromosomes. SNM and MNM also localize to autosomes, where they have a role in maintaining pairing of autosomal homologs (Thomas et al. 2005), which is discussed further below.

Figure 1-3. X–Y chromosome pairing in *Drosophila* male meiosis.

(A) The rDNA transcription unit (TU) and intergenic spacer (IGS) region compose a complete rDNA unit. Each rDNA TU consists of the 18S, 5.8S, 2S and 28S genes, the external transcribed spacer (ETS) and internal transcribed spacers (ITS). Transcription units are separated by IGSs. The IGS comprises several arrays of tandem repeats, including five to ten copies of a 240-bp repeat located immediately upstream of the rDNA TU in each rDNA repeat. (B) The X and Y chromosomes are shown schematically, with heterochromatic regions as rounded rectangles, euchromatin as dotted lines and centromeres as green ovals. rDNA loci are located in the central region of the X heterochromatin and near the base of the short arm of the Y heterochromatin. SNM and MNM, are recruited to 240-bp repeats and mediate stable homologous connections, analogous to chiasmata, throughout meiosis I until anaphase I. XL, the left arm of the X chromosome; XR, the right arm of the X chromosome; YS, the short arm of the Y chromosome; YL, the long arm of the Y chromosome.



DO PCS FUNCTION DIRECTLY AS PAIRING SITES?

The role of the *C. elegans* PC sequences in pairing is not entirely clear. On the one hand, heterologous pairing or synapsis between native chromosomes that share the same PC protein and target sequences, such as chromosomes II and III or chromosomes I and IV, is never observed. On the other hand, multi-copy transgenes comprising large blocks of protein recruitment sequences, where the density of these sequences is much higher than in the wild type, can induce heterologous pairing when located on non-homologous chromosomes (Phillips et al. 2009). Thus, these sites can function as direct pairing sites when artificially concentrated, but they probably do not function in this way on native chromosomes. In the native situation, the PC sequences are interspersed with other unrelated sequences; therefore, these non-PC sequences might function to test for homology. On the basis of this interpretation, PC sequences might function indirectly to promote pairing of nearby sequences, but not to provide the main sites for stable homolog connections (Phillips and Dernburg 2006). This is consistent with the view that the *C. elegans* PCs function in a manner similar to telomeres in the bouquet mechanism. As discussed above, telomeres are thought to promote pairing of nearby sequences rather than to provide homolog recognition sites directly.

By contrast, the 240-bp repeats in *Drosophila* probably function directly as pairing sites, rather than by merely stimulating the pairing of linked non-PC sequences. The best evidence for this comes from studies in which transgenic arrays of the 240-bp repeat were inserted at random sites in the euchromatin (the part of the chromosome most active in gene expression) of an X chromosome

deficient for native rDNA. All such insertions were effective in partially restoring X–Y pairing (McKee 1996). However, because the Y chromosome lacks homology to the X euchromatin it is hard to see how X chromosome euchromatic sequences near the PC insertions could contribute to pairing. A role for nearby non-PC sequences in pairing of normal X and Y chromosomes cannot be ruled out. The 240-bp repeats in these chromosomes are interspersed with longer rDNA transcription unit sequences that are shared between the X and Y chromosomes. These regions could serve as additional sites for pairing interactions, even though those sequences lack autonomous pairing capacity as isolated transgenes (McKee et al. 1992; McKee 1996).

PCS APPEAR TO HAVE DIFFERENT ROLES IN THE CHROMOSOME SEGREGATION PROCESS BETWEEN ORGANISMS

The PCs in both *C. elegans* and *Drosophila* have been shown to function in the stabilization or maintenance of pairing (McKim 2005). However, the term ‘stabilization’ has different meanings in the two systems. As described above, in *C. elegans* the PCs act in early meiotic prophase (zygotene) to stabilize initial pairing interactions and promote synapsis (MacQueen et al. 2005). HIM-8 and the ZIM proteins also act early in meiosis, as shown by the timing of the *him-8* and *zim* mutant phenotypes, which appear as early as zygotene, and by the fact that these proteins are removed from chromosomes by the end of pachytene (Phillips et al.

2005; Phillips and Dernburg 2006). These observations suggest that PC proteins are needed only for pairing and synapsis and are dispensable for later steps in the homolog segregation pathway.

By contrast, analysis of tagged autosomal loci in *mnn* and *snm* mutants in *Drosophila* reveals that SNM and MNM are dispensable for pairing in early prophase; there is no diminution in pairing frequencies in these mutants relative to the wild type (Thomas et al. 2005). Instead, *mnm* and *snm* mutations disrupt chromosome behavior from mid-prophase when chromosome territories – chromosome-specific nuclear domains that contain both homologs of a bivalent – appear more diffuse when compared with that in the wild type. Subsequently, when chromosomes condense at prometaphase I, they do so as univalents in *snm* and *mnm* mutants (Thomas et al. 2005). Moreover, SNM and MNM are retained on chromosomes until anaphase I, indicating a much later role for these proteins in pairing maintenance than that of the HIM-8 and ZIM proteins. Thus, both SNM and MNM and the HIM-8 and ZIM proteins function to stabilize pairing, yet they do so at distinct stages of the homolog segregation process.

OTHER SPECIALIZED SITES IN *DROSOPHILA*

The findings described above show that PCs can perform essential roles in meiotic chromosome pairing. An interesting question is whether such roles are confined to specific isolated cases or whether PCs are general phenomena. In light of the compelling evidence for a PC on the X–Y chromosome pair in *Drosophila*, an

obvious question is whether PCs contribute to pairing of other *Drosophila* chromosomes, in either male or female meiosis.

Autosomes in *Drosophila* male meiosis

In light of the evidence for PC-directed X–Y chromosomal pairing, it has also been suggested that pairing of autosomes in *Drosophila* male meiosis involves specific sites (Vazquez et al. 2002). However, numerous studies involving diverse techniques have failed to provide any convincing evidence for such sites on autosomal chromosomes 2 and 3, which together account for ~80% of the *Drosophila* genome. These studies, which have been extensively reviewed elsewhere (McKee 1998; McKee 2004), demonstrate that the euchromatic regions of chromosomes 2 and 3 pair at multiple interstitial sites in early prophase and that heterochromatic regions lack autonomous pairing capacity. However, they do not rule out the possibility of specific nonautonomous pairing sites (i.e. sites at which connections depend upon prior alignment of homologs in linked euchromatic regions) in centric heterochromatin. A recent fluorescent *in situ* hybridization (FISH) analysis, using probes to chromosome-specific repeated sequences, failed to detect any such stable pairing sites (i.e. sites that remain paired throughout meiosis I) in the heterochromatic regions of the major autosomes (Tsai et al. 2011), thus providing further evidence against PC-based pairing of the major autosomes. However, the small fourth chromosomes did remain paired at a specific heterochromatic site in >90% of spermatocytes throughout prophase I, suggesting that the fourth chromosome contains a PC. It remains to be determined whether

fourth chromosome pairing will map to a specific site or whether it is a chromosome-wide phenomenon.

The failure to detect PCs on the major autosomes during *Drosophila* male meiosis leaves unanswered the important question of how these homologs remain connected after the loss of intimate allelic pairing at the mid-G2 transition. The autosomal homologs share a common territory throughout mid- and late-prophase I and condense into well-aligned bivalents at prometaphase I; thus, this indicates that an unknown factor could keep them together. SNM and MNM are involved in this process, as mutations in both lead to a loss of territory definition (Thomas et al. 2005). SNM and MNM are also observed within autosomal chromatin (Thomas et al. 2005), but the binding sites of SNM and MNM to autosomes remain undefined. Furthermore, recruitment of MNM, and perhaps SNM, to autosomes depends upon the Teflon (TEF) protein, which is required for segregation of the autosomes but not the sex chromosomes (Tomkiel et al. 2001; Thomas et al. 2005). We have suggested, by analogy to chiasmata in recombinational meiosis, that stable connections between autosomal homologs exist at different sites in different meiotic cells (Tsai et al., 2011). Time-lapse analyses in living spermatocytes using GFP-tagged chromosomal sites could be useful for revealing such stable connections; a stable connection site that happened to lie sufficiently close to a tagged chromosomal site should restrict the relative mobility of the tagged homologous alleles, perhaps dramatically so in favorable cases.

Boundary sites appear not to function in pairing in *Drosophila* females

Until recently, *Drosophila* females were also thought to utilize specific sites to pair their chromosomes, but recent findings have discredited this idea. As in *C. elegans*, the 'pairing sites' in *Drosophila* females were identified in flies that were heterozygous for reciprocal translocations. Females that were heterozygous for X; 4 translocations, carrying one normal X chromosome and one that is broken into two pieces each attached to a portion of the tiny fourth chromosome, were utilized. In each genotype in the original study, X recombination was found to be suppressed in a distinct interval around the translocation breakpoint but occurred at normal frequencies elsewhere on the X chromosome (Hawley 1980). Analysis of several such translocations suggested that the X chromosome was subdivided into three discrete autonomously recombining regions defined by four widely distributed 'boundary sites'. As the translocation breakpoints disrupted recombination only within the region they interrupt, it was thought that these sites functioned as alignment sites and that adjacent pairs of boundary sites are required to be in *cis* in order to mediate alignment of the intervening region (Hawley 1980). A recent study found similar behavior for another group of translocations, which led to the mapping of two boundary sites on chromosome arm 3R (Sherizen et al. 2005). However, molecular analysis of pairing in females that were heterozygous for these 3R translocations revealed that both pairing and synapsis in the recombinationally suppressed regions still occurred at normal frequencies (Sherizen et al. 2005). Normal levels of pairing and synapsis were also observed in females that were heterozygous for a normal sequence X chromosome and a multiply rearranged

balancer X chromosome, a genotype in which recombination is completely suppressed (Gong et al. 2005). Thus, if the boundary sites identified in the previous studies do have roles in pairing or synapsis, then this role appears to be more subtle than originally hypothesized. At least within euchromatic intervals, alignment and synapsis in *Drosophila* female meiosis apparently does not rely on specific defined sites but rather on multiple interactions throughout homologous regions.

PAIRING CENTERS: COMMON FEATURES OF MEIOTIC CHROMOSOMES?

As described above, in *Drosophila*, analyses of pairing have failed to identify any additional PCs and have instead indicated that general homology pairing is the predominant pairing mechanism. Moreover, nothing similar to the PCs of *C. elegans* or the X–Y chromosome pair in *Drosophila* has been described in other organisms. Nevertheless, as summarized below, phenomena suggestive of PC-like properties have been described for specialized chromosomal sites, including nucleolus organizer regions (NORs), telomeres and centromeres. As noted above, telomere meiotic function above has similarity to the functioning of *C. elegans* PCs. However, the data on centromere pairing are particularly intriguing and will be analyzed in some depth below.

Nucleolus organizer regions

In general NORs are not thought to have prominent roles in pairing or synapsis. In organisms in which preferential synapsis initiation sites have been mapped, these sites do not coincide with NORs (Page and Hawley 2004). Indeed in budding yeast, NORs are apparently excluded from synapsis (Tsubouchi et al. 2008). However, PC-like behavior has been reported for NORs in *ahp2* mutants of *Arabidopsis thaliana*. AHP2 is a homolog of the homologous-pairing protein 2 (HOP2), which is conserved among yeast, animals and plants and has been shown, in several organisms, to be required for proper homolog partner choice. In *Arabidopsis*, the *ahp2* mutation was found to severely disrupt meiotic pairing and synapsis at most genomic sites. However, the short arms of chromosomes 2 and 4, where the two NORs are located, exhibit normal pairing frequencies and normal SC formation. These findings indicate that the NORs act as cis-acting pairing and synapsis initiation sites in *ahp2* mutants (Stronghill et al. 2010), in a manner reminiscent of *C. elegans* PCs. The extent to which NORs contribute to pairing of chromosomes 2 and 4 in wild-type plants, and whether NORs exhibit similar behavior in other organisms, still remains to be determined.

Centromeres and centric heterochromatin

Centromeres have been reported to pair during meiosis in a wide variety of organisms (reviewed by Stewart and Dawson 2008). In addition to clustering of both homologous and non-homologous centromeres, which is a common feature of

pre-meiotic and early meiotic nuclei, the pairwise association of centromeres before the general onset of pairing and synapsis has also been observed in budding yeast and wheat (Martinez-Perez et al. 1999; Tsubouchi and Roeder 2005). In both of these cases, however, early pairwise associations are not homologous but instead involve apparently random ‘couplings’ of centromeres, with the pairings becoming homologous as cells proceed through meiotic prophase. However, this transition appears to be driven by homologous interactions initiated in other chromosomal regions rather than by any homologous interactions of the centromeres themselves. FISH analyses in wheat show that telomeric and sub-telomeric regions pair earlier than centromeres in meiosis and that the transition from non-homologous to homologous centromere associations is driven by the progression of synapsis from the telomere towards the center of the chromosome. This suggests that the homology at specific sequences near telomeres, rather than at centromeres, is involved in the correct recognition and selection of partners (Corredor et al. 2007). Moreover, when the wheat centromeres are replaced with those from the corresponding rice chromosomes there is no effect on the pairing patterns in wheat nuclei, indicating that centromeres have no role in the sorting of homologous from non-homologous chromosomes. Similarly, interchromosomal ‘centromere swaps’ have no effect on meiotic chromosome pairing and segregation in budding yeast (Clarke and Carbon 1983).

Remarkably, however, in budding yeast, these non-homologous centromere couplings seem to have an important role in synapsis. During zygotene (by which time non-homologous couplings have been largely replaced by homologous

associations), a majority of the segments of the polymerized SC central element proteins (including the molecular zipper ZIP1) either have one end at a centromere or incorporate a centromere within them. This is consistent with the idea that synapsis frequently initiates at centromeres and can propagate either unidirectionally or bidirectionally (Tsubouchi et al. 2008). Moreover, ZIP1 localizes to centromeres before the onset of general synapsis, and the early non-homologous centromere couplings are completely dependent on ZIP1 (Tsubouchi and Roeder 2005). Thus, centromeres in yeast share some similarity to PCs in *C. elegans* and initiate synapsis, but, unlike PCs, they do not appear to have a main role for pairing partner identification.

Centromere pairing has also been reported later in meiotic prophase, after the disassembly of the SC in a number of organisms (Stewart and Dawson 2008). In budding and fission yeast and *Drosophila* females, these interactions are important for segregation of achiasmate chromosomes (i.e. those that lack chiasmata). In budding yeast, the centromeres of achiasmate chromosomes pair with each other during late prophase, irrespective of whether the chromosomes are homologs or non-homologs, and these chromosomes segregate preferentially to opposite poles with moderate efficiency (Kemp et al. 2004). Recent evidence shows that these late-prophase centromere couplings, like the earlyprophase couplings described above, require ZIP1. Moreover, loss of ZIP1 randomizes segregation of achiasmate chromosomes (Gladstone et al. 2009). In the same study, it was also found that centromeres of homologous chromosomes pair in late prophase and that this pairing promotes the orientation of homologous centromeres

to opposite poles. Thus, the budding yeast centromere provides an example of a chromosome pairing site with important roles in both synapsis and segregation, but which does not contribute directly to homologous partner choice.

In *Drosophila* females, centromeric heterochromatin regions pair throughout meiotic prophase and this pairing serves to promote the segregation of achiasmate homolog pairs (Dernburg et al. 1996; Karpen et al. 1996). Unlike in yeast, achiasmate chromosome segregation in *Drosophila* is at least partly homology driven (i.e. non-exchange X chromosomes segregate preferentially from other non-exchange X chromosomes, rather than from a non-exchange non-homolog (Hawley et al. 1992)); this is also more efficient as it yields segregation frequencies of ~100% in many cases. However, achiasmate centric pairing in *Drosophila* is not limited to centromeres. Mapping studies have shown that the pairing ability is diffusely distributed throughout large tracts of centric heterochromatin and that pairing frequency depends on the length of heterochromatic homology (Hawley et al. 1992; Karpen et al. 1996). The involvement of such extensive regions probably explains the homology dependence of achiasmate segregation in *Drosophila* and could also contribute to its high efficiency. Thus, although the *Drosophila* case provides the only compelling example in which centric pairing drives chromosome assortment and segregation on a homologous basis, it is unclear what role the centromeres themselves have in this process. An interesting possibility is that centromere associations do occur, perhaps non-homologously, as in budding yeast, but that these serve to promote homology testing and, eventually, enable the formation of stable connections within flanking heterochromatic domains.

Interestingly, *Drosophila* also provides what is perhaps the only clear example of truly homologous centromere pairing (as opposed to centric heterochromatic pairing), but in male rather than female meiosis. Using a GFP-tagged centromere protein to visualize centromeres in live cells, centromeres have been found to cluster non-specifically in early prophase, when euchromatic sequences are tightly paired, then to sort into pairwise and strictly homologous associations shortly after the loss of homologous pairing in the chromosome arms (Vazquez et al. 2002; Yan et al. 2010). A recent FISH study demonstrated that this pairing is centromere-specific and does not extend even into very nearby pericentromeric heterochromatin (heterochromatin situated near to a centromere) (Tsai et al. 2011), and thus could be an example of PC-like behavior. However, homologous centromere pairing is short-lived; centromeres become unpaired by mid-prophase I and remain unpaired throughout the remainder of meiosis I. The functional significance of these transient pairings and the basis for the homology dependence is unknown. Centromeres of different *Drosophila* chromosomes appear not to share DNA sequence homology (Sun et al. 2003), so there could be a sequence basis for such specificity. Alternatively, the specificity could be entirely adventitious, driven by the homologous pairing of linked arms earlier in prophase. Centromere pairing occurs shortly after the homologous chromosome pairs have resolved into separate nuclear territories, so that, when they pair, it is probable that a centromere only has access to the centromere of its homolog. It will be of interest to determine how centromeres pair in experimental situations in which both homologous and non-homologous pairing partners are available.

Overall, the evidence indicates that centromeres pair actively and specifically with each other, but that such pairings are generally not homology driven. Centromere pairing can nevertheless play important roles in synapsis and homolog segregation.

CONCLUSIONS AND PERSPECTIVES

We have described two prominent examples of the use of PCs to mediate homologous pairing. In both cases, the PCs function as recruitment sites for specialized pairing proteins. However, the pairing proteins and sites function somewhat differently in the two systems. In *C. elegans* PC-mediated homologous pairing is analogous to the telomere-led bouquet in mediating the pairing of linked sequences and the initiation of synapsis between homologs. *Drosophila* males, which lack recombination, synapsis and chiasmata, have evolved a specialized pairing site for the otherwise non-homologous X–Y chromosome pair and a unique protein complex containing SNM and MNM that substitutes for chiasmata, thereby providing stable inter-homolog connections. Surprisingly, however, *Drosophila* apparently does not utilize specific sites to pair any of their other chromosomes (with the possible exception of the tiny fourth chromosome pair) in either sex. Instead, general homology pairing appears to underlie homolog alignment and partner choice in both sexes. Important questions about the male achiasmate segregation system remain unanswered. One such question is how homolog alignment is translated into stable interhomolog connections, particularly on the

major autosomes, which appear to be unconnected throughout most of meiotic prophase despite occupying a common territory. Another is how SNM and MNM are recruited to autosomes. Finally, it remains to be determined how specific connections between the four different pairs of homologs are mediated by SNM and MNM. How chromosome specificity is achieved is also an important unanswered question in the *C. elegans* system. The precise role of the PC sequences and PC proteins in homolog pairing also remains to be established.

Overall, there is little evidence that PCs are widely used as a solution to the pairing problem. In most organisms, cytological and molecular evidence points to multiple sites along chromosomes that are able to initiate homologous interactions (Bozza and Pawlowski 2008; Roeder 1997). Nevertheless, most organisms do seem to rely on specific sites to help with various aspects of chromosome pairing, synapsis and segregation. Telomeres do not pair directly but do have a prominent role in homolog partner choice in many organisms, a role that seems remarkably similar, mechanistically, to that played by PCs in *C. elegans*. However, centromeres often do pair directly with each other, albeit nonhomologously. Centromere pairing has been shown to contribute to synapsis initiation, centromere orientation and achiasmate segregation but, with the possible exception of *Drosophila* females, probably not to homologous partner choice. Further research in a variety of model organisms should shed light on the relationships among these diverse pairing systems.

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CHAPTER 2

HOMOLOG PAIRING AND SISTER CHROMATID COHESION IN HETEROCHROMATIN IN DROSOPHILA MALE MEIOSIS I

A version of this chapter is modified from the manuscript that has been accepted by *Chromosoma* and is in press now. Jui-He Tsai primary contributions: designed FISH probes, carried out FISH experiments, analyzed FISH data, and wrote the manuscript draft.

ABSTRACT

Drosophila males lack meiotic recombination and chiasmata yet homologous chromosomes pair and disjoin regularly. It has been suggested that homologs may remain paired at sites within the heterochromatin of each arm. The X-Y pair utilizes a specific repeated sequence within the heterochromatic ribosomal DNA (rDNA) blocks as a pairing site. No pairing sites have yet been identified for the autosomes. To search for such sites, we utilized probes targeting specific heterochromatic regions to assay heterochromatin pairing sequences and behavior in meiosis by fluorescence *in situ* hybridization (FISH). We found that the small fourth chromosome pairs at heterochromatic region 61 and associates with the X chromosome throughout prophase I. Homolog pairing of the fourth chromosome is disrupted when the homolog conjunction complex is perturbed by mutations in SNM or MNM. On the other hand, six tested heterochromatic regions of the major autosomes proved to be largely unpaired after early prophase I, suggesting that stable homolog pairing sites do not exist in heterochromatin of the major autosomes. Furthermore, FISH analysis revealed two distinct patterns of sister chromatid cohesion in heterochromatin: regions with stable cohesion and regions lacking cohesion. This suggests that meiotic sister chromatid cohesion is incomplete within heterochromatin and may occur at specific preferential sites.

INTRODUCTION

The defining feature of meiosis is pairing and segregation of homologous chromosomes. Homolog segregation occurs during meiosis I and is followed by a second division (meiosis II) in which sister chromatids segregate. Homolog pairing initiates early in meiosis I, during the prophase stage, a lengthy and complex stage that involves extensive changes in chromosome architecture (McKee 2004; Roeder 1997). In most organisms, pairing is accompanied by recombination between the paired homologs in the context of an elaborate proteinaceous structure, known as synaptonemal complex (SC), that connects aligned homologs from end to end (Page and Hawley 2004). After completion of recombination and disassembly of SC, the connections between homologs that result from crossovers, known as chiasmata, serve as stable linkers that enable homologs to orient to opposite poles on the meiotic spindle (Carpenter 1994). Meiosis in male *Drosophila* is unusual in lacking recombination, SC and chiasmata (Meyer 1960; Morgan 1914). Yet homologs are stably associated throughout the meiotic divisions and segregate reliably to opposite poles. As in other eukaryotes, meiotic prophase I in male *Drosophila* is lengthy and entails complex changes in nuclear architecture as well as substantial growth (a 25-fold volume increase from young to mature primary spermatocytes) (Cenci et al. 1994; McKee 2004). However, due to the absence of recombination and SC, the usual substages of prophase I, which are based largely on morphological features of SC and chiasmata, are inapplicable. Instead prophase I is subdivided into stages named S1-S6 that are identified by cell and nuclear size and by various features of nuclear morphology observed in preparations stained

with fluorescent DNA dyes (Cenci et al. 1994). During the earliest stages, S1 and S2a, which follow immediately after premeiotic S phase, the eight chromosomes (representing four pairs, the X–Y, second, third, and fourth chromosomes) are intermingled and occupy most of the volume of the small nuclei. In the subsequent stage, S2b, a dramatic nuclear reorganization takes place in which the chromatin splits into three distinct territories that correspond to the three large chromosome pairs, the X–Y, second, and third chromosome pairs. The small fourth pair can sometimes be distinguished at this and later stages of prophase I but is often obscured by the larger chromosomes. The chromosome territories remain separate and peripherally located throughout the remainder of prophase I. The chromatin is decondensed throughout the latter stages of prophase I and little in the way of structural detail can be discerned. Consequently, although homologs occupy a common territory, it is not clear how they are configured with respect to one another. At prometaphase I, the chromosomes condense rapidly into compact bivalents and align on the metaphase I spindle in preparation for division at anaphase I.

Sex chromosome pairing in male meiosis has been intensively studied and provides an excellent example of the central role of specific *cis*-acting sequences (McKee 1996). The X and Y are largely non-homologous and pair only in a limited region that encompasses the ribosomal DNA (rDNA) loci that are located in the central region of the X heterochromatin and near the base of the short arm of the Y heterochromatin and contain 200–250 tandem copies of the genes for the 18S, 5.8S, and 28S ribosomal RNAs. Deletions of the X chromosome rDNA disrupt X–Y

pairing and result in random X–Y segregation at anaphase I, but transgenes carrying ribosomal RNA genes can significantly restore X–Y pairing and segregation (McKee and Karpen 1990). In addition to the rDNA, X–Y pairing requires two proteins, stromalin in meiosis (SNM) and modifier of Mdg4 in meiosis (MNM), that co-localize to the nucleolus during prophase I, then to the paired region of the X–Y bivalent during prometaphase I and metaphase I. Mutational loss of either protein causes X–Y pairing failure at prometaphase I and random X–Y assortment at anaphase I (Thomas et al. 2005).

Pairing of the autosomes has also been extensively investigated (McKee 1996, 2009). An analysis using the green fluorescent protein (GFP)-Lac repressor/lac operator system to label 13 separate sites on the euchromatic arms of the major autosomes (the second and third chromosomes) showed that homologous euchromatic loci are tightly paired throughout S1 and S2a, exhibiting pairing frequencies in excess of 90%, but that pairing of homologs (and of sister chromatids) disappears suddenly and dramatically at S2b, coincident with the appearance of distinct chromosome territories (Vazquez et al. 2002). Four separate GFP spots are henceforth visible at each lacO array insertion; these spots diffuse freely within the territory throughout middle and late prophase I but remain confined to the territory. Although this analysis was restricted to euchromatic regions, the pairing behavior of centromeres has been analyzed using a GFP tagged version of centromere identifier (CID), a centromere-specific histone. Homologous centromeres were found to be unpaired (although often clustered non-homologously) during S1 and S2, then to pair transiently at S3 (shortly after the loss

of euchromatic pairing), only to come unpaired again by S4 and to remain unpaired throughout the rest of meiosis I. Thus, although homologs experience intimate pairing of both euchromatin and centromeres during early prophase I, all such associations are lost long before the meiotic divisions. Nevertheless, homologs remain together in nuclear territories throughout the latter stages of prophase I, and condense into aligned bivalents at prometaphase I. How they remain stably associated and preserve the information needed to align properly when they condense is a mystery. What is known about these mysterious connections is that they depend on SNM and MNM (Thomas et al. 2005). Both proteins localize to the autosomal territories during prophase I and to the condensed autosomes at prometaphase I and metaphase I as well as to the X–Y pair. Moreover, mutational loss of either protein disrupts the integrity of autosomal territories during middle and late prophase I and leads to pairing failure and random segregation of all four pairs during meiosis I.

It has been suggested that homologs may remain paired at specific sites within the heterochromatin of each arm, analogous to the heterochromatic rDNA pairing sites of the X–Y chromosomes (Vazquez et al. 2002). A problem with this suggestion is that several cytogenetic studies have shown that autosomal heterochromatin is insufficient to promote pairing (Appels and Hilliker 1982; Hawley et al. 1992; McKee et al. 1993; Yamamoto 1979). For example, McKee et al. tested a series of 2-Y transpositions (rearrangements involving insertion of segments of chromosome 2 into the Y) heterozygous with normal X and second chromosomes for formation of quadrivalents involving the X–Y and second chromosome pairs and

for preferential segregation of the Y2 transposition element from the normal second chromosome (McKee et al. 1993). They found that all transpositions involving euchromatin of the second chromosome exhibited both elevated quadrivalent frequencies and preferential segregation, but one involving a large, entirely heterochromatic segment of the second chromosome did not. This, along with similar results from other studies seems to rule out the possibility that autonomous pairing sites are located in the heterochromatin of the major autosomes. However, the possibility of “passive” heterochromatic connections, driven by active pairing in adjacent euchromatin, as suggested by Vazquez et al. (2002), has not been examined. It seems possible that active pairing in euchromatic regions during early prophase leads to alignment of homologs and somehow induces connections between adjacent heterochromatic regions mediated by pairing factors such as SNM and MNM which persist throughout the late stages of meiosis I. If so, it might be possible to find autosomal heterochromatic sites that remain stably associated after loss of euchromatic and centromere pairing. The major goal of this study was to test this prediction using fluorescence *in situ* hybridization (FISH) with heterochromatic probes. The fourth chromosome, also known as the dot chromosome, undergoes achiasmate segregation in both male and female *Drosophila* and never undergoes genetic exchange. Based on its meiotic behavior, the fourth chromosome has been proposed to associate with the X chromosome during meiosis (Sandler and Novitski 1956). In female meiosis, the association of the X and fourth chromosomes has been examined using specific FISH probes, which showed both chromosomes in close proximity in the prophase nucleus.

Furthermore, pairing of the fourth chromosome has been shown to rely primarily on heterochromatic homology in females but on euchromatic homology in males (Dernburg et al. 1996; Hawley et al. 1992). Recently, the fourth homologs in female meiosis have been shown to undergo separation and re-association movements during prometaphase I. The great majority of oocytes showed chromatin threads between the fourth homologs during mid-prometaphase I (Gilliland et al. 2009; Hughes et al. 2009). However, the behavior of the fourth chromosome in male meiosis has not been described in detail. The availability of FISH probes that preferentially target the X, Y, and fourth chromosomes enables us to examine whether the sex and fourth chromosomes are physically associated in meiosis I in males.

Stable connections between segregating chromosomes are necessary to prevent premature separation. Besides homolog pairing, sister chromatid cohesion is also required for the meiotic divisions. Cohesion promotes the formation of SC, stabilizes chiasmata and functions in monoorientation of sister centromeres (Petronczki et al. 2003). Sister chromatid cohesion is established during DNA replication, holds sister chromatids together throughout the chromosome arms and the centromeres, and then is lost in a stepwise manner to achieve successive chromosome segregations. Cohesion is mediated by a multi-subunit cohesin complex which contains one member each of the SMC1, SMC3, SCC1/RAD21/REC8, and SCC3/SA families (Haering et al. 2008). In most eukaryotes, sister chromatid cohesion is disrupted in a two-step process in meiosis: the cleavage of the Rec8 subunit on chromosome arms at anaphase I leads to

homolog segregation, and the cleavage of centromere cohesin at anaphase II allows sister chromatid segregation. A Rec8 homolog has not been identified in *Drosophila*, but meiotic centromere cohesion has been shown to require the Orientation Disruptor and Sisters On the LOose (SOLO) proteins (Miyazaki and Orr-Weaver 1992; Yan et al. 2010). It has previously been shown using the CID-GFP marker that sister centromeres are paired throughout meiosis until anaphase II. However, arm cohesion in euchromatic regions is lost at the S2b/S3 transition, coincident with the loss of homolog pairing. The cohesion of heterochromatic regions outside the centromeres per se has not been examined.

In this study, stable homolog pairing sites for the major autosomes were not found. However, we demonstrate that heterochromatic region 61 (h61) contains stable homolog pairing sites for the fourth chromosome during meiosis and that the fourth chromosome is positioned non-randomly near the sex chromosomes in male meiosis. Furthermore, most heterochromatic sites we tested on the Y, second, and third chromosomes fail to maintain sister chromatid cohesion throughout meiosis, but a particular satellite (1.686 g/cm^3) present in heterochromatin of both second and third chromosomes exhibited stable cohesion throughout meiosis.

MATERIALS AND METHODS

Fly stocks

The *yw* strain was used as a wild-type control. The solo mutations were obtained from the Zuker-2 (Z2) collection of EMS mutagenized second chromosomes and *mn*m and *sn*m mutations were obtained from the Zuker-3 (Z3) collection of EMS mutagenized third chromosomes. The Z2 and Z3 lines used in this study were identified in a screen for paternal fourth chromosome loss and were provided by B. Wakimoto (Koundakjian et al. 2004; Thomas et al. 2005; Wakimoto et al. 2004). All flies were maintained at 23°C on standard cornmeal–sucrose–yeast–agar medium.

FISH probe preparation

The FISH probes were prepared in two ways in this study. The 359 bp repeat probe was amplified by PCR from *Drosophila* genomic DNA (Hsieh and Brutlag 1979) and labeled with fluorescein-12-dUTP using the Fluorescein-High Prime kit (Roche Applied Science). Other probes, including those for the AATAG repeat (AATAG)₆, the AATAC repeat (AATAC)₆, the AACAC repeat (AACAG)₆, the 1.686 g/cm³ satellite (AATAACATAG)₃, the AATAT repeat (AATAT)₆, the dodeca satellite (CCCGTACTGGTCCCGTACTGGTCCCGTACTCGGTCCCGTACTCGGT), and the Responder (Rsp) repeats (GTCAAATGGGTGATTTTTTCGATTTCAAGT), were synthesized as a single-stranded oligonucleotides (Integrated DNA Technologies). A labeling reaction (20 µl volume) contained 1 µl of 100 µM

oligonucleotides, 1 μ l of 1 mM fluorescein-12-dUTP or Alexa Fluor 546-14-dUTP (Molecular Probes), 60 U of terminal deoxynucleotidyl transferase (Promega) and 4 μ l 5 $\times$ buffer. Reactions were carried out for 20 h at 37°C and stopped by heating at 70°C for 10 min. Probes were used without further purification.

FISH analysis

FISH experiments were carried out by the procedure of Thomas and McKee (2009). Briefly, spermatocytes were fixed on the precleaned slides (Fisher Scientific) then passed through an ethanol series and air dried at room temperature. To rehydrate, the slides were incubated three times for 10 min in 2 $\times$ SSCT (2 $\times$ SSC containing 0.1% Tween-20), once for 10 min in 25% formamide/2 $\times$ SSCT and 50% formamide/2 $\times$ SSCT, and prehybridized for 3 h at 37°C in 50% formamide/2 $\times$ SSCT. Of the hybridization buffer, 20 μ l (3 $\times$ SSC, 50% formamide and 10% dextran sulfate) containing 0.1–2 μ l of the labeled probe(s) was added to each slide. The slides were covered with a siliconized coverslip and sealed with rubber cement. Probes and samples were denatured at 95°C for 6 min and hybridized at least 20 h at different temperatures (16°C for the AATAT probe, 37°C for the dodeca probe, and 20°C for the other probes). After hybridization, the slides were washed three times with 50% formamide/2 $\times$ SSCT, once with 25% formamide/2 $\times$ SSCT, and three times with 2 $\times$ SSCT. The slides were counterstained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) and mounted with VECTASHIELD medium (Vector).

Microscopy and image processing

The developmental stages of spermatocytes were distinguished by cell size, shapes of DNA territories and cell number per cyst, following the criteria of Cenci et al. (1994). All testis preparations were examined using an Axioplan (Zeiss) microscope equipped with an HBO 100-W mercury lamp, Plan-Neofluar 100×/1.30 NA oil immersion lenses (Zeiss), and a high-resolution CCD camera (Roper) at room temperature. Grayscale digital images were collected, pseudocolored, and merged using Metamorph Software (Universal Imaging Corporation). Images were processed with Photoshop and Illustrator (CS2; Adobe).

Spots determination and distance measurement

Each FISH probe targets one or two specific chromosome regions. At prometaphase, the signals are presented as discrete spots. However, during prophase I, when the chromosomes were decondensed, the signals sometimes appeared as groups, consisting of one spot of strong intensity and several small faint spots, instead of single spots. To measure pairing, we counted the numbers of signal spots or groups during prophase I, prometaphase I, and prometaphase II. At all meiotic stages, signals were considered as one spot (group) if the boundaries of spots (groups) overlapped. When the chromosomes were condensed, such as at prometaphase I and II, two spots in close proximity could be resolved when the distance between the centers of peak intensities was $\geq 0.7 \mu\text{m}$, similar to the ranges

of the Vazquez et al. (2002) study. We measured the “background” frequency of spot overlap where two unpaired signals in the same territory appear as a paired signal due to overlapping positions using the AATAC and 359 bp probes that target unpaired sites (non-rDNA regions) of the Y and X chromosomes, respectively. The frequency of such overlapping signals at prometaphase I was measured to be 3.4% (N=29).

For distance measurements between heterologous probe signals where more than one spot for each probe was present, an average spot distance was determined for each spermatocyte. For example, in a spermatocyte with two spots (A1 and A2) of the AATAT (h61) probe and two spots (B1 and B2) of the dodeca (h53) probe at prometaphase I, the average of the four distance measurements A1–B1, A1–B2, A2–B1, and A2–B2 was used as the distance between the h61 and h53 probes of this spermatocyte. The distances were further analyzed by JMP 7.02 of the SAS statistical packages (SAS Institute Inc., Cary, NC, USA), followed by Student’s t tests in order to identify whether the distances between the fourth and X chromosomes during meiosis were significantly different from the distances between the fourth and other chromosomes.

RESULTS

Specific heterochromatic regions for homolog pairing in *Drosophila* male meiosis

Since euchromatic and centromere pairing are both lost after stage S3 in male meiosis, the heterochromatin is the priority candidate for homolog pairing. Therefore, we examined pairing between chromosomes by probes that are restricted to one or two heterochromatic region(s).

Pericentromeric pairing does not contribute to homolog conjunction for the second and third chromosomes

Homologous centromeres have been shown to transiently pair at stage S3 but come unpaired at stage S4 by analysis of CIDGFP transgenic flies or immunostaining with anti-CID antibody during spermatocyte development (Vazquez et al. 2002; Yan et al. 2010). Based on the hypothesis that the transient pairing of centromeres might trigger or promote pericentromeric heterochromatin pairing, we tested probes close to the centromeres of chromosome 2 and 3, *Rsp*, and dodeca satellite. The *Rsp* locus was mapped to region h39 on the second chromosome (Brittnacher and Ganetzky 1989; Pimpinelli and Dimitri 1989). The dodeca satellite was discovered and mapped to region h53 on the third chromosome by Villasante et al. (Abad et al. 1992; Fig. 2-1). Several studies have used dodeca sequences as the centromeric probe for salivary gland polytene chromosomes (Andreyeva et al. 2007; Cortes et al. 2003). Furthermore, the dodeca satellite was shown to form

connections between sister chromatids during mitosis (Carmena et al. 1993). We examined pairing of homologs from early prophase I until prometaphase II. Of the spermatocytes, 30–50% showed single spots of the Rsp (h39) or dodeca (h53) probes at stages S1 and S2, indicating that homologs were sometimes paired at the h39 and h53 regions during early prophase I when euchromatic alleles are paired and centromeres are often unpaired although frequently clustered non-specifically (Fig. 2-2 and Table 2-1). Surprisingly, less than 30% of spermatocytes showed single spots of the h39 or h53 probes at stage S3. These low pairing frequencies of pericentromeric sequences are remarkable since this is the stage when homologous centromeres are fully paired, based on analysis of CID-GFP spots. After S3 when homologous centromeres are unpaired, both probes showed less than 10% of spermatocytes with single spots. These low frequencies persisted from S4 to prometaphase I. These results indicate that the h39 and h53 regions are not stable homolog pairing sites. Even though h39 and h53 are close to their respective centromeres, neither transient centromere pairing nor stable centromere cohesion induces stable connections in these regions.

Figure 2-1. Probes to chromosome-specific repetitive sequences in the *Drosophila melanogaster* genome.

The cytogenetic reference map of heterochromatic regions of the chromosomes is modified from that of Pimpinelli et al. (1995) and Hoskins et al. (2007). The five chromosomes are shown schematically, with heterochromatic regions as rectangles, euchromatin as solid lines and centromere as "C". The corresponding heterochromatic regions are labeled underneath the FISH probes. There are three pericentromeric probes: the 359 bp repeat probe targets heterochromatic region 31 (h31) on the X chromosome close to the centromere; the *Rsp* probe targets a site near the centromere of the second chromosome in h39; the dodeca probe targets h53 which is close to the centromere of the third chromosome. Probes for non-centromeric heterochromatin are: the AATAC repeat probe targets h6 on the Y chromosome long arm; the 1.686 g/cm³ satellite probe targets both the second and third chromosomes in h37 and h48; the AATAG repeat probe targets h4 on the Y chromosome long arm and h36 on the second chromosome; the AACAC repeat probe targets h23 on the Y chromosome short arm and h42 on the second chromosome; the AATAT repeat probe targets several sites on all the chromosomes but the most intense hybridization site is h61 on the fourth chromosome.

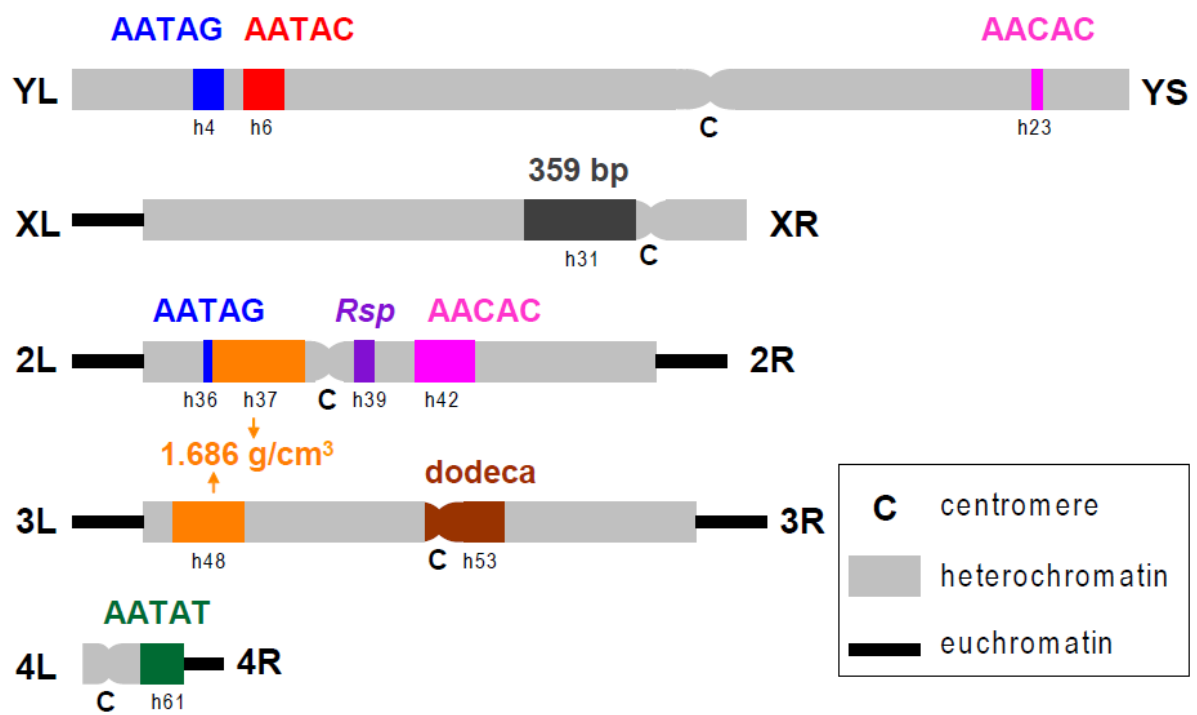


Figure 2-2. The dodeca (h53) probe signals at different stages of spermatocytes.

The spermatocytes from young yw flies were used for FISH screening. Early prophase represents stages S1–S2; middle prophase represents stages S3–S4; late prophase represents stages S5–S6. The h53 probe signals are shown as green and DNA, stained by DAPI, is shown as red. Size bar represents 5 μm .

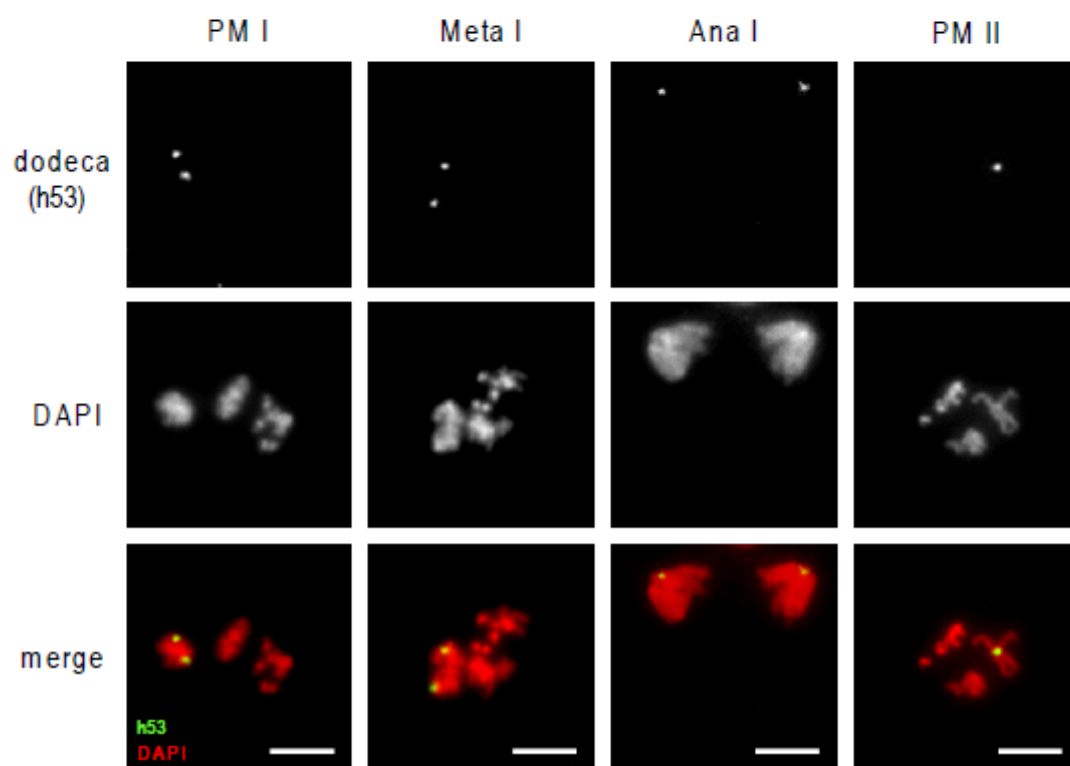
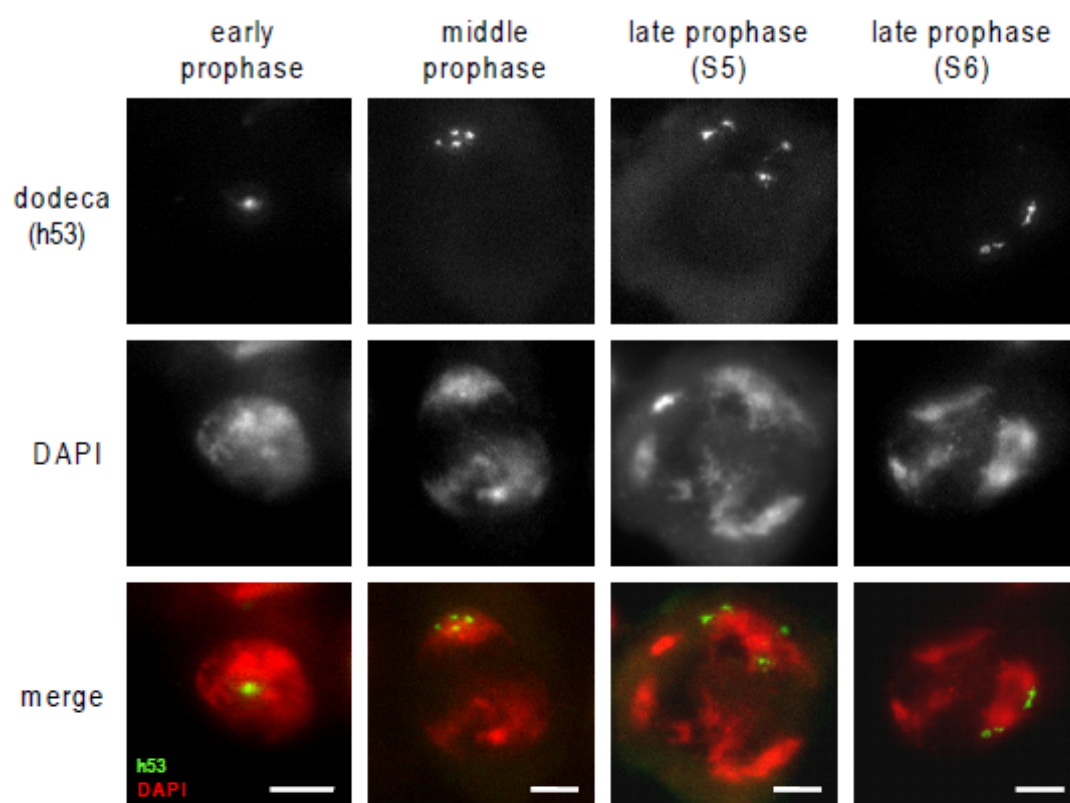


Table 2-1. Pairing percentages of pericentromeric probes.

Meiotic stages	S1	S2a	S2b	S3	S4	S5	S6	PM I	PM II
The <i>Rsp</i> (h39) probe: 2R									
1 Spot	46	40	39	6.0	3.7	1.5	2.2	2.4	83
2 Spots	44	60	61	65	48	49	51	93	17
3 or 4 Spots	9.4	0.0	0.0	27	48	49	47	4.9	0.0
<i>N</i> (cell number)	180	80	18	83	79	135	89	41	66
The dodeca (h53) probe: 3R									
1 Spot	55	31	33	28	9.7	7.8	8.7	0.0	100
2 Spots	35	61	67	69	56	46	74	100	0.0
3 or 4 Spots	9.7	8.0	0.0	2.8	35	46	17	0.0	0.0
<i>N</i> (cell number)	155	62	21	72	113	128	69	79	37

Spot frequencies of the h39 probe for the second chromosome pericentromeric region, the h53 probe for the third chromosome pericentromeric region at prophase I (S1–S6), prometaphase I (PM I), and prometaphase II (PM II) of *yw* flies. One spot indicates that homologs are paired; two spots indicate that homologs are unpaired but sister chromatids are paired; three or four spots indicate that sister chromatids are unpaired from prophase I to prometaphase I. For prometaphase II, one spot indicates that sister chromatids are paired and two spots indicate that sister chromatids are unpaired.

Pairing of non-centromeric heterochromatic regions on the second and third chromosomal arms

The 1.686 g/cm³ satellite consists mainly of AATAACATAG sequences (10 bp repeats), representing about 2.1% of the *Drosophila melanogaster* genome (Lohe and Brutlag 1986). Previous studies with a biotinylated probe showed that these repeats, for the most part, localize to regions h37 on the second chromosome and h48 on the third chromosome (Koryakov et al. 2003; Lohe et al. 1993; Pimpinelli et al. 1995). Therefore, the 1.686 g/cm³ satellite (h37&h48) probe targets non-centromeric heterochromatic sites on chromosomal arms 2L and 3L (Fig. 2-1). In this study, the h37&h48 probe, (AATAACATAG)₃, was labeled at the 3' end with fluorescein-12-dUTP and hybridized to young *yw* fly testes. A h53 probe labeled with Alexa Fluor 546-14-dUTP was applied simultaneously to enable us to distinguish h37 and h48 signals on the second or third chromosome. For region h37, most spermatocytes exhibited one spot at early prophase I (S1–S2), suggesting that homologs are paired at this heterochromatic site during the stages when euchromatic alleles are also paired. However, one-spot frequencies dropped dramatically at region h37 at the S2b/S3 transition when euchromatin comes unpaired, then dropped again at the S3/S4 transition when homologous centromere pairing is lost. One-spot frequencies fell to less than 20% at late prophase I (S5–S6), and to only 10% at prometaphase I (Fig. 2-3 and Table 2-2). Similar results were observed for heterochromatic region 48 on the third chromosome: homolog pairing frequencies were high at early prophase but then dropped to very low levels after loss of euchromatic and centromere pairing (Fig. 2-3 and Table 2-2). We thus

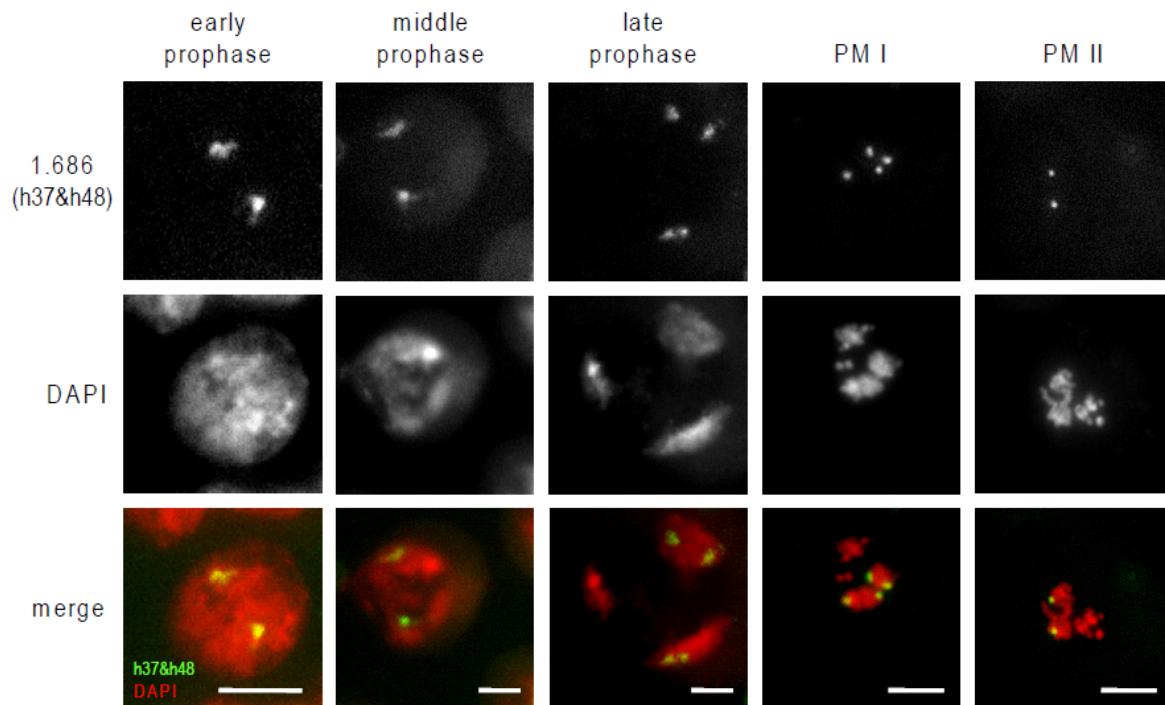


Figure 2-3. The 1.686 g/cm³ (h37&h48) probe signals at different stages of spermatocytes.

The 1.686 g/cm³ (h37&h48) probe signals at different stages of spermatocytes. The h37&h48 probe signals are shown as green and DNA, stained by DAPI, is shown as red. Size bar represents 5 μ m.

Table 2-2. Pairing percentages of the 1.686 g/cm³ (h37&h48) probe.

Meiotic stages	S1	S2a	S2b	S3	S4	S5	S6	PM I	PM II
2L (h37)									
1 Spot	92	89	87	49	22	17	10	10	100
2 Spots	7.6	11	13	50	78	82	87	90	0.0
3 or 4 Spots	0.0	0.0	0.0	0.7	0.0	1.0	2.5	0.0	0.0
<i>N</i> (cell number)	144	53	30	155	78	99	79	30	78
3L (h48)									
1 Spot	94	87	83	45	19	13	6.3	3.3	100
2 Spots	5.6	13	17	54	78	85	94	97	0.0
3 or 4 Spots	0.0	0.0	0.0	0.7	2.6	2.0	0.0	0.0	0.0
<i>N</i> (cell number)	144	53	30	155	78	99	79	30	78

Spot frequencies of the h37&h48 probe for the second chromosome and the third chromosome at prophase I (S1–S6), prometaphase I (PM I), and prometaphase II (PM II) of *yw* flies. One spot indicates that homologs are paired; two spots indicate that homologs are unpaired but sister chromatids are paired; three or four spots indicate that sister chromatids are unpaired from prophase I to prometaphase I. For prometaphase II, one spot indicates that sister chromatids are paired and two spots indicate that sister chromatids are unpaired.

conclude that neither h37 nor h48 contains a stable site for homolog pairing. Since there was no statistically significant difference between the pairing frequencies of h37 and h48 at middle and late prophase I and prometaphase I by chi-square analysis, it appears that the pairings of h37 and h48 are independent. Furthermore, some spermatocytes (<5%) exhibited two separated spots (average distance ~3.8 μm) within one DNA territory that were connected by thread-like signals, suggesting that heterochromatic regions might still interact with each other (Fig. 2-4). Even when heterochromatic regions were connected by thread structures in these spermatocytes, we still counted the signals as two spots since two chromosome spots clearly did not overlap.

The AACAC repeats on the Y chromosome and second chromosome were discovered by Zhimulev et al. and mapped to regions h23 and h42 (Makunin et al. 1995). The AATAG repeats are present at region h4 on the Y chromosome and region h36 on the second chromosome (Lohe et al. 1993; Fig. 2-1). Previous studies demonstrated the existence of at least six genetic elements that map on the Y chromosome: four on the long arm (kl-5, kl-3, kl-2, and kl-1) and two on the short one (ks-1 and ks-2), called fertility factors, which contain substantial amounts of satellite DNAs (Gatti and Pimpinelli 1992; Piergentili and Mencarelli 2008). kl-5, kl-3, and ks-1 have huge physical dimensions, spanning 4,000 kb each and their DNA is involved in the formation of lamp brush-like loops inside primary spermatocyte nuclei (Bonaccorsi et al. 1988). Both probes exhibited massively spread-out signals in prophase I spermatocytes, particularly the AACAC (h23&h42) probe that targets ks-1 (data not shown). These massive signals made it difficult to distinguish

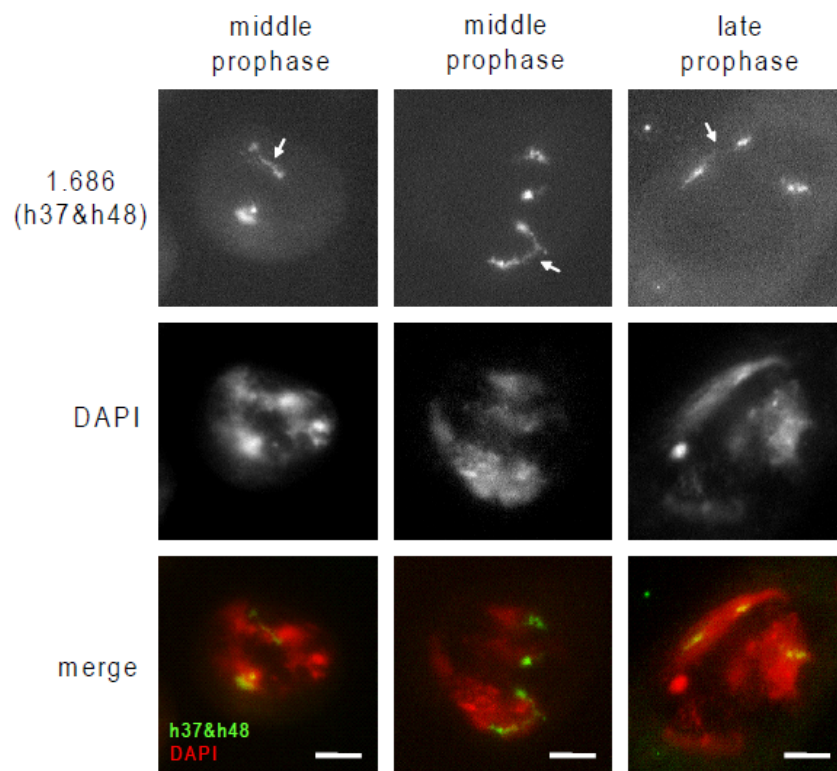


Figure 2-4. Thread-like signals of the 1.686 g/cm³ (h37&h48) probe at middle and late prophase I of spermatocytes.

The h37&h48 probe signals are shown as green and DNA, stained by DAPI, is shown as red. White arrows indicate thread-like signals between homologs. Size bar represents 5 μ m.

the spots in the DNA territories; therefore, we were unable to determine whether h36 and h42 on the second chromosome are homolog pairing sites during prophase I. The signals of both probes became clear at prometaphase I when chromosomes become condensed (Fig. 2-5). At prometaphase I, none of spermatocytes exhibited two spots and around 80% of spermatocytes exhibited four or more spots, indicating homologs are unpaired at h42 on the second chromosome (Table 2-3). For the AATAG (h4&h36) probe, most spermatocytes also exhibited four or more spots at prometaphase I (Table 2-3). These results imply that the h36 and h42 regions are not stable homolog-pairing sites.

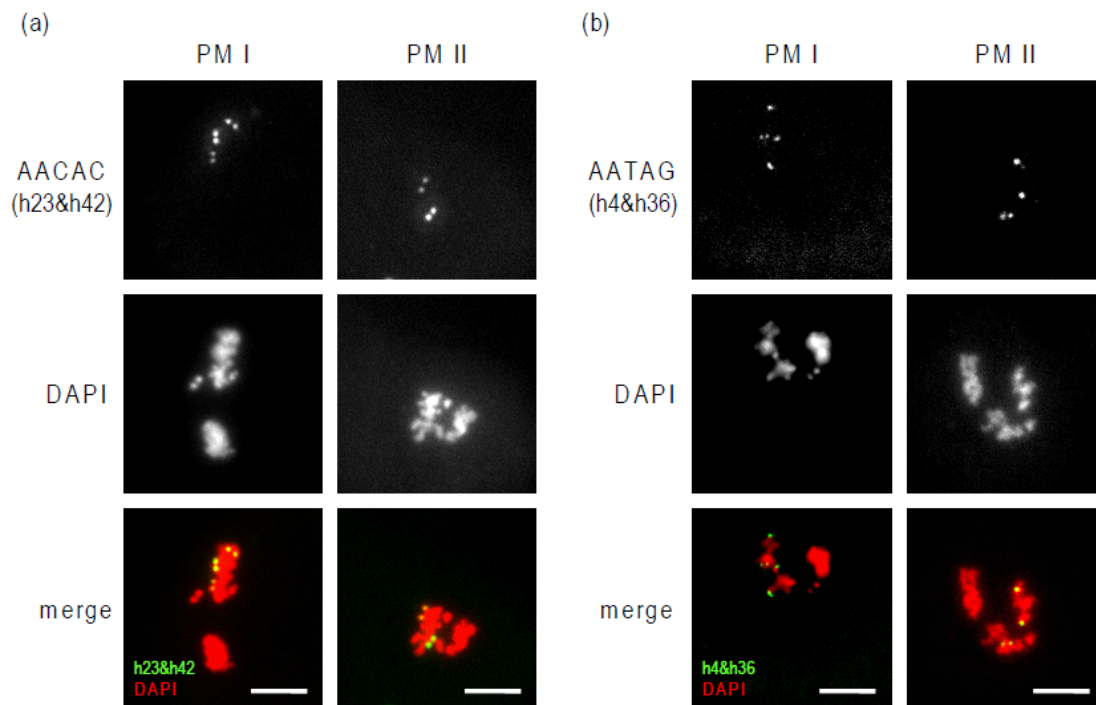


Figure 2-5. The AACAC (h23&h42) and AATAG (h4&h36) probes signals at different stages of spermatocytes.

The (a) h23&h42 probe and (b) h4&h36 probe signals are shown as green and DNA, stained by DAPI, is shown as red. Size bar represents 5 μm .

Table 2-3. Pairing percentages of the AACAC and AATAG probes.

Meiotic stages	PM I	PM II ^a
The AACAC (h23&h42) probe: YS and 2R		
2 Spots	0.0	0.0
3 Spots	20	28
4 Spots	28	72
5 Spots	29	0.0
6 Spots	24	0.0
<i>N</i> (cell number)	88	83
The AATAG (h4&h36) probe: YL and 2L		
2 Spots	0.0	0.0
3 Spots	11	32
4 Spots	26	68
5 Spots	26	0.0
6 Spots	37	0.0
<i>N</i> (cell number)	38	40

Spot frequencies of the h23&h42 probe for the Y chromosome and second chromosome and the h4&h36 probe for the Y chromosome and second chromosome at prometaphase I (PM I) and prometaphase II (PM II) of *yw* flies. For both the h23&h42 and h4&h36 probes signals, two spots indicate that the second homologs are paired and Y sister chromatids are paired; three spots indicate that either the second homologs are paired or Y sister chromatids are paired; four spots indicate that the second homologs and Y sister chromatids are unpaired; five or six spots indicate that second and Y sister chromatids are unpaired at PMI. For prometaphase II, three spots indicate that either second sister chromatids or Y sister chromatids are unpaired; four spots indicate that both second and Y sister chromatids are unpaired. ^a Only the spermatocytes containing the Y chromosome were counted for the PM II stage

Heterochromatic region 61 on the fourth chromosome contains a homolog pairing site

In addition to the major autosomes, we also tested a probe for the fourth chromosome, which has been shown to pair in the heterochromatin during female meiosis (Dernburg et al. 1996). The AATAT repeat (h61) probe targets several sites including h1, h2, h4-9, h15, h22, and h24 on the Y chromosome, h33 on the X chromosome, and h61 on the fourth chromosome, but the strongest hybridization region is h61 (Pimpinelli et al. 1995). Indeed, we observed a bright single spot on the fourth chromosome that co-localized with a small, bright condensed DNA mass (Fig. 2-6a). The h61 region is likely to contain a stable pairing site based on the observation that greater than 90% of spermatocytes during all stages of prophase I showed the fourth homologs paired as a single strong hybridization signal (Table 2-4). It is noteworthy that the fourth chromosome forms a compact DNA territory starting in early prophase I with particularly prominent DAPI staining (Fig. 2-6a, middle panel). The single h61 spot separated into two spots at prometaphase I.

However, these two spots were always very close to one another, within $1.07 \pm 0.2 \mu\text{m}$ (N=50), whereas unpaired homologs were usually separated by more than $2.0 \mu\text{m}$. For instance, the distances between two spots of the h53 probe averaged $3.27 \pm 0.57 \mu\text{m}$ (N=79) at prometaphase I. The fourth homologs may be connected in some way at prometaphase I. However, we could not find any threadlike structures between the fourth homologs either by FISH probe or DAPI staining. Furthermore, the fourth homologs were found unpaired at prometaphase I in *mn*m (Fig. 2-6b) and *sn*m mutants (Fig. 2-6c). These results imply that even

though fourth homologs appear disconnected at prometaphase I, they are nevertheless conjoined in some way by the SNM/MNM complex.

Association between the X and fourth chromosomes during meiosis

Previous studies showed that the segregation of sex chromosomes can be affected by the fourth chromosome, and vice versa, and that the fourth chromosome associates with the X chromosome in female *Drosophila* (Dernburg et al. 1996; Sandler and Novitski 1956; Sturtevant 1934). In the process of analyzing the h61 probe signals and the DAPI staining, we noticed that the fourth chromosome DNA territory was usually close to another DNA territory during prophase I. In order to examine whether the X chromosome is near to the fourth chromosome and to determine the distances between the fourth and X chromosomes during meiosis, we utilized the h61 probe together with the 359 bp repeat (h31) probe, which hybridizes near the X centromere. The probes presented as clear signals during meiosis and showed that the fourth chromosome was usually near the X chromosome. Sometimes these two territories were overlapping (Fig. 2-7). To determine how regular the X–fourth association is, we measured the distances between the X and fourth chromosome probes in approximately 50 spermatocytes for each meiotic stage. To assess the specificity and relative strength of the association, we also conducted similar measurements for several other pairs of probes from heterologous chromosomes. As expected, the average distances between the h61 (fourth chromosome) and h31 (X chromosome) probes were less than the average

distances between the h61 and h53 (third chromosome) probes (Fig. 2-8). Although both the h31 and h53 probes hybridized close to their respective centromeres, the distances of h61-h53 (fourth-third) were almost twofold greater than h61-h31 (fourth-X; with statistical significance $P < 0.001$) during meiosis I, particularly at late prophase I when nuclear volume expanded (Tables 2-5 and 2-6). Furthermore, the distances of h61- AATAC (h6) (fourth-Y) were similar but slightly greater than the distances of h61-h31 (fourth-X), and the distances of h61-h53 (fourth-third) were similar to the distances of h31-h53 (X-third) during meiosis I. These data provide evidence that the fourth chromosome associates with the sex chromosomes, in particular the X chromosome, during male meiosis I.

Figure 2-6. The AATAT (h61) probe signals at different stages of spermatocytes.

The h61 probe signals are shown as green and DNA, stained by DAPI, is shown as red in (a) *yw*, (b) *mnm* mutant, and (c) *snm* mutant flies. Size bar represents 5 μm . The distances between two spots of the fourth homologs are $1.07 \pm 0.2 \mu\text{m}$ (N=50) for *yw*, $5.79 \pm 4.3 \mu\text{m}$ (N=9) for *mnm* mutants and $3.66 \pm 1.6 \mu\text{m}$ (N=15) for *snm* mutants.

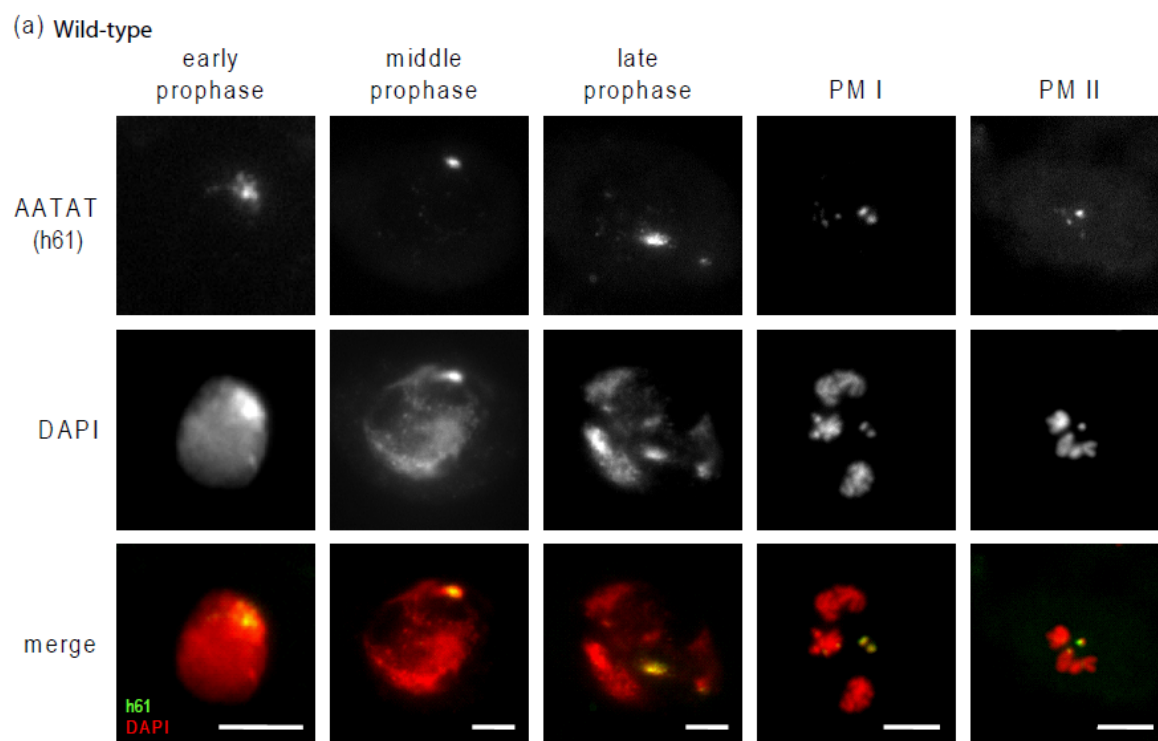
(b) *mn*(c) *sn*

Table 2-4. Pairing percentages of the AATAT (h61) probe.

Meiotic stages	S1	S2a	S2b	S3	S4	S5	S6	PM I	PM II
The AATAT (h61) probe: 4R									
1 Spot	92	95	96	97	96	98	95	0.0	100
2 Spots	8.4	4.6	4.3	2.7	4.0	1.7	4.8	100	0.0
3 or 4 Spots	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>N</i> (cell number)	165	65	23	74	75	56	84	58	39

Spot frequencies of the h61 probe for the fourth chromosome at prophase I (S1-S6), prometaphase I (PM I) and prometaphase II (PM II) of *yw* flies. We counted the strong intensity spots for determining the pairing. One spot indicates that homologs are paired; two spots indicate that homologs are unpaired but sister chromatids are paired; three or four spots indicate that sister chromatids are unpaired from prophase I to prometaphase I. For prometaphase II, one spot indicates that sister chromatids are paired and two spots indicate that sister chromatids are unpaired.

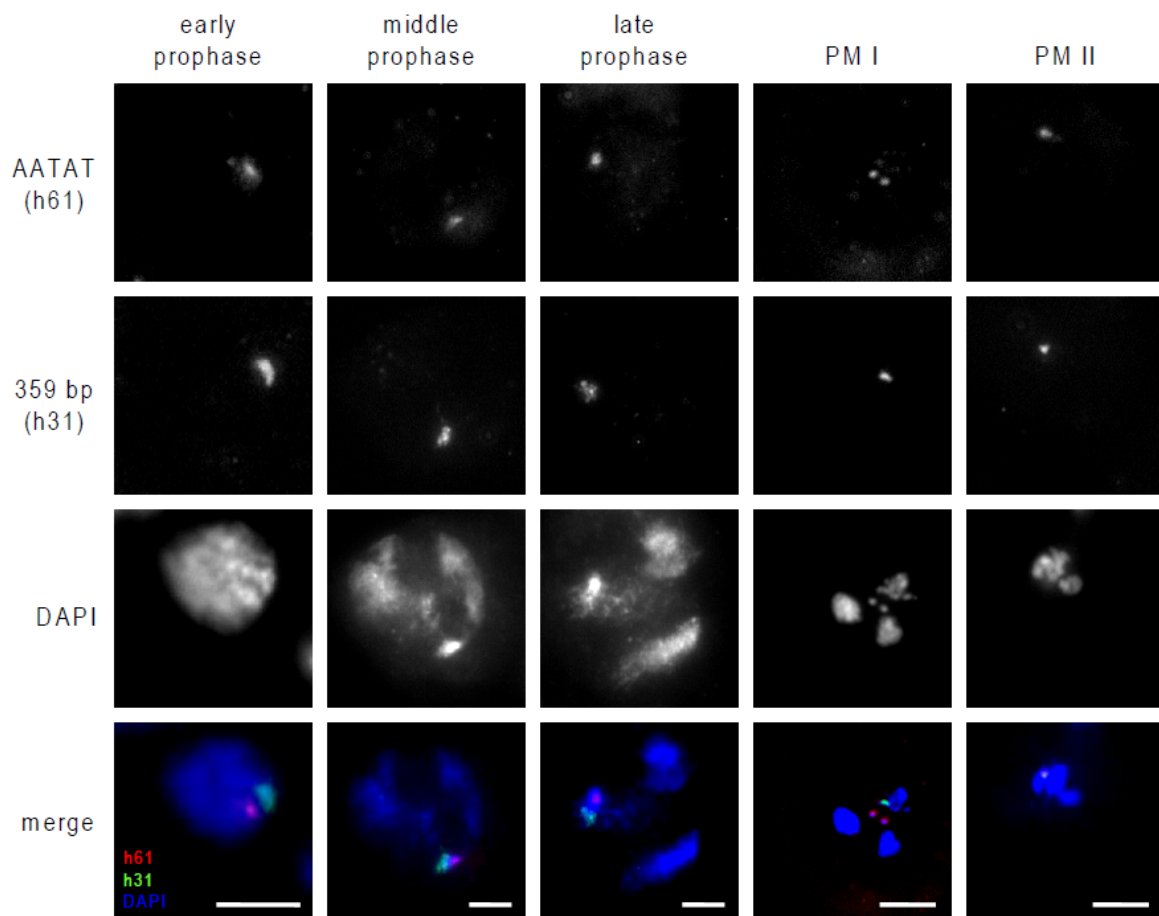


Figure 2-7. Signals of the AATAT (h61) and 359 bp (h31) probes at different stages of spermatocytes.

The h61 probe signals are shown as red, the h31 probe signals are shown as green and DNA, stained by DAPI, is shown as blue. Size bar represents 5 μm .

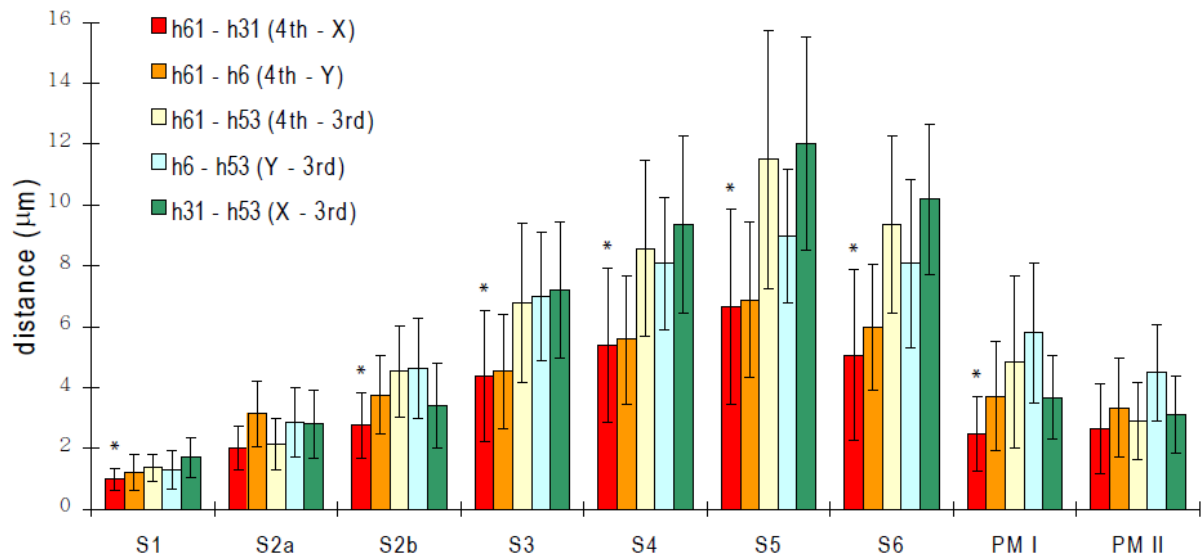


Figure 2-8. The distances between the h6, h31, h53, and h61 probes.

Two probes were used in each experiment. The average distances between two probes are shown as bars at prophase I (S1-S6), prometaphase I (PM I) and prometaphase II (PM II). Around 50–100 spermatocytes were counted for each stage of prophase I except stage S2b, for which around 20–40 spermatocytes were counted; 20–40 spermatocytes were counted for prometaphase I and 10–40 spermatocytes were counted for prometaphase II in each experiment. Because some spermatocytes showed uncondensed and discontinuous h6 probe signals during middle and late prophase I, only the spermatocytes with spots of h6 probe signals were counted for distance measurement. *P value is <0.001 for the distance comparison of fourth–X and fourth–third.

Table 2-5. The ratio for distances of probe combinations.

	4th-3rd	4th-X	4th-Y	Y-3rd	X-3rd
S1	1 (1.37 m)	0.71 (0.98 m)	0.88 (1.21 m)	0.94 (1.29 m)	1.24 (1.7 m)
S2a	1 (2.15 m)	0.94 (2.02 m)	1.46 (3.14 m)	1.33 (2.86 m)	1.31 (2.81 m)
S2b	1 (4.53 m)	0.61 (2.76 m)	0.83 (3.76 m)	1.02 (4.63 m)	0.75 (3.4 m)
S3	1 (6.77 m)	0.65 (4.38 m)	0.67 (4.53 m)	1.03 (6.99 m)	1.06 (7.21 m)
S4	1 (8.57 m)	0.63 (5.41 m)	0.65 (5.58 m)	0.94 (8.07 m)	1.09 (9.36 m)
S5	1 (11.5 m)	0.58 (6.64 m)	0.6 (6.88 m)	0.78 (8.97 m)	1.04 (12.01 m)
S6	1 (9.35 m)	0.54 (5.06 m)	0.64 (5.99 m)	0.86 (8.07 m)	1.08 (10.19 m)
PM I	1 (4.83 m)	0.52 (2.49 m)	0.77 (3.72 m)	1.2 (5.8 m)	0.76 (3.68 m)
PM II	1 (2.91 m)	0.91 (2.65 m)	1.14 (3.34 m)	1.54 (4.49 m)	1.07 (3.12 m)

We set the distances between the 4th and 3rd chromosomes (h61-h53) to 1 for each meiotic stage. The numbers in the table represent the ratio of the distance between the two indicated chromosomes to the 4th-3rd distances for the same meiotic stage.

The numbers in parentheses are the raw distances.

Table 2-6. *P*-Values for distance combinations.

	S1	S2a	S2b	S3	S4	S5	S6	PM I	PM II
4th-X vs. 4th-3rd	< 0.001	0.3651	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.4485
4th-X vs. 4th-Y	0.0026	< 0.001	0.0098	0.6426	0.6593	0.6383	0.03	0.0063	0.0739
4th-X vs. 3rd-X	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.022	0.2849
4th-X vs. 3rd-Y	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
X-3rd vs. 4th-3rd	< 0.001	< 0.001	0.0061	0.2139	0.0533	0.2682	0.0678	0.0306	0.639
X-3rd vs. 4th-Y	< 0.001	0.05	0.3806	< 0.001	< 0.001	< 0.001	< 0.001	0.9291	0.6497
X-3rd vs. Y-3rd	< 0.001	0.7282	0.0005	0.5284	0.001	< 0.001	< 0.001	< 0.001	0.0203
4th-3rd vs. Y-3rd	0.305	< 0.001	0.8103	0.4916	0.2135	< 0.001	0.0105	0.0371	0.0027
4th-Y vs. Y-3rd	0.2882	< 0.001	0.0277	< 0.001	< 0.001	< 0.001	< 0.001	0.0179	0.0365
4th-3rd vs. 4th-Y	0.0364	0.0941	0.0852	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.278

The h37, h48, and h61 regions contain stable sister chromatid cohesion sites in male meiosis

The FISH-stained chromosome preparations allowed us to examine the behavior of heterochromatic regions of sister chromatids as well as homologs in male meiosis. For the h61 probe on chromosome 4, none of the spermatocytes exhibited more than two prominent spots during prophase I. All of the cells presented two strong closely adjacent spots at prometaphase I and one spot at prometaphase II. Thus, the fourth sister chromatids appear to pair throughout meiosis I and meiosis II until anaphase II (Fig. 2-6 and Table 2-4). Consequently, the h61 region may contain stable sister chromatid cohesion sites during male meiosis. Moreover, for the h37&h48 probe, greater than 95% of spermatocytes exhibited less than three spots at region h37 and less than three spots at region h48 throughout prophase I to prometaphase I. Only one spot was present at each region at prometaphase II (Fig. 2-3 and Table 2-2). The spot frequencies suggest that the h37 and h48 regions also function as stable sister chromatid cohesion sites in meiosis. The pattern of the h37&h48 probe in solo mutants further supports sister chromatid cohesion at h37 and h48: these spermatocytes always exhibited five to eight spots at prometaphase I and four spots at prometaphase II, indicating that sister chromatid cohesion was lost (Fig. 2-9a). These findings are significant because there is little information about sister chromatid cohesion sites on chromosomal arms in *D. melanogaster*.

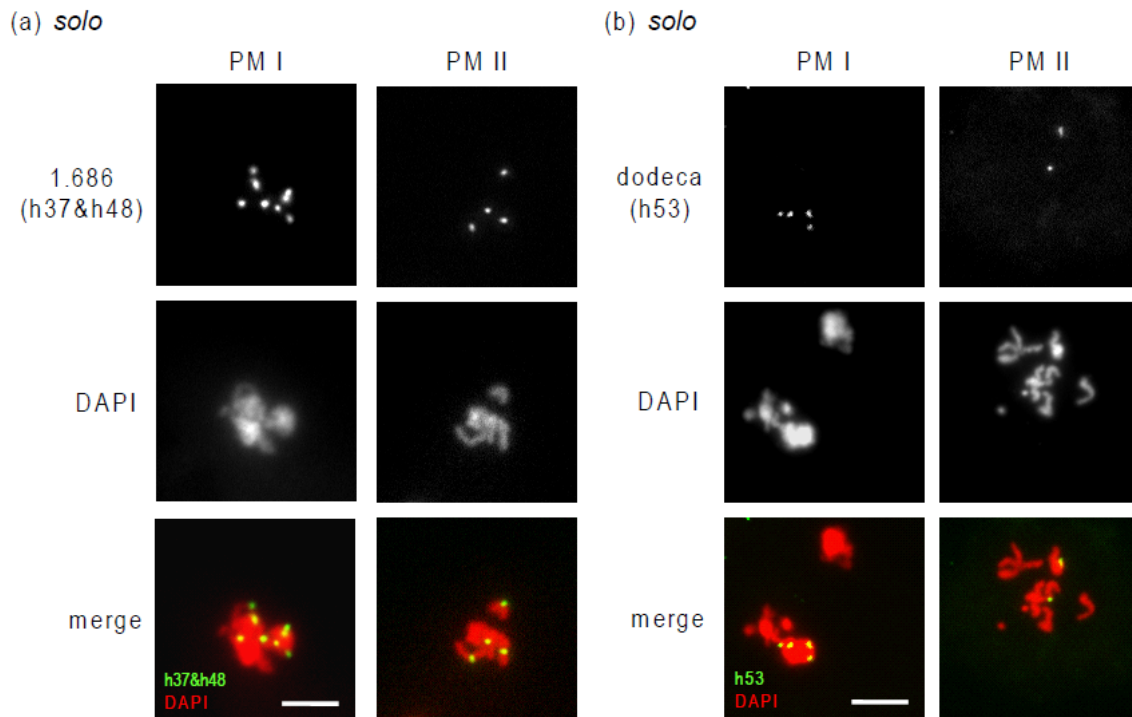


Figure 2-9. The 1.686 g/cm³ (h37&h48) and dodeca (h53) probes signals at different stages of spermatocytes in *solo* mutants.

(a) The h37&h48 probe signals are shown as green and DNA, stained by DAPI, is shown as red. Eight spots at prometaphase I and four spots at prometaphase II indicate that sister chromatids are unpaired.

(b) The h53 probe signals are shown as green and DNA, stained by DAPI, is shown as red. Four spots at prometaphase I and two spots at prometaphase II indicate that sister chromatids are unpaired. Size bar represents 5 μm .

Using CID-GFP to mark centromeres, sister centromeres have been shown to pair throughout meiosis until anaphase II (Vazquez et al. 2002; Yan et al. 2010). Given the proximity of the h39 and h53 regions to their respective centromeres, we expected the FISH probes for these regions to behave similarly. Surprisingly, however, these pericentromeric probes exhibited a more complex and dynamic pattern. At early prophase (stages S1–S2) greater than 90% of spermatocytes exhibited less than three spots at both the h39 and the h53 regions, indicating that sister chromatids were paired at these stages. However, in approximately 50% of stage S3–S6 spermatocytes, three or four spots were observed in the territory, indicating sister chromatid separation in one or both of the homologs. Surprisingly, at both h39 and h53, spot numbers reverted to two in most prometaphase I spermatocytes, consistent with reestablishment of sister chromatid pairing. At prometaphase II, the majority of spermatocytes also exhibited one spot, indicating that sister chromatids were paired (Fig. 2-2 and Table 2-1). Similar to the h37&h48 probe, the pattern of the h53 probe in solo mutants showed that sister chromatid cohesion was permanently lost: these spermatocytes always exhibited four spots at prometaphase I (Yan et al. 2010) and one to three spots (due to meiosis I sister chromatid nondisjunction) at prometaphase II (Fig. 2-9b). Thus the “pair-back” phenomenon observed at h53 in wild-type is dependent on the cohesion protein SOLO.

We also examined other heterochromatic regions using the h4&h36 and h23&h42 probes. For both probes, approximately 50% of spermatocytes showed more than four spots at prometaphase I and at least 65% of spermatocytes showed

four spots at prometaphase II (Fig. 2-5 and Table 2-3). According to the spot frequencies, neither the h36 and h42 regions of the second chromosome nor the h4 and h23 regions of the Y chromosome are sites for stable sister chromatid arm cohesion.

Besides the h4&h36 and h23&h42 probes, another Y chromosome probe examined in this study is the AATAC repeat, which uniquely targets h6 on the Y chromosome (Bonaccorsi and Lohe 1991; Lohe et al. 1993; Fig. 2-1). The AATAC (h6) probe usually showed one or two spots at prophase I (Fig. 2-10), but sometimes with weak or even nonexistent signals at late prophase I. In addition, 30–50% of spermatocytes exhibited uncondensed and discontinuous signals at middle and late prophase I (Fig. 2-11). The h6 region is close to kl-3, which forms a Y-loop during meiosis, suggesting that the h6 region may be involved in kl-3-loop formation during prophase I. However, discrete spots were present at prometaphase I and prometaphase II. The results showed that the h6 region is not a stable sister chromatid cohesion site since around 95% of spermatocytes at prometaphase I and II showed unpaired sister chromatids (Table 2-7).

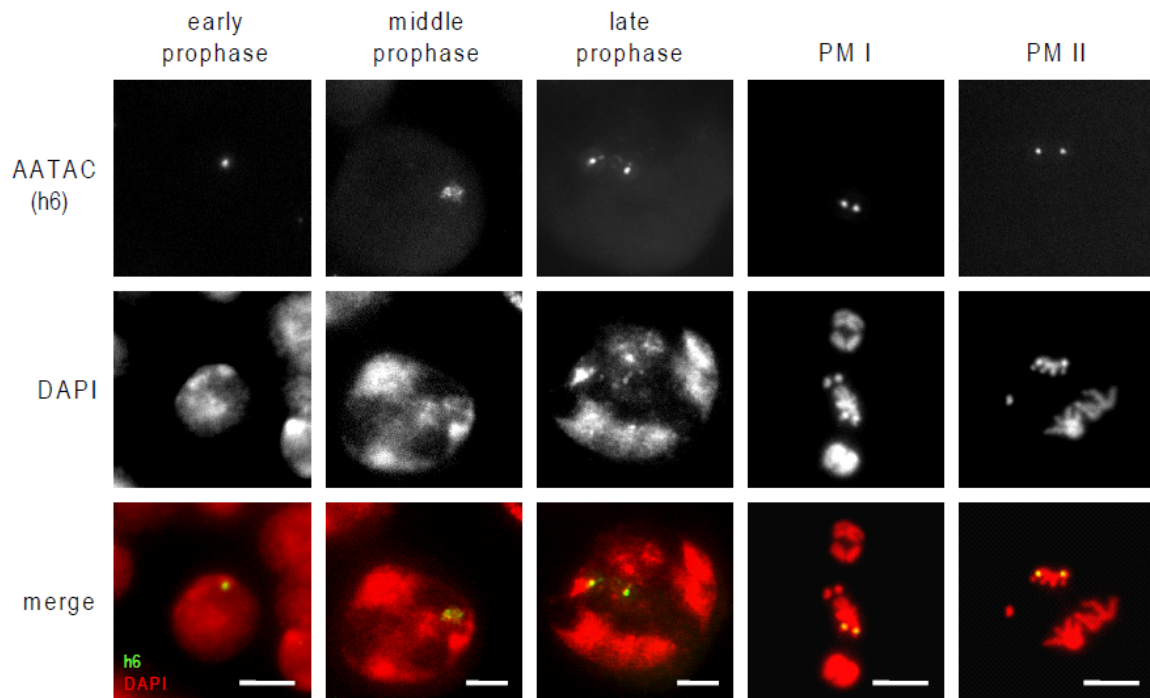


Figure 2-10. The AATAC (h6) probe signals at different stages of spermatocytes.

The h6 probe signals are shown as green and DNA, stained by DAPI, is shown as red. Size bar represents 5 μ m.

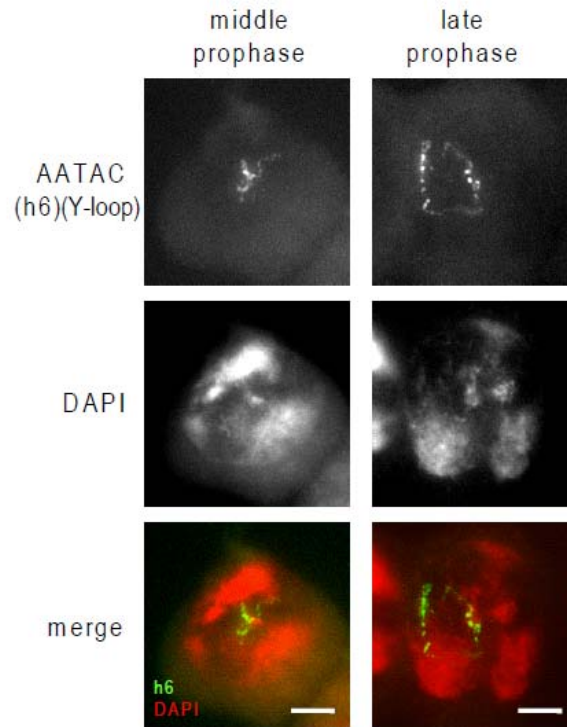


Figure 2-11. Y-loop-like signals of AATAC (h6) probe at middle and late prophase I of spermatocytes.

The h6 probe signals are shown as green and DNA, stained by DAPI, is shown as red. Size bar represents 5 μm .

Table 2-7. Pairing percentages for of the AATAC (h6) probe.

Meiotic stages	PM I	PM II ^a
The AATAC (h6) probe: YL		
1 Spot	5.8	2.8
2 Spots	94	97
<i>N</i> (cell number)	103	36

Spot frequencies of the h6 probe for the Y chromosome at prometaphase I (PM I) and prometaphase II (PM II) of *yw* flies. One spot indicates that sister chromatids are paired and two spots indicate that sister chromatids are unpaired. ^a Only the spermatocytes containing the Y chromosome were counted for the PM II stage.

DISCUSSION

Homolog pairing sites during male meiosis I

Based on the hypothesis that euchromatic pairing during early prophase induces stable associations between homologous heterochromatic regions as suggested by Vazquez et al. (2002), we have attempted to identify specific DNA sequences in heterochromatic regions of the autosomes that serve as stable homolog pairing sites during meiosis. Ideally, we would have liked to screen all heterochromatic regions. However, it is impossible to find specific FISH probes for all heterochromatic regions. Bacterial artificial chromosome (BAC) probes might provide a way to overcome this difficulty. We tested three BAC probes – BACR04P15, BACR11B14, and BACR17M18 – targeting the middle of heterochromatic arms (h43 on the right arm of the second chromosome) or distal heterochromatic arms adjacent to euchromatin (h46 on the right arm of the second chromosome and h47 on the left arm of the third chromosome). Unfortunately, signals of these three probes were very weak or even undetectable during prophase I (data not shown).

Instead of screening all heterochromatic regions, we surveyed the regions for which strong and specific FISH probes were available. Seven out of 27 heterochromatic regions of the autosomes were examined. The FISH probes targeted two pericentromeric regions, three regions in the middle of heterochromatic arms and two distal heterochromatic regions. To determine the level of pairing, the number

of spots was counted. In general, we could expect to observe either one spot (homologs paired), two identical spots (homologs unpaired but sister chromatids paired), one large and two smaller spots (homologs unpaired and sister chromatids unpaired in one homolog), or four identical spots (homologs and sister chromatids all unpaired). All of these combinations were observed. One other possible outcome has to be considered: sister chromatids unpaired despite homologs being paired, which should manifest as either one or two smaller spot(s) fairly close to one large spot. This scenario was rarely observed. When two spots were observed in one DNA territory, they were always identical. When three spots were observed in one DNA territory, the distances between one large spot and two small spots were always more than 2 μm which was greater than the distance between the fourth homologs ($\sim 1.07 \mu\text{m}$ at prometaphase I), suggesting that those three spots were not examples of two liberated sister chromatids with homolog pairing maintained.

In *Drosophila*, homologs pair at substantial frequencies in mitotic as well as meiotic cells. Of immediate relevance, euchromatic alleles pair at frequencies around 50% in spermatogonia, compared to >90% in young primary spermatocytes. Pairing frequencies then fall drastically at the S2b/S3 transition (Vazquez et al. 2002). Pairing frequencies around 50% have also been documented at several heterochromatic sites using FISH probes (Fung et al. 1998). In the present study, homolog pairing frequencies of heterochromatic sites behaved similarly to those at euchromatic sites in that pairing frequencies were generally highest at early prophase I then decreased as meiosis progressed (Tables 2-1 and 2-2).

Interestingly, the early (S1–S2) pairing frequencies for the two pericentromeric probes (Table 2-1) are similar to mitotic frequencies, suggesting that heterochromatic pairing in early meiotic prophase I may represent continuation of mitotic pairing. On the other hand, the early pairing frequencies of the h37&h48 probe, about 90% (Table 2-2), look more like meiotic euchromatic pairing (Vazquez et al. 2002). This suggests that the unknown pairing factors which enhance the euchromatic pairing in early meiosis, also enhance the pairing of part of heterochromatin.

Heterochromatin has been suggested to function in maintenance of homolog pairing. Therefore, when homologous pairing of both euchromatin and centromeres is lost after stage S3, specialized stable heterochromatic pairing sites are expected to remain paired. However, the frequencies of homolog pairing for the tested regions of the major autosomes dropped to the ranges of 1.5–20% after stage S3, as compared to >95% pairing of the h61 region on the fourth chromosome. This indicates that stable homolog pairing sites, if such exist, for both the second and third chromosomes have not been identified by these probes.

In light of these results, the idea of stable pairing sites of the major autosomes should be reconsidered. MNM and SNM, components of the homolog conjunction complex, have been shown to reside on the autosomes throughout meiosis I until anaphase I (Thomas et al. 2005). One possibility is that homolog pairing, mediated by the homolog conjunction complex, occurs at different sites in different cells but is stable once it occurs. This would be analogous to chiasmata which can occur at many different sites along chromosomes but provide stable

connections once established. This hypothesis is supported by our observation of low pairing frequencies at middle and late prophase I (Tables 2-1, 2-2 and 2-3). These frequencies could represent nuclei in which the homolog conjunction complex localized to the region being assayed.

Another possibility is that homologs of the major autosomes, unlike the sex and fourth chromosomes, are connected by heterochromatic threads instead of stable pairing sites to keep the DNA territories during meiosis. Indeed, we observed thread-like structures between unpaired homologs at prophase I although only in a small minority (<5%) of spermatocytes and only for one probe. This could be a substantial underestimate of the true frequency since heterochromatic threads are thin and fragile and therefore difficult to visualize. In this scenario, the homolog conjunction complex, which is required to promote territory formation and chromosome segregation (Thomas et al. 2005; Tomkiel et al. 2001), may function on entangled DNA to generate heterochromatic threads although the mechanism is still unknown.

Fourth chromosome pairing and association with the X chromosome

The fourth chromosome is about 4.2 Mb and is the smallest chromosome in *D. melanogaster* (Locke and McDermid 1993). The effects of chromosome loss are different between the fourth and major autosomes. Loss of a copy of the fourth chromosome impairs viability but loss of either the second or third chromosome leads to embryonic lethality. In males mutant for Cap-H2, a subunit of condensin II,

both the sex and fourth chromosome pairs exhibit normal meiotic segregation whereas the second and third chromosomes exhibit an elevated frequency of nondisjunction (Hartl et al. 2008). Moreover, in studies using anti-CID to mark centromeres, we have observed only about 10% of spermatocytes with eight CID spots at late prophase I. The great majority of spermatocytes exhibit seven CID spots, one associated with the fourth chromosome and two each associated with the major chromosome territories (data not shown). These observations all suggest that the fourth chromosome behaves differently from the major autosomes with respect to pairing.

Pairing of fourth chromosome heterochromatin during female meiosis has been demonstrated (Dernburg et al. 1996). In this study, we showed that greater than 90% of spermatocytes exhibited one strong h61 signal during prophase I, indicating that the fourth homologs are tightly paired in male meiosis. The fourth chromosome forms a condensed DNA mass during prophase I, so one might expect to observe pairing by using any fourth probe because of the small size of the fourth DNA mass relative to the target sites of heterochromatic FISH probes. Furthermore, it is possible that stable pairing in the h59 or h60 regions may lead to fused homologous h61 signals since the fourth chromosome is tightly condensed throughout prophase I (Fig. 2-6a). Using different fourth chromosome-specific probes will be helpful to identify the precise locations and sequences of stable pairing sites. However, we have not yet found other specific probes for the fourth chromosome. In this study, we demonstrate that h61 may contain stable pairing sites but we do not exclude the possibility that h59 and h60 may also contain pairing

sites or that the entire fourth chromosome pairs during meiosis. It will be interesting to determine if other stable pairing sites exist in the fourth chromosome by using flies with fourth chromosome heterochromatin deletions, such as h61 deletion, in future FISH studies.

Recently, SNM and MNM were identified and shown to be essential for segregation of all four homolog pairs in male meiosis I. Here, we provide cytological evidence that SNM and MNM are involved in mediation of fourth homolog pairing in males based on the h61 probe patterns in *yw*, *mnm* mutant and *snm* mutant flies.

Interestingly, the fourth homologs form two close bright spots at prometaphase I in *yw* males. The fourth homologs have been shown to be connected by heterochromatic threads during female meiosis I (Gilliland et al. 2009; Hughes et al. 2009). However, we did not observe threadlike structures between the fourth homologs at prometaphase I either by FISH probe or DAPI staining. The fourth chromosome threads have been observed, although in low frequency of spermatocytes, in Cap-H2 mutants for examining the function of condensins in male meiosis (Hartl et al. 2008). The detection of thread structures was technically very challenging in wild-type *Drosophila* oocytes. Therefore, it is possible that there were heterochromatic threads between these two close homolog spots that were not visualized due to technical difficulty. The homolog conjunction complex or other proteins may associate with heterochromatic threads to connect the fairly close fourth homologs at prometaphase I. Furthermore, unlike females, the fourth homologs reside close to each other instead of moving precociously towards the

opposite poles at prometaphase I in *yw* males. These results are consistent with previous evidence that the mechanism of achiasmate chromosome segregation may differ between male and female meiosis.

Based on the meiotic chromosome segregation pattern in triplo-IV males in which the X and fourth chromosomes did not segregate randomly but preferentially, an X–fourth association was proposed (Gershenson 1940). A similar pattern is observed in triplo-IV females in which the fourth chromosome could pair with the X chromosome and cause occasional X chromosome non-disjunction (Sandler and Novitski 1956). Moreover, in Oregon-R wild-type strain females, the fourth chromosome was associated with and located near the proximal heterochromatin of the X chromosome in prophase I (Dernburg et al. 1996). In this study, we present cytological evidence for preferential X–fourth association during meiosis in males. The distances between the fourth chromosome (center of h61 probe signals) and the X chromosome (center of h31 probe signals) are consistently less than the distances between h61 probe signals and h53 probe (third chromosome) signals, which indicate that the fourth chromosome is positioned non-randomly near the X–Y pair during meiosis. This X–fourth association explains the observation that the chromatin mass often appears to subdivide into three but not four DNA territories after early prophase I. The small fourth chromosome pair is often obscured by its close association with the X–Y pair.

Sister chromatid cohesion

Sister chromatid cohesion is required to hold sister chromatids together and prevent premature separation. Cohesion arises concomitant with DNA replication and is lost at anaphase. In yeast, the cohesin complex is initially loaded onto chromosomes at multiple places, dependent on the Scc2/Scc4 complex, then moves to its more permanent locations (Ciosk et al. 2000). However, how the cohesin complex moves to association sites and the characteristics of those sites are still unclear.

From FISH screening, we observed two distinct sister chromatid cohesion patterns in heterochromatin: regions lacking cohesion and regions with stable cohesion. Based on spot frequencies at prometaphase I and II, the h4, h6, h23, h36, and h42 regions showed lack of sister chromatid cohesion (Tables 2-3 and 2-7). On the other hand, at the h37, h48, and h61 regions, greater than 95% of spermatocytes exhibited stable sister chromatid cohesion throughout meiosis until anaphase II (Tables 2-2 and 2-4). This sister chromatid cohesion was lost in solo mutants (Fig. 2-9a), indicating that sister chromatid cohesion at h37 and h48 is promoted by SOLO. SOLO has been shown to associate closely with the cohesin protein SMC1 (Yan et al. 2010), suggesting that cohesion at regions h37, h48, and h61 is mediated by cohesin. Taken together, these results indicate that sister chromatid cohesion is incomplete within the heterochromatic domains in male *Drosophila*, which is consistent with the pattern in yeast in which the cohesin complex binds to preferential sites on chromosomes (Blat and Kleckner 1999; Glynn et al. 2004). The h37, h48, and h61 regions belong to bright fluorescent blocks of

Hoechst 33258 staining; therefore, stable cohesion may occur preferentially at AT-rich DNA sequences. The proximity to centromeres seems to have little correlation with stable cohesion sites since the regions with stable cohesion as well as regions without cohesion are located in the middle of or near the ends of heterochromatic arms.

With pericentromeric probes, we observed that sister chromatids of the h39 and h53 regions came apart (three or four spots) at middle and late prophase I but then seemed to “pair-back” (two spots) at prometaphase I (Fig. 2-2 and Table 2-1). SOLO, a cohesive molecule of centromeric cohesion, appears to be related to this phenomenon because the “pair-back” behavior is absent in solo mutants (Fig. 2-9b). This behavior is puzzling since sister chromatid cohesion is established at S phase and there is no direct evidence that cohesin complex can reform stable cohesion when DNA is not replicating. One possibility is that the h39 and h53 regions are not sister cohesion sites, but their proximity to centromeres combined with high levels of chromosomal condensation lead to the appearance of cohesion at prometaphase I. Because pericentromeric regions are close to centromeres, condensation of chromosomes could pull unpaired sister pericentromeric regions close enough to each other to appear as “paired” signals. The level of resolution provided by FISH may be insufficient to distinguish these unpaired but close regions. High resolution studies in the future may clarify the behavior of these pericentromeric regions.

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CHAPTER 3

INTERACTION BETWEEN MODIFIER OF MDG4 IN MEIOSIS (MNM) AND STROMALIN IN MEIOSIS (SNM)

This part is intended to be submitted for publication as Jui-He Tsai, Hirotugu Yamada and Bruce D. McKee. Jui-He Tsai performed most the work, Hirotugu Yamada assisted with the NDJ tests.

ABSTRACT

Accurate pairing and segregation of homologous chromosomes at meiosis I are essential steps in sexual reproduction to prevent the generation of aneuploid gametes. The *Modifier of Mdg4 in Meiosis* (MNM) and *Stromalin in Meiosis* (SNM) proteins have been identified to be required for regular pairing and segregation of achiasmate homologous chromosomes in *Drosophila* male meiosis. In this study, we confirm and analyze the interaction between MNM and SNM through co-immunoprecipitation and yeast two-hybrid assay. In particular, we demonstrate the BTB domain of MNM interacts directly with the IRR1 domain and other uncharacterized regions of SNM, whereas the FLYWCH domain of MNM, although essential for MNM-SNM interaction, does not interact directly with SNM. Yeast two-hybrid assay also revealed that MNM forms homodimers and/or multimers. Point mutation analysis showed that L9K replacement of the BTB domain weakens MNM-SNM interaction as well as the formation of MNM homodimers/multimers, resulting in high frequencies of chromosome nondisjunction. These data indicate that MNM and SNM are recruited to the same complex and function together in pairing and segregation of homologous chromosomes during *Drosophila* male meiosis.

INTRODUCTION

Meiosis in male *Drosophila* is a unique meiotic system in which recombination does not occur. Two structures typically associated with homolog segregation are absent: the synaptonemal complex, a protein structure that mediates chromosome pairing, and chiasmata, physical connections between recombined homologous chromosomes (Meyer 1960; Morgan 1914). Nevertheless, these non-exchange homologs can pair and segregate faithfully to opposite spindle poles during meiosis I (Hawley 2002; McKee 2004). Male *Drosophila* has four pairs of chromosomes: one X-Y pair and three pairs of autosomes, numbers 2, 3 and 4. Recently, *Modifier of Mdg4 in Meiosis* (MNM) and *Stromalin in Meiosis* (SNM) were identified and shown to be essential for stable connections between achiasmate homologous chromosomes. These two genes are required for segregation of all four homolog pairs in male *Drosophila* meiosis I, but are unnecessary for female meiotic segregation (Thomas et al. 2005). Both *mnm* and *snm* mutations cause high frequencies of univalents and random segregation of homologs during meiosis I (Soltani-Bejnood et al. 2007; Thomas et al. 2005). MNM and SNM are expressed exclusively in spermatocytes and are present in nuclei throughout meiosis I but disappear suddenly and permanently at anaphase I (Thomas et al. 2005). Immuno-FISH analysis revealed that MNM and SNM co-localize with each other at the 240-bp ribosomal DNA repeats (the X-Y pairing site) both in the nucleolus and on the condensed X-Y bivalent. Both MNM and SNM also localize to all three autosomal pairs, but the sites at their localizations on autosomes have not been determined. MNM and SNM have been shown to be dependent on each other for localization to

chromosomes. MNM-GFP was undetectable at all stages of meiosis I in *snm* null mutants and anti-SNM staining of the X–Y bivalent was absent in *mnm* mutants (Thomas et al. 2005).

MNM is encoded by the *mod(mdg4)* locus, a complex locus which encodes over 30 different proteins by alternative splicing and has effects in several processes, such as male meiotic homolog pairing, female fertility, the properties of insulator sequences, position effect variegation, neurogenesis and apoptosis (Dorn and Krauss 2003; Krauss and Dorn 2004). These Mod(mdg4) protein isoforms share a common 402-residue N-terminus including a Bric-a-brac, Tramtrack, and Broad Complex (BTB) domain and differ from each other at their C-termini. The BTB domain, also known as the Poxvirus zinc finger (POZ) domain, is a versatile protein-protein interaction motif found in many zinc-finger-containing transcriptional regulators and is known for its ability to dimerize, oligomerize and interact with other proteins by using its unique tri-dimensional fold with a large interaction surface formed by approximately 95 core amino acids (Albagli et al. 1995; Bardwell and Treisman 1994; Ghosh et al. 2001; Stogios et al. 2005). Most of the C-termini of these isoforms, including MNM (Fig. 1a), contain an unusually spaced zinc finger like-C2H2 motif embedded in a large FLYWCH domain.(Dorn and Krauss 2003; Labrador and Corces 2003). This domain is named by the conserved hydrophobic amino acids, F/Y-X(n)-L-X(n)-F/Y-X(n)-WXCX(6-12)CX(17-22)HXH (where X presents any amino acid). The FLYWCH zinc finger motif was originally defined through mod(Mdg4) proteins from *Drosophila* and later also found in other proteins in *C. elegans* and humans (Dorn and Krauss 2003; Beaster-Jones and Okkema

2004; Ow et al. 2008). The function of the FLYWCH domain has not been elucidated clearly in *Drosophila*. The only functionally characterized Mod(mdg4) isoform, Mod(mdg4)67.2, interacts via its specific FLYWCH containing C-terminus with the DNA binding protein Su(Hw) as part of the gypsy silencing mechanism, indicating a role for FLYWCH domain in protein-protein interactions (Gause et al. 2001; Ghosh et al. 2001). This domain has also been reported to be required for the DNA binding activity of PEB-1 protein in *C. elegans* (Beaster-Jones and Okkema 2004).

The *snm* gene has not been characterized as well as the *mnm* gene. It is predicted to encode a 973 amino acid protein sharing homology to SCC3 in yeast and Stromalin (SA) in *Drosophila* and various vertebrates (Thomas et al. 2005). SCC3/SA is a component of the cohesion complex required for sister chromatid cohesion in eukaryotes (Prieto et al. 2001; Toth et al. 1999). SNM contains an IRR1 domain, a conserved domain among cohesin proteins (Fig. 3-1). Although SNM is absent in other sequenced insect genomes, paralogs of both SA and SNM are present in *Drosophila* genomes, indicating that SNM arose within the Diptera (Thomas et al. 2005). Unlike other cohesin proteins, SNM mainly functions in stable homologous chromosome pairing and only has minor effects on sister chromatid cohesion (Thomas et al. 2005). SNM is present only from the onset of prophase I to anaphase I and functions in primary spermatocytes during meiosis I (Thomas et al. 2005). On the other hand, SA is expressed ubiquitously and localizes to mitotic centromeres and functions in sister chromatid cohesion (Valdeolmillos et al. 2004; Vass et al. 2003).

Co-localization of MNM and SNM to prominent nucleolar foci and to the pairing region of the X–Y bivalents during meiosis I, as well as mutual dependence of SNM and MNM on each other for localization to chromosomes lead to a hypothesis that these two proteins interact directly with each other. To test this hypothesis, we utilized two fundamental techniques: the yeast two-hybrid system, and immunoprecipitation. In this study, we demonstrate that MNM and SNM interact with each other, in particular, BTB and FLYWCH domains of MNM are responsible for interacting with SNM. Furthermore, a L9K substitution of BTB domain not only reduces the interaction ability of MNM to SNM but impairs MNM function in stable homolog pairing. These findings set the stage for mechanistic studies of the roles of MNM and SNM in *Drosophila* male meiosis.

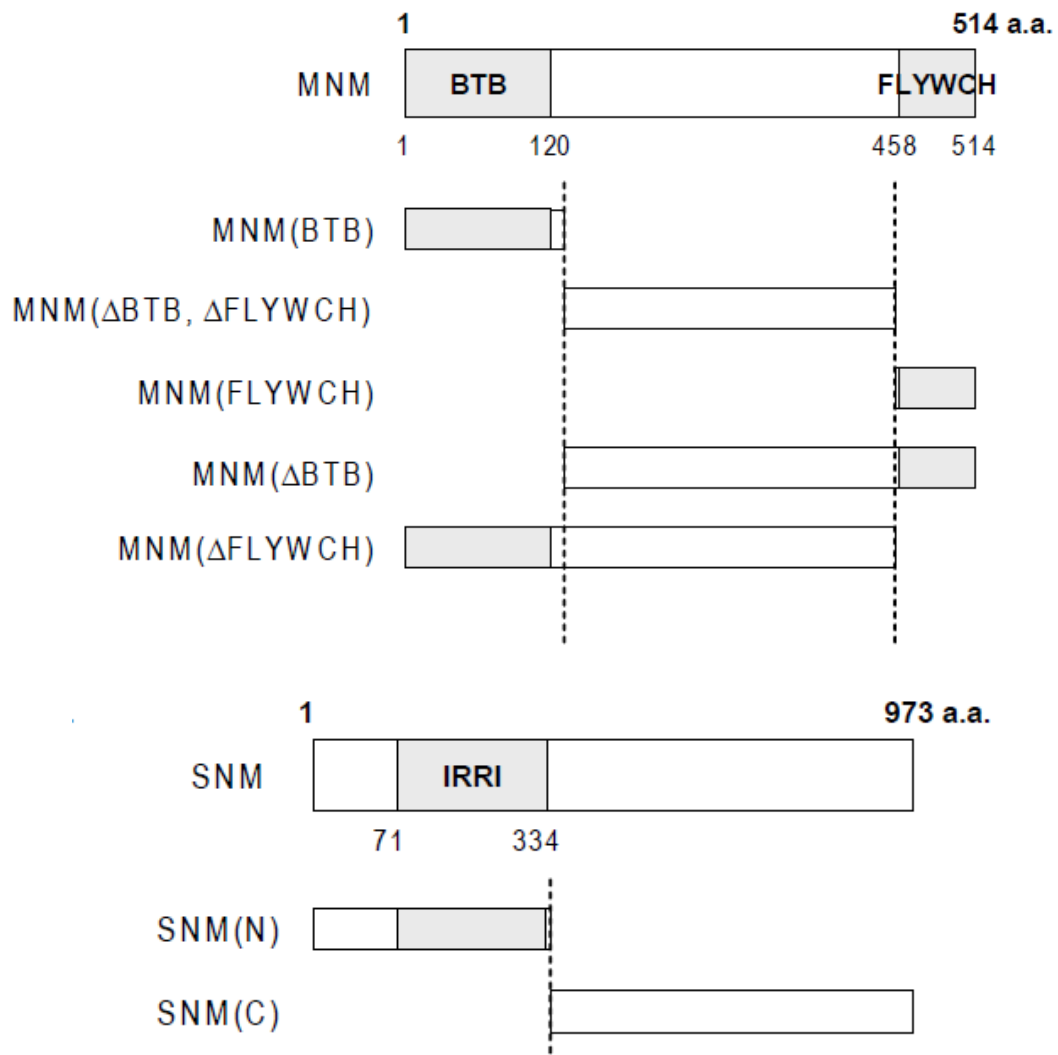


Figure 3-1. Schematic map of the SNM and MNM constructs used in the two-hybrid experiments.

The MNM protein is shown, with its N-terminal BTB domain and C-terminal FLYWCH domain. The SNM protein contains an IRR1 domain at the C-terminus. The numbers represent the length of amino acids. Each deletion construct is aligned to its full-length protein.

MATERIALS AND METHODS

Fly stocks

The *mnm* mutation was obtained from the Zuker-3 (Z3) collection of EMS mutagenized third chromosomes. The Z3 line used in this study was identified in a screen for paternal fourth chromosome loss and was provided by B. Wakimoto (Koundakjian et al. 2004; Thomas et al. 2005; Wakimoto et al. 2004). All flies were maintained at 23°C on standard cornmeal–sucrose–yeast– agar medium.

Construction of MNM fusion clones and generation of transgenic flies

The full-length MNM coding sequence was amplified by using *Pfx* polymerase (Invitrogen) and primers 5'-CACCATGGCGGACGACGAGCAATT-3' and 5'-CAAATGGTTGTGCACGCCTGA-3'. The PCR product was cloned into the pENTR/D-TOPO entry vector (Invitrogen), and the resulting product was sequenced. The insert then was recombined into Gateway P-element vectors pPWF and pPWV (Drosophila Genomics Resource Center), generating the germline transformation vectors P{w[+mC]=UASp-MNM::FLAG} and P{w[+mC]=UASp-MNM::Venus}. Both vectors include mini-white to detect germline transformants, upstream activation sequences for transcriptional activation by GAL4, always with either a FLAG tag or Venus tag. These constructs were transformed into *w*¹¹¹⁸ flies (BestGene Inc.). Transformants were mapped by standard procedures.

The MNM^{L9K} mutation, the MNM cDNA with the residue L9K replacement, was engineered by QuikChange II Site-Directed Mutagenesis Kit (Stratagene) using pENTR/D-TOPO entry vector containing *mnm* full-length cDNA fragment as a template with primers 5'-GGACGACGAGCAATTCAGCAAGTGCTGGAACAACTTC-3' and 5'-GAAGTTGTTCCAGCACTTGCTGAATTGCTCGTCGTCC-3'. Sequence analysis prior to recombine the mutant construct into the pPWV vector confirmed that only the L9K mutation was introduced. The P{w[+mC]=UASp-MNM^{L9K}::Venus} construct was transformed into flies by the aforementioned methods.

Immunoprecipitation

UASp-MNM::FLAG was induced by nos-GAL4::VP16 in *Drosophila* males and 100 pairs of testes were collected in 1× PBS (pH 7.4). Testes were lysed using 150 µl of NP40 Cell Lysis Buffer (Invitrogen). To immunoprecipitate MNM::FLAG, 150 µl of lysates were incubated with 10 µl of anti-SNM rabbit antibody (1.3 mg/ml) or rabbit serum (diluted to 1.3 mg/ml from original serum, sigma) and 340 µl IP solution (1 mM PMSF, 1 mM DTT, 1× Protease Inhibitor (Roche), 10% glycerol, 1× PBS, pH 7.4) rocking for 4 h, the lysates/anti-SNM antibody or serum solutions were then added to 80 µl of washed protein A agarose beads and rocked overnight at 4°C. Lysates/antibody or serum/IP solutions/beads were centrifuged and beads were washed 6× times with wash buffer (1 mM PMSF, 1 mM DTT, 1× Protease Inhibitor (Roche), 10% glycerol, 1× PBS, pH 7.4). 35 µl of loading buffer were added to the beads and heated to release proteins binding to the beads. The

released solutions were used to run 8% SDS-PAGE Acr/Bis electrophoresis. The MNM::FLAG protein was detected by Western blot by using anti-FLAG M2 antibody (1:750, Sigma) and 1:1000 anti-mouse HRP-conjugated with Super-Signal West Pico Chemiluminescent Substrate system (Pierce). For anti-ubiquitin immunoprecipitation, 15 µl of anti-ubiquitin rabbit antibody (1 mg/ml) was used for pulling down. The MNM::FLAG protein was detected by aforementioned methods. As for anti-FLAG immunoprecipitation, before incubation with antibody or serum solutions, a pre-clear process was carried out in which 150 µl of lysate was incubated with 600 µl IP solution and 80 µl of washed protein G agarose beads (Roche) rocking for 3 h at 4°C. The SNM protein was detected by anti-SNM diluted 1:2000, followed by HRP-conjugated goat anti-rabbit at a dilution of 1:7500 and Super-Signal Wes Pico Chemiluminescent Substrate system.

Phosphatase treatment

100 pairs of testes were collected and lysed with 150 µl of NP40 Cell Lysis Buffer. 10 units of shrimp alkaline phosphatase (SAP; Promega) were added to protein extraction. The reaction was continued for 30 min at 37°C and ready for following immunoprecipitation and western blotting.

Genetic assays for meiotic sex chromosome nondisjunction

To measure sex chromosome NDJ, $+/B^sYy^+$ males were crossed singly to two *yw* females in shell vials and maintained at 23°C; the parents were discarded on day 7 and progeny scored through day 21. Fertilization of regular *yw* (haplo-X) eggs by X, Y, XY or O sperm produces: $+♀♀(XX)$, $w B^s♂♂(XY)$, $B^s♀♀(XXY)$, and $yw♂♂(XO)$, progeny respectively. The rescue experiments for {UASp-MNM::FLAG}, {UASp-MNM::Venus} and {UASp-MNM^{L9K}::Venus} transgenic flies were carried out by similar methods.

Venus detection in unfixed spermatocytes

Transgenic {hs-MNM::Venus} and {hs-MNM^{L9K}::Venus} males were subjected to three heat shocks (37°C for 1 h/day). Testes were dissected from young adults in testes buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF) and gently squashed in testes buffer containing 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI). All testis preparations were examined using an Axioplan (Zeiss) microscope equipped with an HBO 100-W mercury lamp, Plan-Neofluar 100×/1.30 NA oil immersion lenses (Zeiss), and a high-resolution CCD camera (Roper) at room temperature. Grayscale digital images were collected, pseudocolored, and merged using Metamorph Software (Universal Imaging Corporation). Images were processed with Photoshop and Illustrator (CS2; Adobe).

Plasmid constructions for two-hybrid assays

The MNM and SNM cDNA were polymerase chain reaction (PCR) amplified from constructs as previously described by Thomas et al. (2005). Translational fusions between full-length MNM and SNM cDNA, and the GAL4 activation domain (AD) and the DNA-binding domain (BD) were constructed in the pGAD-C1 and pGBDU-C1 vectors, respectively (James et al. 1996). Plasmids were transformed into yeast strain PJ69-4A (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4 Δ gal80 Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) by the lithium acetate method described in the yeast protocols handbook (Clontech) and then grown in selective media. We used the HIS3 gene, controlled by Gal4 promoter, as the primary marker for protein-protein interactions.

The MNM fragments: MNM Δ 128-514 (MNM:BTB), MNM Δ 1-127 & Δ 456-514 (MNM:M), MNM Δ 1-455 (MNM:FLYWCH), MNM Δ 1-127 (MNM: Δ BTB) and MNM Δ 456-514 (MNM: Δ FLYWCH) and SNM fragments: SNM Δ 337-973 (SNM:N) and SNM Δ 1-336 (SNM:C) were PCR amplified, cloned into the pGAD-C1 and pGBDU-C1 vectors, and thereafter transformed into PJ69-4A yeast cells by the aforementioned methods.

β -galactosidase liquid assay

The assays were performed following protocols recommended by Clontech. Briefly, transformants were grown under selective conditions and the initial optical densities at a wavelength of 600nm (OD₆₀₀) were recorded. Cells were then re-

suspended in 100 μ l Z-buffer, and underwent 3 freeze/thaw cycles in liquid nitrogen and 37°C water bath. Then 700 μ l Z-buffer containing 1.89 μ l β -mercaptoethanol (Fisher) and 160 μ l Z-buffer containing 640 mg *ortho*-nitrophenyl β -D-galactopyranoside (ONPG) (Sigma) were added to reaction tubes and yellow color was allowed to develop for 30-60 min at 30°C. The reaction was stopped by adding Na_2CO_3 to a final concentration of 0.29 M. The optical density of the supernatant of the reaction was measured at a wavelength of 420nm (OD_{420}). One β -galactosidase unit was defined as the amount which hydrolyzes 1 μ mol ONPG to *ortho* -nitrophenol and D-galactose per minute per cell and calculated according to the formula, $(1000 \times \text{OD}_{420}) / (\text{time} \times \text{concentration factor} \times \text{OD}_{600})$. The activity of β -galactosidase was measured on at least three independent transformants and the average activities with standard deviation were calculated and plotted.

RESULTS

SNM and MNM associate with each other *in vivo*

The two-hybrid system is a molecular genetic technique which is used to study protein-protein interactions under *in vivo* conditions (Young 1998). Physical interactions are reflected by trans-activation of the GAL4-responsive *HIS* and *lacZ* reporter genes. First, we cloned the full-length coding sequence of MNM fused in-frame to the yeast GAL4 DNA binding domain (BD) as a “bait” construct. The full-length coding sequence of SNM was fused in-frame with the transcription activation domain (AD) of GAL4. Both plasmids were then co-transformed into yeast cells. Yeast growth on selective plates lacking histidine indicates interaction of two GAL4 domain fused proteins with each other. Here the MNM BTB domain combination – AD:MNM(BTB) and BD:MNM(BTB) – served as a positive control because the BTB domain of Mod(mdg4) has been shown to mediate the formation of homodimers and heterodimers (Fig. 3-2a)(Albagli et al. 1995; Ghosh et al. 2001; Mazur et al. 2005). Yeast carrying single plasmid or empty vectors served as negative controls. For almost all negative controls, no growth occurred after transformation on the his⁻ plates, except BD:MNM which survived with low viability (Fig. 3-2b and data not shown). It has been documented that the *HIS3* reporter is sensitive and useful for the effective detection of weak interactions but might be somewhat leaky (James et al. 1996), thus, the autonomous expression of *HIS3* reporter might have resulted in growth for this negative control. Notably, yeast harboring plasmids encoding the AD:SNM and BD:MNM exhibited much better growth on the his⁻ plate than did the

BD:MNM only yeast (Fig. 3-2c), indicating that the growth of the yeast with full-length MNM and SNM combination is not due only to leaky expression of *HIS3*. The reciprocal two-hybrid test, where MNM was fused to GAL4AD and SNM was fused to GAL4BD, were also carried out and exhibited growth on the selective plates (data not shown). Furthermore, the β -galactosidase liquid assays also showed that yeast harboring plasmids encoding the full-length MNM and SNM expressed levels of β -galactosidase activity about 2-fold greater than that produced by yeast cells containing BD:MNM alone and at least 7-fold greater than those of the other negative control sets (Fig. 3-2d and Fig. 3-3). Although this expression is lower than the β -galactosidase units produced by the BTB-BTB interaction, these results still imply that MNM and SNM physically interact with each other under *in vivo* condition.

The interaction between SNM and MNM was confirmed by immunoprecipitation of protein extracts from *Drosophila* testes. We generated the transgenic fly *P{w[+mC]=UASp-MNM::FLAG}* that contains tandem 3XFLAG insertions at the C-terminus of MNM. Flies homozygous for the *UASp-MNM::FLAG* transgene can rescue *mnm* male mutant phenotypes completely when it is induced by *nos::GAL4-VP16* (Table 3-1). Western blots stained with anti-FLAG antibody revealed that the MNM::FLAG fusion protein was co-immunoprecipitated from testes extracts of *UASp-MNM::FLAG; nos-GAL4::VP16/MKRS* males by anti-SNM antibody but not by host control serum (Fig. 3-2e). Two bands showed up in both the Input and SNM pull-down lanes, suggesting that MNM protein might undergo post-translational modification. Moreover, the SNM protein was also pulled down by

reciprocal immunoprecipitation with anti-FLAG antibody (Fig. 3-2e), indicating that MNM and SNM function together through physical association.

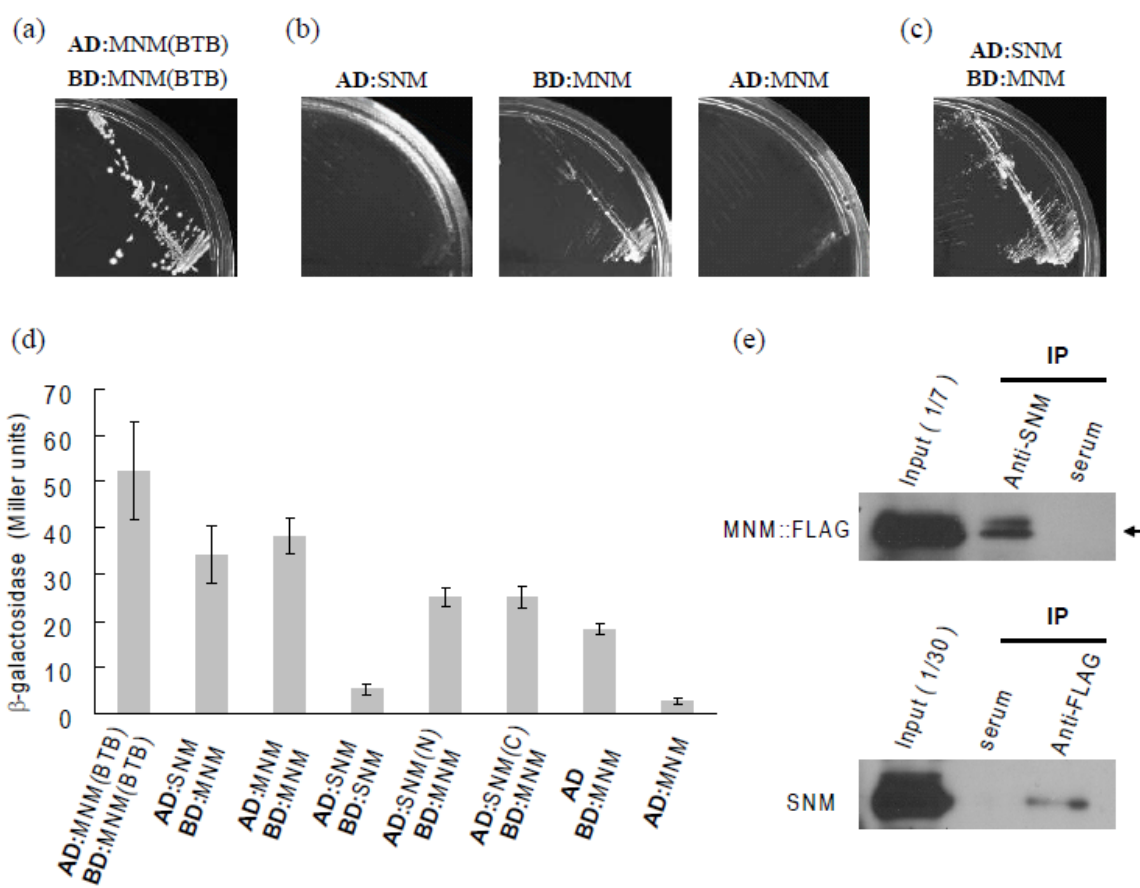
There are various types of post-translational modification. The rapid disappearance of MNM suggests that MNM might undergo degradation at anaphase I. We tested ubiquitination by anti-Ubiquitin pull-down assay, however, MNM::FLAG bands only showed in the Input lane and not in the ubiquitin pull-down or serum control lanes (Fig. 3-4). In order to test whether phosphorylation occurs on MNM::FLAG, proteins extracted from *Drosophila* testes were treated with shrimp alkaline phosphatase (SAP), which catalyzes dephosphorylation, following immunoprecipitation and western blotting. However, two bands still showed in both the Input and pull-down lanes after SAP treatment (Fig. 3-5). Taken together, these results suggest that the double MNM::FLAG bands can not be explained by post-translational modification involving ubiquitination or phosphorylation.

We next attempted to examine whether either MNM or SNM interact with themselves. MNM was a likely candidate since its conserved BTB domain interacts directly and strongly with another BTB domain. The fused constructs, in which full-length MNM was fused to GAL4AD and GAL4BD, were generated and transformed into yeast. Quantification of β -galactosidase levels exhibited that MNM strongly interacts with itself (Fig. 3-2d), which is consistent with previous studies that showed protein containing the BTB domain can form homodimers (Ghosh et al. 2001; Mazur et al. 2005). To test SNM dimerization, we generated similar constructs in which full-length SNM was fused to GAL4AD and GAL4BD and transformed those constructs into yeast. The level of β -galactosidase activity of yeast containing

AD:SNM and BD:SNM was as low as those found in the negative controls (Fig. 3-2d), indicating that SNM is not able to interact with itself.

Figure 3-2. SNM and MNM interact directly with each other.

Growth of yeast carrying different combinations of SNM and/or MNM constructs on selective media plates for the reporter genes used in the yeast two-hybrid experiments. (a) Yeast containing AD:MNMBTB and BD:MNMBTB served as a positive control. (b) Yeast containing MNM or SNM alone served as negative controls. (c) Yeast co-transformation with full-length SNM and full-length MNM grew on the selective plate. (d) β -galactosidase activity, expressed as Miller units, from extracts of yeast strains carrying combinations of SNM and MNM constructs. Values are the means $\pm$ SD of assays on at least three repeat experiments. (e) Testes lysates were prepared from MNM-FLAG flies [UASp::MNMBTB/Cy; nos::GAL4-VP16/MKRS] and used for reciprocal immunoprecipitation with anti-SNM, anti-FLAG antibodies and serum (mimic controls). Immunoprecipitates and lysates (Input controls) were analyzed by Western blot using anti-FLAG or anti-SNM antibody to identify the proteins.



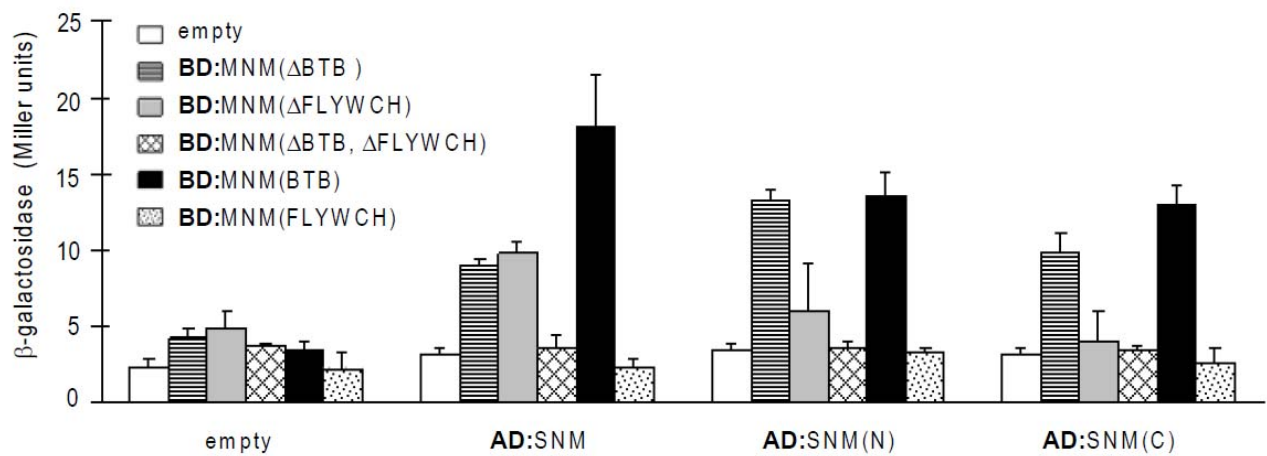


Figure 3-3. The BTB and FLYWCH domains of MNM mediate interaction with SNM.

β -galactosidase activity, expressed as Miller units, of yeast harboring plasmids encoding the indicated GAL4AD fusions in combination with the indicated GAL4BD fusions. Values are the means $\pm$ SD of assays on at least three repeat experiments.

Table 3-1. Sex chromosome nondisjunction in *mnm* mutant and transgenic flies.

Paternal genotypes	Sperm class [*]				N	%ND _J
	X	Y	XY	O		
$B^sYy^+; mnm^{Z3-5578}/Df(3R)T16$	61	45	43	67	216	50.93
$B^sYy^+; MNM::FLAG; nos-gal4::VP16, mnm^{Z3-5578}/Df(3R)T16$	331	313	0	3	647	0.43
$B^sYy^+; MNM::Venus; nos-gal4::VP16, mnm^{Z3-5578}/Df(3R)T16$	142	132	5	11	290	5.5
$B^sYy^+; MNM^{L9K}::Venus; nos-gal4::VP16, mnm^{Z3-5578}/Df(3R)T16$	68	56	23	55	202	38.6
<i>mnm</i> mutant: $mnm^{Z3-5578} = W499STP$						

N: total number of progeny scored. The percentage of X–Y NDJ was calculated as $100 \times (XY + O)/N$. * Sperm genotypes deduced from progeny phenotypes.

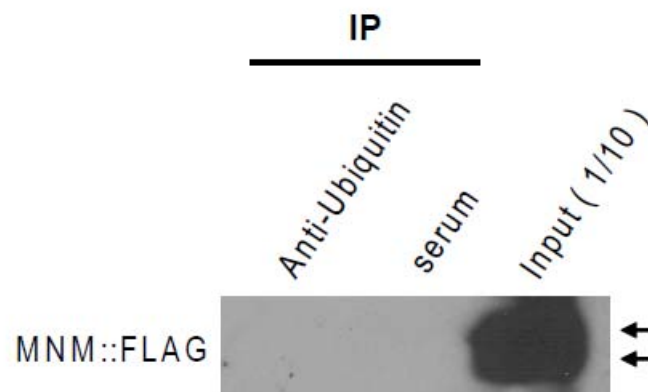


Figure 3- 4 Post-translational modification of MNM is not ubiquitination.

Testes lysates were prepared from MNM::FLAG flies [*UASp-MNM::FLAG; nos-GAL4::VP16/MKRS*] and used for immunoprecipitation with anti-ubiquitin and serum (mimic control). Immunoprecipitates and lysates (Input controls) were analyzed by Western blot using anti-FLAG to identify the MNM protein. No MNM::FLAG protein was detected in both the ubiquitin pull-down and serum control lanes.

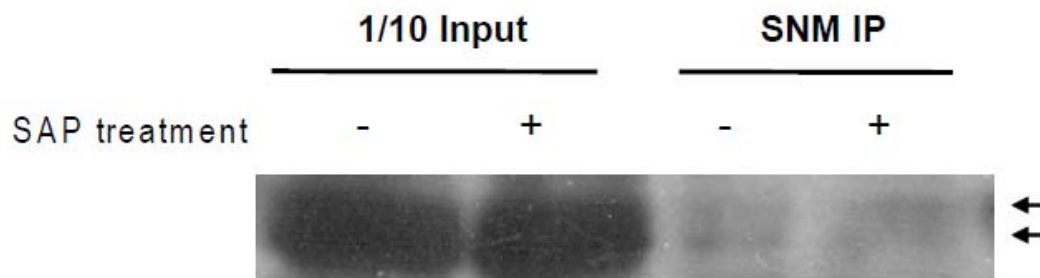


Figure 3- 5. Post-translational modification of MNM is not phosphorylation.

Testes lysates were prepared from MNM::FLAG flies [*UASp-MNM::FLAG; nos-GAL4::VP16/MKRS*]. For SAP treatment: “+” represents proteins were treated with shrimp alkaline phosphatase following immunoprecipitation assay and “-” represents proteins were not treated with shrimp alkaline phosphatase. Arrows indicate two bands still showed in both the Input and SNM pull-down lanes after SAP treatment.

The IRR1 domain is not the only part of SNM to mediate interaction with MNM

After identification of the interaction between SNM and MNM, we set out to identify domains responsible for this interaction. We first tested the IRR1 domain of SNM by making the constructs with and without the N-terminal conserved IRR1 domain fused to the GAL4AD, AD:SNM(N) and AD:SNM(C), respectively (Fig. 1). The levels of β -galactosidase activity of yeast carrying full-length MNM and N-terminus of SNM were higher than all negative control sets, including the combination of AD and BD:MNM, but lower than those of yeast carrying full-length SNM and MNM, indicating that the interaction at the IRR1 domain is somewhat weaker (Fig. 2d). Furthermore, yeast carrying AD:SNM(C) and BD:MNM produced similar levels of β -galactosidase activity as yeast carrying AD:SNM(N) and BD:MNM (Fig. 2d). This revealed that the C-terminus of SNM, lacking the IRR1 domain, is also able to interact with full-length MNM. Taken together, these results suggest that the IRR1 domain does interact with MNM but it might not be the only region of SNM responsible for interacting with MNM.

Interaction between SNM and MNM are mediated by the BTB and FLYWCH domains of MNM

Two domains of MNM – a BTB domain at the N-terminus and the FLYWCH domain at the C-terminus – appeared to be good candidates to be involved in interaction with SNM. We tested these domains by generating a series of MNM deletion constructs for yeast two-hybrid analysis. We made GAL4BD fused

constructs of MNM lacking either the BTB domain [BD:MNM(Δ BTB)], the FLYWCH domain [BD:MNM(Δ FLYWCH)] or both [BD:MNM(Δ BTB, Δ FLYWCH)] (Fig. 3-1) and tested their interactions with full-length SNM. Yeast carrying BD:MNM(Δ BTB) and AD:SNM, as well as yeast carrying BD:MNM(Δ FLYWCH) and AD:SNM, produced similar levels of β -galactosidase activity which were greater than levels produced by the negative control sets but much less than those produced by yeast expressing the intact proteins (Fig. 3-3). Furthermore, yeast cells co-transformed with AD:SNM and BD:MNM(Δ BTB, Δ FLYWCH) yielded very low levels of β -galactosidase units comparable to those of the negative controls (Fig. 3-3), suggesting that both BTB and FLYWCH domains of MNM contribute to interactions with SNM.

We next wanted to test whether these two domains by themselves were capable of interactions with full-length SNM. GAL4BD fused constructs containing only the BTB domain or the FLYWCH domain were co-transformed with AD:SNM into yeast. β -galactosidase analysis showed that the BTB domain by itself was able to interact with full-length SNM and the strength of the interaction to be much greater than the negative controls. Surprisingly, however, the FLYWCH domain failed to interact with the intact SNM protein (Fig. 3-3). This result, as well as the weak interaction found between BD:MNM(Δ FLYWCH) and AD:SNM, suggest the FLYWCH domain of MNM is crucial for the SNM/MNM interaction although it does not directly interact with SNM. An alternative might be that the FLWCH domain alone cannot fold properly. The fact that the BD:MNM(Δ BTB) construct interacts with full-length SNM indicates that there must be at least one interaction motif outside the BTB domain, i.e. either in the FLYWCH domain or in the middle region

of MNM, though neither of those regions can interact with SNM on its own. Perhaps, neither the middle region of MNM nor the FLYWCH domain can fold properly alone but the BD:MNM(Δ BTB) construct folds properly for protein interaction.

We also tested the ability of truncated MNM fused to GAL4BD constructs to interact with SNM fused to GAL4AD constructs either containing or lacking the IRR1 domain (Fig. 3-3). According to the levels of β -galactosidase activity, the construct containing only the BTB domain appeared to interact with both the N-terminus and C-terminus of SNM. Deletion of the BTB domain [BD:MNM(Δ BTB)] diminished the interaction between the MNM protein and SNM truncations [AD:SNM(N) and AD:SNM(C)]. Moreover, neither yeast co-transformed with BD:MNM(Δ BTB, Δ FLYWCH) and AD:SNM(N) nor yeast carrying BD:MNM(Δ BTB, Δ FLYWCH) and AD:SNM(C) produced levels of β -galactosidase greater than those of negative controls. This implies that, similar to interact with full-length SNM, the FLYWCH domain is also important for MNM to interact with the N-terminus and C-terminus of SNM.

When comparing the combination of the isolated BTB domain [MNM(BTB)] and full-length SNM with the combination of the FLYWCH domain deletion [MNM(Δ FLYWCH)] and full-length SNM, we found that the intensity of the interaction between MNM(BTB) and SNM was much stronger than that between MNM(Δ FLYWCH) and SNM. Similar results were also found in the SNM N-terminus and SNM C-terminus sets. These results suggest that when the FLYWCH

domain is missing, the middle region of MNM might interfere with the interaction ability of the BTB domain, possibly due to incorrect protein folding.

L9K mutation of the BTB domain impairs MNM function

Upon identification of the BTB domain of MNM being involved in MNM-MNM and MNM-SNM interactions, we wished to further explore its functions. The X-ray crystal structures of the BTB domains of promyelocytic leukemia zinc finger (PLZF) and B cell lymphoma 6 (BCL6) reveal structural bases for dimerization and oligomerization, which involve anti-parallel β sheet formation (Ahmad et al. 1998; Ahmad et al. 2003; Li et al. 1999). The residue leucine 11 of PLZF contributes to the exposed hydrophobic surface and centrally locates in the dimer-dimer interface (Ahmad et al. 1998). Mutation of this leucine of PLZF strongly reduced formation of high-molecular-weight PLZF complex with DNA (Melnick et al. 2000). Since the equivalent residue of MNM BTB domain (L9) is highly conserved, a MNM construct with L9K replacement was made and tested in the yeast two-hybrid assay. Compared to normal MNM, the levels of β -galactosidase units were greatly reduced in yeast containing the isolated BTB domain with the L9K mutation [AD:MNM(BTB^{L9K}) and BD:MNM(BTB^{L9K})] as well as in the intact MNM protein [AD:MNM^{L9K} and BD:MNM^{L9K}] (Fig. 3-6). These results are consistent with previous findings that the leucine mutation results in a flawed BTB/POZ domain that impedes *in vivo* homodimerization (Melnick et al. 2000). Furthermore, the interaction between MNM and SNM is also affected by this L9K mutation (Fig. 3-6). It is

noteworthy that L9K mutation severely impaired but did not completely destroy the interaction ability of MNM, for yeast containing [AD:MNM^{L9K} and BD:MNM^{L9K}] and [AD:SNM and BD:MNM^{L9K}] showed higher residual levels of β -galactosidase than those produced by the negative controls..

To confirm the results in *Drosophila*, we generated two transgenic fly strains with fluorescence tags, one with full-length MNM – $P\{w[+mC]=UASp-MNM::Venus\}$ – and the other one with L9K mutation – $P\{w[+mC]=UASp-MNM^{L9K}::Venus\}$ – for functional assays. Homozygotes of $UASp-MNM::Venus$ significantly rescued *mnmm* male mutant phenotypes when induced by *nos-GAL4::VP16*, whereas $UASp-MNM^{L9K}::Venus$ males still exhibited high rates of nondisjunction (Table 3-1). Moreover, we failed to detect the signal of MNM^{L9K}::Venus on chromosomes during meiosis I, whereas MNM::Venus signal appeared at the onset of meiosis until anaphase I (Fig. 3-7), confirming that the L9K replacement severely impairs the function of MNM in protein-protein interaction and homologous chromosome pairing. Thus, although the β -galactosidase assay showed that MNM's capacity to interact was not abolished in yeast cells, the interactions might be too weak in spermatocytes to achieve any biological activity.

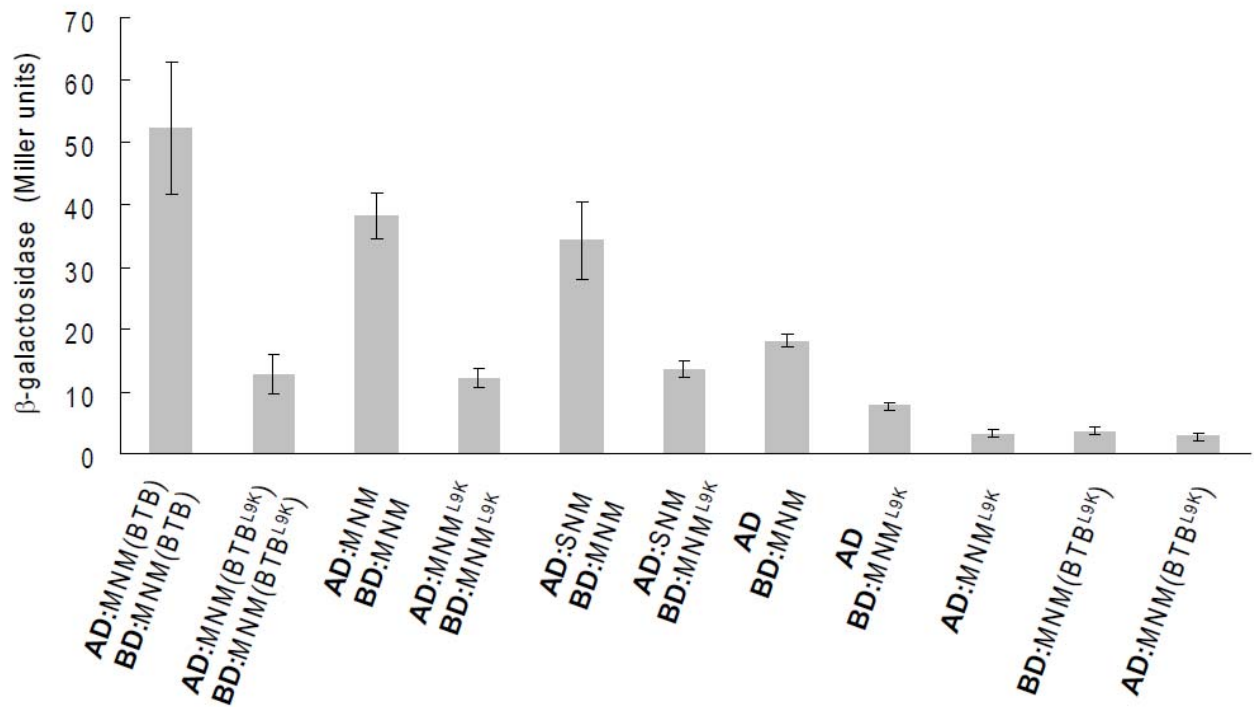


Figure 3- 6. L9K replacement disrupts the ability of the BTB domain of MNM to interact with itself or SNM.

β-galactosidase activity, expressed as Miller units, from extracts of yeast strains carrying combinations of SNM and MNML9K constructs. Values are the means $\pm$ SD of assays on at least three repeat experiments.

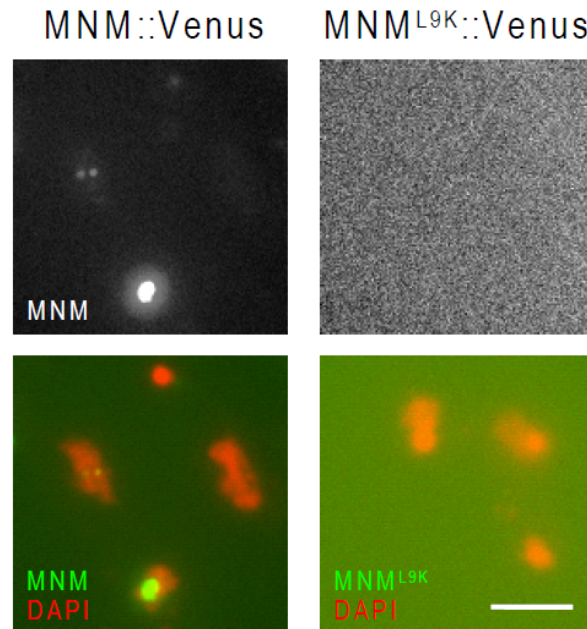


Figure 3- 7. Localizations of MNM::Venus and MNM^{L9K}::Venus to chromosomes in primary spermatocyte nuclei from transgenic {hs-MNM::Venus} and {hs-MNM^{L9K}::Venus} males.

Spermatocytes from transgenic males stained with DAPI and imaged for MNM::Venus and MNM^{L9K}::Venus fluorescence (upper panels: Venus only; lower panels: merge of Venus and DAPI). Each panel shows one nucleus at prometaphase I. All panels are overexposed relative to the nucleolar or X-Y signals to bring out the fainter autosomal signals. Size bar represents 5 μ m.

DISCUSSION

In the yeast two-hybrid experiments we found that one negative control – yeast carrying full-length MNM fused to GAL4 DNA-binding domain in the pGBDU-C1 vector – survived on the selective his⁻ plate, whereas the other negative controls did not. A similar result was observed when full-length MNM was fused to GAL4 DNA-binding domain in another vector, pGBD-C1 (data not shown). Two possibilities might cause the growth of these negative controls: either the *HIS3* reporter is leaky or the MNM protein functions in transcriptional activation. In the case of leaky expression of *HIS3* reporter, the cell viability should be low and β -galactosidase expression should also be low. However, results of liquid assays showed that the β -galactosidase levels of yeast containing BD:MNM construct were about 3-fold higher than the other negative controls (Fig. 3-2d and Fig. 3-3), suggesting intrinsic transcriptional activating function of the BD:MNM fusion. It has been reported that the BTB or FLYWCH domains are present in many transcriptional regulatory proteins, such as GAGA factor in *Drosophila*, FLH-1 in *C. elegans* and PLZF in humans, and are important for proper protein function (Katsani et al. 1999; Melnick et al. 2000; Ow et al. 2008). For example, the GAGA factor utilizes a C-terminal C2H2 zinc-finger motif to bind to DNA and forms large multimeric complexes by BTB-BTB interactions (Espinosa et al. 1999; Katsani et al. 1999). Thus, it is possible that the MNM protein has transcription regulation function. In this study, we showed that deletion or mutation of the BTB domain resulted in weakened intrinsic β -galactosidase expression (Fig. 3-3 left panel and Fig. 3-6). Such results suggest that homodimers or multimers of MNM, not

monomer, function in transcription activation since the BTB domain mediates dimerization and oligomerization. Furthermore, there is an uncharacterized glutamine-rich region in the middle section of MNM. Several transcription factors have been characterized that contain glutamine-rich domains which mediate transcriptional activation (Escher et al. 2000). However, deletion of the glutamine-rich region ($\Delta 128-225$) of MNM did not significantly decrease the level of β -galactosidase expression (data not shown), suggesting that this region might not contribute to transcriptional activation.

To date, along with Teflon (TEF) which has been documented to be required for regular segregation of autosomes (Arya et al. 2006; Thomas et al. 2005; Tomkiel et al. 2001), MNM and SNM have been reported to be essential for stable conjunction and regular segregation of all four homolog pairs in male meiosis I. However, the mechanism by which those proteins mediate homologous chromosome pairing and segregation is still unclear. Here we have shown that MNM and SNM directly interact with each other *in vivo*, which is consistent with our previous observations that both MNM and SNM depend on each other for chromosome localization (Thomas et al. 2005). In this study, we considered an MNM-SNM interaction model where the BTB domain is involved in MNM homodimeric or oligomeric interactions as well as binding to SNM, whereas the FLYWCH domain might be involved in stabilizing the interaction between MNM and SNM. This model is supported by our observations that the BTB domain of MNM is able to interact with the full-length SNM protein in the yeast two-hybrid system, and that deletion of FLYWCH domain results in weakened interaction between MNM

and SNM even though FLYWCH by itself does not interact with the full-length SNM protein (Fig. 3-3).

It has been proposed that multiple MNM-SNM complexes reside on homologous chromosomes to prevent premature dissociation, driven by the self-associative potential of the BTB domain of MNM (Thomas et al. 2005). Here, we found that an MNM protein containing a L9K mutation in the BTB domain failed to localize to chromosomes and caused a meiotic defect in chromosome segregation. This suggests a role for the BTB domain in chromosome association. This suggestion is broadly consistent with the cytological observations of the Mod(mdg4)^{67.2} protein, which is a MNM isoform and is a component of the gypsy insulator. Inactivation of the Mod(mdg4)^{67.2} BTB domain by point mutations or deletion of the BTB domain rendered the mutant proteins unable to localize to chromosomes (Golovnin et al. 2007).

By alternative splicing, all the mod(mdg4) isoforms contain a common N-terminus whereas the C-terminus are variable. Similar to the N-terminus BTB domain, the C-terminus FLYWCH domain of MNM is also essential for functional MNM protein. It has previously been shown that deletion of the FLYWCH domain of MNM disrupts homologous chromosome conjunction in male meiosis (Soltani-Bejnood et al. 2007; Thomas et al. 2005). In this study, we sought and obtained evidence that deletion of the FLYWCH domain impairs MNM-SNM interaction. In light of the compelling evidence that the FLYWCH domain does not directly interact with SNM, an obvious question is how the FLYWCH domain mediates the interaction between MNM and SNM. We speculate that the C2H2 motif which is

embedded in the FLYWCH domain might play an important role in stabilizing the MNM-SNM complex due to the zinc finger motif is being a potential protein-protein interaction region (Wolfe et al. 2000). One possibility is that the C2H2 motif cooperates with the BTB domain to stabilize the formation of MNM homodimers or multimers, enhancing MNM's ability to interact with SNM. In fact, genetic analysis has shown that a mutation of this C2H2 motif caused defective homologous chromosome conjunction in *Drosophila* male meiosis (Soltani-Bejnood et al. 2007).

MNM and SNM are recruited to 240-bp repeat sequences of rDNA for X-Y pairing (Thomas et al. 2005). Autosomes lack rDNA sequences but the homologous pairing is still mediated by MNM and SNM. How MNM and SNM are recruited to autosomes has not been clearly elucidated yet. We showed that both N-terminus and C-terminus of SNM are able to interact with full-length MNM, suggesting that protein-protein interaction motifs might be present in uncharacterized C-terminus as well as the conserved IRR1 domain of SNM. Furthermore, TEF has been suggested to hold autosome bivalents as a bridging molecule during meiosis (Arya et al. 2006). It is highly possible that potential protein-protein interaction motifs of SNM as well as the BTB and FLYWCH domains of MNM directly interact with TEF, perhaps the C2H2 zinc-finger motifs of TEF, resulting in the MNM-SNM-TEF complex residing on autosomes. This model is supported by our previous cytological observations the MNM failed to localize to autosomal bivalents in *tef* mutant spermatocytes (Thomas et al. 2005). A variation of this model is that, rather than directly interacting with TEF, MNM-SNM recruit

chromatin-associated proteins which cooperate with TEF for chromosome localization.

Overall, the identification of specific domains of the MNM and SNM proteins that contribute to intermolecular interaction provides a strong biochemical foundation for elucidating the involvement of these two proteins in mediating homologous chromosome conjunction and segregation. Further research of target sequences in MNM and SNM and identification of other proteins involved with MNM-SNM complex should provide additional evidence and contribute to in-depth understanding of the mechanism of homologous chromosome pairing in male meiosis.

ACKNOWLEDGMENTS

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CHAPTER 4

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

GENERAL CONCLUSIONS

The main purpose of this research is to investigate the mechanism of homolog pairing in *Drosophila* male meiosis. A great number of studies have been performed in yeast, *C. elegans*, plants and mammals to study homologous chromosome pairing and segregation in meiosis; these have revealed many important principles, such as synapsis, recombination, and formation of chiasmata (Carpenter 1994; Page and Hawley, 2004; Roeder 1997; Zickler 2006). However, male *Drosophila* is a unique achiasmatic meiosis in which synaptonemal complex formation and recombination do not occur during meiosis (White 1973). The mechanism of homolog pairing has yet not been elucidated well in *Drosophila* males. In this study, we identified the stable sites for homolog pairing and sister chromatid cohesion on the autosomes, as well as demonstrated the interaction between MNM and SNM, which are essential for all four chromosomes of homolog pairing in *Drosophila* male meiosis.

A new stable pairing site is identified in the heterochromatic region 61 on the fourth chromosome

Previously, the stable homolog pairing site has been only identified in the X–Y pair (McKee and Karpen 1990; McKee 1996). No pairing sites have yet been reported for the autosomes. In our FISH screening, greater than 90% of spermatocytes exhibited the fourth homologs paired as a single strong hybridization signal during prophase I (Fig. 2-6a and Table 2-4). Two probe signals were shown

close to each other and at a fixed distance at prometaphase I in wild-type spermatocytes, whereas probe signals were found unpaired at prometaphase I in *mnm* and *snm* mutants (Fig. 2-6b and 2-6c). Thus the pairing activity of the fourth homolog resides in the heterochromatic region 61. In contrast, six tested heterochromatic regions of the major autosomes, the second and third chromosomes, have proved to be largely unpaired after early prophase I. This suggests that homolog pairing mechanism of major autosomes might differ from the sex and fourth chromosomes.

Sister chromatid cohesion is incomplete along chromosome arms during *Drosophila* male meiosis

Sister chromatid cohesion was first named to refer a physical linkage between two duplicated sister chromatids (Miyazaki and Orr-Weaver 1994). Sister chromatid cohesion is generated during DNA replication stage, holds sister chromatids together throughout the chromosome arms and the centromeres, and then is released in a stepwise manner to achieve chromosome segregation (Nasmyth 2001). In this study, we observed two distinct sister chromatid cohesion patterns within heterochromatin: regions with stable cohesion throughout meiosis and other regions in which cohesion is absent by at least the middle of prophase I. Our results clearly show that sister chromatid cohesion is incomplete within the heterochromatic domains in *Drosophila* males, which is consistent with the pattern in yeast (Blat and Kleckner 1999; Glynn et al. 2004). Furthermore, this sister

chromatid cohesion was severely disrupted in *solo* mutants (Fig. 2-9), implying that sister chromatid cohesion is promoted by SOLO in *Drosophila*.

MNM and SNM are components of the homolog conjunction complex

Both MNM and SNM are essential for homolog pairing in *Drosophila* male meiosis (Soltani-Bejnood 2007; Thomas et al. 2005). It has also been reported that MNM and SNM depend on each other for chromosome localization (Thomas et al. 2005). Our findings demonstrate that MNM and SNM directly interact with each other *in vivo*. We also identified the interaction domains between MNM and SNM. These results indicate that both MNM and SNM are involved in the same complex, called homolog conjunction complex although the other components have not been identified yet. In *Drosophila* males the homolog conjunction complex, instead of synaptonemal complex and chiasmata, might stabilize interhomolog connections during meiosis I. However, how SNM and MNM are recruited to chromosomes has to be further investigated.

BTB domain is crucial for MNM function

The BTB domain is an evolutionary conserved protein-protein interaction motif found throughout the eukaryotes (Perez-Torrado et al. 2006). It has been reported that the disruption of the BTB domain by mutation or deletion resulting in lost of function of BTB-containing proteins (Ghosh et al. 2001; Melnick et al. 2000). Since the BTB domain is highly conserved, one can imagine that dimerization and

oligomerization ability of the BTB domain must be important for MNM. Our data indicates that MNM is able to form homodimer. Dimerization of MNM as well as interaction with SNM was interfered when the BTB domain was mutated by L9K replacement (Fig. 3-4). MNM^{L9K}-Venus fusion protein was nearly undetectable in the transgenic flies, whereas MNM-Venus fusion protein appeared from early prophase I until anaphase I, suggesting that MNM monomer might be unstable. Furthermore, the evidence that MNM^{L9K} mutation cause high frequencies of chromosome nondisjunction further support the idea that the BTB domain plays an important role for functional MNM.

FUTURE DIRECTIONS

The data obtained during the course of research for this dissertation will be important in understanding the homologous chromosome pairing in *Drosophila* male meiosis. The studies about screening of autosomal homolog pairing sites demonstrate that the fourth homologs pair at heterochromatic region 61; however, no specifically stable pairing sites of the major autosomes have been found. We have suggested that homolog pairing of the major autosomes, mediated by the homolog conjunction complex, occurs at different sites in different cells but is stable once it occurs, which is analogous to chiasmata in recombinational meiosis. To prove such “chiasmata-like” stable connections, time-lapse analyses in living spermatocytes using GFP-tagged chromosomal sites could be applied, in which a stable connection site that happened to lie sufficiently close to a tagged chromosomal site should restrict the relative mobility of the tagged homologous alleles. Furthermore, it will be interesting to determine whether MNM/SNM localizes to these “chiasmata-like” pairing sites on the major autosomes, since both MNM and SNM co-localize with X-Y pairing site. Transgenic flies will be used to assess co-localization of MNM-CFP or SNM-CFP foci with GFP-tagged chromosomal sites.

The pairing of homologous centromeres happens transiently during stage S3, but suddenly loses pairing by stage S4 (Vazquez et al. 2002). Although centromere pairing is transient, it may be a prerequisite for establishment of stable homolog connections at other heterochromatic sites. It will be intriguing to determine whether SNM localizes to centromeres during stage S3 since SNM is required for the pairing of homologous centromeres during middle prophase I. If SNM functions

directly to connect homologous centromeres, it might localize on the centromeres. Therefore, the immunocytology will be carried out by using anti-FLAG or anti-HA antibodies against FLAG-HA-SOLO, which is present on all centromeres, in *Drosophila* males expressing SNM-Venus. Exploring the function of SNM related to its localization will be helpful to understand the mechanism of meiotic homolog pairing in *Drosophila* males.

Another very interesting issue is to identify other components of the MNM&SNM complex and other interacting proteins. Yeast two-hybrid will be carried out to test for direct interactions with candidate proteins. The first candidate is TEFLON which is required for autosomal localization of MNM and perhaps SNM (Thomas et al. 2005). Moreover, SNM is a paralog of the highly conserved SCC3/SA family of cohesions. Our preliminary results showed that both SMC1 and SMC3, which form the backbone of all known cohesin complexes, co-immunoprecipitated with anti-SNM antibody. Thus, it is highly possible that SNM interacts with other cohesion proteins, such as the kleisin homologs RAD21 and C(2)M and the meiosis-specific cohesion protein SOLO. In addition, the interactions between MNM and these cohesion proteins will be also examined. If any positive result is obtained from yeast two-hybrid, it will be confirmed again by immunoprecipitation assay or bimolecular fluorescence complementation (BiFC).

According to the results of immunoprecipitation and Western blot, there were two bands showed up in both the Input and anti-SNM pull-down lanes (Fig. 3-2e), suggesting that MNM protein might undergo post-translational modification. There are various types of post-translational modification, such as phosphorylation,

ubiquitination, SUMOylation and glycosylation. We have tested shrimp alkaline phosphatase (SAP) treatment, which catalyzes the dephosphorylation, as well as anti-Ubiquitin pulling-down assay. However, there is no direct evidence that MNM undergo phosphorylation or ubiquitination. It will be helpful to analyze the post-translational modification by Mass Spectrometry. Thus, proteins after dual pulling down of anti-SNM and anti-FLAG will be run on 8% SDS-PAGE followed by Coomassie brilliant blue R250 (Sigma-Aldrich). Protein bands will be excised. Identification of post-translational modification of MNM will be performed by Applied Biomics (Hayward, CA, USA).

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