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Diploid germline cells must undergo two consecutive meiotic divisions before differentiating as haploid sex cells. During meiosis I, homologs pair and remain conjoined until segregation at anaphase. *Drosophila melanogaster* spermatocytes are unique in that the canonical events of meiosis I including synaptonemal complex (SC) formation, double-strand DNA breaks, and chiasmata are absent. Sex chromosomes pair at intergenic spacer sequences within the rDNA. Autosomes pair at numerous euchromatic homologies, but not at heterochromatin, suggesting that pairing may be limited to specific sequences. However, previous work generated from genetic segregation assays or observations of late prophase I/prometaphase I chromosome associations fail to differentiate pairing from maintenance of pairing (conjunction).

To begin, the capability of X euchromatin to pair and conjoin with the Y chromosome was examined using an rDNA-deficient X and a series of *Dp(1;Y)* chromosomes. Genetic assays determined that duplicated X euchromatin can substitute for endogenous rDNA pairing sites; however, segregation was not proportional to homology length. Using fluorescent in situ hybridization (FISH) to early prophase I spermatocytes, pairing was shown to occur with high fidelity at all homologies tested. By comparing genetic and cytological data, we determined that centromere proximal pairings were best at segregation. Segregation was dependent on the conjunction protein Stromalin in Meiosis while the autosomal-specific Teflon was dispensable.

Next, the ability of the X euchromatic homology to pair with and segregate from the heterolog chromosome 3 was examined using *Dp(1;3)* chromosomes containing X euchromatin duplications ranging in size from 21 to 177 Kb. In contrast to duplications of X euchromatin on the Y, duplications of X material on chromosome 3 are not as effective in directing segregation. In early prophase I, however, homologies on the X and chromosome 3 pair. Pairing between homologs is normally released at S2b of prophase I. Using a control probe to only select cells where chromosome 2 has already unpaired, the X and *Dp(1;3)* was unpaired in a significantly higher number of cells than was the X and *Dp(1;Y)*. This result suggests different mechanisms exist to manage pairings between homologs and pairings between heterologs.

The FISH pairing assay was used to score meiotic I nondisjunction (NDJ) and compared to genetic NDJ. Some NDJ frequencies were significantly different between the two methods. Data suggests genetic NDJ calculations are not always a true measure of the meiotic defect. The FISH pairing assay was also used to investigate an uncharacterized male meiotic mutant since the assay provides a rapid identification of the defective meiotic stage. FISH identified a unique defect that caused sister chromatids to segregate to opposite poles during meiosis I. This identification would not have been possible by only monitoring the outcome of meiosis through genetic crosses.

The molecular techniques and approaches described within are suggested to be useful in defining the mechanisms regulating the establishment of conjunction and segregation between paired sequences.

DETERMINING REQUIREMENTS FOR MEIOTIC PAIRING IN *DROSOPHILA MELANOGASTER* SPERMATOGENESIS

by

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Meiosis is comprised of two cellular divisions that follow a single round of DNA replication. The first "reductional" division segregates a replicated chromosome from its homolog and is followed by a second "equational" division that segregates the sister chromosomes (Page and Hawley 2003). The formation of four haploid daughter cells from a single diploid parent cell is highly conserved among many species, including humans (Hassold and Hunt 2001); therefore, an understanding of the mechanisms of meiosis gained from the study of model organisms may be applicable to human meiosis.

Chromosomes must accomplish homologous partner recognition or "pairing" for meiosis to occur with high fidelity. The paired homologs form a structure called a bivalent. In most species, pairing is followed by the assembly of the proteinaceous synaptonemal complex that stabilizes the bivalent while promoting homologous recombination through a biased double-strand break repair pathway (Allers and Lichten 2001). Homologous chromatid arms cross over and establish chiasmata, physical connection points that further aid in the stabilization of bivalents (Carpenter 1994). Bivalents are maintained throughout prophase I and metaphase I until the onset of anaphase I when chiasmata are resolved by the dissolution of sister chromatid arm cohesins (Uhlmann, Lottspeich, and Nasmyth 1999).

In addition to this recombination-dependent pathway, some species have developed recombination-independent mechanisms to ensure proper distribution of chromosomes to gametes. In the roundworm *Caenorhabditis elegans*, specific sites on each chromosome called pairing centers (MacQueen and Villeneuve 2001) are bound by zinc finger proteins and a KASH/SUN-domain protein complex (the LINC complex) to establish connections across the nuclear envelope between chromosomes and the cytoskeleton (Sato *et al.* 2009). Actin-dependent chromosome movements are thought to aid in homolog recognition by bringing together homologous sequences and possibly jostling apart inappropriate, less stable associations between non-homologs.

An alternative strategy to aid in partner recognition is seen in many plant species. In these species, telomeres of all chromosomes are embedded in the inner nuclear membrane and cluster, creating a "bouquet formation" and confining homology searching and pairing to a smaller region of the nucleus (Zickler and Kleckner 1998).

In still other species, such as the budding yeast *Saccharomyces cerevisiae*, DNA sequence-independent associations between homologous centromeres prior to bouquet formation enhance the chances that homologous pairs of kinetochores attach to the correct spindle pole (Kemp *et al.* 2004). Meiotic telomere clustering and centromere association are thought to increase the chance that partners come into contact with one another; however, it is poorly understood how homologs actually recognize and pair with one another in any system.

Understanding the mechanisms of meiotic pairing is important to human health, where meiotic errors leading to aneuploidy (the presence of an abnormal number of

chromosomes) is a particularly common cause of genetic disease. It is estimated that 10-30% of human embryos are aneuploid, which can lead to miscarriages, mental retardation, and other genetic syndromes arising primarily from gene dosage imbalances (Hassold and Hunt 2001). Insight into meiotic mechanisms is also important to understand how environmental hazards impact human aneuploidy. Several chemicals, termed aneugens, are known to cause errors in meiosis. The industrial chemical bisphenol A (BPA), pesticide component trichlorfon, and muscle relaxant diazepam have all been shown to cause chromosome alignment abnormalities and spindle formation defects during meiosis in both mouse and humans (Hunt *et al.* 2003; Cukurcam *et al.* 2004; Baumgartner *et al.* 2001). Because the increase in the human population heightens the likelihood of human-aneugen interactions in the environment, it is critical to assess the dynamic mechanisms of meiosis to better understand the disruptive roles aneugens play.

Drosophila melanogaster is an amenable model for research as it has a short life cycle, is easy and relatively cheap to manage, and has a substantial number of orthologs associated with human cancers, genetic diseases, and aging. Genetic studies of meiotic chromosome transmission are simplified by the fact that the fly has only four pairs of chromosomes and aneuploidy for the sex chromosomes, and the fourth chromosomes are fairly well-tolerated. Because many aneuploid progeny are still viable, even mutations that completely disrupt meiosis and lead to random chromosome segregation can be studied genetically. *Drosophila* are also an interesting model as males and females have each evolved their own mechanisms to accomplish meiosis. Female flies use the

canonical meiotic pathway utilizing the synaptonemal complex to aid in recombination and the formation of chiasmata (Carpenter 1994), while male flies lack synaptonemal complexes, chiasmata, and meiotic recombination (Morgan 1914). This unique aspect of male meiosis in flies has encouraged investigation over decades leading to the development of an array of tools to enhance study. In particular, from a screen for male sterile mutations (Wakimoto, Lindsley, and Herrera 2004), 60 mutations have been identified that perturb chromosome transmission in meiosis and can be used as a starting point to define genes required for meiosis.

Studies of meiosis in male *Drosophila* are aided by the developmental organization of the testis which permits easy identification and examination of meiotic progression. *Drosophila* spermatogenesis begins at the tip of each testis, where a patch of somatic hub cells and both germline and somatic stem cells reside (Hardy *et al.* 1979). Germline stem cells divide by mitosis, after which one remains in contact with the hub cell to maintain stem cell identity, and the other daughter stem cell, now referred to as a gonialblast, transitions out of the tip of the testis surrounded by two somatic cyst cells (Yamashita, Jones, and Fuller 2003). Four synchronous mitotic divisions of the gonialblast with incomplete cytokinesis follow to create a 16-cell meiotic cyst in which individual cells, now termed spermatocytes, are connected by ring canals. The entire cluster of sixteen interconnected spermatocytes are surrounded by the original two somatic cyst cells (Fuller 1993).

Once the mitotic divisions are complete, pre-meiotic S phase initiates quickly and concludes within three hours (Cenci *et al.* 1994), followed by a G2 phase characterized

by a significant 25-fold increase in cellular volume (Lindsley and Tokuyasu 1980). It is at this stage that major differences from recombination-dependent meiosis of other species become evident. The traditional stages of prophase I are not identifiable in *Drosophila* males as the chromosomes remain decondensed from G2 throughout mid-prophase I, and males lack recombination-associated landmarks of prophase progression. Rather, staging of progression from premeiotic S phase to prometaphase of meiosis I relies on the spatial arrangement of chromatin/chromosomes and size of the meiotic cells. This progression has best been defined by Cenci et al. (1994) who divided G2/prophase I into seven stages named S1, S2a, S2b, S3, S4, S5, and S6. Primary spermatocytes in S1 have just completed DNA synthesis in S phase, and as they grow and transition into S2a, the nucleus is visible at one pole of the cell while the mitochondria are clustered at the opposite side of the cell (Cooper 1965). At stage S2a, the sex chromosomes and three pairs of autosomes are indistinguishable and are clustered within the nucleus. As the cell grows and enters S2b phase, chromatin can be seen as a tri-lobular association with the nuclear lamina (Cenci *et al.* 1994) where each "territory" is comprised of a homologous pair of chromosomes. Additionally in S2b phase, the nucleolus is observed adjacent to the sex chromosome territory (Lindsley and Tokuyasu 1980), and the two chromosome 4s can be seen associated with the sex chromosomes or sometimes by themselves in the middle of the nucleus (Cenci *et al.* 1994). As the cell grows, the chromosomes remain in their own territory with the exception of the transcriptionally active Y chromatin often visible in the center of the cell in thread-like structures called Y chromosome loops (S3-S6) (Bonaccorsi *et al.* 1988; Cenci *et al.* 1994).

From late S6, spermatocytes transition into the meiotic divisions marked by the degradation of the nucleolus, and the chromatin condenses while remaining in territories (Cenci *et al.* 1994). Prometaphase I is marked by nuclear membrane breakdown, spindle fiber insertion into the nucleus, and migration of chromosome masses to the center of the cell. At metaphase I, chromatin forms a singular mass in the middle of the cell with chromosomes attached to microtubule bundles arising from each spindle pole. Homologs separate at anaphase I, and the nucleus reforms after telophase I (Cenci *et al.* 1994). At this stage, the meiotic cyst contains 32 secondary spermatocytes, and the onset of meiosis II is almost immediate. A short prophase II occurs where chromosomes re-condense followed by the metaphase II transition, anaphase II separation of sister chromatids, and telophase II division into 64 interconnected spermatids that will eventually elongate and individualize into functional sperm (Cenci *et al.* 1994).

The recombination-deficient meiosis in the male fly is an optimal system to study homolog interactions from pairing to segregation as the complexity of recombination that is required in many systems is absent, potentially permitting easier identification of genes specifically involved in pairing. The majority of mutations that disrupt chromosome segregation in females identify genes involved in recombination while in males, mutations that alter chromosome segregation are more likely to affect pairing, adhesion, or conjunction mechanisms. For these reasons, the male meiotic system has been highly investigated including examination of homolog partner recognition (pairing), association through metaphase I (conjunction), and connection of homologs to opposite spindle poles metaphase I for correct segregation at anaphase I (orientation).

In addition to the evolution of two different systems of meiosis between *Drosophila* sexes, the male has also developed two genetically separable pathways to segregate chromosomes, one for the sex chromosomes and one for the autosomes. Both cis- and trans-acting requirements vary between the sex chromosomes (X and Y) and autosomes (chromosomes 2, 3, and 4) and are summarized below.

Cis-acting Requirements for Autosome Pairing and Conjunction

Several studies have examined the DNA sequences requirements for homolog segregation in male meiosis. Because these studies preceded the technical ability to detect homolog interaction at the stage when pairing initially occurs, they often assessed homolog conjunction rather than pairing. In most studies, condensed chromosomes at prometaphase I or metaphase I were examined for physical associations. Genetic assays of segregation also fail to distinguish between pairing and conjunction, as both events must successfully occur for proper segregation. Thus, while these studies claim to identify sequences important for "pairing," it is not clear if these sequences identified were required for pairing, conjunction, or both.

For the work proposed here, it is important to make the distinction between the two. I will use the term "pairing" only to refer to the initial interactions required for homolog recognition and association. The term "conjunction" will be used to describe the ability of paired homologs to remain connected while undergoing condensation and spindle-mediated forces during prometaphase I and metaphase I. While some sequences may be capable of facilitating both pairing and subsequently conjunction, other sequences may only function in pairing or only in conjunction.

In the most thorough of these early studies, autosomes carrying either duplications, translocations, or deletions were examined at prometaphase and metaphase I for bivalent formation. Autosome pairs were shown to require euchromatic homology for conjunction at metaphase I while autosomes sharing only heterochromatic homology were not associated at metaphase I (Yamamoto 1979). Additionally, genetic tests in males found that autosomes with only heterochromatic homology failed to segregate from each other at meiosis I (Yamamoto 1979). The amount and location of euchromatic homology on the chromosome arm was also found to be important for conjunction. Identical chromosome 2s that were largely heterochromatic do not associate at metaphase I, and deletions of proximal euchromatin have a larger effect on association of chromosome 4 homologs than do distal deletions (Yamamoto 1979). A similar study confirmed that pairs of chromosome 2s with only proximal euchromatic homology also segregated better than chromosome 2s with only distal euchromatic homology (Hilliker, Holm, and Appels 1982).

Different chromosomes were assessed for their ability to both pair and remain conjoined using a collection of transpositions of chromosome 2 material onto the Y chromosome. Conjunction between the transposed segment and its intact chromosome 2 partner was observed by quadrivalent formation, in which X-Y and 2-2 chromosomes were conjoined in a single structure (McKee, Lumsden, and Das 1993). A quadrivalent is formed when the transposed euchromatic region on the Y pairs with chromosome 2, the chromosome 2s pair with each other, and the X and Y chromosomes also pair. At mid-to-late prophase I, all transposed euchromatic regions tested were capable of forming

quadrivalents (McKee, Lumsden, and Das 1993). A euchromatic region more proximal to the centromere were more effective at conjunction and directing proper segregation, and a transposition of only heterochromatic sequences was not capable of forming quadrivalents (McKee, Lumsden, and Das 1993). McKee, Lumsden, and Das (1993) also showed the frequency of quadrivalent formation and the ability to direct segregation increases as length of the transposed homology increases. Based on these results, they suggested that "pairing sites" are widely distributed along the arms of chromosome 2. This conclusion is justified, as these sites must have mediated pairing as well as conjunction, but the differences observed in the efficacy of "pairing" may instead reflect differences in the ability to mediate conjunction. It remains unknown if the same euchromatic sites are required for both pairing and conjunction, or if other homologies (i.e. heterochromatic homologies) can pair.

The first study to truly investigate pairing in early prophase I *Drosophila* spermatocytes utilized the Green Fluorescent Protein (GFP)-Lac repressor/*lac* operator system, first described in yeast (Vazquez, Belmont, and Sedat 2002). In this system, a GFP-Lac I fusion protein is expressed in the male germline and binds to integrated tandem arrays of lac operator (Lac O) sequences. This allows one to visualize associations between the lacO arrays in living cells, thus permitting analysis of both pairing and sister chromatid associations. For 14 euchromatic lacO arrays examined, meiotic pairing was shown to be initiated as early as interphase of early stage S1 spermatocytes, immediately after the mitotic divisions. Surprisingly, association between both homologs and sister chromatids was lost by mid-G2 after the chromosome territories

were formed (Vazquez, Belmont, and Sedat 2002). The major caveat of this study is that pairing was only examined at repetitive arrays of exogenous sequences. One study using fluorescent in situ hybridization (FISH) observed a similar behavior for repetitive, endogenous heterochromatic sequences (Ren *et al.* 1997), but to date, pairing has never been examined at endogenous single copy sequences. Until recently, FISH to single copy sequences was not possible due to the inability to detect probes; however, this technical limitation has been solved by the use of in silico generated oligonucleotide probes (Beliveau *et al.* 2012).

Cis-acting Requirements for Sex Chromosome Pairing and Conjunction

The X and Y sex chromosomes have minimal homology, and none is euchromatic, indicating sex chromosome pairing must occur at heterochromatic sequences. Potential XY pairing sites were originally identified based on the observation that certain regions of the X and Y remain associated at prometaphase I and metaphase I (Cooper 1959). These reside in the repetitive heterochromatic region near the centromere of the long arm of the X chromosome and near the base of the short arm of the Y chromosome. Both of these regions contain sequence homology of the rDNA genes involved in the formation of the nucleolus organizers (NOR) (Ritossa 1976). Males with X chromosomes devoid of this region demonstrate anomalies including high levels of XY nondisjunction (NDJ) and meiotic drive, the differential recovery of equal products of meiosis. In this case, sperm containing less chromatin are recovered more frequently (Sandler and Braver 1954). The rDNA arrays located in this region contain 200-250 tandem copies of the genes for the ribosomal subunits 18S, 28S, and 5.8S responsible for

nucleolus formation (Ritossa 1976). These studies did not examine sequence requirements for X and Y pairing in prophase I, but indicate that the rDNA arrays, or regions surrounding them, are sufficient for XY conjunction.

Studies using rDNA transgenes have shown that it is the rDNA itself that plays a significant role in XY segregation. A single rDNA gene inserted into a heterochromatically-deficient X chromosome increases XY disjunction and reduces meiotic drive. The increase of XY disjunction is correlated to the copy number of rDNA genes inserted on the X (McKee and Karpen 1990). Conversely, insertion of an rDNA gene into an autosome does not restore XY disjunction (McKee and Karpen 1990), indicating that this is a cis-acting effect. Transgene studies showed that the sequences important region for XY conjunction mapped to the 240 bp intergenic spacer (IGS) located upstream of each 18S and 28S rDNA repeat (McKee, Habera, and Vrana 1992). These observations indicate that IGS sequences are capable of promoting conjunction between the X and Y, and thus they have been referred to as sex chromosome "pairing sites." Again, these observations do not rule out that other sequences may pair but fail to effect conjunction.

Using FISH to visualize the heterochromatic IGS regions of the rDNA of condensed XY chromosomes in late-prophase I S6 spermatocytes, it has been confirmed that these regions are conjoined while surrounding regions of heterochromatin are not paired (Thomas *et al.* 2005). Pairing in stages prior to S6 could not be assayed by FISH probes to the rDNA IGS regions because the X and Y chromatin is too diffuse and probe signals are scattered (Thomas *et al.* 2005). Taken all together, these studies indicate that

rDNA is essential for XY conjunction, but do not rule out the possibility of XY pairing at other sequences.

Proteins Required for Homolog Pairing and Conjunction

Mod(mdg)-in-meiosis (MNM) and Stromalin-in-meiosis (SNM), and Teflon are all necessary for conjunction of the autosomes, while sex chromosome conjunction requires only MNM and SNM (Tomkiel, Wakimoto, and Briscoe 2001; Thomas *et al.* 2005). Null alleles of both SNM and MNM lead to increased NDJ of all chromosomes, and anaphase I chromosome separation is disorderly (Thomas *et al.* 2005). Using the GFP-LacI-*lacO* system described above, pairing ability does not seem altered in male flies lacking a functional MNM or SNM protein suggesting the mutant phenotype is associated with the downstream loss of homolog conjunction (Thomas *et al.* 2005). Cytology of *teflon* mutant males revealed a conjunction defect as precocious separation of all three pairs of autosomes at late prophase I/prometaphase I was observed while sex chromosomes behaved normally (Tomkiel, Wakimoto, and Briscoe 2001).

Using antibodies and GFP-tagged proteins, MNM and SNM were shown to localize to the rDNA on the sex chromosomes (Thomas *et al.* 2005) specifically at the rDNA IGS (Thomas and McKee 2007). GFP-labeled MNM localizes to multiple locations along the autosomes with some signal strengths fluorescing brighter than others, and its autosomal localization is dependent on Teflon. To date, neither SNM nor Teflon are detectible on autosomes, but it is likely that the lack of detection is owing to technical reasons. An MNM/SNM/Teflon complex has been proposed to regulate autosome conjunction, while an MNM/SNM complex may regulate sex chromosome conjunction

(Thomas *et al.* 2005; Thomas and McKee 2007). These proteins may function in a manner analogous to chiasmata in female meiosis, to hold paired homologs together until anaphase I segregation once conjunction is lost. The 240 bp IGS repeats on the X and Y and analogous euchromatic sequences dispersed along the arms of the autosomes previously thought of as "pairing sites" may primarily serve as conjunction sites for MNM/SNM/Teflon proteins to bind and hold homologs together.

The possibility remains that other sequence homologies on the sex chromosomes and on the autosomes have the ability to pair, and once the conjunction complex is assembled, fall apart at all locations other than the conjunction complex sites. There appears to be, at least in part, some chromosomal level control over pairing sites since the cis- and trans-acting components vary for the autosomes and the sex chromosomes. The rDNA IGS sequences are perfectly capable of directing XY segregation while rDNA translocated to an autosome does direct segregation from the X. Pairing needs to be investigated in very early prophase I, S1-S2 cells, because by the time chromosomes condense at mid-prophase I, conjunction complex proteins are present and other intimate pairing may have been lost.

Goal of Study

To better understand the relationship between chromosome pairing, conjunction, and segregation by investigating the process of meiotic homolog pairing in a system that is devoid of the recombination-dependent meiotic mechanisms, the male fruit fly *Drosophila melanogaster*.

CHAPTER II

SEX CHROMOSOME PAIRING MEDIATED BY EUCHROMATIC HOMOLOGY IN *DROSOPHILA* MALE MEIOSIS

Christopher A. Hylton, Katie Hansen, Andrew Bourgeois and John E. Tomkiel Dean. 2020. 'Sex Chromosome Pairing Mediated by Euchromatic Homology in *Drosophila* Male Meiosis', *Genetics*, 214(3): 605-616.

Introduction

Meiosis is the highly conserved process comprised of two cell divisions that produce four haploid daughter cells from a single diploid parent cell. To ensure an equal distribution of homologous chromosomes to gametes, homologs must locate each other, pair, conjoin, and segregate with high fidelity. Several events have been identified that aid in homolog pairing, but the mechanisms of partner recognition remain enigmatic. Multiple plant species create a chromosome "bouquet" by clustering and imbedding all telomeres into the inner nuclear membrane thereby confining homolog identification and pairing to a smaller region of the nucleus (Bahler *et al.* 1993). *Caenorhabditis elegans* uses microtubule/dynein-mediated movements through linkages to telomeric chromosomal sites deemed "pairing centers" which are thought to facilitate interactions between homologs (MacQueen *et al.* 2005; Sato *et al.* 2009; Wynne *et al.* 2012). The budding yeast *Saccharomyces cerevisiae* establishes DNA sequence-independent associations between homologous centromeres prior to bouquet formation to enhance the odds that homologous pairs of kinetochores attach to the correct spindle pole

(Kemp *et al.* 2004). Despite progress in understanding the mechanisms that aid in homolog association, the molecular basis of pairing itself remains poorly understood.

Recombination appears to play an essential role in pairing in some systems. During meiosis I of *S. cerevisiae*, the formation of double-stranded breaks, a prerequisite for recombination, occurs prior to homolog synapse initiation. In *spo11* yeast that lack double strand breaks, homologs fail to synapse (Giroux, Dresser, and Tiano 1989; Weiner and Kleckner 1994), which indicates that the homology search achieved by single-stranded DNA during recombination in yeast is required for homolog pairing and synapsis. In contrast, *mei-W68* and *mei-P22 Drosophila* females lack double-strand breaks and assemble SC indicating recombination is not required for pairing and synapsis (McKim *et al.* 1998). Taken together, these results reveal that while some species require recombination for pairing, other species have evolved separate recombination-independent mechanisms to pair and segregate homologs.

Male *Drosophila*, which completely lack recombination, have two genetically separable pathways to pair and segregate chromosomes. One pathway is specific for the sex chromosomes and the other for the autosomes. Sex chromosomes pair at specific sites, originally termed collochores, that were identified based on the observation that certain regions of the X and Y remain associated at prometaphase I and metaphase I (Cooper 1959). Potential pairing sites were identified in the repetitive heterochromatic region near the centromere of the X chromosome and near the base of the short arm of the Y chromosome. These two regions contain sequence homology of the rDNA genes, which contain 200-250 tandem copies of the genes for the ribosomal subunits (Ritossa

1976). Males with rDNA-deficient X chromosomes exhibit high levels of XY nondisjunction (NDJ). A transgenic copy of the rDNA gene on the X restores disjunction (McKee and Karpen 1990). The 240 bp intergenic spacer (IGS) region located upstream of each 18S and 28S rDNA repeat is necessary and sufficient for pairing (McKee, Habera, and Vrana 1992).

In contrast to the sex chromosomes which pair only at the rDNA, autosomes pair at sequences that are distributed throughout the euchromatin, and both the amount and chromosomal location of euchromatic homology may be important for conjunction (McKee, Lumsden, and Das 1993). Cytological and genetic tests show that autosomes with only heterochromatic homology fail to segregate from each other at meiosis I (Yamamoto 1979; Hilliker, Holm, and Appels 1982). These studies suggested that autosomal heterochromatin lacked pairing ability.

Because these conclusions were largely derived from observations of chromosome associations during late prophase I to prometaphase I, sequences were only defined as pairing sites if they had the ability to remain conjoined. The initial interactions needed for homolog recognition and pairing occur premeiotically, however, and at these later stages, many interactions may have already been resolved. Thus, the previously defined "pairing sites" may really represent regions that remain conjoined and may not necessarily represent all sequences involved in pairing.

Direct observations of pairing provide a more accurate assessment of pairing sites. Meiotic pairing is temporally separable from homolog associations that occur in somatic cell ("somatic pairing"). Homologs are not paired at the earliest stage that germline cells

can be distinguished in the embryo, but then begin to associate in gonial cells prior to meiosis (Joyce *et al.* 2013). Examination of early prophase I pairing in vivo using the GFP-Lac repressor/lac operator system, found that homologs were paired at each of 13 different single autosomal loci (Vazquez, Belmont, and Sedat 2002). In agreement with earlier studies, this shows that many autosomal sequences can pair. Heterochromatic homologies also pair with similar kinetics, as shown by *in situ* hybridizations to autosomal satellite repeats (Tsai, Yan, and McKee 2011).

Distinct from pairing, conjunction refers to the ability of paired homologs to remain coupled during prophase I condensation and prometaphase/metaphase I spindle-mediated movements. Teflon (Tef), Modifier of Mdg in Meiosis (MNM), and Stromalin in Meiosis (SNM) have all been shown to be required for conjunction of the autosomes, while sex chromosome conjunction requires only MNM and SNM (Tomkiel, Wakimoto, and Briscoe 2001; Thomas *et al.* 2005). MNM and SNM localize to the rDNA on the sex chromosomes (Thomas *et al.* 2005) specifically at the rDNA IGS (Thomas and McKee 2007). Potential MNM/SNM/Tef and MNM/SNM complexes may regulate autosomal and sex chromosome conjunction respectively, holding paired homologs together until anaphase I (Thomas *et al.* 2005; Thomas and McKee 2007). Recently, super resolution microscopy and temporally expressed transgenes showed that MNM and SNM are required to maintain conjunction but cannot establish pairing themselves (Sun *et al.* 2019). Thus, while the 240 IGS pairing sites on the X and Y certainly have the ability to mediate pairing and may serve as a site for conjunction protein binding, they may not be

the only sequences with the ability to pair. It remains to be examined if other sequence homologies can pair but lack the ability to stabilize conjunction.

Here, we directly examine pairing and its relationship to conjunction. We describe a system to examine sex chromosome pairing during early prophase I at homologies other than the IGS repeats. We show that X euchromatic sequences placed on the Y chromosome are able to pair and in some cases facilitate conjunction and segregation of sex chromosomes in the absence of X chromosome rDNA. This system allowed us to identify sequences capable of pairing, to ask how much homology is sufficient for pairing, and to determine whether the location of homology is important for pairing and conjunction.

Materials and Methods

Drosophila Stocks and Crosses

Drosophila were raised on a standard diet consisting of cornmeal, molasses, agar, and yeast at 23°C. *Dp(1;Y)* chromosomes (Cook *et al.* 2010) and *Df(tef)803Δ15* (Arya *et al.* 2006) are previously described. The tef^{z3455}, snm^{z0317}, snm^{z2138}, mnm^{z5578}, mnm^{z3298}, and mnm^{z3401} alleles were originally obtained from the C. Zuker laboratory at the University of California at San Diego (Wakimoto, Lindsley, and Herrera 2004) and are previously described (Tomkiel, Wakimoto, and Briscoe 2001; Thomas *et al.* 2005). All other stocks were obtained from the Bloomington Stock Center (Gramates *et al.* 2017).

Genetic Assays of Meiotic Chromosome Segregation

In(1)$sc^{4L}sc^{8R}$ and *Df(1)X-1* are X chromosomes that have been reported to be rDNA-deficient. We found that *Df(1)X-1* X resulted in sterility in combination with the

Dp(1;Y) Y chromosomes tested, and therefore the *In(1)sc^{4L}sc^{8R}* X was selected for crosses. Segregation of *In(1)sc^{4L}sc^{8R}* from a *Dp(1;Y)* chromosome was monitored by crossing *In(1)sc^{4L}sc^{8R} y^{1} / Dp(1;Y)B^{S} Y y^{+}* males to *y w sn ; C(4)RM ci ey / 0* females. Offspring are scored as either normal (B^{S} y^{+} sn sons or y^{1} daughters), sex chromosome diplo- (B^{S} y^{+} females), or sex chromosome nullo-exceptions (y w sn males). The midpoint of the duplicated X euchromatin on each *Dp(1;Y)* was calculated by taking the average of the distal- and proximal- most estimations of breakpoints (Cook *et al.* 2010).

Fourth chromosome missegregation was monitored by the recovery of ci ey *nullo-4* progeny. In crosses involving *tef* mutations, males were made homozygous for the fourth chromosome mutation *spa* to allow monitoring of both *nullo-4* and *diplo-4* progeny.

Probe Design

Probe pools were generated to selected sequences at a density of 10 probes/Kb and a complexity of ~10,000 probes per pool (Arbor Biosciences, Ann Arbor, MI). Triple-labeled Atto-594 oligonucleotide probes were generated to sequences present on both *In(1)sc^{4L}sc^{8R}* and the following *Dp(1;Y)* chromosomes: *Dp(1;Y)BSC76* (X salivary gland chromosome bands 2E1-3E4 spanning base pairs 2606837 - 3606837); *Dp(1;Y)BSC185* (X salivary gland chromosome bands 12A4-12F4 spanning base pairs 3824004 - 14826069); and *Dp(1;Y)BSC11* (X salivary gland chromosome bands 16F7-18A7 spanning base pairs 18193946 - 19193592).

A triple-labeled Atto-488 probe was generated to bp 20368577 - 21368577 (56F-57F) on chromosome 2. An Atto-488 probe (Eurofins MWG Operon, Louisville, KY)

was synthesized to the Y-specific AATAC heterochromatic repeat (Lohe and Brutlag 1987).

FISH

Slides of testis tissue were processed for FISH using a modification of the protocol as described (Beliveau, Apostolopoulos, and Wu 2014). Testes from larvae (Pairing Assay) or pharate adults (NDJ Assay) were dissected in Schneider's *Drosophila* media (GIBCO BRL, Gaithersburg, MD). Tissue was transferred to a drop of Schneider's on a silanized coverslip and gently squashed onto a Poly-L-Lysine coated slide (Electron Microscopy Sciences, Hatfield, PA). Coverslips were immediately removed after freezing in liquid nitrogen. Tissue was fixed in 55% methanol/25% acetic acid for 10 min followed by 10 min dehydration in 95% ethanol. Slides were processed immediately or stored for up to 1 week at 4°C.

For hybridizations, slides were rehydrated in 2X saline-sodium citrate/Tween-20 (SSCT) at room temperature for 10 min. Membranes were permeabilized and DNA denatured by incubation in 50% formamide/2X SSCT for 2.5 min at 92°C then 60°C for 20 min. Slides were rinsed in 1X phosphate buffered saline (PBS) for 2 min and allowed to dry. 5 µl of probe master mix containing 12.5 µl hybrid cocktail (50% dextran sulfate, 20X SSCT), 12.5 µl formamide, 1 µl of 10 mg/ml RNase, 2 µl of probe 1 (5 pmol/µl), and 2 µl of probe 2 (5 pmol/µl) was pipetted directly onto a silanized 18 x 18 mm coverslip which was placed on the tissue and sealed with rubber cement. Slides were heated at 92°C for 2 min to denature the DNA then incubated in a damp chamber at 42°C for >18 hours. Following incubation, coverslips were removed, and slides were

incubated in 2X SSCT at 60°C for 20 min, 2X SSCT at RT for 10 min, and 0.2X saline-sodium citrate (SSC) at RT for 10 min to remove unbound probe. DNA was stained with 1 µg/µl 4',6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) and tissues mounted in ProLong Gold antifade (Invitrogen, Carlsbad, CA). Probes were visualized using a Keyence BZ-X700 Fluorescence Microscope. S1-S2 spermatocytes were selected based on size (10 to 20 µm), and signals were scored as paired when within 0.8 µm (Beliveau, Apostolopoulos, and Wu 2014).

Estimation of the Ability of Paired Sequences to Direct Segregation

To determine how frequently pairing led to disjunction, we assumed that chromosomes that did not pair would segregate at random. First, we determined the pairing frequency from FISH assessment of S1-S2 cells (= % Paired). We then cytologically determined the frequency of secondary spermatocytes and spermatids in which the X and Y had segregated to opposite poles at meiosis I (= % NDJ). We assumed that this latter frequency represented meiocytes in which XY pairings underwent normal segregation, plus half the frequency of random disjunctions that resulted when the X and Y failed to pair. Based on this assumption, we calculated the percent of cells in which pairing of XY chromosomes led to normal disjunction as:

Paired then disjoined = (% Paired – [%NDJ – (1/2 % Unpaired)]) / % Paired.

rDNA Magnification Assay

rDNA magnification was assessed by crossing *In(1)sc^{4L}sc^{8R} y^{1} / Y* males (Cross A) or *In(1)sc^{4L}sc^{8R} y^{1} / Dp(1;Y)B^{S} Y y^{+} BSC76* males (Cross B) to *C(1)RM, y w f / y^{+} Y* females. Fifty *In(1)sc^{4L}sc^{8R} y^{1} / y^{+} Y* sons generated from cross A or B were then crossed

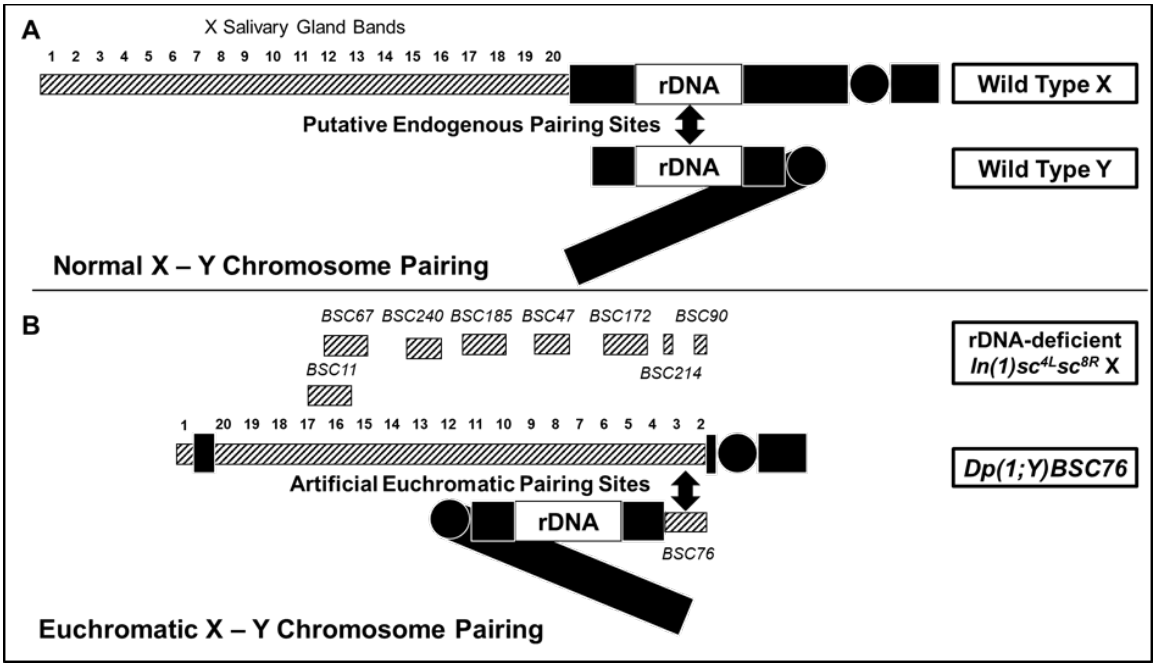
to y w sn females to determine sex chromosome NDJ. NDJ was calculated amongst progeny of each father, and distributions of NDJ frequencies were compared by one-way ANOVA.

Results

Euchromatic Homology Directs Segregation of the X from the Y

We developed a system to ask if euchromatic homologies could direct pairing and segregation of the sex chromosomes utilizing a series of *Dp(1;Y)* chromosomes (Cook *et al.* 2010) and the rDNA-deficient $In(1)sc^{4L}sc^{8R}$ X chromosome that is missing the sex chromosome pairing sites. Each *Dp(1;Y)* chromosome contains a unique segment of X euchromatin. The size and position of the duplicated homology with the X chromosome partner also varies (Figure 1). We reasoned if the euchromatic homology was sufficient to pair, conjoin, and direct segregation of the sex chromosomes, then $In(1)sc^{4L}sc^{8R}$ / *Dp(1;Y)* males would produce fewer exceptional progeny than $In(1)sc^{4L}sc^{8R}$ / *Y* males.

Figure 1. Normal XY Pairing vs. Pairing at Euchromatin (Hatched Boxes). (A) Wildtype showing rDNA pairing sites. (B) *In(1)sc*^{4L}*sc*^{8R} X lacking rDNA. The locations of the X duplications on the collection of *Dp(1;Y)*s tested are indicated above the X. *Dp(1;Y)BSC76* is shown paired with its euchromatic homology on the X.



As a metric of segregation, we monitored NDJ of the sex chromosomes among progeny of *In(1)sc*^{4L}*sc*^{8R} / *Dp(1;Y)* males. Direct comparisons of the behaviors of the different *Dp(1;Y)* males are complicated as the viabilities of *Dp(1;Y)*-bearing sons differ greatly (data not shown), most likely a result of gene dosage imbalance contributed by the X duplications. To directly compare the behaviors of different *Dp(1;Y)* chromosomes, we considered only two classes of progeny that were genetically identical from all crosses. X/0 sons were used as a metric of sex chromosome NDJ, and X/X daughters were used as a metric of normal disjunction. We used the ratio of (X / 0) / (X / X + X / 0) as an estimate for the frequency of missegregation of sex chromosomes in each class of test males, and for the remainder of the manuscript, sex chromosome NDJ

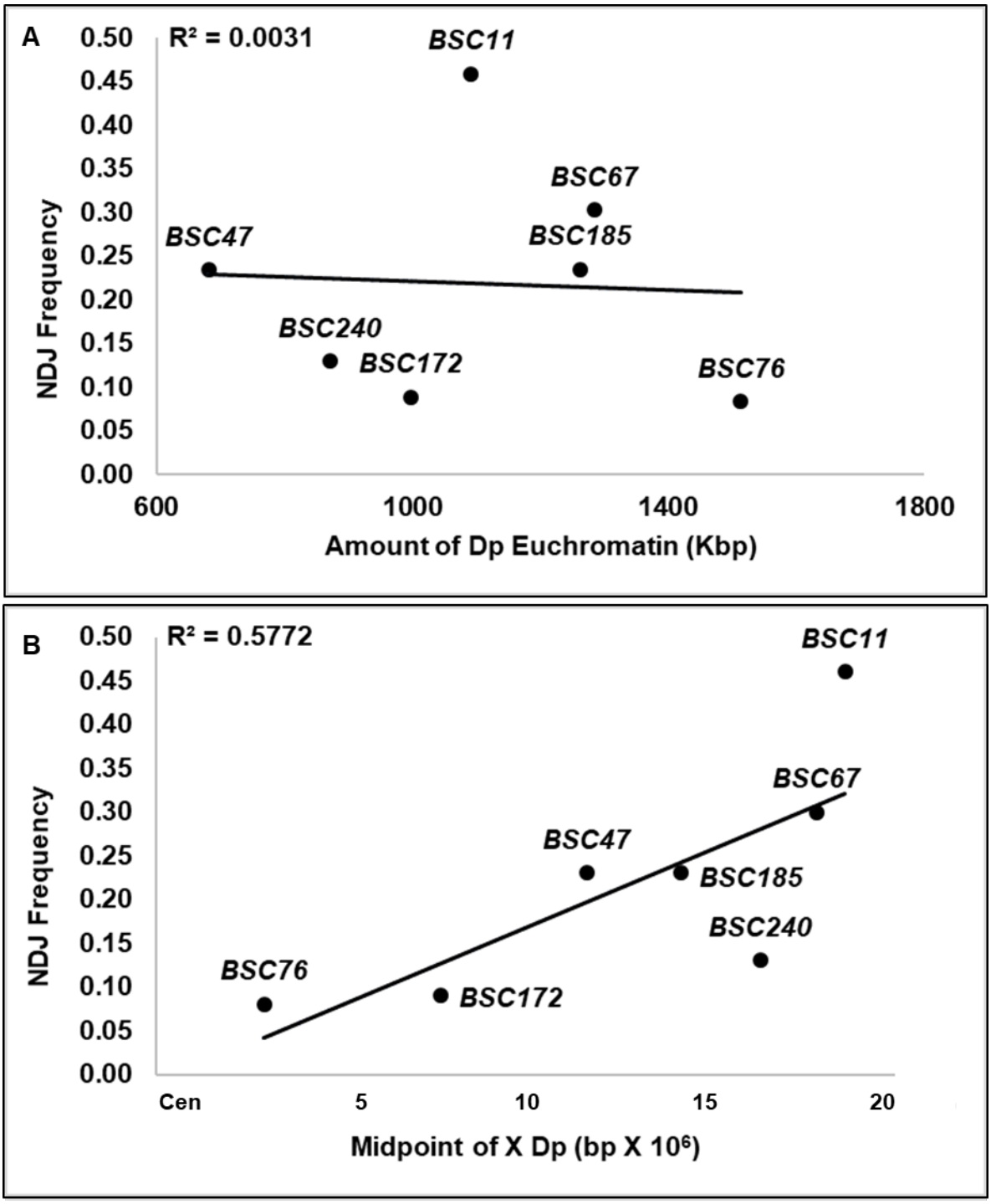
will be determined as such. We found that some of the *Dp(1;Y)*s were better at segregating from the X chromosome (Table 1). The ability to segregate was not related to the length of the duplicated X euchromatin sequence (Figure 2A). In fact, *Dp(1;Y)BSC11*, which contains over 1 Mbp of X euchromatin homology, showed no improvement in segregation relative to y^+Y. However, we noted a relationship between proper XY segregation and the chromosomal location of X homology. When the homologous sequences on the inverted X chromosome were closer to the centromere, less NDJ was observed (Figure 2B). The poorest segregating duplication, *Dp(1;Y)BSC11,* contained the distal-most homology. As a control, chromosome 4 segregation was also monitored to determine if X duplicated material itself generally perturbed chromosome segregation due to effects of aneuploidy. Chromosome 4 NDJ was less than 1% in each of the *Dp(1;Y)*-bearing males tested, indicating that none of the *Dp(1;Y)*s increased autosomal NDJ (data not shown).

Table 1. Frequency of XY NDJ among Progeny from *In(1)sc^{4L}sc^{8R} / Dp(1;Y)* Males.

Sperm genotype:		*X*	*Dp(1;Y)*	*X/Dp(1;Y)*	*0*	*0*/(*X*+*0*)
Paternal Y	X region duplicated on Y*					
y$^+$Y	-	925	434	52	579	0.38
Dp(1;Y)BSC76	2E1--3E4	321	51	0	29	0.08
Dp(1;Y)BSC172	7A3--7D18	319	41	0	31	0.09
Dp(1;Y)BSC47	10B3--11A1	387	35	2	118	0.23
Dp(1;Y)BSC185	12A4--12F4	421	75	2	129	0.23
Dp(1;Y)BSC240	14A1--15A8	660	59	1	98	0.13
Dp(1;Y)BSC67	15F4--17C3	307	35	1	133	0.30
Dp(1;Y)BSC11	16F7--18A7	495	69	19	419	0.46

* Salivary gland chromosome bands.

Figure 2. Sex Chromosome NDJ Frequencies among Progeny of *In(1)sc^{4L}sc^{8R} / Dp(1;Y)BSC* Males Versus (A) Euchromatic Homology Length and (B) Genomic Sequence Position of the X Homology.



We conclude that the duplicated X euchromatin on the Y chromosome is capable of facilitating pairing, conjunction, and segregation of the sex chromosomes, and that the ability to do so is related to underlying sequences and/or chromosomal position. However, a potential caveat to our interpretation is that our genetic metric may be influenced by 'meiotic drive', a phenomenon that results in the unequal recovery of reciprocal meiotic products. Meiotic drive is induced by a failure of sex chromosome pairing in male flies, and drive strength is directly proportional to the pairing frequency (McKee 1984). Although termed 'meiotic drive', this process has been shown to result in a post-meiotic differential elimination of sperm dependent on chromatin content (Peacock, Miklos, and Goodchild 1975). Thus, it was a formal possibility that the differences we had observed could somehow result from differential effects of the various *Dp(1;Y)* chromosomes on meiotic drive. To avoid this potential complication, we turned to a direct cytological assessment of chromosome behavior in meiosis.

We used FISH with X- and Y-specific probes to directly assess the outcomes of meiosis in secondary spermatocytes and onion stage spermatids. An Atto-594 (Red) X chromosome probe labels an X euchromatic sequence, while an Atto-488 (Green) Y chromosome labels the unique AATAC heterochromatic repeat. Segregation frequencies of the sex chromosomes were determined by examining related pairs of secondary spermatocytes, or related tetrads of spermatids (Figure 3). This analysis confirmed our conclusions based on our genetic observations, that the fidelity of segregation from $In(1)sc^{4L}sc^{8R}$ varied among tested *Dp(1;Y)*s, and this variation was related to proximity of the homology to the X centromere (Table 2).

Figure 3. FISH Examination of *In(1)sc^{4L}sc^{8R}* / *Dp(1;Y)BSC76* Disjunction in DAPI-stained Spermatocytes. The X chromatids are marked with a red probe and the Y chromatids are marked with a green probe to the AATAC repeat. (A) Normal XY segregation during meiosis I and (B) meiosis I NDJ. (C) Meiosis II division after a normal meiosis I division and (D) after a meiosis I NDJ. Scale bar = 2 μm.

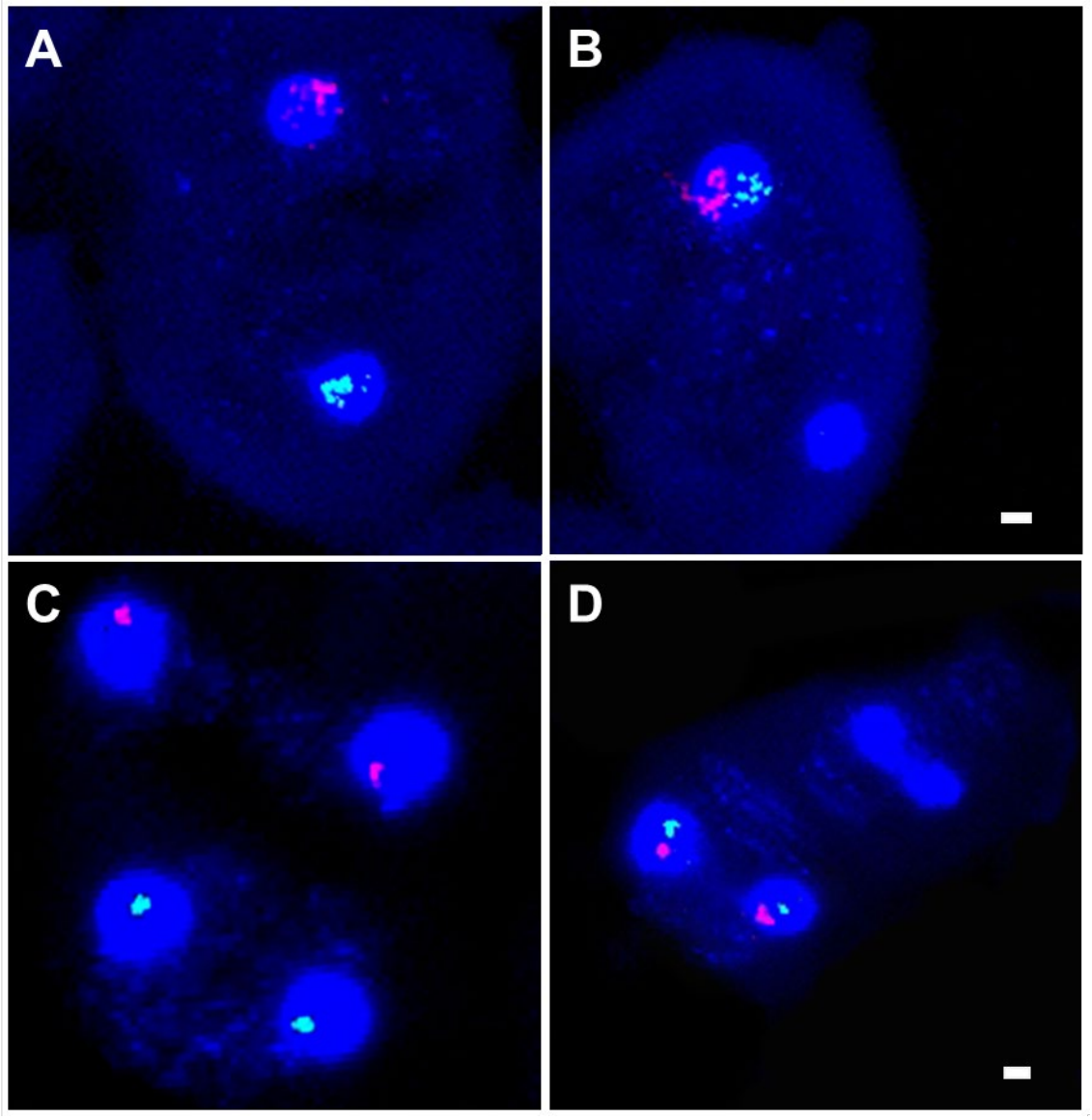


Table 2. XY NDJ Frequencies as Determined by FISH.

X	Y	# Divisions Scored	XY NDJ
Canton S	Canton S	206	0.00
In(1)sc^{4L}sc^{8R}	*y+Y*	200	0.33
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC76*	307	0.11
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC185*	214	0.22
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC11*	237	0.30
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC90*	201	0.12
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC214*	206	0.08

While these observations clearly suggest that the various *Dp(1;Y)* chromosomes were pairing with the rDNA-deficient X, they do not address where this pairing might be occurring. It is known that in the presence of structurally altered Y chromosomes, a process termed rDNA magnification can be induced (Tartof 1974). This process involves stable increases and/or decreases in rDNA copy number on an rDNA-deficient X via unequal sister chromatid exchange (Ritossa 1968). Although the *In(1)sc^{4L}sc^{8R}* chromosome is reportedly deleted for all of the rDNA, one or more cryptic rDNA cistrons could be potentially induced to magnify and restore XY pairing via the endogenous rDNA pairing sites. As few as six copies of the rDNA intergenic spacer repeats may restore pairing between the X and the Y (Ren *et al.* 1997), thus it was important to determine if our results could be explained by rDNA magnification rather than pairing outside the rDNA. To test for rDNA magnification, we provided potential magnification conditions by passing an *In(1)sc^{4L}sc^{8R}* X through a male bearing a *Dp(1;Y)*. We chose the *Dp(1;Y)* that exhibited the highest fidelity of segregation, *Dp(1;Y)BSC76*,

as this would be predicted to show the greatest amount of magnification, if it were indeed occurring. We recovered the potentially amplified X chromosomes in sons, and genetically tested their ability to segregate from the Y. As a control, we tested genetically identical males which had received an *In(1)sc^{4L}sc^{8R}* that had not been exposed to potentially magnifying conditions. If magnification was occurring, then we expected that sons bearing the potentially magnified *In(1)sc^{4L}sc^{8R}* would demonstrate improved segregation of the sex chromosomes relative to the controls. For each test, we scored progeny of 50 males. No statistical difference was found between the two classes (ANOVA, F value = 1.76527; *p* value = 0.17475) (Figure 4, Table 3). We conclude that the ability of a *Dp(1;Y)* to segregate from an rDNA-deficient *In(1)sc^{4L}sc^{8R}* is not a consequence of rDNA magnification and likely reflects pairing between X euchromatic homologies.

Figure 4. Test for rDNA Magnification of *In(1)sc^{4L}sc^{8R}* in *Dp(1;Y)BSC76* Males. Distributions of NDJ frequencies in sons of *In(1)sc^{4L}sc^{8R}* / *Dp(1;Y)BSC76* or *In(1)sc^{4L}sc^{8R}* / *Y* males.

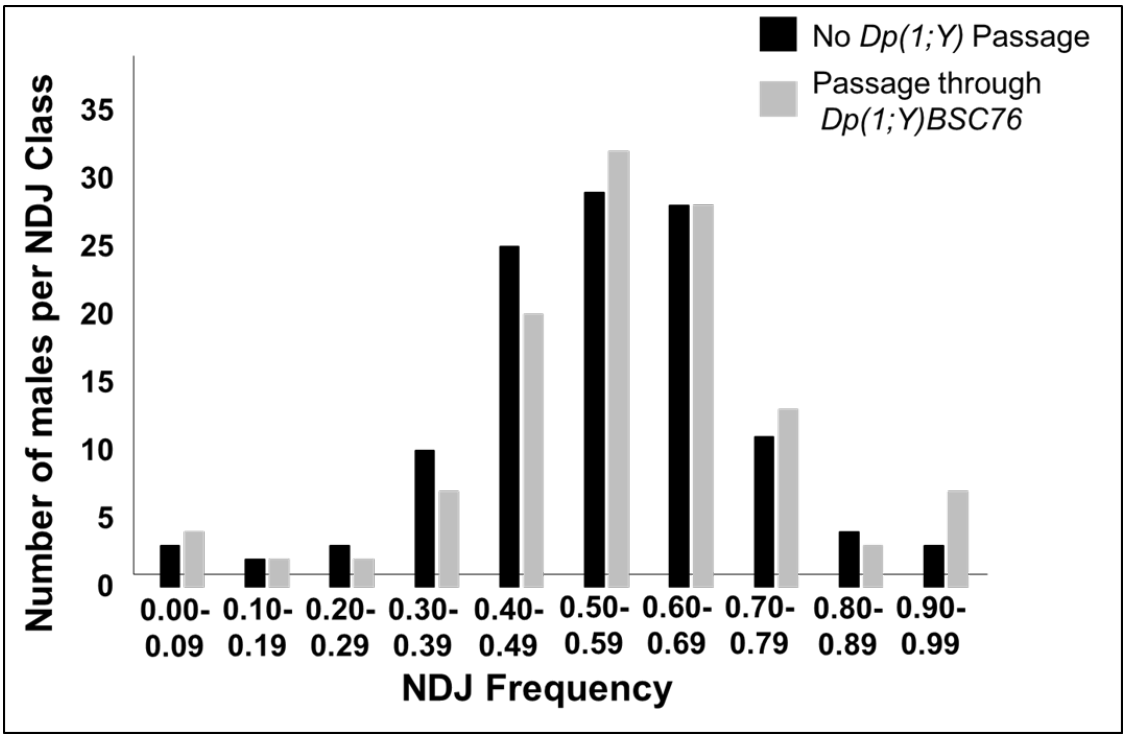


Table 3. Frequency of XY NDJ among Progeny from *In(1)sc^{4L}sc^{8R}* / Y Males after Potential rDNA Magnification.

Sperm genotype:	*X*	*Y*	*X/Y*	*0*	*XY* NDJ
No Magnification	1962	747	86	2146	0.45
Potential Magnification	1668	563	74	2009	0.48

Homologies from various non-overlapping regions of the X chromosome enhanced segregation demonstrating that multiple sequences are capable of acting as pairing sites. Because no relationship between the length of the *Dp(1;Y)* and the ability

to direct segregation was observed, we wanted to determine if these pairing site sequences were distributed randomly throughout the X euchromatin. To ask if we could potentially map a pairing site within a duplicated region, *Dp(1;Y)*s nested within the *Dp(1;Y)BSC76* euchromatic duplication were tested. The two smallest nonoverlaping duplications *Dp(1;Y)BSC90* and *Dp(1;Y)BSC214* were equally proficient at directing XY segregation albeit at a lower frequency than *Dp(1;Y)BSC76* (Table 4). These data suggest at least two different euchromatic segments within this one region are capable of pairing and directing XY segregation.

Table 4. Mapping Segregational Ability within *Dp(1;Y)BSC76*.

Sperm genotype:		*X*	*Dp(1;Y)*	*X/Dp(1;Y)*	*0*	*0*/(*X*+*0*)
Paternal Y	X region duplicated on Y*					
Dp(1;Y)BSC76	2E1-2E2--3E4	1347	196	7	154	0.10
Dp(1;Y)BSC80	3A6-B1--3E4	1286	95	1	226	0.15
Dp(1;Y)BSC83	3B3-B4--3E4	2105	420	14	280	0.12
Dp(1;Y)BSC84	3C2-C3--3E4	1364	241	7	209	0.13
Dp(1;Y)BSC88	3C6-D2--3E4	1834	541	7	190	0.09
Dp(1;Y)BSC90	3D5-3E4--3E4	628	155	20	129	0.17
Dp(1;Y)BSC214	2E2-2F2--2F6	984	252	12	199	0.17

* Salivary gland chromosome bands.

Direct Observation of Pairing between Euchromatic Homology on the X and Y

To directly ask if pairing was occurring between the euchromatic sequences on the *In(1)sc^{4L}sc^{8R}* and *Dp(1;Y)s*, we designed a FISH assay to cytologically visualize sex chromosome pairing in spermatocytes at early prophase I (S1-S2) (Figure 5).

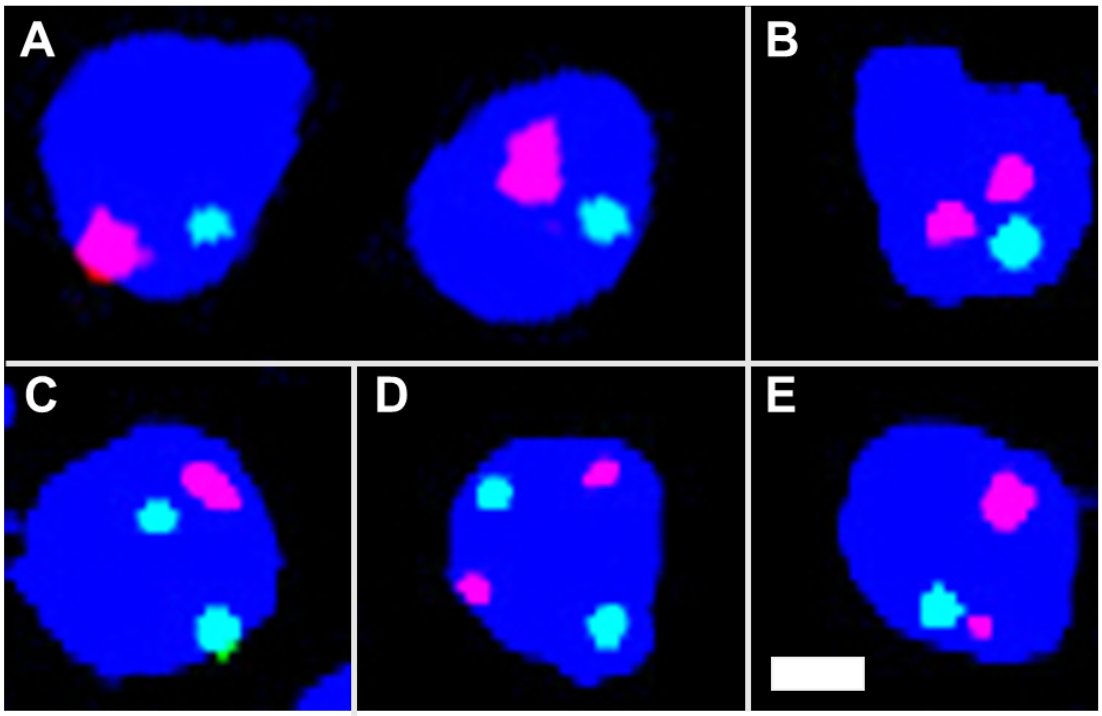
Spermatocytes with diameters between 10 and 20 microns were selected because at this size they are considered to be in S1-S2a stage (Cenci *et al.* 1994) where pairing is observed (Vazquez, Belmont, and Sedat 2002). To assess pairing, a single copy X probe (Atto-594-Red) was hybridized to both the intact X and the X euchromatin duplicated on the *Dp(1;Y)* (Figure 5). Because both pairing and sister chromatid cohesion is lost as spermatocytes mature (Vazquez, Belmont, and Sedat 2002), a control chromosome 2 probe (Atto-488-Green) was used to assure the cells observed had not progressed beyond S2 (Figure 5). Cells with two or more green signals were not scored as they may have already begun their progression to S3 when homologs no longer exhibit pairing. The X and Y were deemed paired when one red signal was present or two distinct signals were present that were less than 0.8 microns apart (Joyce *et al.* 2013).

There are two potential errors in this meiotic pairing assay that must be considered. First, there can be a slight asynchrony in the loss of pairing and sister chromatid cohesion on different chromosomes at the end of S2. Thus, some cells were predicted to be observed in which the X and Y had indeed paired, but sex chromosome pairing or sister chromatid cohesion had been lost prior to loss of pairing at the control autosomal site. This occurrence would have led to a false negative scoring of these cells as unpaired. To estimate how often this occurred, we hybridized the same probes to spermatocytes of males with *wildtype* sex chromosomes, so the red probe would only hybridize to the X. Ten percent of spermatocytes of the selected size in four such males had one autosome signal and two X signals representing sister chromatid separation (n =

188). This means that we may be underestimating pairing frequencies by as much as 10%.

Second, false positives in which pairing is erroneously scored are expected to occur by chance overlap of unpaired X signals. To estimate how often this occurs, from four testes, we counted the number of spermatocytes that had overlap (within 0.8 μm) of the X and autosome signals. Five percent of spermatocytes showed overlap of X and autosome signals (n = 178). Overall, based on these two error rates, our measured frequencies may overestimate pairing by roughly five percent. Considering both sources of error, we expect that our overall estimates of pairing may be up to 5% less than the actual pairing frequencies.

Figure 5. FISH Examination of Pairing in DAPI-stained S1-S2 Primary Spermatocytes. The X chromosomes are labeled red and chromosome 2s are labeled green. (A) Paired XY and paired chromosome 2 bivalents. (B) Unpaired XY and a paired chromosome 2 bivalent. (C) A paired XY bivalent and unpaired chromosome 2. (D) Both unpaired. (E) Sister chromatid separation from a paired XY bivalent. Scale bar = 2 μm.



Although *Dp(1;Y)*s varied in their ability to segregate from *In(1)sc*^{4L}*sc*^{8R}, all duplicated euchromatic sequences showed similar ability to pair with the homologous sequences on the intact X (Table 5). Considering our potential errors in estimation of pairing, some sequences showed nearly complete pairing. These results indicate that the observed differences in segregation of the various *Dp(1;Y)*s from the X could not be accounted for by differences in pairing ability (Table 5), but rather that pairing at some sites led to better segregation, possibly because of a greater ability to remain conjoined. To examine this possibility, we estimated that frequency at which paired chromosomes

ultimately segregated properly for five different *Dp(1;Y)* genotypes. To avoid complications of meiotic drive, these estimates were based on direct measurements of pairing and segregation by FISH (see Materials and Methods). The abilities of the five *Dp(1;Y)*s to disjoin differed and showed the same trend with respect to the centromere proximity (Figure 6). These estimates supported our previous conclusion that the more proximal to the centromere the homology was on the X, the better its ability to direct segregation.

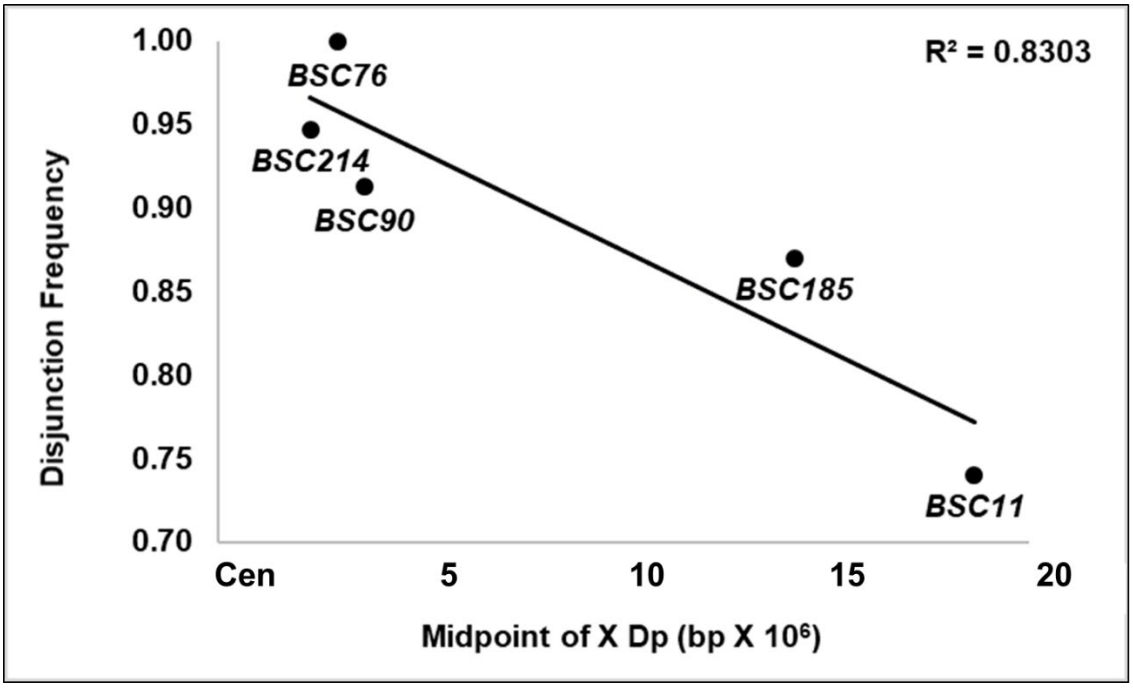
We next asked if the ability of these euchromatic sequences to pair was dependent on the lack of the native X rDNA pairing sites. One possibility was that pairing might normally occur only at the rDNA if it had the ability to outcompete other homologies for limited pairing proteins. To test this possibility, we measured pairing between an X chromosome bearing rDNA and *Dp(1;Y)BSC76* and found that pairing at the euchromatic homology was not diminished (Table 5). This shows pairing at the rDNA did not compete with pairing at the euchromatic homology.

Table 5. XY Pairing in S1-S2 Primary Spermatocytes.

X	Y	# Cells Scored	% Paired	% Paired that Disjoined*
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC76*	202	78.2	100.0
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC185*	215	73.5	87.0
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC11*	213	84.0	74.0
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC90*	204	92.2	91.3
In(1)sc^{4L}sc^{8R}	*Dp(1;Y)BSC214*	236	93.2	94.7
wildtype	*Dp(1;Y)BSC76*	195	91.8	ND

* See Materials and Methods for calculation.

Figure 6. Frequency of Disjunction of Paired *In(1)sc^{4L}sc^{8R}* and *Dp(1;Y)BSC* Chromosomes Versus Genomic Sequence Position of the X Homology.



Effects of *tef* and *snm* on Euchromatin-mediated Sex Chromosome Segregation

We next used our pairing system to examine the requirements for the conjunction proteins Tef, MNM and SNM. Tef is normally required to maintain conjunction between autosomes, has no effects on sex chromosome segregation, and has been proposed to be autosome-specific (Tomkiel, Wakimoto, and Briscoe 2001). However, because autosomal pairing sites are euchromatic and sex chromosome pairing sites are normally heterochromatic, the autosomal specificity of Tef may actually reflect a specificity for euchromatin. To test this possibility, we used the *In(1)sc^{4L}sc^{8R} / Dp(1;Y)* pairing system to determine if Tef was required for euchromatic sex chromosome conjunction and segregation. First, we confirmed that the *In(1)sc^{4L}sc^{8R}* chromosome behavior was not altered in a *tef* background. We monitored sex chromosome NDJ of *In(1)sc^{4L}sc^{8R} / y^{+} Y* males bearing a *tef* mutation and found that sex chromosome missegregation rates were statistically the same for *tef / + vs tef*, ($p > 0.95$), Table 6). Next, we compared sex chromosome NDJ from *In(1)sc^{4L}sc^{8R} / Dp(1;Y)BSC76* males homozygous or heterozygous for *tef*, and results did not differ statistically for *tef / + vs tef*, ($p > 0.50$), Table 6). These results suggest that Tef is indeed autosome-specific and is not required for conjunction of these X euchromatic homologies.

We similarly attempted to test the requirements for MNM and SNM to establish conjunction between X euchromatic homologies. Unfortunately, we were unable to perform the same test. For unknown reasons, *In(1)sc^{4L}sc^{8R} / Dp(1;Y)* males homozygous for *mnm* or *snm* were sterile. This was true for all alleles tested both as homozygotes and transheterozygotes (*snm^{z0317}*, *snm^{z2138}*, *mnm^{z5578}*, *mnm^{z3298}*, and *mnm^{z3401}*). *X / Dp(1;Y) ;*

mnm males were also sterile; however, we were able to assay NDJ in *X / Dp(1;Y) ; snm* males (i.e. males bearing a *wildtype* X). As SNM is necessary for conjunction at the rDNA, we reasoned that any segregation of the X from the *Dp(1;Y)* observed in *snm* males could be attributed to the behavior of the X euchromatic homologies. Therefore, we compared sex chromosome NDJ frequencies from *snm* or *snm* / + males bearing *Dp(1;Y)BSC76* or *Dp(1;Y)BSC67.*

Sex chromosome segregation in *X / Dp(1;Y)BSC76; snm* males was randomized, and not significantly different from control *X / B^s Y y^+; snm* males ($p > 0.75$, Table 7). NDJ in *X / Dp(1;Y)BSC67; snm* males was actually slightly higher than in control *snm* / + males ($p < 0.05$). Whereas in previous crosses, *In(1)$sc^{4L}sc^{8R}$ / Dp(1;Y)BSC76* and *In(1)$sc^{4L}sc^{8R}$ / Dp(1;Y)BSC67* showed different NDJ frequencies, no differences were observed here ($p > 0.25$). These data indicate that SNM is required to mediate conjunction between X chromosome euchromatin.

Table 6. Effect of *tef*^{z3455} / *Df(tef)803Δ15* on XY Segregation in *In(1)sc*^{4L}*sc*^{8R} / *Dp(1;Y)BSC76* Males.

Sperm genotype:	*X;4*	*Y;4*	*0;4*	*X/Y;4*	*X;0*	*X;4/4*	*Y;0*	*Y;4/4*	*0;0*	*0;4/4*	*X/Y;0*	*X/Y;4/4*	4 NDJ	XY NDJ
Paternal Genotype:														
FM7a / y+Y														
tef / +	3148	2813	8	9	0	7	0	4	0	0	0	0	0.00	0.00
tef	1221	996	3	0	514	397	441	403	1	0	0	1	0.44	0.00
In(1)sc^{4L}*sc*^{8R} */ y+Y*														
tef / +	2071	745	1112	90	4	1	2	4	0	18	1	4	0.01	0.30
tef	614	273	341	37	237	144	133	90	112	125	21	12	0.41	0.30
FM7a / Dp(1;Y)BSC76														
tef / +	1048	270	1	2	0	0	0	0	0	0	0	0	0.00	0.00
tef	643	229	2	0	234	116	94	64	0	1	0	0	0.37	0.00
ln(1)sc^{4L}*sc*^{8R} */ Dp(1;Y)BSC76*														
tef / +	1804	240	205	11	1	0	0	0	1	0	0	0	0.00	0.10
tef	280	35	37	6	96	89	22	13	4	6	1	0	0.39	0.09

Progeny of *tef*^{z3455} / + and *Df(tef)803D15* / + did not significantly differ and were combined.

Table 7. Effect of *snm*^{z0317} / *snm*^{z2138} on XY Segregation in *X / Dp(1;Y)BSC* Males.

Sperm genotype:		*X;4*	*Y;4*	*0;4*	*X/Y;4*	*X;0*	*Y;0*	*0;0*	*X/Y;0*	4 NDJ	0/(X+0)
Parental Genotype:											
X / B^{s} *Y y+*	*snm*^{z0317} / +	1204	858	1	0	0	0	0	0	0.00	0.00
	snm	425	274	386	182	112	104	121	98	0.26	0.49
X / Dp(1;Y)BSC76	*snm*^{z0317} / +	1296	385	3	0	0	0	0	0	0.00	0.00
	snm	387	113	413	126	123	37	106	53	0.24	0.50
X / Dp(1;Y)BSC67	*snm*^{z0317} / +	881	217	20	7	0	0	0	0	0.00	0.02
	snm	154	37	170	40	29	10	48	11	0.20	0.54

Discussion

The *Drosophila* male is an interesting model in which to study meiosis because homologs do not recombine, and thus they lack the canonical mechanism of homolog attachment and segregation. It is also of particular interest because it was the first organism in which specific sequences were identified that function as meiotic pairing sites. A 240 bp sequence within the IGS of the rDNA is sufficient for pairing and segregation of the X from the Y (McKee and Karpen 1990; McKee, Lumsden, and Das 1993; McKee, Habera, and Vrana 1992). Although the X and the Y share significant sequence homology other than these IGS sequences in both the rDNA cistrons and at the stellate/crystal loci (Livak 1990), these homologies do not seem to promote pairing and segregation. Lack of pairing at other homologies suggested that there was a unique property of the IGS sequences with respect to sex chromosome meiotic pairing.

Similarly, there appeared to be some specificity to which autosomal sequences could function as "pairing sites". Euchromatic segments of chromosome 2 translocated to the Y are capable of pairing and directing segregation from the intact chromosome 2 homolog, but a translocated segment of chromosome 2 heterochromatin is not (McKee, Lumsden, and Das 1993). Likewise, rearranged autosomal homologs that share only heterochromatic homologies do not pair and segregate from each other (Yamamoto 1979; Hilliker, Holm, and Appels 1982). These studies raised the question as to how the cell restricts pairing to specific sequences.

Are there Specific "Pairing Sites" in Male Meiosis?

Our work here suggests an alternative interpretation of these previous results. Prior observations of meiotic pairing were made during late prophase I, prometaphase I, and/or metaphase I (Yamamoto 1979; McKee, Habera, and Vrana 1992; McKee and Karpen 1990; McKee, Lumsden, and Das 1993). In these studies, chromosomes were judged as paired only if associations were observed in these later stages, and as such, failed to distinguish between the processes of pairing and conjunction.

Here, we have separately examined pairing and segregation (and by inference conjunction) utilizing a series of *Dp(1;Y)*s (Cook *et al.* 2010) and the rDNA-deficient *In(1)sc*^{4L}*sc*^{8R} X chromosome. Using *in situ* hybridization in combination with genetic tests of chromosome transmission, we were able to directly observe meiotic pairing independently of conjunction and assay its relationship to segregation. Our results indicate that 13 different Y chromosome rearrangements bearing X euchromatic homology are capable of pairing with the X. Rather than being limited to specific sequences, we suggest that pairing in males, as in other systems, may simply be homology-based. This possibility is consistent with observations that autosomal heterochromatic repeats are indeed paired in early prophase I (Tsai, Yan, and McKee 2011), and that *lacI* repeats inserted in 13 different euchromatic positions are all paired in early prophase I (Vazquez, Belmont, and Sedat 2002).

We found that all homologous segments tested paired with high fidelity (>74%). No relationship between homology length and pairing ability was observed, which means that either (1) pairing sites are not evenly distributed along the X chromosome (i.e. some

short segments may have as many or more pairing sites as other longer segments) or (2) the duplicated sequences tested (~700 Kbp – 1500 Kbp) were all above the minimum threshold required for efficient pairing. We conclude that either all euchromatin can pair or that pairing sites are distributed throughout the euchromatin.

To further address if there are minimal sequence requirements for XY pairing, we subdivided a duplicated euchromatic sequence into two smaller 120 Kb and 161 Kb fragments. We found that both sequences paired equally well, implying that the subdivided segment contains at least two sequences capable of pairing. Further analysis using deletions of these duplicated regions will be necessary to determine if pairing occurs at all euchromatin or if there are unique pairing sites within each tested region. In the absence of evidence for the latter, the most parsimonious explanation for our data is simply that all homologous sequences have the ability to pair.

What Determines Conjunction in Male Meiosis?

If all homologous sequences can pair but not all remain associated and/or have the ability to direct segregation, then specific sequences may act as conjunction sites. Three proteins necessary for conjunction have been identified to date, MNM, SNM, and Tef. A putative MNM/SNM complex is required for conjunction for all bivalents, whereas Tef only affects conjunction between autosomal homologs (Thomas *et al.* 2005). By examining the pairing behavior of integrated *lacO* sites, it was concluded that mutants in *mnm* and *snm* do not disrupt pairing in S1 (Thomas *et al.* 2005), whereas the effects of *tef* mutants on pairing have not yet been examined. Both MNM and SNM localize to the 240 bp IGS repeats embedded within the rDNA cistrons (Thomas and McKee 2007). Tef

is needed to localize MNM (and presumably SNM) to sites along the autosomes (Thomas *et al.* 2005). Whereas Tef binding sites have yet to be identified, the existence of three canonical C2H2 zinc fingers in Tef suggest that there may indeed be a consensus sequence for establishing conjunction on autosomes (Arya *et al.* 2006).

In our system, we examined the ability of X chromosome homologies to remain conjoined and thereby direct segregation. It was possible that these sequences lacked the MNM/SNM binding sites present in IGS sequences and also the autosomal binding sites potentially recognized by Tef. We wondered which, if any, of these proteins might be involved in mediating conjunction. We first tested if *tef* mutations had any effect on X / *Dp(1;Y)* segregation. Although *tef* mutations show an autosome-specificity, it was possible that this specificity reflected a euchromatin-specific function that did not affect the normally heterochromatic XY conjunction. If this were the case, we might have expected *tef* mutations to disrupt the euchromatin-mediated XY conjunction. We found, however that Tef was not required suggesting that Tef is indeed specific for autosomes.

We next sought to test the requirements for MNM and SNM. While SNM and MNM show binding specificity to IGS sequences (Thomas and McKee 2007), the exact binding sites within the IGS have not been determined. It is not known if potential binding sequences might also be distributed throughout X euchromatin.

Unfortunately, we were unable to test the role of MNM because for an unknown reason, MNM mutants in combination with the sex chromosome rearrangements were sterile. However, we were able to test SNM, and indeed, found it to be required for segregation in our XY euchromatic pairing system. This result shows that SNM is

necessary for conjunction between X euchromatin and suggests that sequences sufficient for SNM binding are present in X euchromatin. Because Tef is not required, the mechanism of SNM binding to the X euchromatin likely differs from the mechanism by which SNM binds to the autosomes. There may be homology to IGS sequences in the X euchromatin that directly bind SNM, although we could not identify extensive homology using BLAST (Altschul *et al.* 1990). Interestingly, there is a cluster of IGS-like sequences present on chromosome 3R that share almost 90% identity to the rDNA IGS repeats (FLYBASE). Polymorphisms that differentiate these sequences from the X rDNA IGS sequences may be critical in determining SNM binding.

An alternative explanation for SNM-mediated conjunction at X euchromatin is that $In(1)sc^{4L}sc^{8R}$ may have a small number of remaining IGS sequences. One or two IGS sequences on their own may not be sufficient for establishing pairing but may be sufficient for mediating conjunction if pairing via euchromatin occurred *in cis*.

Centromere-proximal Sequences are More Effective at Directing Segregation

Interestingly, although we found all homologous sequences paired with similar fidelity, not all sequences behaved the same in the ability to direct segregation. Pairings between centromere proximal sequences were better at directing homolog segregation. The distal-most X and *Dp(1:Y)* pairing observed, in fact, failed to measurably contribute to segregation. A similar observation was made for the segregation of *Dp(2;Y)*s from intact chromosome 2 homologs. Euchromatic homology found to be most effective at directing segregation was the histone locus, which resides on 2R adjacent to the centromere (McKee, Lumsden, and Das 1993).

Why might centromere-proximal association demonstrate a greater frequency of proper segregation? One possibility is that pairing close to the remaining heterochromatin of the *In(1)sc*^{4L}*sc*^{8R} X may be more effective at establishing conjunction at cryptic IGS sequences. Proximal pairing may be better at bringing such sites on homologs close enough to facilitate conjunction. Very distal pairings, as in the case of *Dp(1;Y)BSC11,* may be ineffective. Alternatively, centromere-proximal attachments could simply be better at establishing tension across the bivalent at metaphase I. Tension is important for stabilizing kinetochore attachments necessary for establishing bipolar orientation (Salmon and Bloom 2017). In many systems, when tension is not present at kinetochores because of insufficient microtubule attachment, a metaphase arrest is triggered (Nicklas *et al.* 2001). In male *Drosophila*, however, activation of this checkpoint by unpaired chromosomes merely delays the transition to anaphase I (Rebollo and Gonzalez 2000). It is conceivable that meiosis would proceed through anaphase I even if the XY bivalent had not formed stable bipolar attachments, leading to NDJ. This possibility may explain why the centromere-proximal rDNA locus evolved as the native XY pairing site.

In summary, our examination of XY euchromatic pairing suggests some fundamental differences in the previous models of meiotic pairing and conjunction in male flies. Rather than pairing being limited to specific sequences, we propose that the simplest model is that all homologous sequences can pair, and only a subset of homologies function as conjunction sites during meiosis I. The repeats with the IGS sequences of the rDNA are most likely conjunction sites which serve to bind the

conjunction proteins MNM and SNM (Thomas and McKee 2007), and a putative complex of these proteins with Tef may localize to conjunction sites within autosomal euchromatin. Conjunction sites may be able to pair, but not all pairing sites may be capable of establishing conjunction.

Our assay promises to be useful to further define requirements for meiotic pairing. Deletion analysis of euchromatic region may delimit the minimal sequences required for pairing and determine whether specific sequences are required for pairing and/or conjunction.

CHAPTER III

PAIRING BETWEEN THE X AND *Dp(1;3)*s IN *DROSOPHILA* MALE MEIOSIS

Introduction

When a cell divides meiotically, a diploid parent cell is reduced into four haploid daughter cells. It is of grave importance to perform these divisions flawlessly, as errors in meiosis produce aneuploid gametes, and gene dosage imbalances can lead to offspring that are inviable or have severe genetic syndromes. Homologous chromosomes must first find their partners, pair, and conjoin before they can segregate properly. The process of pairing is critical as all subsequent steps cannot occur without first pairing. Research has identified some requirements of homolog pairing; however, the overall process is not well understood.

The male fly is a good model to characterize pairing as meiosis occurs without the complicated steps of recombination. Most studies that examined pairing requirements for male meiosis have analyzed condensed chromosomes at prometaphase I or metaphase I rather than during early prophase I, when pairing is established. Therefore, the data from these studies do not address pairing per se but are limited to defining requirements for both pairing and conjunction, the ability to maintain association after intimate pairing is released. However, chromosome sequences may exist that are involved in pairing but not maintained through conjunction.

For the autosomes, euchromatic but not heterochromatic, sequence homologies have been shown to be capable of mediating both pairing and conjunction (Yamamoto 1979; Hilliker, Holm, and Appels 1982). Because the X and Y lack euchromatic homology, the sex chromosomes must utilize different sequences for pairing and conjunction. The tandem rDNA repeat embedded in the heterochromatin near the centromere of the X and on the base of the Y short arm have been identified as the required sites (Cooper 1959; Ritossa 1976; McKee and Karpen 1990). Specifically, transgene studies have shown that pairing and conjunction map to a repeated 240 bp intergenic spacer (IGS) region in the promoter of the 18s rDNA genes (McKee, Habera, and Vrana 1992; McKee and Karpen 1990).

To better understand the requirements of pairing, experiments using transpositions and duplications have been used. At mid-to-late prophase I, transpositions of chromosome 2 euchromatin onto the Y chromosome will pair and conjoin with the intact chromosome 2 partner, while transpositions of chromosome 2 heterochromatin will not (McKee, Lumsden, and Das 1993). Similar to what is seen with euchromatic pairing and conjunction between homologs, the longer the duplicated chromosome 2 material is, the more proficient this homology between heterologs is at pairing, conjoining, and directing segregation (McKee, Lumsden, and Das 1993). These experiments performed during late prophase I to metaphase I demonstrate that euchromatic sites along the autosomes and the rDNA on the X and Y are required for pairing and conjunction. Importantly, other potential sites of pairing may occur in early prophase I, but might not *remain* paired through late prophase I.

The first study to assay pairing of native sequences during early prophase I used FISH to euchromatic sequences on *Dp(1;Y)*s and an rDNA-deficient *In(1)sc^{4L}sc^{8R}* X chromosome. All the euchromatic homologies duplicated on the *Dp(1;Y)*s tested had the ability to pair during S1-S2a of prophase I; however, not all duplications were able to segregate chromosomes with the same success (Hylton *et al.* 2020). The closer the duplicated sequences on the *Dp(1;Y)* lie to the centromere on the X, the better at segregation, but no relationship to duplication length was identified (Hylton *et al.* 2020). These results suggest that all homologies can pair, but conjunction is determined by different parameters than pairing. Conjunction may only occur at specific sites, or there may be more extensive homology required for establishing conjunction than is required for pairing.

Together these observations suggest that chromosome level mechanisms exist to regulate pairing and/or conjunction at different sites. With the characterization of pairing during early prophase I possible using FISH, we wanted to test the ability of X euchromatin duplicated on to chromosome 3 to pair and direct segregation from the X. Y chromosomes bearing as few as 120 Kb of X euchromatin were shown to pair and segregate from chromosome 2 (McKee, Lumsden, and Das 1993); therefore, *Dp(1;3)* chromosomes ranging in size from 21 to 177 Kb were selected to determine size and sequence requirements for pairing and segregation.

Next, we wanted to ask if any temporal differences in pairing exist between the two types of rearranged chromosomes: re-establishment of pairing between homologs and pairing between otherwise heterologous chromosomes. We compared *In(1)sc^{4L}sc^{8R}*

X and *Dp(1;Y)* pairing versus chromosome 3 and *Dp(1;3)* pairing in primary spermatocytes during prophase I to determine if a mechanism may exist to resolve inappropriate pairings between similar sequences on non-homologous chromosomes. Our data show that in early prophase I, pairing can occur between homologies on the X and chromosome 3. Pairings between *Dp(1;3)*s and the X are resolved earlier in prophase I than are the pairings between the *Dp(1;Y)*s and $In(1)sc^{4L}sc^{8R}$. Disruption of inappropriate pairing may occur with the formation of chromosome domains, a process by which homologous pairs are isolated in separate locations of the cell during mid-to-late prophase I. As would be expected when pairing between heterologs is resolved prior to anaphase I, duplications of X material on chromosome 3 are not very effective in directing segregation of the X and *Dp(1;3)*. These results suggest that separate mechanisms are in place to resolve homologous and non-homologous pairing.

Materials and Methods

Drosophila Stocks and Crosses

Drosophila were raised on a standard diet consisting of cornmeal, molasses, agar and yeast at 23°C. All *Dp(1;3)* stocks were obtained from the Bloomington Stock Center (Gramates *et al.* 2017).

Genetic Assays of Meiotic Chromosome Segregation

Segregation of a *Dp(1;3)* chromosome from an intact X chromosome was monitored by crossing *y w sn* / Y; *Dp(1;3)* / + males to *y w sn* females. *y w sn* / *y w sn; Dp(1;3)* / + females were crossed to *y w sn* / Y males to control for viability.

The segregation value S is the proportion of euploid progeny from *y w sn* / Y; *Dp(1;3)* / + fathers in which the duplication segregates from the X adjusted for viability difference using segregation data from *y w sn* / *y w sn*; *Dp(1;3)* / + mothers.

S = (X + 3 from Fathers) / [(X + 3 from Fathers) + [(Y + 3 from Fathers) * [(X + 3 from Mothers) / (Y + 3 from Mothers)]]]

Testis Dissection

Testes from larvae or pharate adults were dissected in Schneider's Drosophila media (GIBCO BRL, Gaithersburg, MD). Tissue was transferred to a drop of Schneider's on a silanized coverslip and gently squashed onto a Poly-L-Lysine coated slide (Electron Microscopy Sciences, Hatfield, PA). Coverslips were immediately removed after freezing in liquid nitrogen. Tissue was fixed in 55% methanol / 25% acetic acid for 10 min followed by 10 min dehydration in 95% ethanol. Slides were processed immediately or stored for up to 1 week at 4°C.

Probe Design

Triple-labeled probes pools were generated to selected sequences at a density of 10 probes/Kb and a complexity of ~10,000 probes per pool (Arbor Biosciences, Ann Arbor, MI). ATTO-594 oligonucleotide probes were generated to hybridize to 1,000 Kbp present on the X chromosome and the following regions of X chromosome sequences duplicated on the *Dp(1;3)* chromosome 3s: *Dp(1;3)RC017* (X salivary gland chromosome bands 3D5-3E1 spanning base pairs 3543803 - 3606837); *Dp(1;3)RC029* (X salivary gland chromosome bands 12A4-12D2 spanning base pairs 13824546 - 14001084); and

Dp(1;3)RC035 (X salivary gland chromosome bands 17F2-18A2, spanning base pairs 18900731 - 19062922).

A triple-labeled ATTO-488 probe was generated to bp 20368577 - 21368577 (56F-57F) on chromosome 2.

FISH

Slides of testis tissue were processed for FISH using a slight modification of the protocol as described in Beliveau, Apostolopoulos, and Wu (2014) and as reported in Hylton *et al.* (2020). S1-S2a (10 to 20 μm) and S2b (>20 to 30 μm) spermatocytes were selected based on size, and signals were scored as paired when within 0.8 μm as in Beliveau, Apostolopoulos, and Wu (2014).

Results

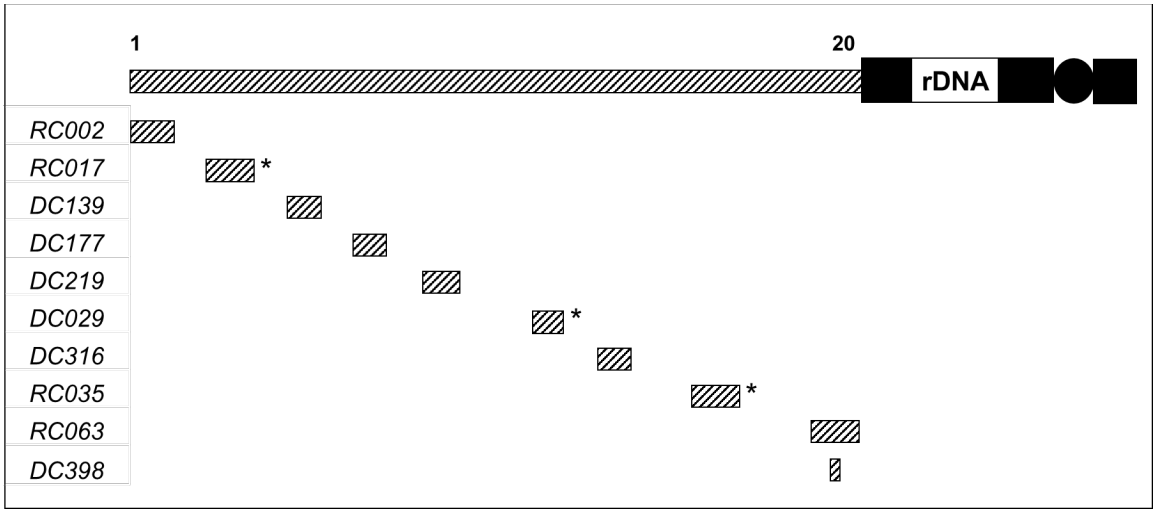
*Dp(1;3)*s Pair with but do not Effectively Segregate Away from the X

To ask if X euchromatic homologies duplicated on chromosome 3 could pair and direct segregation from an intact X, *Dp(1;3)* chromosomes were selected that contain duplicated X euchromatin of different lengths and from different locations all along the arm of the X chromosome (Figure 7). The duplicated X euchromatin homology on each *Dp(1;3)* varies from 21 to 177 Kb, and these *Dp(1;3)*s were selected since they were roughly the same size range as the ~120 Kb of X homology found to be sufficient for the X and Y to pair and segregate (Hylton *et al.* 2020).

Pairing of the *Dp(1;3)*s and the X chromosome were monitored using FISH probes that bind euchromatic sequences on the intact X and the X euchromatic sequences duplicated on the *Dp(1;3)*. Pairing is observed in spermatocytes during early S1-S2

(Cenci *et al.* 1994) of prophase I (Vazquez, Belmont, and Sedat 2002), and these cells were selected based on size (Between 10 and 20 µm). The pairing assay is described at length in (Hylton *et al.* 2020).

Figure 7. Regions of X Euchromatin Duplicated on Each *Dp(1;3)* Chromosome. Euchromatin is marked by hatched boxes. * Pairing examined by FISH.



Of the three *Dp(1;Y)*s analyzed for their abilities to pair with the intact X chromosome, all were paired in greater than 90% of the cells analyzed (Figure 8, Table 8). Pairing did not appear to be affected by the length of the X euchromatin duplicated on the *Dp(1;3)* or by the location of the homology on the X chromosome.

Figure 8. FISH Examination of X / *Dp(1;3)* Pairing in DAPI-stained S1-S2 Primary Spermatocytes. The X and chromosome 3 are labeled red and chromosome 2 pair are labeled green. (A) Paired X / *Dp(1;3)* chromosomes and paired chromosome 2 bivalents. (B) Unpaired X / *Dp(1;3)* chromosomes and a paired chromosome 2 bivalent. (C) Paired X / *Dp(1;3)* bivalent and unpaired chromosome 2s. Scale bar = 2 μm.

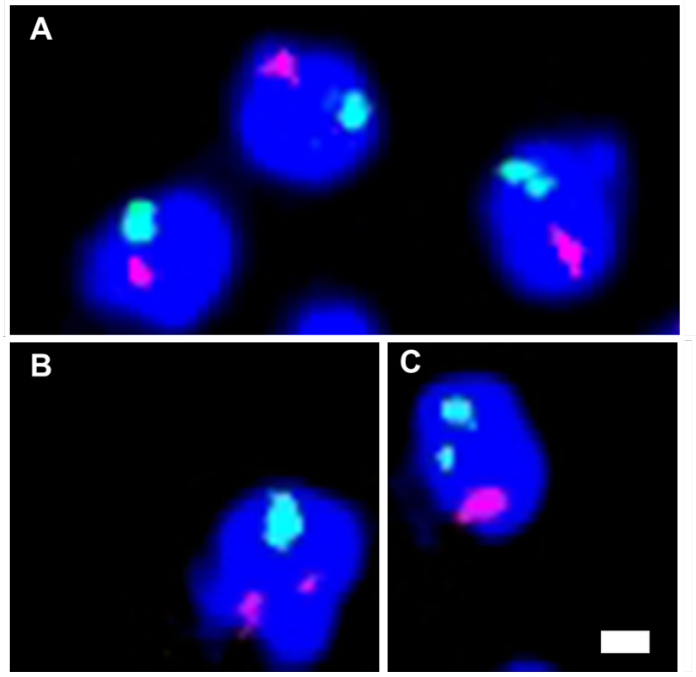


Table 8. X / *Dp(1;3)* Pairing in S1-S2 Primary Spermatocytes.

X	3	# Cells Scored	% Paired
wildtype	*Dp(1;3)RC017*	212	92.9
wildtype	*Dp(1;3)RC029*	204	93.1
wildtype	*Dp(1;3)RC035*	383	90.3

X / *Y*; *Dp(1;3)* / + fathers used in the crosses produce four sperm classes (*X;* +, *Y;* +, *X; Dp(1;3),* and *Y; Dp(1;3)*). The latter two will form a zygote triploid for the duplicated region after fertilization, and the viability of such zygotes greatly depends on the

duplicated region. To eliminate these effects of the duplication on viability, only the euploid classes were considered in this analysis. To control for any other potential viability differences between the resulting classes, the viabilities of the identical progeny generated from *X* / *X; Dp(1;3)* / 3 females was measured. In these females, the *Dp(1;3)* must segregate with one of the two identical Xs, so any variability in the recovery of X; + sons versus daughters will reflect any viability differences. Using the viability differences from the female data, the segregation frequency at which the X segregated from the *Dp(1;3)* in males could be calculated (Segregation Ratio, S) (See Materials and Methods).

Data show that the *Dp(1;3)*s do not consistently segregate from the X (Table 9). Some of the calculated S values for the *Dp(1;3)*s are significantly greater than 0.5, which is the expected frequency if the *Dp(1;3)* and X chromosome segregated randomly (Table 9). None of the *Dp(1;3)*s segregated from the X as effective as the *Dp(1;Y)*s segregated from the $In(1)sc^{4L}sc^{8R}$ X (Hylton *et al.* 2020), which in some cases was greater than 0.9. The *Dp(1;3)* that exhibited the best S value was only 0.6.

Table 9. Segregation of *Dp(1;3)* Chromosomes from an Intact X Chromosome.

		Transmission:	Paternal		Maternal		
		Genotype:	*X;TM6C*	*Y;TM6C*	*X;TM6C*	*Y;TM6C*	S
Chromosome 3[†]	Length of *Dp(1;3)* (Kb)	X Region Duplicated on 3[††]					
Dp(1;3)RC002	140	1Lt--1A1	691	674	533	532	0.506
Dp(1;3)RC017	163	3D5--3E5	1298	1105	420	422	0.541*
Dp(1;3)DC139	94	5A1--5A5	729	787	244	232	0.468
Dp(1;3)DC177	94	7B3--7B4	611	612	227	292	0.562*
Dp(1;3)DC219	105	9A5--9B2	704	669	330	280	0.472
Dp(1;3)RC029	177	12C6--12D2	648	581	262	351	0.599*
Dp(1;3)DC316	94	14E1--14F2	775	683	408	471	0.567*
Dp(1;3)RC035	162	17F2--18A2	837	739	350	426	0.580*
Dp(1;3)RC063	160	20F1--20F4	735	655	450	528	0.568*
Dp(1;3)DC398	21	20F3--20F3	678	619	405	453	0.551*

S = paternal segregation ratio adjusted for viability by the maternal segregation ratio (see Materials and Methods).
* Indicates significance at $p < 0.05$. † All *Dp(1;3)* chromosomes arose from BAC insertions of X euchromatin at 65C.
†† Salivary gland chromosome bands.

Dp(1;3) and X Pairings are Resolved Earlier than are *Dp(1;Y)* and X Pairings

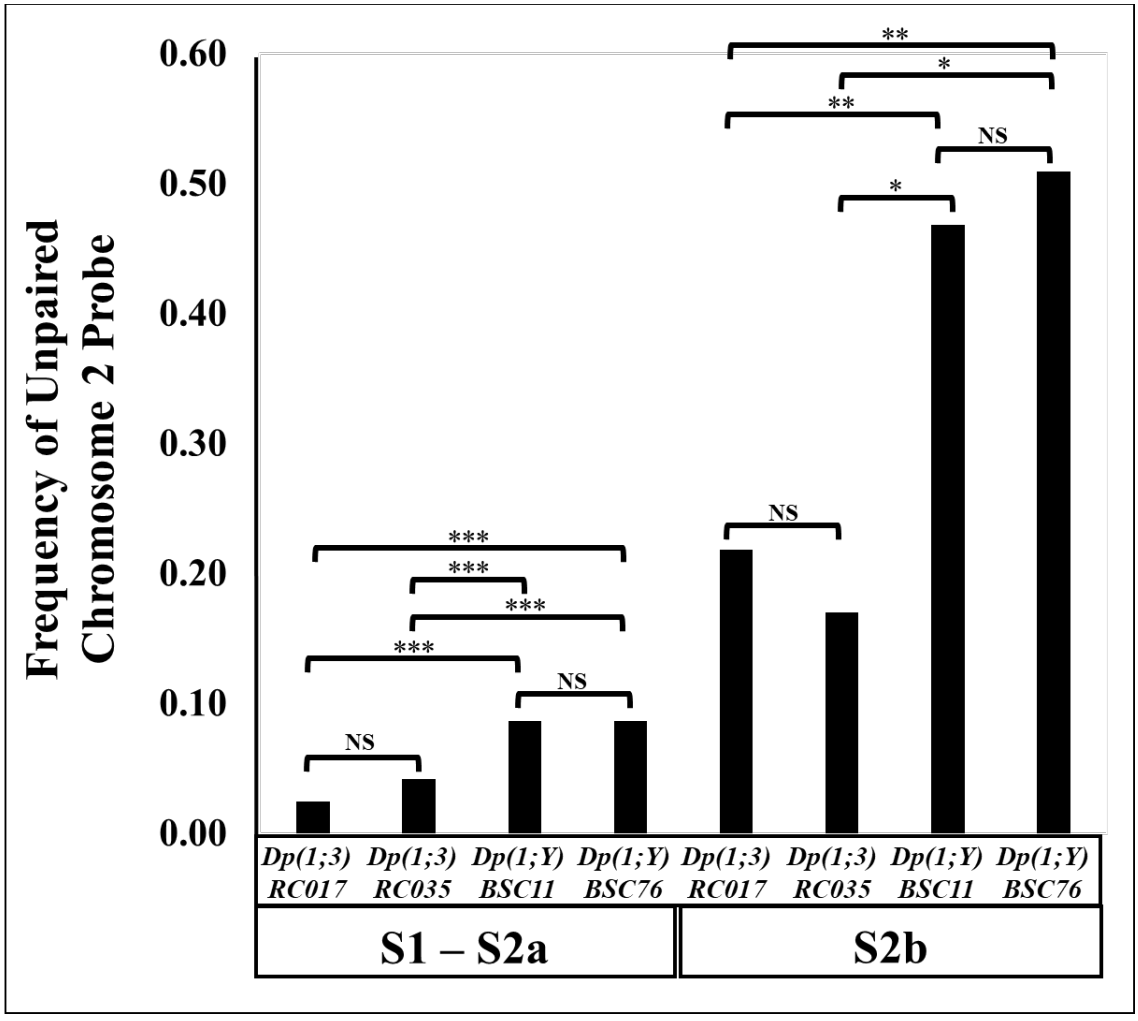
The *Dp(1;3)*s paired very efficiently with the X chromosome, but unlike pairings between similarly sized X duplications on *Dp(1;Y)*s, the X / *Dp(1;3)* pairings did not efficiently direct merotelic segregation of the paired chromosomes. This lack of segregation suggested that X / *Dp(1;3)* pairings might be disrupted before affecting orientation at meiosis I. Previous work examining pairing at inserted *LacO* arrays found that homologs separate during S2b of prophase I (Vazquez, Belmont, and Sedat 2002). At this stage, the formation of three chromosome domains can first be visualized around the periphery of the cell, each of which contain a homologous major chromosome pair (Cenci *et al.* 1994; Vazquez, Belmont, and Sedat 2002). It has been theorized that the formation of chromosome domains sorts out heterologous pairings while maintaining proper homologous pairings (Vazquez, Belmont, and Sedat 2002). We wondered if the X and *Dp(1;3)* pairings were disrupted as a consequence of domain formation.

The FISH pairing assay allows direct visualization of chromosomes during the early stages of prophase I. We used FISH to compare the dynamics of pairing between the X and *Dp(1;3)*s versus the X and *Dp(1;Y)*s. We expected that heterologous pairings may be resolved earlier than homologous pairings. A probe to chromosome 2 was used to monitor the progression of unpairing between homologs. Because there is some asynchrony in unpairing, we expect that unpairing of chromosome 2 might proceed the unpairing of X and *Dp*s in a fraction of the cells. Because we wanted to avoid including cells where the X and *Dp* had failed to pair, we only scored cells in which the X sequences were paired. In S2b cells where the chromosome 2 probe was unpaired, we

scored the fraction of the *Dp*s remaining paired. We reasoned that if the *Dp* remained paired later into prophase I, then the fraction of cells in which the autosome probe was unpaired would increase. Conversely, if pairing at the *Dp* was disrupted early, we would find very few cells in which unpairing of the autosome probe proceeded the unpairing of the *Dp*.

The S1-S2a cells were selected by size (10 to 20 μm) based on criteria from Cenci *et al*. (1994). In a small frequency of these cells, the chromosome 2 pairings had already been resolved prior to reaching the S2b size (>20 to 30 μm). In such cells, there were significantly more X and *Dp(1;3)* unpairings than X and *Dp(1;Y)* unpairings (Figure 9). This difference in the behavior of *Dp(1;3)*s and *Dp(1;Y)*s was even more striking in S2b cells where unpairing had further progressed. At this stage, there was a significantly higher fraction of cells where the X and *Dp(1;3)* was unpaired than were cells where the X and *Dp(1;Y)* was unpaired (Figure 9). This finding suggests that the timing of X and *Dp(1;3)* unpairing is earlier than the X and *Dp(1;Y)*, and this unpairing may occur concomitantly with domain formation.

Figure 9. Dissolution of Pairing at X Euchromatin on *Dp(1;Y)*s vs. *Dp(1;3)*s Relative to Autosomal Pairing. Frequencies of X / *Dp(1;3)* pairings versus X / *Dp(1;Y)* pairings in cells where chromosome 2s have unpaired. NS = No significance. Significant difference at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Discussion

The *Drosophila* male is the first organism in which meiotic pairing sites were identified. XY pairing was thought to primarily occur at the 240 bp sequence within the rDNA IGS because this region is sufficient for XY segregation (McKee and Karpen 1990; McKee, Lumsden, and Das 1993; McKee, Habera, and Vrana 1992) while other

homologies such as the stellate/crystal loci (Livak 1990) are not. However, prior experiments monitored pairing during late prophase I when conjunction is already in effect. At this stage, pairing at sites that do not conjoin may have been lost.

With the development of the FISH pairing assay, intimate pairing can be studied during early prophase I and the steps of pairing and conjunction can be successfully separated (Hylton *et al.* 2020). Using this assay, we have previously shown that euchromatic X duplications as small as 120 Kb on the Y are sufficient for X / *Dp(1;Y)* pairing and segregation (Hylton *et al.* 2020). Next, we aimed to revisit the topic of pairing between heterologous chromosomes using the FISH pairing assay. Transpositions of chromosome 2 material to the Y chromosome, *Tp(2;Y)*s, have been shown to pair and conjoin from an intact chromosome 2 at mid-to-late prophase I (McKee, Lumsden, and Das 1993). In addition, many of the *Tp(2;Y)*s tested were able to direct segregation albeit at a frequency not much higher than random. Unfortunately, we were unable to use the *Tp(2;Y)* chromosomes for our experiments because many of the stocks are no longer available. Instead, we used *Dp(1;3)*s which are chromosome 3s with a duplicated sequence of X euchromatin.

Three *Dp(1;3)*s were monitored for their abilities to pair with the X during S1-S2a. We found the X and *Dp(1;3)* paired in over 90% of the cells scored. These three *Dp(1;3)*s along with seven others were tested for their ability to direct segregation from the X. Some *Dp(1;3)*s were able to segregate away from the X; however, similarly to the X and *Tp(2;Y)* segregation frequencies, their segregation was at best only 10% better than random.

Since it was evident that homologous sequences duplicated on a heterologous chromosome can pair at a frequency > 0.90, we wondered what was interfering with X and *Dp(1;3)* segregation. After all, most X and *Dp(1;Y)* segregation frequencies were very high, including *Dp(1;Y)BSC76* which exhibited a segregation frequency of 0.92 (Hylton *et al.* 2020). It has been suggested that pairings between heterologous chromosomes are sorted out during domain formation (Vazquez, Belmont, and Sedat 2002). We used the FISH pairing assay to monitor pairing between the X and *Dp(1;Y)* and the X and *Dp(1;3)* during S2b of prophase I, the stage when chromosomes begin to unpair and domain formation is first evident (Vazquez, Belmont, and Sedat 2002; Cenci *et al.* 1994). In S2b cells where the control chromosome 2 bivalent had already unpaired, the X and *Dp(1;Y)* were remained paired in a significantly higher number of cells than were the X and *Dp(1;3)*.

Since we monitored both pairing between homologs and pairing between heterologs using the same sequence homologies for the pairing, our results suggest that two separate mechanisms exist to separate chromosomes. The X and *Dp(1;3)* unpairing appears to occur concomitantly with domain formation as suggested by Vazquez, Belmont, and Sedat (2002). This possibility would also explain why the X and *Dp(1;Y)* stay paired longer and can segregate. The paired duplicated X euchromatin on *Dp(1;Y)* is able to conjoin with the X, migrate to the same domain, and segregate at anaphase I. Perhaps, the paired duplicated X euchromatin on *Dp(1;3)* is unable to conjoin with the X or if it does conjoin, not strong enough to maintain conjunction when heterologs migrate to different domains.

Drosophila male meiosis is unique because it lacks many aspects of the traditional meiotic script including the synaptonemal complex, formation of chiasmata between homologs, and recombination. Thus, separate mechanisms had to evolve for successful meiosis, including how to resolve inappropriate pairings between heterologs. The formation of domains may occur to resolve heterologous pairing by quarantining homologs to different sites in the cell. This possibility is similar to the one proposed in the *C. elegans* system. A KASH/SUN-domain protein complex connects chromosomal "pairing centers" to the cytoskeleton, and chromosome movements are thought to jostle apart inappropriate pairings between heterologs while maintaining homolog pairings (Sato *et al.* 2009; MacQueen and Villeneuve 2001). The movement of chromosomes in *C. elegans* and the formation of chromosome domains in *Drosophila* may both serve the same purpose to minimize heterologs from interfering with homolog segregation in meiosis I. Real time analysis of domain formation using translocations and duplications should be used to better characterize this mechanism in the male fly.

CHAPTER IV

APPLICATIONS OF THE FISH PAIRING ASSAY

Introduction

Historically, *Drosophila* male meiotic mutants have been characterized by tests of genetic segregation and cytology; however, there are drawbacks to these methods. Genetic crosses are somewhat limited as aneuploid progeny can often be inviable, and gametes with less chromatin are often recovered at a higher frequency than those with more chromatin. This unequal recovery of gametes is termed 'meiotic drive' (Sandler and Braver 1954), and the cause is not well understood. Traditional cytological methods such as DAPI or orcein staining require chromosomes to be in a condensed state for visualization limiting assessment of cells in late prophase I to metaphase I. Today, highly selective FISH probes to single-copy sequences can be generated at a moderate cost (Beliveau *et al.* 2012), and scoring meiotic divisions using FISH can eliminate these issues of viability and meiotic drive. Additionally, a single generation genetic test in flies requires 20 days to complete while FISH only requires about 36 hours to process.

Analyzing spermatocytes and spermatids cytologically with single-copy FISH probes can provide better insight into which aspects of meiosis are disrupted in a mutant. For example, the mutation *orientation disruptor* (*ord*) (Sandler *et al.* 1968) causes an increase in reductional and equational NDJ of the sex chromosomes and chromosome 4s in both males and females (Mason 1976). Genetic inference from this data suggested that

defects at both MI and MII occur. Later, cytology using orcein staining suggested that precocious separation of sister chromatids during early MI was the cause of the defect in females and leads to random segregation of sisters at MII (Goldstein 1980; Miyazaki and Orr-Weaver 1992). Finally, FISH probes to satellite repeats near the centromere were used to confirm the cohesion defect as the source of the mutant phenotype in females (Bickel, Orr-Weaver, and Balicky 2002). In the case of *ord*, using FISH to directly label chromosomes was a more direct way of rapidly assessing male meiotic mutants to determine which meiotic stage is affected.

As a proof of concept, I aimed to address the two aforementioned applications of FISH to directly score the outcomes of XY segregation after meiosis I and II. First, I selected male fly lines that in segregation assays exhibit a range of NDJ and used FISH to score the cytological XY NDJ. Cytological NDJ was then compared to NDJ calculated by genetic testing. Second, I selected a previously uncharacterized male meiotic mutant, from a collection of 60, to analyze (Wakimoto, Lindsley, and Herrera 2004). Genetic segregation data of this mutant was collected and FISH used to score sex chromosome segregation in secondary spermatocytes.

Results

FISH Eliminates Viability Differences and Meiotic Drive in the Calculation of XY NDJ

Dp(1;Y) chromosomes along with wildtype and *In(1)sc*^{4L}*sc*^{8R} X chromosomes were used for this study. Genetic crosses were set up as described in Chapter 2 and progenies scored for XY NDJ. Progeny bearing *Dp(1;Y)*s may express viability differences due to the *Dp(1;Y)* itself, and this difference could potentially alter NDJ

frequency. To avoid this possibility previously, sex chromosome segregation was calculated by ignoring the *Dp(1;Y)* classes and using the formula NDJ = *X / (X+0)* (See Chapter 2). Here, however, sex chromosome NDJ was calculated using all progeny classes because all sperm classes are scored in the cytological analysis. Meiotic divisions were monitored by scoring secondary spermatocytes and spermatids for XY content using FISH probes to the AATAC repeat on the Y and the 2E1-3E4 on the X.

Results indicate that the cytological and genetic NDJ frequency calculations are significantly different for seven of the twelve tested genotypes (Table 10). Males bearing an *In(1)sc*^{4L}*sc*^{8R} X exhibit higher levels of XY missegregation because of the missing rDNA on the X, and consequently will have a more pronounced effect of meiotic drive. Data shows that many of these males with higher NDJ frequencies exhibit larger variances between NDJ calculation methods (Table 10). Cytological observation is clearly more accurate for monitoring chromosome segregation. FISH is more direct than measuring outcome because it eliminates the effects of meiotic drive and aneuploid-related viability differences.

Table 10. NDJ Frequency Calculations: FISH vs. Progeny Recoveries.

X	Y	By FISH # Divisions Scored	By FISH XY NDJ	Genetic XY NDJ
Canton S	y^+Y	206	0.01	0.01
$In(1)sc^{4L}sc^{8R}$	y^+Y	200	0.33	0.32
+	*Dp(1;Y)BSC76*	207	0.03	0.04*
$In(1)sc^{4L}sc^{8R}$	*Dp(1;Y)BSC76*	307	0.11	0.07*
+	*Dp(1;Y)BSC185*	224	0.02	0.00*
$In(1)sc^{4L}sc^{8R}$	*Dp(1;Y)BSC185*	214	0.22	0.21
+	*Dp(1;Y)BSC11*	200	0.02	0.00
$In(1)sc^{4L}sc^{8R}$	*Dp(1;Y)BSC11*	237	0.30	0.44*
+	*Dp(1;Y)BSC90*	204	0.05	0.00*
$In(1)sc^{4L}sc^{8R}$	*Dp(1;Y)BSC90*	201	0.12	0.16
+	*Dp(1;Y)BSC214*	200	0.01	0.00*
$In(1)sc^{4L}sc^{8R}$	*Dp(1;Y)BSC214*	206	0.08	0.15*

* Indicates a significant difference between cytological and genetic NDJ frequencies at $p < 0.05$.

Using FISH to Screen Uncharacterized Male Meiotic Mutants

Male meiotic mutant *CG38303*$^{Z265\text{-}61}$ has not been fully characterized and is part of a collection of ethyl methane sulfate-induced mutations (Wakimoto, Lindsley, and Herrera 2004). NDJ was calculated genetically through crosses and cytologically using X and Y FISH probes. NDJ frequencies were almost 50%, suggesting that chromosome segregation for both the XY and 4th chromosome were nearly random at MI (Table 11). FISH analysis of the mutant revealed that sister chromatids separate precociously during MI and segregate to opposite poles in over 75% of the cells scored (Figure 10, Table 12).

The directed segregation of sisters to opposite poles at MI is characteristic of mutants in the components of the monopolin complex in yeast, previously undescribed in *Drosophila*. In *S. cerevisiae*, the monopolin complex is involved in chromosome segregation during meiosis I by crosslinking and mono-orienting sister chromatids to ensure segregation of homologs (Monje-Casas *et al.* 2007; Corbett *et al.* 2010). The core of the complex, including Csm1 and Lrs4, has been shown to bind the kinetochore-associated protein DSN1 which is broadly conserved among many eukaryotes (Rabitsch *et al.* 2003; Plowman *et al.* 2019). Homologs of these proteins cannot be identified in flies by homology searches, but it is believed that flies must have an analogous system since chromosomes perform the same segregation. Perhaps this protein could be the first component of the complex identified in flies. This monopolin-like phenotype in *CG38303*$^{Z265\text{-}61}$ identified by FISH would not have been possible by strictly analyzing the genetic data.

Table 11. Effect of *CG38303*$^{Z265\text{-}61}$ on Sex and Chromosome 4 Segregation in Males.

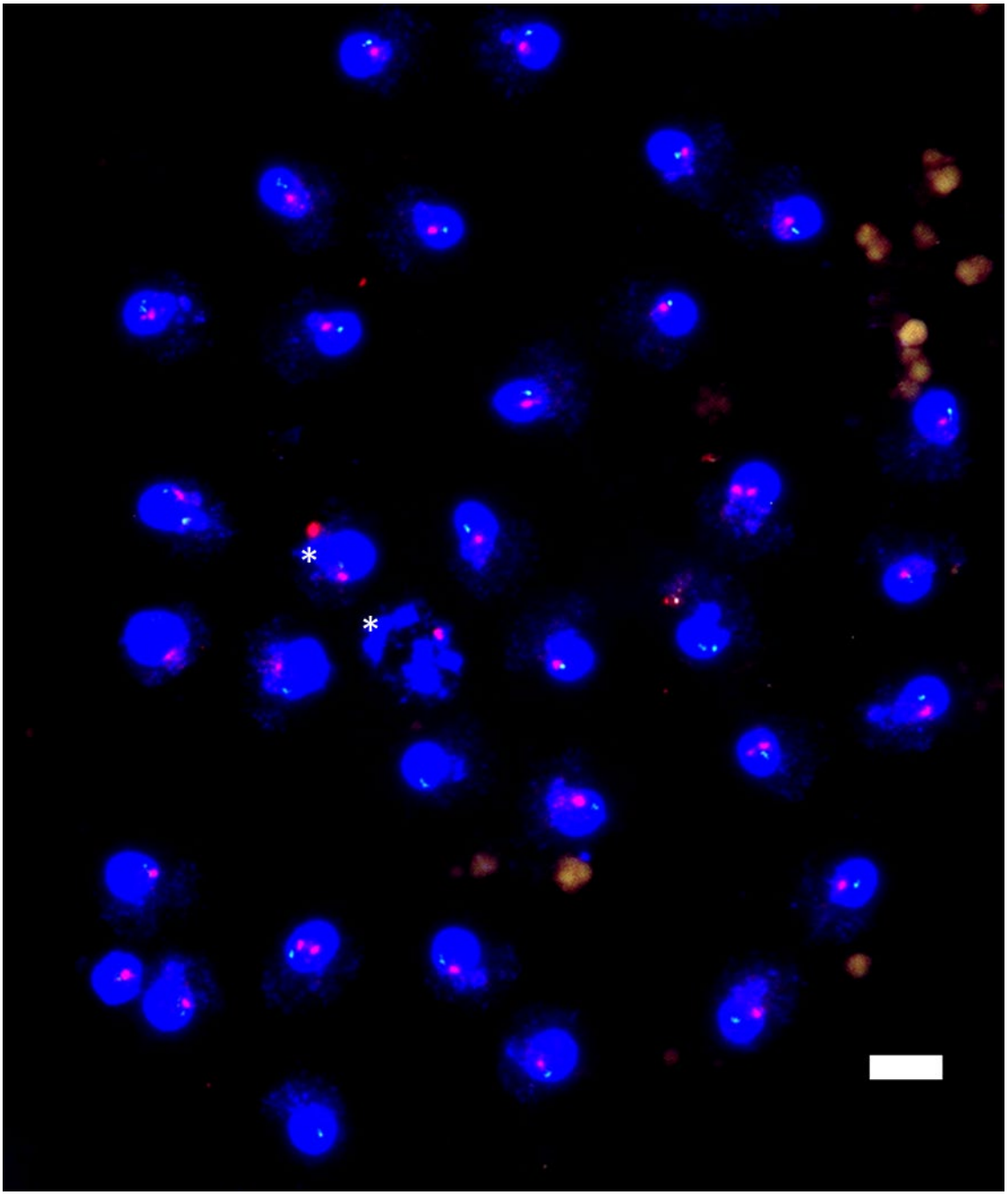
Sperm Genotype:	*X;4*	*Y;4*	*0;4*	*X/Y;4*	*X;0*	*X;4/4*	*Y;0*	*Y;4/4*	*0;0*	*0;4/4*	*X/Y;0*	*X/Y;4/4*	4 NDJ	XY NDJ
Paternal Genotype:														
CG38303	99	73	104	28	32	34	32	44	56	31	15	18	0.46	0.45
CG38303 / *Cy*	474	726	5	0	0	0	0	0	0	0	0	0	0.00	0.00

Table 12. FISH Analysis of Sex Chromosome Segregation Patterns in *CG38303*$^{Z265\text{-}61}$ Males.

MI Divisions:	*XX<->YY*	*X<->XYY*	*XXY<->Y*	*X<->/XY*	*XXYY<->0*	*XY<->X*	*X<->X*	Reductional *X*	Reductional *Y*	Equational *X*	Equational *Y*
Paternal Genotype:											
CG38303	23	1	5	70	0	1	1	0.28*	0.24*	0.72*	0.76*
CG38303 / *Cy*	35	0	0	0	0	0	0	1.00	1.00	0.00	0.00

* Indicates significance at $p < 0.001$.

Figure 10. FISH Analysis of Secondary Spermatocytes in *CG38303*$^{Z265\text{-}61}$ Males. The red probe binds the X and the green probe binds the Y. Spermatocytes that contain both a red and green signal are products of a meiosis I in which sister chromatids segregated merotelically. Scale bar = 10 μm. * Indicates cyst cells.



Conclusion

The benefits of using FISH for analysis of meiosis are plentiful, especially since the cost of probes is more affordable. Off-targeting has become less of an issue with the production of oligopaint probes (Beliveau *et al.* 2012). In addition, the entire process of FISH takes less than 36 hours from tissue dissection to fluorescence analysis. By avoiding analysis of outcome, both meiotic drive and viability differences due to aneuploidy are eliminated. FISH is a much more direct analysis of chromosome behavior and can be used in the future to more completely and efficiently describe meiotic mutants.

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CHAPTER V

CONCLUSIONS AND FUTURE WORK

The research described here contributes to the field of meiotic chromosome pairing. For the first time, we were able to examine chromosome pairing at native *Drosophila* sequence homologies when pairing is initiated during early prophase I. Using the FISH pairing assay we developed, we found that euchromatic homology between the X and Y is sufficient for pairing. Not all pairings are created equal as some pairings were capable of directing segregation from their partners while other pairings did not direct segregation. When the duplicated X euchromatin on the Y lies closer to centromere on the X, segregation occurs more consistently. We speculated the reason this segregation occurs is because the tension required to bypass the metaphase checkpoint is satisfied when paired closer to the centromere. In fact, this may be what drove the evolution of the centromere-proximal rDNA as the native XY pairing sites.

FISH also revealed that duplications of X euchromatin on chromosome 3 are sufficient to pair the heterologs X and chromosome 3; however, segregation does not effectively occur regardless of where the homology lies on the X. This result contrasts the ability of the *Dp(1;Y)*s to direct segregation from the X chromosome and shows that the ability to direct segregation is not explainable merely by size of the homologies involved in the pairings. Instead, it suggests that there may be some fundamental difference in the manner in which a cell deals with pairings between homologous versus

heterologous chromosomes. By analyzing pairing at the S2b stage of prophase I, we found that more of the *Dp(1;3)* and chromosome 3 pairings were resolved than were the *Dp(1;Y)* and X pairings. This earlier release of pairing and lack of segregation that occurs when homology is duplicated between heterologs provides evidence of chromosome level regulation of pairing.

These findings provide the first evidence to support the model, first proposed by Vasquez, Belmont, and Sedat (2002), that the formation of chromosome domains acts as a means of "sorting" out the chromosomes into domains of homologous pairs. In *C. elegans*, pairing centers on chromosomes are bound by a KASH/SUN-domain protein complex which connects the chromosomes and the cytoskeleton through the nuclear envelope (Sato *et al.* 2009; MacQueen and Villeneuve 2001). Chromosome movements are thought to jostle apart inappropriate, heterologous connections while leaving homologous chromosomes attached. It is important to resolve pairing between non-homologs before segregation to avoid possible aneuploid gametes. For example, when a chromosome translocation occurs, a piece or all of one chromosome fuses to another chromosome which can cause aneuploidy. Approximately 5% of trisomy 21 cases in humans are due to adjacent segregation of translocations (Flores-Ramirez *et al.* 2015), and this segregation pattern is likely a result of unresolved conjunction between the transposed sequences.

Now that we have developed a system to segregate chromosomes with only a small duplicated region of homology, experiments can be designed to determine the minimum requirements for pairing. The shortest duplicated sequence that was shown to

pair was 120 Kb for XY pairing and 162 Kb for X and chromosome 3 pairing. The methodology described within now provides a way to further delimit requirements for pairing and segregation. The next steps should be to subdivide these sequences into smaller fragments. CRISPR-Cas9 technology is the most current and efficient method to create deletions in the genome and could be used to target the duplicated euchromatin. By creating smaller nested fragments within the duplicated regions, one can determine whether there is a threshold of homology required for pairing, or perhaps specific sequences lie within these fragments that are required for pairing.

Using the FISH pairing assay, we can, for the first time, clearly separate pairing from conjunction. We have identified that these sequences can participate in conjunction, so a next step could be to determine if the sequences are bound by conjunction proteins. A possibility to explain the pairing between euchromatic homology on the X and Y is that conjunction proteins may bind to specific sites within the duplicated X regions. On autosomes, conjunction proteins bind all along the arm, and this pattern may also occur in the X euchromatin. We found further evidence for this possibility as SNM is required for the segregation of *Dp(1;Y)*s from an X chromosome. We have already demonstrated that the conjunction protein Teflon, which is normally required for segregation of the autosomes, is not needed for XY segregation using euchromatic homology. The dispensability of Teflon at euchromatin-mediated XY conjunction sites indicates that Teflon is specific for the autosomes and not for euchromatin. Using FISH in combination with antibodies to the conjunction proteins MNM and SNM would reveal if MNM/SNM are binding to the paired euchromatic sequences on the X and Y.

We have demonstrated that many different X euchromatic sequences can facilitate pairing both between homologs and heterologs. The rDNA IGS sequences have long been referred to as "pairing sites", which they are, yet our work is important in that it shows that other homologies can also pair. That is, the rDNA is not unique in its ability to pair. This finding leads to the conclusion that the rDNA is more likely a "pairing and conjunction" site.

Our work supports the implications on the suitability of the male fly as a model for meiosis. The identification of the rDNA as a special "pairing site" led to the conclusion that male flies might be unique with respect to pairing, and therefore not an appropriate system to study pairing in general. However, this work has shed light on the fact that pairing in male *Drosophila* may be more similar to pairing in the female and other organisms than was previously suggested.

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